STUDIES ON

N-ACETYL-β-D-HEXOSAMINIDASES IN HUMAN PLACENTA

A Thesis submitted by

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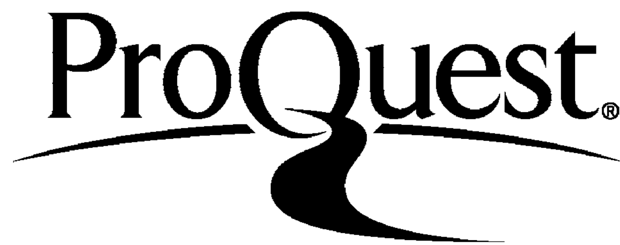

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ABSTRACT

The distribution, biosynthesis and secretion of multiple forms of N-acetyl-β-D-hexosaminidase have been studied in first trimester and term human placentas.

Hexosaminidases A, B, I_1 and I_2 were identified in extracts from whole term placental tissue and the major multiple forms, A and B, were purified 1400-fold and 1000-fold, respectively. Antisera raised to purified hexosaminidase A and B cross-reacted with the A, B, I_1 and I_2 forms and confirmed their structural relatedness.

Total hexosaminidase specific activities and multiple form profiles were compared in well-defined anatomical regions of first trimester and term placentas. Most regions showed a decrease in specific activity with gestation except for the chorion laeve and umbilical cord which showed an increase and no change, respectively. The profiles differed both with respect to placental region and developmental stage.

A placental slice system was used to study the biosynthesis and secretion of hexosaminidase *in vitro*. Evidence for the *de novo* synthesis of hexosaminidase was obtained by immunological isolation and analysis of radiolabelled enzyme. Comparative studies showed that rates of hexosaminidase and total protein synthesis were greater in first trimester than term placental villi. Hexosaminidase was secreted into the incubation medium by slices from first trimester and term placental villi, chorion laeve and amnion. Differences in the amount of enzyme and in the nature of the multiple forms secreted were found both with respect to placental region and developmental stage. The low levels of secreted I_2 suggested that the placenta was unlikely to provide the major source of elevated I_2 levels in pregnancy serum. Inhibitor studies suggested the involvement of microtubules and microfilaments in the secretion of hexosaminidase from placental villi.

Hexosaminidase mRNA was identified in the poly (A)-containing RNA isolated from term placental total RNA preparations. mRNAs coding for the α-and β-subunit polypeptides have been tentatively identified using anti-(hexosaminidase B) and specific anti-(hexosaminidase A) prepared by the absorption of anti-(hexosaminidase A) with hexosaminidase B.

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ABBREVIATIONS

With the exceptions listed below, the abbreviations used are those recognised by the Biochemical Journal (1978) 169, 1-27.

ConA	Concanavalin A
cDNA	Complementary DNA
IgG etc.	Immunoglobulin G etc.
PPO	2,5-Diphenyloxazole
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid

INTRODUCTION

There is now a considerable amount of evidence to implicate lysosomal hydrolases in a variety of biological and pathological situations which involve the breakdown of cellular or extracellular components. This evidence is extensively reviewed in the series of volumes entitled "Lysosomes in Biology and Pathology" which are edited currently by Dingle and Dean and published by North-Holland/ Elsevier.

In spite of their importance, several aspects of lysosomal enzyme biochemistry remain to be elucidated. For example, although many lysosomal enzymes have been shown to exist as multiple forms, the precise nature of the structural and functional relationships between these forms is not clear in most cases. There is also a lack of information about the factors which control lysosomal enzyme activity. Increases or decreases in catalytic activity are associated with both normal and pathological processes but the precise mechanisms by which these changes are brought about are mostly unknown. Indeed, the complete picture of the complicated steps in the biosynthesis of any single lysosomal enzyme has yet to be described.

This study deals with an investigation of the lysosomal hydrolase N-acetyl-β-D-hexosaminidase (EC 3.2.1.52). Like most other lysosomal enzymes, hexosaminidase is a glycoprotein and exists in multiple forms. Because of the known deficiency of some of these forms in several inherited lysosomal storage disorders, particularly Tay-Sach's disease and Sandhoffs' disease, hexosaminidase has been extensively studied and therefore well characterised, especially compared to other lysosomal enzymes. It was felt that hexosaminidase would be an interesting enzyme to study in relation to the problems outlined above, with particular emphasis on hexosaminidase biosynthesis. A review of the multiple forms of hexosaminidase and other lysosomal enzymes has been written by Dean (1975) and Barrett and Heath (1977).

Throughout this report, the term hexosaminidase is used to denote N-acetyl-β-D-hexosaminidase. Most hexosaminidases are so-named because they catalyse the hydrolysis of both terminal N-acetylgalactosaminyl and terminal N-acetylglucosaminyl moieties. However, where this dual specificity has not been examined or where a particular multiple form shows a specificity for only one of the two hexosaminyl residues, the terms β-galactosaminidase or β-glucosaminidase will be used as appropriate.

1. N-acetyl-β-D-hexosaminidase

1.1 Occurrence

The demonstration of hexosaminidase-like activity was first reported by Helfereich and Iloff (1933) who observed that an almond seed extract was capable of hydrolysing the synthetic substrate, phenyl-N-acetyl-β-D-glucosaminide to yield free N-acetyl-β-D-glucosamine. This activity was not related to either β-glucosidase or α-mannosidase since the three activities did not co-purify and had different sensitivities to heat treatment. Shortly after this and again using synthetic substrates, Watnabe (1936) reported finding β-glucosaminidase activity in extracts of bovine liver, muscle, pancreas, adrenal cortex and cerebrum.

Subsequent studies have demonstrated hexosaminidase activity in most mammalian tissues. For example, the enzyme was found in other bovine tissues such as uterus (Coleman *et al*., 1967), epididymis (East *et al*., 1941), aorta (Buddecke and Werries, 1965) and spleen (Verpoorte, 1972) and in sheep tissues such as thyroid (Chabaud *et al*., 1971) and brain (Bishayee and Bachawat, 1974). Relatively high amounts of hexosaminidase were found in ram spermatozoa (Allison and Hartree, 1970). Conchie *et al*. (1956) examined several rat tissues and found the highest hexosaminidase specific activity in the epididymis. The human placenta was also found to have a relatively high hexosaminidase specific activity (Walker *et al*., 1960). The enzyme has also been detected in the alimentary tract secretions of several mammalian species (Conchie and Macdonald, 1959) and in other body fluids such as serum (Walker *et al*., 1960; Woollen *et al*., 1965), cerebro-spinal fluid (Wiesmann *et al*., 1971), semen (Allison and Hartree, 1970) and tears (Ikonne and Ellis, 1973; Goldberg *et al*., 1977).

A variety of other organisms have been shown to contain hexosaminidase activity and include lobster (Neuberger and Pitt-Rivers, 1939; Bhorgava and Gottschalk, 1966), cellular slime mould (Coomis, 1969; Every and Ashworth, 1973), *Aspergillus niger* (Bahl and Agarwal, 1969), *Aspergillus oryzae* (Mega *et al*., 1970) and *Staphylococcus aureus* (Wadstrom and Hisatsune, 1970).

In virtually all of the instances described above, artificial substrates rather than natural substrates were used to assay hexosaminidase activity. Of these substrates the most commonly used are

4-nitrophenyl N-acetyl-β-D-hexosaminides (Pugh *et al*., 1957) and 4-methylumbelliferyl N-acetyl-β-D-hexosaminides (Leaback and Walker, 1961). When hydrolysed, the former releases free 4-nitrophenol which is measured spectrophotometrically while the latter releases 4-methylumbelliferone which is measured fluorimetrically. The fluorimetric assay is about twenty-times more sensitive than the spectrophotometric method and has been used to determine hexosaminidase activity in single neurones (Hirsch, 1972).

1.2 Properties

Based on tissue distribution and heat inactivation studies, Roseman and Dorfman (1951) showed that β-glucosaminidase and α-glucosaminidase activities were not related. Also, by studying the hydrolysis of various carbohydrates by sheep liver and epididymal extracts, Linker *et al*. (1955) concluded that β-glucosaminidase only hydrolysed terminal glucosaminidic bonds of odd-numbered oligosaccharides. Heyworth *et al*. (1957) investigated β-glucosaminidase and β-galactosaminidase in extracts of ram testes. They showed that both activities had a pH optimum of 4.4 and that the ratio of glucosaminidase to galactosaminidase activity remained constant at 4:1 even after several purification steps and progressive heat inactivation. Furthermore, N-acetylglucosamine and N-acetylgalactosamine competitively inhibited both activities. On the basis of these findings, the authors suggested that one enzyme, i.e. hexosaminidase, was responsible for the hydrolysis of both glucosaminide- and galactosaminide-containing substrates. This conclusion was also reached by Findlay *et al*. (1958), who showed that β-glucosaminidase activity from pig epididymis was competitively inhibited by both N-acetyl-glucosaminolactone and N-acetylgalactosaminolactone, and by Walker *et al*. (1961), who were unable to separate rat kidney β-glucosaminidase and β-galactosaminidase activities by a variety of fractionation procedures.

Although it had been observed frequently that mammalian hexosaminidases had an acid pH optimum, the significance of this fact was not fully realised until Sellinger *et al*. (1960) studied the intracellular distribution and properties of the rat liver enzyme. They found that hexosaminidase activity was largely associated with a specific particulate fraction and could be released from the particles by treatment with digitonin or by freeze-thawing. In this respect hexosaminidase behaved in a similar manner to other enzymes which had acid pH optima and which

had previously been shown by de Duve et al. (1955) to be located in subcellular organelles termed lysosomes. These results were confirmed by Conchie and Hay (1963) who studied the distribution of several acid hydrolases, including hexosaminidase, in subcellular fractions from rat and mouse tissues. All the enzymes showed the structure-linked latency described above. However, with respect to hexosaminidase, some of the activity was tightly bound to the particulate material, even after osmotic or mechanical disruption and treatment with Triton X-100. Weissmann et al (1967) and Robinson et al. (1972) found that much of the hexosaminidase activity from rat liver and calf brain, respectively, was firmly bound to disrupted lysosomal membranes. This feature is not unique to hexosaminidase since other lysosomal enzymes have been shown to be bound to the lysosomal membrane eg., β-glucosidase (Beck and Tappel, 1968) and β-glucuronidase (Henning et al. 1973). The interaction between enzyme and membrane appears to be largely electrostatic since the bound activities can be solubilized by changing the pH or increasing the ionic strength.

Although the bulk of the hexosaminidase activity in the above cases was located in the lysosomal fraction and bound to the lysosomal membrane, small, but significant, amounts of activity were often found in other subcellular fractions. Conchie and Hay (1963) commented on the heterogeneity of the subcellular distribution of several lysosomal enzymes in rat and mouse tissues. Hexosaminidase showed a bimodal distribution in that relatively large amounts of activity were found in the microsomal as well as the lysosomal fraction.

Frowein and Gatt (1966,1967a) turned their attention to the small amount of hexosaminidase activity in the 100,000g supernatant fraction from a calf brain homogenate. This soluble enzyme differed from the particulate form in several respects, and most importantly in relation to its glucosaminidase/galactosaminidase activity. In agreement with other workers, the particulate enzyme showed both β-glucosaminidase and β-galactosaminidase activities and all attempts to separate them were unsuccessful. On the other hand, the soluble fraction seemed to contain two separate enzymes, a β-glucosaminidase and a β-galactosaminidase. The results which led to this conclusion were that the glucosaminidase/galactosaminidase ratio changed during various purification steps and that free N-acetylgalactosamine did not interfere with galactosaminidase and glucosaminidase activities, respectively. It was also observed that

the supernatant enzymes were inactivated at, or below, pH 4.0 while the particulate enzyme showed optimum activity under these conditions. Robinson et al (1972) also found β-glucosaminidase and β-galactosaminidase activities in a 100,000g supernatant from calf-brain but were unable to separate them by a variety of purification procedures. However, there have been several reports of a specific β-glucosaminidase, with a pH optimum of 6.5, in the soluble fraction of brain and other human tissues (Hooghwinkel et al. 1972; Poenaru and Dreyfus, 1973). An isolated report of a specific β-galactosaminidase in human tissue has also been published (Overdijk et al. 1975).

1.2.1. Multiple forms of hexosaminidase in non-human tissues

The findings of Frowein and Gatt (1967a) which have just been described in section 1.2 indicated that more than one form of hexosaminidase could be separated from calf brain by subcellular fractionation. At about the same time, Caygill et al. (1966) reported that hexosaminidase from ram testes could be separated into two forms by chromatography on CM-cellulose at pH 4.5 and elution by stepwise increases in pH. Shortly afterwards, Leaback and Walker (1967) demonstrated two forms of pig epididymal hexosaminidase by chromatography on CM-cellulose and DEAE-cellulose. Apart from the apparent difference in charge, the two forms had similar molecular weights, heat stabilities and kinetic properties.

The first detailed report of multiple forms of hexosaminidase was made by Robinson and Stirling (1968) who showed that the enzyme from human spleen could be resolved into two forms by starch-gel electrophoresis and DEAE-cellulose chromatography. Although multiple forms of human hexosaminidase are the subject of the next section, it is relevant to discuss these workers findings at this point because they formed the basis of many subsequent investigations. The form which moved rapidly towards the anode on electrophoresis at pH 7 was termed hexosaminidase A and the slower moving, cathodic form as hexosaminidase B. Hexosaminidase A was retained by DEAE-cellulose at pH 6 while the B form was eluted with the void volume. Other differences were evident. For example, hexosaminidase B was more stable than hexosaminidase A when subjected to changes in pH and increases in temperature. Both forms were found in lysosomes but only the A form in the supernatant fraction. Hexosaminidase A and B had the same K_m values and pH optima when tested with synthetic substrates and both showed β-glucosaminidase and β-galactosaminidase activity.

After this report on the human spleen enzyme, various animal and plant tissues were shown to possess multiple forms of hexosaminidase, although only one form was found in Jack bean (Li and Li, 1970) and Aspergillus oryzae (Mega et al. 1970).

Two forms of hexosaminidase, having properties similar to the human spleen enzyme, were identified in bovine spleen (Verpoorte, 1972; Werries and Fressman, 1974). Other tissues having two forms include rat liver (Robinson and Stirling, 1969), rat kidney (Price and Dance, 1967), porcine kidney (Wetmore and Verpoorte, 1972) and hen oviduct (Tarentino and Maley, 1971). In addition, two forms of hexosaminidase were separated from Physarum polycephalum (Kilpatrick and Stirling, 1975) and bull seminal plasma (Khar and Anand, 1977). In all the above instances, separation of the multiple forms was achieved by either ion-exchange chromatography or electrophoresis and, where tested, both forms had β-glucosaminidase and β-galactosaminidase activities.

Although starch-gel electrophoretic analysis of calf brain homogenates revealed two forms of hexosaminidase, chromatography on DEAE-cellulose resolved two major forms and an intermediate, minor form (Robinson et al. 1972). More than two multiple forms of hexosaminidase have been reported for some other tissues. For example, using DEAE-cellulose chromatography, three and four forms of the enzyme were identified in ram testis and epididymis, respectively (Bullock and Winchester, 1973). All these forms had identical molecular weights and similar activity towards synthetic substrates. Weissmann and Hinrischen (1969) found at least four forms of hexosaminidase in pig liver using an isoelectric focusing technique, and, using polyacrylamide gel electrophoresis, Hayase et al. (1973) were able to demonstrate at least twelve forms of the enzyme in bull epididymal homogenates.

The nature of the structural relationship between the various multiple forms of hexosaminidase has proved difficult to study, largely due to the unavailability of highly purified enzyme preparations. Nevertheless, during their studies on the human spleen enzyme, Robinson and Stirling (1968) showed that treatment of partially purified hexosaminidase A with neuraminidase generated a form which had similar electrophoretic properties to hexosaminidase B. Treatment of hexosaminidase B with neuraminidase had no detectable effect. Similar results for neuraminidase treatment were reported for the acidic formsof hexosaminidase from rat kidney (Goldstone et al. 1971), rat neuronal cell-bodies

(Sellinger et al. 1973) and bull epididymus (Hayase et al. 1973). From these observations it was generally concluded that the acidic form (hexosaminidase A) was derived from the basic form (hexosaminidase B) by the addition of sialic acid residues. Consistent with this conclusion, Verpoorte (1972) showed that the amino acid compositions of the two forms purified from bovine spleen were very similar, but that the A form contained more sialic acid and neutral carbohydrate than the B form. Similar findings were also reported for the A and B forms purified from bull seminal plasma (Khar and Anand, 1977). Evidence for the glycoprotein nature of sheep brain hexosaminidase was provided by Bishayee and Bachawat (1974). They showed that several lysosomal enzymes, including hexosaminidase, were bound by the phyto-lectin, concanavalin A. This protein specifically binds components which contain either α-mannose or α-glucose residues (Goldstein et al. 1965).

An important finding was made by Verpoorte (1972). He observed reductions in the molecular weights of purified bovine spleen hexosaminidase A and B after treatment with guanidine hydrochloride and dithiothreitol, suggesting a subunit composition for both forms. A subunit structure is also indicated for the multiple forms from porcine kidney (Wetmore and Verpoorte, 1972) and the limpet (Bannister and Phizackerly, 1973). In the latter case, the two multiple forms had molecular weights of 217,000 and 136,000 respectively. Since the subunits (α and β) had molecular weights of 82,000 and 54,000 the authors proposed that the 217,000 molecular weight enzyme had the structure $\alpha_2\beta$ while the 136,000 molecular weight form was $\alpha\beta$. The A and B forms from equine kidney both had a molecular weight of 125,000 while a third form, termed A', had a molecular weight of 250,000. It was suggested that the A' form was an aggregate of the A and B forms (Seyama and Yamakawa, 1974).

1.2.2 Multiple forms of hexosaminidase in human tissues

After the observation by Robinson and Stirling (1969) that human spleen hexosaminidase could be separated into two forms, termed A and B, Okada and O'Brien (1969) reported that the activity of the A form was greatly diminished or even undetectable in tissues from patients with classical Tay-Sach's disease. This autosomal recessive disorder is characterised by the storage of certain glycosphingolipids and predominantly

neuronal GM_2-ganglioside. It results in progressive mental and physical deterioration and finally death, generally between the ages of 2-3 years. Two variants of this GM_2-gangliosidosis with similar genetics and clinical symptoms have also been described. In one of these, Sandhoff's disease or the O variant, both hexosaminidase A and B are deficient and in the other, the AB variant, both activities are normal or even elevated (Sandhoff, 1969; Young et al. 1970). These observations have generated an intense interest in human hexosaminidase, particularly with respect to elucidating the structure and function of the A and B forms as well as the other forms which have been discovered.

Generally, hexosaminidase A and B constitute the major components, most tissues containing only small amounts of the other forms. However, in certain instances, some of these normally minor forms predominate. The tissue distribution, identification and properties of the multiple forms of human hexosaminidase will be described first, followed by a discussion of their possible structural relationships and physiological significance.

1.2.2.1 Hexosaminidase A and B

Hexosaminidase A and B have been found in many human tissues, including brain (Sandhoff, 1969; Young et al. 1970; Robinson et al. 1972), kidney (Dance et al. 1969; Marinkovic and Marinkovic, 1977) leucocytes (Friedland et al., 1970), skin fibroblasts (Kanfer and Spielvogel, 1973) lung fibroblasts (Dillard and Tappel, 1974), placenta (Huddleston et al. 1971; Stirling, (1972) and liver (Carroll and Robinson, 1973). In addition both forms were found in serum (Okada and O'Brien, 1969; Price and Dance, 1972; Ikonne and Ellis, 1973). There are conflicting reports concerning the presence of A and B in urine. Grebner and Tucker (1973) found both forms in normal urine while Price et al. (1970) and Banerjee and Basu (1975) found only hexosaminidase A.

Various methods have been used for the separation of the A and B forms including isoelectric focusing (Sandhoff, 1969; Hayase and Kritchevsky, 1973), polyacrylamidegel electrophoresis (Friedland et al. 1970), cellulose acetate electrophoresis (Hooghwinkel et al. 1972) and DEAE-cellulose chromatography (Young et al. 1970). These methods take advantage of the differences in isoelectric point or charge of the respective forms. In some cases, A and B have been distinguished on

the basis of their differential heat stability (Dance *et al.* 1970; Hirsch, 1972; Dillard and Tappel, 1974).

The deficiency of hexosaminidase A in Tay-Sach's disease and of both A and B in Sandhoff's disease is generalised and not restricted to any particular tissue. This indicates that hexosaminidases from different tissues share similar polypeptide back bones. Nonetheless, tissue-specific differences in certain properties of hexosaminidase have been reported, presumably due to variations in post-translational modification. For example, placental A and B forms migrate faster on agar gel electrophoresis at pH 7 than the corresponding forms from brain and liver (Srivastava *et al.* 1974). Ikonne and Ellis (1973) used DEAE-cellulose chromatography to compare hexosaminidase A from various tissues with that from serum. The serum A form was resolved into two subcomponents, the minor one of which eluted with the single A form found in tissue extracts. Treatment of serum with neuraminidase altered the elution characteristics of the major hexosaminidase A component, suggesting that differences in sialic acid content might be responsible for the observed microheterogeneity. Similar observations were reported by Swallow *et al.* (1974) using starch-gel electrophoresis. Subsequent investigations have shown that the corresponding tissue and serum forms of some other lysosomal enzymes can be distinguished by differences in their elution properties when chromatographed on DEAE-cellulose (Ellis *et al.* 1975b; Willcox and Renwick, 1977).

Differences in the antigenic properties of hexosaminidase A and B from different tissues have also been reported. Srivastava *et al.* (1974b) observed that antisera raised against the placental A and B forms reacted much more readily with the corresponding placenta and liver enzymes than with the enzyme from fibroblasts. Carroll and Robinson (1973) have reported that antibodies to liver hexosaminidase A and B cross-reacted equally well with hexosaminidase prepared from liver, spleen and brain. In contrast, Jones *et al.* (1975) reported that antisera raised against the A and B forms from placenta did not cross-react with hexosaminidase from liver, spleen, kidney, intestine or normal serum in immunodiffusion systems. Double-immunodiffusion experiments carried out by Verpoorte and Coombs (1977) using an antiserum to highly purified human plasma hexosaminidase A gave a reaction of partial identity between plasma and placental hexosaminidase A. Therefore, although similar, the two forms were not identical.

The basis for these tissue-specific differences is not known with certainty . With respect to hexosaminidase A from plasma and placenta, amino acid analyses and circular dichroism studies confirmed that the two forms were very similar and indicated that conformational differences were unlikely to be responsible for the observed immunological heterogeneity. It has already been suggested that the difference in the chromatographic behaviour of serum and tissue hexosaminidase A may be due to variations in carbohydrate content and a similar explanation may also account for the tissue-specific immunological differences described above.

That hexosaminidase A and B might be glycoproteins was suggested by Robinson and Stirling (1968). Banerjee and Basu (1975) showed that hexosaminidase A from urine was bound by immobilised concanavalin A and could be specifically eluted with α-methyl mannoside, indicating the presence of α-mannose and/or α-glucose residues on the enzyme. Similar results were obtained for human brain hexosaminidase A and hexosaminidase A and B from the placenta (Aruna and Basu, 1975; Beutler et al., 1975; Geiger et al., 1975). Convincing evidence for the glycoprotein nature of placental hexosaminidase A and B has been presented by Lee and Yoshida (1976). Using highly purified enzyme preparations they found neutral carbohydrate and hexosamine residues in both forms, but could detect no sialic acid. Geiger and Arnon (1976), also using highly purified placental enzymes, did find sialic acid residues but only on hexosaminidase A.

1.2.2.2 Hexosaminidase I_1 and I_2

At least two minor forms of hexosaminidase were eluted after hexosaminidase B and before hexosaminidase A on DEAE-cellulose chromatography of human brain extracts (Young et al., 1970). Using the same technique, Price and Dance (1972) showed that plasma and serum also contained two hexosaminidase components which eluted between the major A and B forms. These intermediate forms were termed I_1 and I_2 (Price and Dance, 1972) and were found subsequently in other human tissues in varying amounts. For example, low levels of I_1 and I_2 relative to A and B were detected in liver (Carroll and Robinson, 1973; Ikonne and Ellis, 1973), brain (Ellis et al., 1975a) and skin fibroblasts (Ellis et al., 1975b). Stirling (1972) found no detectable intermediate forms in extracts of placental tissue although data presented by Srivastava

et al., (1974b) indicated the presence of small amounts of I_1 and I_2. The discrepancy here could be due to the two different separation techniques used; i.e. starch gel electrophoresis versus DEAE-cellulose chromatography, respectively. Hexosaminidase I_1 and I_2 were not found in urine samples (Price and Dance, 1970; Grebner and Tucker, 1973).

In some situations the activities of the intermediate forms, and in particular hexosaminidase I_2, are elevated such that A and B are no longer the major forms present. Stirling (1972) found relatively high levels of an intermediate form which he termed hexosaminidase P in pregnancy serum. The P form accounted for about 60% of the total hexosaminidase activity of maternal serum from the fourth week of pregnancy until term. Based on DEAE-cellulose chromatographic analysis, the P form was indistinguishable from the hexosaminidase I_2 of normal serum making it highly probable that the form found in pregnancy serum simply represents elevated I_2 activity (Price and Dance, 1972). Using polyacrylamide gel isoelectric focusing, Hayase and Kritchevsky (1973) confirmed the results of Stirling (1972) in that pregnancy serum contained elevated levels of an intermediate form of hexosaminidase. Their technique resolved the A, B and I forms into a number of sub-components, the significance of which is not clear. Potier et al. (1977) reported that, towards the end of normal pregnancy, amniotic fluid contained high levels of I_2 compared to the A and B forms.

The I_1 and I_2 forms from serum possessed both β-glucosaminidase and β-galactosaminidase activities and had pH optima of 4.5. Furthermore, like hexosaminidase B, but unlike hexosaminidase A, they were stable on heating at 50°C (Price and Dance, 1972; Hayase and Kritchevsky, 1973). Hexosaminidases I_2 and A co-eluted when subjected to chromatography on Sephadex G-200, indicating that they had similar molecular weights (Stirling, 1973).

1.2.2.3 Other forms

Hooghwinkel et al. (1972) reported that human brain contained a specific β-glucosaminidase separable from the A and B forms by cellulose acetate electrophoresis. Initially, this activity was only observed in brain tissue but subsequent investigations showed it to be present in liver, lung and placental tissue (Poenaru and Dreyfus, 1973; Penton et al. 1975). Relatively large amounts of a specific β-glucosaminidase were also demonstrated in foetal tissue but none could be detected in adult urine or serum (Poenaru and Dreyfus, 1973). Although not strictly accurate, this form is generally referred to as hexosaminidase C.

The inability of some workers to detect hexosaminidase C can be accounted for in several ways. Firstly, hexosaminidase C is more anodal than hexosaminidase A on electrophoresis at pH 7 and therefore could be lost after relatively long electrophoretic separations. Secondly, unlike hexosaminidase A and B, the C form is optimally active at pH 6.5 and shows minimal activity at pH 4.5, the usual pH for assaying hexosaminidase. Hexosaminidase C is more labile than either hexosaminidase A or B, thus making it unlikely to be detectable in autopsy specimens or in samples stored for a long time. Finally, Srivastava et al. (1975) were unable to find hexosaminidace C in samples of liver and kidney which had been chromatographed on DEAE-cellulose. This can be explained by the finding that hexosaminidase C is either inactivated or strongly bound by the anionic cellulose exchanger (Braidman et al., 1974).

The identification of hexosaminidase C is complicated by the presence of hexosaminidase S, which has similar electrophoretic mobility (Beutler et al., 1975; Ikonne et al., 1975). However, it is possible to distinguish between hexosaminidase C and S in other ways. For example, hexosaminidase C has a pH optimum of 6.5 and a molecular weight of about 200,000 compared to hexosaminidase S which has an acid pH optimum and a molecular weight of between 100,000 - 140,000 (Penton et al., 1975; Braidman et al., 1974; Beutler et al., 1975). Additionally, hexosaminidase S shows both β-glucosaminidase and β-galactosaminidase activities and can be distinguished immunologically from the C form (Reuser and Galjaard, 1976).

Overdijk et al. (1975) have reported the presence of a specific β-galactosaminidase, termed hexosaminidase D, in brain extracts. This form had a similar molecular weight to the A and B forms and co-eluted with hexosaminidase A on DEAE-cellulose chromatography. It could be separated from the A, B and C forms by cellulose acetate electrophoresis at pH 8.

Grebner and Tucker (1973) described a minor peak of hexosaminidase activity which eluted after hexosaminidase A on DEAE-cellulose chromatographic analysis of adult urine samples. Since this form was found predominantly in male urine, it was termed hexosaminidase M. Similar to the A, B, I and S forms, hexosaminidase M had both β-glucosaminidase and β-galactosaminidase activities and a pH optimum of about 4.3. Several other workers have analysed urine samples by DEAE-cellulose chromatography

and failed to find a hexosaminidase M component (Price et al., 1970; Banerjee and Basu, 1975).

Stirling (1974) found an enzyme in liver extracts which hydrolysed a natural, N-acetyl-β-D-glucosamine-containing dissacharide called N,N' diacetylchitobiose, but which had no activity towards synthetic substrates. This chitobiase was not retained by DEAE-cellulose at pH 7, similar to hexosaminidase B. However, chromatography on Sephadex G-100 separated hexosaminidase (determined using synthetic substrates) and chitobiase activities. The former had a molecular weight of about 100,000 while the latter had a molecular weight of between 25,000 and 50,000.

1.2.2.4 Subcellular distribution

The subcellular distribution of the above multiple forms has been largely neglected and, even where studied, tissues consisting of more than one cell type have usually been used. Robinson and Stirling (1968) established that the A and B forms from spleen were located in lysosomes. Hexosaminidase from brain and placenta was also found predominantly in the lysosomal fraction, although no attempt was made to separate the multiple forms (Tallman et al., 1972; Corash and Gross, 1974).

The acid hydrolases of polymorphonuclear leucocytes are associated with at least three classes of cytoplasmic granules which are separable by density gradient centrifugation (Bagglioni et al., 1969; Bretz and Bagglioni, 1974). Ellis and Patrick (1975) used DEAE-cellulose chromatography to analyse the multiple forms of hexosaminidase in the particulate fractions obtained by density gradient centrifugation of post-nuclear supernatants from human polymorphonuclear leucocytes. Hexosaminidase A, B, I_1 and I_2 were found in virtually all fractions but the relative proportions of these components varied from fraction to fraction. In addition, at least three sub-forms of hexosaminidase A were resolved, each preferentially associated with particulate fractions of different density. Thus, the lysosome-like granules in human polymorphonuclear leucocytes can be distinguished on the basis of their characteristic densities and on the basis of their content of hexosaminidase multiple forms.

The acid pH optimum reported for both hexosaminidase S (Beutler et al., 1975) and chitobiase (Stirling, 1974) suggests a lysosomal

localisation but no results have been published to substantiate this possibility. Hexosaminidase C was not detected in either the lysosomal or microsomal fractions but was found in the soluble fraction (Penton *et al.*, 1975).

1.3 Structural Relationships of human hexosaminidases

A close structural relationship between hexosaminidase A and B was initially suggested by the report of Robinson and Stirling(1968) that hexosaminidase A could be converted to hexosaminidase B by neuraminidase treatment or by storage at room temperature. The observation that both hexosaminidases A and B were deficient in the O varient of GM_2-gangliosidosis (Sandhoffs' disease) confirmed that the two forms were interrelated. Although these observations made it unlikely that hexosaminidases A and B were totally unrelated proteins, the deficiency of hexosaminidase A, but not hexosaminidase B, in the B varient of GM_2-gangliosidosis (classical Tay-Sachs' disease) indicated at least some point of difference.

Several models have been put forward to explain the structural relationship between the A and B forms. The first of these was proposed by Robinson and Stirling (1968) and, subsequently, by others (Goldstone *et al.*, 1971; Murphy and Craig, 1972 and Snyder *et al.*, 1972). Based on the neuraminidase-dependent conversion of A to B, these workers suggested that hexosaminidase A might be derived from hexosaminidase B by the attachment of sialic acid residues. Early studies did not refute this model but rather confirmed that hexosaminidase A and B were very similar. For example, the two forms had identical molecular weights and identical pH optima and kinetic constants for the hydrolysis of synthetic substrates (Robinson and Stirling, 1968; Sandhoff and Wässle, 1975; Wenger *et al.*, 1972). Furthermore, antibodies to hexosaminidases A and B cross-reacted with both forms (Srivastava and Beuther, 1972; Carroll and Robinson, 1973).

Although these findings were consistent with hexosaminidases A and B being the same protein differing only in carbohydrate content, other observations were not readily explained by this model. Firstly, some workers were unable to show conversion of A to B by neuraminidase treatment (Hayase *et al.*, 1973; O'Brien, 1973; Ikonne and Ellis, 1973) and, secondly, the conversion was demonstrated by storage at room temperature, controlled heating and even by using heat-denatured neuraminidase (Robinson and Stirling, 1968; Sandhoff, 1973; Carroll and Robinson,

1973; Tallman et al., 1974). It was also necessary to explain the results of Srivastava et al. (1973) and Bartholomew and Ratazzi (1974) in that absorption of anti-(hexosaminidase A) with hexosaminidase B produced an antiserum which reacted with hexosaminidase A but not hexosaminidase B, while absorption of anti-(hexosaminidase B) with hexosaminidase A removed all anti-hexosaminidase activity.

Most of the above discrepancies were explained by the studies of Carmody and Ratazzi (1974) which showed that conversion of A to B only occurred with certain commercial neuraminidase preparations and never when purified neuraminidase was used. The converting factor was found to be the sulphydryl agent merthiolate, added as a preservative to certain commercial neuraminidase preparations. The absence of this compound from some batches of neuraminidase and from purified preparations explained the inability of some workers to show conversion. Although it did not exclude the possibility that hexosaminidase A and B were sialoglycoproteins, this finding clearly indicated that conversion of A to B did not depend on the removal of sialic acid residues.

Further insight into the structures of hexosaminidases A and B was gained by Srivastava et al. (1974a, 1974b) who obtained highly purified preparations from human placenta. Sedimentation equilibrium analyses gave a molecular weight of about 100,000 for both forms and treatment with guanidine hydrochloride or maleic anhydride, followed by SDS/polyacrylamide gel electrophoresis, revealed the presence of subunits. Hexosaminidase B dissociated into a band corresponding to a protein of molecular weight 18,000 whereas hexosaminidase A dissociated into three bands corresponding to a major species of molecular weight 18,000 and two minor bands of molecular weights 36,000 and 65,000, respectively. Since the two minor bands were integral multiples of 18,000 the authors interpreted these as aggregates of the smaller subunits or incompletely dissociated hexosaminidase A and concluded that hexosaminidases A and B were likely to be hexamers.

In view of the above data and based on the observations that hexosaminidase A possessed antigenic determinants not present on hexosaminidase B and that both A and B shared common antigenic determinants, Srivastava and Beutler (1974) proposed a "common and unique" subunit model in which hexosaminidase A was $(\alpha\beta)_3$ and hexosaminidase B was β_6. This model was supported by the report of Srivastava et al. (1974b) which suggested that A and B had different amino acid compositions.

It should be noted that a structure of $(\beta Y)_3$ for hexosaminidase B was not ruled out by any of the above findings. According to the "common and unique" subunit model, Tay-Sachs' disease could be explained by a mutation affecting the α subunit and Sandhoffs' disease by a mutation affecting the β subunit.

The existence of subunits for hexosaminidase A and B was confirmed by Tallman et al. (1974), who also studied highly purified preparations from human placenta. Treatment of either the A or B form with urea, dithiothreitol and mercaptoethanol followed by electrophoresis in SDS/polyacrylamide gels at pH 10.5 produced a single band corresponding to a molecular weight of 33,000. No proteins of lower molecular weight were detected. Sephadex G-200 chromatography gave a molecular weight of 127,000 for both A and B and the authors concluded that both forms were tetramers consisting of 33,000 molecular weight subunits. If treatment with urea and reducing agents was not included, no 33,000 molecular weight component could be detected upon SDS-polyacrylamide gel electrophoresis of either A or B. Instead, hexosaminidase A appeared predominantly as a 65,000 molecular weight species and hexosaminidase B as a complex mixture of proteins of molecular weights between 120,000 and 140,000. These observations indicated that disulphide bonds played an important part in the association of the subunits to form the native enzymes, but the precise nature of the association was unclear. No amino acid analyses or immunological studies were reported but, nonetheless, these workers interpreted their own data and that of others in terms of hexosaminidase A and B representing different conformational states of the same protein. This model was not precluded by any of the available evidence. For example, the ability to prepare a specific anti-(hexosaminidase A) antiserum could be explained by the existance of conformationally-dependent antigenic determinants. Also, the different amino acid compositions for the A and B forms reported by Srivastava et al. (1974b) were certainly not great enough to invalidate the "conformer" model. Finally, classical Tay-Sachs' disease was explained as a mutation affecting an amino acid important for promoting the hexosaminidase A conformational state and Sandhoffs' disease as a mutation affecting the enzyme active site.

The discrepancy in that Srivastava et al. (1974b) obtained a subunit molecular weight of 18,000 whereas Tallman et al. (1974) obtained a value of 33,000 is not readily explained. The problem of establishing

the actual number of subunits comprising hexosaminidase A and B is discussed further towards the end of this section.

Compelling evidence in favour of the "common and unique" subunit model was provided by Beutler and Kuhl (1975) who were able to convert highly purified placental hexosaminidase A into two enzymatically active components by freeze-thawing in the presence of 3M sodium chloride. One of these components was electrophoretically indistinguishable from authentic hexosaminidase B and reacted with both anti-hexosaminidase A and anti-hexosaminidase B. The other component was electrophoretically indistinguishable from authentic hexosaminidase S. As with authentic hexosaminidase S, the conversion product reacted with anti-hexosaminidase A but not with anti-hexosaminidase B. It was concluded that hexosaminidase A, ($\alpha\beta$)n, had dissociated into its component subunits and that these had recombined to produce two different homopolymers, i.e. hexosaminidase B, ($\beta\beta$)n, and hexosaminidase S, ($\alpha\alpha$)n. Further proof that hexosaminidase B and S were different homopolymers was provided by the observation that freeze-thawing either B or S failed to generate any other forms while freeze-thawing a mixture of B and S gave rise to active hexosaminidase A. It is difficult to reconcile these results with the conformer model.. Freeze-thawing in the presence of sodium chloride is known to cause the dissociation of non-covalently bound subunits and has, for example, been used to show interconversion of the multiple forms of lactate dehydrogenase (Markert, 1963; Vessel, 1965). These forms arise through different tetrameric combinations of two distinct subunits.

Interconversion of hexosaminidase multiple forms was also demonstrated by treatment of hexosaminidase A with p-hydroxymercuribenzoate. Beutler _et al_. (1975, 1976) and Srivastava _et al_. (1976) treated placental and kidney hexosaminidase A, respectively, with p-hydroxymercuribenzoate to yield a product which was electrophoretically indistinguishable from authentic hexosaminidase B and another product which was inactive and more anodal than hexosaminidase A on electrophoresis at pH 7. It was supposed that p-hydroxymercuribenzoate dissociated hexosaminidase A into its α and β components which subsequently recombined to give active hexosaminidase B, ($\beta\beta$)n and inactive, alkylated α-chain polymers. Treatment of the separated conversion products with dithiothreitol to remove p-hydroxymercuribenzoate, failed to produce hexosaminidase A. However, as in the freeze-thawing experiment

described above, hexosaminidase A was regenerated if both conversion products were present together. These observations added more weight to the "common and unique" subunit model. Furthermore, the effect of p-hydroxymercuribenzoate suggested a possible role for sulphydryl groups in the non-covalent association of the α and β subunits in hexosaminidase A.

Conclusive evidence that both hexosaminidase A and B share a common subunit and that hexosaminidase A has, in addition, a unique subunit was provided by Geiger and Arnon (1976) who characterised the A and B forms from human placenta. Treatment of either A or B with guanidine hydrochloride gave rise to a 50,000 molecular weight species. In agreement with Tallman et al. (1974), further dissociation was only achieved by reduction and alkylation, whereupon a 25,000 molecular weight species was formed. Analysis of these polypeptide chains by DEAE-cellulose chromatography in the presence of urea revealed that hexosaminidase B was composed of a single basic protein (β) while hexosaminidase A was composed of equal amounts of acidic (α) and basic (β) components. The β chains derived from hexosaminidases A and B had identical amino acid compositions but, and more importantly, the amino acid compositions of the separated α and β chains were significantly different. This clearly invalidated the proposition that hexosaminidase A and B represented different conformational states of the same protein. Other differences were also discovered. Hexosaminidase A contained six disulphide linkages and at least one free sulphydryl group whereas hexosaminidase B contained ten disulphide linkages and no detectable free sulphydryl groups. Since the native A and B forms both had a molecular weight of about 104,000 and their component subunits had a molecular weight of 25,000, Geiger and Arnon (1976) put forward tetrameric structures of $\alpha_2\beta_2$ for hexosaminidase A and β_4 for hexosaminidase B. The exact molecular structures proposed by these workers on the basis of the information presented above, are shown in Figure 1.1. Tetrameric structures for hexosaminidase A and B were also suggested by Beutler et al. (1976), based on their studies with highly purified placental enzymes. Different amino acid compositions for hexosaminidase A and B, again from human placenta, were reported by Lee and Yoshida (1976).

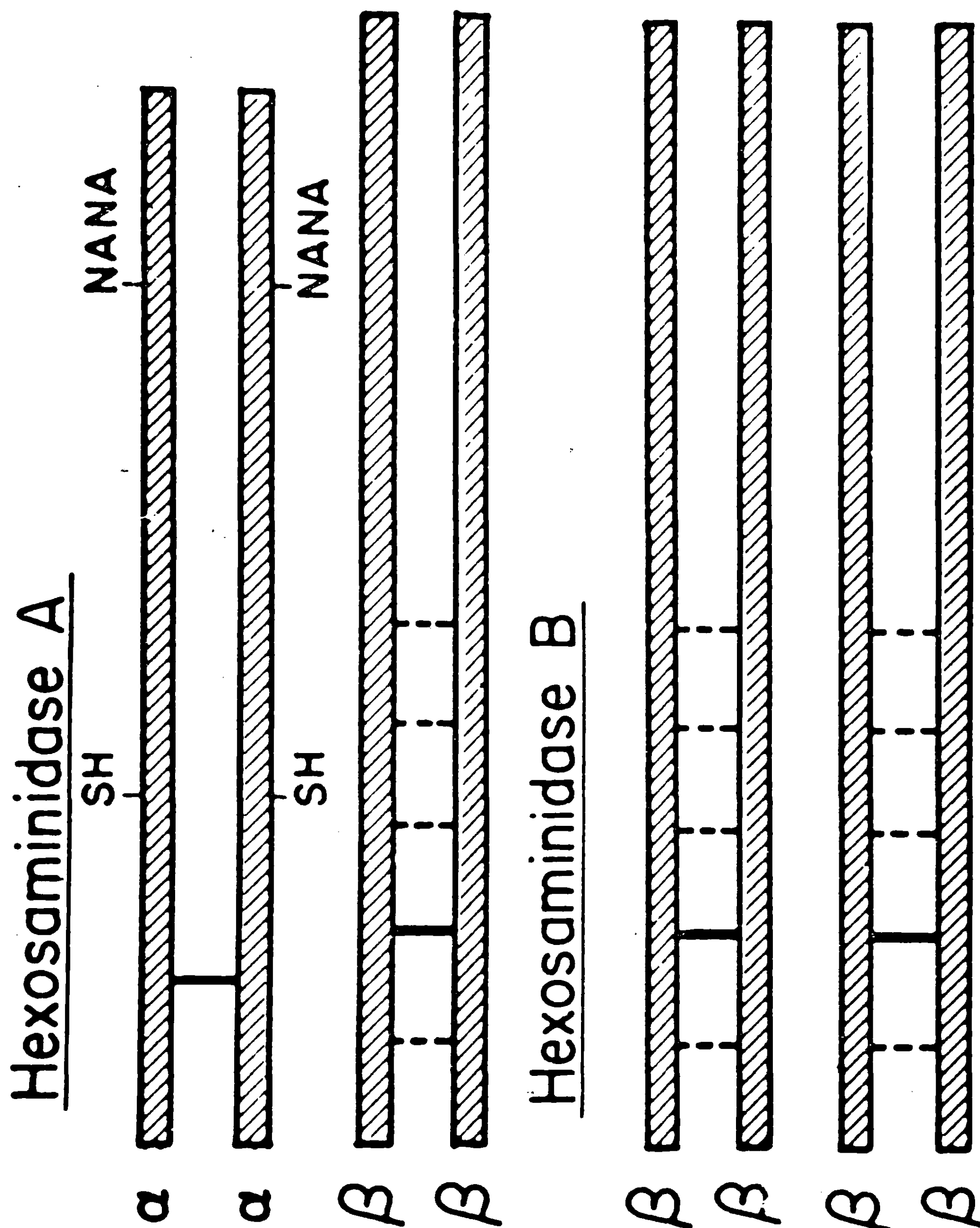
In contrast to these findings for the placental enzymes, studies using purified kidney hexosaminidases indicated that the A and B forms were hexamers with structures $\alpha_3\beta_3$ and β_6, respectively (Srivastava et al.,1976)

The discrepancy was due to the fact that Srivastava et al. (1976) obtained a molecular weight of 18,000 for the dissociated kidney enzyme α and β chains compared to the value of 27,000 obtained by Geiger and Arnon (1976) and Beutler et al. (1976) for the placental enzyme subunits. A subunit molecular weight of 18,000 was also obtained for kidney hexosaminidase by Marinkovic and Marinkovic (1977). The molecular weights of the kidney enzyme subunits were derived, in both cases, by urea/SDS/gel electrophoresis. On the other hand, the data regarding the placental enzyme subunits were obtained by both SDS/gel electrophoresis and sedimentation equilibrium centrifugation and are, therefore, likely to be more reliable. Segrest and Jackson (1972) have commented on the anomalous behaviour of glycoproteins during SDS/gel electrophoresis and it is not inconceivable that organ-specific variations in carbohydrate content could account for the different results obtained for the kidney and placental subunits. Slight electrophoretic and immunological differences between hexosaminidases from placenta and other tissues, possibly due to carbohydrate heterogeneity, have already been described (see section 1.2.2.1). It is clear that further studies are required to resolve the precise number of subunits which comprise hexosaminidase A and B.

There were also some other discrepancies in the above structural studies which require clarification. Geiger and Arnon (1976) found that placental hexosaminidase A contained sialic acid residues whereas hexosaminidase B contained none. Lee and Yoshida (1976), on the other hand, could find no sialic acid in either the A or B forms from placenta. Furthermore, the specific activities reported by these two groups for the A and B forms did not agree, in spite of the fact that all the preparations were judged to be homogeneous by several criteria. The presence of a minor contaminant or the possibility of partial degradation or inactivation of the enzymes during isolation cannot be ruled out as explanations. Since different purification procedures were used by the two groups it is conceivable that a particular step could have temporarily exposed the enzymes to conditions which favoured the removal of, for example, carbohydrate residues.

An alternative approach, using the technique of somatic cell hybridisation, has been used to investigate the structural relationship between hexosaminidase A and B. These experiments took advantage of the fact that when human and rodent cells were fused together, some human

Fig.1.1 PROPOSED STRUCTURES FOR HEXOSAMINIDASES A AND B (FROM GEIGER AND ARNON,1976)



Solid lines represent interchain S-S bridges and broken lines represent either intra- or interchain S-S bridges.

NANA represents sialic acid.

chromosomes, but no rodent chromosomes, were lost. In early experiments, when the resulting cell hybrids were screened for the presence of hexosaminidases, cells were found which contained hexosaminidase A only and hexosaminidase B only (van Someren and Heuegoven, 1973; Gilbert _et al._, 1975). These results showed that hexosaminidase A and B were expressed independently of each other, a concept incompatible with the "common and unique" subunit theory. Results of other experiments contradicted the above data in that cell hybrids containing exclusively hexosaminidase A were never found (Lalley _et al._ 1974; Lalley _et al._, 1976; Hoeksma _et al._, 1977a,b). These same workers showed, by detailed electrophoretic and immunological analyses, that the apparent independent expression of hexosaminidase A was due to the formation of a "hybrid" hexosaminidase consisting of both rodent and human hexosaminidase subunits. The correct observation was, therefore, that while hexosaminidase B was expressed independently of hexosaminidase A, authentic human hexosaminidase A was never found in the absence of hexosaminidase B. By comparing the expression of hexosaminidase A and B with the expression of chromosomal marker enzymes it was possible to assign the gene for hexosaminidase B to chromosome number 5 and the genes for hexosaminidase A to chromosomes 5 and 15. Although these results were most readily explained by the "common and unique" subunit model, the remote possibility that a gene on chromosome 15 was responsible for the production of a component necessary for converting one conformational state to another was not precluded. However, considered in the light of the structural studies described above, which strongly indicate that the former model is correct, it can be concluded that the gene coding for the β-subunit is located on chromosome 5 while that coding for the α-subunit is on chromosome 15.

It is proposed now to review the evidence relating to the other forms of hexosaminidase found in human tissues. Earlier, it was described that the absorption of anti-(hexosaminidase A) antiserum with hexosaminidase B removed all the anti-hexosaminidase B activity leaving an antiserum reactive only against hexosaminidase A. Based on subunit structures of $(\alpha\beta)_n$ and $(\beta\beta)_n$ for hexosaminidase A and B, respectively, it is assumed that absorbed anti-(hexosaminidase A) antiserum contains antibodies to the α-subunit only and that anti-(hexosaminidase B) antiserum contains antibodies to the β-subunit only. These terms should be borne in mind during the following discussion.

It has already been stated that hexosaminidase S is likely to be

an α-chain homopolymer. This was based on the fact that hexosaminidase S reacted with absorbed anti-(hexosaminidase A) and anti-(hexosaminidase A) antisera but not with anti-(hexosaminidase B) antiserum (Ikonne *et al.*, 1975; Beutler *et al.*, 1975) and also by the interconversion experiments of Beutler and Kuhl (1975) and Beutler *et al.* (1976). Further evidence in favour of this subunit structure was provided by the finding that the S form was present in Sandhoff's disease (a β-chain mutation) but was not detectable in Tay-Sachs' disease (an α-chain mutation). Srivastava *et al.* (1976) suggested that hexosaminidase S might have an altered ratio of α-and β-subunits, eg. $\alpha_5\beta_1$. This seems unlikely in view of the above data, but elucidation of the precise subunit composition of hexosaminidase S will require either amino acid analysis or more stringent immunological investigations. The relatively low amounts of the S form present in tissues renders the former approach less feasible but the immunological approach remains a practical possibility.

Similar to hexosaminidase S, the low tissue concentrations of the I_1, I_2, C and D forms have prevented their purification in sufficient amounts to allow detailed examination of their molecular structure. Nonetheless, some aspects of their structural interrelationships with the A, B and S forms have been derived from immunological studies and from studies on patients with different variants of GM_2-gangliosidosis.

Detailed immunological studies on the I_1 and I_2 forms have not been carried out, although Carroll and Robinson (1973) reported that a hexosaminidase I fraction (presumably a mixture of I_1 and I_2 forms) from human liver gave a reaction of complete immunological identity with antisera produced against hexosaminidase A and B. This, together with the observations that they both showed β-glucosaminidase and β-galactosaminidase activities and had acid pH optima, indicate that the I_1 and I_2 forms are structurally related to hexosaminidase A and B. Furthermore, since both I_1 and I_2 were undetectable in Sandhoff's disease (Young *et al.*, 1970) but were present in classical Tay-Sachs' disease (Young *et al.*, 1970; Price and Dance, 1972), it seems likely that these intermediate forms are more closely related to hexosaminidase B than hexosaminidase A. The I_1 and I_2 forms were also stable on heating at 50°C, a property shared with hexosaminidase B but not A. It is possible that I_1 and I_2 represent manifestations of differences in the attachment of carbohydrate to hexosaminidase B and are, therefore, β-chain homopolymers (Beutler and Kuhl, 1975). However, Srivastava *et al.* (1976) have proposed an altered ratio

of α-and β-subunits, eg. $\alpha_1\beta_5$ for I_1 and $\alpha_2\beta_4$ for I_2, as an alternative possibility. Immunological studies, particularly involving the absorbed anti-(hexosaminidase A) antiserum, might yield information to discriminate between these two proposals.

Hexosaminidase C differs from the other forms of hexosaminidase in several respects such as pH optimum, molecular weight and subcellular distribution. Further, it does not represent a true hexosaminidase since it only shows β-glucosaminidase activity. Antiserum raised against either the A or B forms did not cross-react with the C form (Penton et al. 1975; Reuser and Galjaard, 1976). Considered with the observations that it was present in both Tay-Sachs' and Sandhoff's disease, these results strongly suggest that hexosaminidase C is not structurally related to the A and B forms. A similar conclusion could also be reached with respect to chitobiase since it, too, was reported to be present in both Tay-Sachs' and Sandhoff's disease (Stirling, 1974b).

Relatively little information is available concerning hexosaminidase D and more fundamental studies are required before anything conclusive can be stated regarding its structural relatedness to the other hexosaminidases.

1.4 Functions of hexosaminidases

As stated earlier, hexosaminidase catalyses the hydrolysis of terminal, β-glycosidically linked N-acetylhexosaminyl residues. The naturally occurring compounds which are potential substrates for the enzyme therefore include many polysaccharides, glycoproteins and glycolipids. Hexosaminidase preparations from bovine spleen and aorta (Buddecke and Werries, 1964, 1965), human fibroblasts (Thompson et al. 1973; Cantz and Kresse, 1974) and canine liver (Hayasi, 1977) were shown to release free N-acetylhexosamines from chondroitin sulphate and hyaluronic acid. Bhavanandan et al. (1964) showed the release of N-acetylgalactosamine from several naturally occurring glycopeptides using purified bovine spleen hexosaminidase and Li and Li (1970) reported that hexosaminidase purified from Jack bean was active against glycoproteins such as ovomucoid and ovalbumin. Frowein and Gatt (1967) isolated three glycosphingolipids from calf brain, each of which had a terminal N-acetylgalactosaminyl residue, and investigated their rates of hydrolysis by the particulate, ie. lysosomal, and soluble forms of brain hexosaminidase. All three substrates were hydrolysed by the lysosomal hexosaminidase, but the

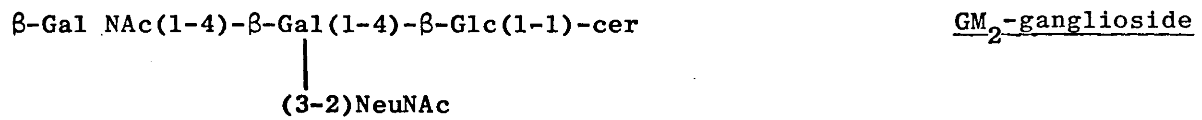
soluble enzymes were completely inactive over a wide pH range. However, although all the substrates were attacked by the lysosomal hexosaminidase, the actual rates of hydrolysis were different. The lowest rate of hydrolysis was recorded for ganglioside-GM_2 which differed from the other compounds tested in having a sialic acid moiety adjacent to the terminal N-acetylgalactosamyl residue (see Figure 1.2). Hexosaminidase preparations from Jack bean and bovine spleen were also reported to show greatly reduced activity towards natural, sialic acid-containing substrates compared to the respective asialo-derivatives (Li and Li, 1970; Bhavanandan et al. 1964). This effect is generally attributed to steric hindrance by the sialic acid moiety.

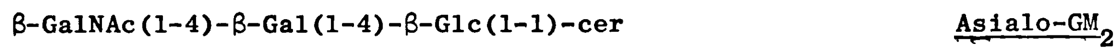
The existence of multiple forms of hexosaminidase clearly raises questions as to their substrate specificities. In this context, the involvement of human hexosaminidase A and B in the sequential degradation of glycolipids has been the subject of much investigation, particularly after the finding that hexosaminidase deficiencies were associated with the abnormal storage of GM_2-ganglioside and related compounds. Indeed, some clues as to the substrate specificities of hexosaminidase A and B were provided by the characteristic glycolipid storage patterns in patients suffering from different variants of GM_2-gangliosidosis. A summary of various GM_2-gangliosidoses is given in Table 1.1 and the storage products associated with them are shown schematically in Fig. 1.2.

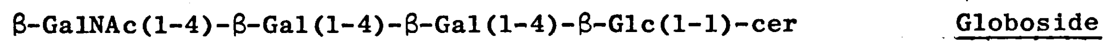
GM_2-ganglioside is stored in neuronal tissue, and to a lesser extent in the visceral organs, of patients with classical Tay-Sachs' disease (Eeg-Olofsson et al. 1966; Sandhoff et al. 1969). Small amounts of asialo-GM_2 are also found. Sandhoff's disease is similar, but visceral involvement, particularly in the kidneys, is more prominent. In addition to storage of GM_2 there is a more pronounced storage of asialo-GM_1 and globoside than in Tay-Sachs' disease (Sandhoff, 1968). Based on these findings and the respective hexosaminidase deficiencies it was suggested that hexosaminidase A was responsible for the hydrolysis of the terminal N-acetylgalactosaminyl residue from GM_2 while hexosaminidase B and possibly hexosaminidase A were both capable of hydrolysing asialo-GM_2 and globoside.

It should be noted that the alternative pathway of GM_2 degradation by the sequential action of neuraminidase and hexosaminidase B (reaction 2, Figure 1.3) was operative in vitro when Tay-Sachs' tissue was tested (Kolodny et al. 1969; Tallman et al. 1972). This alternative route, which can by-pass the hexosaminidase A-catalysed reaction (reaction 1, Figure 1.3),

FIG. 1.2 SOME NATURALLY OCCURRING SUBSTRATES FOR HEXOSAMINIDASE





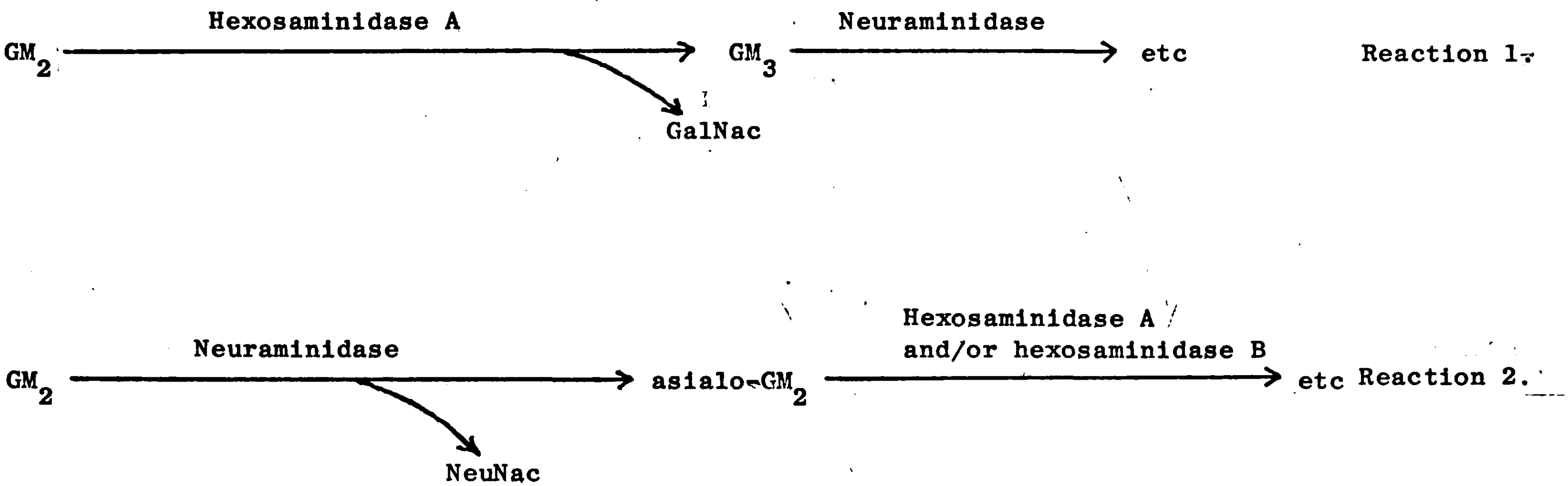


cer = ceramide (N - acylsphingosine)

TABLE1.1 GM_2 GANGLIOSIDOSIS AND ITS VARIANTS

VARIANT	MAJOR STORAGE PRODUCT	HEXOSAMINIDASE ACTIVITY	REFERENCES
O	GM_2, asialo-GM_2, globoside	Deficiency of A and B	Sandhoff (1969)
B	GM_2	Deficiency of A. B activity normal or elevated.	Okada and O'Brien (1969)
AB	GM_2	Both A and B normal or elevated	Young *et al* (1970) Sandhoff *et al* (1971)
Juvenile	Relatively mild accumulation of GM_2	Partial deficiency of A	Suzuki and Suzuki (1970)

FIGURE 1.3 INITIATION OF GANGLIOSIDE GM_2 BREAKDOWN BY HEXOSAMINIDASE A AND NEURAMINIDASE



probably accounts for the relatively low GM_2 accumulation in non-neuronal tissue in Tay-Sachs' disease. However, the higher levels of glycosphingolipids characteristic of normal neuronal tissue are such that in the absence of hexosaminidase A the alternative pathway is unlikely to cope with the increased amounts of GM_2. In addition, there is evidence to suggest that the activity of GM_2-neuraminidase is lower in neuronal than in other tissue (Kolodny et al. 1971).

As stated above, small, but above normal, amounts of asialo-GM_2 are stored in Tay-Sachs' disease indicating that, although active in vitro, the hexosaminidase B-catalysed hydrolysis of asialo-GM_2 is somehow impaired in vivo. In order to explain this observation it has been suggested that the increased amounts of GM_2 might inhibit the hydrolysis of asialo-GM_2 by hexosaminidase B (Sloan and Fredrickson, 1972) or that asialo-GM_2 becomes embedded in the lamellae of membranous cytoplasmic bodies found in neuronal tissue making it inaccessible to enzyme action (Sandhoff et al. 1971). The deficiency of both hexosaminidase A and B in Sandhoff's disease means that both pathways of GM_2 catabolism are blocked and, as expected, this disorder is characterised by a more rapid accumulation of GM_2, greater accumulation of asialo-GM_2 and greater involvement of the visceral organs than classical Tay-Sachs' disease.

