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RABBIT LIVER  $\beta$ -N-ACETYLHEXOSAMINIDASES  
-- PURIFICATION, PHYSICAL PROPERTIES AND  
SUBSTRATE SPECIFICITY

BY BARRY R. HOFFMAN

Thesis presented to the School of Graduate  
Studies in partial fulfillment of the  
requirements for the Ph.D. degree  
in Biochemistry



UNIVERSITY OF OTTAWA

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## SUMMARY

Previous work has shown that estrogens are principally eliminated by the rabbit via the urine in the form of a double conjugate of 17 $\alpha$ -estradiol with glucuronic acid at position 3 and N-acetylglucosamine at position 17. Both sugars are attached to the steroid through  $\beta$ -glycosidic linkages. Conjugation with N-acetylglucosamine, as well as with glucuronic acid, is effected primarily in the liver through the action of specific microsomal transferases. The N-acetylglucosaminyl transferase is highly specific for the 17 $\alpha$  position and transfers N-acetylglucosamine from UDP-N-acetylglucosamine to 17 $\alpha$ -hydroxy estrogens which have undergone prior conjugation at the phenolic three position with glucuronic acid or sulfate.

The in vivo synthesis of estrogen  $\beta$ -N-acetylglucosaminides in the rabbit has led to speculation about the physiological significance of these conjugates. They undoubtedly serve an excretory function since they are found in the urine and bile. However, they are also hydrolysed when incubated with rabbit tissue homogenates. This indicated the presence of  $\beta$ -N-acetylglucosaminidase activities which could effectively cleave the  $\beta$ -N-acetylglucosaminide bond of steroid conjugates and led to the work reported in this thesis. This included a detailed study of the nature and purification of the multiple forms of the  $\beta$ -N-acetylhexosaminidase present in rabbit liver as well as an exploration of their activity toward estrogen  $\beta$ -N-acetylglucosaminides in comparison with that towards other substrates.

Three substrates principally were used in the present study, namely 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide, p-nitrophenyl- $\beta$ -N-acetylglucosaminide and p-nitrophenyl- $\beta$ -N-acetylgalactosaminide. The  $\beta$ -N-acetylhexosaminidase activity in crude liver extracts hydrolysed the two N-acetylglucosaminide compounds optimally in citrate buffer at pH 4.4 and showed a pH optimum of 4.1 towards the N-acetylgalactosaminide. Under the assay conditions used, the enzyme liberated free aglycone from the p-nitrophenyl substrates at a constant rate for at least 75 minutes while initial rates were maintained for at least 3 1/2 hours with the steroid substrate.

Nine differently charged forms of the enzyme were found in crude liver extracts, as revealed by isoelectric focussing. These species separated into two main populations following gel filtration -- a large MW population containing the five isomers with the most basic isoelectric pH values (forms E to I) and a small MW population composed of the three isomers with moderately acidic isoelectric pH values (forms B to D). Form A, the most negatively charged isomer, belonged to neither population and was uniquely characterized by its extreme sensitivity to low pH and its substrate specificity. Large forms of the enzyme were estimated to weigh 270,000 Daltons and small forms 150,000 Daltons. While multiple forms of the enzyme which differ in charge have been commonly found in the tissues of higher as well as many lower animals, isomers which differ appreciably in size have been rarely encountered. The interplay between size and charge among the multiple forms of the rabbit liver enzyme was significantly different from that characterizing the multiple forms of any other  $\beta$ -N-acetylhexosaminidase previously studied.