Although several workers have confirmed that tissues from patients with Tay-Sachs' disease and Sandhoff's disease were defective in their ability to cleave the terminal N-acetylgalactosaminyl residue from GM_2 (Kolodny et al. 1969; Sandhoff et al. 1971; Tallman et al. 1972), verification of the predicted substrate specificities of the different hexosaminidases proved difficult to study. Attempts to demonstrate the hydrolysis of GM_2 by hexosaminidase preparations from normal individuals gave contradictory results. Crude preparations of hexosaminidase from muscle (Kolodny et al. 1969), liver (Sandhoff et al. 1971; Li et al. 1973) and placenta (Srivastava et al. 1974) were able to catalyse the hydrolysis of GM_2, as were intact lysosomes isolated from fresh brain (Tallman et al. 1972). On the other hand, hexosaminidase which had been isolated from frozen liver and placenta was unable to hydrolyse GM_2 (Wenger et al. 1972; Srivastava et al. 1974). Highly purified hexosaminidase A and B from liver (Li et al. 1973) and placenta (Srivastava et al. 1974) and hexosaminidase A from urine (Banerjee et al. 1977) were also inactive against GM_2. Sandhoff (1970) reported that purified liver hexosaminidase A had

GM_2 hydrolase activity in the presence of sodium taurocholate while hexosaminidase B showed no GM_2 hydrolase activity in the presence or absence of detergent. However, Tallman et al. (1974) found that both hexosaminidase A and B, purified from placenta, were able to attack GM_2 in the presence of sodium taurocholate. Although the latter finding is somewhat difficult to explain, the activation of GM_2 hydrolase activity is probably related to the ability of the bile salt to disperse the GM_2 aggregates into smaller micelles, thus rendering them more susceptible to hexosaminidase attack.

A possible explanation for the above discrepancy between crude hexosaminidase preparations on the one hand and of purified enzyme on the other was suggested by the results of experiments carried out by Li et al. (1973). They found that the hydrolysis of GM_2 by purified hexosaminidase A could be stimulated by a heat stable, non-dialysable factor found in liver homogenates. Hexosaminidase B did not hydrolyse GM_2 even in the presence of the factor. Although present in liver homogenates, this factor was lost upon purification of hexosaminidase. The GM_2 hydrolase activating factor was purified from liver by Hechtman (1977) and Hechtman and le Blanc (1977). It was found to be a heat stable protein with a molecular weight between 36,000 - 39,000 and devoid of any hexosaminidase activity. Electrophoresis on polyacrylamide gels revealed the presence of two protein bands, the significance of which is not clear at present. As found by Li et al. (1973), the factor stimulated the hydrolysis of GM_2 by purified hexosaminidase A while hexosaminidase B showed no such activity in the presence or absence of the factor. Kinetic experiments suggested the formation of a complex between GM_2, hexosaminidase A and the factor, although it was not clear whether the precise mode of action of the factor was by effecting physicochemical changes in the substrate or whether it altered the binding or catalytic properties of the enzyme.

Bach and Suzuki (1975) observed that fractionation of a crude liver homogenate by isoelectric focusing separated hexosaminidase A into two subfractions, one active towards synthetic substrates and the other active towards GM_2. However, the separation was not complete and there was considerable overlap of the two activities. These results were interpreted as an indication of the presence of two components with different specificities towards synthetic and natural substrates. In the light of the findings described by Li et al. (1973) and Hechtman (1977), it seems

more probable that the isoelectric focusing procedure had achieved a partial separation of hexosaminidase A and the activating protein. Thus, only the fractions which contained both the enzyme and the activator would be able to attack GM_2. Consistent with this alternative explanation, Hechtman (1977) showed that a partial separation of hexosaminidase A and the activating protein could be brought about by DEAE-sephadex chromatography. Nonetheless, it would be interesting to repeat the experiment described by Bach and Suzuki (1975) using purified hexosaminidase and adding purified activating protein to the individual hexosaminidase fractions.

Although the existence of the activating factor explained the inability of some workers to show significant hydrolysis of GM_2 using purified hexosaminidase A, the rate of hydrolysis in the presence of the factor was not as great as that achieved by crude hexosaminidase preparations or by intact lysosomes. It is, therefore, possible that optimal GM_2-hydrolase activity requires the lysosomal environment and/or some other cofactor. This idea is further supported by the observation that sonic disruption of lysosomes greatly diminished their GM_2-hydrolase activity (Quirk _et al_. 1972).

The existance of a GM_2-hexosaminidase activator also raises the possibility of mutations affecting the activator protein rather than hexosaminidase and it will be particularly interesting to establish whether such mutations can be associated with any of the variants of GM_2-gangliosidosis. For example, the apparently unaccountable reports of individuals who possess normal levels of hexosaminidase A and B, as determined with synthetic substrates, but who show storage of GM_2 and other symptoms of GM_2-gangliosidosis, could be explained by a deficiency of the activating protein. Similarly, it may be informative to analyse the activating protein, or the ability of hexosaminidase A to interact with the protein, in individuals with the Juvenile form of GM_2-gangliosidosis and in the less frequently encountered adults who show a typical Tay-Sachs'-type hexosaminidase A deficiency but who are otherwise healthy. In this context, it will be helpful to clarify the precise mode of action of the activating protein in normal individuals.

The role of hexosaminidase A and B in the degradation of asialo-GM_2 and globoside proved more straightforward to verify and _in vitro_ experiments confirmed that both forms showed asialo-GM_2 and globoside hydrolase activities. In contrast to GM_2-hydrolase activity, the latter

activities were demonstrated using either crude or purified hexosaminidase preparations and were not affected by the activating protein (Sandhoff et al. 1971; Li et al. 1973; Bach and Suzuki, 1975; Hechtman and le Blanc, 1977).

In summary, experimental evidence has confirmed the substrate specificities of hexosaminidases A and B predicted by the glycolipid storage patterns in Tay-Sachs' and Sandhoff's diseases.

Very little attention has been paid to the activities of the other multiple forms of hexosaminidase towards natural substrates. The presence in Sandhoff's disease of hexosaminidases S and C, and of chitobiase indicates that these forms do not contribute significantly to the hydrolysis of GM_2, asialo-GM_2 or globoside. Indeed, Frowein and Gatt (1967b) showed that both the specific β-glucosaminidase and β-galactosaminidase isolated from calf brain supernatant were unable to cleave the terminal N-acetylgalactosaminyl residue from GM_2 or asialo-GM_2. These forms possibly relate to hexosaminidase C and D from human tissues. There is a notable absence of glycosaminoglycan storage in both Tay-Sachs' and Sandhoff's diseases. This most likely reflects the presence of chitobiase which, as described earlier, is active towards such compounds.

2. <u>The nature of the molecular defects in Tay-Sachs' and Sandhoff's disease.</u>

In tissues from patients with Tay-Sachs' disease, hexosaminidase A activity is usually less than 5% of that found in normal controls and in some cases may be undetectable altogether. Hexosaminidase B activity, on the other hand, is often elevated above normal levels (Okada and O'Brien, 1969; Sandhoff et al. 1971). In cases of Sandhoff's disease, hexosaminidase A and B activities are both diminished and are very often undetectable. Although some residual activity can sometimes be demonstrated this seems to represent increased amounts of hexosaminidase S (Sandhoff et al. 1971; Carroll and Robinson, 1973; Srivastava et al. 1975; Beutler et al. 1975).

Two possible explanations for the enzyme deficiencies described above can be considered. One is that the deficient enzymes are present in reduced amounts or are totally absent and the other is that affected individuals possess structurally altered enzymes with reduced catalytic activities. Since it is now generally held that hexosaminidases A and B

have subunit structures of $(\alpha\beta)_n$ and $(\beta\beta)_n$, respectively, the deficiency of hexosaminidase A in Tay-Sachs' disease can be more specifically ascribed to a mutation affecting the amount or structure of the α-subunit and the deficiency of both the A and B forms in Sandhoff's disease to a mutation affecting the amount or structure of the common β-subunit.

When fibroblasts from Tay-Sachs' and Sandhoff's disease patients were allowed to fuse together, the enzyme deficiencies of the respective parental cells were corrected in the resulting hybrid cells (Galjaard et al. 1974; Thomas et al. 1974). This is easily explained by the "common and unique" subunit theory by assuming that the Tay-Sachs cells provided the genetic information for the β-subunit while the Sandhoff cells provided the information for the α-subunit. Although this intergenic complementation confirmed that the two disorders were caused by different gene mutations, it did not give any indication as to the nature of the defects.

In theory, the most logical and direct method of distinguishing between the presence of reduced amounts of enzyme and the presence of a structurally altered enzyme would be to isolate the residual deficient hexosaminidases and compare their primary structures with that of normal hexosaminidase. This approach, using tryptic digestion and amino acid sequence analysis, has been most frequently applied in the cases of the haemoglobinopathies of which over eighty variants have now been shown to be due to the substitution of a single amino acid in either the α or β-globin chains (see Lehnmann and Carrell, 1969). These discoveries have been made possible by the fact that haemoglobin can easily be purified and in sufficient quantities for detailed structural analyses. On the other hand, the state of knowledge about hexosaminidase structure is still at a relatively rudimentary level, mainly due to the practical problems encountered in preparing sufficient quantities of purified enzyme. For this reason, it is unlikely that amino acid sequence analysis of hexosaminidase is feasible, for the present time at least.

Two other approaches are commonly used to gain insight into the underlying defect in cases of inherited enzyme deficiency. The first of these assumes the presence of residual activity in the enzyme deficient state and involves comparing physicochemical properties of the residual and normal enzymes. This can include measurement of their electrophoretic mobilities, kinetic constants, thermal stabilities and pH optima. Changes

in these parameters are normally taken to suggest the presence of a structurally altered enzyme rather than a reduction in enzyme amount. For example, a combination of these measurements has led to the identification of several variants of glucose-6-phosphate dehydrogenase deficiency and subsequent amino acid analysis has confirmed that a single amino acid substitution was responsible in at least one of these cases (Yoshida, 1967; Motulsky and Yoshida, 1969). Similarly, the residual argininosuccinate synthetase in some patients with citrullinaemia and the residual α-fucosidase in a patient with fucosidosis had altered kinetic properties compared to their respective normal counterparts (Tedesco and Mellman, 1967; Matteo et al., 1976).

Comparisons of the physicochemical properties of hexosaminidase from patients with Tay-Sachs' or Sandhoff's disease with those of normal hexosaminidase have not been frequently reported. This is most likely due to the fact that in most cases the residual activity is too low for adequate comparison or indeed is absent altogether. However, results presented by Sandhoff et al. (1971) indicate that the residual hexosaminidase A and the elevated hexosaminidase B from patients with Tay-Sachs' disease had the same isoelectric-points, pH optima and thermostabilities as their counterparts from normal tissues. These data could be taken as evidence for the presence of reduced amounts of normal hexosaminidase A and increased amounts of normal hexosaminidase B in Tay-Sachs' disease. It should be noted that some workers have reported slight differences in the kinetic and electrophoretic properties of hexosaminidase B from patients with Tay-Sachs' disease (Wenger et al. 1972; Carroll and Robinson, 1973). Also, Price and Dance (1972) and Grebner and Tucker (1973) discovered that the heat-stable hexosaminidase activity in their patients with Tay-Sachs' disease was not hexosaminidase B but seemed to represent an intermediate (I) form. These findings are not readily explained but could perhaps reflect the general metabolic disorganisation caused by the grossly abnormal accumulation of glycolipid in Tay-Sachs' disease. With respect to Sandhoff's disease, the residual A and B forms from cases studied by Sandhoff et al. (1971) had normal pH optima and thermal stabilities.

The other approach involves testing tissue extracts obtained from patients with a particular enzyme deficiency for immunologically cross-reacting material using a specific antiserum raised against the appropriate normal enzyme. The presence of catalytically inactive but immunologically

cross-reactive material is generally taken to indicate the presence of a structurally altered enzyme. For example, patients with galactosaemia in which galactose-1-phosphate uridyl transferase activity was deficient, and patients with metachromatic leukodystrophy, in which arylsulphatase A activity was deficient, were found to possess cross-reactive material when tested with anti-enzyme antisera (Tedesco and Mellman, 1971; Neuwelt et al., 1971).

When this method was applied to tissue extracts from patients with Tay-Sachs' disease in which hexosaminidase A activity could not be demonstrated, anti-(hexosaminidase B) cross-reactive material was found but no component which reacted with absorbed anti-(hexosaminidase A) could be detected. (Srivastava and Beutler, 1974; Bartholomew and Rattazzi, 1974; Geiger et al., 1975). The lack of absorbed anti-(hexosaminidase A) cross-reactive material suggested that the deficiency of hexosaminidase A in these cases could be due to reduced amounts or even to the absence of α-chains. Consistent with this idea is the observation that hexosaminidase B activity is elevated in some cases of Tay-Sachs' disease. This would be expected in view of the presumed increase in the availability of free β-chains. In two unrelated cases of Sandhoff's disease, hexosaminidase A and B activities were undetectable but immunologically cross-reactive material was demonstrated using either absorbed anti-(hexosaminidase A) or anti-(hexosaminidase B) antiserum (Srivastava and Beutler, 1974). These results suggested a structural mutation affecting the common β-subunit of hexosaminidase A and B. On the other hand, Carroll and Robinson (1973) reported a case of Sandhoff's disease in which A and B activities were undetectable and in which no anti-(hexosaminidase B) cross-reactive material was present. An alternative, though less plausible, explanation for those instances in which no cross-reactive material was detected could be the presence of structurally different polypeptides with altered antigenic properties.

In summary, in those cases of Tay-Sachs' disease studied to date, the limited physiochemical or immunological evidence suggests a quantitative deficiency of the α-chains in hexosaminidase A. In Sandhoff's disease there is evidence for both quantitative and qualitative deficiencies of the β-chains common to hexosaminidase A and B.

Some workers have speculated that the quantitative defect in cases of Tay-Sachs' disease might reflect reduced or absent α-chain synthesis (Carroll and Robinson, 1973, 1974). Other disorders such as fucosidosis

(Alhadeff et al., 1975), Fabry's disease (Sutton and Omenn, 1972) and a variant form of phenylketonuria (Milstein and Kaufman, 1975). have also been offered as possible examples of inherited deficiencies of protein synthesis. Tissue protein levels are determined by a combination of several factors. A defect at any of the stages in the transcription and translation of the genetic material could alter the amount of protein synthesised. It should also be noted that there is evidence from studies of certain haemoglobinopathics that amino acid substitution mutations can change the conformation of a protein so as to render it less stable than its normal counterpart (Perutz and Lehmann, 1968). Such a protein might be more easily degraded and hence have a shorter half-life in vivo compared to the normal protein.

A discussion of a group of inherited anaemias known as the thalassaemias is particularly relevent at this point. These disorders have provided a model system in which to study the different molecular lesions which can give rise to a quantitative protein deficiency and, therefore, should give some indication of the type of experiments which could be applied to other situations, such as in Tay-Sachs' disease, where a quantitative deficiency is suspected.

The thalassaemias are characterised either by a reduced amount or by a total absence of the α, β or γ chains which comprise adult haemoglobin. Thus, patients with α^+ or β^+ thalassaemia have reduced amounts of α-or β-globin in their red blood cells, respectively, while in patients with α^o or β^o thalassaemia no α-or β-chains can be detected. Other variants will be described below.

In cases where the effected globin chain was present in reduced amounts, amino acid sequencing studies revealed no evidence of an amino acid substitution and led to the suggestion that there was defective globin synthesis. Early studies examined the kinetics of globin chain synthesis in reticulocytes taken from thalassaemic individuals. No abnormality of globin chain initiation, elongation or termination was found and this, in turn, pointed to a deficiency in the mRNA-ribosome complex (Bank and Marks, 1966; Clegg et al., 1968).

Further insight was made possible by the development of cell-free protein synthesising systems and by the development of techniques for isolating and assaying globin mRNA. This meant that individual components of the protein synthesising machinery of thalassaemic reticulocytes could be tested.

Ribosomes and initiation factors isolated from affected cases were substituted in cell-free systems and found to function normally (Nienhuis et al., 1971; Nathan et al., 1971). However, when mRNA preparations from α^{+} and β^{+}-thalassaemics were added to cell-free systems derived from rabbit reticulocytes or mouse ascites tumour cells very little α-or β-globin chains were synthesised, thus mirroring the respective in vivo conditions (Nienhuis and Anderson, 1971; Benz and Forget, 1971; Grossbard et al., 1973). Although these experiments confirmed that the mRNA coding for the α-or β-globin chains was deficient, they did not allow a distinction to be made between reduced amounts of mRNA or the presence of functionally abnormal mRNA.

Such a distinction was soon made possible by the introduction of the technique of molecular hybridisation. This technique is based on the fact that by using an RNA-dependent DNA polymerase and radioactive precursor nucleoside triphosphates it is possible to make a labelled, complementary DNA copy of a specific mRNA template. This cDNA can recognise and hybridise with other nucleic acids having a complementary nucleotide sequence and can therefore be used to identify and quantitate specific mRNAs and specific regions of DNA. Using DNA to normal α-globin mRNA it was discovered that reticulocytes from patients with α^{+}-thalassaemia contained reduced amounts of α-chain mRNA (Housman et al., 1973) and that this was due to the deletion of one of the pair of haploid α-chain genes (Taylor et al., 1974). In patients with α^{o}-thalassaemia it was shown that total absence of α-chain mRNA was due to the deletion of both haploid α-chain genes (Ottolenghi et al., 1974; Taylor et al., 1974).

A different molecular lesion was found to be responsible for the decreased amounts of α-globin found in certain other thalassaemics. Here, amino acid sequence analysis revealed that the affected α-chains had an additional amino acid sequence on their C-terminal ends when compared to normal α-globin (Milner et al., 1971). Further studies showed that the extra amino acid sequence corresponded with a normally untranslated region at the 3' end of α-globin mRNA (Forget et al., 1974; Wilson et al., 1977). This suggested that the disorder was due to a mutation affecting the nucleotide sequence usually responsible for terminating translation. The elongated α-chains produced as a result of this chain-termination mutation are inherently unstable and hence have a shorter half-life in vivo than their normal counterparts.

Overall, this results in a reduction in the amount of α-chains.

Application of the molecular hybridisation technique to the β-thalassaemias did not give the clear-cut answers which had been obtained for α-thalassaemia. The reduced amounts of β-globin in β^{+}-thalassaemics were shown to be due to the presence of diminished amounts of β-chain mRNA (Kacian et al., 1973; Housman et al., 1973) but analysis of DNA from affected individuals revealed that the β-globin genes were intact (Ramirez et al., 1975, 1976). Studies on β^{o}-thalassaemia revealed considerable heterogeneity at the mRNA level. In some cases no hybridisable β-chain mRNA was detected (Forget et al., 1974; Tolstoshev et al., 1976; Comi et al., 1977) while limited hybridisation was observed in others (Kan et al., 1975; Ramirez et al., 1976; Ottolenghi et al., 1977). Theβ-globin genes were apparently intact in all these instances and so the precise nature of the molecular defect in each case remains uncertain. Further insight should be possible by the application of nucleotide sequencing techniques. Conconi et al. (1975) reported cases of β^{o}-thalassaemia in which the β-globin mRNA could be activated in vitro by the addition of post-ribosomal supernatant from normal reticulocytes. Further studies on the translational capabilities of effected reticulocytes might be informative.

Although it is tempting to ascribe a quantitative protein deficiency to a regulator or controller gene mutation rather than to a structural mutation, this must be done with caution. The example of the thalassaemias clearly illustrates that diverse molecular mechanisms, including structural mutations, can give rise to just such a deficiency. Interestingly, there is, as yet, no direct evidence for a regulator gene mutation amongst these disorders.

Application of the methodology described above for the thalassaemias to other disorders should be equally informative. However, there are problems in doing this, most of which are practical and related to the fact that, unlike haemoglobin, the majority of enzymes which may be associated with quantitative deficiencies are only present in tissues in trace amounts. For example, based on the specific activity data of Geiger and Arnon (1976), hexosaminidase accounts for about 0.02% of total placental protein. This contrasts with globin which represents about 90% of reticulocyte protein. Accordingly, this probably means that the mRNA's coding for these relatively minor proteins will, themselves, only represent

a small proportion of the total mRNA population. This makes both the assay of mRNA activity and the purification of specific mRNAs a relatively difficult task. Nonetheless, an increasing number of mRNAs coding for proteins which represent less than 1% of the total cellular protein are being studied and successfully translated _in vitro_. Examples include tryptophan oxygenase from rat liver (Schutz _et al._, 1975), glutamine synthetase from chick retina (Sarkar and Griffith, 1976), aldolase from rat ventral prostate (Mainwaring _et al._, 1974) and cellulase from pea epicotyl (Verma _et al._, 1975). The main reason for this has been the introduction of highly sensitive and specific immunological procedures for identifying mRNA translation products. It can only be predicted that the improvement of techniques will enable other disorders to be investigated to the same depth as the thalassaemias.

The deficiency of hexosaminidase A in Tay-Sachs' disease may be particularly amenable to further study. Although some detailed aspects of its structure remain unclear, human hexosaminidase is probably the best characterised lysosomal enzyme. Furthermore, based on the available evidence, Tay-Sachs' disease bears some superficial similarity to the thalassaemias. First of all, the deficiency of hexosaminidase A appears to be due to decreased amounts or even to the absence of α-chains. This compares with the characteristic absence of α-or β-globin in certain thalassaemias. Secondly, because of the greater availability of free β-chains in Tay-Sachs' disease, affected individuals have elevated levels of hexosaminidase B. This, for example, compares with the relatively increased amounts of free β-globin chains which accumulate in α-thalassaemia.

3. The biosynthesis of lysosomal enzymes

3.1 Evidence for the synthesis of lysosomal enzymes

As already noted in previous sections a major limitation is studying hexosaminidase and, indeed other lysosomal enzymes, is the fact that, generally, they only represent a small proportion of total cellular protein. This characteristic is a particular problem in the study of lysosomal enzyme synthesis and is probably the main reason for the relatively small amount of information which is available regarding this subject. Nonetheless, in a few instances indirect or direct evidence has been obtained to show that increases in lysosomal enzyme activities are due, at least partly, to _de novo_ enzyme synthesis.

Studies have shown that the stimulation of various lysosomal enzyme activities in several different experimental systems by a variety of environmental stimuli can be abolished by the addition of protein synthesis inhibitors. For example, both cychoheximide and puromycin inhibited sucrose-stimulated cathepsin D activity in chick limb-bone rudiments in organ culture (Hille et al. 1970). Puromycin also inhibited the stimulation of several lysosomal enzymes which occurred in mouse peritoneal macrophages in response to the endocytosis of digestible materials (Axline and Cohn, 1970). Similarly, the increase in the activities of β-glucuronidase, acid phophatase and cathepsin normally associated with the differentiation of rabbit alveolar macrophages in cell-culture was prevented by incubation with puromycin or DL- p - fluorophenylalanine (Cohn and Benson, 1965).

This type of experiment suggests that protein synthesis is a prerequisite for the observed increased lysosomal enzyme activity but does not necessarily indicate that de novo synthesis of the enzyme in question has occurred. Although there is very little evidence for the existence of inactive precursors of lysosomal enzymes (Dean and Barrett, 1976), it is still conceivable that the protein synthesis inhibitors blocked the production of specific activators or factors responsible for converting inactive zymogens to active enzyme forms.

Direct evidence for the synthesis of some lysosomal enzymes has been obtained by demonstrating the incorporation of radioactive amino acid into enzyme protein. Kato et al. (1970) studied the effect of gonadotrophin administration in vivo on mouse kidney β-glucuronidase and found that the hormone increased both the enzyme activity and the amount of radioactivity incorporated into β-glucuronidase-specific protein. The labelled enzyme was isolated from kidney homogenates by standard purification techniques. Similar findings were reported by Swank et al. (1973), but in this case the radiolabelled β-glucuronidase was isolated from crude tissue extracts by precipitation with specific anti-(β-glucuronidase) antiserum.

Evidence for the biosynthesis of hexosaminidase by rat neuronal cell bodies incubated in vitro has been presented by Khawaja and Sellinger (1976). After incubation of the cell-bodies with radioactive amino acids, subcellular fractions were obtained by differential centrifugation and solubilised extracts of these fractions were analysed by DEAE-cellulose chromatography and by cellulose acetate electrophoresis.

The evidence that hexosaminidase had been synthesised was that hexosaminidase catalytic activity and TCA-insoluble radioactivity co-fractionated. This experimental approach is the least satisfactory of those described, especially since the enzyme preparations were not pure and presumably contained significant amounts of contaminating radioactive material.

3.2 Turnover of lysosomal enzymes

The experiments described in section 3.1 indicated that *de novo* synthesis of lysosomal enzymes was involved in situations in which enzyme activity was elevated. However, protein levels are determined by the rates at which proteins are degraded into their component amino acids as well as by the rates of protein synthesis. Studies on the turnover of lysosomal enzymes have been restricted largely to those enzymes for which relatively straightforward purification methods exist.

In an attempt to investigate the mechanism of the increased ribonuclease activity during the regression of hormone-dependent rat mammary gland tumours, Cho-Chung and Gallino (1973) determined the rates of ribonuclease synthesis and degradation both during the growth and regression of these tumours. Rates of synthesis were derived from the rate of incorporation of radioactive amino acid into specific antibody precipitable material. In order to estimate the rate of ribonuclease degradation, rats were injected with a pulse of radioactive amino acid followed by a chase of unlabelled amino acid. The loss of radioactivity with time from immunoprecipitable ribonuclease was then used to calculate the degradation rate. The actual rate constants obtained by these workers probably do not reflect the true values because free, labelled amino acid derived from protein degradation can be re-used for another round of protein synthesis. Nonetheless, the results clearly indicated that the accumulation of ribonuclease in regressing tumours was the net result of both increased enzyme synthesis and increased degradation. The pulse-chase method was also used by Warburton and Wynn (1976) who showed that the sucrose-stimulated increase in arylsulphatase B activity in cultured hamster fibroblasts was accompanied by increased rates of enzyme synthesis and degradation. As found for ribonuclease, the rate of arylsulphatase B synthesis was greater than the rate of degradation with the net

result that the half-life of the enzyme decreased from 30 to 10 days and enzyme activity increased in the culture system. The problem of re-utilisation of radioactive amino acids is greatly minimised in studies using cultured cells mainly because of the rapid influx of unlabelled amino acid from the culture medium to the cells during the chase period. Thus, the data relating to the turnover of arylsulphatase B probably reflect the true situation.

Several workers have studied the turnover of β-glucuronidase. Wang and Touster (1975) examined the half-life of rat liver β-glucuronidase using a double-isotope technique. This involved injecting rats with ^{3}H- labelled amino acid followed some days later by the injection of the same amino acid but labelled with ^{14}C. From the ratio of ^{14}C : ^{3}H radioactivity present in immunoprecipitated β-glucuronidase they were able to calculate a half-life for β-glucuronidase of about 29 days. However, because of the possibility of isotope re-utilisation, this value may be an overestimate of the true half-life. Using single isotope pulse-chase methods, Smith and Ganschow (1975) and Warburton and Wynn (1977) obtained half-lives of between 4 and 7 days and between 4 and 5 days for the mouse liver and hamster fibroblast enzymes, respectively.

Bosman (1972) calculated the half-lives of several lysosomal enzymes, including hexosaminidase, in a mouse leukaemic cell line based on the decay of enzyme activities following treatment of the cells with cycloheximide. Different enzymes had different half-lives. Similar experiments were carried out with cultured rat embryo fibroblasts (Amenta _et al_. 1977). In this instance cathepsin D activity decreased to 70% of the control value within 4 hours whereas the activities of acid phosphatase and β-glucuronidase remained more or less constant throughout a 3 day culture period in the presence of the drug. Interpretation of these data is subject to certain complications. Firstly, cycloheximide treatment has been shown to decrease the rate of protein degradation in several systems (Hershko and Tomkins, 1971; Ballard and Hopgood, 1973). Paradoxically, there is evidence to suggest that this reduction in protein degradation is due to inhibition of the synthesis of rapidly turning-over lysosomal proteases (Wildenthal and Griffin, 1976). Secondly, measurement of the loss of enzyme activity does not necessarily indicate degradation of enzyme polypeptides but could reflect denaturation or inactivation due to loss of an activator.

In spite of the problems associated with the measurement of rates of synthesis and degradation, the available data indicates that lysosomal enzymes have heterogenous turnover rates. In this respect they are similar to proteins found in other cellular compartments and which also show a spectrum of turnover rates (Dehlinger and Schimke, 1970; Dehlinger and Schimke, 1971).

In addition to the investigations carried out on individual lysosomal enzymes, some workers have turned their attention to the turnover of total lysosomal protein and to lysosomes themselves. Goldstone and Koenig (1974) determined the half-life of the total soluble glycoprotein isolated from rat kidney lysosomes after the injection into rats of radioactive N-acetylmannosamine, a precursor of N-acetylneuraminic acid. Interestingly, they found that the N-acetylneuraminic acid moieties of the glycoproteins turned over more rapidly than protein components. Segal (1975) reported half-lives of 30, 24 and 29 hours for rat liver lysosomes, lysosomal membranes and soluble lysosomal protein, respectively. These values were obtained by injecting rats with $[^{14}C]$ arginine labelled in the guanido group and measuring the loss of radioactivity from the appropriate fraction. The use of this particular radioactive amino acid is believed to minimise the problem of re-cycling of isotope especially in liver where arginase levels are relatively high (Swick and Handa, 1955).

Although the experiments described in this section provide some information about the turnover of lysosomal enzymes and of lysosomes themselves, it is not yet clear how these processes are regulated. With respect to lysosomal enzyme degradation it is a matter of speculation as to why lysosomal enzymes are not rapidly broken down by other lysosomal enzymes. It has been suggested that the interaction of the enzymes with their substrates or the binding of the enzymes to the lysosomal membrane may afford some degree of protection, perhaps by temporarily increasing enzyme stability (van Hoof and Hers, 1972; Dean and Barrett, 1976).

3.3 The role of subcellular organelles in lysosomal enzyme synthesis

The lysosomal localisation of hexosaminidase, as well as other enzymes, raises the question as to the site of enzyme synthesis. Unlike certain other cell organelles, the lysosome contains neither genetic information nor protein synthetic machinery. Therefore,

lysosomal enzymes are synthesised outside the lysosome and the major problem concerns the mechanism by which the appropriate enzymes are packaged in the lysosomal structure. For example, are the enzymes taken up by preformed lysosomes or, alternatively, associated as the lysosomes themselves are organised?

In addition, enzyme synthesis may be further complicated by the finding that many lysosomal enzymes are multisubunit and glycoprotein in structure. Thus, the appearance of an active enzyme requires that the appropriate mRNA be translated at the correct intracellular site, that the newly synthesised polypeptides assemble to form the multimeric enzyme complex and that the appropriate carbohydrates are added.

3.3.1 The site of lysosomal enzyme synthesis

Electron microscopy and autoradiography of mouse peritoneal macrophages given a pulse of radioactivity followed by an unlabelled amino acid chase showed that radioactivity appeared first in the rough endoplasmic reticulum, then in the Golgi apparatus and finally in lysosomes (Cohn and Fedorko, 1969). Similar observations were reported for rat kidney tubule cells (Nayyar and Koenig, 1972). Although no attempt was made to follow the translocation of an individual lysosomal protein, these studies suggested that lysosomal enzymes were probably synthesised on ribosomes attached to the rough endoplasmic reticulum, transported via the lumen of the endoplasmic reticulum to the Golgi apparatus, and finally sequestered in lysosomes. Kinetic studies on the incorporation of radioactive amino acids into β-glucuronidase in different subcellular fractions from rat liver (van Lancker and Lentz, 1970) and mouse kidney (Kato _et al_., 1970; Golstone and Koenig, 1972) supported the above general scheme in that radiolabelled enzyme was detected first in the microsomal and later in the lysosomal fractions. It should be noted that an alternative scheme for the synthesis of lysosomes which by-passes the Golgi apparatus has been proposed by Novikoff (1973). Electron microscopic and cytochemical studies revealed a specific region of the smooth endoplasmic reticulum near the Golgi vesicles which stained for acid phosphatase and which had certain morphological features in common with lysosomes (Novikoff, 1964). Furthermore, this region was observed to give rise to lysosome-like structures directly without the involvement of the Golgi apparatus (Novikoff, 1975).

The ultrastructural and subcellular fractionation studies described above provide evidence that acid hydrolases are synthesised on membrane-bound ribosomes. Further evidence was provided by Khawaja and Sellinger (1976) who showed that hexosaminidase activity was associated with polyribosomes attached to the microsomal fraction from rat neuronal cell bodies. It should be noted that the possibility of the polyribosomes being contaminated with lysosomal hexosaminidase released during the isolation procedure was not ruled out in this investigation. Goldstone et al. (1973b) separated the rough microsomal fraction from rat kidney into two sub-fractions by isopycnic centrifugation and found that one was particularly rich in several acid hydrolases. From this they concluded that lysosomal enzymes were only synthesised on certain restricted regions of the rough endoplasmic reticulum. Since catalytic activity alone was used to detect enzyme molecules, these experiments only provided indirect evidence that the rough endoplasmic reticulum was the site of lysosomal enzyme synthesis. No report could be found of experiments aimed at establishing the role of free ribosomes in lysosomal enzyme synthesis.

The question as to what determines whether a protein will be synthesised on membrane-bound or free ribosomes is currently receiving much attention (see Shore and Tata, 1977). There is evidence that certain of the components of the polyribosome complex, i.e. the nascent polypeptide chain, the 60_S ribosomal subunit and the mRNA, can interact directly with the membrane of the endoplasmic reticulum and hence could possibly be involved in the selection process.

That a nascent polypeptide chain might determine its own site of synthesis is embodied in the "signal" hypothesis of Blobel and Sabatini (1971). Proteins for secretion are synthesised predominantly on membrane-bound ribosomes and studies of their biosynthesis have provided much evidence in support of this hypothesis. When mRNAs coding for secretory proteins were translated in cell-free systems it was discovered that the primary translation products were larger than the final secreted forms. This difference in molecular weight was due to the presence of an additional amino acid sequence on the NH_2-terminal ends of the newly synthesised products which was proteolytically removed during or after translation in vivo or in vitro in the presence of microsomal membranes (see Campbell and Blobel, 1976; Shore and Tata, 1977). It was thought that the terminal peptide might provide a "signal"

which could be recognised by a receptor on the endoplasmic reticulum and so result in the binding of the polyribosome complex to the membrane where synthesis could continue. Furthermore, the fact that the NH_2-terminal sequences consisted largely of hydrophobic residues provided a mechanism whereby the nascent chain could transverse membrane and so reach the lumen of the endoplasmic reticulum.

A role for the ribosome in selecting the intracellular site of protein synthesis was suggested by the fact that polyribosomes remained attached to the microsomal membrane after release of the nascent chain by puromycin treatment and could only be detached by increasing the KCl concentration (Blobel, 1971; Rosbash and Penman, 1971; Adelman et al., 1973). Several workers have reported differences in the protein content of isolated free and membrane-bound ribosomes and used this as evidence for the existence of two distinct classes of ribosome (Borgese et al., 1973; McConkey and Hauber, 1975; Ramsey and Steele, 1977). However, these results should be interpreted in the light of experiments which showed that free monoribosomes and polyribosomes were reversibly associated with a cytoplasmic protein (Blobel, 1976) and that released membrane-bound polyribosomes retained membrane-specific proteins (Kreibich et al., 1975; Ojakian et al., 1977; Fujita et al., 1977). This raises the possibility that there is only one type of ribosome and that the protein heterogeneity described above simply reflected the binding of different extrinsic proteins. Consistent with the view that there is only one class of ribosome, it was demonstrated that isolated free and bound polyribosomes had an equal affinity for binding to stripped microsomal membranes (Nolan and Munro, 1972; Shires et al., 1973). These considerations make it unlikely that the ribosome is directly involved in initiating binding to the endoplasmic reticulum. However, after binding has been initiated, the ribosome may assist in anchoring the polyribosome complex to the membrane.

It was suggested by Shires et al. (1974) that the selection process might involve the recognition by the endoplasmic reticulum of a specific polynucleotide sequence on the mRNA. Studies on cultured cells such as HeLa and mouse myeloma showed that the majority of membrane-bound polyribosomes were attached to the endoplasmic reticulum by a pancreatic ribonuclease-sensitive linkage (Rosbash and Penmann, 1971; Mechler and Vassalli, 1975; Faiferman et al., 1973). Thus, in these cells, mRNA-membrane interaction plays a major role in binding the

polyribosomes to the endoplasmic reticulum. However, the fact that polyribosome detachment could also be brought about by releasing the nascent chains with puromycin makes it impossible to say whether binding was initiated by nascent chain-membrane interaction or mRNA-membrane interaction. Several workers demonstrated that mRNA remained bound to microsomal membranes via the poly(A) region at the 3' terminus after removal of ribosomes and nascent polypeptide chains (Milcarek and Penman 1974; Lande et al., 1975). It is difficult to see how this binding could be selective since many mRNAs, including those attached to free polyribosomes, possess a poly(A) tract. However, the report of Cardelli et al. (1976) provided some evidence that polynucleotide sequences adjacent to the poly(A) region of rat liver mRNA might be involved in binding and this could, conceivably, introduce a degree of specificity to the process. It remains to be determined whether binding occurs directly between polynucleotide sequences and membranes or whether ribonucleoprotein-membrane interactions are involved.

Of the three mechanisms considered above the results of some recent experiments on virus-infected cells favour the "signal" hypothesis. In the Sindbis virus, two envelope proteins, E_1 and E_2, and a soluble or core protein are encoded by a 26_S polycistronic mRNA with a single initiation site. The three proteins are derived by proteolytic cleavage of the primary translation product. In sindbis-virus-infected chick fibroblasts, the 26_S RNA became associated with membrane-bound ribosomes and the two envelope proteins were found embedded in the membrane of the endoplasmic reticulum (Wirth et al., 1977). However, the core protein, which was synthesised first, appeared in the cell supernatant fraction. Since the same ribosomes and the same messenger were responsible for the synthesis of all three proteins it was concluded that the specificity for attachment of the polyribosomes to the membrane lay with the NH_2-terminus of the emerging polypeptide chain.

It is implicit in the above conclusion and in the "signal" hypothesis itself that translation must begin before attachment of the polyribosome to the endoplasmic reticulum. Studies on the synthesis of the vesicular stomatitis virus envelope glycoprotein G provided direct evidence for this proposition. The G protein is synthesised on membrane-bound ribosomes of virus-infected cells and carbohydrate residues are added before the completed glycoprotein is incorporated into the viral envelope. Synthesis of the complete glycoprotein can also be demonstrated

by translating viral mRNA in a wheat germ cell-free protein synthesising system in the presence of rough microsomal vesicles. Rothman and Lodish (1977) showed that translation of the mRNA coding for the G protein was initiated in a wheat germ system in the absence of microsomal membranes, i.e. on free polyribosomes. However, when further initiation was prevented by the addition of 7-methylguanosine-5'-phosphate and then rough microsomal vesicles added after different time intervals, the nascent G polypeptides were inserted into the membrane and glycosylated to yield authentic G glycoprotein. This demonstrated that membrane-bound ribosomes were derived from free ribosomes after the initiation of protein synthesis. Further it was shown that the timing of the membrane addition was important for correct processing of the protein. No insertion or glycosylation occurred if the membranes were added too early and reduced insertion and glycosylation occurred if the addition took place beyond a certain critical time. These kinetic studies provided direct evidence that binding of a polyribosome complex to the endoplasmic reticulum was determined solely by a certain amino acid sequence at the NH_2-terminus of a nascent polypeptide chain. Estimation of the length of nascent chain protruding from the polyribosome at the time of optimum binding and glycosylation was found to be about 40 amino acid residues. In theory, this chain length was just sufficient to accommodate the transient "signal" peptide found on nascent secretory proteins and believed to be involved in initiating membrane binding (see Campbell and Blobel, 1976). Although the precise amino acid sequence of the G protein is not known, its NH_2-terminal end was reported to be rich in hydrophobic residues (Schloemer and Wagner, 1975). As described earlier, this feature is also common to typical "signal" peptides and is thought to enable the emerging polypeptide chain to penetrate the endoplasmic reticulum membrane.

The above experiments provide persuasive evidence in support of the "signal" hypothesis. However, further experiments are needed before it can be stated that a similar mechanism applies to all systems. Caution is necessary, particularly in the light of work which showed that ovalbumin, a secretory protein synthesised by chick oviduct, was synthesised without the expected NH_2-terminal "signal" sequence while other proteins secreted by the oviduct were (Palmiter _et al._, 1977a, 1977b). Thus, different secretory mechanisms may operate even within the same system.

Nothing is known of the factors which determine the site of

lysosomal enzyme synthesis. This information will only be obtained when mRNAs coding for lysosomal enzymes are isolated and translated *in vitro* so that the early stages in their synthesis can be examined.

3.3.2. Translocation and post-translational processing

As already stated, the biosynthesis of many lysosomal enzymes involves the addition of carbohydrate residues and the assembly of polypeptide subunits. The co-ordination of these events results in the appearance of catalytically active acid hydrolases at various stages during transit from their site of polypeptide synthesis to the lysosome. Generally, activity is acquired gradually with the highest levels found in the lysosomal fraction.

β-Glucuronidase is the only acid hydrolase whose intracellular transport has been studied in any detail and some aspects of this have been described at the beginning of section 3.3. However, β-glucuronidase is unusual in that, unlike other acid hydrolases, substantial activity is found in both the microsomal and lysosomal fractions. The discovery of a single gene mutation in the mouse resulting in a deficiency of microsomal and lysosomal β-glucuronidase indicated that both forms were derived from the same structural gene (Paigen, 1961). This idea was supported by investigations in the mouse and the rabbit which revealed that the microsomal and lysosomal enzymes were catalytically and immunologically identical (Paigen, 1961; Dean, 1974). However, this biosynthetic relationship was complicated by the description by Ganschow and Paigen (1967) of a strain of mouse carrying a mutation at a locus unlinked to the β-glucuronidase structural gene and which prevented the appearance of microsomal but not lysosomal β-glucuronidase activity. On the basis of these results, Paigen (1971) proposed that there was an "architectural" gene which produced a protein responsible for the association of β-glucuronidase with the endoplasmic reticulum membrane. Swank and Paigen (1973) noted that β-glucuronidase extracted from mouse kidney microsomes had a higher molecular weight than the lysosomal enzyme and suggested that the additional protein component(s) represented the "architectural" gene product. However, the precise nature of this accessory material remains to be established.

Further insight into the relationship between microsomal and lysosomal β-glucuronidase was provided recently. Mandell and Stahl (1977) studied the liver-dependent increase in plasma β-glucuronidase activity

caused by organophosphate administration to rats. They showed that di-isopropyl phosphofluoridate caused a decrease in liver microsomal β-glucuronidase activity followed by a short-term increase in the lysosomal enzyme activity. Subsequently, the lysosomal activity also decreased and was accompanied by an increase in plasma β-glucuronidase level. This temporal sequence of events was unaffected by chemical in hibitors of protein synthesis and is consistent with the microsomal enzyme being the precursor of the lysosomal and plasma enzyme. The results did not exclude the presence of some unknown precursor pool for β-glucuronidase but further studies aimed at following the intracellular pathway of pre-labelled β-glucuronidase in the absence of new enzyme synthesis should help to resolve the problem.

Various investigations have provided direct and indirect evidence to support the idea that carbohydrate residues are added sequentially to newly synthesised proteins during their passage from the rough endoplasmic reticulum to the lysosome. Kinetic studies of the incorporation of radioactive carbohydrates showed that glucose, N-acetylglucosamine and mannose were added in the rough endoplasmic reticulum while sialic acid and additional N-acetylglucosamine were added in the Golgi apparatus. The radioactive carbohydrate-labelled proteins were eventually detected in the lysosomal fraction (Goldstone and Koenig, 1972; Nayyar and Koenig, 1972).

Needleman et al. (1976) determined the isoelectric points of acid phosphatase, β-glucuronidase, hexosaminidase, β-galactosidase and arylsulphatase in subcellular fractions from rat kidney. In the microsomal fraction all the enzymes existed as basic glycoproteins with isoelectric points between 7.5 and 9. In the Golgi and lysosomal fractions, both acidic forms with isoelectric points of about 4.4 and basic forms with isoelectric points between 6.2 and 8 were found. The hydrolases in the smooth microsomal fraction had isoelectric points intermediate between those in the rough microsomal and Golgi fractions. This decrease in the electronegativity of several acid hydrolases during intracellular transport is consistent with the sequential addition of acidic carbohydrate residues, and in particular sialic acid.

There have been very few studies on the incorporation of carbohydrate into individual lysosomal enzymes. Kato et al. (1970) demonstrated that radioactive glucosamine was incorporated into β-glucuronidase in the rough endoplasmic reticulum. Indirect evidence presented by

Khawaja and Sellinger (1976) suggested that glucose and/or mannose were added to hexosaminidase in the rough endoplasmic reticulum since hexosaminidase solubilised from a rat neuronal cell-body microsomal fraction was precipitated by concanavalin A.

The first appearance of detectable acid hydrolase activity in a subcellular fraction could reflect the addition of a functionally important sugar component. The involvement of covalently attached carbohydrate in determining the catalytic activity of lysosomal enzymes has not been studied systematically. Treatment of human hexosaminidase with purified neuraminidase did not alter hexosaminidase activity (Ikonne and Ellis, 1973) and the removal of phosphohexose residues from human β-glucuronidase by alkaline phosphatase treatment was also without any detectable effect (Kaplan _et al._, 1977). However, periodate oxidation of purified rat liver β-glucuronidase and hexosaminidase did result in losses of about 10% and 50% of their activities, respectively, indicating that certain carbohydrate residues could be of functional importance (Stahl _et al._, 1976). However, the generalised specificity of periodate to most carbohydrates and the fact that the reaction end products were not analysed precludes any definite conclusions as to the identity of the particular sugars involved.

4. Secretion of lysosomal enzymes

Although acid hydrolases are located predominantly in lysosomes, trace amounts have been detected in mammalian body fluids such as serum and cerebrospinal fluid (see Section 1.1). The extracellular appearance of enzymes could be due to leakage from damaged or dead cells or to exocytosis from healthy cells. In recent years evidence has accumulated which indicates that lysosomal enzymes are released from a variety of intact mammalian cells either continuously or in response to environmental stimuli (Davies and Allison, 1976). In some cases this release forms part of a normal physiological process (eg. the remodelling of bone during growth) while in others it has serious pathological consequences (eg. the chronic inflammation associated with rheumatoid arthritis).

Some of the circumstances under which lysosomal enzymes are secreted will be described below, followed by a discussion of possible secretory mechanisms. Finally, the mechanisms by which secreted acid hydrolases are recognised and taken up by cells will be considered.

4.1 Conditions for lysosomal enzyme release from cells and tissues in vitro.

The secretion of lysosomal enzymes has been demonstrated using various experimental systems including organ culture, tissue slices and cultured cells. Chick limb-bone rudiments in organ culture released acid hydrolases into the incubation medium in response to the presence of retinol and to the endocytosis of non-digestible sugars (Fell and Dingle, 1963; Dingle et al., 1969). The involvement of parathyroid hormone in bone resorption was clarified by Vaes (1969) who reported that the hormone caused the secretion of acid hydrolases from specialised bone mononuclear phagocytes called osteoclasts. The release of acid phosphatase from rat thyroid glands in organ culture and from slices of rat liver was stimulated by thyroid stimulating hormone and rabbit serum, respectively (Dingle, 1969; Weiss and Dingle, 1964). Cultured fibroblasts derived from human and other mammalian sources were shown to continuously release lysosomal enzymes into the incubation media (Wiesmann et al., 1971; Hickman and Neufeld, 1972; von Figura, 1977).

By far the greatest amount of information concerning lysosomal enzyme secretion has come from studies of phagocytic cells. Polymorphonuclear leucocytes and macrophages, obtained from peritoneal exudates or peripheral blood, release relatively large quantities of acid hydrolases in response to a variety of environmental stimuli. This contrasts with the continual secretion of acid hydrolases by cultured fibroblasts. Interest in phagocytic cells was generated because of their involvement in both acute and chronic inflammatory lesions (see Henson, 1971; Davies and Allison, 1976). Exposure of polymorphonuclear leucocytes or macrophages to antigen-antibody complexes caused the cells to secrete a large proportion of their lysosomal enzyme content (Henson, 1973; Cardella et al., 1974). Similarly, several components of the complement system interacted with phagocytic cells and brought about the selective release of acid hydrolases to the medium (Henson, 1976). Weissmannet al. (1971) showed that phagocytosis of latex or zymosan particles by human peripheral blood leucocytes and mouse peritoneal macrophages was accompanpanied by the selective release of lysosomal enzymes. With respect to macrophages, group A streptococcal cell walls (Davies et al., 1974a), dental plaque (Page et al., 1973), carrageenan (Allison and Davies, 1975) and asbestos (Davies et al., 1974b) all induced lysosomal enzyme secretion. Most of the agents described above which induce lysosomal enzyme secretion *in vitro* also cause chronic inflammation when injected into experimental

animals, thus providing strong evidence that the two events are directly connected.

4.2 Possible mechanisms of lysosomal enzyme secretion

The experiments described below relate, for the most part, to the secretion of acid hydrolases from phagocytic cells. During phagocytosis, enzyme release is thought to result from the liberation of lysosomal constituents into partially formed endocytotic vacuoles. Evidence for this came from electron and light microscopic examination of rabbit and human polymorphonuclear leucocytes engaged in the phagocytosis of bacteria and immune complexes. Lysosomes were observed fusing with phagocytic vacuoles arising from invaginations of the plasma membrane. Cytochemical staining, using the appropriate substrates, showed that lysosomal enzymes were discharged into the vacuolar spaces (Zucker-Franklin and Hirsch, 1964; Zurier et al., 1973; Bainton, 1973).

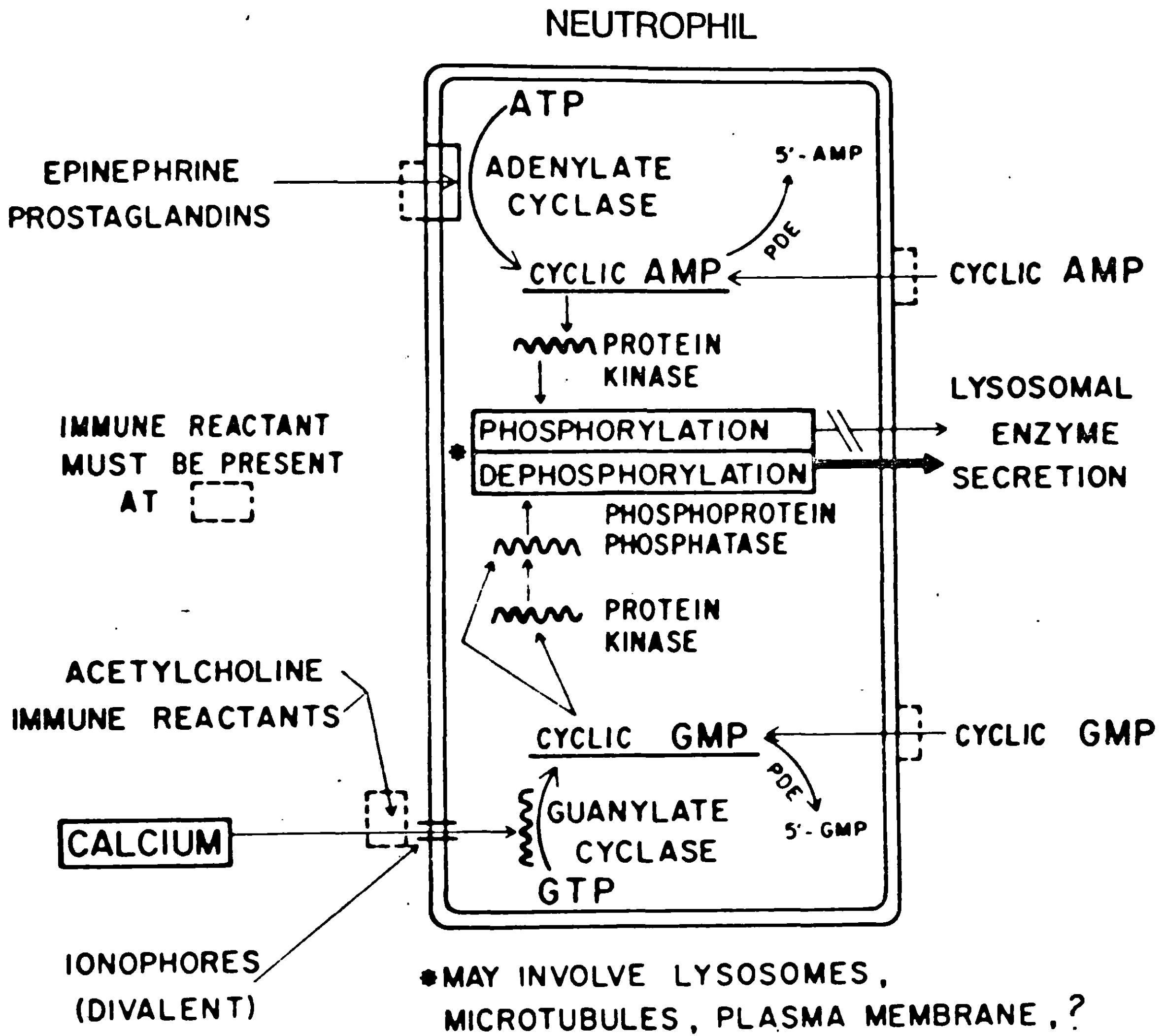
Elucidation of the biochemical events involved in initiating the lysosomal enzyme secretory process has been the object of many investigations. Various factors were shown to modulate the secretion of hydrolytic enzymes from phagocytosis-stimulated polymorphonuclear leucocytes so giving an indication of the underlying molecular mechanisms involved.

The release of lysosomal enzymes from polymorphonuclear leucocytes in response to immune reactants was reported to be dependent on the presence of extracellular calcium (Ignarro, 1974; Ignarro and George, 1974). This requirement for extracellular calcium is typical of stimulus-dependent secretory systems, eg. the release of insulin from pancreatic β-cells (Grodsky and Bennet, 1966). Cyclic GMP and pharmacological agents known to elevate intracellular cyclic GMP levels (acetylcholine, Prostaglandin $F_{2\alpha}$) also stimulated phagocytosis-induced lysosomal enzyme secretion whereas cyclic AMP and agents known to elevate its intracellular levels (adrenalin, prostaglandins E_1 and A_2, theophylline) inhibited enzyme release (Ignarro, 1973; Ignarro and George, 1974; Smith, 1977). Smith and Ignarro (1975) reported that the exposure of human polymorphonuclear leucocytes to an immune reactant in the presence of extracellular calcium resulted in the association of calcium with the cells, the intracellular accummulation of cyclic GMP and finally the release of lysosomal β-glucuronidase. Similar observations were made when the cells were exposed to the lipid-soluble calcium carrier, A 23187,

in the absence of an immune reactant. The temporal relationship between stimulation, calcium mobilisation, cyclic GMP accumulation and enzyme secretion suggested a cause and effect situation. Furthermore, the effect of the calcium ionophore, A23187, suggested that the mode of action of the immune reactant, and indeed other environmental stimuli, might be to cause the movement of calcium into cells where it could influence cyclic nucleotide metabolism and result in lysosomal enzyme release (see Fig. 4.1). It was recently reported that both A23187 and the complement component C5a increased the rate of calcium influx into rabbit polymorphonuclear leucocytes and that the rate of calcium influx was directly proportional to the amount of lysosomal enzyme activity released (Nacchache et al., 1977; Boucek and Snyderman, 1976). The precise mechanism by which an environmental stimulus causes the entry of calcium into cells is not known. Polymorphonuclear leucocytes and macrophages were shown to possess plasma membrane receptors for immunoglobulin (Lay and Nussenzweig, 1968; Messner and Jelinek, 1970). Treatment of polymorphonuclear leucocytes with trypsin or neuraminidase did not affect receptor activity and led to the suggestion that membrane lipid moieties might be involved (Metzger, 1974). In this context, there is evidence from a variety of experimental systems to suggest that phosphatidyl inositol breakdown may be an intrinsic part of the plasma membrane receptor activation sequence which leads to an increase in intracellular calcium concentration (Michell, 1975).

Many of the reactions implicated by the model depicted in Fig. 4.1 have yet to be substantiated for phagocytic cells but supportive evidence can be drawn from other systems. For example, calcium was reported to stimulate guanylate cyclase activity (White et al., 1973; Hardman et al., 1971) and to inhibit adenylate cyclase in avian erythrocytes (Steer and Levitzki, 1975; Campbell and Siddle, 1976). These observations could explain the calcium-dependent accumulation of cyclic GMP described above for phagocytosis-stimulated leucocytes. The mechanisms by which cyclic nucleotides control lysosomal enzyme secretion have not been demonstrated directly. Generally, cyclic AMP and cyclic GMP exert opposing actions in biological systems and there is evidence to indicate that cyclic nucleotide-dependent protein kinases mediate altered cyclic nucleotide levels and altered cellular function (Goldberg et al., 1973, Berridge, 1975). The observations that cyclic GMP

FIG.4.1 PROPOSED MODEL OF THE REGULATION OF LYSOSOMAL ENZYME SECRETION FROM HUMAN POLYMORPHONUCLEAR LEUCOCYTES (FROM SMITH AND IGNARRO, 1975)



PDE represents phosphodiesterase

stimulated and cyclic AMP inhibited the release of acid hydrolases from polymorphonuclear leucocytes and that these cells contained a cyclic AMP-dependent protein kinase (Tsung et al., 1972) are in keeping with the above idea.

It has been suggested that cyclic nucleotide-dependent protein kinases might act directly or indirectly on microtubules, the plasma membrane and/or the lysosomal membrane and so modulate lysosomal enzyme secretion (Zurier, 1974; Smith and Ignarro, 1975; Smith, 1977). Weissmann et al. (1975) showed that cyclic nucleotide-dependent fluctuations in the release of β-glucuronidase from polymorphonuclear leucocytes were correlated with changes in microtubule assembly, but, to date, there is very little direct evidence connecting cyclic nucleotides and microtubules. Goodman et al. (1970) reported that isolated microtubule subunits could act as phosphate receptors for a cyclic nucleotide-dependent protein kinase and Sandoval and Cuatrecasas (1976) observed that cyclic GMP caused the dephosphorylation of partially purified rat brain microtubule subunits. The latter result could reflect the activation of a phosphoprotein phosphatase by a cyclic GMP-dependent protein kinase. Evidence that microtubules are involved in lysosomal enzyme secretion has been obtained for both polymorphonuclear leucocytes and macrophages (Weissmann et al., 1971; Wright and Malawista, 1973; Zurier et al., 1974). The evidence was that colchicine, a drug known to bind to tubulin and prevent its polymerisation into microtubules, inhibited the release of acid hydrolases. A combined electron microscopic and biochemical study of polymorphonuclear leucocytes showed that concentrations of colchicine which inhibited microtubule assembly also inhibited lysosomal enzyme secretion in a dose-related manner (Hoffstein et al., 1977). Ultrastructural investigations also demonstrated that exposure of polymorphonuclear leucocytes to C5a resulted in the rapid assembly of microtubules and the fusion of lysosomes with phagocytic vacuoles (Goldstein et al., 1973, Hoffstein et al., 1977). These stimulated cells showed frequent invaginations of the plasma membrane in areas associated with assembled microtubules. On the other hand, colchicine-treated cells in which microtubule assembly was prevented showed plasma membrane invaginations at random sites and intracellular disorganisation. Based on these observations, it was proposed that microtubule assembly brought about the secretion of lysosomal enzymes by promoting intracellular conditions which favoured the translocation of phagosomes, ie. stimulated

regions of plasma membrane, into lysosome-rich regions of the cytoplasm (Hoffstein *et al.*, 1977). An alternative possibility, that functional microtubules are directly required for lysosome-phagosome fusion, seems unlikely since phagocytosed bacteria were digested normally by colchicine-treated polymorphonuclear leucocytes (Pesanti and Axline, 1975).

Evidence for the involvement of the microfilament system in lysosomal enzyme secretion has also been obtained. When polymorphonuclear leucocytes and macrophages were incubated in the presence of cytochalasin B and a phagocytic stimulating agent, phagocytosis was inhibited but lysosomal enzyme release was enhanced (Davies *et al.*, 1973; Temple *et al.*, 1973). Cytochalasin B is believed to interact with contractile microfilaments and so affect a variety of cellular functions including exocytosis (Allison, 1973) Davies *et al.* (1973) suggested that microfilaments normally serve to confine subcellular organelles, including lysosomes, within the inner cytoplasm and hence prevent or reduce their interaction with the plasma membrane. Inhibition of microfilament function by cytochalasin B could allow this interaction to occur with the concommitant release of lysosomal enzymes at the sites of fusion.

Results obtained with colchicine or cytochalasin B should be interpreted with caution since both drugs have been reported to affect other cellular systems in addition to microtubules and microfilaments. For example, colchicine was reported to have a hypocalcaemic action and to inhibit protein synthesis (see Allison, 1973; Pesanti and Axline, 1975). So far, the involvement of microtubules and microfilaments has been considered only with respect to exocytosis. Interpretation of data is further complicated by the observation that cytochalasin B inhibited the endocytosis of secreted lysosomal enzymes by cultured fibroblasts (von Figura and Kresse, 1975). The net result was an accumulation of extracellular enzymes and diminution of intracellular levels. It is not known whether phagocytic cells are capable of sequestering extracellular enzymes, but the possibility should be borne in mind when considering experiments involving cytochalasin B. A role for microtubules in endocytosis has not been evaluated but, again, this should be considered when examining data. The release and subsequent re-uptake of acid hydrolases by fibroblasts is discussed further in section 4.3.

Much of the discussion in this section has been concerned with

polymorphonuclear leucocytes. Whether the process of lysosomal enzyme secretion is similar in all cell types is not known with certainty. It is interesting that cyclic nucleotides, or agents affecting their intracellular levels, do not influence the release of acid hydrolases from macrophages to the same extent as polymorphonuclear leucocytes (Ringrose *et al.*, 1975) and in some cases have no detectable effects at all (Ackerman and Beebe, 1975). Such differences are perhaps not surprising, since lysosomal enzyme release from leucocytes occurs within minutes of exposure to the appropriate stimulus whereas macrophage secretion takes place over several hours or even days. Similarly, other secretory mechanisms may be operative in fibroblasts, where, at least in culture, the secretion of lysosomal enzymes occurs continuously. There is limited information about the mechanism by which parathyroid hormone induces the secretion of hydrolytic enzymes from osteoclasts during bone organ culture. It has been suggested that hormone treatment stimulates the synthesis of prostaglandins which, in turn, provide the signal for enzyme release. The evidence was that the addition of prostaglandin E_1 to a bone culture system caused the initiation of osteolytic activity and that aspirin, an inhibitor of prostaglandin synthesis, prevented parathyroid hormone-induced osteolysis, (Powles *et al.*, 1973).

4.3 Cellular uptake of acid hydrolases

The possibility that acid hydrolases secreted by cells might be taken up from the extracellular medium by other cells was suggested by observations on cultured cells derived from patients with certain inherited lysosomal enzyme deficiencies. Fibroblasts from patients with Hurler's syndrome are deficient in lysosomal α-L-iduronidase activity. When these cells were grown with normal fibroblasts the enzymic defect was corrected suggesting that α-iduronidase released by the normal cells had somehow been taken up by the mutant cells. Indeed, when α-iduronidase was added to the culture medium surrounding Hurler fibroblasts, about half of the enzyme activity was recovered intracellularly 48 hours later (Neufeld *et.al.*(1975). Similarly, fibroblasts from patients with inherited β-glucuronidase deficiency accumulate excessive amounts of acid mucopolysaccharides when grown in cell culture. This biochemical abnormality was corrected by the addition of bovine or human β-glucuronidase to the culture medium, again indicating that

extracellular enzyme had been taken up by the cells (Hall et al., 1973; Hickman and Neufeld, 1972). Histochemical studies showed that human β-glucuronidase was taken up by fibroblasts and eventually sequestered in lysosomes (Lagunoff, 1973).