The bulk of the  $\beta$ -N-acetylhexosaminidase activity present in liver homogenates was recovered in the 100,000 xg supernatant following centrifugation. The addition of sodium deoxycholate to the isotonic homogenizing medium enhanced recovery of soluble enzyme activity. Large and small forms of the enzyme present in this high speed supernatant have been purified to near homogeneity using the following combination of techniques: acid precipitation at pH 2.85, ammonium sulfate fractionation, Concanavalin A Sepharose affinity chromatography, Sephadex G-200 superfine gel filtration and DEAE-Cellulose anion exchange chromatography. Form A was unstable to low pH and was lost from the crude supernatant following acid precipitation. Large and small MW forms remained intact as one unit throughout the first three steps of purification but were separated by gel filtration into two distinct populations. DEAE-Cellulose chromatography resolved both populations into a number of subfractions and yielded highly purified preparations of the moderately charged isomers of the small MW population, as well as the more positively charged isomers of the large MW population. Approximately a 4850-fold purification over similarly sized forms in the crude supernatant was achieved with the former and 23,800-fold with the latter. Polyacrylamide gel electrophoresis revealed some contamination of enzyme species in these fractions by protein not originating from the enzyme. The enzyme forms examined in this work were endogenously constituted components of the  $\beta$ -N-acetylhexosaminidase activity of rabbit liver; they were shown not to be artifacts generated by either the extraction or purification procedures.

Large and small forms of the enzyme were specific for the  $\beta$ -

glycosidic bond of N-acetylglucosaminides and N-acetylgalactosaminides, and showed no activity in cleaving other glycosidic linkages.  $\beta$ -N-Acetylglucosaminidase activity towards p-nitrophenyl- $\beta$ -N-acetylglucosaminide exceeded  $\beta$ -N-acetylgalactosaminidase activity towards p-nitrophenyl- $\beta$ -N-acetylgalactosaminide. This was due to the fact that the  $\beta$ -glycosidic bond of the N-acetylglucosaminide derivative was hydrolysed more rapidly than that of the corresponding N-acetylgalactosaminide following substrate binding in the enzyme-substrate complex.  $K_m$  values for the latter compound, however, were slightly lower than those for the former. Large and small forms of the enzyme gave closely similar N-acetylglucosaminidase/N-acetylgalactosaminidase activity ratios with the two p-nitrophenyl compounds.

Large and small forms of the enzyme are glycoproteins. There were about six times as many small forms, by weight, as large forms in the crude supernatant; extracts from ten livers were estimated to contain 33.3 mg of the former species and 5.7 mg of the latter. Both sized populations exhibited roughly equal stability during purification. In a somewhat analogous fashion to corresponding human isoenzyme forms, negatively charged isomers of the rabbit liver enzyme (small MW forms) partially converted to positively charged isomers (large MW forms) when frozen and thawed in 3M NaCl. The reverse transformation, however, did not occur following disruption of the quaternary structure of large MW forms by a similar procedure. In terms of subunit composition, such conversion behaviour suggested that large forms are composed of all the types of polypeptide subunits present in small forms, while the latter also contain additional types of polypeptide chains absent from the former. Small forms were more susceptible than large forms

to physical inactivation by heat.

Large MW forms of the enzyme were more active than small MW forms in hydrolysing both steroid and p-nitrophenyl  $\beta$ -N-acetylglucosaminide substrates. On a per molecule enzyme basis, large forms were 13.2 times more active with 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide and 3.5 times more active with p-nitrophenyl- $\beta$ -N-acetylglucosaminide. Large forms also hydrolysed 17 $\alpha$ -estradiol 3- $\beta$ -glucuronyl-17- $\beta$ -N-acetylglucosaminide, 17 $\alpha$ -estradiol 3-sulfate-17- $\beta$ -N-acetylglucosaminide, 17 $\beta$ -estradiol 3- $\beta$ -N-acetylglucosaminide and estrone 3- $\beta$ -N-acetylglucosaminide more rapidly than did small forms. Differences in absolute activity between the two populations were more pronounced with the 17-N-acetylglucosaminide conjugates than with the 3-N-acetylglucosaminides. Kinetic studies revealed that this enhanced activity of large enzyme forms stemmed from a more efficient cleavage of the  $\beta$ -N-acetylhexosaminide bond once substrate was bound in the enzyme-substrate complex. Km values given by large MW forms were closely similar to those of small MW forms for each substrate tested.

With both populations hydrolysis of the estrogen  $\beta$ -N-acetylglucosaminide linkage was not significantly affected by the presence of other non-amino sugar hydrophilic substituents on the steroid nucleus. In addition, unconjugated steroids had no effect on the steroid  $\beta$ -hexosaminidase activity of either population. Both populations showed appreciably higher activity towards the 3-N-acetylglucosaminide estrogen conjugates than towards the 17-N-acetylglucosaminides.