Further information about the uptake of lysosomal enzymes was provided by studies on another genetic disorder called I-cell disease. Fibroblasts from affected individuals are deficient in several acid hydrolases whereas the culture media in which they are grown have elevated levels of the same enzymes (Wiesmann et al., 1971a; Leroy et al., 1972). The latter observation correlates with the increased acid hydrolase activities found in serum and other body fluids from patients with the disorder (Wiesmann et al., 1971b; den Tandt et al., 1974). To account for these findings, Wiesmann et al. (1971a, b) proposed that the lysosomal membrane was somehow defective thus allowing leakage of acid hydrolases. Evidence against this idea was presented by Hickman and Neufeld (1972) who showed that I-cell disease fibroblasts were capable of normal endocytosis and also retained acid hydrolases added to the culture medium. However, normal fibroblasts did not readily take up acid hydrolases secreted by I-cell fibroblasts. These experiments suggested that the defect in I-cell disease resided in the enzymes themselves and not in the lysosomal membrane. Hickman and Neufeld (1972) proposed that the endocytosis of acid hydrolases required the cellular recognition of a specific structural marker common to each of several enzymes. The mutation in I-cell disease could affect this marker so that the secreted acid hydrolases would not be recognised and taken up.

Several workers have compared the cellular uptake of acid hydrolases obtained from different tissue sources. Hexosaminidase secreted by normal fibroblasts and β-glucuronidase from platelets were rapdily endocytosed whereas the same enzymes from placenta, urine and liver were not (Hickman et al., 1974; Brot et al., 1974). Fibroblasts deficient in the appropriate enzyme were used for these studies since internalised enzyme was more easily detected. Brot et al. (1974) termed the rapidly endocytosed and the poorly endocytosed enzymes as "high uptake" and "low uptake" forms, respectively. Nicol et al. (1974) used DEAE-cellulose chromatography and polyacrylamide gel electrophoresis to separate two forms of β-glucuronidase from human spleen. The acidic form showed "high uptake" activity and the basic form "low uptake" activity when tested with fibroblasts.

In an attempt to establish the nature of the specific marker mediating the uptake of acid hydrolases and to account for the variation in uptake activity, Hickman et al. (1974) subjected hexosaminidase from normal fibroblast culture medium to periodate oxidation. Under the conditions used, catalytic activity was not affected but the "high uptake" activity of the enzyme was abolished. The authors concluded that carbohydrate residues formed at least part of the specific recognition marker on hexosaminidase. Furthermore, differences in carbohydrate content could explain the presence of "low and high uptake" forms of this and other acid hydrolases. Recently, more information about the uptake marker has been obtained. Kaplan et al. (1977) studied the endocytosis of β-glucuronidase by human fibroblasts in the presence of different carbohydrates. Enzyme uptake was competitively inhibited by phosphorylated sugars, and in particular by mannose-6-phosphate. Inhibition of β-glucuronidase uptake was also observed with certain phosphate-containing yeast mannans. On the other hand, non-phosphorylated sugars were only weakly inhibitive or did not inhibit at all. On the basis of these results it was proposed that fibroblasts had a plasma membrane receptor which recognised a phosphohexose-containing marker on acid hydrolases. This idea was supported by the additional observation that alkaline phosphatase treatment of platelet β-glucuronidase abolished its "high uptake" activity without affecting its catalytic activity. Similar conclusions were reached by Sando and Neufeld (1977) following detailed kinetic studies of α-iduronidase uptake and its inhibition by phosphorylated carbohydrates. Similar to β-glucuronidase, the uptake of α-iduronidase was abolished by treatment with alkaline phosphatase.

The possibility that another type of receptor might be involved in addition to the phosphohexose-specific receptor described above was suggested by Hieber et al. (1976, 1977). They discovered that β-galactosidase from bovine testis was a glycoprotein containing mannose and glucosamine residues but no sialic acid, fucose or galactose. The enzyme was rapidly taken up by human fibroblasts but this could be diminished by pre-treating the enzyme with α-mannosidase or by adding mannose-containing compounds to the culture medium. It was inferred that uptake involved the recognition of α-mannosyl markers in the β-galactosidase molecules. Although it was not clear whether the mannose was phosphorylated, the observation that β-galactosidase uptake was not

inhibited by the presence of β-glucuronidase strongly suggested that distinct receptors were responsible for mediating the uptake of the two enzymes. *In vivo* studies also provided evidence that the recognition and internalisation of acid hydrolases could be determined by non-phosphorylated carbohydrate moieties on the enzymes. When acid hydrolases were injected intravenously in rats they were cleared from the circulation within minutes. The enzymes were mainly taken up by the liver and sequestered in lysosomes (Thorpe et al., 1974; Stahl *et al.*, 1975b; 1976). The circulation clearance time of β-glucuronidase was greatly increased by the infusion of glycoproteins containing either terminal mannosyl or N-acetylglucosaminyl residues suggesting that similar residues were present on the enzyme and mediated its uptake by the liver (Stahl *et al.*, 1975a, 1976; Sly *et al.*, 1977). Both "low" and "high uptake" forms of β-glucuronidase were cleared by the liver at more or less equal rates in contrast to the situation with fibroblasts. This liver uptake system may be similar to the fibroblast system described above with respect to β-galactosidase. However, studies with other enzymes are required in order to clarify this possibility. From the foregoing information it is evident that the uptake of secreted lysosomal enzymes is complicated by the fact that different cells probably recognise different structural features on the enzyme molecules.

The characteristics of lysosomal enzyme recognition and uptake can be compared with other mammalian uptake systems. Glycoproteins with terminal galactosyl residues are specifically recognised by a hepatocyte plasma membrane receptor and endocytosed (see Ashwell and Morrell, 1974). That the uptake of acid hydrolases is mediated by a different system or systems was indicated by the observations that galactosyl-terminal glycoproteins did not interfere with acid hydrolase uptake. This was demonstrated by *in vitro* experiments with fibroblasts and by *in vivo* circulation clearance experiments (Hickman and Neufeld, 1974; Kaplan *et al.*, 1977; Sando and Neufeld, 1977; Stahl *et al.*, 1975a). Rat liver Kupfer cells possess a receptor which recognises glycoproteins with either terminal N-acetylglucosaminyl or mannosyl residues (Sly *et al.*, 1977; Stockert *et al.*, 1976). There may be some relationship between this system and those described by Hieber *et al.* (1976), Stahl *et al.* (1976) and Sly *et al.* (1977) in which the uptake of acid hydrolases was reduced by both mannosyl- and N-acetylglucosaminyl-terminal compounds. On the other hand, the fibroblast receptor which appeared to specifically

recognise phosphohexosyl residues on acid hydrolases (Kaplan _et al._, 1977; Sando and Neufeld, 1977) represents a novel system and it remains to be discovered whether it operates for other glycoproteins.

It was stated in section 1.2.2.1 that there are subtle differences in charge between multiple forms of acid hydrolases from body fluids and their intracellular counterparts. These differences will now be considered in the light of their possible significance to the uptake of acid hydrolases. Hexosaminidase A from human serum was partially resolved into a major and minor component by DEAE-cellulose chromatography. The minor serum hexosaminidase A component identified with the single liver hexosaminidase A component whereas the major and more basic component was unique to serum and other body fluids (Ikonne and Ellis, 1973). Neuraminidase treatment converted the major serum component to a form held less tightly by DEAE-cellulose. Similar treatment of the minor serum hexosaminidase A component or liver hexosaminidase A was without any detectable effect. These results led to the suggestion that the major serum form possessed terminal sialic acid residues while the form common to both serum and liver had no such residues or that they were inaccessible to neuraminidase action. Subsequent studies of several other acid hydrolases from human liver and serum and from human fibroblasts and fibroblast culture media confirmed the above observations in that the extracellular forms were distinguished from the intracellular forms by charge differences (Willcox and Renwick, 1977; Ellis _et al._, 1975). Also, in most cases, treatment with neuraminidase converted the unique serum components to more basic forms. The exceptions were human serum acid phosphatase, β-glucosidase and β-xylosidase which were not altered detectably by this treatment.

The general conclusion that certain extracellular forms of acid hydrolases possess terminal sialic acid residues may be relevant to the uptake of these enzymes by cells. It is known that the presence of terminal sialic acid residues on plasma glycoproteins tends to prevent their clearance from the circulation. Removal of sialic acid exposes the specific recognition markers described earlier and results in the rapid uptake of the asialoglycoproteins into hepatocytes (Ashwell and Morell, 1974). Sialic acid may play a similar role in regulating the uptake of acid hydrolases. It will be particularly interesting to see whether the neuraminidase-sensitive forms of acid hydrolases are related

to the "low uptake" forms discussed above. There is already some indirect evidence that this may be so. DEAE-cellulose chromatographic analysis and cellulose acetate electrophoretic analysis of normal fibroblast culture media and culture media surrounding I-cell disease fibroblasts revealed that, whereas the normal media contained both neuraminidase-sensitive and neuraminidase-insensitive forms of acid hydrolases, the I-cell media contained almost exclusively neuroaminidase-sensitive forms (Ellis et al., 1975; Vladitu and Rattazzi, 1975). As described at the beginning of this section, acid hydrolases released by I-cell disease fibroblasts were not readily taken up by normal fibroblasts. Considered together, these observations make it likely that the neuraminidase-sensitive forms of acid hydrolases, ie. those with terminal sialic acid residues, represent "low uptake" enzymes as described by Brot et al. (1974).

With respect to I-cell disease, Hickman and Neufeld (1972) postulated that the increased extracellular levels of acid hydrolases characteristic of the disorder were caused by the secretion of defective enzymes that could not be endocytosed (see beginning of this section). Based on their studies of the intracellular and extracellular forms of acid hydrolases in I-cell disease, Ellis et al. (1975) offered an alternative explanation for this defect. They proposed that during the biosynthesis of several acid hydrolases there was a common reaction which gave rise to "low uptake" and "high uptake" forms. A fault at this step, such that only the "low uptake" forms were completed and secreted, could explain the biochemical findings in cases of I-cell disease. They also speculated that the common-biosynthetic reaction, and hence the site of the defect in I-cell disease, might involve a sialyl-transferase-catalysed addition of sialic acid to enzyme precursors. At about the same time, Vladitu and Rattazzi (1975) suggested that the defect might lie in the failure to remove sialic acid residues from certain enzymes before secretion. This latter hypothesis was also favoured by Thomas et al. (1976) who showed that I-cell disease fibroblasts contained increased amounts of sialic acid-rich material and were deficient in an acid neuraminidase. However, studies on other patients with I-cell disease and their families will be necessary in order to establish that the neuraminidase deficiency is the primary lesion and not a secondary effect.

Although it remains to be seen whether lysosomal enzyme uptake

is a normal function of cell types other than fibroblasts or liver cells, the concept has some important implications, particularly with respect to the regulation of extracellular enzyme levels. As a result of the studies described in this and previous sections it is apparent that extracellular levels depend on rates of enzyme secretion, inherent enzyme stabilities and, presumably, the rates of enzyme uptake. Attempts to correct lysosomal enzyme deficiency disorders by giving patients intravenous injections of the appropriate hydrolase have been largely unsuccessful due to the rapid clearance of the infused enzymes from the circulation into the liver (see Desnick et al., 1976). The ability to modulate uptake and even to direct the enzyme to specific sites could be useful and might be possible by chemical modification of the recognition markers on the enzymes themselves. Hickman and Neufeld (1972) suggested that secretion and subsequent re-uptake might provide an alternative route for transporting acid hydrolases from their site of synthesis to the lysosome. The possible significance of such a scheme was demonstrated by the experiments of von Figura and Kresse (1975). Fibroblasts from patients with Sandhoff's disease (which lacked hexosaminidase A and B activity) were shown to take up exogeneous hexosaminidase from the culture medium. However, if the cells were incubated with cytochalasin B, hexosaminidase uptake was abolished. Similarly, incubation of normal fibroblasts with cytochalasin B caused an accumulation of extracellular acid hydrolase activity and a diminution of intracellular levels. These latter results were interpreted as inhibition of endocytosis by cytochalasin B with the outcome that acid hydrolases were secreted by the cells but not taken up again This experiment showed that the maintainance of intracellular acid hydrolase levels, at least in cultured fibroblasts, was dependent to a large extent on secretion and subsequent re-uptake of the same enzymes.

5. The human placenta

It has been emphasised throughout this introduction that a major obstacle in purifying lysosomal enzymes and investigating their biosynthesis is the low tissue concentrations of these enzymes. The decision to use the placenta for the study of human hexosaminidases was based on several considerations. Firstly, a survey of several mammalian tissues revealed that the human placenta was one of the richest sources of hexosaminidase activity (Walker et al., 1960). Secondly, the placenta

has been used as the starting material for the purification of hexosaminidases A and B (see section 1.3). Thirdly, the placenta was interesting in its own right, particularly with respect to the functioning of the lysosomal system during placental development. Pregnancy-associated changes in the activities of placental and serum hexosaminidase have been reported (see section 5.4). Finally, the placenta was one of the few human tissues which could be obtained in virtually unlimited quantities and in a fresh condition.

Before outlining the aims of the present project, a description of placental structure and function will be given.

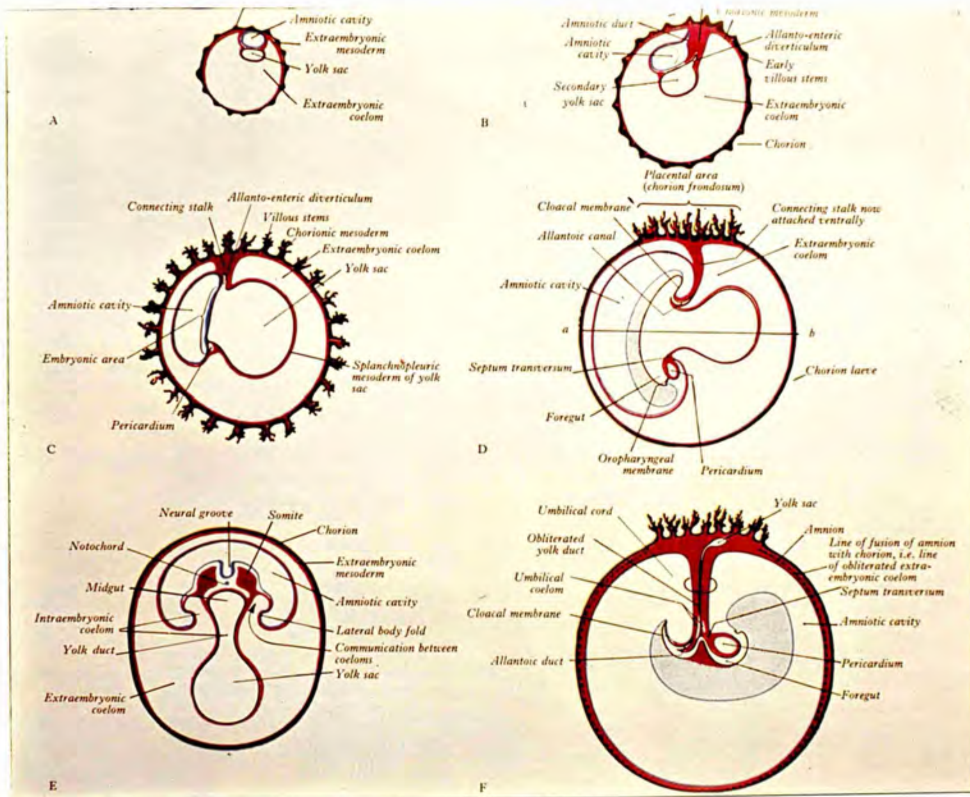
5.1 Structural development of the human placenta

In comparison to other mammals, relatively few detailed studies have been published on the early stages of development of the human placenta. This is due primarily to the difficulties encountered in obtaining experimental material at the correct developmental stage and under controlled conditions. Most human pregnancies are discovered several weeks after implantation has occurred. Nevertheless, light and electron microscopic examinations of pre- and post-implantation fertilised ova were reported by Hertig and Rock (1941, 1945), Hertig *et al.* (1956) and Hamilton and Boyd (1960) and these formed the basis of the descriptions given below.

During the six or seven days preceding implantation the fertilised human ovum develops into a spherical structure called the blastocyst (Fig. 5.1A). This consists of an outer layer of cells called the trophoblast surrounding a fluid-filled cavity or extra embryonic coelom. Within the extraembryonic coelom and attached to a region of trophoblast is an inner cell mass or embryonic region which eventually gives rise to the foetus. Foetal development will not be considered here. On the other hand, the trophoblast differentiates to form the various structures comprising the placenta and is the subject of the present section.

At the time of implantation, around the seventh day after fertilisation, the trophoblast of the blastocyst comes into contact with, and adheres to, the epithelium of the uterine wall. There is a rapid proliferation of trophoblast cells at the point of contact, many of which fuse together to form a multinucleated mass of cytoplasm termed the syncitiotrophoblast. Simultaneously, maternal tissue is engulfed and eroded by the invading trophoblast such that by the tenth day after

FIG. 5.1 EARLY STAGES IN THE DEVELOPMENT OF THE FOETO-PLACENTAL UNIT



fertilisation the blastocyst is completely embedded in the wall of the uterus. At this stage the trophoblast consists of two distinct layers, an inner or formative layer comprising well-defined cells and termed the cytotrophoblast and an outer layer of syncitiotrophoblast. By about the fifteenth day after fertilisation spaces or lacunae become visible in the syncitiotrophoblast and, as a result of the erosion of maternal blood vessels, these become filled with maternal blood. The strands of trophoblast left between the enlarging lacunae are called villi and each villus consists of a cytotrophoblast core covered with an outer layer of syncitiotrophoblast (Fig. 5.2). The villi grow rapidly due to the proliferation of the cytotrophoblast and the concomitant formation of more syncitiotrophoblast and ramify deeply into the uterine tissue. Each villus soon acquires its own blood vessel which connects to the circulatory system of the developing embryo. Since the lacunae between the villi are filled with maternal blood and the capillaries of the villi with foetal blood, transfer of dissolved substances between mother and foetus can occur via the syncitiotrophoblast and cytotrophoblast layers of the villi (Fig. 5.3). Although the structural changes just described take place over the entire surface of the blastocyst, the greatest proliferation of villi occurs at that region which initially made contact with the uterine wall and which supports the embryo, ie. the embryonic pole of the blastocyst. Continued growth and branching of the villi produces the thick mass of villous tissue which comprises the bulk of the placenta. The region of trophoblast from which these villi arise acquires a lining of mesoderm and is termed the chorion frondosum. In contrast to the villi formed at the embryonic pole, the villi covering the remainder of the blastocyst gradually atrophy and by the twelth week after fertilisation are absent altogether. The area of trophoblast denuded of villi in this way is termed the chorion laeve (Fig. 5.1D).

As well as the differentiation of the villi, other placental structures are formed during the first few weeks after implantation. The amnion is apparent at the time of implantation as a layer of cells partially covering the inner cell mass and separating it from the outer wall of the blastocyst. However, as the foetus develops and the amniotic sac fills with fluid, the amnion itself begins to grow and distends outwards, gradually surrounding the foetus and coming in close contact with the innermost surface of the chorion laeve (Fig. 5.1F). Apposition

FIG. 5.2 TRANSVERSE SECTION OF A TERMINAL VILLUS STAINED WITH HAEMATOXYLIN AND EOSIN

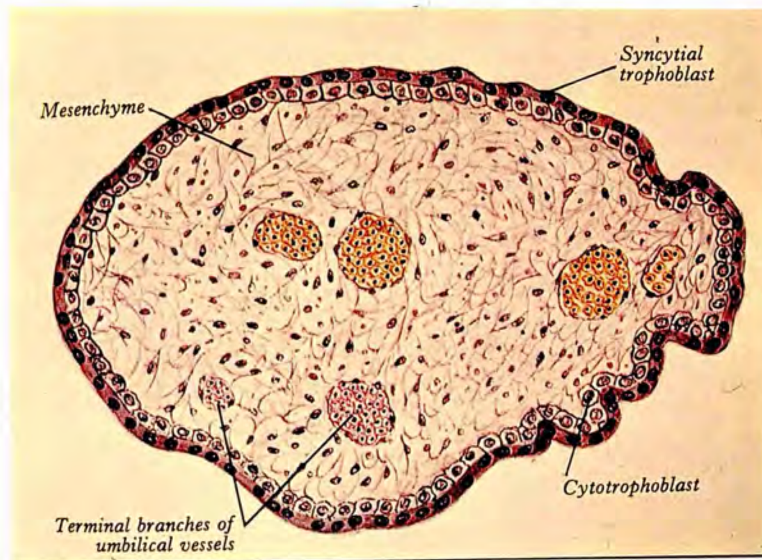
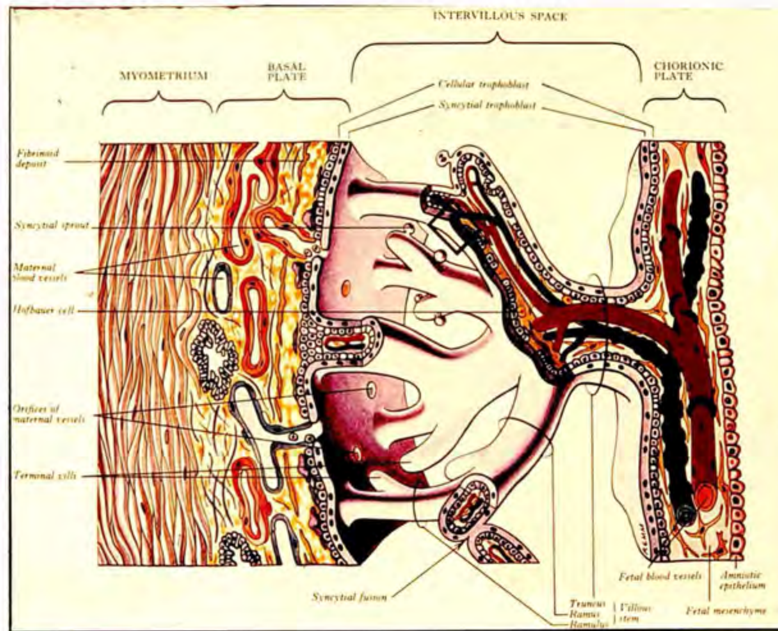


FIG. 5.3 A SCHEMATIC DIAGRAM SHOWING THE RELATIONSHIP BETWEEN PLACENTAL AND MATERNAL TISSUES



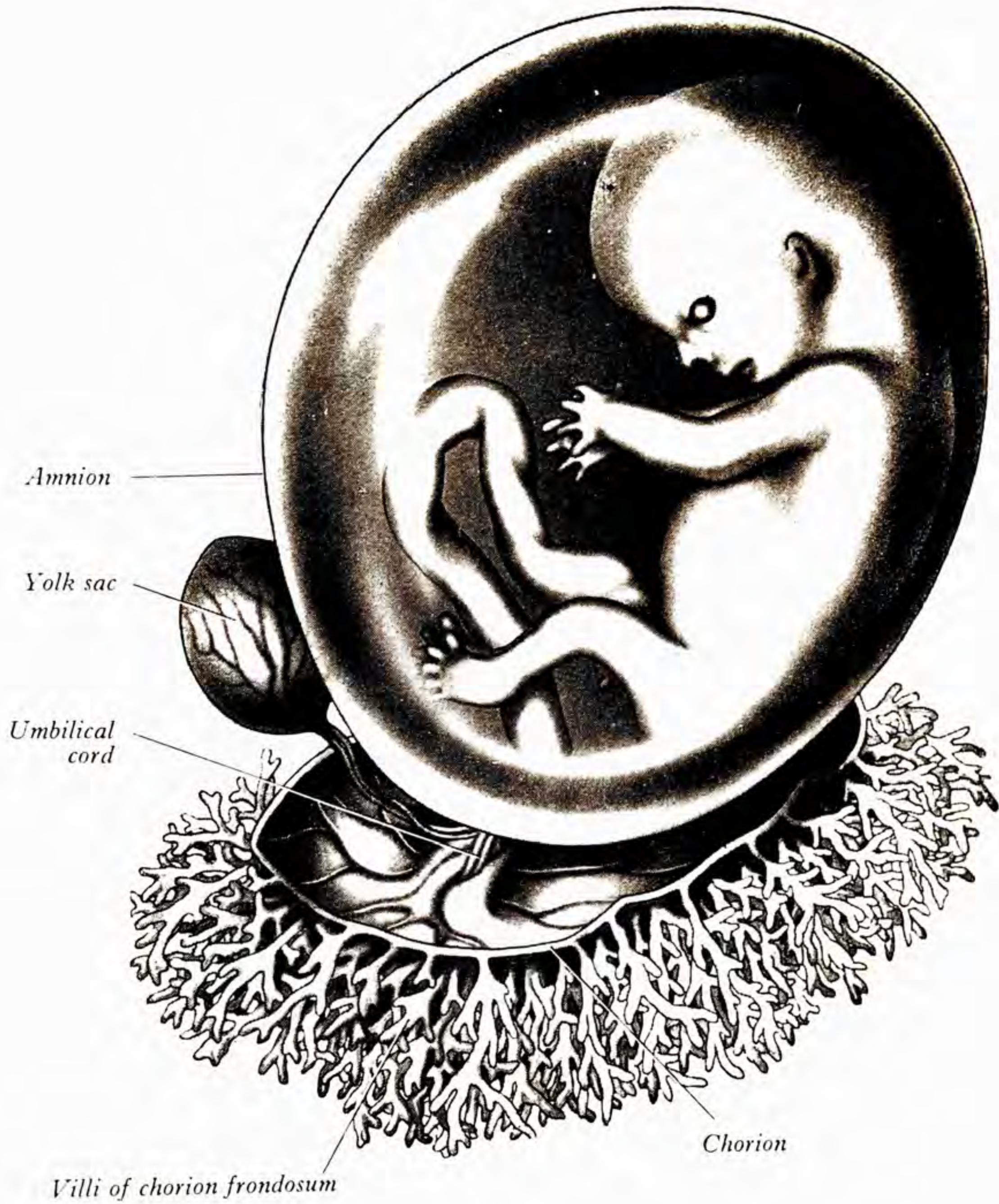
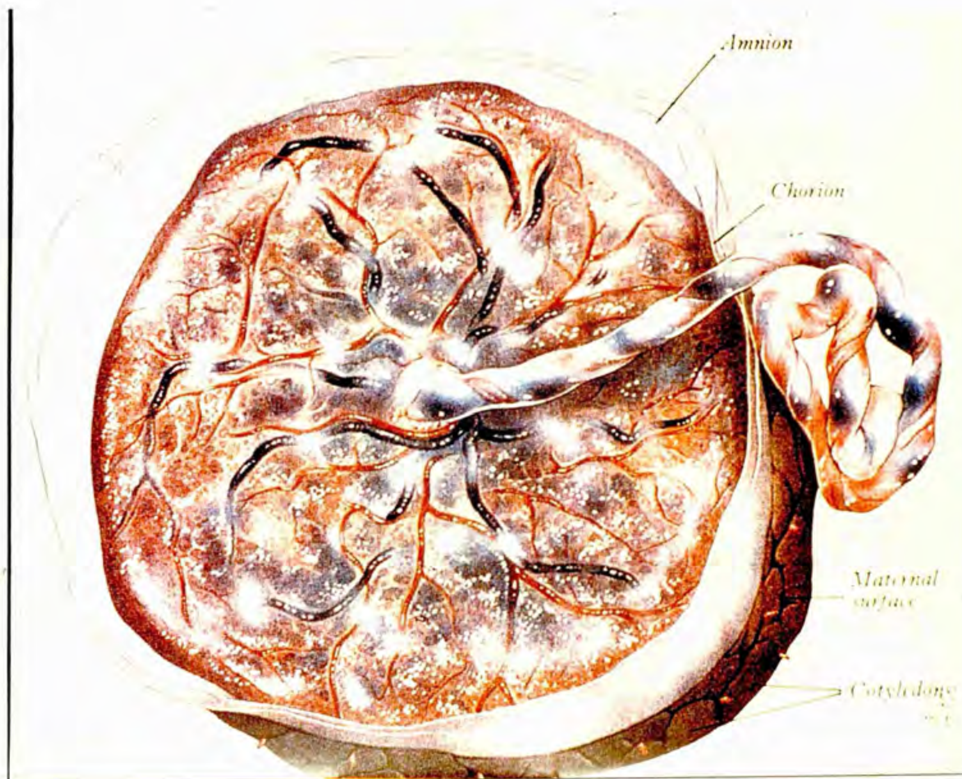


Fig 5.4 The foeto-placental unit at about 8 weeks of gestation

FIG. 5.5 A HUMAN PLACENTA AT TERM



of the amnion and the chorion laeve generally occurs within fifteen to twenty weeks of gestation. Distension of the amniotic sac also causes the amnion to converge on the stalk of mesoderm which connects the foetus to the trophoblast. The connecting stalk with its covering of amnion is termed the umbilical cord. Within the mesoderm of the cord are embedded two endodermal tubes which differentiate to form theumbilical blood vessels. These carry blood from the placental villi to the foetus and vice versa.

By the twelth week of gestation, ie. by the end of the first trimester, the placental unit is considered to consist of the chorion frondosum and associated villi, the chorion laeve and the amnion. The umbilical cord is also included since it is the only direct link between the foetus and the placenta proper. Although the placenta undergoes no major structural differentiation after the first trimester, there is a considerable increase in size and weight. For example, at the eight to ten week stage the amniotic sac contains about 50ml of amniotic fluid and the villi of the chorion frondosum take the form of a disc about 4cm in diameter and weighing only a few grammes (Fig. 5.4). At term, ie. about forty weeks, there is about one litre of amniotic fluid and the placental villi form a discoid mass about 20cm in diameter, 5cm thick and weighing 400-500g (Fig. 5.5).

5.2 The placenta at term

After delivery of the foetus the placenta becomes separated from the uterine wall and is expelled from the uterine cavity. Apart from the ruptured amnion and chorion laeve and the loss of amniotic fluid, the placenta is structurally intact when shed. Thin patches of uterine tissue may remain adherent to its maternal surface. A typical term placenta is shown in Fig. 5.5 and some of its physical properties were described at the end of section 5.1. The comparative ease with which term human placentas can be obtained has enabled numerous structural and ultrastructural studies to be carried out (see Dempsey and Wislocki, 1953; Boyd and Hughes, 1954; Wislocki and Dempsey, 1955; Wynn, 1972; Boyd and Hamilton, 1970). The various anatomically distinct regions of the placental unit are described below.

5.2.1 The chorionic villi

The bulk of the term placenta consists of villi which arise from

the chorion frondosum. Each villous stem forms a number of branches which in turn form sub-branches, all of which are interwoven. The intervillous space is filled with maternal blood. This arrangement is shown schematically in Fig. 5.3 and a transverse section of a villus is shown in Fig. 5.2. Each villus is composed of a mesenchyme core ensheathed by a layer of cytotrophoblast and a layer of syncitiotrophoblast.

The syncitiotrophoblast is a true multinuclear syncitium with no dividing cell walls. Its cytoplasm contains numerous secretory vacuoles, lysosomes and both free and endoplasmic reticulum-bound ribosomes. In addition there is a network of microfilaments. The surface of the syncitiotrophoblast in direct contact with the maternal blood has a microvillous border and beneath the surface are pinocytotic vacuoles. In contrast, the cytotrophoblast contains no secretory vacuoles and comparatively few lysosomes and membrane-bound ribosomes. However, free ribosomes, mitochondria and microfilaments are present. It should be noted that the covering of syncitiotrophoblast may be thinned and that the cytotrophoblast may be absent over certain areas. The villous core consists of connective tissue in which are found blood capillaries, fibroblasts and specialised macrophages called Hofbauer cells.

The plate of chorionic tissue from which the villi arise, ie. the chorion frondosum, is covered on its foetal aspect by amniotic epithelium. Beneath this is a layer of connective tissue carrying blood vessels, then a layer of cytotrophoblast and finally a layer of syncitiotrophoblast. The connective tissue is particularly prominent in the chorionic plate and contains mostly fibrous material with few cells.

5.2.2 The amnion

This is the innermost of the two foetal membranes and is about 0.2mm thick and composed of several distinct layers. The epithelial layer consists of cuboidal cells with surface microvilli which, in vivo, are in contact with the amniotic fluid. The amniotic epithelium is supported by a compact layer of reticular fibres which form a complicated meshwork and probably account for the strength of the amnion. Beneath this is a layer of fibroblasts and Hofbauer cells in a matrix of mucus-like material.

5.2.3 The chorion laeve

This is the outermost of the two foetal membranes and separates the amnion from the uterine wall. Although they are in direct contact with each other and, indeed, may appear to be one, the chorion laeve and the amnion can be easily separated, even at term. The chorion laeve is about 0.1mm thick and, similar to the amnion, is composed of several distinct layers. Adjacent to the amnion is a layer of fibroblasts, followed by a reticular layer consisting of fibroblasts, Hofbauer cells and blood vessels in a meshwork of reticulin fibres. Finally, there is a layer of cytotrophoblast which, *in vivo*, is in contact with the uterine wall. Syncitiotrophoblast is largely absent from term chorion laeve or is considerably thinned.

5.2.4 The umbilical cord

At term, the cord is about 50cm long and about 2cm in diameter. It consists of an outer covering of amniotic epithelium which encloses a mass of mesoderm. The mesoderm itself is composed of widely scattered fibroblasts separated by an extensive matrix of collagen fibres and mucopolysaccharide. An artery and vein run the length of the cord thus connecting the foetal and placental circulations.

5.3 Placental function

Reduced to its simplest terms the placenta can be regarded as a barrier which allows the selective transfer of materials between maternal and foetal circulations. The villi, and particularly the terminal villi, are the main functional units of the placenta and their structural characteristics are such that they present an enormous surface area over which the various transfers can occur. The "placental barrier" separating foetal and maternal blood consists of consecutive layers of vascular endothelium, villous mesenchyme, cytotrophoblast and syncitiotrophoblast. In some cases transfer across these layers can be explained by simple diffusion while in others more complicated processes are involved. The materno-foetal transport of various substances is discussed below together with aspects of the endocrine and immunological functions of the placenta. It is not the purpose of this section to review placental function in detail but rather to illustrate the functional complexity of the organ. Where possible, examples are drawn from experiments which used human material but some information obtained from studies of other

mammals is also included. This distinction is pointed out because the placenta, perhaps more so than any other organ, shows considerable structural variation between species and hence identity of specific functions cannot necessarily be assumed.

5.3.1 Transfer function

Maternal and foetal blood do not mix at any stage of gestation. Materials present in maternal blood that are required for the growth and development of the foetus are transferred via the placental villi to the foetal circulation.

Studies suggested that oxygen and water were transferred from the maternal to the foetal circulation by simple diffusion (Bartels et al., 1962; Battaglia et al., 1960). Administration of deuteriated water to pregnant human subjects revealed that the net rate of movement of water across the placenta to the foetal circulation increased with gestation (Hellman et al., 1948). The net rate of sodium transfer was also shown to increase with gestation (Flexner et al., 1948). The progressive rise in the rate of transfer of these materials probably reflects the increase in surface area of the villi caused by extensive villous branching and by the thinning of the syncitiotrophoblast. However, simple diffusion is not sufficient to explain the transfer of potassium. Potassium levels fell in pregnant rats maintained on a potassium-free diet whereas placental and foetal levels remained more or less normal (Dancis and Springer, 1970). It was suggested that the placenta actively protected the foetus from potassium deprivation but the precise mechanism remains unknown. In human subjects, calcium concentration was found to be higher in the foetal than in the maternal blood suggesting the presence of an active transport system in the placenta (Delivoria - Papadopoulos, et al., 1967). Foetal blood levels for most amino acids were also shown to be higher than maternal (Ghadimi and Pecora, 1964; Young and Prenton, 1969). Thus, as for calcium, the net transfer towards the foetus was against a concentration gradient. In addition, metabolic poisons were reported to inhibit the uptake of amino acids by human placental slices incubated in vitro (Longo et al., 1973; Smith et al., 1973). Both these observations provide strong evidence that the transfer of amino acids across the placenta is by active transport. On the other hand, maternal blood glucose levels were shown to be higher than in the foetus (Dawes, 1968). Furthermore, the net transfer of glucose towards the foetus was

more rapid than could be accounted for by simple diffusion (Widdas, 1952; Holmberg et al., 1956) and could not proceed against a concentration gradient (Chinard et al., 1956). It is now generally held that glucose transfer across the placenta is by facilitated diffusion. Interpretation of data relating to the transfer of carbohydrates and amino acids is complicated by the ability of the placenta to metabolise these and other compounds (Szabo and Grimaldi, 1970).

The transplacental transport of proteins is less well understood than that of simpler molecules such as oxygen and amino acids. Certain maternal plasma proteins were shown to be transferred to the foetal circulation (Gitlin et al., 1964; Dancis et al., 1961). The process was selective and although albumin and immunoglobulin IgG were transferred, virtually no transfer of IgM, IgA, IgD or IgE was detected. The significance of this observation is discussed in section 5.3.3. Specific receptors for IgG were demonstrated on the microvillus surface of the human syncitiotrophoblast ie. the surface of the placenta in direct contact with maternal blood (Gitlin and Gitlin, 1974; Balfour and Jones, 1977). This finding was consistent with the proposal of Brambell (1970) that the binding of IgG to the placental surface could be a prerequisite to IgG pinocytosis and transplacental transport. However, it is not yet clear how the IgG molecules are transported across the placenta without being degraded by proteolytic enzymes. IgG-specific receptors were also detected in first trimester placental cell membranes (Gitlin and Gitlin, 1974). This is surprising because there is very little transplacental transport of IgG during early pregnancy, most occurring towards term (Gitlin and Biasucci, 1969).

In the examples given above the transfer of substances from mother to foetus was emphasised. It is also generally believed that the end products of foetal metabolism are transferred across the placenta to the maternal circulation. Studies of carbon dioxide transfer in humans (Bartels et al., 1962) and of bilirubin and urea transfer in non-human primates (Bashore et al., 1969; Battaglia et al., 1968) suggested that net movement towards the maternal blood was by simple diffusion.

5.3.2 Endocrine function

The placenta is unique among endocrine organs in being able to synthesise both steroid and polypeptide hormones. Solomon, (1960) and Pion et al. (1966) showed that the perfused human placenta was capable

of synthesising progesterone from pregnenolone or cholesterol but not from acetate. Similarly, perfusion experiments provided evidence for the conversion of cholesterol to oestrogen by the placenta (Telgedy *et al.*, 1970). The inability of the placenta to use acetate for steroid biosynthesis suggested that the normal production of these hormones was dependent on a supply of the appropriate precursor from the foetal and maternal circulations.

There is good evidence that the placenta is capable of *de novo* synthesis of certain polypeptide hormones. Gusden and Yen (1967) and Suwa and Friesen (1969) showed the incorporation of radioactive amino acids into immunoreactive human placental lactogen by cultured placental explants and placental slices, respectively. The same *in vitro* systems were used by Benagiano *et al.* (1972) and Maruo *et al.* (1974) to demonstrate the incorporation of radioactive amino acids into immunoprecipitable human chorionic gonadotrophin. Immunohistochemical studies suggested that both these hormones were synthesised by the syncitiotrophoblast (Midgley and Pierce, 1962; Thiede and Choate, 1964; Sciarra *et al.* 1973). Progressively increasing amounts of placental lactogen are secreted into the maternal circulation during normal pregnancy (Genazzani *et al.*, 1972). Chorionic gonadotrophin is also released into the maternal circulation reaching a peak level at around 60 days after fertilisation followed by a decline to a lower level which is maintained until term (Saxena, 1971). Other polypeptide hormones including adrenocorticotrophic hormone, chorionic thyrotrophin, insulin and oxytocin have been identified in placental tissue extracts but experimental evidence that the placenta is their site of synthesis is lacking.

5.3.3 Immunological function

The placenta has two immunological functions. First of all, it allows the selective transfer of certain maternal antibodies such that the foetus is born with some degree of passive immunity. Secondly, the placenta protects both itself and the foetus, which are histoincompatable with the mother, from immunological rejection during gestation. The precise way in which this is brought about remains unclear but the available information suggests that several different mechanisms may be involved.

The basis of foeto-maternal histoincompatability is the paternal component in the genetic make-up of the foetus and the placenta.

Although the trophoblast is in direct contact with maternal tissue there is no visible tissue response by the mother, as might be expected. The apparent immunological inertness of the placenta could reflect a lack of antigenic sites or their masking by a barrier. In this context several workers have observed an amorphous, mucopolysaccharide-like layer covering the surface of the syncitiotrophoblast of human and other mammalian placentas (Wynn, 1967; Kirby et al., 1964; Currie and Bagshawe, 1967). Moreover, neuraminidase treatment of the mouse trophoblast rendered it antigenic when transplanted to an adult host suggesting that sialic acid residues on the trophoblast surface might mask potential antigenic sites. Another way in which the placenta is thought to protect the foetus from immunological attack by the mother is by preventing the transfer to the foetal circulation of certain maternal antibodies and in particular IgM (Gitlin et al., 1964). Unlike IgG, which is transferred, antibodies of the IgM class are known to have potent cytotoxic effects on tissues and would almost certainly damage the foetus.

There is some evidence that the mother may become immunologically tolerant to the foeto-placental unit. For example, in humans, the survival of paternal-derived skin grafts on the mother was prolonged after pregnancy had occurred (Beer et al., 1971). Small clusters of trophoblast cells are normally shed into the maternal circulation during the course of pregnancy and may be responsible for inducing this tolerance to paternal antigens (Attwood and Park, 1961). The placental proteins, human chorionic gonadotrophin, human placental lactogen, pregnancy associated α_2-glycoprotein and pregnancy specific β_1-glycoprotein have each been reported to have immunosuppressive properties *in vitro* (Han, 1975; Contractor and Davies, 1973; von Schoultz et al., 1973; Horne et al., 1976). It is thought that the release of these proteins into the maternal circulation or their presence on the surface of the syncitiotrophoblast might suppress a maternal lymphocyte-mediated attack on the foeto-placental unit.

In summary, during its lifespan of about 40 weeks the human placenta performs a wide variety of metabolic functions which can be compared to those normally carried out by the lungs, the intestinal tract, the kidneys and the liver. In addition, the placenta is a complex endocrine organ and is probably immunologically competent. Szabo and Grimaldi (1970) stated that the placenta might well be the most complicated

mammalian organ and, with the possible exception of nervous tissue, this could be true.

5.4 The lysosomal system of the placenta

Studies using a combination of light or electron microscopy and enzyme cytochemistry revealed the presence of acid hydrolase-containing vacuoles in the syncitiotrophoblast and, to a lesser extent, in the cytotrophoblast of both first trimester and term human placentas (Terzakis, 1963; Lister, 1964; Tighe et al., 1967; Christie, 1968). These vacuoles were interpreted as lysosomes and, generally, more were observed in the first trimester than in the term samples. Christie (1968) noted that the cytochemical reaction for acid phosphatase was greater in first trimester than term syncitiotrophoblast whereas trace amounts of β-glucuronidase were detectable throughout gestation. As described in section 5.2 Hofbauer cells have been identified in several regions of the placenta including the villi, the amnion and the cord. These cells are now considered to be a specialised type of phagocyte, probably related to the macrophages. Their cytoplasm contained numerous vacuoles and they were shown to have a relatively high content of hydrolytic enzymes (Wislocki and Dempsey, 1948; Fox and Khargongor, 1969, 1970). Bourne (1962) was unable to detect acid phosphatase activity in the Hofbauer cells of term amnion and chorion laeve and Wislocki and Dempsey (1948) and McKay et al. (1958) reported that the acid phosphatase activity of syncitial Hofbauer cells was high in first and second trimester samples and decreased towards term.

In comparison to other tissues such as the liver, there have been few reports of the isolation of lysosomes from placental tissue. Differential centrifugation was used to partially purify lysosomes from human placental villi (Contractor, 1969; Contractor and Shane, 1972; Lewicki and Trzeciak, 1972b) and amnion (Schwartz et al., 1976a). Detailed reports of the subcellular distribution of some acid hydrolases in both first trimester and term villi were presented by Corash and Gross (1973, 1974). They used differential centrifugation to separate two lysosome populations as identified by the structure-linked latency of acid hydrolases and by electron microscopy. The bulk of acid hydrolase activity was associated with lysosomes in the classic lysosomal-mitochondrial fraction but significant activity was also associated with particles in the soluble-microsomal fraction. Further complexity was

revealed when the above lysosome-containing fractions were subjected to isopycnic centrifugation. Each fraction showed a bimodal density distribution with respect to latent acid hydrolase activity, suggesting a spectrum of lysosomes of different size. This heterogeneity is perhaps not surprising in view of the many different cell types which comprise the placental villi.

In addition to the ultrastructural and subcellular distribution studies described above, several workers have carried out quantitative assays for a number of acid hydrolases in crude placental extracts. Hexosaminidase and β-glucuronidase specific activities were determined in samples of villi, foetal membranes and cord from term placentas. The specific activities of both enzymes were highest in the foetal membranes and lowest in the cord (Walker et al., 1960; Platt and Platt, 1969b). Cathepsin B_1 specific activity was higher in the villi than in the amnion (Warwas and Dobryszycka, 1976). Changes in the specific activities of acid hydrolases as a function of placental development have been reported. Hexosaminidase, acid phosphatase and arylsulphatase activities decreased with gestation (Edlow et al., 1971; Corash and Gross, 1974). In each of these cases the enzyme assays were carried out on poorly-defined homogenates probably consisting of different placental regions. Also, only first trimester and term samples were examined. Lewicki and Trzeciak (1972a) studied arylsulphatase in placentas obtained during the second trimester as well as during the first trimester and at term. Their data showed that arylsulphatase specific activity increased to a peak at around 24 weeks of gestation and decreased towards term.

Although lysosomal enzymes have been detected in each of the distinct anatomical regions of the human placenta, the physiological significance of these observations remains largely hypothetical. It is generally held that lysosomes are involved in the turnover of cellular material which occurs during placental growth and development (Christie, 1968; Corash and Gross, 1974, Urbani, 1964). The establishment of the foeto-placental unit in the uterus requires the destruction of some maternal tissue, a function also attributed to placental lysosomes. Brambell (1966, 1970) proposed that placental lysosomes might be responsible for the selective destruction of certain potentially harmful maternal antibodies and so could assist in the protection of the foetus from immunological attack by the mother. Recently, Schwartz et al. (1976a) demonstrated phospholipase A_2 in the lysosomal fraction obtained from

human foetal membranes and suggested that the enzyme might initiate the biochemical events which result in the onset of labour. This hypothesis was based on the ability of phospholipase A_2 to cleave glycerophospholipids to yield arachidonic acid which , in turn, serves as a precursor for prostaglandin synthesis. The administration of either arachidonic acid or certain prostaglandins to pregnant human subjects is known to result in abortion or premature delivery (Macdonald et al., 1974; Karim, 1972). In a subsequent paper, Schwartz et al. (1976b) further proposed that the activation of phospholipase A_2 might be mediated by progesterone and showed that a progesterone-binding protein appeared in human foetal membranes towards term.

Several workers reported that maternal serum hexosaminidase activity increased throughout gestation and decreased to normal levels within hours of birth (Walker et al., 1960; Furaya and Kishinami, 1964; Woollen and Turner, 1965; Platt and Platt, 1969a). These same workers postulated that the placenta might be the source of the increased serum enzyme activity. Using starch gel electrophoresis, Stirling (1972) and Price and Dance (1972) showed that the rise in the pregnancy serum hexosaminidase level was largely due to the appearance of hexosaminidase I_2. However, Stirling (1972) failed to detect this intermediate form in extracts of villi or foetal membranes and concluded that the placenta was not the tissue source. Leung et al. (1977) compared the hexosaminidase activities of maternal serum and the placenta. Although polyacrylamide gel electrophoretic analysis revealed the presence of significant amounts of an intermediate form in both serum and placenta, the kinetic properties of total hexosaminidases from these two sources were different and led the authors to conclude that the placenta was not the origin of the serum I_2 activity. The validity of this conclusion is open to question since the kinetic studies were carried out on total hexosaminidase rather than individual multiple forms. Further doubt is raised by the inability of these workers to detect hexosaminidase B in placental extracts by polyacrylamide gel electrophoresis. This form is widely reported to be present in substantial amounts in the placenta (Huddleston et al., 1971; Stirling, 1972; Tallman et al., 1974; Geiger and Arnon, 1976). In an attempt to establish the origin of pregnancy serum I_2, Jones et al. (1975) raised antisera to the A and B forms from placenta and studied their reactivity towards hexosaminidase from maternal tissues and serum. The antisera did not cross-react with

maternal tissue hexosaminidase in immunodiffusion studies. On the other hand, anti-(placental hexosaminidase B) serum decreased pregnancy serum hexosaminidase activity to a normal level in an immunoprecipitation experiment. Although only total hexosaminidase activity was assayed and the ability of the antiserum to precipitate hexosaminidase from normal serum was not reported, the authors interpreted their results as evidence in favour of a placental origin for pregnancy serum I_2. The total lack of cross-reactivity between the antisera and the maternal tissue hexosaminidases is difficult to reconcile with other reports in the literature. In cases where placental hexosaminidases have been used as antigens, the resulting antisera reacted with adult tissue enzymes (Carroll and Robinson, 1973; Srivastava _et al._, 1974b). Thus, the available information does not permit any definite conclusion as to the origin of pregnancy serum hexosaminidase activity.

There is some evidence that the placenta might be the source of the multiple forms of pregnancy serum alkaline phosphatase and leucine-aminopeptidase. In both these instances electrophoretic and kinetic studies revealed that the multiple forms from serum identified with forms present in placental extracts and not with the corresponding enzymes from maternal tissues (Boyer, 1961; Meade and Rosalki, 1964). As yet, other than a report of the release of certain proteases from first trimester trophoblast in culture (Ayavou _et al._, 1966), the role of the placenta in the secretion of hydrolytic enzymes has not been investigated directly.

5.5 The human placenta as an experimental system

Although the human placenta has been perfused _in situ_ (Jaffe and Paterson, 1966), this type of study is not suitable for routine use. On the other hand, a variety of _in vitro_ systems have been prepared from placental tissue. For example, cultured explants of placental villi were used to investigate the biosynthesis of placental lactogen (Gusden and Yen, 1967) and chorionic gonadotrophin (Maruo _et al._, 1974). Certain placental cells were established in monolayer culture by Fox and Khargonkor (1970) and Harpaz _et al._ (1975). Slices of placental villi were used to study the synthesis of cholesterol (Villee, 1968), the uptake of amino acids (Dancis _et al._, 1968, Gussek _et al._, 1975) and the synthesis and secretion of placental lactogen (Suwa and Friesen, 1969).

Several workers have reported the preparation of cell-free protein synthesising systems from the placenta (Mori, 1965; Laga _et al._, 1970; Laga _et al._, 1973; Boime _et al._, 1974). Although the placenta-derived system was able to translate synthetic messages and globin mRNA, its amino acid incorporating activity was not as great as the comparable system prepared from rat liver. Also, it was noted that cell-free systems prepared from first trimester tissue were more active than those from term tissue and studies suggested that a deficiency of some component in the post-ribosomal supernatant was probably responsible. Procedures are available for the preparation of free and membrane-bound polyribosomes and of ribosomal subunits from the placenta (Laga _et al._, 1970; Boime _et al._, 1974; Vanduffel _et al._, 1975). The mRNAs coding for lactogen and chorionic gonadotrophin have been isolated from both first trimester and term placental villi and successfully translated in heterologous cell-free systems (Boime _et al._, 1976; Chatterjee _et al._, 1976; Cox _et al._, 1976). The availability of these methods made it possible to investigate the mechanisms which control the amounts of these polypeptide hormones produced by the placenta during pregnancy. For example, the greater synthesis of placental lactogen by term compared to first trimester placentas correlated with increased amounts of translatable lactogen mRNA (Boime _et al._, 1976). This was confirmed by McWilliams _et al._ (1977) who prepared a cDNA copy of lactogen mRNA and found more hybridisable material in term than first trimester villi. In addition, by using the cDNA probe, they were able to quantitate the lactogen genes in placentas obtained at different stages of gestation. The results indicated that gene amplification was not responsible for the increased production of lactogen mRNA and pointed to a transcriptional control mechanism.

It should be noted that there are certain inherent disadvantages in the use of the placenta as an experimental system. As described earlier, the placenta contains many different cell types which, in most cases, cannot be separated. This cellular heterogeneity could complicate the interpretation of some data, particularly where drug treatments have been involved. An observed effect may reflect the net response of several cells. In certain other tissues, such as the liver, isolated cell preparations are used to overcome some of the problems associated with cellular heterogeneity. However, because of the comparatively

large variety of different placental cells and the particular structural characteristics of the syncitiotrophoblast, it is unlikely that this approach will be applicable to the placenta. During pregnancy and even during birth, the placenta can be exposed to a diverse array of drugs and to physical stresses. The effects of these factors on the placenta are difficult to assess and control, and may very from case to case. Therefore, the preparation of reproducible in vitro systems may be difficult to attain.

6. Aims

Comparatively little has been published on the biosynthesis of complex enzymes. To date, most studies have been concerned with simple and abundant proteins, usually from non-human sources. As stated in the foregoing introduction the enzyme hexosaminidase is extremely interesting from a number of standpoints. Firstly, it is a complex glycoprotein which exists in a number of different structural forms varying in both protein and carbohydrate compositions. Secondly, the enzyme has a particular intracellular localisation, the lysosome. Thirdly, several well characterised diseases are associated with deficiencies in one or more of these enzyme forms. Finally, the human placenta is a relatively rich source of the enzyme and pregnancy-associated changes in the activities of placental and serum hexosaminidase have been reported.

The study of human hexosaminidase biosynthesis was feasible because of the availability of large quantities of placental tissue. General problems of interest are those concerned with subunit synthesis and assembly, the addition of carbohydrate, sites of synthesis and the packaging of the enzyme in lysosomes. Of additional interest is the distribution and function of this enzyme in relation to placental structure, function and development. The project has been divided into two main areas of study: firstly, hexosaminidase in relation to placental structure and development and, secondly, aspects of hexosaminidase biosynthesis.

MATERIALS AND METHODS

General laboratory chemicals were obtained from B.D.H. Ltd., Poole, Dorset and were of "AnalaR" grade wherever possible. Substrates for the assay and location of hydrolytic enzymes, α-D-methylmannoside, Triton X-405 and Triton X-100 were purchased from Koch-Light Ltd., Colbrook, Bucks. Colchicine, heparin, cytochalasin B, cycloheximide, agarose, human albumin (fraction V), bovine serum albumin (fraction V), enzymes and co-factors for cell-free protein synthesis were obtained from Sigma(London) Chemical Co., Kingston-upon-Thames, Surrey. The Boehringer Corp. (U.K.), Lewes, East Sussex supplied micrococcal nuclease and standard proteins for molecular weight estimation. Sephadex G-200, DEAE-Sephadex, Cm-Sephadex, concanavalin A-Sepharose, cyanogen bromide-activated Sepharose 4B were obtained from Pharmacia (G.B.) Ltd., London. DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were obtained from Whatman Biochemicals, Maidstone, Kent. International Enzymes Ltd., Windsor, Berks supplied oligo(dT)-cellulose. Sepharose-bound 2-acetamido-2-deoxy-D-mannono-1,4-lactone was a gift from Dr. J.L. Stirling, Biochemistry Department, Queen Elizabeth College, London. L-[4,5(n)-^{3}H] lysine, L-[U-^{14}C]lysine and L-[^{35}S]methionine were purchased from the Radiochemical Centre, Amersham, Bucks and NCS solubiliser was purchased from Amersham/Searle. Human immunoglobulins, rabbit antiserum to human immunoglobulins and goat antiserum to rabbit immunoglobulin G were obtained from Miles Laboratories, Slough, Bucks.

Unless stated otherwise, Tris-containing buffers are expressed with respect to the concentration of the Tris component and were prepared by titrating to the desired pH with 3M HCl. Krebs-Ringer bicarbonate (KRB) buffer was prepared as described by Krebs and Henseleit (1932). Citrate/phosphate buffer was prepared as described by McIlvaine (1921). The composition of phosphate-buffered saline (PBS) is given by Starkey and Barrett (1976).

All operations, including centrifugations and column chromatography, were carried out at 4°C unless stated otherwise. Large volumes ($>$10ml) were concentrated using an Amicon Ultrafiltration system (PM 10 membrane) and small volumes were concentrated by vacuum dialysis.

7 General techniques

7.1 Tissues

Term human placentas were obtained from the maternity units at Ashford General Hospital, Ashford, Middlesex and Heatherwood Hospital, Ascot, Berks. Only placentas from uncomplicated pregnancies and from spontaneous deliveries were used. First trimester placental tissue was obtained from pregnancies terminated for social reasons and the collection and use of this material was approved by the Ethical Committees of the hospitals concerned.

Samples of sera from pregnant and non-pregnant women and samples of normal human cortex were obtained from the Institute of Child Health, Guilford Street, London.

7.2 Enzyme assays

7.2.1 N-acetyl-β-D-hexosaminidase (EC 3.2.1.52)

7.2.1.1 Quantitative assay

Hexosaminidase activity was determined using the fluorescent assay method described by Leaback and Walker (1961). The stock substrate solution consisted of 0.4mM 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside in citrate/phosphate buffer, pH 4.5, and containing human serum albumin (0.1mg/ml). Tubes, each containing stock substrate (100μl), were set up in a water bath at 37°C. Suitably diluted enzyme solution (20μl) was added to each tube and incubations were carried out for 30 min at the same temperature. Enzyme blanks were found not to be necessary but substrate blanks were routinely included. The reaction was stopped and the fluorescence developed by the addition of 0.2M sodium hydroxide/glycine buffer, pH 10.3 (2.0ml). The fluorescence was measured using a Locarte digital fluorimeter with an excitory wavelength between 340-360nm and an emission wavelength set at 440nm. Initially, the fluorimeter was calibrated using standard solutions of 4-methylumbelliferone in 0.2M sodium hydroxide/glycine buffer, pH 10.3 (0.1 - 10nMols /2.1ml) and thereafter by using a reference solution of quinine sulphate (4μg/ml in 0.1M sulphuric acid). In this way, the amount of 4-methylumbelliferone liberated during the assay (in nMols) could be read directly from the instrument. One unit of enzyme activity liberated 1.0μMol of 4-methylumbelliferone/min under the above conditions.

7.2.1.2 Determination of heat stable and heat labile activities

The method used was that of O'Brien et al. (1970). The samples to be tested were diluted with citrate/phosphate buffer, pH 4.5, each to a final volume of 0.5ml and incubated in sealed tubes for times up to 3 hours at 50°C. At the end of the incubation times the appropriate tubes were removed and stored at -20°C. An identical set of tubes was also prepared and stored at -20°C without the heat treatment. At the end of the experiment all the samples were thawed and hexosaminidase activity was determined as described in section 7.2.1.1. Heat stable activity was defined as that activity remaining after heating to 50°C. Heat labile activity was estimated by comparing the activity remaining after heating with the activity present in the corresponding unheated sample.

7.2.1.3 Detection of hexosaminidase activity after analytical electrophoresis

After polyacrylamide gel electrophoresis or immunoelectrophoresis, hexosaminidase activity was located using one of two methods. The first of these used the fluorescent substrate, 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside. Polyacrylamide gels or agarose-covered slides were covered with a piece of Whatman number 2 paper previously soaked in a solution of 0.4mM substrate. After incubation for 10 min at 37°C, the paper was removed and replaced with another piece soaked in 0.2M sodium hydroxide/glycine buffer, pH 10.3. This stopped the reaction and developed the fluorescence. On removal of this latter piece of paper, hexosaminidase activity was located by viewing the slides or gels under an ultra violet light.

The other method of detecting hexosaminidase used the histochemical substrate naphthol AS-B1-2-acetamido-2-deoxy-β-D-glucopyranoside as described by Hayashi (1965). The following stock solutions were prepared:

Solution 1. 4% (w/v) p-rosanaline in 0.4M HCl

Solution 2. 4% (w/v) sodium nitrite in water

Solution 3. 1mM substrate in citrate/phosphate buffer, pH 4.5.

0.3ml of solution 1 and 0.3ml of solution 2 were mixed and left to stand for 5 min. To this mixture was added 5.5ml of substrate solution and water to a final volume of 10.0ml. Pieces of Whatman number 2 paper were soaked in this solution and incubated with the gels or slides for 15-20 min at 37°C. After this time the gels or slides were rinsed in water and viewed.

The positions of hexosaminidase activity were indicated by a deep red colour.

7.2.2 α-D-glucosidase (EC 3.2.1.20)

α-Glucosidase activity was determined using a fluorescent assay method (de Barsy et al., 1972). The stock substrate solution consisted of 0.45mM 4-methylumbelliferyl-α-D-glucopyranoside in 0.2M sodium acetate buffer, pH 4.0. Tubes, each containing stock substrate solution (100µl), were set up in a water both at 37°C. Suitably diluted enzyme solutions (20µl) were added and the tubes incubated for 30 min at the same temperature. The reaction was stopped and the fluorescence developed by the addition of 0.2M sodium hydroxide/glycine buffer, pH 10.3 (2.0ml). The amount of 4-methylumbelliferone released was measured as described in section 7.2.1.1. One unit of enzyme activity liberated 1.0µMol of 4-methylumbelliferone/min under the above conditions.

7.2.3 β-D-glucuronidase (EC 3.2.1.31)

β-Glucuronidase activity was determined using a fluorescent assay method (Corash and Gross, 1974). The stock substrate solution consisted of 0.4mM 4-methylumbelliferyl-β-D-glucuronide in 20mM sodium acetate buffer, pH 5.0. Tubes, each containing stock substrate solution (100µl), were set up at 37°C. Diluted enzyme solutions (20µl) were added and the tubes incubated for 30 min at 37°C. The reaction was stopped and the fluorescence developed by the addition of 0.2M sodium hydroxide/glycine buffer, pH 10.3, (2.0ml). Free 4-methylumbelliferone was measured as described in section 7.2.1.1. One unit of enzyme activity liberated 1.0µMol of 4-methylumbelliferone/min under the above conditions.

7.2.4. Lactate dehydrogenase (EC 1.1.1.27)

Lactate dehydrogenase was assayed by following the disappearance of NADH at 340nm upon the conversion of pyruvate to lactate, as described by Bergmeyer et al. (1963). The following stock solutions were mixed in a 3.5ml plastic cuvette in the proportions shown:

Solution 1.	0.1M sodium phosphate buffer, pH 7.0	2.85ml
Solution 2.	23mM sodium pyruvate	0.10ml
Solution 3.	12mM NADH	0.05ml

This mixture was pre-incubated at 25°C. Then suitably diluted

enzyme solution (20μl) was added and the incubation was continued at the same temperature for 5.0 min. The change in absorbance at 340nm which occurred during this time was monitored using a Pye-Unicam SP 1800 recording spectrophotometer. One unit of enzyme activity transformed 1μMol of substrate/min under the above conditions.