Large MW forms exhibited a greater preference than small MW forms for steroid substrates as compared with p-nitrophenyl substrates. All of the species comprising the large MW population showed the

same steroid specificity and this was also true for members of the small MW population. Differences in population ratios of steroid to p-nitrophenyl  $\beta$ -N-acetylglucosaminidase activity were most pronounced with the 17- $\beta$ -N-acetylglucosaminide estrogen conjugates and somewhat muted with the 3- $\beta$ -N-acetylglucosaminides. These latter conjugates, as opposed to the former, contained an amino sugar positioned adjacent to an aromatic ring, as is the case with p-nitrophenyl- $\beta$ -N-acetylglucosaminide, and this common structural feature may have accounted for the closer equivalence of steroid to p-nitrophenyl  $\beta$ -N-acetylglucosaminidase activity seen with the 3-N-acetylglucosaminide steroid conjugates as opposed to the 17-N-acetylglucosaminides.

The limited tests performed with form A pointed to an extremely high specificity for steroid conjugates as compared with p-nitrophenyl substrates. p-Nitrophenyl- $\beta$ -N-acetylglucosaminide/17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide activity ratios were far lower for this isomer than for any of the other multiple forms of the rabbit liver enzyme.

N-acetylglucosamino-lactone and N-acetylgalactosamino-lactone inhibited the hydrolytic activity of both large and small MW enzyme forms. Inhibition against the p-nitrophenyl substrates was competitive in nature whereas that against 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide appeared to be non-competitive. Each lactone gave  $K_i$  values in the range of  $10^{-6}$  M against all three substrates; those against the  $\beta$ -N-acetylglucosaminide and  $\beta$ -N-acetylgalactosaminide of p-nitrophenyl were very similar and this added to the evidence that large and small forms of the enzyme possess both  $\beta$ -N-acetylgluco-

saminidase and  $\beta$ -N-acetylgalactosaminidase activity and, therefore, lack absolute specificity towards the configuration of the fourth carbon of the amino sugar ring. Lactones corresponding in configuration to sugar acids other than N-acetylhexosaminic acid did not inhibit the  $\beta$ -N-acetylhexosaminidase activity of either population.

N-Acetylglucosamine and glucosamine were considerably weaker inhibitors of enzyme activity than the two amino sugar lactones. Nevertheless, they caused an appreciable reduction in hydrolysis when present in millimolar concentrations. N-acetylglucosamine was a somewhat better inhibitor than glucosamine of the hydrolytic activity of both enzyme populations.

Both enzyme populations failed to show any but trace amounts of transglycosylase activity in systems where p-nitrophenyl- $\beta$ -N-acetylglucosaminide functioned as the sugar donor and tetrasaccharides derived from hyaluronic acid and chondroitin sulfate were present as the sugar acceptor. Pentasaccharides were not synthesized but it appeared likely that trace amounts of the di-N-acetylglucosaminide of p-nitrophenol were formed.

It is concluded from this work that multiple forms of  $\beta$ -N-acetylhexosaminidase are present in rabbit liver. Large and small forms of the enzyme, as well as form A, differ in absolute and relative activity towards steroid and p-nitrophenyl substrates, and probably differ in subunit composition. Nevertheless, none of the forms exclusively catalysed the hydrolysis of any of the substrates tested. All forms of the enzyme possess steroid  $\beta$ -N-acetylhexosaminidase activity in vitro and could be expected to hydrolyse estrogen  $\beta$ -N-acetylhexosaminide conjugates in vivo as well. However, a full

assessment of the impact which the  $\beta$ -N-acetylhexosaminidases of the liver and other tissues exert on steroid conjugate metabolism in the living rabbit will require further studies on the hydrolysis of steroid conjugates in vivo, particularly that of  $17\alpha$ -estradiol 3- $\beta$ -glucuronyl-17- $\beta$ -N-acetylglucosaminide.

GLOSSARY

The following compounds have been referred to in this thesis by their trivial name or written in abbreviated form.