7.3 Measurement of protein

Protein amount was either determined by the method of Lowry et al. (1951), using crystalline bovine albumin as the standard or, in the case of purified samples, by the ultra violet absorbtion method described by Kalckar (1947).

7.4 Column chromatography

Sephadex G-200, DEAE-Sephadex, CM-Sephadex, DEAE-cellulose, CM-cellulose, concanavalin A-Sepharose and oligo (dT)-cellulose were prepared as described in the manufacturers' instructions. Details of column dimensions, flow rates, etc., are given in the appropriate sections. Although most columns were obtained commercially, it was often found convenient to use 5ml or 10ml plastic syringes fitted with a porous polyethylene gel support disc. Unless stated otherwise, all columns were run at 4°C using downward-flow, gravity feed elution.

7.5 Polyacrylamide gel electrophoresis systems

Polyacrylamide gel electrophoresis was carried out under anionic and cationic conditions as described by Gabriel (1971) and in the presence of sodium dodecyl sulphate (SDS) as described by Weber et al. (1972). Separating gels were polymerised in the presence of ammonium persulphate and N,N,N',N'-tetramethethylene diamine (Temed) and stacking gels were polymerised in the presence of riboflavin and Temed. Gel tubes measuring 75mm x 5.0mm internal diameter were used for all the gel systems.

7.5.1 Under anionic conditions

Electrophoresis was performed in gels consisting of 7.5% (w/v) acrylamide, 0.2% (w/v) N,N'-methylenebisacrylamide, 0.03% (v/v) Temed, 0.07% (w/v) ammonium persulphate in 0.4M Tris buffer, pH 8.9. The stacking gels consisted of 2.5% (w/v) acrylamide, 0.8% (w/v) N,N'-methylenebisacrylamide, 0.05% (v/v) Temed, riboflavin (5μg/ml) and 20% (w/v) sucrose in 50mM Tris buffer, pH 6.7. Prepared gel tubes were inserted in a Shandon polyacrylamide gel electrophoresis apparatus and

5mM Tris/0.3M glycine buffer, pH 8.3, was placed in the electrode compartments. Up to 250μg of protein in up to 100μl of 5mM Tris/ 0.3M glycine buffer, pH 8.3, containing 0.2% (w/v) surcrose was applied to each gel along with 3μl of 0.05% Bromophenol blue. Electrophoresis was carried out at 2.5mA/gel for 4 hours or until the tracking dye approached the lower end of the gels. The gels were removed from the tubes by rimming with a syringe needle and stained for hexosaminidase activity using the fluorescent assay method described in section 7.2.1.3. After enzyme staining, the gels were stained for protein with 0.25% (w/v) Coomassie brilliant blue in 50% methanol/acetic acid (10:1, v/v) and then destained with a solution of methanol/acetic acid/water (1:1.5:17.5, by vol.).

7.5.2 <u>Under cationic conditions</u>

Electrophoresis was performed in gels consisting of 7.5% (w/v) acrylamide, 0.2% N,N'-methylenebisacrylamide, 0.5% Temed, 0.14% (w/v) ammonium persulphate, 0.1M potassium acetate buffer, pH 4.3. The stacking gels consisted of 2.5% (w/v) acrylamide, 0.6% (w/v) N,N'-methylenebis-acrylamide, 0.06% (v/v) Temed, riboflavin (5μg/ml) in 12mM potassium acetate buffer, pH 6.7. The electrode compartments (anode at top) contained 35mM β-alanine, 13mM acetic acid, pH 4.5. Sample application was as described in section 7.5.1 except that the sample buffer was 35mM β-alanine, 13mM acetic acid, pH 4.5, and the tracking dye was methyl green. Electrophoresis was carried out at 2.5mA/gel at 4°C for about 1.5 hours. The gels were then removed and stained for enzyme activity and protein as described in section 7.5.1.

7.5.3. <u>In the presence of sodium dodecyl sulphate</u>

Each gel contained 10% (w/v) acrylamide, 0.3% (w/v) N,N'-methylenebis-acrylamide, 0.15% (v/v) Temed and 0.1% (w/v) SDS in 0.1M sodium phosphate buffer, pH 7.2. No stacking gels were used. The electrode compartments (anode at the bottom) were filled with 0.1M sodium phosphate buffer, pH 7.2, containing 0.05% SDS. Protein samples were dissolved in 10mM sodium phosphate buffer, pH 7.2, containing 2% (w/v) SDS, 2% (w/v)dithio-threitol and 25% (v/v) glycerol and heated for 5 min at 100°C as described by Hopgood <u>et al</u>. (1973). Sample volumes were not greater than 100μl and details are given in the relevant figure legends. After cooling, a trace of Bromophenol blue was added and the samples were applied to the gels.

Electrophoresis was performed at 8mA/gel for about 4.0 hours or until the tracking dye was within 1.0cm from the end of the gel. After removing the gels from the tubes, the positions of the Bromophenol blue fronts were marked with Indian ink. The gels were then stained for protein using coomassie brilliant blue as described in section 7.5.1.

Molecular weight estimations were made by comparing the relative mobilities of standard proteins of known molecular weight with Bromophenol blue. The protein standards were obtained as a kit from Boehringer and consisted of: Trypsin inhibitor (mol.wt. 21,000), bovine serum albumin (mol.wt. 68,000), RNA polymerase α-subunit (mol.wt. 39,000), RNA polymerase β-subunit (mol.wt. 155,000).

7.6 Counting radioactivity

Low background glass vials were used. Radioactive samples were incubated with NCS solubiliser (0.5ml) at 45°C for 2-4 hours or at 37°C overnight. On cooling, the samples were neutralised by the addition of 25µl of glacial acetic acid/water (17:3, v/v). To each sample was added 10ml of scintillation fluid made by dissolving 5.0g of PPO in 1.0 litre of toluene. The vials were placed in a Packard Tri-Carb liquid scintillation spectrometer and, after an equilibration period of about 4 hours, the radioactivity was counted. Efficiency determinations were made by the external standard method.

8. Purification of hexosaminidases A and B from human placenta

Two methods were used. The first was based on the procedures described by Srivastava *et al.* (1974a) and Tallman *et al.* (1974) and is shown schematically in Fig. 8.1. The second method (Fig. 8.2) was essentially that described by Geiger *et al.* (1975) except that affinity chromatography was done using Sepharose-bound 2-acetamido-2-deoxy-D-mannono-1,4-lactone and not sepharose-bound 2-acetamido-N-(ε-aminohexanoyl)-2-deoxy-β-D-glucopyranosylamine.

Term placentas were obtained at delivery, transported to the laboratory on ice and processed within 1-5 hours or stored at -20°C until needed.

8.1 Method I

Up to four placentas were processed at one time. The foetal membranes and the cords were removed and the remaining tissue (for four

Fig. 8.1 Purification of hexosaminidases A and B from human placenta - Method I

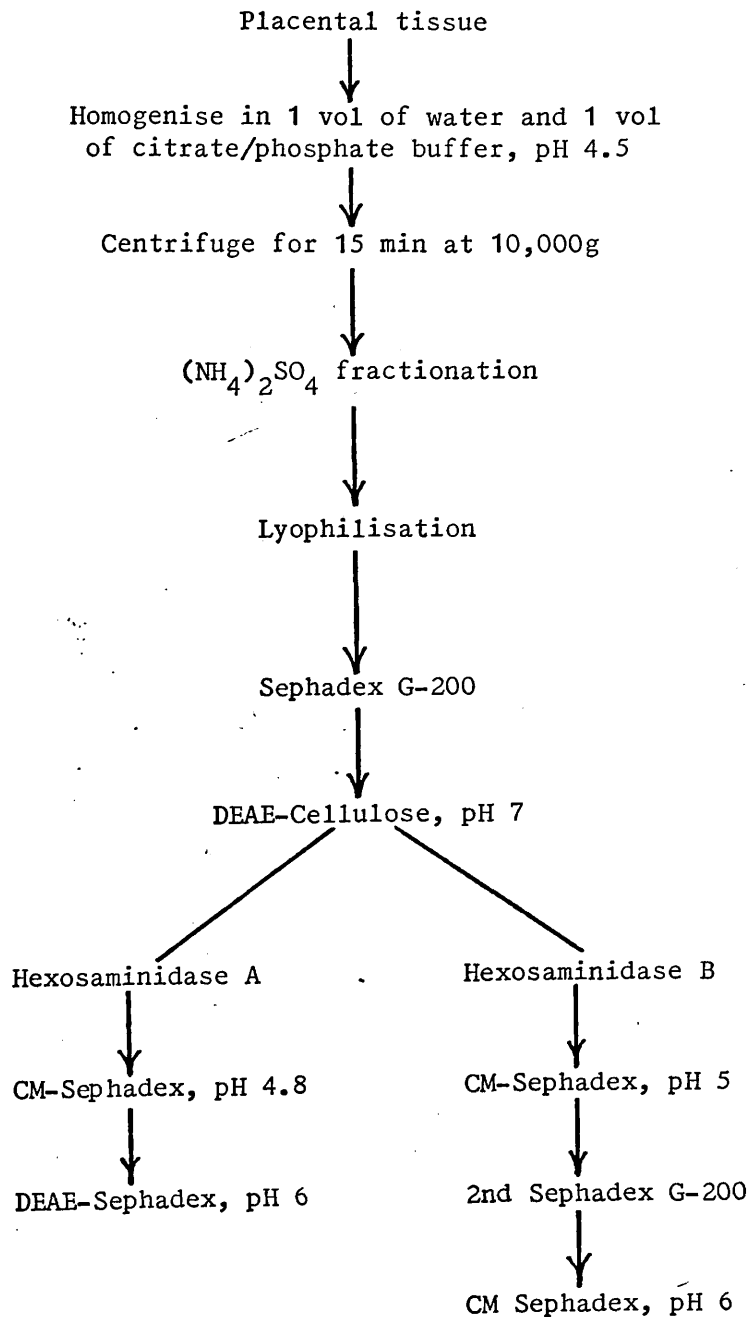
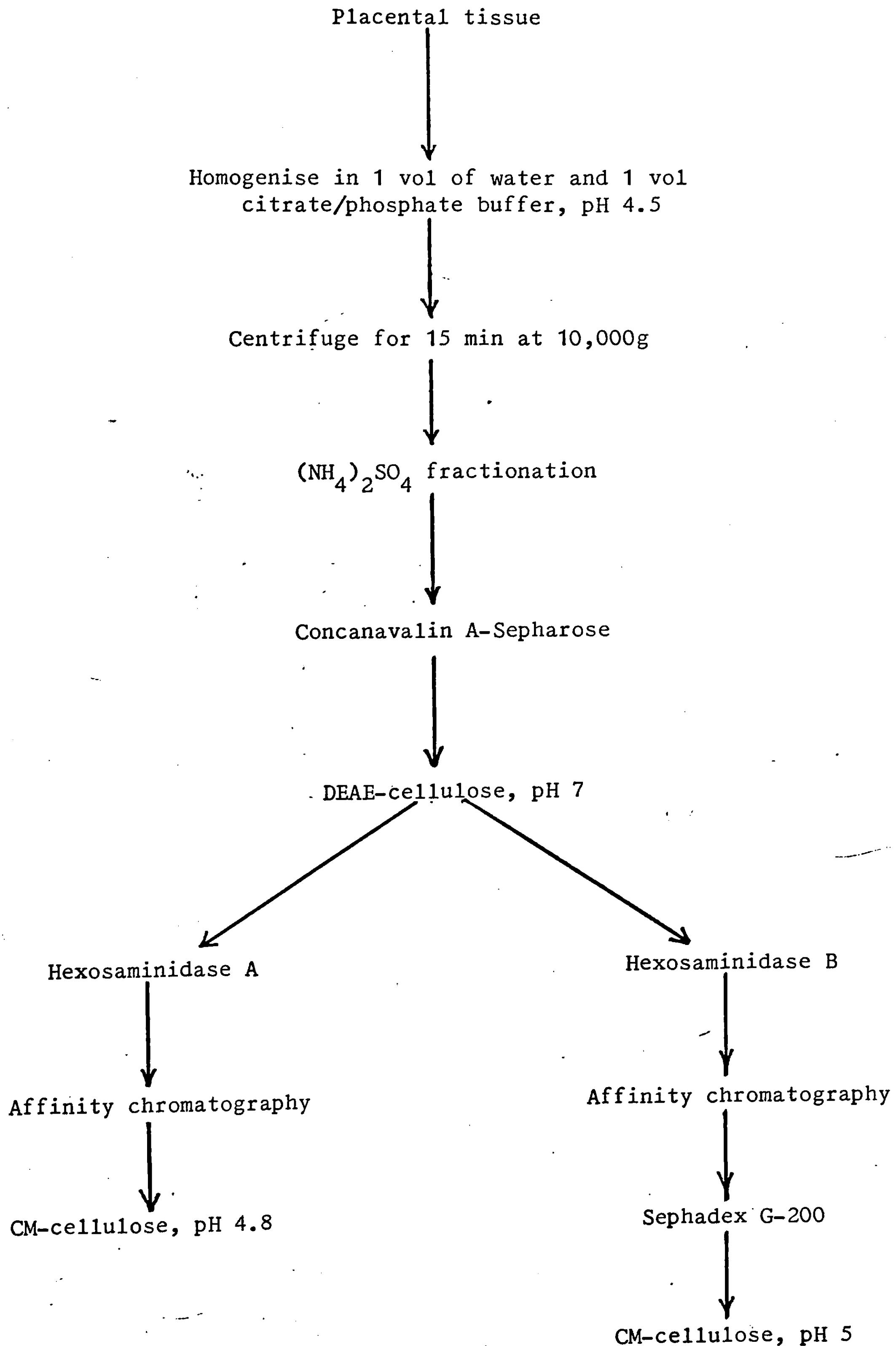


Fig. 8.2 Purification of hexosaminidases A and B from human placenta - Method II



placentas this was about 2kg wet weight) was cut into pieces with scissors. Batches of chopped tissue were placed in a one litre Ata-mix blender and one volume (v/w) of ice-cold water was added. The mixture was homogenised (4 x 15 second bursts at maximum speed) after which an equal volume of cold citrate/phosphate buffer, pH 4.5, was added and the mixture re-homogenised (2 x 15 second bursts at maximum speed). The pooled homogenates were stirred overnight at 4°C, filtered through two layers of cheesecloth, and centrifuged at 10,000g_{av} for 15 min in a 6 x 250 MSE 18 rotor. The supernatants were pooled, made 25% saturated in ammonium sulphate by the addition of the solid salt and stirred overnight at 4°C. Precipitated material was collected by centrifugation at 10,000g_{av} for 15 min and discarded. The supernatants were again pooled and made 65% saturated in ammonium sulphate. After stirring for 6 hours at 4°C, the precipitated material was collected by centrifugation as before. The supernatants were discarded and the pellets suspended in ice-cold water (total volume, 750ml) and dialysed against water overnight. This "25-65% ammonium sulphate fraction" was freeze-dried and the resulting powder re-suspended in cold water (100ml). The suspension was centrifug d at 10,000g_{av} for 15 min in a 6 x 100 MSE 18 rotor and the supernatants kept. The pellet was extracted twice more by re-suspension in water and re-centrifugation and the three supernatants were pooled (total volume, 300ml) and designated as the "freeze-dried fraction".

The "freeze-dried fraction" was concentrated to 90ml using an Amicon ultrafiltration system (PM10 membrane), applied to a 5.0cm x 90cm Sephadex G-200 column and eluted with 10mM sodium phosphate buffer, pH 5.0, containing 0.1M ammonium sulphate. Fractions (10ml) were collected, assayed for hexosaminidase activity and monitored for absorbance at 280nm. Enzyme active fractions were pooled, dialysed against 10mM sodium phosphate buffer, pH 7.0, and concentrated to 25ml. This "Sephadex fraction" was then applied to a 4.4cm x 90cm DEAE-cellulose column equilibrated in 10mM sodium phosphate buffer, pH 7.0, and eluted with the same buffer. Hexosaminidase B passed straight through the column and the 10ml fractions containig this form were pooled. Elution of hexosaminidase A was achieved by the application of a linear sodium chloride gradient which was prepared using a mixing chamber containing 10mM sodium phosphate buffer, pH 6.0 and a reservoir containing 0.3M sodium chloride in the same buffer. From this point on, hexosaminidase

A and B were purified separately.

The hexosaminidase A fraction from the DEAE-cellulose column was dialysed against 20mM sodium acetate buffer pH 4.8 and concentrated to 80ml by ultrafiltration. Purification was continued by applying the enzyme solution to a 2.5cm x 30cm CM-Sephadex column equilibrated in 20mM sodium acetate buffer, pH 4.8. The column was washed with the acetate buffer until the E_{280} of the eluate was negligible and then the enzyme was eluted with a linear sodium chloride gradient prepared using a mixing chamber containing the acetate buffer (1.0 litre) and a reservoir containing 0.5M sodium chloride in the same buffer (1.0 litre). The active fractions were pooled, dialysed against 25mM sodium phosphate buffer, pH 6.0, and concentrated to 12ml. Final purification of hexosaminidase A was achieved by chromatography on a 2.5cm x 30cm DEAE-Sephadex column equilibrated in 25mM sodium phosphate buffer, pH 6.8. The enzyme was eluted with a linear salt gradient prepared using the phosphate buffer (1.0 litre) in the mixing chamber and 0.3M sodium chloride in the same buffer (1.0 litre) in the reservoir.

The hexosaminidase B fraction from the DEAE-cellulose column was dialysed against 20mM sodium acetate buffer, pH 5.0, and concentrated to 25ml by ultrafiltration. The enzyme solution was applied to a 2.5cm x 30cm CM-Sephadex column equilibrated in the same acetate buffer. Hexosaminidase B was eluted with a linear sodium chloride gradient prepared using 20mM sodium acetate buffer, pH 5.0 (1.0 litre) in the mixing chamber and 0.6M sodium chloride in the same buffer (1.0 litre) in the reservoir. Further purification was achieved by applying the enzume solution to a 2.5cm x 30cm Sephadex-G-200 column and eluting using 10mM sodium phosphate buffer, pH 5.0, containing 0.1M ammonium sulphate. The pooled active fractions were dialysed against 25mM sodium phosphate buffer pH 6.0 and concentrated to 5ml. This "second Sephadex G-200 fraction" was finally purified by applying to a 1.0cm x 20cm CM-Sephadex column equilibrated in the same phosphate buffer. Hexosaminidase B was eluted with a linear sodium chloride gradient prepared using a mixing chamber containing 25mM sodium phosphate buffer, pH 6.0, (100ml) and a reservoir containig 0.2M sodium chloride in the same buffer (100ml).

8.2 Method II

Typically, two placentas were processed together. After removal of the membranes and cords this represented about 800g of tissue. The homogenisation and ammonium sulphate fractionation steps were as described for Method I. The "25-65% ammonium sulphate fraction" was dialysed first against water, then against 0.1M potassium phosphate buffer pH 7.0, containing 0.5M sodium chloride. The sample was then pumped through a 10ml plastic syringe containing 4ml of concanavalin A-Sepharose at a flow rate of 60ml/hour and at room temperature. After the sample had been applied, the column was washed with 0.1M potassium phosphate buffer, pH 7.0, containing 0.5M sodium chloride until the E_{280} of the eluate became negligible. Hexosaminidase was then eluted by the application of 0.5M α-methylmannoside in the same buffer. The active fractions were pooled, dialysed first against water, then against 10mM sodium phosphate buffer, pH 7.0 and concentrated to 15ml.

The concentrated "concanavalin A fraction" was applied to a 2.5cm x 40cm DEAE-cellulose column equilibrated in the 10mM sodium phosphate buffer, pH 7.0. Hexosaminidase B passed straight through the column and active fractions were pooled. Hexosaminidase A was eluted by the application of a linear sodium chloride gradient prepared using a mixing chamber containing 10mM sodium phosphate buffer, pH 6.0, (1.0 litre) and a reservoir containing 0.3M sodium chloride in the same buffer (1.0 litre). From this point on, hexosaminidases A and B were purified separately.

Hexosaminidase A from the DEAE-cellulose column was dialysed against 50mM sodium phosphate buffer, pH 5.5, concentrated to 15ml and pumped through a 10ml plastic syringe containig 6ml of sepharose-bound 2-acetamido-2-deoxy-D-mannono-1,4-lactone. Stepwise elution was carried out, firstly with 50mM sodium phosphate buffer, pH 5.5, (100ml), secondly with 0.2M sodium chloride in the same buffer (200ml) and finally with 0.2M sodium borate buffer, pH 8.0, (150ml). Enzyme active fractions were pooled, dialysed against 25mM sodium citrate buffer, pH 4.5 and concentrated to 2ml by vacuum dialysis. Final purification of hexosaminidase A was achieved by applying the enzyme solution to a CM-cellulose column equilibrated with 25mM sodium citrate buffer, pH 4.5. When the E_{280} of the eluate became negligible, hexosaminidase A was eluted by the application of a linear sodium chloride gradient prepared using a mixing chamber containing 25mM sodium citrate buffer, pH 4.5 (250ml) and a reservoir containing 0.5M sodium chloride in the same buffer (250ml). Enzyme active

fractions were pooled, dialysed against 10mM sodium phosphate buffer, pH 7.4, and concentrated to 1ml by vacuum dialysis. The enzyme preparation was dispensed into 50µl aliquots and stored at -70°C. Although further large scale purification of hexosaminidase A was not attempted, small amounts (not more than 100µg) were purified by electrophoresis in analytical polyacrylamide gels under cationic conditions (see section 7.5.2). After staining the gels with the fluorescent substrate (section 7.2.1.3), the enzyme active bands were exised and homogenised in water (0.5ml) using a hand-operated teflon/glass homogeniser. The homogenates were left at 4°C for 3 hours and then centrifuged in the microcentrifuge for 6 min. The pooled supernatants containing the extracted hexosaminidase A were concentrated to about 250µl by vacuum dialysis and either used immediately or stored at -70°C.

Hexosaminidase B from the DEAE-cellulose step was dialysed against 50mM sodium phosphate buffer, pH 5.5, concentrated to 12ml and pumped through the affinity column as described for hexosaminidase A. The active fractions were pooled, dialysed against 10mM sodium phosphate, pH 5.0, and concentrated to 2ml by vacuum dialysis. The hexosaminidase B solution was then chromatographed on a 2.5cm x 30cm column of Sephadex G-200. Elution was carried out using 10mM sodium phosphate buffer, pH 5.0, containing 0.1M ammonium sulphate. Enzyme active fractions were pooled, dialysed against 10mM sodium phosphate, pH 5.0, to remove ammonium sulphate, and concentrated to 1ml by vacuum dialysis. Final purification of hexosaminidase B was achieved by applying the enzyme solution to a column of CM-cellulose equilibrated with 10mM sodium phosphate buffer, pH 5.0. When the E_{280} of the eluate became negligible, hexosaminidase B was eluted by the application of a linear sodium chloride gradient prepared using a mixing chamber containing 10mM sodium phosphate buffer, pH 5.0 (250ml) and a reservoir containing 0.6M sodium chloride in the same buffer (250ml). Active fractions were pooled, dialysed against 10mM sodium phosphate buffer, pH 7.4 and concentrated to 1ml. This preparation was dispensed into 100µl aliquots and stored at -70°C.

9. Immunological methods

9.1 Production of anti-(hexosaminidase) antisera

All antisera were raised in rabbits of the lop variety and control sera were collected before the immunisation schedules were started.

Antisera were stored in 0.5ml aliquots at -20°C without preservative.

Two different approaches were used to prepare antibodies to hexosaminidases A and B. For the production of anti-(hexosaminidase B) antibodies, 70μg of purified hexosaminidase B in 10mM sodium phosphate buffer, pH 7.4 (100μl) was mixed with Freund's complete adjuvant (100μl) and emulsified by sonication using the MSE ultrasonic disintegrator (2 x 10 second bursts at full power). On day 1, this emulsion was injected subcutaneously into a rabbit at a site on the shoulder. The rabbit was given successive injections on days 14, 28 and 42, each consisting of 70μg of emulsified hexosaminidase B. Subcutaneous and intramuscular sites were alternated. On day 52, blood was collected from a marginal ear vein, allowed to stand at room temperature for 1 hour and at 4°C for 4 hours. The serum was withdrawn using a Pasteur pipette and centrifuged at maximum speed for 15 minutes in an MSE bench centrifuge. The supernatant was removed and tested for the presence of anti-(hexosaminidase) antibodies by immunoprecipitation and immunoelectrophoresis as described below (sections 9.2 and 9.3).

Anti-(hexosaminidase A) antibodies were raised by the injection of precipitin lines consisting of hexosaminidase A/anti-(hexosaminidase B) complexes. Precipitin lines were prepared as follows. Microscope slides were covered with 1% (w/v) agarose as described in section 9.3 and two parallel troughs, each measuring 0.2cm x 6.0cm, were excised from each slide. Purified hexosaminidase A (30μl) was placed in one trough and anti-(hexosaminidase B) antiserum (30μl) in the other. The slides were incubated at room temperature for 24 hours during which time straight precipitin lines became visible. Non-precipitated material was removed by immersing the slides in 0.15M sodium chloride for 48 hours. The slides were then removed and stained for hexosaminidase activity using the fluorescent substrate as described in section 7.2.1.3. The luorescent hexosaminidase A/anti-(hexosaminidase B) precipitin lines were visualised under an ultra violet lamp, carefully excised, and stored at -20°C until needed. For immunisation, a single precipitin line was suspended in sterile PBS (150μl) and emulsified by sonication with Freund's complete adjuvant, (150μl). On day 1, portions of this emulsion were injected intramuscularly and subcutaneously into a rabbit. Similar injections, each consisting of a single precipitin line, were given on days 14 and 28 and a trial bleed was taken on day 38.

Blood was collected and the antiserum prepared as described above. The rabbit was given booster injections when the antibody titre began to fall.

Both the anti-(hexosaminidase B) and the anti-(hexosaminidase A) antisera were purified by ammonium sulphate fractionation. After the addition of solid ammonium sulphate to 40% saturation, the precipitate was collected by centrifugation at $10{,}000g_{av}$ for 15 min in an 8 x 50 MSE 18 rotor, re-suspended in PBS and dialysed against PBS.

9.1.1 Absorption of anti-(hexosaminidase A) antiserum by immunoaffinity chromatography

This was undertaken in order to obtain antibodies reactive only against the hexosaminidase α-subunit. The basic procedure consisted of passing anti-(hexosaminidase A) antiserum from section 9.1 through a column of Sepharose-bound hexosaminidase B to remove antibodies reactive against the hexosaminidase β-subunit.

The following procedure was used for the preparation of Sepharose-bound hexosaminidase B. Cyanogen bromide-activated Sepharose 4B (1.0g) was washed over a Buchner funnel with 250ml of 1mM hydrochloric acid for 15 min. 5ml of hexosaminidase B solution from the DEAE-cellulose step of purification method II was added to the washed gel. The enzyme solution consisted of 8mg of protein in 0.5M sodium phosphate buffer, pH 8.0, containing 1M sodium chloride and represented about 70 units of hexosaminidase. The resulting enzyme/Sepharose suspension was mixed gently end-over-end in a stoppered glass tube for 20 hours at 4°C. After washing with 200ml of 0.5M sodium phosphate buffer pH 6, containing 1M sodium chloride, the gel was mixed with 25ml of 1M ethanolamine, pH 8.0, for 3 hours at 4°C and then washed alternatively with 0.1M sodium acetate buffer, pH 4.0, containing 1M sodium chloride, and with 0.1M sodium borate buffer, pH 8.0, also containing 1M sodium chloride. The acetate/borate washing cycle was repeated a further five times. Finally, the gel was re-suspended in PBS containing 20mM sodium azide (10ml) and stored at 4°C.

Anti-(hexosaminidase A) antiserum was absorbed by repeated passage through a 10ml plastic syringe containing the immobilised hexosaminidase B until the eluate no longer precipitated hexosaminidase B from solution. This indicated that antibodies to β-subunit determinants had been removed. The absorbed anti-(hexosaminidase A) was concentrated to 1ml by vacuum

dialysis, dispensed into 0.2ml aliquots, and stored at -20°C. The column was regenerated by washing firstly with 10mM sodium phosphate buffer, pH 6.0, containing 8M urea and then with PBS.

9.2 Quantitative immunoprecipitation

Enzyme solutions were dialysed against PBS and, in addition, all antisera and enzyme solutions were centrifuged for 12 min in a microcentrifuge (Quickfit instruments) to remove any insoluble material. Immunoprecipitation was carried out in 1.2ml capacity plastic microcentrifuge tubes by adding variable amounts of antiserum to constant amounts of hexosaminidase activity. It was found convenient to use 25munits of hexosaminidase per tube and to add increasing amounts of antiserum (5-50µl). Final volumes were made to 0.3ml by the addition of PBS. Where enzyme or antibody were limiting, the above amounts were scaled down. After adding the components and mixing, the tubes were incubated for 1 hour at room temperature followed by incubation at 4°C for at least 6 hours. Finally, the tubes were centrifuged for 12 min in a microcentrifuge. The supernatants were assayed for hexosaminidase activity as described in section 7.2.1.1.

9.3 Immunoelectrophoresis

This was carried out by the microtechnique of Scheidegger (1955) using the buffer system described by Lalley et al. (1974). Glass microscope slides (25mm x 75mm) were overlayed with 1.8ml of molten 1.0% (w/v) agarose in 25mM sodium citrate buffer, pH 6.0. One or two antigen wells were cut in the agarose using a 4.0mm well cutter. Antiserum troughs measuring approximately 0.2cm x 6.0cm were cut with a scalpel but the agarose was not removed at this time. Prepared slides were placed in an electrophoresis tank (Shandon/Southern) and enzyme solution (4µl) was added to each antigen well. The electrode compartments contained 25mM sodium citrate buffer, pH 6.0. Electrophoresis was performed at 6V/cm and constant current for 4 hours at 4°C. At the end of electrophoresis, the slides were taken from the tank and the pre-cut agarose troughs removed. The appropriate antiserum was added to each trough and the slides were incubated at room temperature in a humid atmosphere for 48 hours. After this time, non-precipitated proteins were removed by immersing the slides in 0.15M sodium chloride for 24 hours at 4°C and then for 2 hours in water at the same temperature.

The slides were then stained for hexosaminidase activity as described in section 7.2.1.3. After enzyme staining the slides were pressed, dried under a stream of hot air, and stained for protein with 0.25% (w/v) Coomassie brilliant blue in 50% methanol/acetic acid (10:1, v/v) for 10 min. Destaining was carried out with a solution of acetic acid/ ethanol/water (1:4.5:4.5, by vol.).

10 Analysis of hexosaminidase multiple forms by automated DEAE-cellulose chromatography

10.1 Sample preparation

Portions of villous tissue were cut at a depth of about 0.5cm into the maternal surface of a freshly delivered term placenta. These tissue samples were designated "terminal villi". Villous tissue deeper than 0.5cm from the maternal surface was also dissected out and designated "inner villi". Placental villi were homogenised in 5 volumes (v/w) of cold water using a motor-driven teflon/glass homogeniser (6 down and up strokes at 1500 rev/min, clearance 0.4mm). The homogenate was centrifuged maximum speed in an MSE bench centrifuge for 5 min and the supernatant used for the analysis of hexosaminidase components. Samples of chorion laeve, chorion frondosum, amnion and cord were prepared slightly differently because of their more fibrous nature. Individual samples were washed with PBS, blotted dry, wrapped in aluminium foil and immersed in liquid nitrogen for a few seconds. Immediately on removing from the nitrogen, the samples were pulverised to a powder with a pestle and mortar and then homogenised with 2.5 volumes (v/w) of water as described above for the villi. The samples were diluted with 10mM sodium phosphate buffer, pH 6.0, before loading onto the automated chromatography system.

10.2 Automated DEAE-cellulose chromatography

Sample analysis was carried out in the MRC Clinical Genetics Unit, Institute of Child Health, London, under the supervision of Dr. R.B. Ellis. The operation of the automated system was as described by Ellis *et al.* (1975). Briefly at zero time a suitably diluted sample was applied to a 5cm x 0.5cm DEAE-cellulose column and elution continued using 10mM sodium phosphate buffer, pH 6.0. After 10 min the salt gradient (0-0.3MKCl) was started. After leaving the column the eluate was mixed with

substrate and incubated by passage through a heating coil at 37°C. The reaction was stopped and the fluorescence developed by the addition of 0.5M sodium hydroxide/glycine buffer, pH 10.4. The eluate was then passed through the flow cell of a recording fluorimeter and was, therefore, continuously monitored for hexosaminidase activity. The sensitivity of the fluorimeter was adjusted depending on the activity of the sample being analysed and the relative sensitivity settings are given in the appropriate figure legends.

11 The placental slice in vitro system

11.1 Preparation and incubation of placental slices

The following procedure was used to prepare placental slices. A placenta was obtained at delivery, transported to the laboratory on ice and processed within 1-5 hours. A cube of villous tissue (about 2.5cm^3) was cut from the maternal surface of the organ and any obvious connective tissue or adherent maternal tissue was removed. Sufficient slices for most experiments were obtained from three or four such cubes. Slices of 1mm thickness were prepared from these cubes using a McIlwain tissue chopper (Mickle engineering Co.) according to the manufacturers instructions. Slices were lifted with forceps and pooled in a plastic petrie dish containing KRB buffer. When sufficient slices had been collected they were washed over a nylon mesh with fresh KRB buffer and divided into portions (each of about 100mg wet weight) which were placed in plastic tubes containing 2ml of KRB buffer supplemented with glucose (2mg/ml), streptomycin sulphate (40μg/ml) and penicillin G (40 μunits/ml). [^{3}H]Lysine or [^{14}C]lysine and other components such as cycloheximide or colchicine were added to the incubation media as appropriate. The actual amounts used are given in the figure legends. The slices were incubated at 37°C under an atmosphere of O_2/CO_2 (19:1) in a water bath shaking at 20 cycles/min. Incubation times up to 24 hours were used depending on the nature of the experiment.

Essentially the same procedure was used for the preparation of slices from first trimester placentas except that, because of the smaller size of the placenta at this stage of development, it was often necessary to use all of the available villous tissue. The tissue chopper was not used for experiments involving the foetal membranes. Instead, pieces of tissue approximately 0.5cm x 1.0cm were cut with scissors and incubated as described above.

11.2 Treatment of slices at the end of the incubation period

At the end of the incubation period, the tubes containing the slices were removed from the water bath and centrifuged at maximum speed in an MSE bench centrifuge. After centrifugation, the incubation media were decanted off and either kept on ice or stored at -20°C until needed. The slices were re-suspended in ice-cold PBS (containing 10mM unlabelled lysine in the case of protein synthesis experiments) and washed over a nylon mesh with the same buffer. The washed slices were blotted dry, wrapped in aluminium foil and stored at -20°C.

The following procedure was used for extracting soluble protein from the slices. The frozen samples were chopped with scissors while still thawing. Ice-cold water (0.8ml) was added to each sample and the mixtures were sonicated using the MSE ultrasonic disintegrator (2 x 10 sec bursts at full power). The sonicated tissue was then homogenised with a motor-driven teflon/glass homogeniser (4 down and up strokes at 1500 rev/min, clearance 0.3mm). A further volume of cold water (0.2ml) was added and the homogenate centrifuged in the microcentrifuge for 6 min. The resulting supernatant was used for the determination of enzyme activity, protein content and the incorporation of radioactivity into protein, as appropriate. The separated incubation media were also subjected to similar analyses.

It should be noted that, in a few experiments, the slices and the incubation media were not separated but were homogenised and extracted together.

11.2.1 Estimation of total protein synthesis

Total protein synthesis was estimated by determining the incorporation of radioactivity into trichloroacetic acid (TCA)-insoluble material. The solubilised tissue extracts and the incubation media from section 11.2 were treated identically. Aliquots (100μl) were spotted onto 2.1cm diameter discs of Whatman grade 1 paper, dried, and dropped into ice-cold 10% (w/v) TCA for 15 min as described by Mans and Novelli (1961). The discs were then placed in 5% TCA for 30 min at 90°C, washed in fresh, cold 5% TCA and placed in ethanol/ether (3:1 v/v) for 30 min at 37°C. After washing in fresh ethanol/ether, the discs were placed in ether for 15 min at room temperature and then dried. The paper discs were then placed in glass scintillation vials, treated with NCS solubiliser and processed for counting as described in section 7.6.

11.2.2 Estimation of hexosaminidase synthesis

Hexosaminidase synthesis was determined by measuring the incorporation of radioactivity into immunoprecipitable protein. Total hexosaminidase activity in the solubilised tissue extract or incubation medium was determined and an aliquot (usually between 200-300µl) containing 70munits of enzyme activity was removed for analysis. Various procedures were used to reduce the non-specific radioactivity associated with the resulting immunoprecipitate. The details and results of such procedures are given in section 16.2.2. Only the most successful method is described here. The aliquot to be analysed was made 10mM with respect to lysine and 0.7% (v/v) with respect to Triton X-405 by the addition of 0.1 vols of a solution of 0.11M lysine, 7.7% Triton X-405 in PBS. A pre-immunoprecipitation was then carried out by adding human immunoglobulins (10µg of a mixture of IgG, IgM and IgA) and sufficient rabbit antiserum to human immunoglobulins for complete precipitation. After incubation at room temperature for 1 hour and at $4^{\circ}C$ for 4 hours the precipitate was collected by centrifugation in a microcentrifuge for 12 min. The treated supernatant was decanted off and to it was added anti-(hexosaminidase A) antiserum. The volume of antiserum used was calculated to be 30% in excess of that required for complete precipitation of all the hexosaminidase activity present. After incubation at room temperature for 1 hour and at $4^{\circ}C$ overnight, the precipitate was collected by centrifugation in the microcentrifuge for 12 min. The supernatant was discarded and the inside of the tube carefully wiped with a tissue. The immunoprecipitate was then washed four times by resuspension in PBS (75µl) containing 0.7% (v/v) Triton X-405 and 10mM lysine and re-centrifugation in the microcentrifuge. After the last centrifugation, the washed immunoprecipitate was dissolved in 75µl of 10mM sodium phosphate buffer, pH 7.2, containing 2% (w/v) SDS, 2% (w/v) dithiothreitol and 25% (v/v) glycerol and heated at $100^{\circ}C$ for 5 min. After cooling, 5µl of bromophenol blue was added and the sample was subjected to electrophoresis in a 10% (w/v) sodium dodecyl sulphate/ polyacrylamide gel as described in section 7.5.3.

After electrophoresis, the gel was immediately frozen in a bath of methanol/solid CO_2 and sliced into 1mm segments using a Mickle gel slicer (Mickle Engineering Co.). Gel segments were placed in glass scintillation vials (2 gel segments per vial) and 0.5ml of a mixture of NCS/water (9:1 v/v) was added to each. Vials were incubated at $45^{\circ}C$

for 4 hours and, after cooling, 20µl of acetic acid/water (17:3 v/v) was added. Scintillation fluid (10ml) was then added and the vials counted for radioactivity as described in section 7.6. Incorporation into hexosaminidase was estimated by adding the radioactivity in the hexosaminidase fractions and subtracting from these a background value obtained by averaging the radioactivity in two gel slices on either side of the enzyme peak.

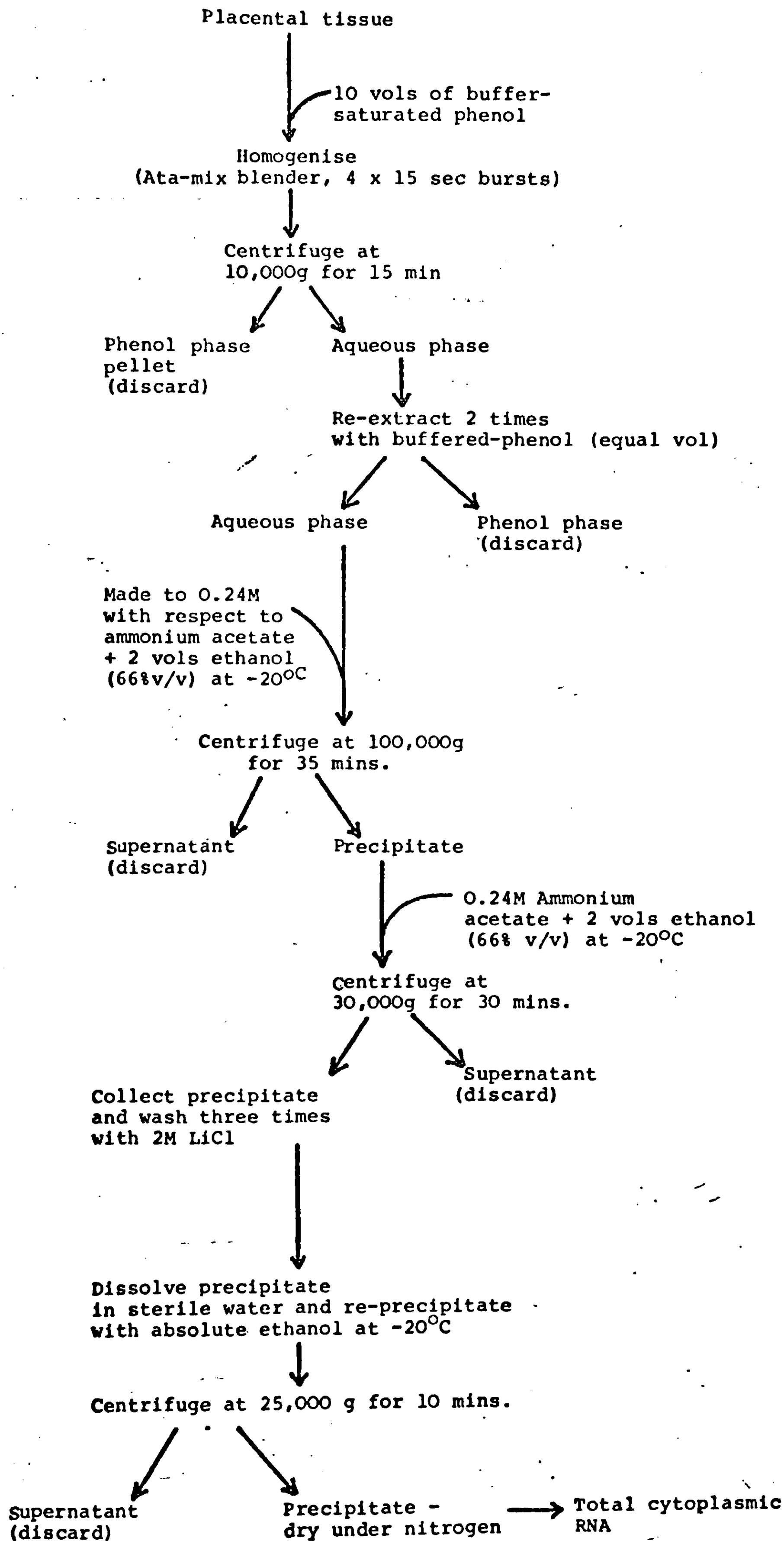
12 Preparation of placental RNA

Most solutions and plastiware were autoclaved at 15lb/in^2 (121°C) for 20 min. Certain solutions, such as those containing enzymes, were prepared using double glass-distilled, autoclaved water (sterile water). Glassware was sterilised by heating overnight in an oven at 180°C. Wherever possible, rubber gloves were worn when carrying out experiments. Heparin (1mg/ml) was added to those solutions used at the beginning of the RNA extraction procedure but was omitted from solutions used at later stages since it has been reported to inhibit protein synthesis in cell-free systems. The RNA extraction procedure was based on methods described by Lomedico and Saunders (1976) and Cox et al. (1976). The ammonium acetate/ethanol precipitation method was originally reported by Osterburg et al. (1975)

12.1 Extraction of total placental RNA

The extraction procedure is shown schematically in Fig. 12 Term placentas were transported from the hospital to the laboratory on ice and processed within 1-5 hours of delivery. Villous tissue (50g wet weight) was cut from the maternal surface and washed with sterile PBS to remove as much blood as possible. The tissue was chopped with scissors and placed in a 1 litre capacity Ata-mix blender. To this was added 300ml of 0.1M Tris buffer, pH 8.5, containing 25mM EDTA, 0.1M LiCl and 1% (w/v) SDS and 150ml of a solution of phenol saturated with the same buffer. The mixture was homogenised (4 x 15 sec bursts at full power) and the resulting homogenate stirred vigorously for 30 min. The homogenate was centrifuged at 10,000g_{av} for 15 min in the 6 x 250 MSE 18 rotor to separate the aqueous and phenol phases. The upper aqueous phase was pipetted off and to it was added an equal volume of buffer saturated phenol. This extraction procedure was repeated a further two times and on the last occasion, a solution of buffer-saturated phenol/chloroform

FIG.12 PREPARATION OF TOTAL CYTOPLASMIC RNA FROM PLACENTAL VILLI



(1:1, v/v) was used. In order to precipitate the RNA, the final aqueous phase was made 0.24M with respect to ammonium acetate and then 2 volumes of 66% (v/v) ethanol, pre-cooled to $-20^{o}C$, were added. The RNA precipitate was allowed to form overnight at $-20^{o}C$ and was collected by centrifugation at $100,000g_{av}$ for 35 min in the 3 x 75 swing-out MSE 65 rotor. The pellets were dissolved in 0.24M ammonium acetate (30ml) and re-precipitated with 2 volumes of 66% (v/v) ethanol. The RNA precipitate was collected by centrifugation at $30,000g_{av}$ for 30 min in the 8 x 50 MSE 18 rotor and the pellets were washed three times by re-suspension in 2M LiCl containing 10mM EDTA (10ml) and re-centrifugation at $25,000g_{av}$ for 10 min in the 6 x 16.5 swing-out MSE Prepspin rotor. The RNA pellet was washed with absolute ethanol and either stored at $-20^{o}C$ or dissolved in 10mM Tris buffer, pH 7.4, containing 0.5M potassium chloride (10ml) in preparation for chromatography on oligo (dT)-cellulose.

12.2 Isolation of placental mRNA

This was achieved by chromatographing the total placental RNA fraction on oligo (dT)-cellulose according to the method described by Aviv and Leder (1972). About 150 E_{260} units of RNA dissolved in 10mM Tris buffer, pH 7.4, containing 0.5M potassium chloride were applied to a 5ml plastic syringe containing oligo(dT)-cellulose (1.5ml bed volume) at room temperature and at a flow rate of 30ml/hour. The column was washed with the same buffer until the E_{260} of the eluate became negligible. The material which was eluted under these conditions was designated non-poly(A)-containing RNA. Poly(A)-containing RNA was eluted from the column with 10mM Tris buffer, pH 7.5. The RNA was precipitated with 2 volumes of absolute ethanol at $-20^{o}C$. The precipitate was collected by centrifugation at $25,000g_{av}$ for 10 min in the 8 x 50 MSE 18 rotor and finally dissolved in a small volume (1-2ml) of sterile water. Aliquots were stored at $-20^{o}C$ until needed.

13 Cell-free systems for the assay of placental mRNA activity

13.1 Preparation of rabbit reticulocyte lysates

New Zealand white rabbits were given subcutaneous injections of neutralised 2.5% (w/v) phenylhydrazine (0.35ml/kg body weight) daily on days 1 to 5, inclusively. On day 8, the rabbits were anaesthetised

with "nembutal" containing heparin (2mg) and bled out by cardiac puncture. The blood was collected into ice-cold PBS (1 litre) containing heparin (10mg) and then centrifuged at 2,500 g_{av} for 10 min in the 6 x 250 MSE 18 rotor. The supernatant and as much of the "buffy coat" as possible were aspirated. The pellet of cells was re-suspended in 20 volumes (v/v) of ice-cold PBS minus heparin and centrifuged as above. This washing procedure was repeated twice, after which the packed cells were weighed. The recovery of cells was between 5-10g wet weight per kg body weight. The cells were lysed by the addition of 1 volume (v/w) of ice-cold sterile water and shaken for 2 min. The resulting suspension was centrifuged at 10,000g_{av} for 15 min in the 8 x 50 MSE 18 rotor and the supernatant (ie. the lysate) was removed and stored in liquid nitrogen in 0.5ml aliquots.

13.2 Preparation of the standard reticulocyte lysate cell-free system

Immediately on thawing, lysates were supplemented as follows:

Lysate	10 volumes
Creatine kinase (5mg/ml in 50% aq.glycerol)	0.1 volumes
Haemin (1mM in 90% ethylene glycol, 20mM Tris, pH 8.2)	0.25volumes

A master mix was made from the following stock solutions by adding them in the proportions shown. The stock solutions were stored at -20°C until required:

Solution 1.	Creatine phosphate (0.2M)	1 volume
Solution 2.	KCl/Mg/Cl_2 (2M/0.01M)	1 volume
Solution 3.	ATP/GTP (20mM/4mM)	1 volume
Solution 4.	Tris buffer, pH 7.5, (1M)	1 volume
Solution 5.	Amino acid mix	1 volume

The amino acid mix consisted of 19 of the following 20 L-amino acids, each at a concentration of 2mM:

Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val.

The amino acid to be used for carrying the radioactive label (either lysine or methionine) was omitted as appropriate.

From these solutions, a reaction mix was prepared as follows:

Supplemented lysate	0.75 volumes
Master mix	0.25 volumes
RNA/radioactive amino acid/water	0.20 volumes

The amounts of RNA and radioactivity varied, and are given in the appropriate figure legends. Where necessary, reaction mixes were made to their final volumes by the addition of sterile water.

13.1 Preparation of mRNA-dependent system from reticulocyte lysates

This system was prepared using the solutions described in section 13.2 with the addition of $CaCl_2$, micrococcal nuclease and EGTA, as reported by Pelham and Jackson (1976). The nuclease-treated lysate was prepared as follows:

Supplemented lysate	0.75 volumes
Master mix	0.25 volumes
$CaCl_2$ (0.1M)	0.01 volumes
Nuclease (1mg/ml)/water	0.01 volumes

This mixture (not more than 0.5ml total volume per tube) was incubated at 20°C for 5 min. In trial experiments, the amounts of nuclease added were varied and these are given in the figure legends. The reaction was stopped by the addition of 0.1M EGTA (0.02 volumes) to give the nuclease-treated lysate.

The reaction mix was prepared as follows:

Nuclease-treated lysate	1 volume
RNA/radioactive amino acid/water	0.2 volumes

13.4 Protein synthesis in the cell-free systems

Reaction mixtures of various volumes, depending on the nature of the experiment, were incubated at 27°C for times up to 120min, as indicated in the figure legends. At the end of the incubation period, the mixtures were placed on ice and made 10mM with respect to unlabelled lysine or methionine and to 0.7% with respect to Triton X-405. Aliquots were removed for estimation of total protein synthesis and hexosaminidase synthesis as appropriate.

13.4.1 Estimation of total protein synthesis

Aliquots (10μl) of reaction mix were removed and spotted onto 2.1cm discs of Whatman 3MM paper and put through the TCA washing procedure of Mans and Novelli (1961) as described in section 11.2.1. The samples were then processed and counted as described in section 7.6.

13.4.2 Assay of hexosaminidase mRNA activity

The method developed was based on immunoprecipitation of labelled hexosaminidase polypeptide chains. Although similar to the assay of hexosaminidase synthesis described in section 11.2.2, the method used here for the analysis of the cell-free translation products differed in that a double antibody method was used as reported by von der Helm and Duesberg (1974). After determining the incorporation of radioactivity into total protein, aliquots containing 500,000 c.p.m. were removed from the reaction mix and made to 0.7% with respect to Triton X-405 and 10mM with respect to methionine. A pre-immunoprecipitation step was then carried out using the immunoglobulin/anti-(immunoglobulin) system described in section 11.2.2. To each of the supernatants obtained after this step was added the appropriate anti-(hexosaminidase) antiserum (3µl) as indicated in the figure legends. After incubation at 4°C for 6 hours, goat anti-(rabbit IgG) antiserum (100µl) was added and incubation continued for 1 hour at room temperature and overnight at 4°C. The immunoprecipitates were collected and analysed essentially as in section 11.2.2 except that 100µl of the buffer stated was routinely used to dissolve the immunoprecipitate prior to electrophoresis.

RESULTS AND DISCUSSION

14 Purification of hexosaminidases A and B from human placenta and production of anti-(hexosaminidase) antisera

The availability of specific anti-(enzyme) antibodies has extended the studies which can be made on the structure and function of enzymes. For example, antibodies raised against α-amylase were used to investigate the structural relationship between the enzymes from different rat tissues (Messer and Dean, 1975) and the inhibition of cartilage autolysis by anti-(cathepsin D) antibodies in cultured chick tendon cells has helped to define the role of this enzyme in this particular process, (Dingle *et al.*, 1971). Immunohistochemical techniques permit the localisation of enzymes in cells and tissues (Goldschneider *et al.*, 1977). Finally, and of particular relevance to this study, the specific immunoprecipitation of radiolabelled enzyme is being increasingly used in studying the synthesis and degradation of enzymes (Hopgood *et al.*, 1973; Obenrader and Prouty, 1977).

In order to study the structural relationships and biosynthesis of placental hexosaminidases, specific antisera were first raised against purified placental hexosaminidase A and hexosaminidase B. In view of the low amounts present, no attempt was made to raise antisera to the minor hexosaminidase forms.

14.1 Purification of hexosaminidases A and B

When this study was initiated, two published methods for the large scale purification of human placenta hexosaminidase A and hexosaminidase B were available (Srivastava *et al.*, 1974a, Tallman *et al.*, 1974). Both were mult -step procedures involving conventional preparative techniques and both had been reported to yield highly purified enzyme preparations. Originally, a combination of both methods was used in this study (Method I) and is shown schematically in Fig. 8.1. Results of the purification are presented in Table 14.1 and elution profiles are shown in Figs. 14.1 - 14.4. Only some of these individual steps will be commented on here.

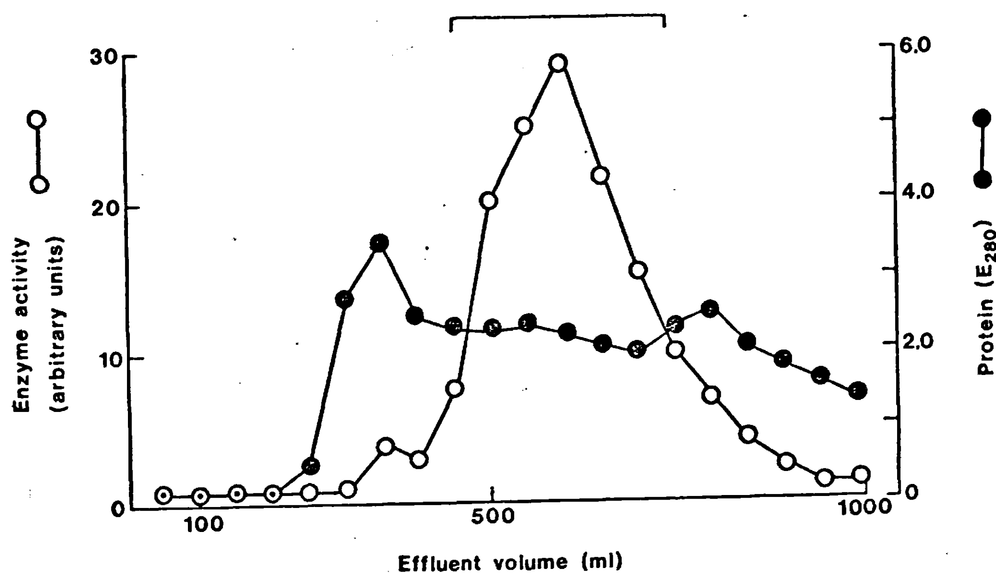
Fig. 14.1 shows the column profile obtained after Sephadex G-200 chromatography of a concentrated "ammonium sulphate fraction" and therefore represents total hexosaminidase activity. Two distinct peaks of enzyme activity were separated by this method. The first, and smallest in terms of activity, being eluted at about 350ml while the bulk of the hexosaminidase activity was eluted shortly afterwards between 450 and 800ml. Since this technique separates proteins based on molecular weight differences,

TABLE 14.1 PURIFICATION OF HUMAN PLACENTAL HEXOSAMINIDASES A AND B BY METHOD I

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification -fold	Yield (%)
1 Homogenate	4590	66000	0.069	-	100
2 Ammonium sulphate fractionation	2690	14600	0.18	2.7	59
3 Freeze-dry	1800	5830	0.31	4.4	39
4 Sephadex G-200	1780	2020	0.88	13	39
5 DEAE-cellulose:					
Hexosaminidase A	522	113	4.6	67	11
Hexosaminidase B	310	170	1.8	26	7
Hexosaminidase A					
6 CM-Sephadex	198	4.12	48.8	695	4
7 DEAE-Sephadex	56.5	0.70	80	1100	1
Hexosaminidase B					
8 CM-Sephadex	172	34	5	72	4
9 Sephadex G-200	57.5	5.71	10	14	1
10 CM-Sephadex	32.7	0.61	55	78	0.7

Experimental details are given in section 8.1

FIG. 14.1 SEPHADEX G-200 CHROMATOGRAPHY OF THE HEXOSAMINIDASE PREPARATION OBTAINED FROM STEP 3 OF METHOD I



Column dimensions = 5cm X 90cm

Sample added = 6g protein

Flow rate = 30ml/hour

Fraction volume = 10ml

Buffer = 10mM sodium phosphate, pH 5.0

The horizontal bar shows the pooled fractions.

FIG. 14.2 DEAE-CELLULOSE CHROMATOGRAPHY OF THE HEXOSAMINIDASE PREPARATION FROM STEP 4 OF METHOD I

Column dimensions	=	**4.4cm X 90cm**
Sample added	=	**2g protein**
Flow rate	=	**35ml/hour**
Fraction volume	=	**10ml**
Gradient	=	**0-0.3M sodium chloride in 10mM sodium phosphate buffer, pH6.0**

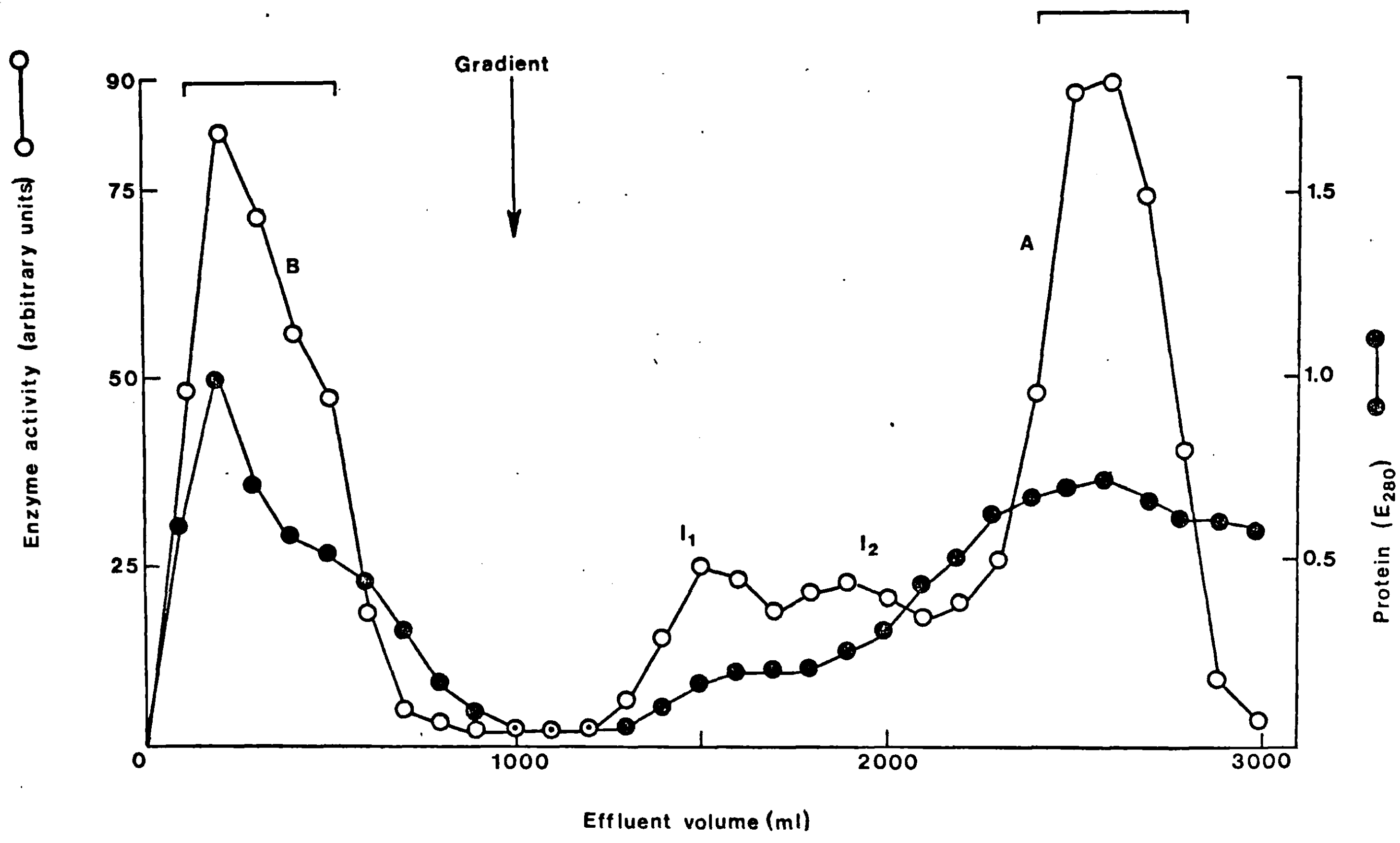

Enzyme activity (arbitrary units)
Protein (E_{280})
Gradient
B
A
I_1
I_2
90
75
50
25
0
1000
2000
3000
1.5
1.0
0.5
Effluent volume (ml)

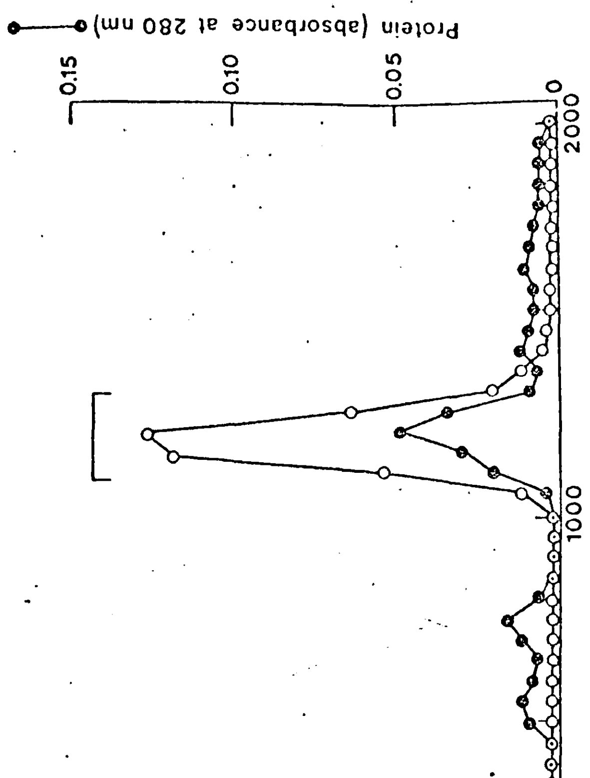
Fig. 14.3 Chromatography of hexosaminidase A on CM-Sephadex and DEAE-Sephadex

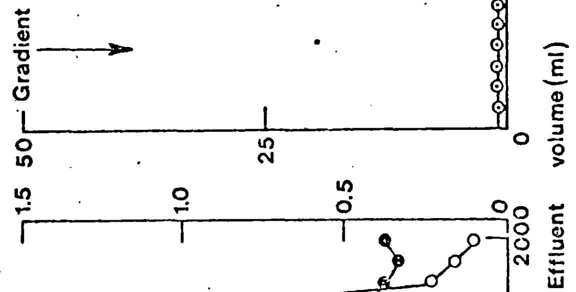
A. CM-Sephadex

Column dimensions	=	2.5cm x 30cm
Sample added	=	113mg protein
Flow rate	=	20ml/hour
Fraction volume	=	10ml
Gradient	=	0-0.5M NaCl in 20mM sodium acetate buffer, pH 4.8

B. DEAE-Sephadex

Column dimensions	=	2.5cm x 30cm
Sample added	=	4mg protein
Flow rate	=	20ml/hour
Fraction volume	=	10ml
Gradient	=	0-0.3M NaCl in 25mM sodium phosphate buffer, pH 6.0


Protein (absorbance at 280 nm)
0.15
0.10
0.05
0
2000
1000
Gradient
B.


50
25
0
volume (ml)
1.5
1.0
0.5
0
2000
Effluent

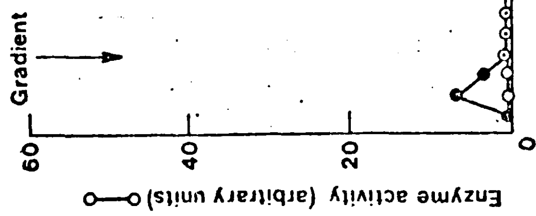

60
40
20
0
Gradient
A.
Enzyme activity (arbitrary units)

Fig. 14.4 Chromatography of hexosaminidase B on CM-Sephadex and Sephadex G-200

A. CM-Sephadex

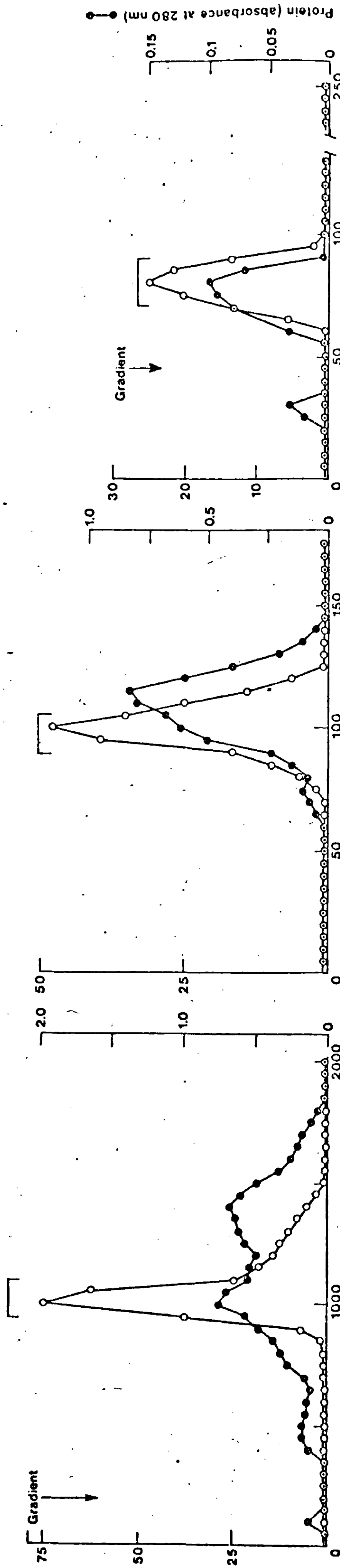
Column dimensions	=	2.5cm x 30cm
Sample added	=	170mg of protein
Flow rate	=	30ml/hour
Fraction volume	=	10ml
Gradient	=	0-0.6M NaCl in 20mM sodium acetate buffer, pH 5.0

B. Sephadex G-200

Column dimensions	=	2.5cm x 30cm
Sample added	=	30mg protein
Flow rate	=	20ml/hour
Fraction volume	=	10ml
Buffer	=	10mM sodium phosphate, pH 5.0, containing 0.1M ammonium sulphate

C. CM-Sephadex

Column dimensions	=	1cm x 20cm
Sample added	=	6mg protein
Flow rate	=	10ml/hour
Fraction volume	=	5ml
Gradient	=	0-0.2M NaCl in sodium phosphate buffer, pH 6.0


Protein (absorbance at 280 nm)
Gradient
Effluent volume (ml)
A.
B.
C.
0
25
50
75
1000
2000
1.0
2.0
0.5
100
150
10
20
30
0.05
0.1
0.15
250

it is likely that the first peak represented hexosaminidase C. This form has a molecular weight of about 200,000 compared to the value of about 100,000 reported for hexosaminidases A and B. Available evidence indicates that hexosaminidase C is not structurally related to the other hexosaminidases (see section 1.3) and since it was not intended to study this enzyme, fractions containing it were discarded. Separation of hexosaminidases A and B was achieved by chromatography on DEAE-cellulose (see Fig. 14.2). It can be seen that hexosaminidase B was not retained by the column at pH 7.0. Hexosaminidases I_1, I_2 and A were eluted sequentially by the application of a linear salt gradient. Hexosaminidase B was not eluted as a symmetrical peak but had a distinct shoulder on its trailing edge. This behaviour was noted on all column runs and, on runs where the column was underloaded, two peaks were often resolved. Heterogeneity of hexosaminidase B has not been widely reported although Leaback and Robinson (1975) described the separation of more than one form of pig epididymal hexosaminidase B by ampholyte displacement chromatography and Grebner and Tucker (1973) observed that hexosaminidase B from human urine often appeared as two peaks on DEAE-cellulose chromatography.

Further purification was carried out as outlined in Fig. 8.1. The final hexosaminidase A and hexosaminidase B preparations had specific activities of 81 and 55 units/mg protein, respectively. Overall yields were low; 1.0% for hexosaminidase A and 0.7% for hexosaminidase B. In absolute terms this meant that 700µg of hexosaminidase A and 600µg of hexosaminidase B were recovered from a total of four term placentas. Attempts to raise specific antisera to these preparations were largely unsuccessful. Injection of hexosaminidase A produced a polyvalent antiserum with low anti-(hexosaminidase) titre and injection of hexosaminidase B failed to elicit any response. The former result showed that the hexosaminidase A preparation had not been sufficiently purified prior to injection.

It was clear from the above results that before another attempt was made to raise anti-(enzyme) antisera some means of improving the overall yields and purity of the final preparations should be sought.

At this time several affinity chromatography procedures for the purification of hexosaminidase from a variety of tissue sources appeared in the literature (Dawson _et al._, 1973; Geiger _et al._, 1974, 1975; Grebner and Parikh, 1974; Pokorny and Glaudemans, 1975). None of these

methods were effective in yielding pure enzyme when used alone but more success was achieved when used in combination with other established techniques (eg. Geiger _et al._, 1975). It should be noted that the term affinity chromatography only properly applies to situations in which elution of the material of interest is brought about by application of a specific competitive inhibitor. In all the above cases, elution was achieved by changing the salt concentration or the pH. Attempts to use specific enzyme inhibitors for this purpose have been relatively unsuccessful (Koshy _et al._, 1975) and suggest that much of the binding of the enzyme to the ligand is due to non-specific forces. The procedure described by Geiger _et al._ (1975) for the purification of placental hexosaminidase A and hexosaminidase B resulted in a high degree of purity with good yields. As well as an affinity chromatography step the main feature of this method was the inclusion of chromatography on Sepharose-bound concanavalin A. Concanavalin A is a phytolectin and has the property of binding molecules possessing either α-D-mannose or α-D-glucose residues (Goldstein et al., 1965). Lysosomal glycosidases were shown to bind to this material, presumably by virtue of the glycoprotein nature, and were eluted using α-methylmannoside or α-methylglucoside (Beutler _et al._, 1975).

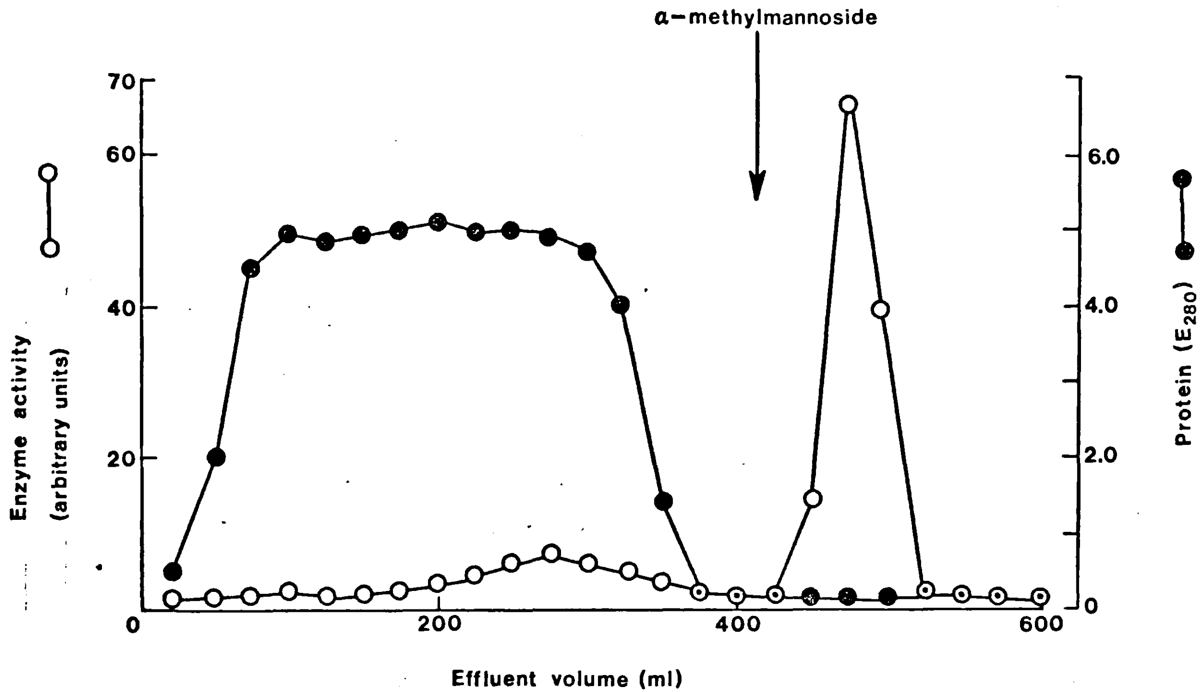
In view of the relatively good yields and high purity reported for the procedure described above (Geiger _et al._, 1975) subsequent purification of hexosaminidase A and hexosaminidase B was carried out by this method with the modification that 2-acetamido-2-deoxy-D-mannono-1,4-lactone was used as the ligand for affinity chromatography instead of 2-acetamido-N-(ε-aminohexanoyl)-2-deoxy-β-D-glucopyranosylamine. Pokorny and Glaudemans (1975) demonstrated that the former gave a 300-fold purification with about 90% yield compared to the 150-fold purification and variable yields reported for the other ligands. An outline of this procedure (Method II) is given in Fig. 8.2 and column profiles and purification data are given in Figs 14.5 - 14.10 and Table 14.2, respectively. The advantage of using the concanavalin A step can be seen from Fig. 14.5 and the data in Table 14.2. Firstly, large volumes of virtually crude placental extract could be applied to a small concanavalin A-Sepharose column. For example, all of the hexosaminidase activity extracted from two placentas was bound by 4ml of the immobilised lectin. Secondly, the enzyme was eluted in a small volume with a recovery of 55% and a 75-fold increase in specific activity. After

TABLE 14.2 PURIFICATION OF HUMAN PLACENTAL HEXOSAMINIDASES A AND B BY METHOD II

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification -fold	Yield (%)
1 Homogenate	2480	40500	0.063	-	100
2 Ammonium sulphate fractionation	1140	11700	0.11	1.8	45
3 Con A-Sepharose	625	75	8.3	138	25
4 DEAE-Cellulose					
Hexosaminidase A	310	20	15	250	13
Hexosaminidase B	212	22	10	160	9
Hexosaminidase A					
5 Affinity chromatography	208	6.2	34	570	8
6 CM-Cellulose	102	1.2	85	1420	4
Hexosaminidase B					
7 Affinity chromatography	96	4.0	24	380	4
8 Sephadex G-200	72	1.5	48	770	3
9 CM-cellulose	50	0.81	62	1000	2

Experimental details are given in section 8.2

FIG. 14.5 CONCANAVALIN A-SEPHAROSE CHROMATOGRAPHY OF THE HEXOSAMINIDASE PREPARATION FROM STEP 2 OF METHOD II



Column dimensions	=	Plastic syringe containing 4ml of concanavalin A-Sepharose
Sample added	=	11g protein
Flow rate	=	60ml/hour
Fraction volume	=	25ml

The enzyme was eluted with α-methylmannoside in 0.1M potassium phosphate buffer, pH7.0, at the point shown.

FIG. 14.6 DEAE-CELLULOSE CHROMATOGRAPHY OF THE HEXOSAMINIDASE PREPARATION FROM STEP 3 OF METHOD II

Column dimensions	=	2.5cm X 40cm
Sample added	=	45mg protein
Flow rate	=	30ml/hour
Fraction volume	=	10ml
Gradient	=	0-0.5M sodium chloride in 10mM sodium phosphate buffer, pH6.0

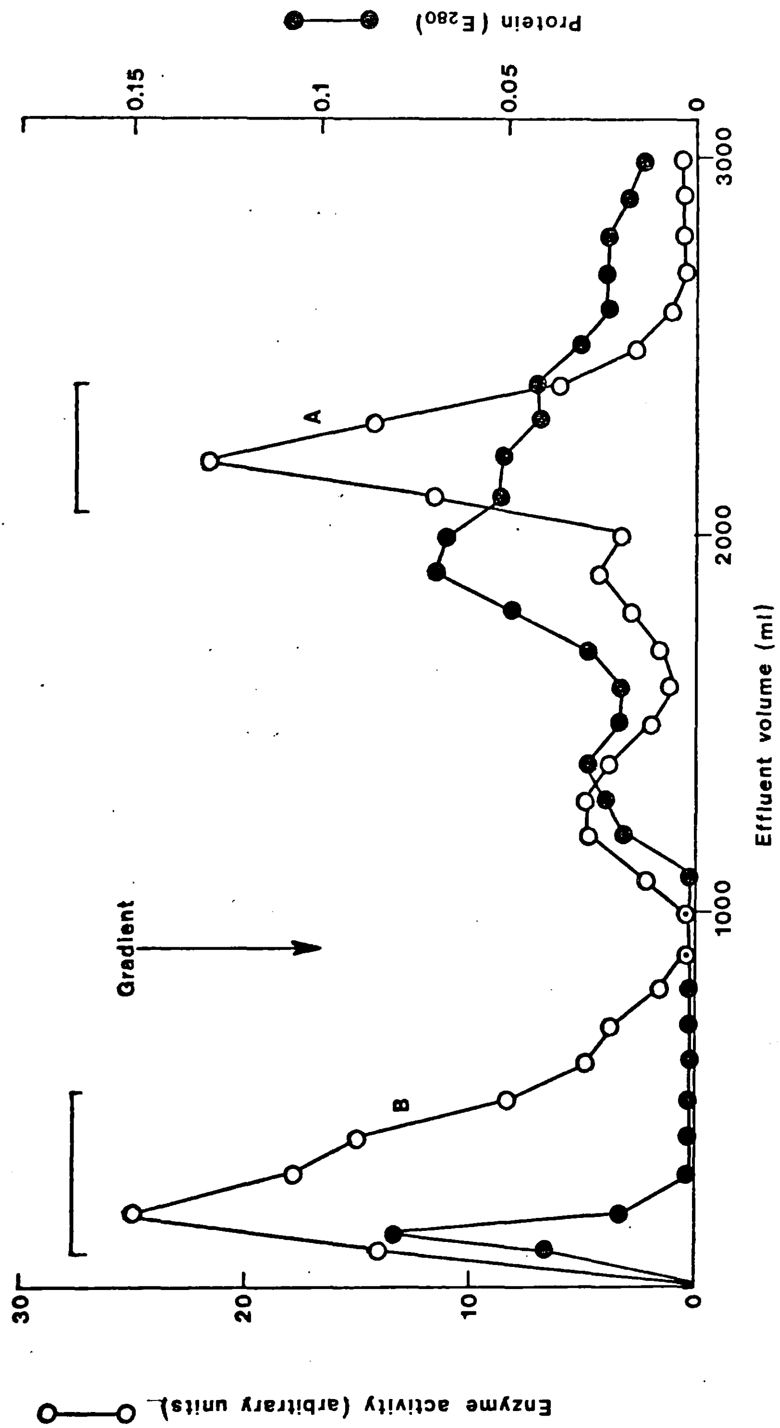
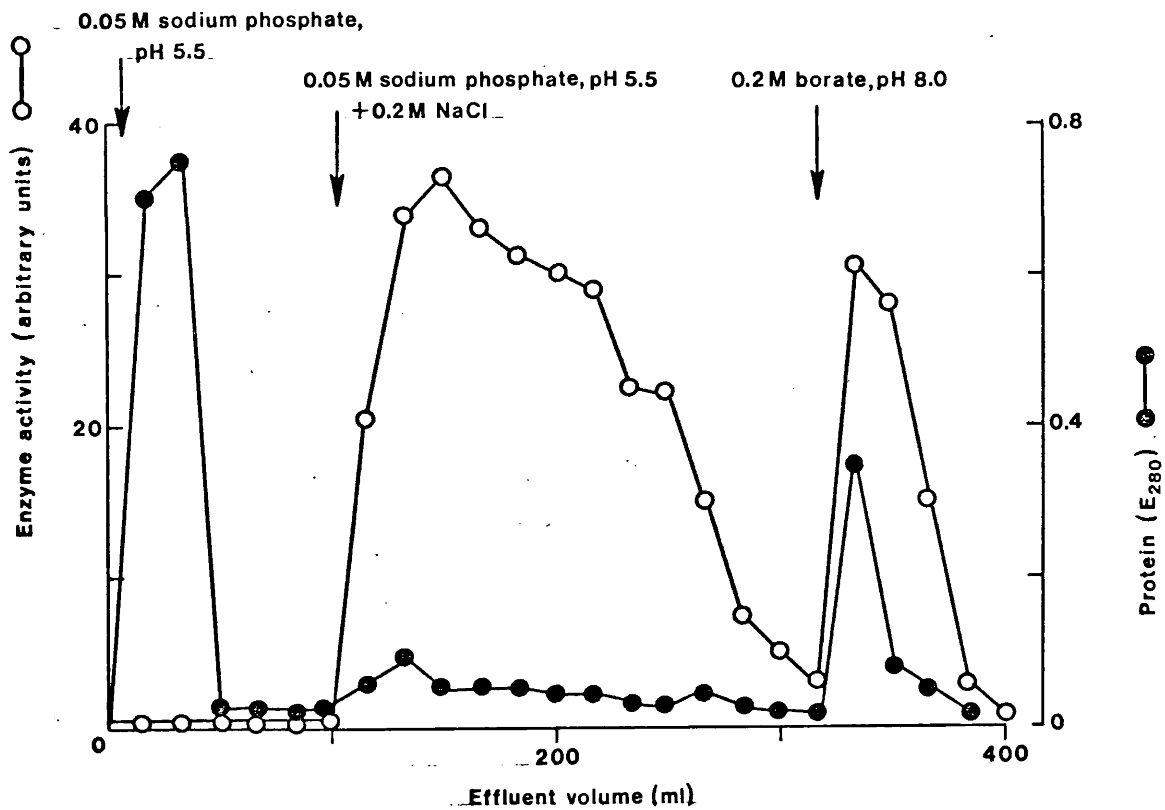

Protein (E_{280})
0.15
0.1
0.05
0
3000
2000
1000
0
A
B
Gradient
Effluent volume (ml)
30
20
10
Enzyme activity (arbitrary units)

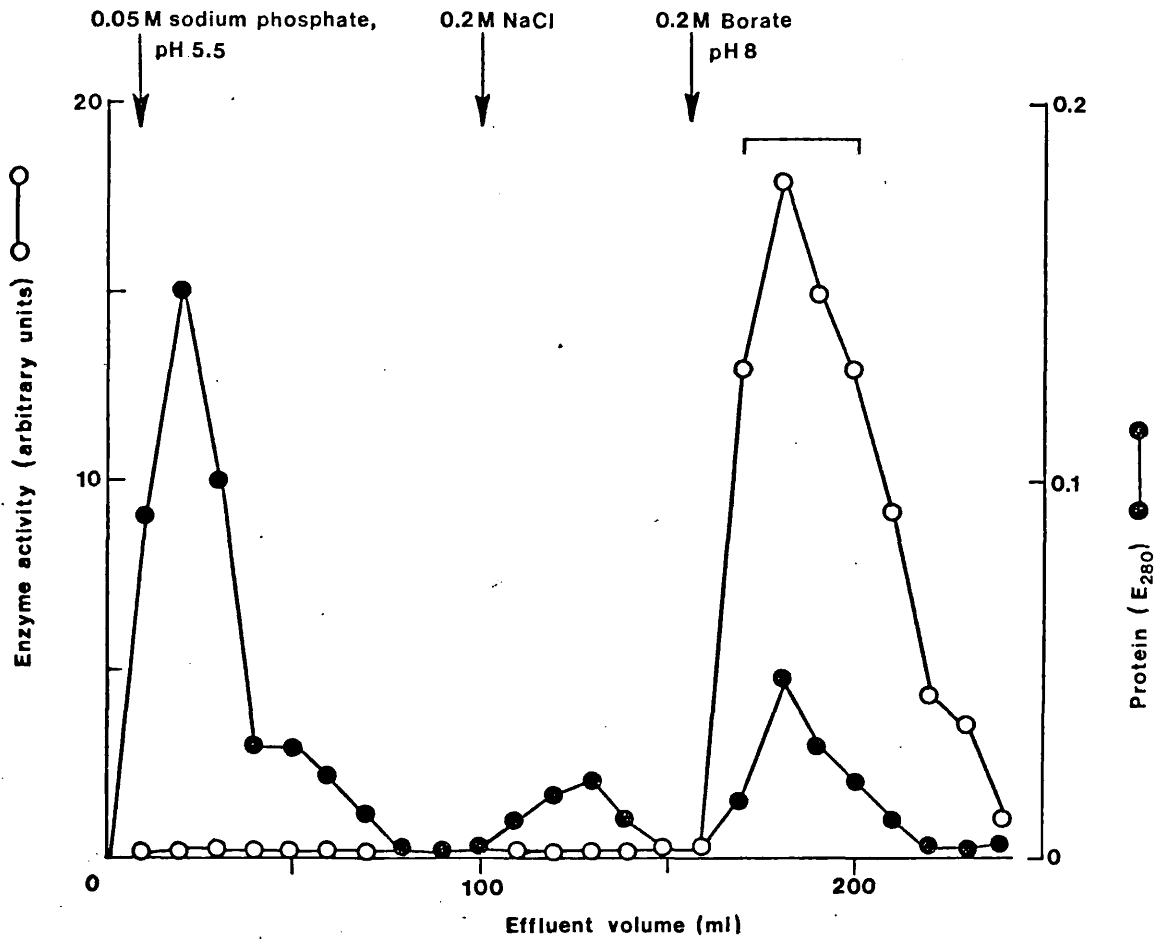
FIG. 14.7 CHROMATOGRAPHY OF THE HEXOSAMINIDASE A PREPARATION FROM STEP 4 OF METHOD II ON 2-ACETAMIDO-2-DEOXY-D-MANNONO-1,4-LACTONE SEPHAROSE



Column dimensions	=	Plastic syringe containing 6ml of the affinity gel
Sample added	=	20mg protein
Flow rate	=	50ml/hour
Fraction volume	=	10ml

The sample was applied to the column and eluted in a stepwise manner using the buffers indicated above.

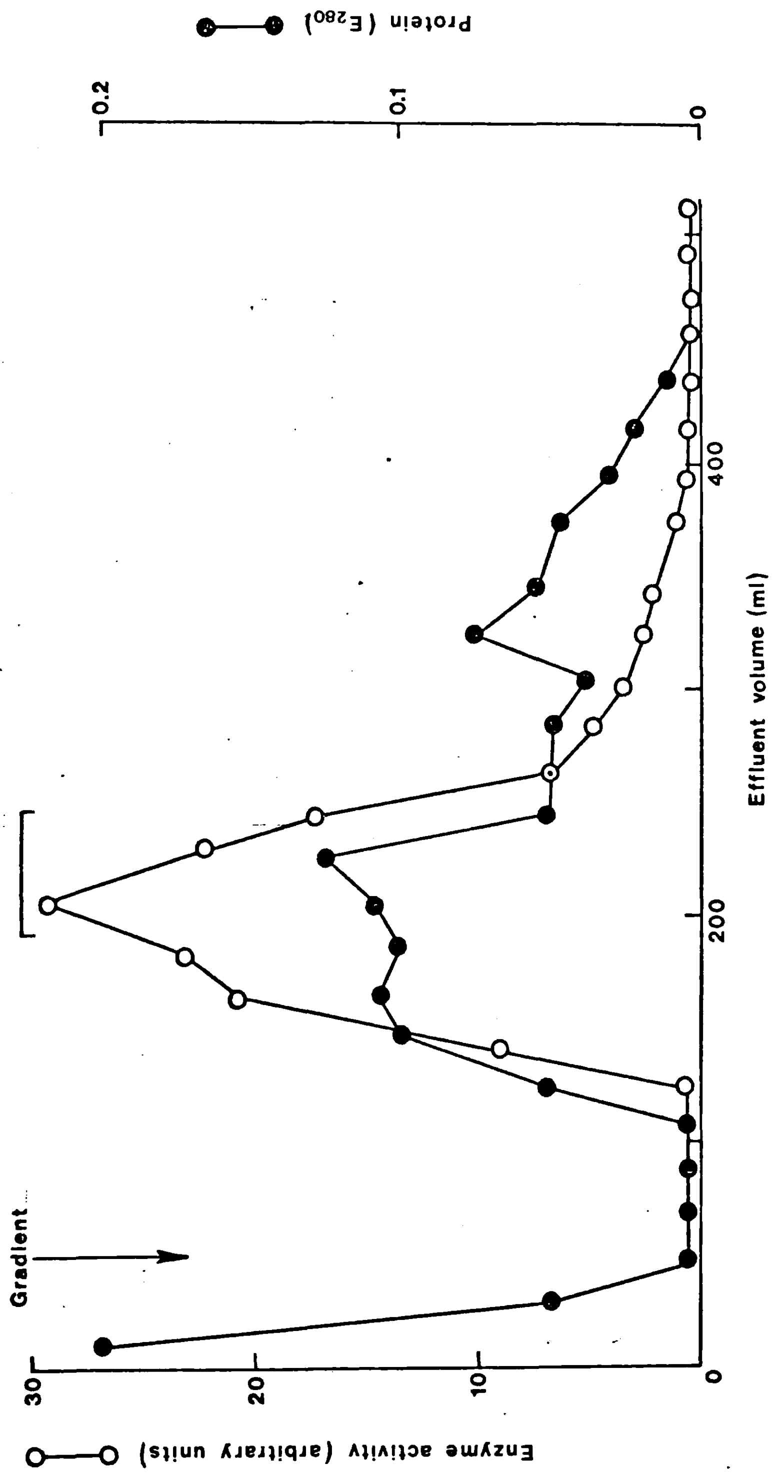
FIG. 14.8 CHROMATOGRAPHY OF THE HEXOSAMINIDASE B PREPARATION FROM STEP 4 OF METHOD II ON 2-ACETAMIDO-2-DEOXY-D-MANNONO-1,4-LACTONE SEPHAROSE



Details as for Fig. 14.7 except that the sample consisted of 22mg protein

FIG. 14.9 CM-CELLULOSE CHROMATOGRAPHY OF HEXOSAMINIDASE A

Column dimensions	=	Plastic syringe containing 10ml of CM-cellulose
Sample added	=	6mg protein
Flow rate	=	20ml/hour
Fraction volume	=	5ml
Gradient	=	0-0.5M sodium chloride in 25mM sodium citrate buffer, pH4.5



Protein (E_{280})
0.2
0.1
0
Gradient
Enzyme activity (arbitrary units)
30
20
10
0
200
400
Effluent volume (ml)

Fig. 14.10 Chromatography of hexosaminidase B on Sephadex G-200 and CM-cellulose

A. Sephadex G-200

Column dimensions	=	2.5cm x 30cm
Sample added	=	4mg protein
Flow rate	=	20ml/hour
Fraction volume	=	5ml
Buffer	=	10mM sodium phosphate buffer, pH 5.0 containing 0.1M ammonium sulphate

B. CM-cellulose

Column dimensions	=	plastic syringe (10ml) bed vol = 10ml
Sample added	=	1.5mg protein
Flow rate	=	20ml/hour
Fraction volume	=	5ml
Gradient	=	0-0.6M NaCl in 10mM sodium phosphate buffer, pH 5.0

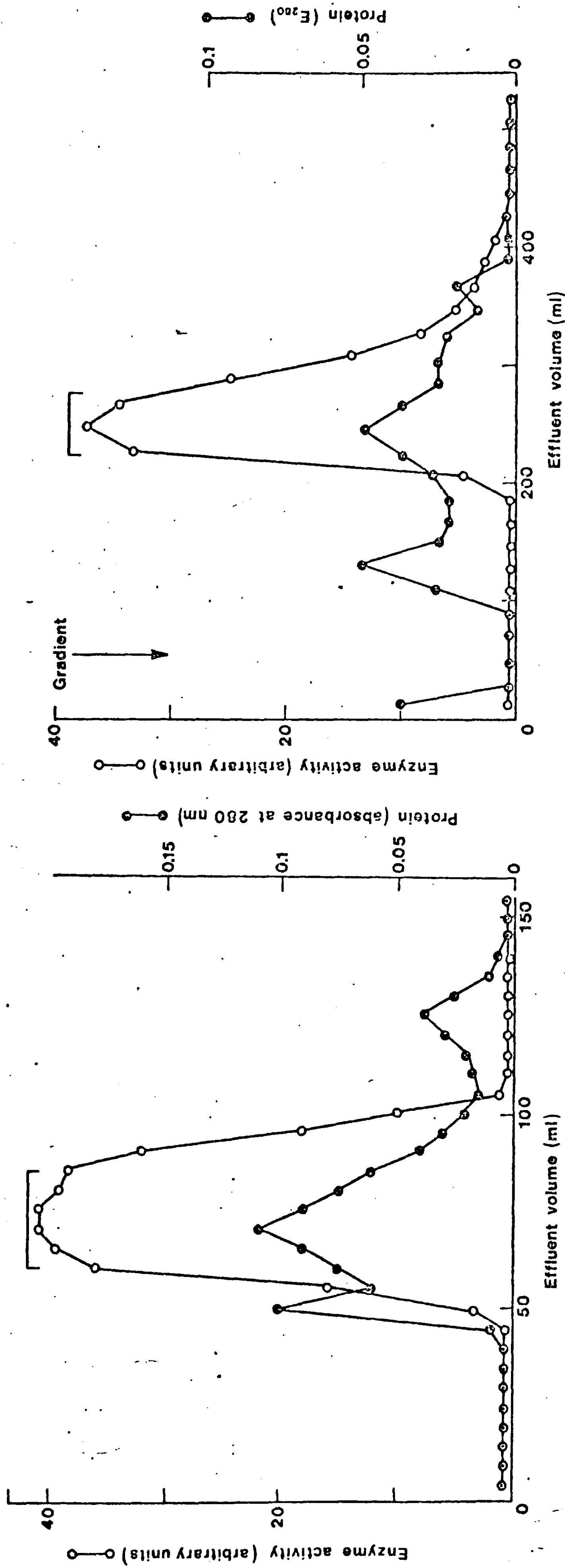
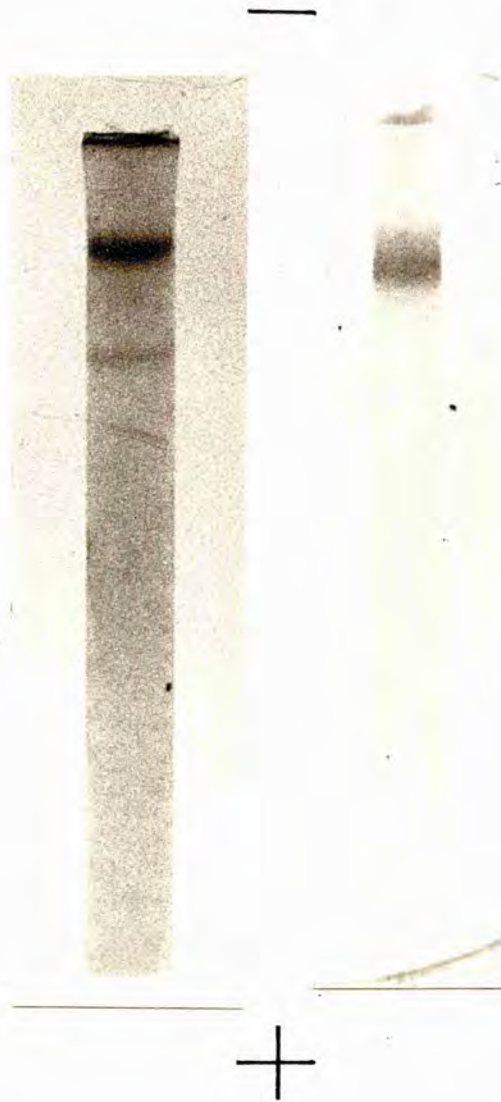

Gradient
Enzyme activity (arbitrary units)
Protein (E_{280})
Effluent volume (ml)
0
20
40
0.05
0.1
200
400
B.
Enzyme activity (arbitrary units)
Protein (absorbance at 280 nm)
Effluent volume (ml)
0
20
40
0.05
0.1
0.15
50
100
150
A.

FIG. 14.11 POLYACRYLAMIDE GEL ELECTROPHORESIS OF PLACENTAL HEXOSAMINIDASES A AND B



About 100μg each of purified hexosaminidase A (left) and hexosaminidase B (right) obtained by purification methodII were subjected to polyacrylamide gel electrophoresis as described in section 7.5.1. The photographs show the gels after staining for protein as described in section 7.5.1. The single band obtained for hexosaminidase B and the major (less anodal) band obtained for hexosaminidase A also stained positively for hexosaminidase activity (notshown).

separation on DEAE-cellulose (Fig. 14.6) hexosaminidases A and B were further purified by affinity chromatography. All of the hexosaminidase B activity was bound by the affinity column and about 50% of the applied activity was recovered in the borate buffer wash with a 2.5-fold increase in specific activity (Fig. 14.8). Hexosaminidase A behaved differently, as can be seen from Fig. 14.7. Most of the activity was eluted in the sodium chloride wash and considerable trailing was observed. A small amount of activity was eluted with the borate buffer wash. Electrophoresis of the two peaks on polyacrylamide gels at pH 4.5 showed that the activity eluted by the borate wash was not due to the presence of contaminating hexosaminidase B. These differences in behaviour between hexosaminidase A and hexosaminidase B when chromatographed on immobilised 2-acetamido-2-deoxy-D-mannono-1,4-lactone were not investigated further but provide additional evidence relating to the heterogeneity of these forms. These results also suggest that the above method could be used for the separation of hexosaminidase A and B as an alternative to ion-exchange chromatography and electrophoresis.

Final purification of hexosaminidase A and hexosaminidase B was carried out as outlined in Fig. 8.2 and the specific activities of the preparations were 85 and 62 units/mg protein respectively. When analysed by polyacrylamide gel electrophoresis hexosaminidase B showed one protein band while hexosaminidase A showed one major and one minor protein band (see Fig. 14.11). No further large-scale purification of hexosaminidase A was attempted because of the small amounts of protein remaining at this stage. Two placentas yielded about 1.2mg of hexosaminidase A and about 0.8mg of hexosaminidase B.

In conclusion, it can be seen that method II gave better overall recoveries than method I and, because of the smaller volumes involved, sample processing was considerably faster. With few exceptions, such as α-fucosidase (Opheim and Touster, 1977) and α-glucosidase (de Barsy et al., 1972) the large scale purification of lysosomal enzymes has proved difficult. This is probably due to their relatively low concentrations in most tissues. For example, hexosaminidase constitutes less than 0.1% of total soluble protein in the term placenta based on the specific activity data given in Table 14.2. The specific activities obtained for hexosaminidase A and hexosaminidase B by both method I and method II were similar and compare reasonably well with the figures

obtained by Srivastava et al. (1974a) but not with those obtained by others. For example, Lee and Yoshida (1976) reported a specific activity of 150 units/mg protein for hexosaminidase A and 207 units/mg protein for hexosaminidase B while Geiger and Arnon (1976) obtained values of 23.3 and 22.6 units/mg protein for hexosaminidase A and hexosaminidase B respectively. It is difficult to find reasons for these discrepancies. All the above enzyme preparations were judged to be homogenous on the basis of polyacrylamide gel electrophoresis and sedimentation equilibrium experiments and, as far as can be seen, the assay conditions were the same in each case. The presence of unresolved contaminants, loss of specific activators during purification, or loss of activity due to gradual denaturation are all possible explanations. It is perhaps notable that Geiger and Arnon (1976) found sialic acid in their hexosaminidase A preparation and none in hexosaminidase B while Lee and Yoshida (1976) found no sialic acid in either form. Loss of certain functionally important carbohydrate residues during the purification could account for these differences and may possibly explain the differing specific activities obtained. These discrepancies suggest that, although beneficial in monitoring individual purification steps, measurement of hexosaminidase specific activity for the purpose of assessing the absolute purity of the preparations is of little value.

14.2 Production of antisera to placental hexosaminidases A and B

The hexosaminidase B preparation obtained by method II was used directly for immunisation. The treatment of the enzyme prior to injection and details of the immunisation schedule are described in Materials and Methods, section 9.1. Rabbit anti-(hexosaminidase B) antibodies were detected by immunoprecipitation analysis and immunoelectrophoresis after a total of 280µg of hexosaminidase B had been injected. Fig. 14.12 shows the result of the immunoprecipitation analysis in which increasing amounts of antiserum were incubated with a fixed volume of crude placental extract containing a constant amount of hexosaminidase activity. It can be seen that 1ml of anti-(hexosaminidase B) serum precipitated from solution about 0.75 units of hexosaminidase, equivalent to about 12µg of enzyme protein (based on the specific activity data in Table 14.2). The residual activity (about 5% of the total initial activity) remaining after precipitation was probably due to the presence of hexosaminidase C

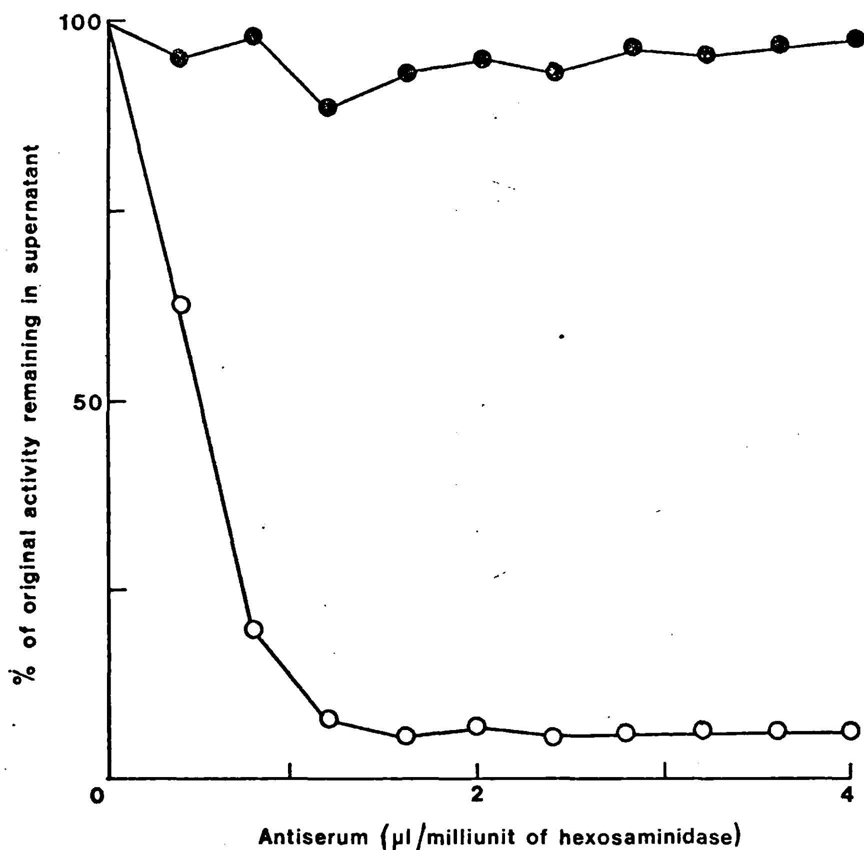
in the placental extract. This enzyme has already been discussed in the introduction (see section 1.3) and shown by other workers to be immunologically distinct from the A and B forms. Immunoelectrophoretic analysis (Fig. 14.13) showed that the anti-(hexosaminidase B) serum reacted with both hexosaminidase A and hexosaminidase B. This is in agreement with published results (Srivastava and Beutler, 1972; Carroll and Robinson, 1973 and Bartholomew and Rattazzi, 1974).

Because the hexosaminidase A preparation from method II was known to be impure, a different strategy was employed for antibody production in order to minimise the risk of producing antibodies to the contaminant(s). Smith _et al_. (1964) described an immunological method for obtaining pure antigens for immunisation purposes from a mixture of different proteins. The procedure involved reacting the mixture of proteins (containing the antigen of interest) with a poly-valent antiserum (containing antibodies against the antigen) in a gel diffusion system. The only further requirement was that the specific precipitin line formed between the required antigen-antibody complex be distinguishable from other precipitin lines. Having identified the precipitin line of interest, this was carefully excised from the gel and used as the immunising material. In the present situation, the availability of anti-(hexosaminidase B) serum and the fact that it reacted with hexosaminidase A to produce an identifiable precipitin line suggested that the above methodology could be applied to obtain anti-(hexosaminidase A) serum.

Purified hexosaminidase A was allowed to react with anti-(hexosaminidase B) serum in a gel diffusion system, as described in Materials and Methods (see section 9.1). The photograph shown in Fig. 14.14 was obtained after the gel had been stained for hexosaminidase activity. Two precipitin lines were visible only one of which stained for enzyme activity (the second precipitin line does not show up in the photograph in Fig. 14.14). The active precipitin line was carefully removed and used for immunisation. After the injection of four such lines, corresponding at most to a total of about 2μg of enzyme protein, anti-(hexosaminidase A) antibodies were detected by immunoprecipitation analysis (Fig. 14.15) and immunoelectrophoresis (Fig. 14.16). One ml of the anti-(hexosaminidase A) serum could precipitate about 0.70 units of hexosaminidase, equivalent to about 11μg of enzyme protein.

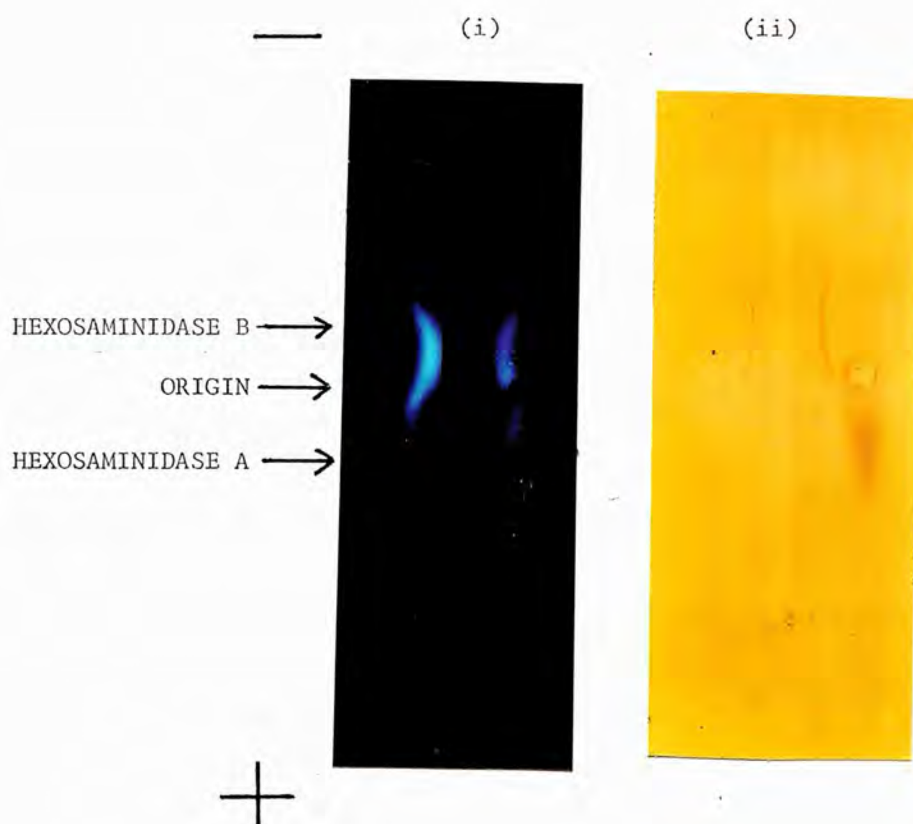
In spite of the fact that about 100 times less antigen was used

FIG. 14.12 IMMUNOPRECIPITATION OF PLACENTAL HEXOSAMINIDASE WITH ANTI-(HEXOSAMINIDASE B) SERUM



Increasing volumes of antiserum were added to tubes containing 25munits of a crude hexosaminidase preparation from step 1 of Method II. After incubation and centrifugation as described in section 9.2, hexosaminidase activity was determined for each of the supernatants. The points shown represent means of duplicate incubations.

FIG. 14.13 IMMUNOELECTROPHORESIS OF A PLACENTAL EXTRACT WITH ANTI-(HEXOSAMINIDASE B) ANTISERUM

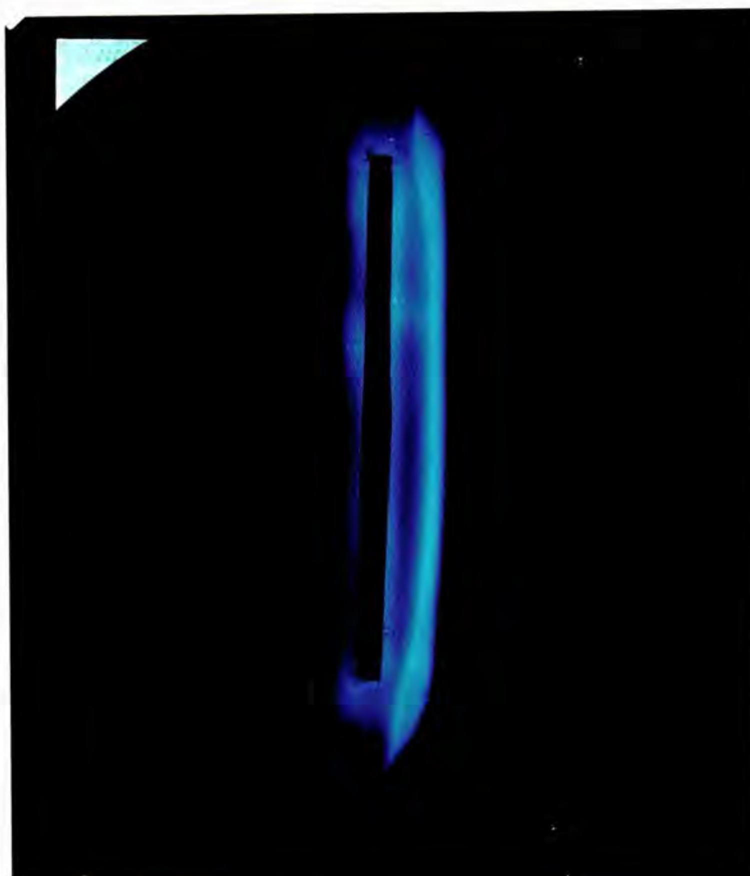


Experimental details are given in section 9.3. The antigen wells (origin) contained crude placental supernatant and the trough contained anti-(hexosaminidase B) serum. Under the conditions used hexosaminidase A migrated towards the anode and hexosaminidase B to the cathode.

(i): stained for hexosaminidase activity as described in section 7.2.1.3.

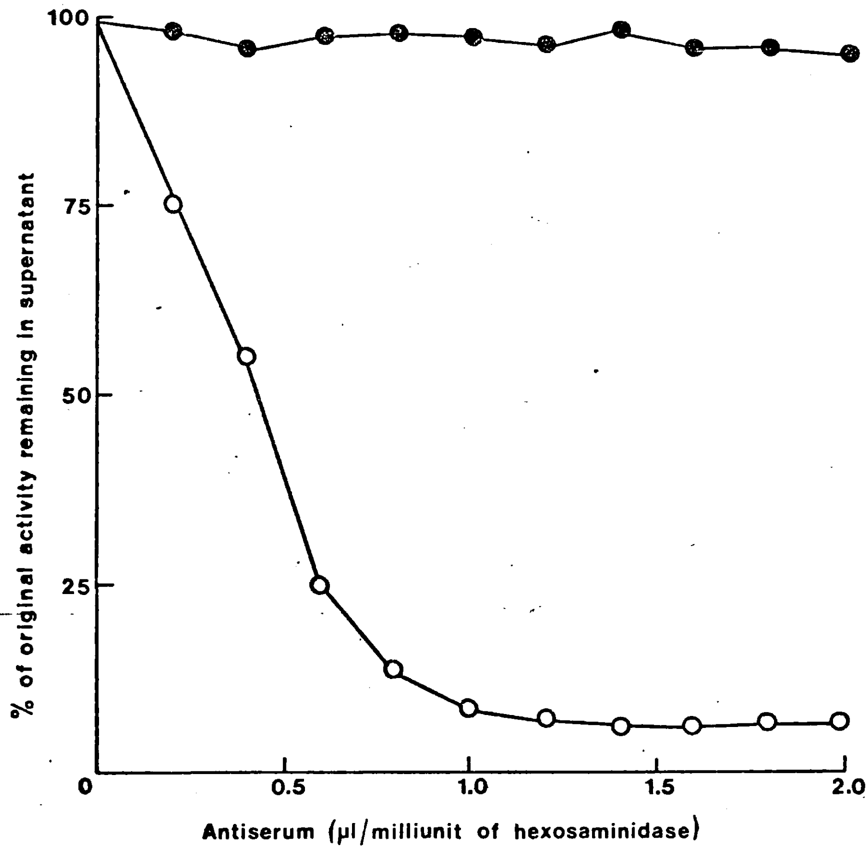
(ii): stained for protein as described in section 7.5.1.

FIG. 14.14 IMMUNODIFFUSION OF HEXOSAMINIDASE A WITH ANTI-(HEXOSAMINIDASE B) ANTISERUM



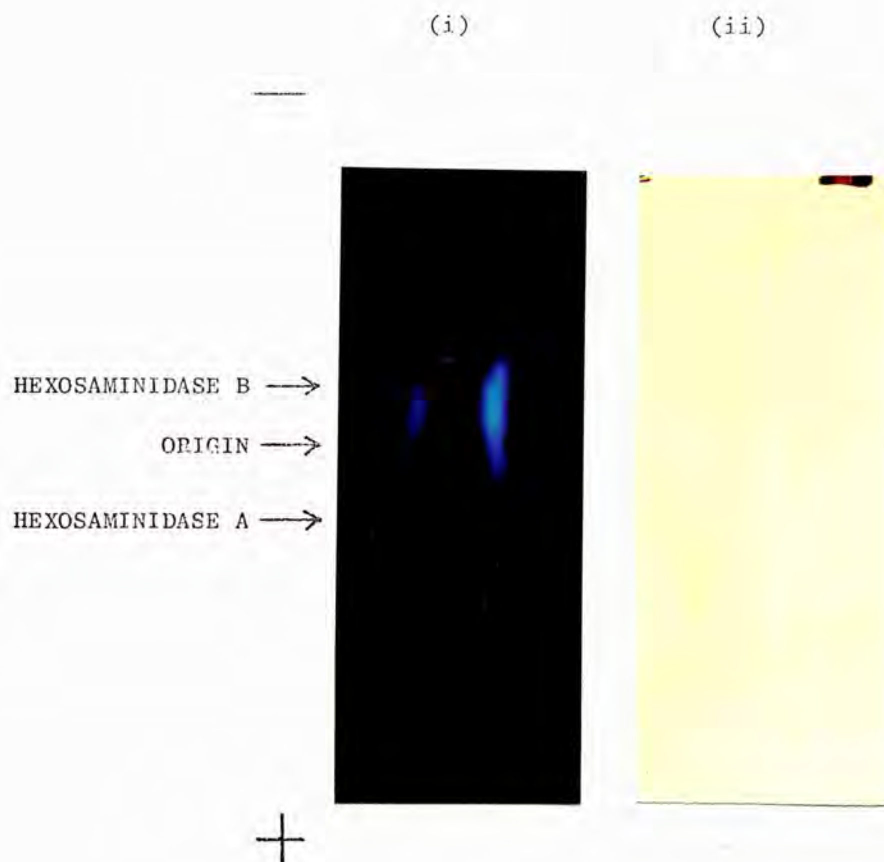
Purified hexosaminidase A (30µl) was placed in the trough on the left and anti-(hexosaminidase B) antiserum (30µl) in the trough on the right and immunodiffusion was carried out as described in section 9.1. The slide was then stained for hexosaminidase activity as described in section 7.2.1.3.

FIG. 14.15 IMMUNOPRECIPITATION OF PLACENTAL HEXOSAMINIDASE WITH ANTI-(HEXOSAMINIDASE A) SERUM



Details as for Fig. 14.12 except that anti-(hexosaminidase A) serum was used.

FIG. 14.16 IMMUNOELECTROPHORESIS OF A PLACENTAL EXTRACT WITH ANTI-(HEXOSAMINIDASE A) ANTISERUM

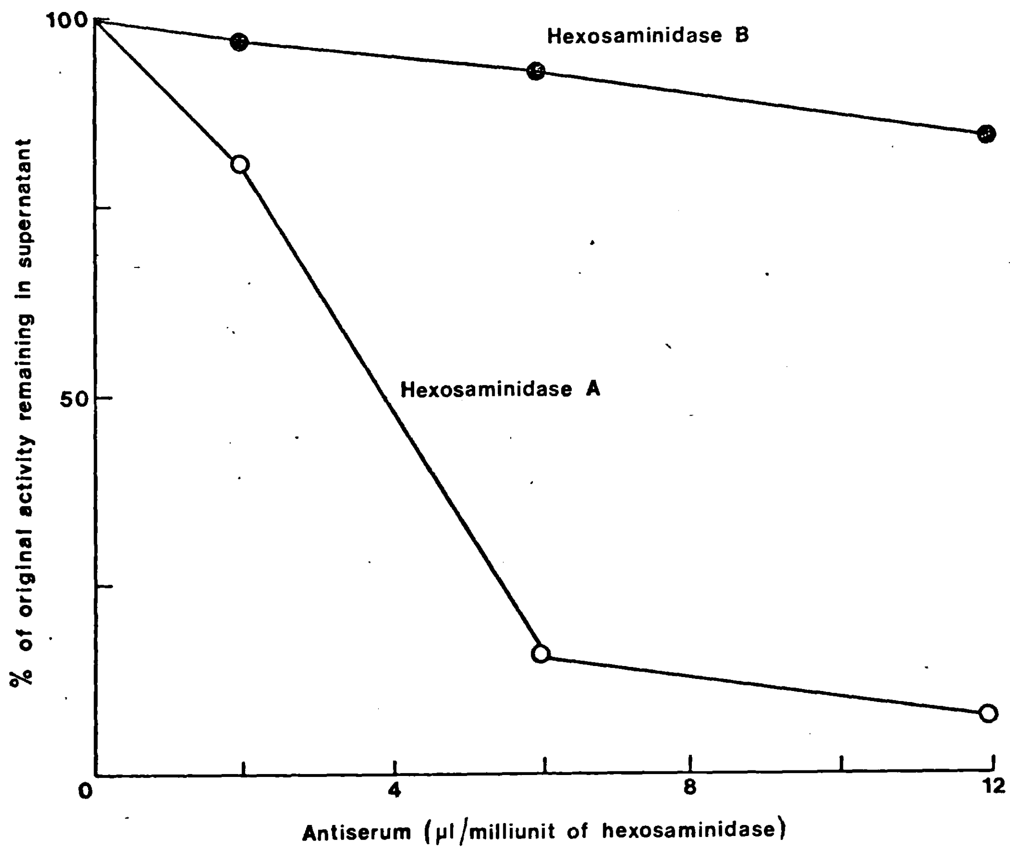


Experimental details are given in section 9.3. The antigen wells (origin) contained crude placental supernatant and the trough contained anti-(hexosaminidase A) serum. Under the conditions used hexosaminidase A migrated towards the anode and hexosaminidase B to the cathode.

(i): stained for hexosaminidase activity as described in section 7.2.1.3.

(ii): stained for protein as described in section 7.5.1.

FIG. 14.17 IMMUNOPRECIPITATION OF PLACENTAL HEXOSAMINDASES A AND B WITH ABSORBED ANTI-(HEXOSAMINIDASE A) SERUM



Increasing volumes of antiserum were added to tubes containing 25munits of hexosaminidase A or hexosaminidase B from step 4 of Method II. After incubation and centrifugation as described in section 9.2, hexosaminidase activity was determined for each of the supernatants. The points are means of duplicate incubations.

for the production of the anti-(hexosaminidase A) serum, the titres of the antisera raised against hexosaminidase A and B were very similar. The reason for the enhanced immune response against immuno-complexed antigens is unknown. Interestingly, chemical polymerisation of weak antigens also causes an increase in antigenicity (Gill, 1972). Although the approach described above has not seen widespread application, specific antisera against cathepsin D (Weston, 1969), elastase (Starkey and Barrett, 1976) and plasma proteins (Krøll and Anderson, 1976) were raised by the injection of precipitin lines and, in each case, a response was obtained by the injection of nanogram amounts of antigen.

The immunoelectrophoresis studies showed that the only visible precipitin arcs stained for both protein and hexosaminidase activity (Figs. 14.13 and 14.16). Similar results were obtained when different dilutions of antisera and antigens were used. These results suggested that the two anti-(hexosaminidase) antisera were monospecific. Frequently, precipitin arcs were visualised when stained for hexosaminidase activity but were not detectable by protein staining. This probably reflected the relatively low amounts of antigen-antibody complex present and the greater sensitivity of the fluorometric assay compared to the protein staining method. This phenomenon has been observed for anti-(hexosaminidase)/hexosaminidase complexes by other workers (Carroll and Robinson, 1973). Although the results described above suggested monospecificity of the anti-(hexosaminidase) sera, the existence of other antigen-antibody complexes not detectable by the methods used here remains a possibility.

14.2.1 Absorption of anti-(hexosaminidase A) serum by hexosaminidase B

The antisera described above reacted with both hexosaminidase A and B. Srivastava and Beutler (1973) were able to prepare an antiserum reactive only against hexosaminidase A by absorbing anti-(hexosaminidase A) serum with hexosaminidase B. This has subsequently been confirmed by other workers (Bartholomew and Rattazzi, 1974 and Ben-Yoseph *et al.*, 1975). When the anti-(hexosaminidase A) serum obtained in the present study was repeatedly passed through a column of partially-purified hexosaminidase B covalently attached to Sepharose 4B, hexosaminidase B precipitating activity was gradually reduced until none was detectable. However, in agreement with Srivastava and Beutler (1973) the absorbed antiserum still

retained activity towards hexosaminidase A. Immunoprecipitation analysis of the absorbed anti-(hexosaminidase A) serum with hexosaminidase A and B is shown in Fig. 14.17. The titre of the absorbed serum was considerably lower than that of either the unabsorbed anti-(hexosaminidase A) or anti-(hexosaminidase B) sera. One ml of absorbed antiserum could precipitate about 80munits of hexosaminidase A corresponding to little more than 1μg of enzyme protein. This suggests that the original anti-(hexosaminidase A) antiserum may have contained more antibodies to the β- than to the α-subunits.

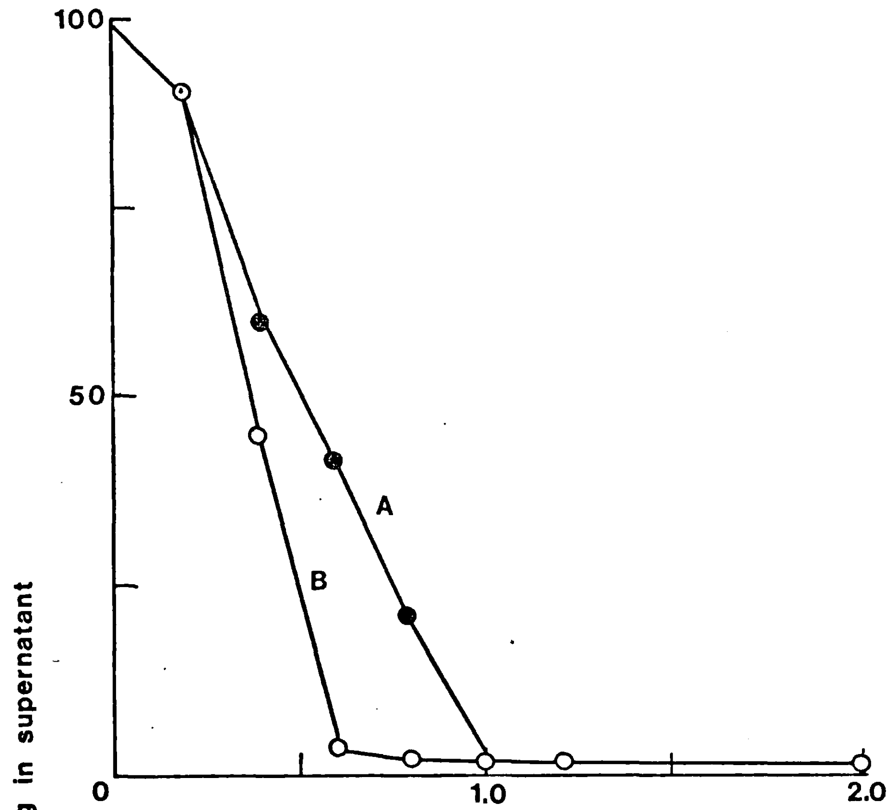
14.3 Immunological relatedness of hexosaminidase multiple forms

The experiments to be described here were carried out to compare the reactivity of hexosaminidases A, B, I_1 and I_2 with the different anti-(hexosaminidase) antisera. The enzyme forms used for these studies were obtained from the DEAE-cellulose step of purification method II. Increasing amounts of the different antisera were added to tubes containing a constant amount of hexosaminidase activity. After incubation, the samples were centrifuged and the hexosaminidase activities in the supernatants were determined.