<u>Trivial Name</u>	<u>Compound</u>
BIS	N,N'-methylenebisacrylamide
BSA	Bovine serum albumin
Con A	Concanavalin A
DEAE-Cellulose	Diethylaminoethylcellulose
Dehydroepiandrosterone 3- $\beta$ -N-acetylglucosaminide	5-Androsten-17-one-3 $\beta$ -yl-2'- acetamido-2'-deoxy- $\beta$ -D-glucopy- ranoside
17-Epiestriol	Estra-1,3,5(10)-trien-3,16 $\alpha$ ,17 $\alpha$ - triol
16,17-Epiestriol	Estra-1,3,5(10)-trien-3,16 $\beta$ ,17 $\alpha$ - triol
17 $\alpha$ -Estradiol	Estra-1,3,5(10)-trien-3,17 $\alpha$ -diol
17 $\alpha$ -Estradiol 17- $\beta$ -N- acetylglucosaminide (E <sub>2</sub> $\alpha$ -17NAcglc)	3-Hydroxyestra-1,3,5(10)-trien- 17 $\alpha$ -yl-2'-acetamido-2'-deoxy- $\beta$ - D-glucopyranoside
17 $\alpha$ -Estradiol 17-gluco- side	3-Hydroxyestra-1,3,5(10)-trien- 17 $\alpha$ -yl- $\beta$ -D-glucopyranoside
17 $\alpha$ -Estradiol 3- $\beta$ -glu- curonide	17 $\alpha$ -Hydroxyestra-1,3,5(10)-trien- 3-yl- $\beta$ -D-glucopyranosiduronic acid
17 $\alpha$ -Estradiol 3- $\beta$ -glu- curonyl-17- $\beta$ -N-acetyl- glucosaminide (E <sub>2</sub> $\alpha$ -3GA- 17NAcglc)	Estra-1,3,5(10)-trien-3,17 $\alpha$ -di- yl-3- $\beta$ -D-glucopyranosiduronic acid-17-2'-acetamido-2'-deoxy- $\beta$ -D-glucopyranoside
17 $\alpha$ -Estradiol 3-sulfate- 17- $\beta$ -N-acetylglucosaminide (E <sub>2</sub> $\alpha$ -3SO <sub>4</sub> -17NAcglc)	Estra-1,3,5(10)-trien-3,17 $\alpha$ -di- yl-3-sulfate-17-2'-acetamido- 2'-deoxy- $\beta$ -D-glucopyranoside

<u>Trivial Name</u>	<u>Compound</u>
17 $\beta$ -Estradiol	Estra-1,3,5(10)-trien-3,17 $\beta$ -diol
17 $\beta$ -Estradiol 3- $\beta$ -N-acetylglucosaminide (E <sub>2</sub> $\beta$ -3NAcglc)	17 $\beta$ -Hydroxyestra-1,3,5(10)-trien-3-yl-2'-acetamido-2'-deoxy- $\beta$ -D-glucopyranoside
Estriol	Estra-1,3,5(10)-trien-3,16 $\alpha$ ,17 $\beta$ -triol
Estrone	3-Hydroxyestra-1,3,5(10)-trien-17-one
Estrone 3- $\beta$ -N-acetylglucosaminide (E <sub>1</sub> -3NAcglc)	17-Oxoestra-1,3,5(10)-trien-3-yl-2'-acetamido-2'-deoxy- $\beta$ -D-glucopyranoside
Estrone 3-sulfate	17-Oxoestra-1,3,5(10)-trien-3-yl-sulfate
15 $\alpha$ -Hydroxyestradiol-17 $\beta$	Estra-1,3,5(10)-trien-3,15 $\alpha$ ,17 $\beta$ -triol
15 $\alpha$ -Hydroxyestrone	Estra-1,3,5(10)-trien-3,15 $\alpha$ -diol-17-one
15 $\alpha$ -Hydroxyestrone 3-sulfate	17-Oxoestra-1,3,5(10)-trien-15 $\alpha$ -ol-3-yl-sulfate
15 $\alpha$ -Hydroxyestrone 3-sulfate-15- $\beta$ -N-acetylglucosaminide	17-Oxoestra-1,3,5(10)-trien-3,15 $\alpha$ -di-yl-3-sulfate-15-2'-acetamido-2'-deoxy- $\beta$ -D-glucopyranoside
KBr	Potassium bromide
KCl	Potassium chloride
17 $\beta$ -Methyl-17 $\alpha$ -estradiol	Estra-1,3,5(10)-trien-17 $\beta$ -methyl-3,17 $\alpha$ -diol
NaCl	Sodium Chloride
Naphthol AS-BI-NAcglc	7-Bromo-3(-hydroxy-2'-acetamido-2'-deoxy- $\beta$ -D-glucopyranoside)-2-naphth-o-anisidide
Naphthol AS-BI- $\beta$ -glucuronide	7-Bromo-3(-hydroxy- $\beta$ -D-glucopyranosiduronic acid)-2-naphth-o-anisidide