The titration curves for anti-(hexosaminidase A) with hexosaminidases A, B, I_1 and I_2 are shown in Fig. 14.18 and those for anti-(hexosaminidase B) with hexosaminidases A, B, I_1 and I_2 are shown in Fig. 14.19. It can be seen that both antisera precipitated all the enzyme forms from solution. This was expected for hexosaminidases A and B since the immunological and structural studies of other workers (see introduction, section 1.3) indicated subunit structures of $(\alpha\beta)_n$ and $(\beta\beta)_n$ for these two forms, respectively. Thus, the precipitation of A and B by both anti-(hexosaminidase A) and anti-(hexosaminidase B) antisera occurs by virtue of the β-subunit common to both forms. On the other hand, to date, there have been no detailed immunological or structural studies of the I_1 and I_2 forms. Carroll and Robinson (1973) reported that an intermediate form of human liver hexosaminidase (probably a mixture of I_1 and I_2) gave a reaction of complete identity with the A and B forms as judged by immunodiffusion against anti-(hexosaminidase) serum. The results obtained in the present study (Figs. 14.18 and 14.19) suggest that I_1 and I_2 share common antigenic determinants with the A and B forms. That the I_1 and I_2 forms possess β-subunits was indicated by their precipitation with anti-(hexosaminidase B).

FIG. 14.18 IMMUNOPRECIPITATION OF HEXOSAMINIDASES A, B, I_1 AND I_2 WITH ANTI-(HEXOSAMINIDASE A) SERUM

Experimental details are given in section 9.2. The hexosaminidase fractions were obtained from step 4 of Method II.


100
50
0
1.0
2.0
A
B

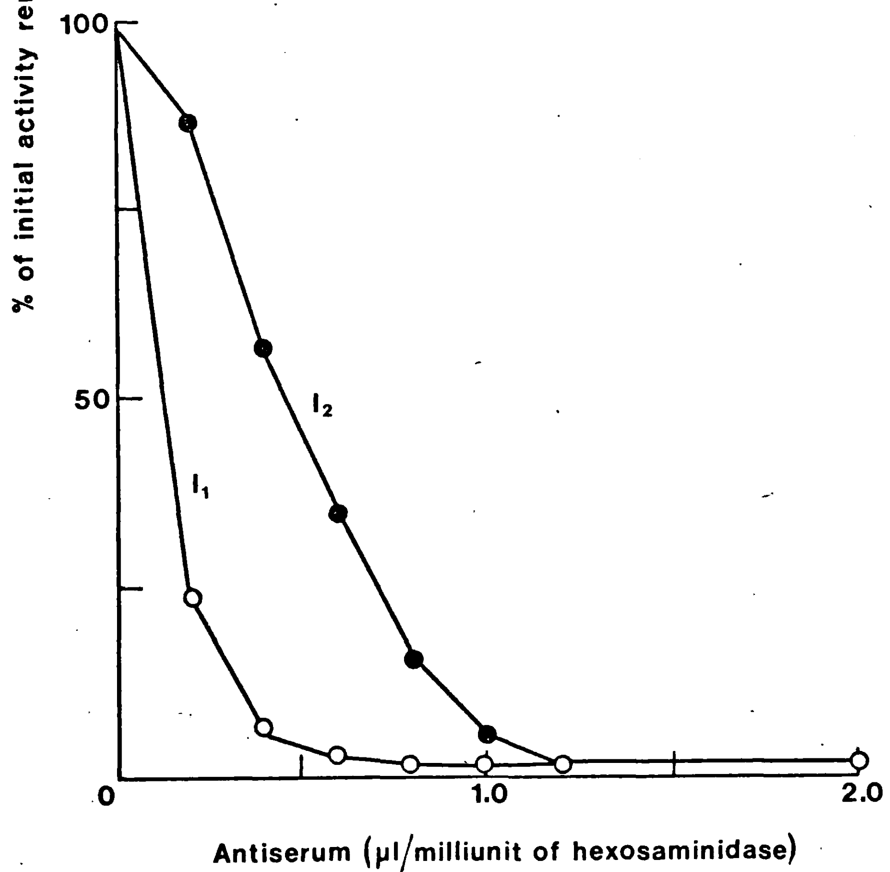
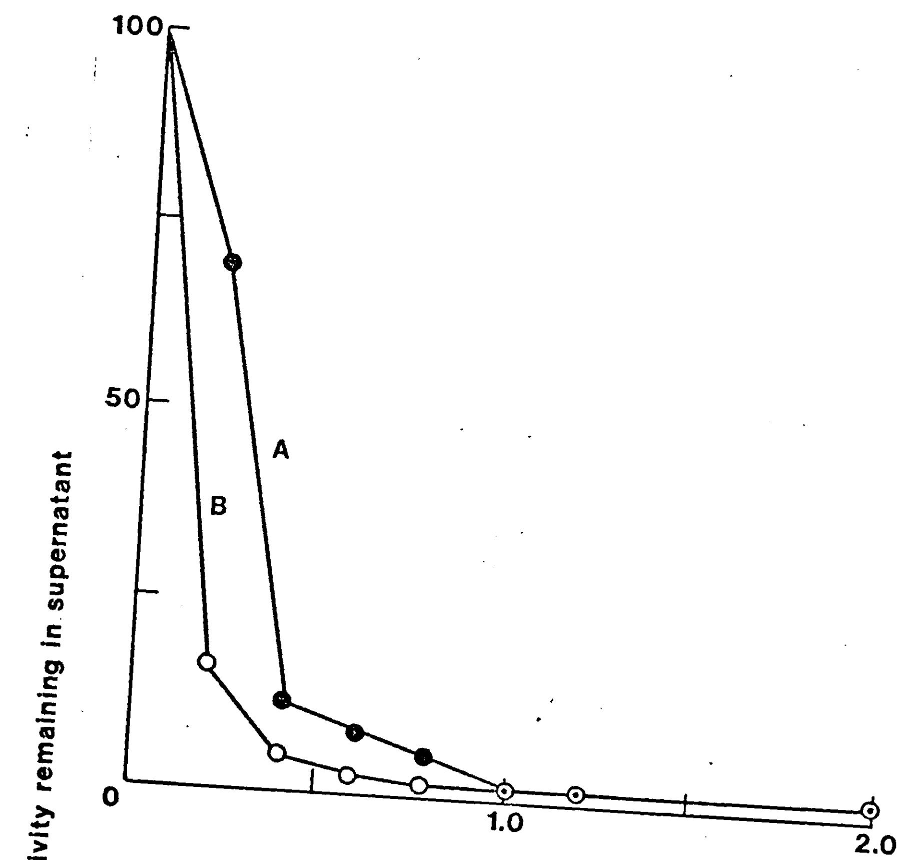
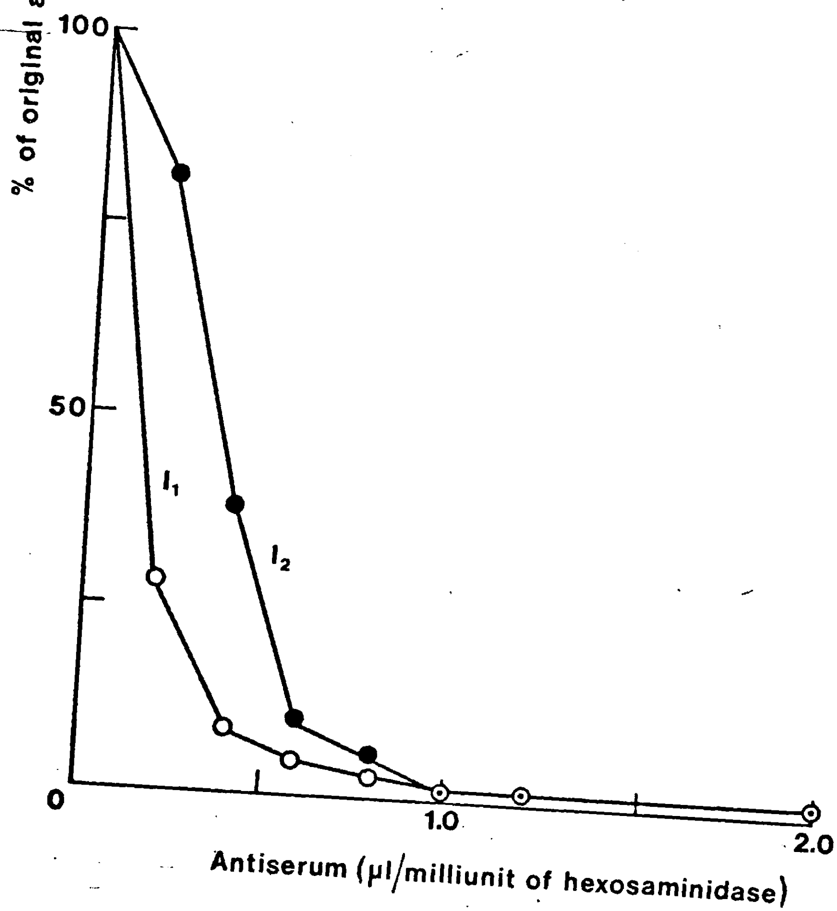

% of initial activity remaining in supernatant
100
50
0
1.0
2.0
I2
I1
Antiserum (µl/milliunit of hexosaminidase)

FIG. 14.19 IMMUNOPRECIPITATION OF HEXOSAMINIDASES A, B, I_1 AND I_2 WITH ANTI-(HEXOSAMINDASE B) SERUM

Experimental details are given in section 9.2.



100
50
0
A
B
1.0
2.0
% of original activity remaining in supernatant



100
50
0
I_1
I_2
1.0
2.0
Antiserum (µl/milliunit of hexosaminidase)

However, since the anti-(hexosaminidase A) serum contained antibodies to both α and β-subunit determinants it could not be stated whether I_1 and I_2 also contained α-subunits. Attempts to test this possibility were made by adding absorbed anti-(hexosaminidase A) serum to solutions of hexosaminidases A, B, I_1 and I_2. It was not possible to obtain complete titration curves because of the small quantity of absorbed antiserum which was available. Instead, a fixed volume of the antiserum, calculated to be 25% in excess of that required for complete precipitation of hexosaminidase A, was added to a constant amount (by activity) of the appropriate hexosaminidase component. The results of this experiment failed to demonstrate the presence of α-subunit determinants on I_1 but some precipitation of hexosaminidase I_2 was observed although this was not complete (see Table 14.3). As already noted in section 14.2 and in agreement with others, the absorbed anti-(hexosaminidase A) serum reacted with hexosaminidase A but not hexosaminidase B. The above results with respect to I_1 and I_2 should be considered in the light of evidence from studies of patients with Tay-Sachs' and Sandhoff's diseases. Both I_1 and I_2 were present in tissue extracts from patients with Tay-Sachs' disease, which is believed to be an α-subunit mutation (Young _et al._, 1970; Price and Dance, 1972). On the other hand, both I_1 and I_2 were undetectable in tissues from patients with Sandhoff's disease, which is believed to be a β-subunit mutation (Young _et al._, 1970). These findings suggest that I_1 and I_2 contain β-subunits but no α-subunits. The immunological results obtained in the present study support the idea that hexosaminidase I_1 is a β-subunit homopolymer. However, the presence of α-subunits in I_2, as suggested here (Table 14.3), is difficult to reconcile with the observation that I_2 was present in Tay-Sachs' disease. A possible explanation for the apparent presence of α-subunits might be that the I_2 preparation used for the experiment was contaminated with hexosaminidase A. This is not unlikely in view of the incomplete separation of these two forms on DEAE-cellulose (see Fig. 14.6).

Further comments can be made regarding the titration curves shown in Figs. 14.18 and 14.19. It can be seen that hexosaminidases B and I_1 were more readily precipitated by the antisera than were the A and I_2 forms. This difference was particularly evident at low antiserum concentrations. For example, under identical conditions, the same volume of anti-(hexosaminidase B) serum precipitated about 85% of hexosaminidase B

TABLE 14.3 IMMUNOPRECIPITATION OF PLACENTAL HEXOSAMINIDASES A,B,I_1 AND I_2 BY ABSORBED ANTI-(HEXOSAMINIDASE A) SERUM

Hexosaminidase fraction	% of initial activity remaining in supernatant: Absorbed anti-(hexosaminidase A)	Control
A	11	94
B	85	90
I_1	91	93
I_2	62	96

Hexosaminidase fractions were obtained from step 4 of purification method II and the absorbed antiserum was prepared as described in section 9.1.1. A fixed volume (150μl) of absorbed antiserum or control serum was mixed with 10 munits of the appropriate hexosaminidase preparation and final volumes were adjusted to 200μl with phosphate-buffered saline. After incubation and centrifugation as described in section 9.2, the hexosaminidase activity in the supernatants was determined. Values are the means of duplicate incubations.

but only about 30% of hexosaminidase A. In agreement with the results presented here, Carroll and Robinson (1973) noted that human liver hexosaminidase B was more readily precipitated than hexosaminidase B by anti-(hexosaminidase) antiserum. However, unlike the present study, these workers did not examine the immunoprecipitation of hexosaminidases I_1 or I_2. This difference between hexosaminidases B and I_1 on the one hand and hexosaminidases A and I_2 on the other is probably related to the presence or absence of the α-subunit and to the availability of certain antigenic determinants. As stated above, results obtained using the absorbed anti-(hexosaminidase A) serum indicated the absence of α-subunits for both B and I_1. Thus, anti-(hexosaminidase B) serum, which contains antibodies to the β-subunit, would react readily with the β-subunit homopolymers B and I_1, but less readily with the α- andβ-subunit heteropolymer A. It is conceivable that the α-subunits could mask some of the antigenic sites on the β-subunits of hexosaminidase A and so hinder the formation of precipitable hexosaminidase A - anti-(hexosaminidase B) complexes. It is more difficult to explain the ready precipitation of B and I_1 by anti-(hexosaminidase A) since this antiserum contained antibodies to both α- and β-subunits. This might be explained by the earlier observation (section 14.2.1) that the anti-(hexosaminidase A) serum contained mostly antibodies to the β-subunit and relatively few to the α-subunit. Thus, at low antiserum to antigen ratios, the anti-(hexosaminidase A) serum would have the same effect as the anti-(hexosaminidase B) serum.

In summary, the results obtained in this study support the idea that hexosaminidases A and B have subunit structures of $(\alpha\beta)_n$ and $(\beta\beta)_n$, respectively. In addition, it is likely that hexosaminidase I_1 represents a β-subunit homopolymer, probably differing from hexosaminidase B in its carbohydrate content. Although the results suggest that I_2 contains both β-and α-subunits, the presence of the latter is questionable due to reservations regarding the purity of the I_2 preparation used. Further studies using a more highly purified preparation or at least one which was free of any hexosaminidase A would clarify this point.

15 Hexosaminidase activity in first trimester and term placentas

The following experiments were designed firstly, to examine the pattern of hexosaminidase multiple forms in the various anatomically distinct regions of first trimester and term human placentas and, secondly, to obtain specific activities for the major multiple forms in these same regions.

Several analytical methods have been used to resolve hexosaminidase into its isozyme forms including electrophoresis in polyacrylamide gel (Friedland *et al.*, 1970; Murphy and Craig, 1972), isoelectric focusing in polyacrylamide gel (Hayase and Kritchevsky, 1973), ion-exchange chromatography on DEAE-cellulose (Young *et al.*, 1970), and electrophoresis on either cellulose acetate (Hooghwinkel *et al.*, 1972) or starch gel (Robinson and Stirling, 1968). The resolving power and reproducibility of the electrophoretic methods have been criticised (Hayase and Kritchevsky, 1973). Greater resolution seemed possible with the iso-electric focusing technique, although there have been reports that many of the apparent multiple forms of enzymes resolved by this method might be artifacts produced by ampholine-protein interaction, (Kaplan and Foster, 1971 and Illingworth, 1972). Recently, Ellis *et al.* (1975) described a rapid, sensitive and highly reproducible method for separating multiple forms of hexosaminidase which involved the use of analytical DEAE-cellulose microcolumn chromatography coupled with continuous automated assay of the column eluate. Since many samples were to be analysed and reliable information was required concerning relatively minor forms, this method was selected for routine use. The profiles so obtained can be used to give quantitative data by relating the total activity to the areas under individual peaks. However, although reported to provide reliable quantitative information about the A and I_2 forms, values for the B form were less reproducible due to the fact that this component is not retained by the DEAE-cellulose (Ellis *et al.*, 1975). For this reason the relatively simple procedure of heat inactivation was employed for quantitating hexosaminidase A and hexosaminidase B. This method (O'Brien *et al.*, 1970) takes advantage of the fact that hexosaminidase B is stable for several hours at 50°C in citrate/phosphate buffer, pH 4.5, while hexosaminidase A is rapidly inactivated. Price and Dance (1972) demonstrated that the I_1 and I_2 forms were also stable under these conditions and therefore the activity remaining after heat treatment was actually the total of hexosaminidase B, I_1 and I_2 activities.

The placental regions selected for study were the amnion, the chorion laeve, the chorion frondosum, the umbilical cord and the chorionic villi. In addition, it was possible to distinguish between terminal villi and inner villi in the term placentas. The spacial relationship of the different placental regions is shown in Figs. 5.4,5.5 and a discussion of placental structure, development and function has been given in section 5.

15.1 Multiple forms of hexosaminidase in different regions of first trimester and term placentas

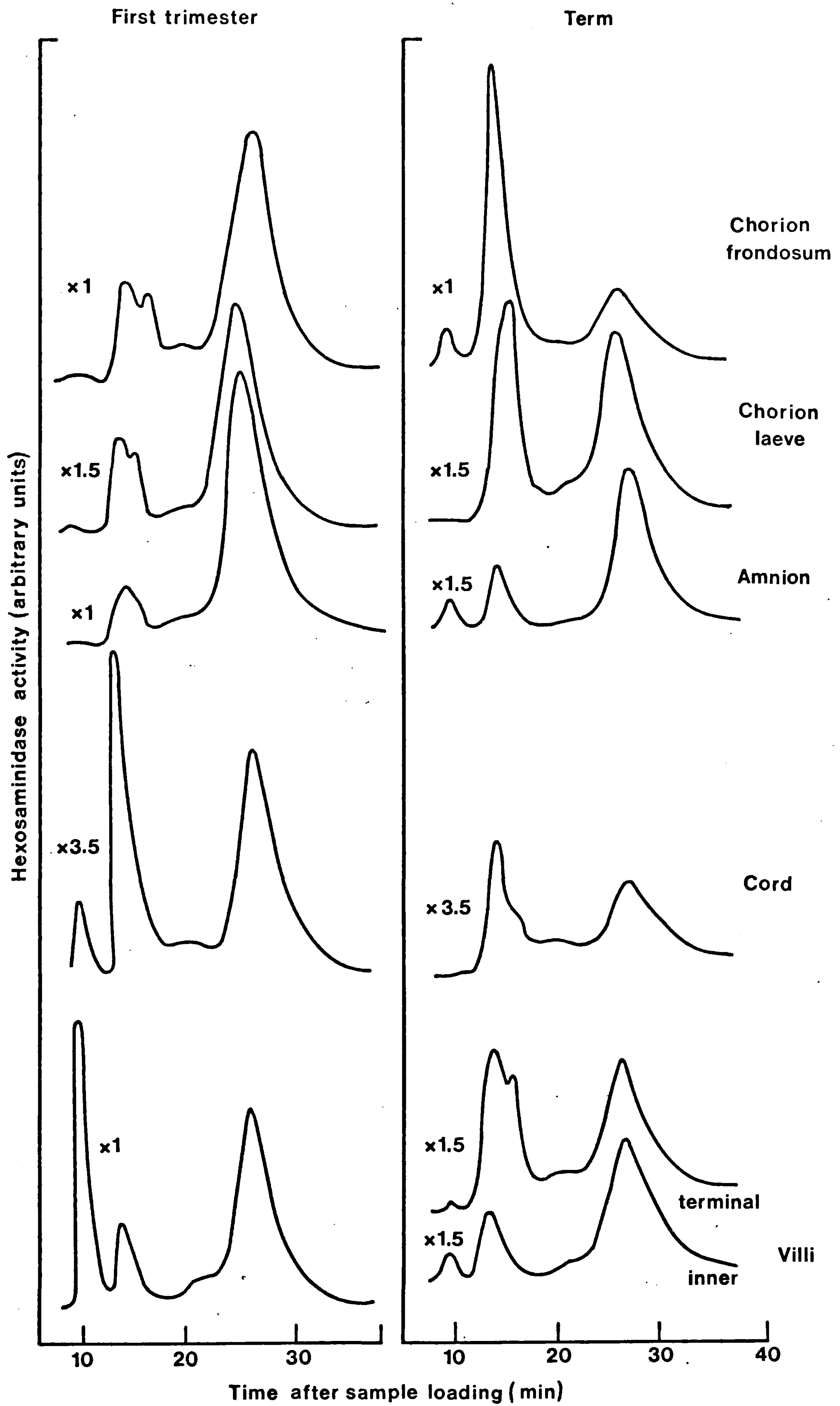
The multiple form patterns for the different placental regions were obtained by the automated DEAE-cellulose microcolumn technique (see Materials and Methods, section 10) and the chart recorder traces are shown in Fig. 15.1. The results shown represent one first trimester and one term placenta and were qualitatively similar to profiles obtained with the other placentas examined. The discussion which follows is based entirely on a comparison of peak height ratios. For example, it is possible to compare the ratio of peak A to peak B in any one sample with the ratio of A to B in any other. It is not possible to compare individual peaks in different samples with each other. This is due to the fact that different fluorimeter sensitivity settings were used.

The different forms were eluted at specific times. Under the experimental conditions used the elution times were typically as follows: B, 10 and 14 min; I_1, 16 min; I_2, 21 min; A, 27 min. The heterogeneity associated with hexosaminidase B was also noticed during the enzyme purification procedure and has been discussed in section 14.1. The I_1 form was only poorly resolved from the second hexosaminidase B peak, sometimes indicated as a change in slope of the trailing edge at about 16 min, in others as a shoulder and in others as a partially separated peak. Resolution was determined, in part, by the relative quantities of the B and I_1 forms.

Examination of the profiles, either for the first trimester or term placentas (Fig. 15.1) shows that the different anatomical regions were characterised by a particular pattern of multiple forms. In the case of the first trimester placenta the chorion frondosum, chorion laeve and the amnion were similar in that the A form was predominant and the I_1 form was present as a partially resolved peak, but differed from the cord and the villi where the A and B forms were co-dominant and

FIG. 15.1 ANALYSIS OF PLACENTAL HEXOSAMINIDASES BY AUTOMATED DEAE-CELLULOSE CHROMATOGRAPHY

Tissue preparation and sample processing are described in section 10. The numbers shown alongside each profile represent the relative fluorimeter sensitivities.


First trimester
Term
Chorion
frondosum
Chorion
laeve
Amnion
Cord
terminal
Villi
inner
×1
×1.5
×1
×3.5
×1
×1
×1.5
×1.5
×3.5
×1.5
×1.5
Hexosaminidase activity (arbitrary units)
10
20
30
10
20
30
40
Time after sample loading (min)

I_2 was virtually absent. The differences between regions were even more apparent in the term placenta where the A form was predominant in the amnion and inner villi and the B form in the chorion frondosum. Both forms were equally present in the chorion laeve, cord and peripheral villi. I_1 was detectable in the cord and terminal villi and especially in the chorion laeve. Further, it is possible to compare the different placental regions as a function of gestation and it can be seen that the patterns changed during placental development. For example, the predominance of the A and B forms was completely reversed in the chorion frondosum and the I_1 form was no longer detectable at term. In the chorion laeve, the profiles changed from the predominance of hexosaminidase A in first trimester to the predominance of the I_1 form and equal proportions of hexosaminidase A and B at term. Finally, the dominant B form eluting at 10 min became a relatively minor peak in the term villi. Since it was not possible to clearly distinguish between types of villi in the first trimester placenta, it is not known whether the different multiple form patterns for the inner and terminal villi in the term placenta developed from a common pattern represented in the first trimester villi or whether the latter represents a mixture of different patterns. The discussion which follows pertains, for the most part, to the I_2 form. A more detailed discussion of the other forms and their possible functional significance during placental development, will be given in section 15.2.

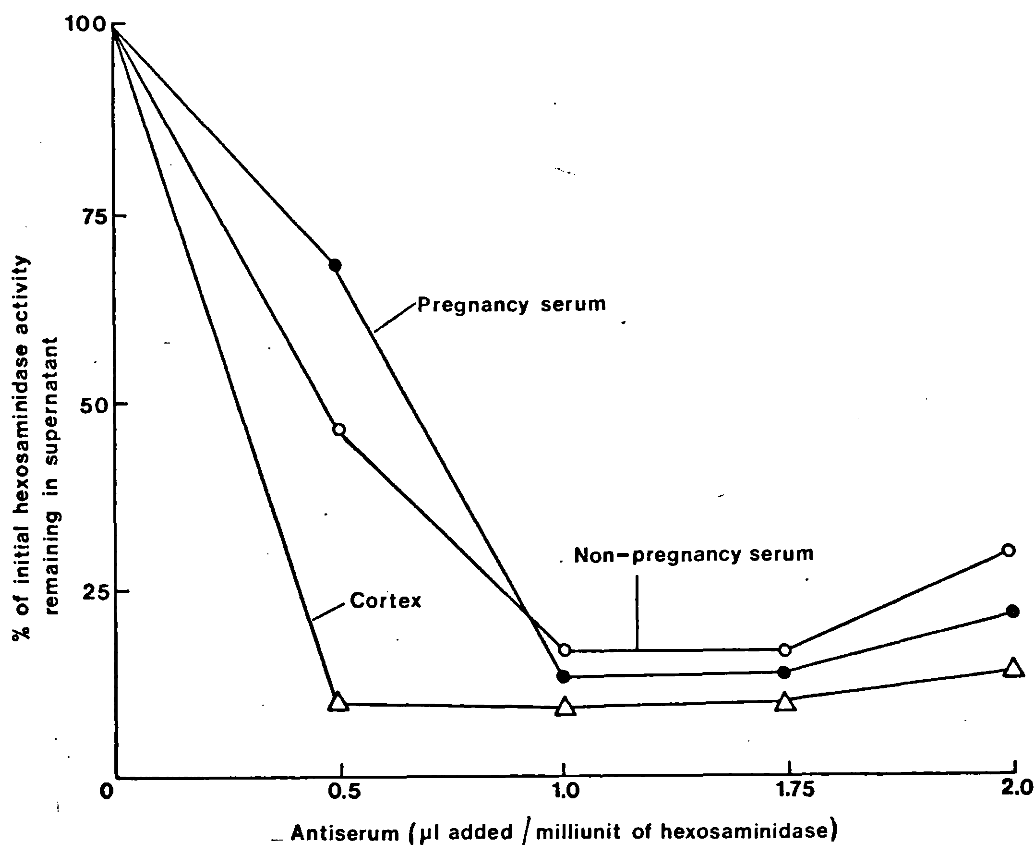
The possibility that the placenta might be the source of the elevated I_2 level found in pregnancy serum has been discussed in the introduction (Section 5.4). In the present study, only extremely low levels of the I_2 form were detectable in the various placental regions. Therefore it is unlikely that the placenta is the major source of pregnancy serum I_2. This conclusion was also reached by Stirling (1972) and Huddleston _et al_. (1971) who were unable to detect I_2 in placental homogenates using starch gel electrophoresis and the appropriate histochemical stain. The relatively low activity of I_2 found in the present study may explain the inability of these workers to detect this form using less sensitive histochemical techniques.

Most workers, as here, have based their conclusion that pregnancy serum I_2 does not originate from the placenta on their inability to detect it in placental extracts. However, other explanations should be considered before this conclusion is accepted. It is possible that

serum I_2 could be formed extracellularly from another hexosaminidase form released by the placenta. It has been suggested that the I_1 and I_2 forms are derived from hexosaminidase B by modification of its carbohydrate content or subunit composition (Beutler and Kuhl, 1975; Srivastava et al., 1976). If this modification occurred extracellularly, then I_2 would not be found in the placenta. The extracellular modification of certain other proteins is known. For example, the conversion of procollagen to collagen by proteolytic cleavage is believed to take place extracellularly or on the outer surface of the cell (Davidson et al., 1975). There is also evidence for the post-secretory deidonination of thyroxine (Bidey et al., 1976). In order to test the possibility of extracellular modification with respect to the formation of I_2, partially purified hexosaminidase B was incubated with 20 week pregnancy serum which had previously been treated with anti-(hexosaminidase B) serum to remove endogenous hexosaminidase activity (including I_2). Analysis of the incubation mixture by DEAE-cellulose chromatography revealed only hexosaminidase B suggesting that, under the conditions used, there was no component in pregnancy serum which was capable of converting placental hexosaminidase B to the I_2 form. Stirling (1972) carried out a similar experiment and reached the same conclusion but using non-pregnancy serum. Evidence for a relationship between placental hexosaminidase B and pregnancy serum I_2 has been suggested by Jones et al., (1975) who reported that an antiserum raised against placental hexosaminidase B reduced the hexosaminidase activity in pregnancy serum to levels found in non-pregnancy serum but did not react with hexosaminidases A and B from adult liver or brain extracts. In contrast to the results of these workers the anti-(hexosaminidase B) serum used in the present study was equally effective in precipitating hexosaminidase from both pregnancy and non-pregnancy serum and was even more effective in precipitating hexosaminidase from a crude extract of human cortex (Fig. 15.2). These observations provided no indication of a unique relationship between placental hexosaminidase B and pregnancy serum hexosaminidase.

Summarising, the experiments described in this section suggest that it is unlikely that the placenta is the source of pregnancy serum hexosaminidase. It is conceivable that hexosaminidase I_2 is formed by the placenta just before secretion making it difficult to detect in the tissue itself. The serum form of albumin is formed in the liver by proteolytic cleavage of a proalbumin precursor at the time of secretion (Edwards et al., 1976; Ikehara et al., 1976). The secretion of

FIG. 15.2 IMMUNOPRECIPITATION OF HEXOSAMINIDASE FROM NORMAL HUMAN SERUM, HUMAN PREGNANCY SERUM AND HUMAN CEREBRAL CORTEX WITH ANTI-(HEXOSAMINIDASE B) SERUM



Increasing volumes of antiserum were added to tubes containing 25munits of the appropriate hexosaminidase preparation. After incubation and centrifugation as described in section 9.2, hexosaminidase activity was determined for each supernatant, Each point is the mean of duplicate incubations, Pregnancy serum was obtained from a patient at about 20weeks of gestation, A crude extract of human cerebral cortex was prepared from post-mortem tissue by homogenisation in 5 volumes (w/v) of water.

hexosaminidase by placental tissue incubated *in vitro* is the subject of experiments described in section 17.

15.2 Specific activities of hexosaminidase, α-glucosidase and β-glucuronidase in first trimester and term placental tissue

In order to determine whether the differences in the multiple form profiles obtained for the various regions in first trimester and term placentas were accompanied by quantitative differences in enzyme activity, the specific activities of total hexosaminidase and hexosaminidase A and B were measured in homogenates obtained from similar regions of first trimester and term placentas. Hexosaminidase A and B activities were determined by the heat inactivation method as described in section 7.2.1.2. A preliminary experiment designed to determine a covenient incubation time for the complete inactivation of hexosaminidase A with minimum inactivation of hexosaminidase B showed that most of the thermolabile activity was lost within the first hour of incubation at 50°C in citrate/phosphate buffer, pH 4.5, with very little further decrease during the next 2 hours (Fig. 15.3). A 2 hour incubation time was used routinely in subsequent experiments. Price and Dance (1972) have shown that hexosaminidase B, I_1 and I_2 activities are only minimally reduced under the conditions used for the heat inactivation assay and that the loss in activity observed is attributable to hexosaminidase A. From the column profiles shown in Fig. 14.1 it can be seen that for most samples, the I_1 and I_2 contribution is negligible and therefore the thermostable activity can be assumed to be hexosaminidase B. Exceptions to this generalisation, for example term chorion laeve and first trimester amnion and chorion laeve, will be discussed where appropriate.

The specific activities of α-glucosidase and β-glucuronidase were also determined in order to establish whether any changes observed for hexosaminidase could be correlated with changes in other lysosomal enzymes. On the other hand, the classic lysosomal marker enzyme, acid phosphatase, was not included since Corash and Gross (1973) have shown that this enzyme is not specifically located in lysosomes in placental tissue.

Comparison of the different anatomical regions in the first trimester and term placentas showed that hexosaminidase, α-glucosidase and β-glucuronidase were not uniformly distributed throughout the placenta but were differentially distributed with respect to the specific regions

examined (Tables 15.1 and 15.2). The specific activities of hexosaminidase ranged from 463 munits/mg protein in the amnion to 63munits/mg protein in the chorion laeve for first trimester placentas and from 225munits/mg protein in the chorion laeve to 40munits/mg protein in the chorion frondosum for term placentas. However, the intraplacental distribution of the other two enzymes, α-glucosidase and β-glucuronidase, did not appear to parallel that of hexosaminidase. With respect to hexosaminidase A and B the results confirmed the multiple form profiles in that the relative proportions of these two forms varied from region to region in both first trimester and term placentas.

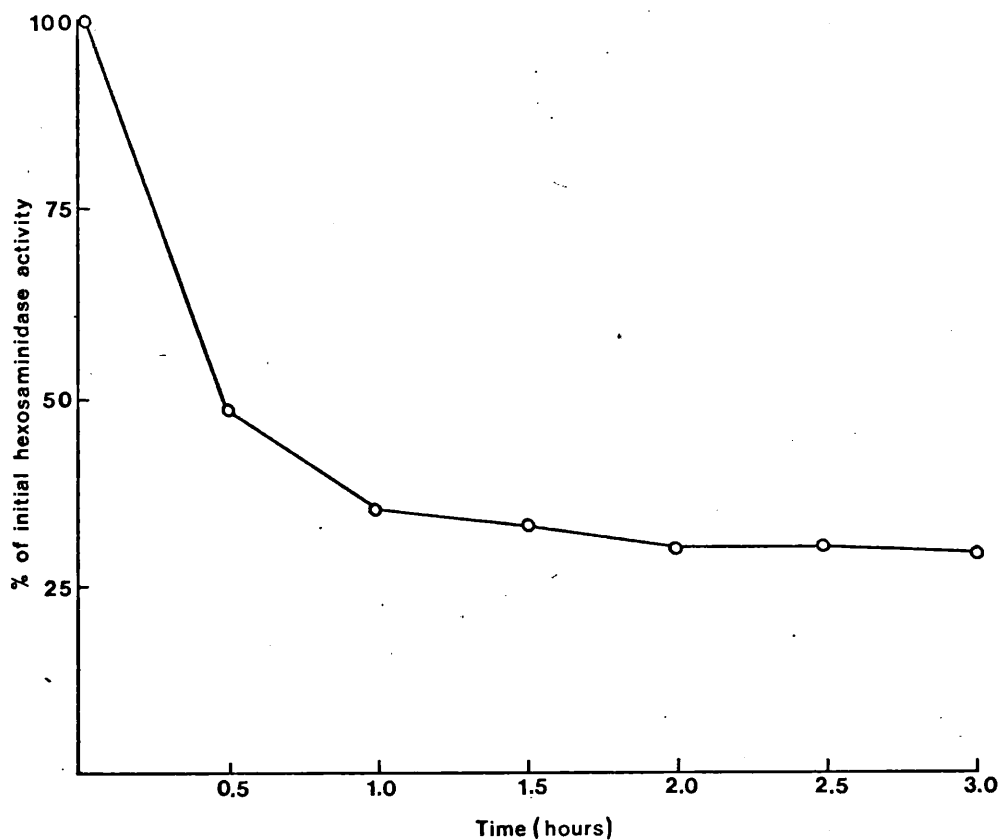
Examination of the different placental regions as a function of gestation shows that the specific activities of the enzymes considered changed during placental development. The specific activities of all the enzymes tended to decrease with gestation. Exceptions were the chorion laeve and the cord. The former showed an increase in specific activity of all enzymes studied while the latter changed very little with respect to gestation. The higher hexosaminidase activity found in term compared with first trimester chorion laeve was attributable to an increase in both hexosaminidase A and B, the greater increase apparently being with respect to hexosaminidase B. Comparison with the appropriate profiles (Fig. 15.1) shows that the increase in thermostable activity just described was, in fact, mainly due to an increase in hexosaminidase I_1. The amnion showed the most noticeable change in hexosaminidase activity from 460-336munits/mg protein in first trimester tissue to 70-50munits/mg protein in term tissue. This decrease in hexosaminidase activity was the result of an unequal decrease in hexosaminidase A and B, hexosaminidase A showing the greater decrease in specific activity. A similar pattern was also seen in the chorion frondosum. In the case of the villi, both hexosaminidase A and B decreased with gestation when compared with either the inner or preipheral villi of term placentas, although this time hexosaminidase B showed the greater decrease. Therefore, the quantitative data for hexosaminidase A and B confirm the earlier semi-quantitative profiles (Fig. 15.1) in that differential expression of the multiple forms occurs in the different placental regions during development.

α-Glucosidase and β-glucuronidase showed the same general trend as hexosaminidase, first trimester tissues having higher specific activities than term tissues. Once again, the chorion laeve proved the

exception in that term samples had higher specific activities than first trimester samples. Also, no change in the cord activities was observed and there was only a slight decrease (less than 1.5-fold) in the villi samples.

Comparison of the results for hexosaminidase with those for α-glucosidase and β-glucuronidase revealed three interesting points. Firstly, although the specific activities of all three enzymes generally decreased as a function of gestation, the magnitude of the change differed considerably depending on the enzyme under consideration. For example, it was particularly noticeable in the amnion where the specific activity of hexosaminidase showed an approximate 7-fold decrease, while that of α-glucosidase and β-glucuronidase showed only an approximate 3-fold decrease. Secondly, the regions with the highest hexosaminidase specific activities (first trimester amnion and term chorion laeve) did not show the highest α-glucosidase and β-glucuronidase specific activities (first trimester villi and chorion frondosum for both enzymes). Thirdly, the absolute specific activities obtained for the three enzymes were widely different. α-glucosidase and β-glucuronidase had similar specific activities within the range 0.1-6munits/mg protein while hexosaminidase ranged from 38-462munits/mg protein. These ranges did not overlap even when hexosaminidase was expressed in terms of hexosaminidase A and hexosaminidase B specific activities. It is possible that this difference reflects the greater functional significance of hexosaminidase compared with that of α-glucosidase and β-glucuronidase. However, it must be remembered that in these experiments artificial substrates were used to assay each enzyme and therefore the specific activities obtained might not reflect the extent to which natural substrates would be hydrolysed. Robinson (1974) has stressed caution when interpreting data obtained using artificial substrates. Very often, enzyme activities obtained with artificial substrates give an overestimate of the activity obtained with natural substrates. For example, Hechtman (1977), using a crude extract of human liver hexosaminidase A, compared the specific hydrolysis of the artificial substrate, 4-methylumbelliferyl-N-acetyl-glucosaminide, with that of the natural substrate, N-acetylgalactosaminyl (N-acetyl-neuraminyl)galactosyl-glucosylceramide, and found that the ratio of activities was 2.5×10^{-6} mol natural substrate hydrolysed per mol of synthetic substrate hydrolysed. Nevertheless, the use of artificial

FIG. 15.3 TIME COURSE OF HEAT INACTIVATION OF PLACENTAL HEXOSAMINIDASE



Details of the procedure used are given in section 7.2.1.2. The enzyme samples consisted of a crude extract of placental villi prepared as described in section 10.1. Each point is the mean of twoobservations.

TABLE 15.1 SPECIFIC ACTIVITIES OF HEXOSAMINIDASE, α-GLUCOSIDASE AND β-GLUCURONIDASE IN FIRST TRIMESTER PLACENTAL TISSUES.

Enzyme	Placental region				
	Cord	Amnion	Chorion laeve	Chorion frondosum	Villi
Total hexosaminidase	72.1 (57.7-86.4)	399 (336-463)	69.6 (62.5-76.7)	138 (60.0-215)	188 (151-225)
Hexosaminidase A	44.4 (36.3-52.2)	231 (184-277)	43.9 (38.1-49.8)	74.2 (33.4-115)	113 (91.9-90.0)
Hexosaminidase B	26.1 (21.4-30.1)	168 (151-185)	25.6 (24.4-26.8)	63 (27.3-100)	75.0 (59.9-90.0)
α-Glucosidase	0.38 (0.36-0.40)	2.51 (2.37-2.65)	0.72 (0.44-1.01)	2.1 (0.68-3.55)	2.79 (2.29-3.28)
β-Glucuronidase	1.2 (0.97-1.36)	2.53 (2.22-2.84)	1.0 (0.81-1.31)	3.86 (2.73-5.00)	4.49 (2.43-6.54)

Tissues were prepared as described in section 10.1. Duplicate samples were taken from the appropriate regions of two first trimester placentas and used to obtain two mean specific activities for each enzyme. These two means are given in parentheses and an overall mean is given above, in each case. Enzyme assay conditions are described in section 7.2. Hexosaminidases A and B were determined by the heat inactivation method (section. 7.2.1.2). Specific activities are expressed as munits of enzyme/mg protein

TABLE 15.2 SPECIFIC ACTIVITIES OF HEXOSAMINIDASE, α-GLUCOSIDASE AND β-GLUCURONIDASE IN TERM PLACENTAL TISSUES

Enzyme	Placental region					
	Cord	Amnion	Chorion laeve	Chorion frondosum	Inner villi	Terminal villi
Total Hexosaminidase	55.1 (44.9-63.4)	62.1 (47.2-73.7)	175 (128-225)	40.5 (38.6-42.2)	46.1 (41.9-50.8)	82.3 (79.3-85.6)
Hexosaminidase A	30.8 (28.6-32.5)	37.3 (27.8-54.2)	68.3 (60.9-78.7)	16-7 (13.9-19.4)	29.6 (28.2-32.3)	59.5 (57.3-61.4)
Hexosaminidase B	22.8 (9.21-31.6)	25.3 (17.2-39.1)	78 (62.3-163)	23.8 (19.2-28.3)	16.5 (9.65-22.4)	22.5 (20.1-25.7)
α-Glucosidase	0.35 (0.12-0.59)	0.85 (0.77-1.06)	1.81 (1.29-2.45)	0.67 (0.28-1.02)	1.52 (1.37-2.72)	3.49 (3.02-3.83)
β-Glucuronidase	1.1 (0.62-1.68)	0.97 (0.71-1.39)	2.9 (2.0-4.9)	1.2 (0.56-1.81)	4.55 (2.69-7.46)	3.55 (3.49-6.24)

Tissues were prepared as described in section 10.1. Duplicate samples were taken from the appropriate regions of three term placentas and used to obtain three mean specific activities for each enzyme. The ranges of these means are given in parentheses and an overall mean is given above, in each case. Enzyme assay conditions are described in section 7.2. Specific activities are expressed as munits of enzyme/mg protein

substrates does not invalidate the apparent heterogeneity found for the distribution of the enzymes and for the extent to which their activities altered with gestation. only limited published information is available on the intraplacental distribution of lysosomal hydrolases. Walker _et al_. (1960) determined the hexosaminidase specific activities in term placental villi and membranes. Highest specific activity was found in the chorion laeve. This result is in agreement with the data shown in Table 15.2. No attempt to quantitate individual multiple forms of hexosaminidase in different placental regions has been reported. However, the intraplacental distribution of multiple forms of cathepsin has been studied in term tissue (Warwas and Dobryszycka, 1976). Unlike hexosaminidase, the highest specific activity of cathepsin was found in the villi.

Slightly more information is available on the changes in placental lysosomal hydrolase activities as a function of gestation although in all published cases enzyme determinations were carried out with ill-defined placental homogenates rather than homogenates from specific anatomical regions. Corash and Gross (1974) and Edlow _et al_. (1971) measured the specific activities of hexosaminidase and β-glucuronidase in homogenates obtained from first trimester and term placentas. While both reported a decrease in hexosaminidase specific activity with gestation, conflicting results for β-glucuronidase were reported in that only Corash and Gross (1974) found a parallel decrease in β-glucuronidase specific activity. In the present study, different placental regions were analysed and the general result that hexosaminidase and β-glucuronidase specific activities decreased with gestation confirmed the results of Corash and Gross (1974) and the hexosaminidase data of Edlow et al. (1971).

Although there is little doubt as to the existence of multiple forms of hexosaminidase in the placenta, conflicting results have been published concerning β-glucuronidase. Huddleston _et al_. (1971), Brot _et al_. (1974) and Edlow _et al_. (1971) have reported the separation of multiple forms of placental β-glucuronidase by starch gel electrophoresis. On the other hand, Contractor and Shane (1972) found only one form of β-glucuronidase in the term placenta using DEAE-cellulose chromatography. As yet, only a single form of α-glucosidase has been purified from term placenta (de Barsy _et al_., 1972). Edlow _et al_. (1971) reported no changes in placental hexosaminidase and β-glucuronidase multiple form

patterns during gestation. However, changes in hexosaminidase multiple forms were observed during development when different placental regions were analysed (see Fig. 15.1 and Tables 15.1 and 15.2). This discrepancy in results may be explained by the previous authors use of ill-defined placental homogenates. Similarly, the interesting observation that the specific activities of hexosaminidase, α-glucosidase and β-glucuronidase all increased in the chorion laeve with respect to gestation would also be undetectable in the analyses of total placental homogenates.

Considerable information is available concerning the structural and functional changes which occur during placental development. From the time of blastocyst implantation up to about the eighteenth week of pregnancy, the trophoblast is primarily concerned with establishing the foeto-placental unit in the uterine wall. During this period the trophoblast grows rapidly, differentiating into the structures known as placental villi. It is the trophoblast, and subsequently the villi, which are believed to be responsible for the extensive erosion and destruction of maternal tissue which occurs during the first trimester. Clearly, these processes must involve considerable turnover of cellular material as well as some means of bringing about localised tissue destruction. The placenta is firmly established by the second trimester and growth and invasion are much reduced, although placental size and weight increase throughout gestation. Studies using light and electron microscopy showed that first trimester villi contained a variety of vacuoles and granules, of which lysosomes and pinocytotic vesicles were prominent (Tighe et al., 1967). This tissue also contained phagocytosed uterine tissue, red blood cells, leucocytes and general cell debris, (Brewer, 1937). However, Lister (1964) observed very few vacuoles in the term villi and, indeed, in the cytotrophoblastic region of the villi lysosomes were completely absent.

Comparatively little is known about the biochemistry associated with the structural and functional changes described above. The experiments discussed deal with specific biochemical changes related to certain lysosomal hydrolases and it is possible to speculate as to their involvement in placental development. The higher specific activities of hexosaminidase, α-glucosidase and β-glucuronidase in first trimester compared with term villi (Tables 15.1 and 15.2) suggest that the lysosomal system is functionally more significant during the period when placental

growth is most rapid and maternal tissue is being eroded. Similarly, the lower specific activities of the lysosomal enzymes in term villi may reflect the reduction in placental invasiveness at term. These enzyme data are also consistent with the observed changes in the ultrastructure of the villi. There is already direct evidence to implicate the lysosomal system in other biological situations which require degradation and re-modelling of tissue, e.g. the involvement of lysosomal cathepsin D in cartilage matrix breakdown (Dingle, 1971) and in the macrophage directed degradation of phagocytosed protein (Dingle et al., 1973). It is interesting to compare observations made in tumour cells with those described above for first trimester villi. The invasion of normal tissues by malignant cells is accompanied by the destruction of normal cells in immediate contact with the invading cancer cells (Holmgren and Marchant, 1968). Furthermore, the specific activities of certain lysosomal enzymes were increased in tumour cells compared to the normal cells from which they were derived (Poole, 1973). For example, the increase in β-glucuronidase specific activity in human cervical tumour cells coincided with the increase invasiveness of cervical cancers (Watts and Goldberg, 1969). Therefore, it is commonly assumed that lysosomal hydrolases play an important causative role in invasive tumour growth. Considering the similarities between certain tumours and the placental trophoblast, it seems likely that the increased activities of lysosomal enzymes found in first trimester villi are directly related to the invasiveness of the trophoblast during the establishment of the foeto-placental unit.

As described above, extensive penetration of the uterus does not occur after about the eighteenth week of pregnancy. However the villi continue to increase in number by repeated branching at their apices. The results in Table 15.2 show that the specific activities of the three lysosomal enzymes examined were higher in the terminal regions than in the stem regions of the villi, particularly in the case of hexosaminidase. This difference may be related to the continued growth and perhaps continued moderate invasiveness by the terminal villous regions while those villous stems located within the placental mass are involved mainly in absorption and transport between mother and foetus. Again, a comparison with tumour cells is interesting in that studies with experimentally induced tumours (Sylven and Malmgren, 1957) showed that the younger actively growing tumour cells had higher specific activities for cathepsin D and

dipeptidase than the older, more slowly growing cells within the tumour mass.

It is difficult to correlate the observations found in this study with the structural and functional properties of the amnion and chorion laeve. As described earlier, the first trimester amnion samples had higher specific activities for all three enzymes studied than the term samples while the converse situation was found for the chorion laeve. These observations suggest that the lysosomal system, or at least these three particular hydrolases, are functionally more significant during the first trimester in the case of the amnion and at term in the case of the chorion laeve. Although lysosome-like structures have been identified in human placental membranes (Bourne, 1962; Schwartz et al., 1976a) no investigation of the lysosomal system as a function of gestation was found in the literature. Except for the proposed involvement of placental membrane phospholipase A_2 in the initiation of labour (Macdonald et al., 1974; Schwartz et al., 1976b) no other lysosomal enzymes have been systematically examined or tentatively related to any specific placental function.

Finally, and in more general terms, the placenta has one of the highest hexosaminidase specific activities of any mammalian tissue (Walker et al., 1960) suggesting that it is an important site for the metabolism of compounds containing N-acetylhexosamine residues. Such compounds include many glycoproteins, glycolipids and polysaccharides. The best studied natural substrates for hexosaminidase are certain gangliosides (see introduction, section 1.4) and these are found predominantly in nervous tissue. Svennerholm (1968) found that, with the exception of the spleen, the concentration of gangliosides, expressed as a percentage of tissue dry weight, was higher in the human placenta than any other extraneural tissue. Other potential substrates, such as mucopolysaccharides, are found in placental connective tissue and in the umbilical cord (Majewski and Siwinski, 1963 and Palma et al., 1963). Furthermore, developmental studies have shown that the placental concentrations of mucoprotein, hyaluronic acid and collagen increase with gestation (Azuma, 1961; Brusilovskii, 1964; Lovell et al., 1967). This may be due, partly at least, to the decrease in hexosaminidase and other lysosomal hydrolase activities described above.

The fact that it is likely that hexosaminidase A and B have different substrate specificities (see introduction, section 1.4) and

that these forms vary, not only with respect to intraplacental distribution but also during the developmental process, would suggest that the metabolism of the respective substrates is important in different regions at different times. However, no systematic study aimed at quantitating these substrates in the different placental regions was found.

16 Synthesis of hexosaminidase by slices of placental villi incubated in vitro

The experiments to be described in this and the subsequent section were carried out for two main reasons. Firstly, to develop a method which could be used for studying the synthesis of hexosaminidase and secondly, to investigate the ability of the placenta to secrete hexosaminidase. A suitable experimental system derived from the human placenta had to be selected. Organ perfusion, tissue culture and tissue slice techniques are the most frequently used placental systems and have already been discussed in the introduction. The perfusion system was limited to providing information about the placenta as a whole and not, as was required, to providing information concerning the activity of individual placental regions. Explants of placental villi have been maintained in culture (Gusdon and Yen, 1967, Maruo *et al.*, 1974) and a placental cell line has been established in culture (Harpaz *et al.*, 1975). Explants of human amnion have also been successfully cultured (Bourne, 1962). Nonetheless, the tissue culture technique did not seem to offer any advantage over the more commonly used tissue slice system for studies on protein synthesis and secretion. Indeed, slices of placental villi incorporated amino acid into protein in a linear fashion and showed few histological changes for up to 96 hours when incubated *in vitro* (Suwa and Friesen, 1969; Hou *et al.*, 1968). Tissue slices are relatively straightforward to prepare and can be easily manipulated *in vitro*. Furthermore, the experimental system can be scaled down to a manageable size and individual placental regions can be studied. The only real disadvantage of the technique is that each slice consists of many cell types and hence the activity of individual cells cannot be determined. Based on the above considerations it was decided to employ the placental slice system for the proposed investigations.

16.1 Characterisation of the placental slice in vitro system

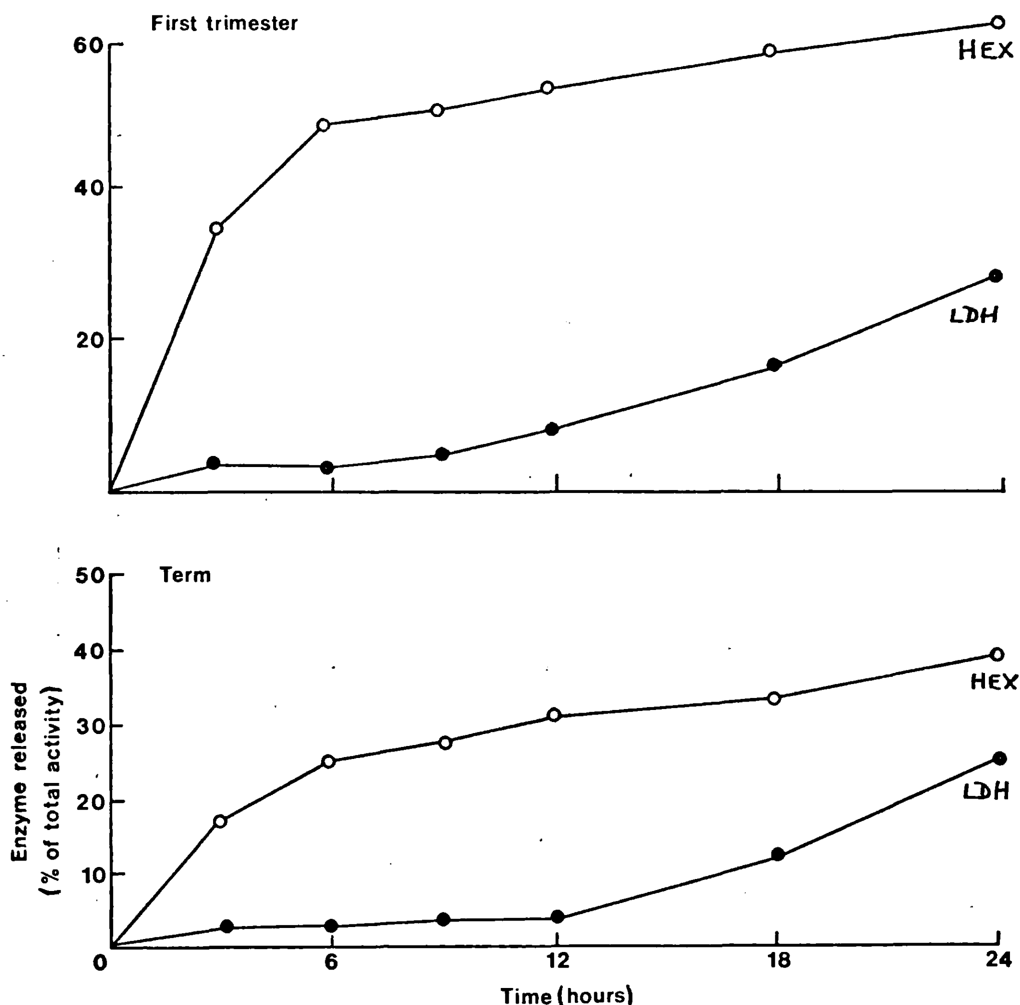
In spite of the suitability of placental slices for the proposed studies, tissue slice systems in general are subject to certain inherent disadvantages. Firstly, the preparation of the tissue inevitably causes a degree of cellular damage with the resultant loss to the incubation medium of enzymes and metabolites normally found in the cytoplasm. Secondly, the relative thickness of each slice means that although cells on the outside of the slice are accessible to oxygen, those within the

slice are effectively subjected to hypoxic conditions. Some preliminary experiments aimed at assessing the extent of these problems in the placental slice system were therefore carried out.

Lactate dehydrogenase is a cytoplasmic enzyme and is not normally released from cells. For this reason, assay of lactate dehydrogenase activity in cell or tissue incubation medium is often used as an indication of the state of viability of the cells (for example, see references by Baur _et al._, 1975 and Davies _et al._, 1973). Initial experiments were carried out in which slices of placental villi were prepared, rinsed briefly in KRB buffer, and then incubated in the same buffer. Under these conditions, considerable amounts of lactate dehydrogenase and hexosaminidase activities were found in the medium at the beginning of the incubation period. This suggested leakage of cytoplasmic components from damaged placental cells and, possibly, contamination of the slices with blood. However, the introduction of a half-hour pre-incubation in KRB buffer, followed by thorough washing over a nylon mesh, was found to be effective in reducing the zero time lactate dehydrogenase and hexosaminidase activities in the medium to non-detectable levels. Fig. 16.1 shows the result of a time course experiment obtained after pre-incubating the tissue as described above. It can be seen that less than 5% of the total lactate dehydrogenase activity appeared in the medium after 6 hours incubation and increased to 8% after 12 hours and to 24% after 18 hours. These data indicated minimal loss of cellular integrity during the first half of the experiment but increasing cell damage after 12 hours incubation. On the other hand, hexosaminidase rapidly appeared in the incubation medium, approximately 20% being released after only 3 hours. Thus, slices of placental tissue incubated _in vitro_ showed a preferential release of hexosaminidase compared to lactate dehydrogenase, suggesting that while a small proportion of the released hexosaminidase was due to leakage from damaged cells most of the activity released into the medium was due to an active secretory process. However, this only held for the first few hours of incubation as increasing amounts of lactate dehydrogenase appeared in the medium after 12 hours.

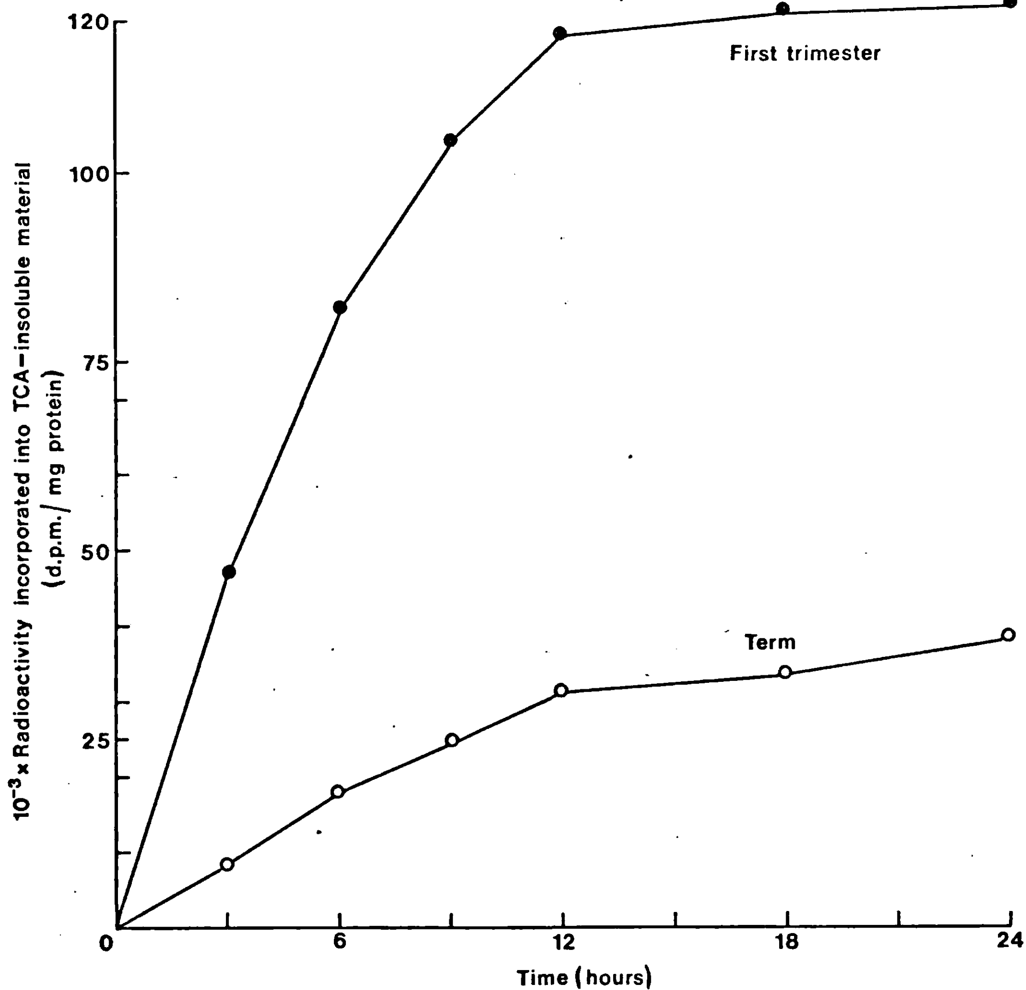
Viability of the placental slice system was also demonstrated by incubating slices with radioactive amino acid and determining the incorporation of radioactivity into TCA insoluble material. Fig. 16.2 shows that this process proceeded in a linear manner for about 12 hours

FIG. 16.1 TIME COURSE OF HEXOSAMINIDASE AND LACTATE DEHYDROGENASE RELEASE FROM SLICES OF FIRST TRIMESTER AND TERM PLACENTAL VILLI



Placental slices were prepared and incubated for the times shown as described in section 11.1. At the appropriate times, tubes were removed and the samples processed as described in section 11,2. Hexosaminidase and lactate dehydrogenase activities were then determined for both the slices and the incubation media as described in section 7.2. Each point represents the mean of duplicate incubations.

FIG. 16.2 TIME COURSE OF THE INCORPORATION OF RADIOACTIVITY INTO TCA-INSOLUBLE MATERIAL IN SLICES OF FIRST TRIMESTER AND TERM PLACENTAL VILLI



Slices of placental villi were incubated with 4μCi of $[^3H]$lysine for the times shown above as described in section 11.1. At the appropriate times, tubes were removed and the samples processed as described in section 11.2. The incorporation of radioactivity into TCA-insoluble material was determined for each incubation system as described in section 11.2.1. Each point is the mean of duplicate incubations.

after which a plateau was reached suggesting that the placental slices were metabolically active with respect to protein synthesis for at least half the duration of the experiment. Two explanations for the reduction in incorporation of radioactivity seen after 12 hours are possible. Firstly, the loss of cell viability and, secondly, reduction of the specific activity of the radioactive precursor due to dilution with endogenous amino acid derived from protein degradation. The relatively long incubation times used for these studies and, in particular, the incorporation studies, required the supplementation of the medium with penicillin-G and streptomycin sulphate to prevent any bacterial growth during the experiment. The effectiveness of this treatment was shown by the absence of bacterial growth when agar plates were inoculated with spent incubation medium. Also, control incubations which contained buffer, labelled amino acid and antibiotics but no tissue were routinely included and incorporation here was never greater than zero time values.

Experiments, similar to those described above for term placental villi, were also carried out using slices of first trimester villi. Results were qualitatively similar to the above in that there was a selective release of hexosaminidase and incorporation of radioactivity increased steadily for at least 12 hours (Figs. 16.1 and 16.2). However, both the amount of enzyme released and the rate of incorporation were greater in the first trimester than term slices. These differences will be discussed further in section 17 and section 16.3.

As stated above, the other objection to the use of tissue slice systems is that only those cells on the outer surface of the slice have access to dissolved oxygen and other materials. In this context, Tay (1977) has shown that the levels of certain metabolites in rat liver slices incubated in the presence of oxygen increased to values found for intact ischaemic animals suggesting a degree of hypoxia in the slice system. Also, autoradiography of rat pancreas slices which had been incubated with radioactive amino acid revealed that most of the incorporation had taken place in the cells on the outer surface of the slices (Venroolj _et al._, 1972). Although no experiments were carried out to show whether oxygen deprivation was a problem with the placental slice system used here, the following features suggested that the placental system may be largely free of the problems associated with the rat liver and pancreas systems. The placental villi have a large surface area/volume

ratio and are therefore well adapted for the absorption of materials, including oxygen, from the maternal blood. That this situation persisted when placental slices were incubated in vitro was suggested by microscopic examination of slices which had been incubated in KRB buffer for 3 hours. Each villus was well separated from the others and should therefore have been in immediate contact with the buffer throughout the incubation period. Furthermore, the main functional cells are located on the outer surface of each villus while connective tissue takes up most of the inner layers. This situation is in contrast to rat liver slices where each slice consists of a compact cellular structure with relatively few cells in direct contact with the incubation medium.

Based on the results discussed above, several points can be concluded regarding the placental slice system used here. Firstly, although damage to some cells was unavoidable the inclusion of a pre-incubation and washing step was effective in minimising the problems associated with cell damage. Secondly, the undamaged tissue remained reasonably viable for up to 12 hours, although some evidence of loss of cellular integrity was obtained for tissue incubated beyond this time. It was decided that judicious selection of incubation times would allow the proposed experiments to be carried out.

Suwa and Friesen (1969) demonstrated that placental slices remained metabolically active, as judged by glucose consumption and amino acid incorporation into protein, for up to 4 days when incubated in KRB buffer. Results obtained in this study do not entirely agree with those of the above workers in that amino acid incorporation only increased during the first 12 hours of incubation in KRB buffer. This is probably explained by the fact that the above workers replaced the incubation medium with fresh medium at 24 hour intervals. It is interesting to note that KRB buffer is not supplemented with an amino acid mixture yet active incorporation of radioactivity was observed for several hours in the system used here and for several days in the system used by Suwa and Friesen. Furthermore, Suwa and Friesen (1969) could find no difference in the rate of amino acid incorporation whether KRB buffer or medium 199 (which does contain free amino acids) was used. They suggested that this reflected the efficiency with which the placenta re-utilised free amino acids derived from endogenous protein degradation.

16.2 Evidence for the synthesis of hexosaminidase by slices of placental villi incubated in vitro

As already stated, one of the main objectives of this investigation was to develop methods for studying the synthesis of hexosaminidase *in vitro*. Two approaches were used to demonstrate hexosaminidase synthesis. Firstly, hexosaminidase activity was measured in placental slices incubated in the presence and absence of the protein synthesis inhibitor, cycloheximide. Secondly, newly synthesised hexosaminidase was identified by specific immunoprecipitation of isotopically labelled protein.

16.2.1 Effect of cycloheximide

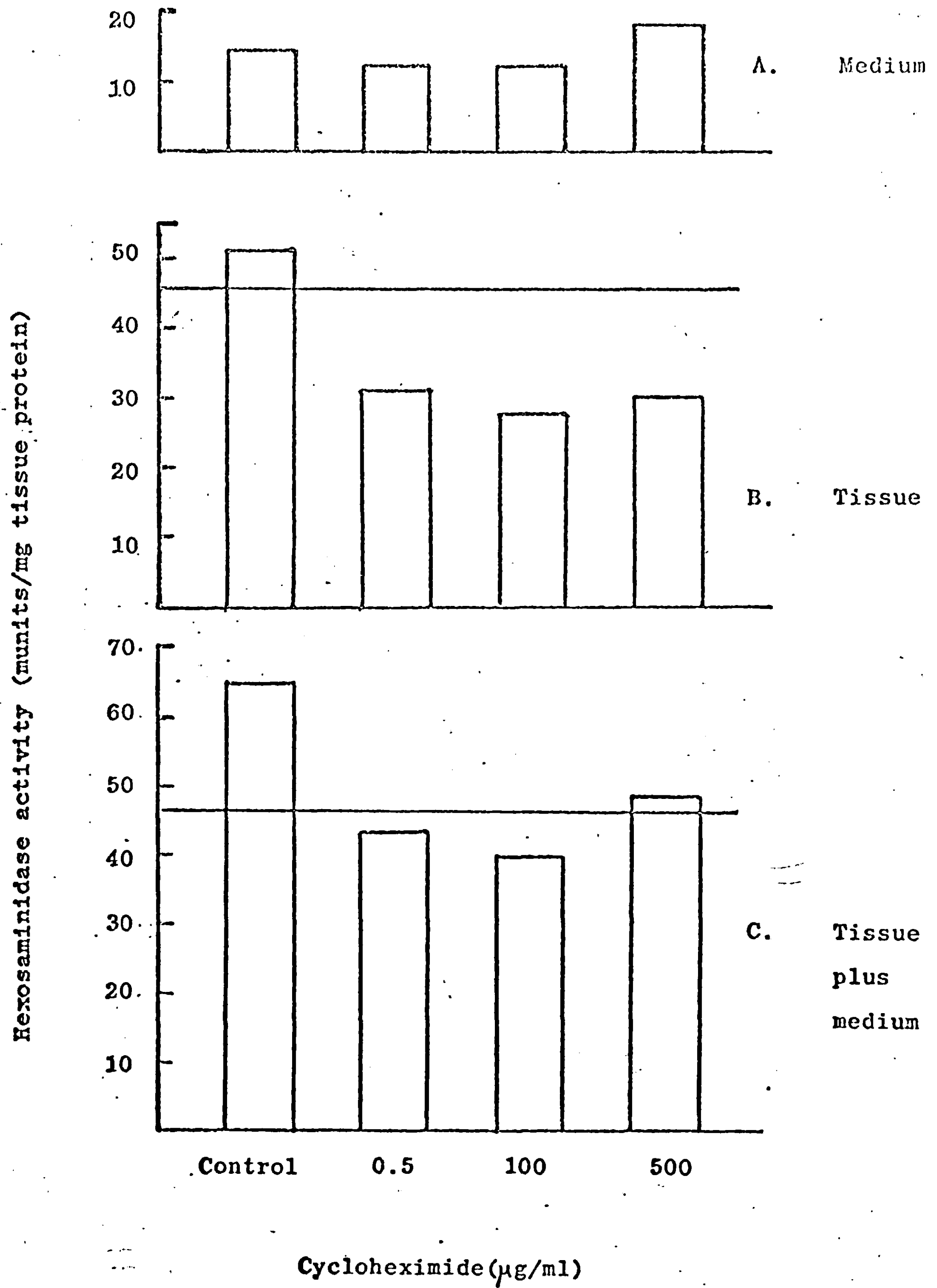
For the experiments using cycloheximide, slices of term placental villi were incubated with different concentrations of the drug for 12 hours. At the end of the incubation period, the samples were centrifuged and hexosaminidase activity was determined separately for both the tissues and the media. The results of this experiment are shown in Fig. 16.3. It can be seen from Fig. 16.3c that hexosaminidase activity increased in the total incubation system during the 12 hour period and that this increase was prevented by cycloheximide. All of the cycloheximide concentrations used were equally effective in preventing enzyme accumulation. In the same experiments, the three increasing drug concentrations inhibited total protein synthesis by 80%, 94% and 96%, respectively, as indicated by the incorporation of radioactive amino acid into TCA-insoluble material. Cycloheximide is known to inhibit protein synthesis in a number of *in vivo* and *in vitro* systems (Pestka, 1971) and the results described above may be interpreted as cycloheximide-mediated inhibition of hexosaminidase synthesis. An alternative, though less likely, explanation could be that cycloheximide inhibited the synthesis of a specific enzyme activator.

Consideration of Fig. 16.3A and 16.3B shows that the increase in hexosaminidase activity observed during incubation can be accounted for by the release of hexosaminidase to the incubation medium. The control tissue hexosaminidase activity after 12 hours incubation was the same as the zero time tissue value. Although cycloheximide treatment reduced tissue hexosaminidase activities to below the zero time value, it had no detectable effect on the release of the enzyme. These results suggest that as newly synthesised hexosaminidase appeared, tissue activities were

FIG. 16.3 EFFECT OF CYCLOHEXIMIDE ON HEXOSAMINIDASE ACTIVITY IN A PLACENTAL SLICE SYSTEM

Slices of term placental villi were prepared and incubated(in duplicate) with different concentrations of cycloheximide for 12 hours as described in section 11.1. Tissue and medium hexosaminidase activities were then determined. The results are given as means of two experiments representing two different placentas. Analysis of variance was carried out using a computer and the results showed that while there was no significant difference between the effects at different dose levels, the treatments did have a significant ($p<0.01$) effect on enzyme levels in the tissue (B) and in the total incubation system (C).

The horizontal line shows the tissue hexosaminidase activity at zero time.


20
10
A. Medium
50
40
30
20
10
B. Tissue
70
60
50
40
30
20
10
C. Tissue plus medium
Hexosaminidase activity (munits/mg tissue protein)
Control
0.5
100
500
Cycloheximide(μg/ml)

maintained at a constant level by the loss of some enzyme to the medium. It seems unlikely that the degradation of intracellular hexosaminidase was a major factor in regulating tissue enzyme levels since the tissue activity lost as a result of cycloheximide treatment was recovered in the incubation medium. However, it should be remembered that cycloheximide treatment tends to decrease degradation rates (Epstein et al., 1975) and hence the apparent insignificance of enzyme breakdown in the present case may not reflect the true situation.

The finding that the release of hexosaminidase from the placental tissue was not dependent on protein synthesis, at least for 12 hours, is in agreement with results obtained for other secreted proteins. For example, cycloheximide had no effect on the intracellular transport and secretion of proteins in a rat pancreas slice system (Jamieson and Palade, 1968; Jamieson and Palade, 1971). Similarly, Hille et al. (1970) demonstrated that the release of lysosomal cathepsin D from cultured cartilage cells was not affected by cycloheximide. Further studies and discussion relating to the release of hexosaminidase from placental slices are given in section 17.

16.2.2 Incorporation of radioactivity into hexosaminidase

At best, the above experiment only suggests that new enzyme synthesis was responsible for the net accumulation of hexosaminidase activity observed during the incubation of placental slices in vitro. Direct evidence for the synthesis of a specific protein can be obtained by demonstrating the incorporation of a radioactive amino acid into the protein of interest. This approach requires that the specific labelled product be isolated or distinguished from the many other labelled proteins inevitably present at the end of the incubation period. The remaining experiments to be discussed in this section are concerned with the development of a method for product analysis based on immunoprecipitation using anti-(hexosaminidase) serum. The alternative method of product analysis based on chemical purification of the labelled product was less feasible for two reasons. Firstly, hexosaminidase constitutes less than 0.1% of total soluble placental protein and, secondly, the available purification procedures consisted of many steps and had relatively low recoveries. Immunoprecipitation, on the other hand, was well suited to microscale enzyme isolation since it was virtually a one step procedure and recoveries were relatively high.

For these studies, it was decided to use the anti-(hexosaminidase A) serum since this was known to cross react with hexosaminidase A, hexosaminidase I_1, hexosaminidase I_2 and hexosaminidase B (see section 14.3). Furthermore, anti-(hexosaminidase A) but not anti-(hexosaminidase B) serum possessed antibodies to both the α- and β-subunits of hexosaminidase. The relatively low titre and small quantities available precluded the use of the absorbed anti-(hexosaminidase A) serum in these studies. For these reasons, the results which follow relate to total hexosaminidase (ie. α- and β-subunit) synthesis only.

Slices of term placental villi were prepared and incubated for 12 hours in the presence of [^{3}H]lysine as described in section 11.1. The amount of radioactivity used in the incubations was based on the known capacity of placental slices to incorporate radioactivity into total protein and by assuming that hexosaminidase probably represented less than 0.1% of the soluble tissue protein. After incubation, the slices were homogenised and the soluble extract was subjected to analysis by specific immunoprecipitation and SDS/gel electrophoresis. Complete precipitation of hexosaminidase was assured by the absence of detectable activity in the supernatant. In initial experiments the immunoprecipitate was washed with phosphate-buffered saline containing unlabelled lysine and Triton X-100 before SDS/gel electrophoresis. A typical gel profile obtained after this washing procedure is shown in Fig. 16.4A and it can be seen that the radioactivity was distributed throughout the gel, most being found near the cathode. At least four peaks were distinguished but resolution was poor due to the relatively high background radioactivity. This result indicated either incomplete dissociation of hexosaminidase or the presence of contaminating radioactive material. Incomplete dissociation of hexosaminidase into its component subunits has been noted by some workers under certain conditions and was dependent on the concentration of reducing agent (Tallman et al., 1974; Srivastava et al., 1974a). Geiger and Arnon (1976) observed incomplete dissociation at low concentrations of reducing agent but complete dissociation at concentrations of dithiothreitol greater than 20mM. When about 10μg of the purified hexosaminidase B preparation obtained in the present study (section 14.1) was treated as described in section 7.5.3 and then subjected to SDS/gel electrophoresis, a single protein band was seen. Comparison of its mobility with the mobilities of standard proteins of

Fig. 16.4 Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of radiolabelled anti-(hexosaminidase)-precipitable material obtained after the incubation of slices of term placental villi in vitro

Slices of term villi were prepared and incubated for 12 hours with 60µCi of [^{3}H]lysine, specific activity 30Ci/mMol, as described in section 11.1. At the end of the incubation period, the samples were centrifuged and the slices homogenised as described in section 11.2. Aliquots of the tissue extracts were then treated as follows:-

A. Addition of anti-(hexosaminidase A) followed by washing of the immunoprecipitate with PBS containing 10mM lysine and 1% (w/v) Triton X-100.

B. As for (A) except that the immunoprecipitate was washed with PBS containing 10mM lysine and 0.7% (w/v) Triton X-405.

C. Addition of Triton X-405 followed by anti-(hexosaminidase A). The immunoprecipitate was washed as described in (B).

D. Addition of Triton X-405 followed by pre-immunoprecipitation with rabbit anti-(human immunoglobulins). The resulting supernatant was then treated as described in (C).

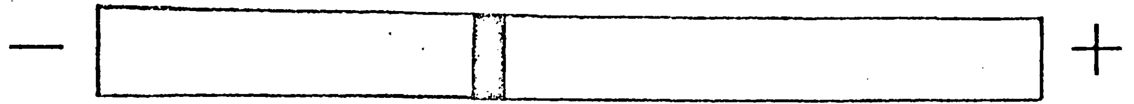
E. Addition of control rabbit serum. The precipitate was washed with PBS containing 10mM lysine and Triton X-405.

F. As for (C) except that the tissue slices had been incubated with cycloheximide (25µg/ml).

The precipitates were then subjected to sodium dodecyl sulphate/ polyacrylamide gel electrophoresis as described in section 7.5.3 and 1.1mm gel slices were processed for radioactivity counting as described in section 11.2.2. Each fraction represents two gel slices.

The vertical arrows show the relative mobilities of: 1, RNA polymerase α-subunit, mol.wt. 39,000; 2, immunoglobulin light chain, mol.wt. 25,000; 3, trypsin inhibitor, mol.wt. 21,000; 4, bromophenol blue.

The figure at the top shows a drawing of an SDS/polyacrylamide gel after electrophoresis of purified hexosaminidase A and staining for protein.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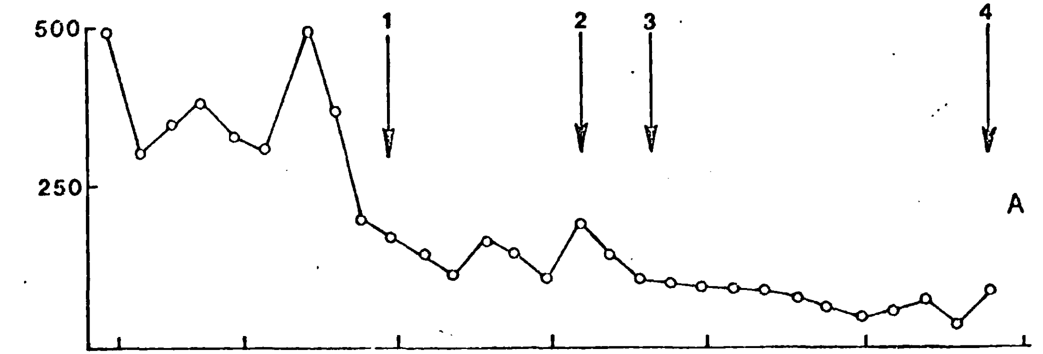
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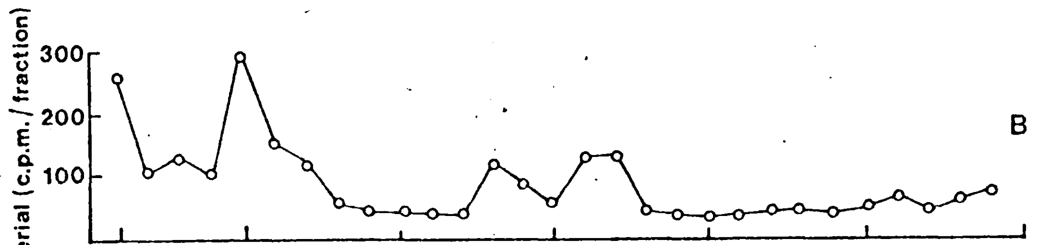
A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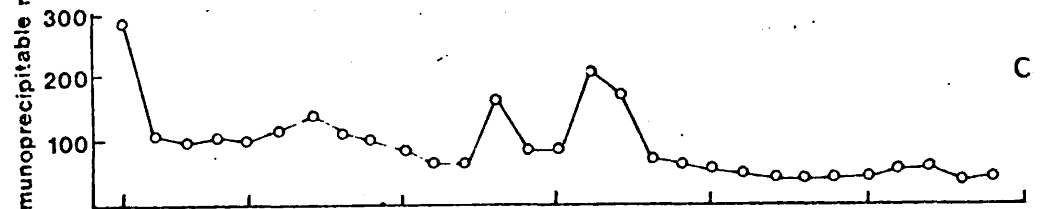
B


300
200
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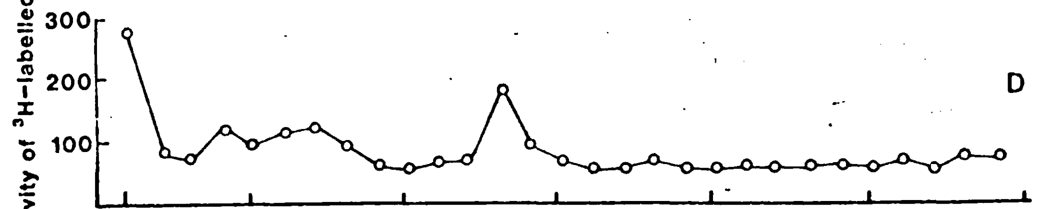
C
Radioactivity of ^{3}H-labelled immunoprecipitable material (c.p.m./fraction)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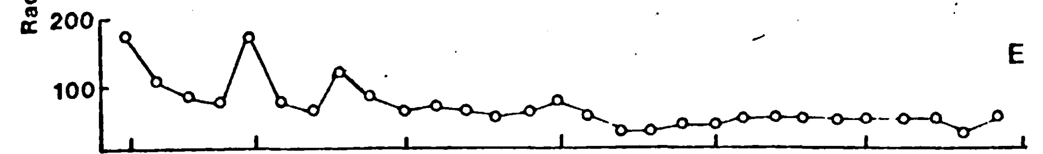
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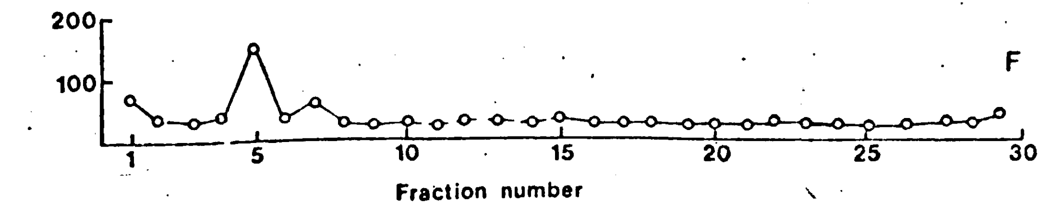

200
100
F
1
5
10
15
20
25
30
Fraction number

known molecular weight indicated a molecular weight of about 29,000. Because the hexosaminidase A preparation was impure (see section 14.1 and Fig. 14.11) it had to be further purified in order to obtain a sample suitable for SDS/gel electrophoresis. Small amounts of hexosaminidase A were electrophoresed in polyacrylamide gels under cationic conditions and the enzyme extracted from the gels as described in section 8.2. When the extracted hexosaminidase A was analysed by SDS/gel electrophoresis a single, feint protein band was observed corresponding to a component of molecular weight of 29,000. These results confirmed the findings of Geiger and Arnon (1976) that the α- and β-subunits of placental hexosaminidase have a similar molecular weight (see section 1.3) and made it unlikely that the radioactive profile shown in Fig. 16.4A was the result of incomplete dissociation of hexosaminidase. The molecular weight of 29,000 obtained in the present study was, however, slightly greater than the value of 25,000 reported by Geiger and Arnon (1976).

The explanation based on the presence of contaminating radioactive material seemed more plausible and two possibilities were considered. Firstly, that radioactive proteins, peptides and/or free amino acid had become non-specifically associated with the antigen-antibody complex and, secondly, that the antiserum contained antibodies to other proteins in addition to hexosaminidase. The former seems to be a characteristic of antigen-antibody reactions in solution and has been noted by other workers (Ballard et al., 1974; Scragg et al., 1975). This phenomenon is a particular problem when, as here, the protein being studied represents only a minor labelled product. In this situation, the contaminating radioactivity can be greater than the radioactivity incorporated into the specific product. Therefore, a number of methods, as indicated in the legend for Fig. 16.4, were attempted in order to remove the supposed radioactive contamination.

Although washing immunoprecipitates with buffer containing Triton X-100 is frequently employed to minimise the problem of non-specific association of radioactivity, other detergents such as Brij 35 and Tween 20 have been reported to be superior in certain circumstances (Palmiter et al., 1971). Ballard et al. (1974) reported that Triton X-405 was more effective than any of the above detergents in preventing the non-specific adsorption of radioactivity to immunoprecipitates formed between rat liver phosphoenolpyruvate carboxykinase and its respective

antibody. Accordingly, Fig. 16.4B shows the effect of washing the hexosaminidase-anti(hexosaminidase A)-complex with Triton X-405 instead of Triton X-100. Comparison with Fig. 16.4A indicates that this washing procedure removed much of the radioactivity near the cathode and generally lowered the background. Even better results were obtained if Triton X-405 was added to the soluble placental extract prior to the addition of the anti(hexosaminidase A) serum (Fig. 16.4C). Two small peaks of radioactivity were distinguishable but a diffuse region of radioactivity was still seen near the cathode.

The above experiments indicated that much of the radioactivity present in unwashed immunoprecipitates was due to the adsorption of non-specific material, probably during the initial formation of the antigen-antibody complex. Although the use of Triton X-405 greatly improved the resolution of radioactive components, some unidentified radioactivity still remained suggesting the presence of strongly bound non-specific material or, as speculated above, a contaminating antigen-antibody system. Some workers have included a pre-immunoprecipitation step using an antigen-antibody system unrelated to the system being studied as an alternative means of overcoming the problem of the co-precipitation of non-specific radioactivity (Scragg _et al._, 1975; Yeoh and Morgan, 1974). Consideration of Fig. 16.4C showed that one of the radioactive peaks co-migrated with the light chain of rabbit immunoglobulin suggesting the non-specific association of radioactivity with this component and/or the presence of rabbit anti-(human immunoglobulin) antibodies in the anti-(hexosaminidase A) serum. Fig. 16.4D shows the effect of pre-treating the placental tissue extract with rabbit anti-(human immunoglobulin) serum in an attempt to remove any human gammaglobulin prior to the addition of the anti-(hexosaminidase A) serum. It can be seen that the peak corresponding to the position of the immunoglobulin light chain was removed by this procedure while the peak corresponding to the position of the dissociated α- and β-subunits of hexosaminidase was unaffected. In order to confirm that the radioactive peak corresponding to the position expected for the hexosaminidase subunits truely represented newly synthesised protein and not just non-specific adsorption of radioactive material to endogenous enzyme, control experiments were carried out as indicated in Fig. 16.4E and Fig. 16.4F. No such peak was observed when tissues were incubated with cycloheximide (Fig. 16.4F). Furthermore, treatment of a

placental tissue extract, previously labelled in the absence of cycloheximide, with control rabbit serum also failed to precipitate radioactive material corresponding in mobility to the enzyme subunits (Fig. 16.4E). Although the absolute amount of radioactivity incorporated into the hexosaminidase peak was relatively low, the appearance of similar peaks at the same position in several repeat electrophoretic runs, representing at least three different term placentas, permit the conclusion that slices of term placental villi incubated *in vitro* were able to direct the synthesis of a radioactive product that was precipitated with anti-(hexosaminidase A) serum and which co-migrated with authentic hexosaminidase subunits. These data were taken as evidence for the *de novo* synthesis of hexosaminidase by slices of placental villi and the procedure described in Materials and Methods, section 11.2.2, was adopted for subsequent estimations of hexosaminidase synthesis.

The above findings also emphasised the importance of analysing the immunoprecipitates by SDS/gel electrophoresis. Direct determination of radioactivity in solubilised precipitates would have been unreliable because of the relatively low degree of incorporation into hexosaminidase and the high background radioactivity. Analysis by SDS/gel electrophoresis had the advantage that not only could the specific product be identified and quantitated but contaminants could be recognised and attempts at improving the specificity of the immunoprecipitation technique could be easily monitored.

16.3 Incorporation of radioactivity into hexosaminidase by slices of first trimester and term placental villi

It was hoped to compare the ability of first trimester and term villi slices to synthesise hexosaminidase as determined by their ability to incorporate radioactivity into anti-(hexosaminidase A) precipitable material.

Dunlop *et al.* (1974) stressed that assessment of protein synthesis based on the incorporation of radioactivity into protein is subject to several sources of error of which alterations in the specific radioactivity of the precursor amino acid pool are the most important. This can be extended to the situation where two different tissues are being compared. Thus, it is quite possible to obtain an erroneous indication of a difference in protein synthesis due to differences in rates of amino

acid transport, amino acid compartmentation or rates of protein degradation, all of which can effect precursor specific activity. With respect to tissue slices, the above authors have proposed that sufficiently high concentrations of precursor in the incubation medium should largely overcome these problems without the need to actually measure specific activities in the tissue samples. The rationale here is that the high concentration of amino acid in the medium causes a rapid equilibration between the extracellular and intracellular pools minimising problems due to differences in amino acid transport and intracellular pool sizes. Any differences in precursor specific activity between two tissues therefore become negligible allowing an accurate comparison of rates of amino acid incorporation based only on the incorporation of radioactivity and the known specific radioactivity of the incubation medium. Although this approach is particularly useful for measuring total protein synthesis, its application to measuring the synthesis of minor protein components is much more limited since this would require extremely high levels of radioactivity to maintain the high specific activity necessary for detection.

Before attempting to compare the incorporation of radioactivity into hexosaminidase by first trimester and term villi slices it was necessary to ascertain whether these measurements would be subject to the type of problems described above. Since this was most easily determined by comparing the incorporation of radioactivity into TCA-insoluble material at high and low concentrations of precursor, for both first trimester and term slices, the following experiment was carried out. Slices of first trimester and term villi were incubated with [^{14}C]lysine for 12 hours in the presence and absence of 1mM carrier lysine. Cycloheximide controls were also included since much of the incorporation of radioactivity into TCA-insoluble material at high amino acid concentrations was insensitive to this drug (Dunlop _et al._, 1974). [^{14}C]lysine was used because the incorporation of ^{14}C-labelled amino acids into TCA-insoluble material showed greater sensitivity to cycloheximide than ^{3}H-labelled compounds (Dunlop _et al._, 1974). At the end of the incubation period, the incorporation of radioactivity into TCA-insoluble material was determined and corrected for cycloheximide insensitive incorporation.

The results given in Table 16.1 show that although the absolute amounts of radioactivity incorporated by first trimester and term villi at a concentration of 1mM lysine were less than the amounts incorporated

TABLE 16.1 INCORPORATION OF RADIOACTIVITY INTO TCA-INSOLUBLE MATERIAL IN SLICES OF FIRST TRIMESTER AND TERM PLACENTAL VILLI AT HIGH AND LOW CONCENTRATIONS OF AMINO ACID.

		Incorporation of radioactivity into TCA-insoluble material (d.p.m/mg protein/hour) (a)	(b) corrected*
First trimester	1mM lysine	2710	2586
	1mM lysine plus cycloheximide	123	
	Trace lysine	12250	11810
	Trace lysine plus cycloheximide	440	
Term	1mM lysine	338	154
	1mM lysine plus cycloheximide	184	
	Trace lysine	2981	2700
	Trace lysine plus cycloheximide	281	

Slices of villi were prepared and incubated for 12 hours with 2µCi of L -[U-^{14}C] lysine, specific activity 320 Ci/mmol, and in the presence and absence of 1mM unlabelled lysine as described in section 11.1. Controls incubated with cycloheximide (25µg/ml) were also included. At the end of the incubation period the samples were processed as described in section 11.2 and TCA-insoluble radioactivity was determined as described in section 11.2.1. Values given are means of triplicate incubations from one first trimester and one term placenta.

* data from column (a) corrected for cycloheximide insensitive incorporation.

at trace levels, the difference in incorporation between the two tissues was greater when measured at the higher amino acid concentration. Thus, at trace levels the first trimester villi incorporated about four times more radioactivity into TCA-insoluble material than term tissue, whereas at a lysine concentration of 1mM the first trimester villi incorporated about seventeen times more radioactivity than term tissue. Although showing the same general trend, in that the first trimester villi incorporated more radioactivity than the term samples, these results showed that comparison of rates of amino acid incorporation based on the incorporation of radioactivity at trace levels of extracellular precursor gave an underestimate of the true difference between the two tissues. The results further implied that the underestimation was due to the lower precursor specific activity in first trimester compared to term villi. No experiments were carried out to establish whether this was, in turn, the result of differences in amino acid transport, amino acid compartmentation, protein degradation or differential leakage by the two tissues.

Based on the above experiment, it was concluded that any attempt to compare hexosaminidase synthesis in first trimester and term villi slices would be meangingless if carried out at trace levels of amino acid. The obvious way of obtaining accurate data was to determine the incorporation of radioactivity into hexosaminidase at a high extracellular precursor concentration. Although such an experiment was theoretically possible, high concentrations of extracellular amino acid reduce the absolute amount of radioactivity incorporated into protein and therefore dictated that increased amounts of $[^{14}C]$lysine be used to obtain measurable and reliable incorporation into hexosaminidase. This meant that a total of at least 250μCi of $[^{14}C]$lysine would be needed per incubation at a concentration of 1mM lysine compared to 60μCi at trace levels. This particular experiment was therefore precluded because of financial limitations.

As an alternative means of comparing hexosaminidase synthesis in first trimester and term villi slices it was felt that a reasonably reliable basis for comparison could be obtained from calculated rates of amino acid incorporation into hexosaminidase based on computed specific activities derived from Table 16.1. Briefly, since the precursor specific activity was known for the tissue incubated in 1mM lysine it was possible to calculate rates of protein synthesis for both

first trimester and term villi slices. Using these rates of protein synthesis and the known incorporation of radioactivity into protein at trace levels of precursor, estimates of the mean precursor specific activity at trace levels were calculated. It only remained to determine the incorporation of radioactivity into hexosaminidase at trace levels for both first trimester and term tissue and to relate these values to the estimated mean precursor specific activities to obtain estimated rates of amino acid incorporation into enzyme protein. Accordingly, slices of first trimester and term villi were incubated with [^{3}H]lysine for 12 hours after which the labelled products in both the tissue and the medium were subjected to product analysis by specific immunoprecipitation and electrophoresis in SDS/gels. The medium samples contained no detectable radiolabelled hexosaminidase, thus substantiating the results of the cycloheximide experiment (section 16.2.1) which suggested that the release of hexosaminidase was not dependent on new enzyme synthesis for at least 12 hours. Quantitation of the incorporation into hexosaminidase was therefore based entirely on analysis of the radiolabelled tissue enzyme. Recovery of radioactivity from the gels was about 87% and results were corrected for this by the appropriate factor.

The results of this experiment are given in Table 16.2 and show that, at trace levels, the first trimester tissue incorporated about three times more radioactivity into anti-(hexosaminidase) precipitable material than term tissue. The calculated rates of amino acid incorporation into hexosaminidase derived from the data in Table 16.1 and Table 16.2 are given in Table 16.3. As expected, these data show an even greater difference between the two tissues in that the first trimester villi incorporated about seventeen times more amino acid into hexosaminidase than term tissue.

The validity of the above calculations rests, to a large extent, on the strength of several assumptions. First of all, it has been assumed that a lysine concentration of 1mM was sufficient to saturate the placental slice system and thus swamp the diluting effect of endogenous amino acids. Whether this was true or not cannot be stated with certainty since no measurement of tissue specific radioactivity was made and no information relating to the amino acid concentration of placental villi could be found. Nonetheless, it is perhaps noteworthy that Dunlop <u>et al</u>. (1974) found that the specific activity in rat brain slices remained constant and was similar to that of the incubation

TABLE.16.2 INCORPORATION OF RADIOACTIVITY INTO HEXOSAMINIDASE IN SLICES OF FIRST TRIMESTER AND TERM PLACENTAL VILLI

	Radioactivity (d.p.m./mg protein/hour) in :		
	TCA-insoluble material	Immunoprecipitable hexosaminidase	% incorporation into hexosaminidase
First trimester	252500 ±27500	81±20	0.032
Term	87500 ±1583	27±5	0.031

Slices of placental villi were prepared and incubated with 60μCi of [^{3}H] lysine, specific activity 33 Ci/mmol for 12 hours as described in section 11.1. At the end of the incubation period the samples were processed as described in section 11.2. Incorporation of radioactivity into TCA-insoluble material was determined as described in section 11.2.1.and incorporation of radioactivity into hexosamidase was estimated as described in section 11.2.2. Values represent means ± S.E.M. for six observations from two first trimester and two term placentas.

TABLE 16.3 ESTIMATED RATES OF PROTEIN AND HEXOSAMINIDASE SYNTHESIS IN SLICES OF FIRST TRIMESTER AND TERM PLACENTAL VILLI

	(a) Apparent rate of protein synthesis (pmols lysine incorporated into TCA-insoluble material/mg protein/hour)	(b) Rate of incorporation of radioactivity into TCA-insoluble material at trace levels of amino acid (d.p.m/mg protein/hour)	(c) Estimate of precursor specific activity at trace levels of amino acid (d.p.m./pmol lysine)	(d) Rate of incorporation of radioactivity into hexosaminidase (d.p.m/mg protein/hour)	(e) Apparent rate of hexosaminidase synthesis (pmols lysine incorporated into immunoprecipitable material/mg protein/hour)
First trimester	1175	252500	215	81	0.38
Term	70	87500	1230	27	0.022
	Calculated from data in Table 16.1 and assuming a precursor specific activity of 1µCi/µmol	From Table 16.2	b/a	From Table 16.2	d/c

medium when amino acid concentrations of 1mM were used. A similar effect was also noted by Khairallah and Mortimore (1976) for a rat liver perfusion system using concentrations of amino acid between 0.5 and 5mM. Since there is evidence to suggest that intracellular amino acids do not constitute a single, well-mixed pool, but rather that they are compartmented, it is necessary to further assume that amino acids can exchange freely between these compartments and that, under the conditions used here, the specific activity of the incubation medium was a good indication of the specific activity of each pool. Once again, experimental evidence to support this assumption has been obtained using other systems (Khairallah and Mortimore, 1976; Mortimore et al., 1972). At worst, the conditions used in the present study could only result in an underestimate, but it is stressed that the overall trend would be unaffected.

Secondly, it is important that the high concentration of lysine used did not interfere with protein synthesis or protein degradation. Again, although this was not tested for in the placental system, results from _in vivo_ and _in vitro_ experiments using other systems indicate that high concentrations of amino acids do not affect either of these processes (Conde and Scornik, 1977; Dunlop _et al._, 1974; Khairallah and Mortimore, 1976). Thirdly, it has been assumed that protein degradation had a minimal effect on the amount of radioactivity incorporated into protein. Nonetheless, because no direct measurement of rates of protein degradation were made, total protein synthesis and hexosaminidase synthesis have been expressed as net rather than absolute rates. That protein degradation was not a major factor in determining the amount of radioactivity incorporated into TCA-insoluble material was suggested by the observation that incorporation proceeded in a linear manner for at least 12 hours at trace levels of amino acid (see Fig. 16.2). The same cannot be said with certainty about hexosaminidase since no attempt was made to measure the time course of incorporation into enzyme protein.

Finally, the respective rates of protein synthesis in the first trimester and term placenta have been considered to be reproducible and the author recognises that the calculations are based on a single experiment using 1mM lysine.

The seventeen-fold greater incorporation of amino acids into protein by first trimester compared to term placental villi found in

this study (Table 16.3) does not agree with other published data obtained using placental slice systems. In all these other reports only trace levels of amino acids were used and, in all cases, the first trimester tissue incorporated between two and three times more radioactivity than the term tissue (Suwa and Friesen, 1969; Sybulski and Tremblay, 1967; Beaconsfield et al., 1964; Mori, 1968). The published data do, however, agree reasonably well with the three to four-fold difference in incorporation rates obtained at trace levels in the present study (Table 16.3). The most likely reason for the discrepancy between the flooding and the trace experiments has been stated above in terms of a difference in precursor specific activity between first trimester and term tissue which is exaggerated when incorporation is determined at trace levels of amino acid.

The rates of protein synthesis obtained in the present study suggest that the production of protein may be even greater during the early stages of placental development than previously believed. This is perhaps not surprising in view of the considerable differentiation and rapid increase in size which the placenta undergoes during this time. Protein synthesis during the first trimester is probably directed towards the production of new tissue and the establishment of the foeto-placental unit. After about the sixteenth week of gestation no major increase in size occurs and all placental tissues have formed. Protein synthesis after this time is, most likely, primarily directed towards placental function and the maintainence of pregnancy. The results found here are also consistent with other structural and biochemical data already described in the introduction which indicate that, in general, placental protein synthesis is greater during the first trimester. Hubert et al. (1974) have provided some evidence that term placentas show deficiencies in tRNA acceptor capacity and in aminoacyl-tRNA synthetase activity. However, it should be remembered that individual proteins may prove exceptions to the above trend. For example, while the production of the placental protein hormone, chorionic gonadotropin, is greater during the first trimester compared to term, the converse is true for another placental protein hormone, lactogen. (Boime and Boguslawski, 1974 and Chattergee et al., 1976). With respect to hexosaminidase, it can be seen that the amount of radioactivity incorporated into anti-(hexosaminidase A) precipitable material expressed as a percentage of total TCA-insoluble radioactivity was the same for both

first trimester and term villi (Table 16.2). Further, the seventeen-fold decrease in the apparent rate of protein synthesis was accompanied by a similar decrease in the apparent rate of hexosaminidase synthesis. These results suggest that hexosaminidase synthesis is regulated by a general rather than a specific control mechanism.

A comparison of the enzyme activities and rates of hexosaminidase synthesis in first trimester and term placental villi is presented in Table 16.4. Although the ratios of hexosaminidase activity to hexosaminidase synthesis have no precise functional significance, nevertheless, the fact that these ratios are widely different in first trimester and term tissues suggests that there is not a direct relationship between catalytic activity and the rate of subunit synthesis. The first possibility is that there is such a relationship but it is masked by the presence of different amounts of activators/inhibitors at different developmental stages. However, in an experiment in which soluble extracts of first trimester and term villi were mixed together it was found that the amount of hexosaminidase activity was purely additive. This strongly suggests that the presence of regulatory factors was not important in explaining the tissue differences.

Another possibility is that the two tissues contain different amounts of inactive subunits and that some mechanism exists for converting inactive subunits to active enzyme molecules. The radiolabelled protein precipitated by the anti-(hexosaminidase) serum does not necessarily represent active enzyme protein. For example, Carroll and Robinson (1974) reported the existence of a low molecular weight inactive protein in human liver extracts which cross-reacted with anti-(hexosamindase) serum. It is possible that the two placental tissues differ with respect to some component responsible for regulating the rates of subunit assembly. Any interpretation is further complicated by the possibility of differential hexosaminidase turnover.

TABLE 16.4. COMPARISON OF HEXOSAMINIDASE CATALYTIC ACTIVITY AND THE RATE OF HEXOSAMINIDASE SYNTHESIS AS A FUNCTION OF PLACENTAL DEVELOPMENT.

	(a) Hexosaminidase activity munits/mg protein	(b)* Rate of hexosaminidase synthesis	Ratio of b : a
First trimester villi	130	0.38	$2.9X10^{-3}$
Term villi	58	0.022	$0.38X10^{-3}$

*From Table 16.3

17 The release of hexosaminidase from placental tissue in vitro

That slices of placental villi incubated *in vitro* were capable of releasing hexosaminidase into the medium has already been shown in section 16.1. The purposes of the present experiments were twofold. Firstly, to compare the ability of slices of villi, chorion laeve and amnion from both first trimester and term placentas to release hexosaminidase and, in particular, to analyse the nature of the hexosaminidase forms released by these different tissues. Secondly, it was intended to investigate the possible involvement of the microtubule and microfilament systems in the release of placental hexosaminidase by using specific inhibitors.

17.1 Hexosaminidase release from tissue slices prepared from first trimester and term placentas

In section 16.1 it was shown that the release of hexosaminidase from both term and first trimester villi was linear for at least 6 hours. For the experiments described here, an incubation time of 4 hours was used. In the time course experiment just described the appearance of the cytoplasmic enzyme, lactate dehydrogenase, was used as an indicator of loss of cellular integrity. This criterion was also employed throughout the present studies and in all cases lactate dehydrogenase release represented less than 6% of the total lactate dehydrogenase activity. At the end of the incubation period the tissues and the media were separated by centrifugation and assayed for hexosaminidase activity. The results are expressed as hexosaminidase activity released per mg of tissue protein.

Table 17.1 shows the distribution of hexosaminidase activity between the tissue samples and their respective media. It can be seen that all the tissues released hexosaminidase into the incubation medium but that different tissues released different amounts. In the term samples, the amounts of hexosaminidase released seemed to be closely related to the tissue enzyme levels. Thus, although the absolute amounts released were different it can be seen from the percentage release figures that these amounts represented a similar proportion of the total hexosaminidase activities. In the first trimester samples, however, no such relationship between tissue and medium enzyme levels was seen. For example, although the hexosaminidase activity in the amnion was several times greater than that in the villi, both tissues released approximately

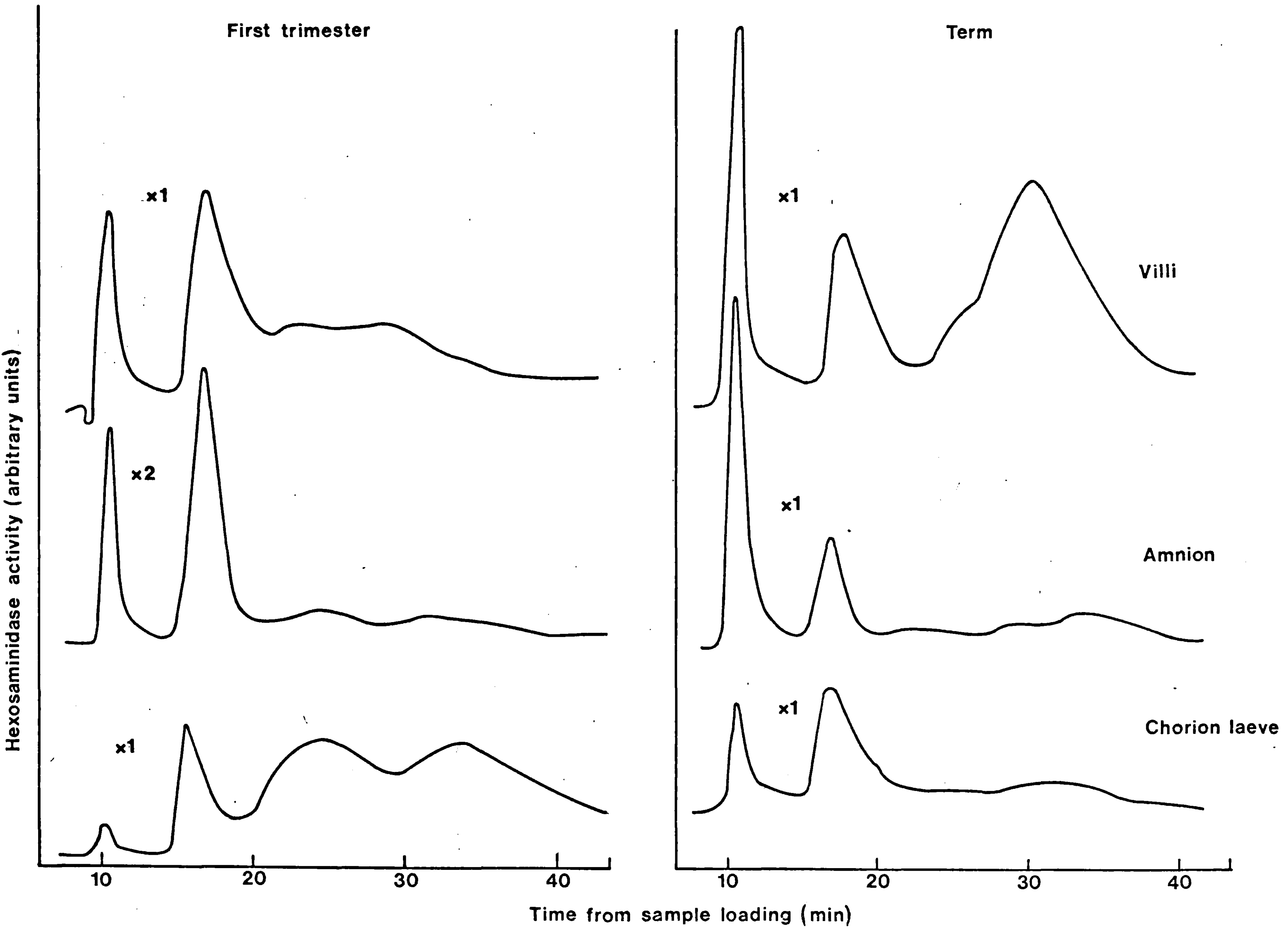
TABLE 17.1 COMPARISON OF HEXOSAMINIDASE RELEASE FROM TISSUE SLICES PREPARED FROM DIFFERENT REGIONS OF FIRST TRIMESTER AND TERM PLACENTAS

		Hexosaminidase activity (munits /mg tissue protein)		
		Villi	Chorion laeve	Amnion
First trimester	Tissue	94± 6	164 ± 4	312±37
	Medium	77±28	34.1±0.8	81 ±18
	% Release	43	35	21
Term	Tissue	80±11	169 ± 6	38 ± 3
	Medium	23±3	43 ± 6	15 ± 2
	% Release	23	24	28

Tissue slices were prepared and incubated for 4 hours as described in section 11.1 after which hexosaminidase activities were determined for both the tissues and the incubation media. Values represent means ± S.E.M. for four observations from two first and two term placentas.

FIG. 17.1 DEAE-CELLULOSE CHROMATOGRAPHIC ANALYSIS OF HEXOSAMINIDASE FORMS RELEASED BY DIFFERENT REGIONS OF FIRST TRIMESTER AND TERM PLACENTAS

Tissue slices were prepared and incubated for 4hours as described in section 11.1. At the end of the incubation period aliquots of the incubation media were analysed by automated DEAE-cellulose chromatography as described in section 10.2. The numbers shown alongside each profile represent the relative fluorimeter sensitivities.



First trimester
Term
x1
x2
x1
x1
Villi
x1
Amnion
x1
Chorion laeve
Hexosaminidase activity (arbitrary units)
10
20
30
40
10
20
30
40
Time from sample loading (min)

the same amount of activity. These results provide evidence that enzyme release was selective and possibly subject to some form of control mechanism. Further, consideration of the data in Table 17.1 indicates that the control of hexosaminidase release changed with placental development and that this was not strictly related to the changes in tissue enzyme levels. Both the villi and the amnion samples showed a decrease in the amount of hexosaminidase released while the chorion laeve showed an increase with gestation. These developmental changes followed the same general trend as, but did not exactly parallel, the changes in the tissue enzyme activities. For example, the 5-fold decrease in the amount of hexosaminidase activity released by the term compared to the first trimester amnion was accompanied by an almost 8-fold decrease in the tissue activity.

In summary, the above results show that placental slices incubated in vitro were able to release hexosaminidase, but to varying extents depending on both the identity and the age of the tissue. In order to establish whether these quantitative changes were accompanied by changes in the nature of the multiple forms released, the different incubation media were analysed by the automated DEAE-cellulose chromatographic technique already described in section 15.1.

The elution profiles given in Fig. 17.1 represent tracings of the chart recorder paper and show the multiple forms of hexosaminidase in the incubation media. Hexosaminidase B was detectable in all samples as two peaks eluting at 10 min and 17 min, respectively. The I_1 form was only ever apparent as a shoulder on the trailing edge of the second hexosaminidase B peak and was not detectable at all in the medium from either the first trimester or the term amnion samples. Hexosaminidase I_2 was usually only apparent as a slight change in slope at about 24 min but in the medium from the first trimester chorion laeve and amnion it was seen as a distinct peak. Hexosaminidase A was generally well resolved although it was only apparent as a change in slope in the medium from term chorion laeve and amnion and first trimester villi.

Generally, hexosaminidase B was the predominant form released by the different tissues. With the exception of the first trimester chorion laeve and the term villi, which both released approximately equal proportions of hexosaminidases A and B, all the other samples showed a high B to A ratio. The first trimester chorion laeve and amnion both released equal proportions of I_2 and A, which distinguished them from the other

samples where the I_2 form was always the most minor component.

It is also interesting to consider the elution profiles with respect to placental age. Apart from the amnion, the other regions showed developmental changes in the pattern of hexosaminidase multiple forms released into the medium. The medium from the first trimester villi had a high B to A ratio whereas in the term villi medium approximately equal proportions of A and B were found. First trimester chorion laeve released more or less equal proportions of hexosaminidases B, I_2 and A while at term the medium contained predominantly the B form.

When tissue samples which had been incubated for 4 hours were analysed by DEAE-cellulose chromatography, the resulting profiles were similar to the tissue profiles already discussed in section 15.1. It is therefore possible to compare the hexosaminidase patterns in the incubation media (Fig. 17.1) with the patterns of their tissues of origin (Fig. 15.1). Accordingly, in all but one case, the media profiles were different from their respective tissues. The exceptions were the term villi samples where both the media and the tissue hexosaminidase patterns were similar. In contrast to this, for example, the first trimester amniotic tissue contained predominantly hexosaminidase A whereas the incubation medium contained predominantly hexosaminidase B, suggesting selective release of this component compared to hexosaminidase A.

The results in Table 17.1 and Fig. 17.1 show that placental tissues were able to exert some degree of control over both the amount and the nature of the hexosaminidase forms released to the extracellular medium. Speculation as to the possible mechanism by which hexosaminidase release is controlled is difficult because of the heterogenous nature of the placental tissues. Each of the tissues studied consist of several different cell-types (see section 5) and the observed developmental variation in hexosaminidase release could reflect changing cell-populations rather than changes in the secretory activities of individual cells. Although the amount of hexosaminidase activity in the incubation media has been attributed to enzyme release by placental cells it should be noted that there is evidence from other systems to suggest that extracellular enzyme levels may be determined by both secretion and cellular uptake (see section 4). Indeed, certain enzyme forms appear to be preferentially taken up by some cells. The experiments carried out in the present study do not allow a distinction to be made between enzyme release and possible enzyme re-uptake.

In spite of the difficulty of interpretation, it is possible to re-evaluate the role of the placenta as a source of pregnancy serum hexosaminidase. As described in the Introduction (section 5) hexosaminidase activity in maternal serum rises steadily throughout pregnancy such that at term, serum levels are between 5 and 10 times greater than during the first trimester. Most of this increase in activity can be attributed to the appearance of the I_2 form, although it is evident that the A form also increases with gestation. It has been proposed that the placenta might be the origin of the elevated hexosaminidase levels, and in particular of the I_2 form. Results already presented in section 15.1 showed that tissue samples from different regions of first trimester and term placentas contained extremely low or non-detectable levels of the I_2 form. Furthermore, incubation of placental hexosaminidase B with pregnancy serum failed to generate hexosaminidase I_2. Although these results suggested that the placenta was not the origin of pregnancy serum I_2 they did not rule out the possibility that the placenta selectively secreted only the I_2 form or that the I_2 form was derived from some other placental hexosaminidase component just prior to secretion. These latter possibilities have been tested in the present studies by examining the release of hexosaminidase from placental tissue incubated in vitro.

The fact that placental slices were able to release hexosaminidase (see Table 17.1) suggested that they could be potential sources of pregnancy serum hexosaminidase. In general the first trimester tissue slices released more hexosaminidase than did the term slices. However, it must be remembered that towards the end of the first trimester the placenta has a wet weight of about 15g compared to a wet weight of about 400g at term. Thus, if the in vitro situation is a reflection of the in vivo situation, the net result would be a greater release of hexosaminidase by term compared to first trimester placentas. This correlates with the increase in maternal serum hexosaminidase activity.

Since the bulk of the placenta consists of villous tissue and since, in vivo, the villi are constantly bathed in maternal blood thus allowing secreted placental products to be transported directly into the maternal circulation, the villi may be considered as the most likely source of serum hexosaminidase I_2. However, analysis of the incubation medium from both first trimester and term villi slices by automated DEAE-cellulose chromatography revealed only low levels of the I_2 form and

suggested that the villi were unlikely to be the source of the respective serum activity. The possible role of the villi as a source of pregnancy serum hexosaminidase A and B was not precluded.

Similar examination of the media from first trimester chorion laeve and amnion revealed the presence of distinct I_2 peaks but only trace levels were detected in the term samples. These findings, together with the fact that the chorion laeve and the amnion line the wall of the uterus and are not in direct contact with the maternal circulation, make it difficult to see how these tissues could contribite significantly to maternal serum hexosaminidase activity.

The above discussions assume that the _in vitro_ observations reflect the _in vivo_ activities of the different tissues. It is possible that some hormonal or other physiological stimulus is necessary for the correct processing and secretion of the I_2 and, indeed, other forms of hexosaminidase by the placenta. The likelyhood that other cells or tissues could be the source of the elevated pregnancy serum hexosaminidase levels should also be considered. Increased levels of hexosaminidase are found in serum samples taken from male and non-pregnant female patients suffering from diabetes mellitus, myocardial infarction, infective hepatitis and certain cancers (Woollen and Turner, 1965). Also, patients suffering from the genetic disorder, I-cell disease, have elevated serum levels of several lysosomal enzymes, including hexosaminidase (Wiesmann _et al._, 1971). The tissue sources in the above instances are not known with certainty. Day _et al._ (1972) have suggested that blood platelets can contribute as much as 33% of the serum hexosaminidase activity. Other possible sources include the liver, the kidney and phagocytic cells such as macrophages and leucocytes. With respect to the increased levels of several lysosomal enzymes found in serum from streptozotocin-induced diabetic rats, Tulsaini _et al._ (1977) provided evidence in favour of a liver source while Fushimi and Tarui (1976) favoured the kidney. The latter workers showed that insulin treatment of diabetic rats returned the elevated serum and reduced kidney hexosaminidase activities to normal levels. Interestingly, the increased serum activity seen in diabetic rats was mainly due to the appearance of an intermediate form corresponding to hexoaminidase I_2 of human tissues. Insulin treatment reduced this form to normal levels.

Although the results obtained in this study make it unlikely that the placenta is the major source of pregnancy serum I_2, substantial

amounts of the A and B forms were released, particularly by the villi. The possible physiological significance of this finding may be related to the proposed destructive capacity of the developing placenta. Thus, the findings with respect to hexosaminidase make it tempting to speculate that this may indicate a general phenomenon and that other lysosomal enzymes are secreted by the placenta *in vivo*. As described earlier, electron and light microscope studies indicate that the placental villi are able to phagocytose and subsequently digest maternal tissue components. The likely involvement of the lysosomal system in this process has also been suggested. The present findings extend this idea and suggest that there may be considerable extracellular digestion of maternal tissue during placental growth, again involving lysosomal enzymes. This may further facilitate placental invasion and erosion and assist in the establishment of the foeto-placental unit. As in section 15.2 it is interesting to compare the findings made here with certain characteristics of tumour growth. Not only do invading tumour cells have high specific activities of several lysosomal enzymes but, also, they are able to release substantial amounts of these enzymes. For example, Guerin T8 tumour cells cultured *in vitro* released both cathepsin D and β-glucuronidase (Poole, 1973) and the fluid immediately surrounding invasive tumour cells has been shown to contain increased levels of cathepsin D and B_1 compared to normal plasma and intraperitoneal fluid (Sylven and Bois-Svennson, 1965). It has been suggested that this extracellular pool of lysosomal enzymes may favour tumour invasiveness (Poole, 1973) and similar inferences can be made with regards the placenta.

17.2 Effect of colchicine and cytochalasin B on the release of hexosaminidase from placental villi slices

In order to investigate the possible involvement of microtubules and microfilaments in the release of hexosaminidase, experiments were carried out in which slices of placental villi were incubated with colchicine and cytochalasin B, drugs known to interfere with microtubule and microfilament function, respectively. For these experiments, 4 hour incubations were used after which hexosaminidase activities were determined in both the tissue slices and the incubation media. Two other lysosomal hydrolases, α-glucosidase and β-glucuronidase, were also assayed. The concentrations of drugs used had no detectable effect on the enzyme catalytic activities. Further, neither colchicine nor cytochalasin B had

any significant effect on the intracellular and extracellular activities of lactate dehydrogenase in the tissue slice system (see Table 17.2). Therefore, these drugs appeared to have no effect on the cellular integrity of the slices under the experimental conditions used.

17.2.1 Colchicine

Table 17.3 shows the effect of 1μM colchicine on the release of hexosaminidase, α-glucosidase and β-glucuronidase from slices of term placental villi. The results of two experiments are presented because of the variability which was found with different placentas. Nonetheless, the experiments showed the same general trend in that while the release of α-glucosidase and β-glucuronidase was partially inhibited the release of hexosaminidase was stimulated by colchicine treatment.

Colchicine has been shown to bind to the protein tubulin and prevent its polymerisation into functional microtubules (Wilson, 1975). Although no published reports on the effect of colchicine on placental tissue slices has been found, the drug is known to inhibit secretion in a wide variety of other systems (see Allison, 1973). A specific example includes the release of β-glucuronidase from human polymorphonuclear leucocytes (Weissmann et al., 1971, Wright and Malawista, 1973). Recently, Hoffstein et al. (1977) provided evidence that concentrations of colchicine which inhibit lysosomal enzyme release from polymorphonuclear leucocytes also prevent microtubule assembly. These observations have led to the general conclusion that secretion is dependent on the presence of microtubules.

Thus, with respect to α-glucosidase and β-glucuronidase, the results presented in Table 17.3 are in agreement with the observation that colchicine inhibits secretion. Furthermore, since the concentration of colchicine used by Hoffstein et al. (1977) and Weissmann et al. (1971) was similar to that used in the present studies it is concluded that the release of α-glucosidase and β-glucuronidase from slices of placental villi was at least partly dependent on an active microtubule system.

The stimulatory effect of colchicine on hexosaminidase release is possibly somewhat surprising since the assumption may have been that all lysosomal enzymes would share a common secretory mechanism. Nevertheless, the observation that colchicine does have an effect on hexosaminidase release suggests that microtubular proteins may be involved

in secretion but not in the expected manner. Colchicine has been reported to stimulate the release of other secretory products. Temple and Wolff (1973) reported that colchicine stimulated steroid secretion from adrenal tumour cells in culture and Gordon and Werb (1976) found that the secretion of elastase and lysozyme from mouse peritoneal macrophages was enhanced and inhibited, respectively, by 1μM colchicine. As yet no adequate explanation for this phenomenon has been reported.

Since the different hexosaminidase forms appear to be selectively released from the villi slices, it was of interest to test the specificity of the colchicine stimulation. As before, the incubation media were analysed by automated DEAE-cellulose chromatography and the results are presented in Fig. 17.2. Colchicine treatment stimulated the release of all detectable forms to the same extent and, therefore, showed no degree of specificity.

The precise mechanism by which microtubules are involved in lysosomal enzyme secretion is not clearly understood. However, it seems likely from recent investigations (Hoffstein et al., 1977) that microtubules are involved in maintaining the internal organisation of cells and, in particular, the topologic relationship between organelles that most favours fusion of phagosomes (ie. invaginated plasma membrane) with lysosomes and the subsequent release of lysosomal constituents. Consequently, it is believed that colchicine inhibits secretion by preventing microtubule assembly and so causing intracellular disorganisation. Under these conditions, the number of phagosome/lysosome interactions and, hence, the release of lysosomal constituents would be diminished.

While it is possible to interpret the α-glucosidase and β-glucuronidase data in terms of the above proposed mechanism, the hexosaminidase results are not so readily explained. The present studies did not indicate whether the differential effects of colchicine could be attributed to the same placental cells. As described in the Introduction (section 5), each placental villus consists of several different cell types, each of which could respond differently to colchicine treatment. Another possibility is that the net release of hexosaminidase represents a balance between release and uptake and that the latter is inhibited by colchicine. To date, there is no experimental evidence to support the effect of colchicine on such an uptake mechanism.

In the experiments described by Gordon and Werb (1976) the

TABLE 17.2 EFFECT OF COLCHICINE AND CYTOCHALASIN B ON THE RELEASE OF LACTATE DEHYDROGENASE FROM SLICES OF TERM PLACENTAL VILLI

Treatment	Lactate dehydrogenase activity (munits/mg protein) Tissue	Medium	% Released
Control	834±36	34±2	4
Colchicine	818±85	37±9	4
	N.S.	N.S.	
Control	311±6	26±2	8
Cytochalasin B	328±29	25.0±0.2	7
	N.S.	N.S.	