<u>Trivial Name</u>	<u>Compound</u>
PNP- $\alpha$ -NAcglc	p-Nitrophenyl-2'-acetamido-2'-deoxy- $\alpha$ -D-glucopyranoside
p-Nitrophenyl- $\beta$ -N-acetylgalactosaminide (PNP- $\beta$ -NAcgal) (PNP-NAcgal)	p-Nitrophenyl-2'-acetamido-2'-deoxy- $\beta$ -D-galactopyranoside
p-Nitrophenyl- $\beta$ -N-acetylglucosaminide (PNP- $\beta$ -NAcglc) (PNP-NAcglc)	p-Nitrophenyl-2'-acetamido-2'-deoxy- $\beta$ -D-glucopyranoside
PNP- $\alpha$ -gal	p-Nitrophenyl- $\alpha$ -D-galactopyranoside
PNP- $\beta$ -gal	p-Nitrophenyl- $\beta$ -D-galactopyranoside
PNP- $\alpha$ -glc	p-Nitrophenyl- $\alpha$ -D-glucopyranoside
PNP- $\beta$ -glc	p-Nitrophenyl- $\beta$ -D-glucopyranoside
p-Nitrophenyl- $\beta$ -glucuronide (PNP- $\beta$ -gluc)	p-Nitrophenyl- $\beta$ -D-glucopyranosiduronic acid
PNP- $\alpha$ -man	p-Nitrophenyl- $\alpha$ -D-mannopyranoside
PNP-SO <sub>4</sub>	p-Nitrophenyl-sulfate
Phenyl- $\beta$ -N-acetylglucosaminide	phenyl-2'-acetamido-2'-deoxy- $\beta$ -D-glucopyranoside
Pregnenolone	5-Pregnen-3 $\beta$ -ol-20-one
SDS	Sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylenediamine
Testosterone 17- $\beta$ -N-acetylglucosaminide	4-Androsten-3-one-17 $\beta$ -yl-2'-acetamido-2'-deoxy- $\beta$ -D-glucopyranoside
TRIS	2-Amino-2(hydroxymethyl)-1,3-propanediol

Trivial Name

Compound

Uridine diphosphate N-  
acetylglucosamine  
(UDP-N-acetylglucosamine)

Uridine-5'-diphospho-2''-acetamido-  
2''-deoxy-D-glucofuranoside

UDP-glucose

Uridine-5'-diphosphoglucofuranoside

Uridine diphosphate  
glucuronic acid

Uridine-5'-diphosphoglucofuranosiduronic acid

The following enzymes have been referred to in this thesis by their trivial name.

<u>Trivial Name</u>	<u>Enzyme (Enzyme Number)</u>
$\alpha$ -N-Acetylglucosaminidase	$\alpha$ -2-Acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase (E.C.3.2.1._)
$\beta$ -N-Acetylgalactosaminidase*	$\beta$ -2-Acetamido-2-deoxy-D-galactoside acetamidodeoxygalactohydrolase (E.C.3.2.1._)
$\beta$ -N-Acetylglucosaminidase*	$\beta$ -2-Acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase (E.C.3.2.1.30)
Acid phosphatase	Orthophosphoric monoester phosphohydrolase (E.C.3.1.3.2)
Aldolase	Fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase (E.C.4.1.2.b)
Chitinase	Chitin glycanohydrolase (E.C.3.2.1.14)
Chymotrypsin A	Peptide peptidohydrolase (E.C.3.4.4.5)
$\alpha$ -Galactosidase	$\alpha$ -D-Galactoside galactohydrolase (E.C.3.2.1.22)
$\beta$ -Galactosidase	$\beta$ -D-Galactoside galactohydrolase (E.C.3.2.1.23)
$\alpha$ -Glucosidase	$\alpha$ -D-Glucoside glucohydrolase (E.C.3.2.1.20)
$\beta$ -Glucosidase	$\beta$ -D-Glucoside glucohydrolase (E.C.3.2.1.21)