Slices of term placental villi were incubated in the presence and absence of 1μM colchicine or 5μM cytochalasin B as described in section 11.1. At the end of the incubation period lactate dehydrogenase activity was determined for both the slices and the media. Each value represents the mean for three observations. Tissue slices for the colchicine experiment and for the cytochalasin experiment were prepared from different placentas. Values for p were calculated using Student's t-test. N.S., not significant ($p > 0.05$)

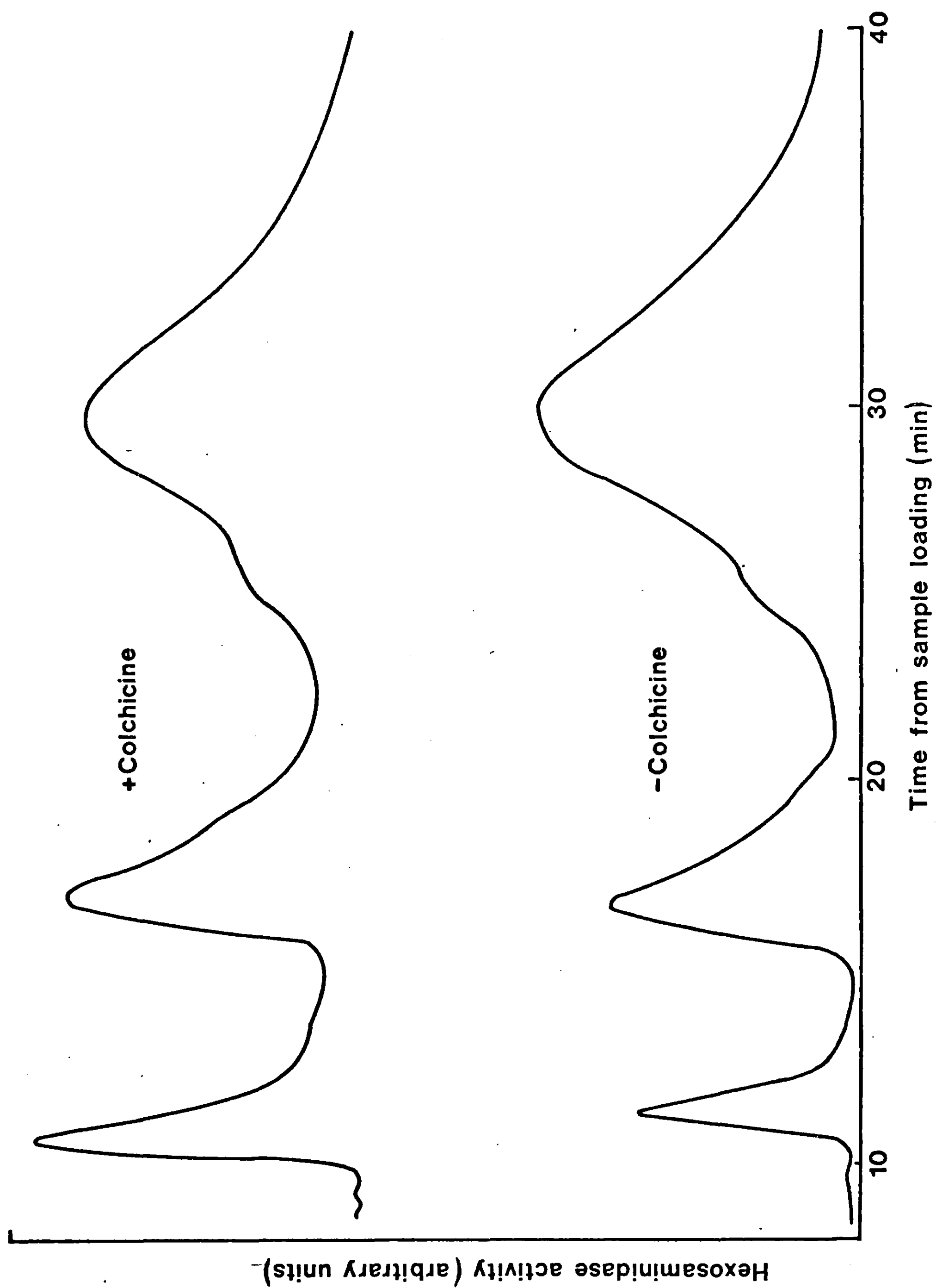
TABLE 17.3 EFFECT OF COLCHICINE ON THE RELEASE OF HEXOSAMINIDASE, α-GLUCOSIDASE AND β-GLUCURONIDASE FROM SLICES OF TERM PLACENTAL VILLI.

Slices of term villi were prepared and incubated for 4hours with 1μM colchicine as described in section 11.1. At the end of the incubation period the samples were processed as described in section 11.2 and enzyme activities were determined for the slices and the media as described in section 7.2. Each experiment represents a different placenta and the results are given as means S.E.M. for three observations. Values for p were calculated using Student's t-test. N.S., not significant ($p > 0.05$)

	Treatment	Enzyme activity (munits/mg tissue protein) Tissue	Medium	% Released	
Hexosaminidase	Control	83.7±10.2	25.2±1.9	23	
	Colchicine	91.1±12.4	44.7±3.6	33	
		N.S.	p<0.01		
α-Glucosidase	Control	2.71±0.11	1.52±0.29	36	Expt.1.
	Colchicine	2.53±0.09	0.99±0.10	28	
		N.S.	N.S.		
β-Glucuronidase	Control	1.45±0.07	0.82±0.04	39	
	Colchicine	1.17±0.12	0.50±0.02	30	
		N.S.	p<0.001		
Hexosaminidase	Control	99.3±5.4	28.1±0.2	21	
	Colchicine	105 ± 7	45 ± 3	30	
		N.S.	p<0.001		
α-Glucosidase	Control	1.59±0.07	0.53±0.06	25	Expt.2.
	Colchicine	2.16±0.08	0.31±0.03	13	
		p<0.01	p<0.02		
β-Glucuronidase	Control	1.32±0.16	0.50±0.07	27	
	Colchicine	1.80±0.17	0.37±0.02	17	
		N.S.	N.S.		

FIG. 17.2 EFFECT OF COLCHICINE ON THE PATTERN OF HEXOSAMINIDASE FORMS RELEASED BY SLICES OF TERM PLACENTAL VILLI

Slices of term placental villi were incubated with 1 μM colchicine for 4 hours as described in section 11.1.At the end of the incubation period aliquots of media were analysed by automated DEAE-cellulose chromatography as described in section 10.2



+Colchicine
-Colchicine
Hexosaminidase activity (arbitrary units)
10
20
30
40
Time from sample loading (min)

colchicine-dependent stimulation of elastase release was accompanied by an increase in intracellular elastase activity while the colchicine-dependent inhibition of lysozyme release was accompanied by a decrease in intracellular activity. These results contrast with other published data in which the colchicine-dependent inhibition of secretion was accompanied by intracellular accumulation of the product under consideration (eg. Dehm and Prockop, 1972). In the present studies, colchicine treatment had no detectable effect on the tissue levels of hexosaminidase, α-glucosidase or β-glucuronidase.

The above results have been attributed to the action of colchicine on tubulin assembly. In view of the relatively low concentration used, it is unlikely that the results obtained were due to the effect of colchicine on some other cellular process. Nonetheless, it should be borne in mind that colchicine has been shown to influence a variety of phenomena. For example, colchicine inhibits amino acid and nucleotide transport across cell membranes (Ukena and Berlin, 1972; Pesanti and Axline, 1975; Mizel and Wilson, 1972) and has a hypocalcaemic action (Heath et al., 1972). Inhibition of protein synthesis (Ehrlich et al., 1974) and stimulation of cellular autophagy (Hiysimäki et al., 1975) by colchicine have also been reported.

17.2.2 Cytochalasin B

Table 17.4 shows the effect of cytochalasin B on the release of hexosaminidase, α-glucosidase and β-glucuronidase from slices of term placental villi. As before, the results of two experiments are given. It can be seen that, under the conditions used, the extracellular activities of all three enzymes were increased by cytochalasin B treatment compared to the controls. Once again the effect was selective since the drug did not stimulate lactate dehydrogenase release (see Table 17.2).

As described in the introduction, the effects of cytochalasin B on the release of secretory products are variable, depending on the system and conditions used. Thus, while inhibiting the release of α-amylase and growth hromone from rat salivary gland and bovine pituitary gland, respectively, (Butcher and Goldman, 1972; Schofield, 1971), cytochalasin B has also been widely reported to enhance the release of lysosomal enzymes from phagocytic cells, such as polymorphonuclear leucocytes and macrophages (Temple et al., 1973; Davies et al., 1973) and from fibroblasts (von Figura and Kresse, 1974). No report on the effect of

TABLE 17.4 EFFECT OF CYTOCHALASIN B ON THE RELEASE OF HEXOSAMINIDASE, α-GLUCOSIDASE AND β-GLUCURONIDASE FROM SLICES OF TERM PLACENTAL VILLI

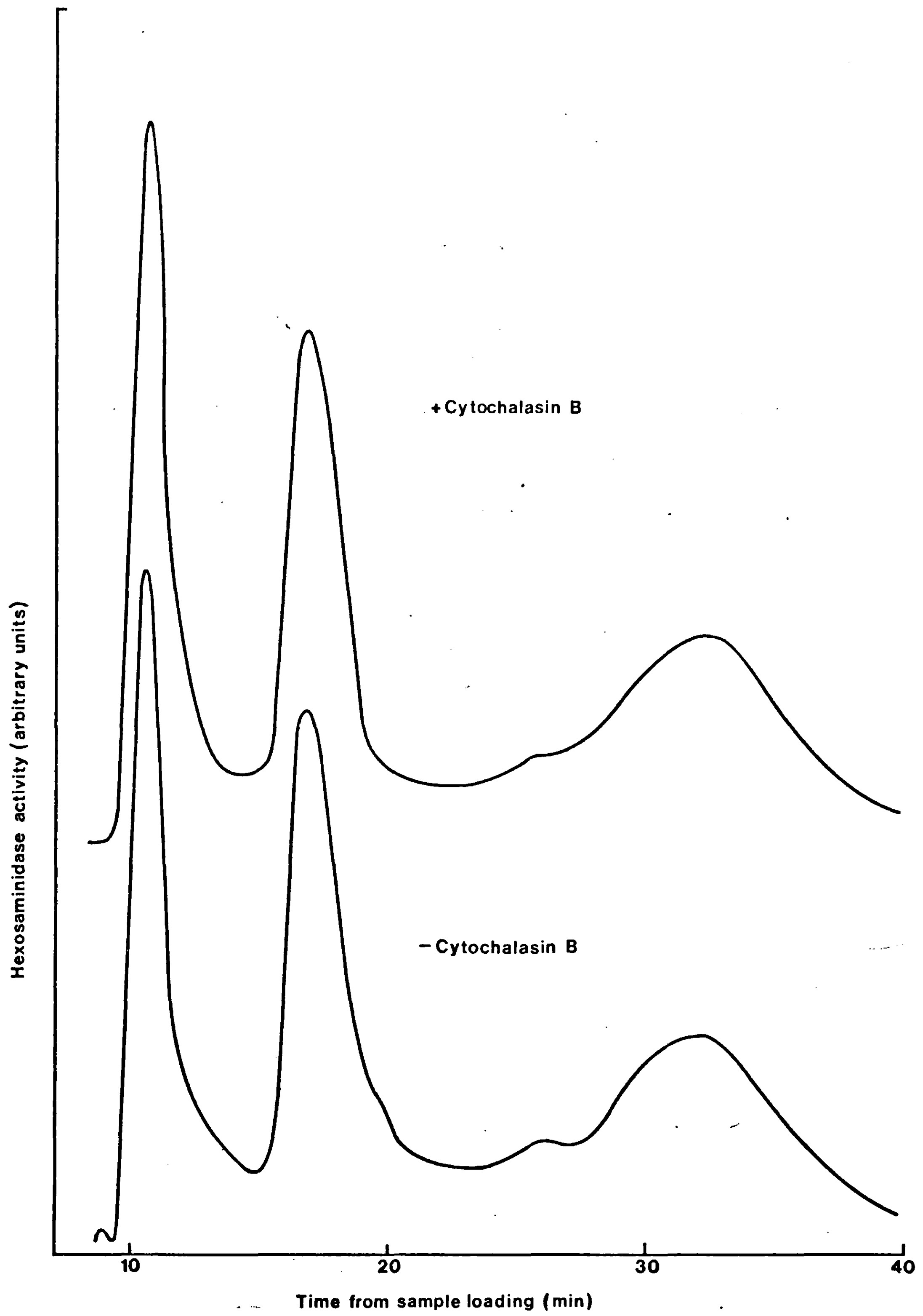
Details as for Fig. 17.3 except that the slices were incubated with 5 μM cytochalasin B. Each experiment represents a different placenta and the results are given as means S.E.M. for three observations. Values for p were determined using Student's t-test, N.S., not significant. ($p > 0.05$)

TABLE 17.4 EFFECT OF CYTOCHALASIN B ON THE RELEASE OF HEXOSAMINIDASE, α-GLUCOSIDASE AND β-GLUCURONIDASE FROM SLICES OF TERM PLACENTAL VILLI.

		Enzyme activity (munits/mg tissue protein)			
	Treatment	Tissue	Medium	% Released	
Hexosaminidase	Control	62.9 ± 2.8	23.6 ± 2.2	27	Expt.1.
	Cytochalasin B	60.2 ± 5.7	33.4 ± 0.9	36	
		N.S.	p<0.01		
α-Glucosidase	Control	1.07± 0.05	0.21± 0.01	16	
	Cytochalasin B	1.12± 0.08	0.25± 0.01	18	
		N.S.	p<0.05		
β-Glucuronidase	Control	3.43± 0.21	2.60± 0.20	43	
	Cytochalasin B	3.53± 0.13	3.13± 0.21	47	
		N.S.	N.S.		
Hexosaminidase	Control	44.7 ± 4.3	15.6 + 2.3	26	Expt.2.
	Cytochalasin B	91.7 ±12.6	44.0 ± 7.0	32	
		p<0.02	p<0.01		
α-Glucosidase	Control	1.80± 0.10	0.23± 0.05	11	
	Cytochalasin B	2.16± 0.27	0.77± 0.07	26	
		N.S.	p<0.01		
β-Glucuronidase	Control	6.40± 0.49	2.43± 0,29	28	
	Cytochalasin B	7.43± 0.35	4.26± 0.36	36	
		N.S.	p<0.01		

FIG. 17.3 EFFECT OF CYTOCHALASIN B ON THE PATTERN OF HEXOSAMINIDASE FORMS RELEASED BY SLICES OF TERM PLACENTAL VILLI

Details as for Fig. 17.2 except that the slices were incubated with 5 μM cytochalasin B.


+Cytochalasin B
-Cytochalasin B
Hexosaminidase activity (arbitrary units)
10
20
30
40
Time from sample loading (min)

cytochalasin B on human placental tissue has been found in the literature.

The results presented in Table 17.4 are in agreement with the latter observations described above in that cytochalasin B stimulates lysosomal enzyme release. However, the degree to which enzyme release was stimulated varied from enzyme to enzyme and from experiment to experiment. This variability could be due to the cellular heterogeneity of the villi. As described in section 4 the release of secretory products from cells can be continuous or discontinuous. In the latter case, release occurs in response to certain stimuli. The continuous release of immunoglobulin from mouse plasmacytoma cells and of lysozyme from mouse peritoneal macrophages was unaffected by cytochalasin B (Parkhouse and Allison, 1972; Gordon and Werb, 1976). On the other hand, cytochalasin B enhanced the release of lysosomal enzymes from both stimulated and unstimulated phagocytes (Davies et al., 1973). Thus, it is not inconceivable that different placental cells could differ in their secretory activity and, hence, in their response to cytochalasin B. The chance inclusion of a predominance of one cell type in an incubation could account for the variability observed.

In the experiments described by Gordon and Werb (1976) it was noticed that cytochalasin B stimulated both intracellular and extracellular elastase activity in cultured peritoneal macrophages. Similar observations were made by Davies et al. (1973) in their studies on lysosomal enzyme release by polymorphonuclear leucocytes, although in the majority of experiments the increased extracellular levels seemed to be due to a redistribution of intracellular enzyme. As with colchicine, cytochalasin B had little or no detectable effect on the tissue levels of all three enzymes in the present studies. Similarly, examination of the incubation media by automated DEAE-cellulose chromatography revealed that cytochalasin B did not alter the pattern of hexosaminidase forms released by the villi slices (Fig. 17.3).

Cytochalasin B has been shown to react directly with isolated contractile protein complexes (Spudich and Lin, 1972). This fact, along with the observations that cytochalasin B disrupted the regular microfilament network in several cell types (Schroeder, 1972; Lash et al., 1970) and had an effect on the release of several secretory products, led to the suggestion that microfilaments were involved in secretion.

Davies et al. (1973) have proposed that the microfilament system forms a network round the cell periphery preventing lysosomes and secretory vacuoles from fusing with the plasma membrane. Such an arrangement of microfilaments has been observed in phagocytic cells (Allison et al., 1971). Cytochalasin B treatment would disrupt this confining network allowing the fusion of organelles and plasma membrane with subsequent release of secretory products.

The increased extracellular enzyme activities obtained in the present study in the presence of cytochalasin B can be partly explained by the above mechanism and suggest that microfilaments, as well as microtubules, are involved in the release of hexosaminidase, α-glucosidase and β-glucuronidase from term placental villi. Consistent with this supposition, actin, a fundamental component of microfilaments has been found to be particularly abundant in the trophoblast cells of human placental villi (Faulk et al., 1974).

Several aspects of cytochalasin B action have not been adequately explained. For example, the increase in intracellular enzyme activities reported by others and the inhibition of secretion observed for some systems are more difficult to reconcile with the above proposed mechanism of microfilament function. Although cytochalasin B does not appear to affect amino acid transport or protein synthesis (Parkhouse and Allison, 1972) it has been reported to inhibit glucose transport and the incorporation of glucosamine into glycoproteins (Mizel and Wilson, 1972; Holzer and Sanger, 1972). In addition, the phagocytosis of various particles by macrophages and polymorphonuclear leucocytes can be completely inhibited by cytochalasin B (Allison et al., 1971).

18 Isolation and in vitro translation of placental mRNA

The experiments already described have indicated that the human placenta should provide a convenient tissue for studying certain aspects of the biosynthesis and secretion of hexosaminidase. However, in order to investigate in detail the earlier stages of hexosaminidase synthesis it is necessary to be able to isolate and translate in a cell-free protein synthesising system, the RNA fraction coding for the enzyme polypeptides. The experiments described here were therefore undertaken in order to establish whether the placenta would also provide a suitable source of active hexosaminidase mRNA.

18.1 Extraction of total placental RNA and isolation of mRNA

Total cytoplasmic RNA was prepared from human placental villi essentially by the method described by Cox *et al.* (1976). Details of the methods and an outline of the procedure used are given in section 12 and Fig. 12, respectively. Since certain steps of the original procedure were modified, these will be discussed below. The basic method involved direct homogenisation of the tissue in buffered phenol. This method was originally used by Lomedico and Saunders (1976) for the preparation of pancreatic mRNA and was reported to be beneficial in overcoming the problems associated with the relatively high RNase content of that tissue. The above workers have stated that RNase inhibitors such as heparin were not needed when RNA was prepared in this way but, in the present studies, active mRNA was only obtained if heparin was used. However, because heparin has been reported to inhibit cell-free protein synthesis (Palmiter, 1973), its use was restricted to the initial stages of the isolation procedure.

After separation of the phenol and aqueous phases, the RNA was precipitated from the latter by the addition of ammonium acetate in combination with cold ethanol followed by high-speed centrifugation. This precipitation technique was used, rather than those employing sodium chloride or potassium acetate, because it was reported to be effective at precipitating RNA from very dilute solutions (Osterburg *et al.*, 1975). Its use was particularly applicable in the present case because yields of RNA were relatively low and initial volumes were high. Once the RNA precipitate had been collected, it was routinely washed several times with 2mLiCl. Palmiter (1973) reported that this treatment removed DNA and low molecular weight RNA as well as heparin.

Using the procedure outlined in Fig. 12 total RNA yields of about 6 E_{260} units/g wet weight of tissue were obtained and preparations had E_{260}/E_{280} ratios of between 1.6 - 1.9.

Since most eukaryotic mRNAs are characterised by a sequence of polyadenylic acid residues at their 3' end, chromatography on oligo (dT)-cellulose affords a convenient means for their isolation and was the method used here. Total placental RNA was applied to a column of oligo (dT)-cellulose and the poly (A)-containing fraction was eluted with low ionic strength buffer. The poly (A)-containing RNA represented between 1 and 2% of the total RNA applied to the column.

18.2 Assay of placental mRNA in cell-free systems derived from rabbit reticulocytes

18.2.1 Total mRNA activity

In order to establish whether the RNA prepared by the above procedure was active a suitable cell-free protein synthesising system sensitive to added mRNA was required.

Recently, Pelham and Jackson (1976) described the preparation of a mRNA-dependent cell-free system which involved treating standard rabbit reticulocyte lysates with micrococcal nuclease thus removing the endogenons mRNA activity. Since the particular nuclease used was dependent on calcium, its activity could subsequently be inhibited by the addition of EGTA, leaving a protein-synthesising system sensitive to added mRNA. This system appeared to be superior to other available mRNA-dependent systems. For example, its background activity in the absence of added message was lower than that of the Krebs II ascites tumour cell system and it was more sensitive to added mRNA than either the wheat germ or the fractionated reticulocyte lysate systems. Furthermore, the efficiency of the nuclease-treated lysate in translating added mRNA was comparable to that of the original lysate and greater than that of the other systems named above. Accordingly, it was decided to use this system for the routine assay of total mRNA activity. Details of the method used for preparing the standard rabbit reticulocyte lysates and the subsequent nuclease treatment have been described in section 13.

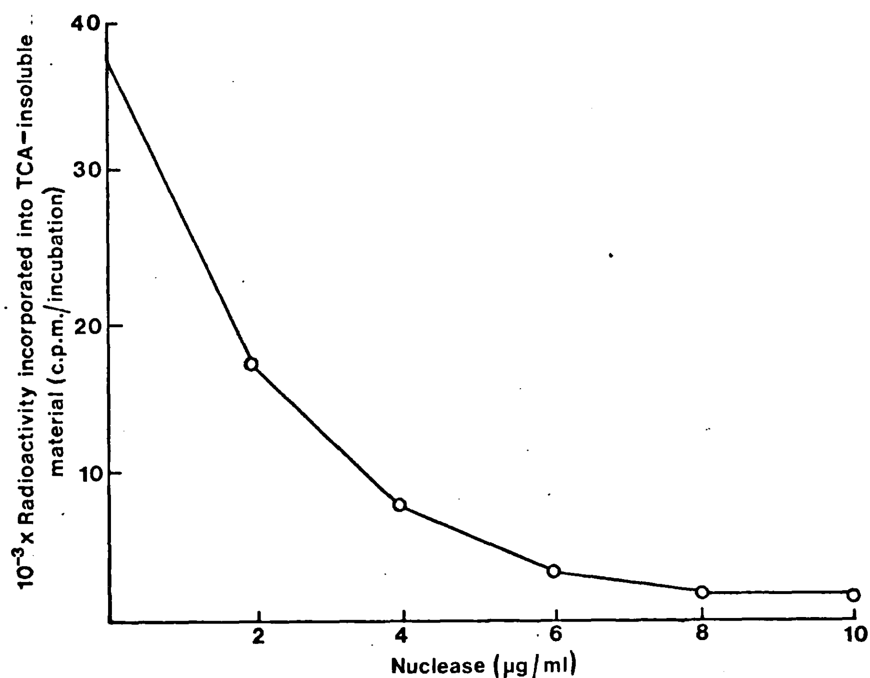
Fig. 18.1 shows the result of an experiment designed to find the optimum concentration of nuclease for removing endogenous amino acid

incorporating activity in one particular lysate. This type of experiment was necessary because different batches of lysate were found to vary in their response to nuclease treatment. The reason for this was not known but may have been done to differences in levels of endogenous calcium in the lysates. In the experiment illustrated in Fig. 18.1 it can be seen that nuclease concentrations between 8 and 10μg/ml reduced endogenons activity by about 95%, while lower concentrations were less effective. Similar observations were made by Pelham and Jackson (1976) and they have suggested that the residual nuclease insensitive incorporation was probably due to a ribosome-independent process although the precise nature of this was not known. Further investigation revealed that the lysate treated with nuclease (8μg/ml) was the most active in response to added globin mRNA (a gift from S. Bonnanou-Tzedaki, Biochemistry Department, King's College, London). Each new lysate was subsequently optimised for the appropriate nuclease concentration in this manner.

The ability of the placental mRNA preparation to stimulate the incorporation of amino acids into protein was assessed by incubating different amounts of the poly (A)-containing RNA in the mRNA-dependent lysate system. Fig. 18.2 shows a typical dose response experiment using placental poly (A)-containing RNA. As can be seen, optimum incorporation of radioactivity into TCA-insoluble material was achieved at an RNA concentration of 50μg/ml. However, incorporation was linear with respect to amount of RNA only within the concentration range up to 10μg/ml.

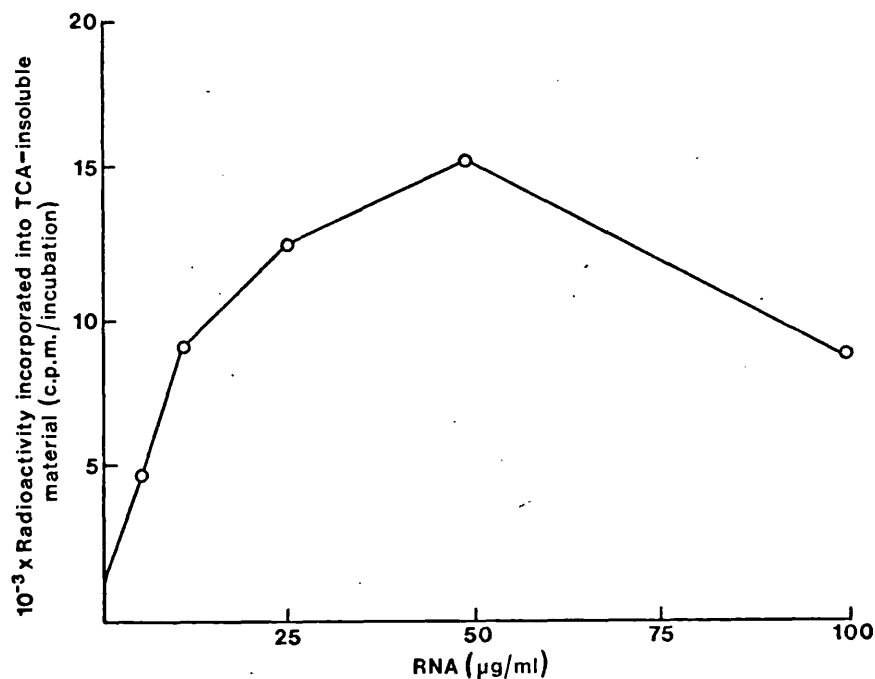
Fig. 18.3 shows a time course experiment in which mRNA-dependent lysates were incubated with and without placental poly (A)-containing RNA (at a final concentration of 25μg/ml) for times up to 60 minutes. A standard (ie non-nuclease treated) lysate was also included. It can be seen that, in the presence of placental poly (A)-containing RNA, the nuclease treated lysate incorporated radioactivity into TCA-insoluble material at a rate about 50% of that seen in the corresponding untreated lysate. In both cases, incorporation was reasonably linear for about 30 min but levelled off after this time. It can also be seen that incorporation by the nuclease-treated lysate was stimulated about 3.5-fold by the addition of the placental RNA.

FIG. 18.1 EFFECT OF MICROCOCCAL NUCLEASE ON PROTEIN SYNTHESIS IN RABBIT RETICULOCYTE LYSATES



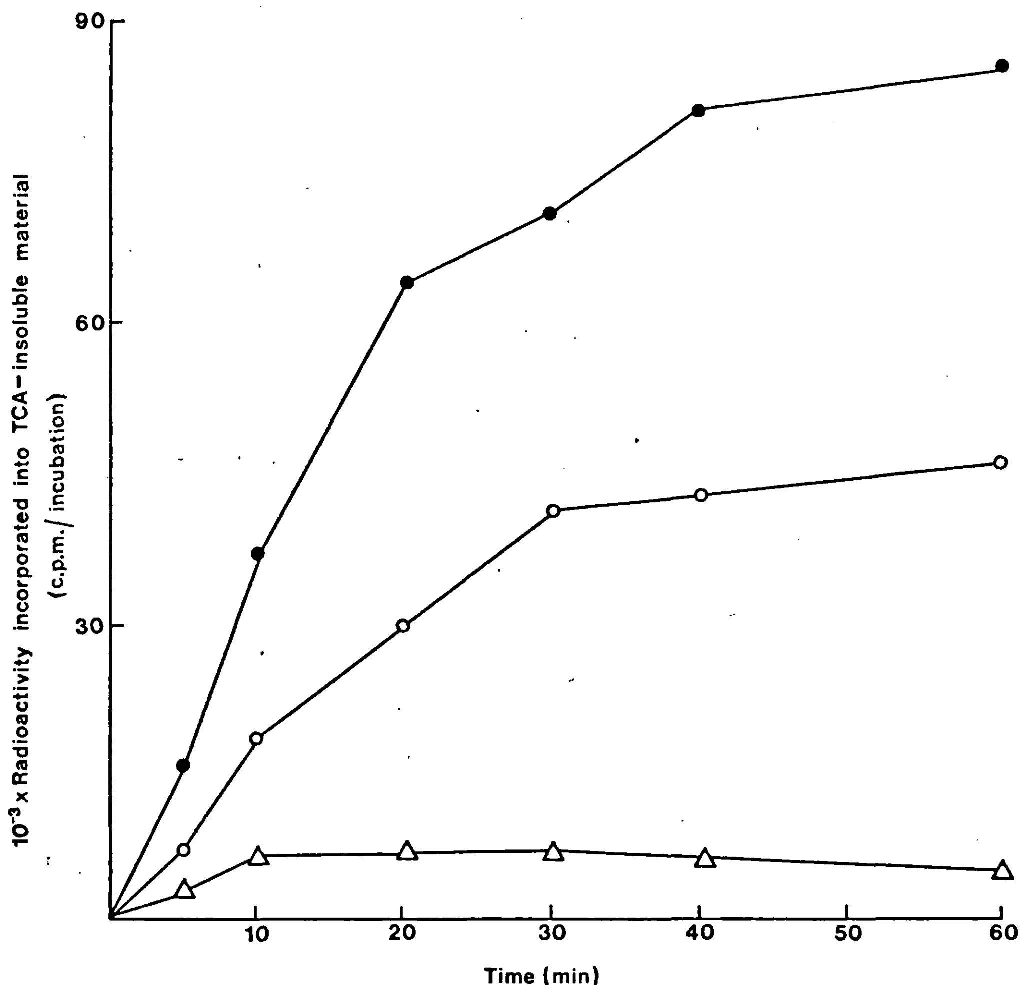
Reticulocyte lysates were treated with different amounts of nuclease as described in section 13.3. Treated lysates (100μl) were incubated with 10μCi [^{3}H]lysine, specific activity 25 Ci/mMol, for 30min at 27°C. Aliquots (20μl) were removed and used to determine the incorporation of radioactivity into TCA-insoluble material as described in section 11.2.1. Each point is the mean of duplicate incubations and values have been corrected for a final reaction volume of 100μl.

FIG. 18.2 DOSE RESPONSE OF A NUCLEASE-TREATED RETICULOCYTE LYSATE TO PLACENTAL POLY(A)-CONTAINING RNA



Nuclease-treated lysates were incubated with 5μCi of [^{3}H]lysine, specific activity 25 Ci/mMol, and different amounts of placental poly(A)-containing RNA for 30min at 27°C. Total reaction volumes were 50μl. Aliquots (20μl) were removed and used to determine the incorporation of radioactivity into TCA-insoluble material. Each point is the mean of duplicate incubations and values have been corrected for a final volume of 50μl.

FIG. 18.3 TIME COURSE OF ^{3}H-LABELLED AMINO ACID INCORPORATION IN RETICULOCYTE LYSATES IN THE PRESENCE OF PLACENTAL POLY(A)-CONTAINING RNA



Standard reticulocyte lysates (section 13.2) and nuclease-treated lysates (section 13.3) were incubated with 15 μCi of [^{3}H]lysine, specific activity 25 Ci/mMol, and 4 μg of placental poly(A)-containing RNA at 27°C. Total reaction volumes were 150 μl. At the times indicated, aliquots (20 μl) were removed and used to determine the incorporation of radioactivity into TCA-insoluble material. The values shown represent means of duplicate incubations and have been corrected for a reaction volume of 150 μl.

●——● Standard lysate minus poly(A)-containing RNA

○——○ Nuclease-treated lysate plus poly(A)-containing RNA

△——△ Nuclease-treated lysate minus poly(A)-containing RNA

18.2.2 Hexosaminidase mRNA activity

Attempts to identify hexosaminidase mRNA were made by adding placental RNA fractions to a rabbit reticulocyte lysate cell-free system and analysing the reaction products by specific immunoprecipitation. Although the mRNA-dependent lysate was useful for assessing total mRNA activity, a problem of reproducibility was sometimes encountered. This inconsistency was never completely overcome even by careful standardisation of conditions. Since product analysis was to be carried out by immunoprecipitation it was decided to translate the RNA fractions in the standard rather than the nuclease-treated lysate.

In previous experiments, assessment of placental mRNA activity has been carried out by determining the incorporation of [^{3}H]lysine into TCA-insoluble material. However, since hexosaminidase represented only a small percentage of the total protein synthesised by slices of term placental villi _in vitro_, it seemed likely that the mRNA coding for the enzyme would also represent a small proportion of total placental mRNA. In order to maximise the chances of detecting the translation product(s) it was decided to use [^{35}S]methionine instead of [^{3}H]lysine. The former had the advantage of a specific activity about 40 times greater than that available for ^{3}H- or ^{14}C-labelled compounds. For the same reason it was also decided to employ the double-antibody immunoprecipitation technique (von der Helm and Duesberg, 1975) rather than the single-antibody method used for the tissue slice studies. This involved the addition of relatively small amounts of anti-(hexosaminidase) antiserum to the cell-free incubation followed by an excess of goat anti-(rabbit IgG) antiserum. The resulting precipitate was then washed and analysed by SDS/polyacrylamide-gel electrophoresis as described in section 7.5.3.

Fig. 18.4 shows the SDS/gel electrophoretic analysis of the labelled anti-(hexosaminidase) precipitable products synthesised in standard reticulocyte lysates supplemented with different placental RNA fractions. Immunoprecipitation was carried out using the three available anti-(hexosaminidase) antisera, namely, anti-(hexosaminidase A), anti-(hexosaminidase B) and absorbed anti-(hexosaminidase A). In the discussion which follows it should be borne in mind that the anti-(hexosaminidase A) serum contained antibodies to both α- and β-subunit determinants while anti-(hexosaminidase B) and absorbed anti-(hexosaminidase A) contained

FIG. 18.4 SODIUM DODECYL SULPHATE/POLYACRYLAMIDE GEL ELECTROPHORESIS OF THE RADIOLABELLED ANTI-(HEXOSAMINIDASE)-PRECIPITABLE PRODUCTS OBTAINED AFTER TRANSLATION OF PLACENTAL RNA FRACTIONS IN A RETICULOCYTE LYSATE CELL-FREE SYSTEM

Standard reticulocyte lysates (400µl) were incubated with 5µCi of [^{35}S]methionine, specific activity 1040 Ci/mMol, and with the appropriate placental RNA fraction (10µg) for 60 min at 27°C. At the end of the incubation period, hexosaminidase mRNA activity was assayed by specific immunoprecipitation followed by SDS/polyacrylamide gel electrophoresis as described in section 13. Each fraction represents two 1.1mm gel slices.

A: Lysate incubated with poly (A)-containing RNA. Immunoprecipitation carried out with anti-(hexosaminidase A)

B: As for A) except that immunoprecipitation was carried out with absorbed anti-(hexosaminidase A)

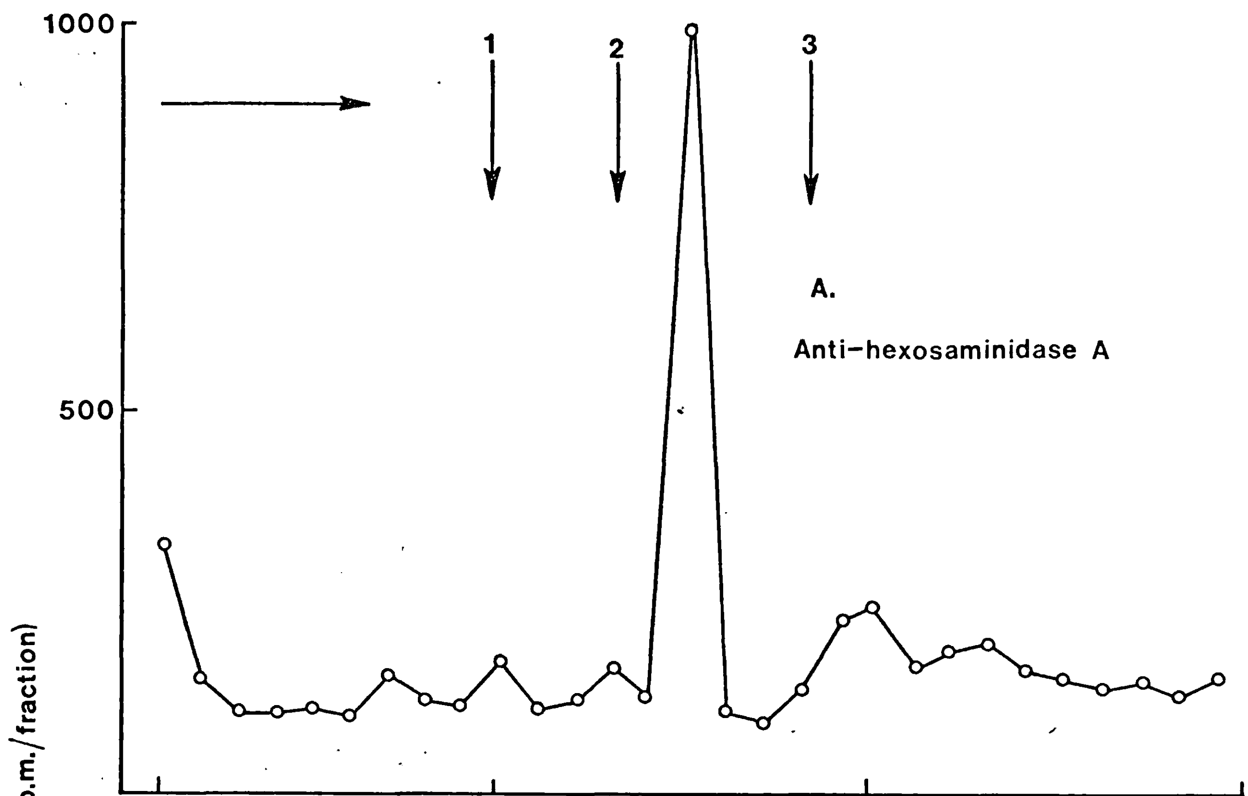
C: As for A) except that anti-(hexosaminidase B) was used

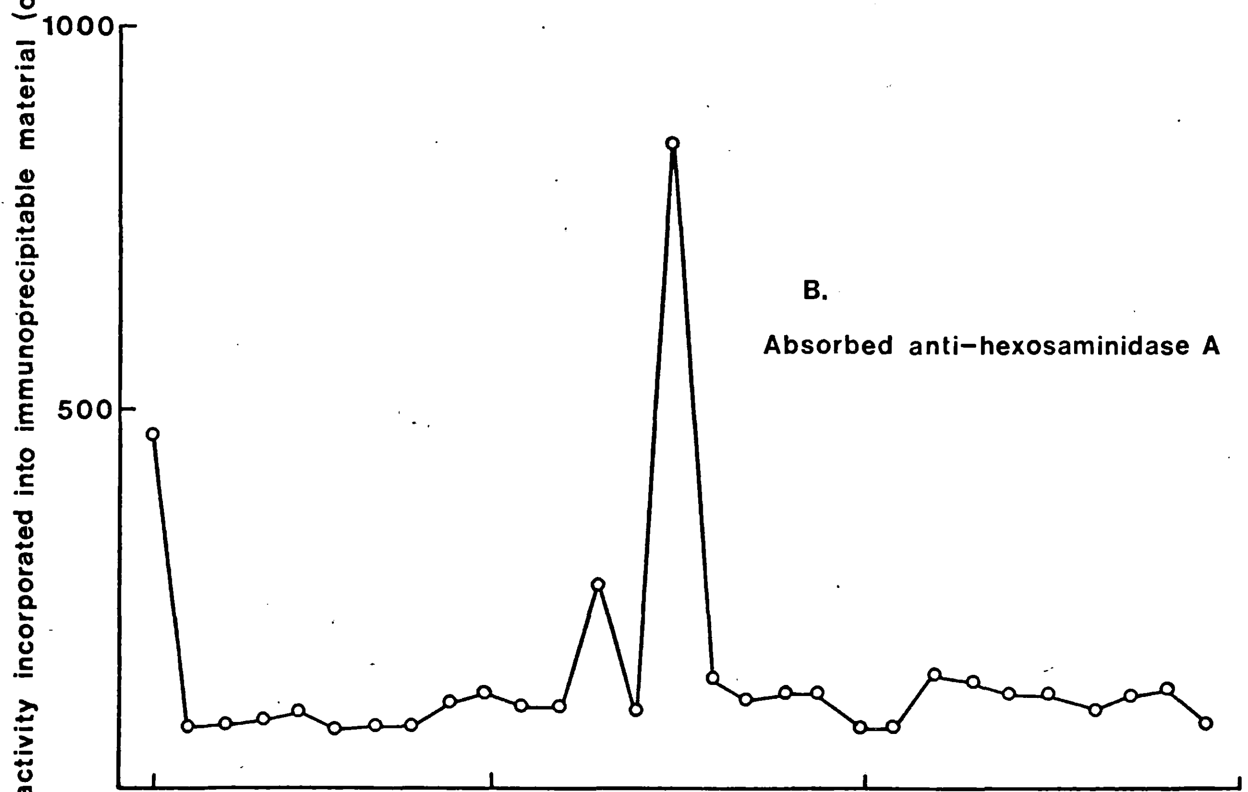
D: Lysate incubated with non-poly (A)-containing RNA. Immunoprecipitation carried out with anti-(hexosaminidase A).

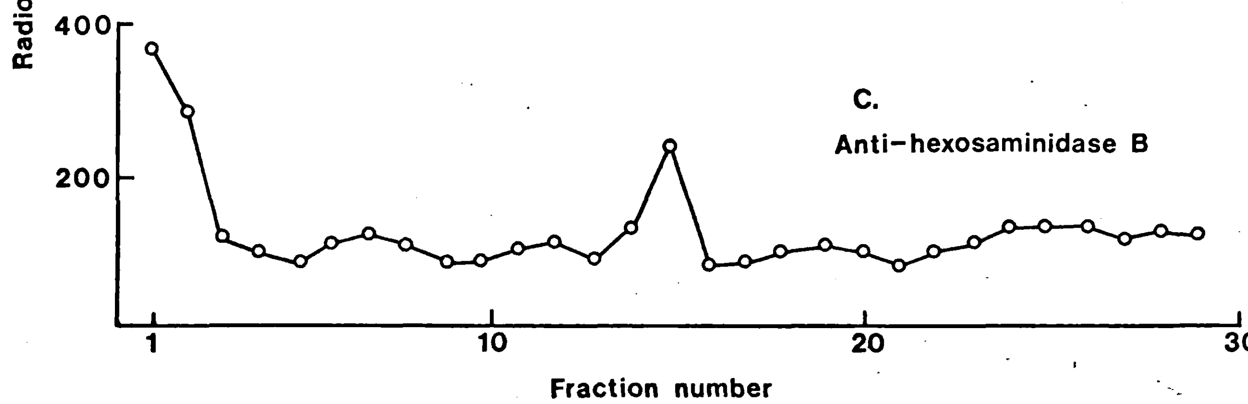
E: Lysate incubated without added RNA. Immunoprecipitation with anti-(hexosaminidase A)

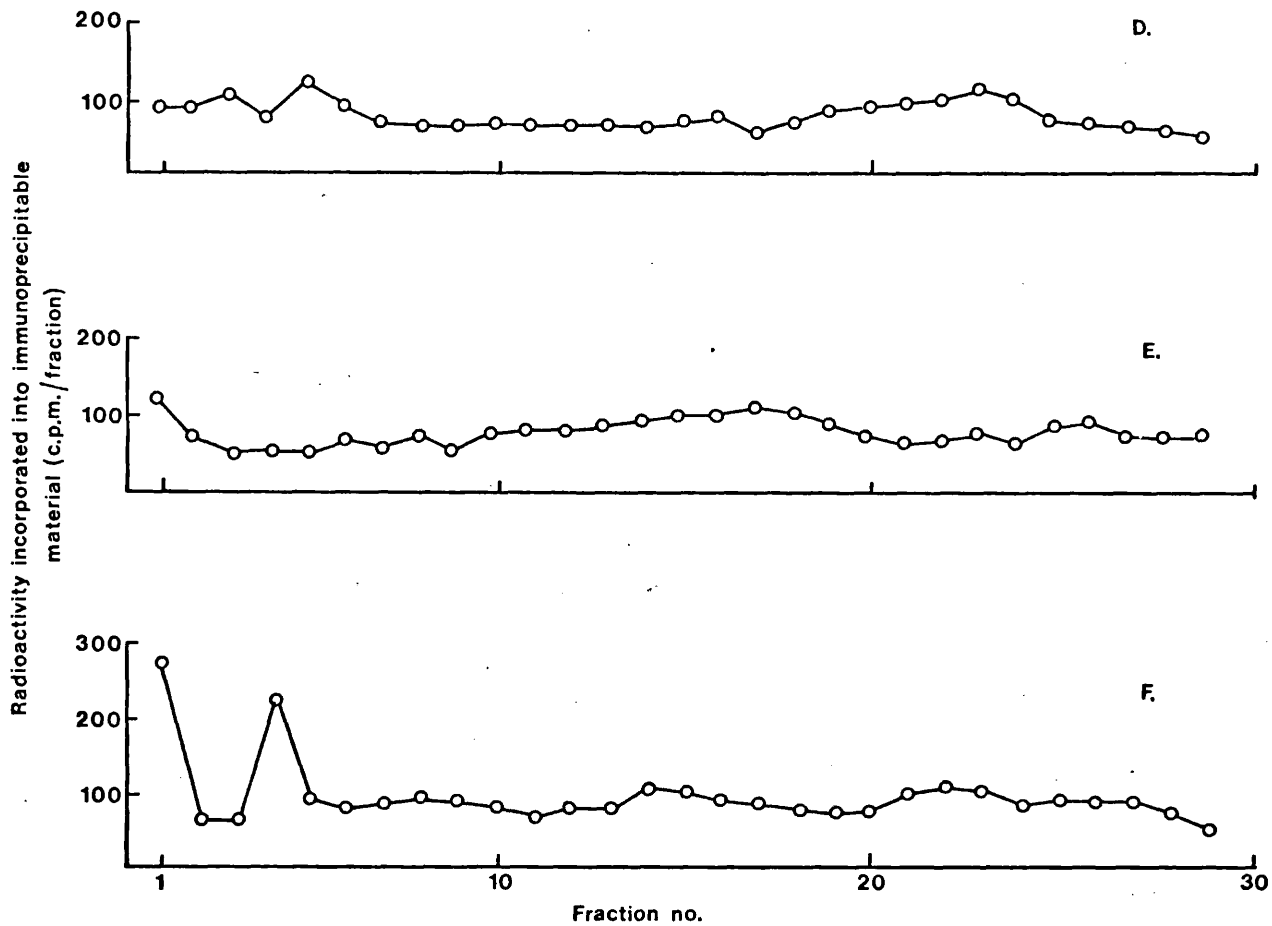
F: As for A) except that control rabbit serum was used

The vertical arrows show the relative mobilities of 1, RNA polymerase α-subunit, mol.wt. 39,000; 2, hexosaminidase subunits synthesised in the placental slice system, mol.wt. 29,000; 3, trypsin inhibitor, mol.wt. 21,000. The direction of migration is shown by the horizontal arrow.


1000
500
1
2
3
A.
Anti-hexosaminidase A
1
10
20
Radioactivity incorporated into immunoprecipitable material (c.p.m./fraction)


1000
500
B.
Absorbed anti-hexosaminidase A
1
10
20


400
200
C.
Anti-hexosaminidase B
1
10
20
30
Fraction number


200
100
D.
Radioactivity incorporated into immunoprecipitable material (c.p.m./fraction)
200
100
E.
300
200
100
F.
1
10
20
30
Fraction no.

antibodies to β-subunit and α-subunit determinants, respectively (see section 14.2). Consideration of Figs. 18.4A to 18.4C shows that placental poly (A)-containing RNA directed the synthesis of radioactive products which reacted with all three anti-(hexosaminidase) antisera and which had mobilities similar to but not identical with free α- and β-subunits. Synthesis of these products was entirely dependent on the presence of placental poly (A)-containing RNA since lysates incubated with non-poly (A)-containing RNA or without any exogenous RNA failed to incorporate radioactivity into an identifiable immunoreactive product (Figs. 18.4D) E)). Specificity of the immunoprecipitation technique was assured by analysis of an immunoprecipitate obtained using control rabbit serum in which no radioactive peak corresponding to the hexosaminidase subunits was detected (Fig. 18.4F). These findings are in agreement with the general observation that eukaryotic mRNA is characterised by the presence of a poly (A)-sequence.

The above data provided evidence for the translation in vitro of mRNA coding for hexosaminidase polypeptides or, at least, the respective α- and β-antigenic determinants. Furthermore, they confirmed that the human placenta would provide a convenient source of this particular mRNA.

Further consideration of Fig. 18.4 reveals that the products synthesised in the cell-free system were of a slightly lower molecular weight than either authentic hexosaminidase subunits or the products synthesised in the placental slice system. This effect may be due to the fact that the cell-free translation products were not glycosylated whereas the products synthesised in the slice system were. The abnormal behaviour of glycoproteins on SDS/gels has been reported by Segrest and Jackson (1966). Struck and Lennarz (1977) noted that the glycoprotein ovalbumin synthesised in vivo did not migrate as fast as the ovalbumin synthesised in the presence of tunicamycin, an inhibitor of glycosylation. Also, the vesicular stomatitis virus glycoprotein G had an apparently higher molecular weight than the product synthesised in a cell-free system (Toneguzzo & Ghosh 1977). As described in section 3, the initial translation products of mRNAs coding for many secreted proteins have slightly higher molecular weights than the products finally secreted. This difference in molecular weight is due to the presence of short amino acid sequences on the N-terminii of the primary translation products which are proteolytically cleaved either during or after translation on membrane-bound ribosomes. Since there is evidence to indicate that lysosomal enzymes

are synthesised and processed via the same intracellular membrane system (see section 3.3.1) it would be interesting to see whether they, too, are initially synthesised as larger precursor molecules. However, in view of the problem of comparing the molecular weights of the hexosaminidase subunits in their glycosylated and non-glycosylated forms, this information could not be obtained in the present study. The lower molecular weight of the cell-free translation products does not preclude the existence of a higher molecular weight precursor.

It can also be seen from Fig. 18.4 that although the specific cell-free translation products precipitated by the three antisera had identical mobilities, they were quantitatively different. Thus, while the anti-(hexosaminidase A) and absorbed anti-(hexosaminidase A) precipitable products were identifiable as distinct peaks, the anti-(hexosaminidase B) precipitable product was apparent as a smaller peak, barely resolved from the background radioactivity. Although the amount of radioactivity associated with this latter component was relatively low, it should be noted that no corresponding peak was observed in any of the control experiments. The lower incorporation into anti-(hexosaminidase B) precipitable material compared to anti-(hexosaminidase A) precipitable material may be partly, but not entirely, explained by the fact that the β-subunit has only 4 methionine residues compared to 6 for the α-subunit (Lee and Yoshida, 1976). Assuming all the newly synthesised β-chains were immunoreactive and hence precipitated under the conditions used, then other explanations may be considered. These may have been less β-chain relative to α-chain mRNA in the placental RNA preparation. This could be indicative of the *in vivo* situation or could be due to differential mRNA degradation during the isolation procedure. The results can also be explained by the preferential translation of the α-chain relative to the β-chain mRNA. There is some evidence for the unequal translation of closely related mRNAs in cell-free systems. For example, under certain conditions, such as high salt concentrations or the presence of inhibitors of initiation, the mRNA coding for the β-chain of haemoglobin is translated more effectively than that coding for the α-chain (McKeehan, 1974; Lodish and Nathan, 1972). Also, under similar circumstances, the translation of immunoglobulin light-chain mRNA has been reported to be favoured compared to the heavy-chain mRNA (Sonenshein and Brawerman, 1976).

Finally, although the results shown in Fig. 18.4 suggest that both α- and β-subunits are synthesised *in vitro*, the possibility that subunit aggregation also occurs is not eliminated by this data. If it could be shown that the immunoprecipitates obtained with the absorbed anti-(hexosaminidase A) and the anti-(hexosaminidase B) antisera contained exclusively α- and β-subunits, respectively, at least the presence of heteropolymers would be excluded. This would require the development of methods for separating α- and β-subunits after immunoprecipitation.

19 Concluding remarks

The human placenta has been used as the tissue source for the development of *in vitro* systems for studying the synthesis and secretion of the lysosomal enzyme, N-acetyl-β-D-hexosaminidase. Studies of hexosaminidase biosynthesis were made possible by the production of antisera to hexosaminidases A and B, the major multiple enzyme forms. Purified enzymes for antibody production were prepared from term placentas using combinations of published procedures. Two purification methods were used. The first employed both gel filtration and ion-exchange chromatography (Srivastava *et al.*, 1974a, Tallman *et al.*, 1974). Sample processing was long and laborious and, because of the low yields, it was difficult to maintain a constant supply of purified material. About 600μg each of hexosaminidases A and B were obtained from four placentas. Some of the above problems were reduced by the adoption of a second purification method which included chromatography on concanavalin A-Sepharose and an affinity chromatography step (Geiger *et al.*, 1975, Pokorny and Glaudemans, 1975). This method was more successful in terms of yield, mainly due to the inclusion of concanavalin A-Sepharose chromatography at an early stage of enzyme purification. Not only did this step result in considerable purification with good recoveries but it was also effective in reducing sample volumes making subsequent steps more manageable. However, in spite of these advantages, it is doubtful whether the second purification method resulted in any improvement in the purity of the final enzyme preparations. Hexosaminidases A and B prepared by the first method had specific activities of 81 and 55 units/mg, respectively, whereas the samples prepared by the second method had specific activities of 85 and 62 units/mg, respectively. When either hexosaminidase A or hexosaminidase B was dissociated under reducing conditions subunits of molecular weight 29,000 were produced, as revealed by SDS/polyacrylamide-gel electrophoresis. This can be compared to the molecular weight of 25,000 reported by Geiger and Arnon (1976) and to the value of between 25,000 and 27,000 reported by Beutler *et al.* (1976). Although the molecular weights of the native enzyme forms were not determined, the results of others indicate that they have similar molecular weights in the range 100,000-140,000 (Tallman *et al.*, 1974; Srivastava *et al.*, 1974b; Lee and Yashida, 1976). Thus, the subunit molecular weight in the present study is consistent with a tetrameric structure for both hexosaminidase A and hexosaminidase B.

Three different anti-(hexosaminidase) antisera were produced during the course of the present studies. The structural and immunochemical studies of others (Beutler and Kuhl, 1975; Beutler *et al.*, 1976; Geiger and Arnon, 1976) have already indicated subunit structures of $(\alpha\beta)_n$ and $(\beta\beta)_n$ for hexosaminidases A and B, respectively. Antibodies to the β-subunit were raised in rabbits by the injection of hexosaminidase B. Antibodies to both α- and β-subunits were subsequently raised by the injection of immune complexes formed between hexosaminidase A and anti-(hexosaminidase B) antibodies. In agreement with other workers (Srivastava et al., 1973; Bartholomew and Rattazzi, 1974) both the anti-(hexosaminidase A) and the anti-(hexosaminidase B) antisera cross-reacted with hexosaminidase A and hexosaminidase B. Also in agreement with other workers (Bartholomew and Ratazzi, 1974; Ben-Yoseph *et al.*, 1975), a third antiserum, reactive only against α-subunit determinants, was produced by absorbing anti-(hexosaminidase A) with partially purified hexosaminidase B. The three anti-(hexosaminidase) antisera were used to compare the structural relatedness of placental hexosaminidases. That hexosaminidases A, B, I_1 and I_2 are structurally related was indicated by the observations that all four forms were precipitated by both the anti-(hexosaminidase A) and the anti-(hexosaminidase B) antisera. The absorbed anti-(hexosaminidase A) failed to precipitate either hexosaminidase B or hexosaminidase I_1 but did react with hexosaminidase A and, to a lesser extent, the I_2 form. Although the I_1 and I_2 forms have been subject to little or no structural studies, the results just described would suggest that the I_1 form, as with hexosaminidase B, is a β-subunit homopolymer. Thus, the different physicochemical properties of the B and I_1 forms are probably the result of carbohydrate differences. The finding that the I_2 form apparently contained α- as well as β-subunits must be viewed with caution since the possibility of contamination with hexosaminidase A could not be ruled out.

Evidence for the *de novo* synthesis of hexosaminidase in a placental slice *in vitro* system was obtained by showing the incorporation of radiolabelled amino acid into anti-(hexosaminidase)-precipitable material. Although the amount of radioactivity incorporated into hexosaminidase polypeptides was relatively low, the data shows that the apparent rate of hexosaminidase synthesis in first trimester placental slices was at least sixteen times that of the term tissue. This decrease in the apparent rate of hexosaminidase synthesis with development was accompanied

by a similar but less extensive decrease in hexosaminidase catalytic activity. Although this descrepancy has been discussed in terms of differences in the amounts of catalytically inactive immunologically reactive subunits present at different stages of development and in the rates at which these subunits combine to form active enzyme complexes, clarification of the underlying mechanisms involved will require the measurement of absolute rates of synthesis and degradation for both α- and β-subunits. It should be possible to assess the presence of free enzyme subunits in placental tissue using an antibody consumption method. With this information it might then be possible to correlate changes in subunit production with the developmental changes in the pattern of hexosaminidase multiple forms. The relatively high rate of hexosaminidase synthesis and the high catalytic activity found for first trimester villi are probably directly related to the rapid growth and destructive activity characteristic of the placenta at this stage of development.

Term placental villi were found to be a convenient source of hexosaminidase mRNA. Messenger RNAs coding for the α- and β-subunits were identified by translating placental poly(A)-containing RNA in a rabbit reticulocyte system and analysing the products by specific immunoprecipitation. The subunits had a molecular weight of 26,000 in contrast to the molecular weight of 29,000 found for the subunits synthesised in the placental slice system. This difference can be most simply explained on the basis that the tissue slice products, but not the cell-free products, were glycosylated. However, the possibility that the tissue slice products are modified by both post-translational addition of carbohydrate and post-translational proteolytic cleavage could not be excluded. The presence of covalently attached carbohydrate could possibility be confirmed by subjecting the products to concanavalin A-Sepharose chromatography. Glycosylated subunits would be bound by this material whereas non-glycosylated subunits would not. With the exception of ovalbumin (Palmiter et al., 1977) other proteins synthesised on membrane-bound ribosomes are initially synthesised as larger pre-proteins. To date the products studied represent primarily secreted proteins and there is no information relating to lysosomal components. Although the results obtained in the present study do not shed any light on this question, they nevertheless confirm that the placenta would be a suitable source of hexosaminidase mRNA for use in future experiments. The problem

of comparing the molecular weights of proteins in their glycosylated and non-glycosylated states could perhaps be resolved by incubating placental slices with tunicamycin, an inhibitor of glycosylation and comparing the molecular weight of the non-glycosylated products with that of the cell-free translation products. An alternative approach would be to subject the different in vitro products to micro-scale amino acid analysis. This would reveal differences in primary structure and hence yield information regarding the existance of precursor molecules.

The different anti-(hexosaminidase) antisera precipitated different amounts of radiolabelled translation products suggesting that more α- than β-subunits were synthesised in the reticulocyte cell-free system. Although it is tempting to ascribe this observation to differences in the levels of mRNAs coding for the α- and β-subunits, it should be borne in mind that many parameters can affect the ratio of products in a cell-free system in which two mRNA species are being assayed. These include the presence of suitable initiation factors (Hall and Arnstein, 1973), the amount of mRNA added (Metafora et al., 1972) and the ionic conditions (Matthews et al., 1972). Quantitation of a specific mRNA based on its functional activity is, therefore, not necessarily reliable and other methods of quantitation which take advantage of the chemical and/or physical properties of the mRNA should be considered. For example, the development of a means of separating and purifying the α- and β-subunit mRNAs would enable quantitation to be carried out by the technique of molecular hybridisation. Application of this methodology would allow quantitation of hexosaminidase mRNAs in both first trimester and term placentas thus helping to elucidate the mechanisms which control hexosaminidase levels during development. Finally, the techniques developed during the present investigation of placental hexosaminidase biosynthesis, particularly at the level of mRNA, could possibly be applied to study hexosaminidase synthesis in tissues from patients suffering from GM_2-gangliosidoses in which hexosaminidase activities are deficient.

The placental slice system has been used to investigate the ability of the placenta to secrete hexosaminidase. Different placental regions selectively released different amounts of hexosaminidase into the incubation media and these amounts were not strictly related to the tissue enzyme levels. Similar studies of first trimester placental tissues showed that, in general, the amount of hexosaminidase released decreased

with placental development but not to the same extent as the decrease in the tissue activities. These observations suggest that the release of hexosaminidase is subject to some form of control. Further examination of the incubation media surrounding tissue slices obtained from different regions of first trimester and term placentas revealed that not only did they release different amounts of hexosaminidase multiple forms but also the pattern of multiple forms released changed with development. In addition, the patterns of multiple forms in the incubation media were, in most cases, different from the respective tissue patterns providing further evidence of a differential release mechanism. Although inhibitor studies have implicated the microtubule and microfilament systems in hexosaminidase release from the placental villi, the cellular heterogeneity of the tissue and the inability to measure possible cellular re-uptake of enzyme makes further discussion difficult. The ability of the placenta to secrete hexosaminidase and other lysosomal hydrolases may be correlated with its previously mentioned ability to invade and destroy maternal tissues.

Throughout the studies just described, although varying amounts of hexosaminidases A, B and I_1 were found in both tissue extracts and incubation media, the I_2 form was never present in more than trace amounts. On the basis of these experiments it is difficult to see how the placenta could be the source of pregnancy serum I_2. On the other hand, the results do suggest that the placenta might contribute to serum hexosaminidase A and hexosaminidase B activities. It is not easy to devise experiments which could provide additional information as to the source of the elevated pregnancy serum hexosaminidase I_2, especially in human subjects. If a laboratory animal could be shown to undergo similar changes in serum hexosaminidase activities during pregnancy, then this might provide an alternative experimental model.

In spite of the fact that the human placenta can be considered a relatively rich source of hexosaminidase, the major problems encountered during this project have, nevertheless, been related to the low tissue enzyme levels. Both enzyme purification and estimations of hexosaminidase synthesis were made difficult for this reason. The cellular heterogeneity of the placenta has complicated the interpretation of some results, particularly where inhibitor treatments were involved. Although it has not been possible to investigate all of the questions originally posed, the work reported here suggests that the placenta is a convenient tissue

for studying many aspects of the biosynthesis of hexosaminidase and, indeed, other lysosomal enzymes. Its use as a source of lysosomal enzyme mRNA warrants particular attention.

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