\*Also referred to as (exo-) $\beta$ -N-acetylhexosaminidase ( $\beta$ -hexosaminidase).  $\beta$ -N-Acetylglucosaminidase and  $\beta$ -N-acetylgalactosaminidase have often been shown to be identical enzymes.

<u>Trivial Name</u>	<u>Compound</u>
$\beta$ -Glucuronidase	$\beta$ -D-Glucuronide glucuronohydrolase (E.C.3.2.1.31)
Hyaluronidase	Hyaluronate glycanohydrolase (E.C.3.2.1.d)
17 $\alpha$ -Hydroxysteroid dehydrogenase	Steroid: NAD 17 $\alpha$ -oxidoreductase (E.C.1.1.1._)
17 $\beta$ -Hydroxysteroid dehydrogenase	Steroid: NAD(P) 17 $\beta$ -oxidoreductase (E.C.1.1.1._)
Lysozyme (endo-N-acetylmuraminidase)	N-Acetylmuramide glycanohydrolase (E.C.3.2.1.17)
$\alpha$ -Mannosidase	$\alpha$ -D-Mannoside mannohydrolase (E.C.3.2.1.24)
Neuraminidase	N-Acetyl-neuraminate glycohydrolase (E.C.3.2.1.18)
Phenolsulfatase	Aryl-sulfate sulfohydrolase (E.C.3.1.6.1)
Ribonuclease A	Polyribonucleotide 2-oligonucleotido- transferase (cyclizing) (E.C.2.7.7.16)
Steroid N-acetylglucosaminyl transferase	UDP-2''-acetamido-2''-deoxy-D-glucose: steroid acetamidodeoxyglucosyl-trans- ferase (E.C.2.4.1._)
Steroid glucuronyl transferase	UDP-glucuronic acid: steroid glucuronyl- transferase (E.C.2.4.1._)

The following is a list explaining some of the abbreviations and symbols used in this thesis.

Ci	Curie
DPM	Disintegrations per minute
$^3\text{H}$	tritium
HSS	high speed supernatant
Ki	inhibition constant
Km	Michaelis constant
$\lambda$	$10^{-6}$ liter
$\mu$	micro or micron
MW	molecular weight
psi	pounds per square inch
$R_f$	relative distance of migration with respect to the dye or solvent front
Vmax	velocity of enzyme reaction at infinite concentration of substrate
TLC	thin layer chromatography

LIST OF FIGURES

<u>Figure</u>	<u>Title</u>	<u>Page</u>
1	pH activity curves	61
2	Progress curves	62
3	Ammonium sulfate fractionation of $\beta$ -hexosaminidase in High Speed Supernatant	63
4	Sephadex G-200 fine gel filtration of partially purified enzyme preparation	68
5a	DEAE-Cellulose chromatography pH 6.0	69
5b	DEAE-Cellulose chromatography pH 7.9	70
6	Isoelectric focussing pH 4-8: High Speed Supernatant	72
7	Electrophoresis of charge isomers of $\beta$ -hexosaminidase separated by isoelectric focussing	74
8	Schematic representation of the relative distance of migration with respect to the dye front on 5.5% polyacrylamide gels of the multiple forms of rabbit liver $\beta$ -hexosaminidase	75
9	Monitoring enzyme purification by polyacrylamide gel electrophoresis	102
10	Isoelectric focussing pH 4-8: Con A Sepharose fraction	104
11	Sephadex G-200 superfine filtration of Con A Sepharose fraction	106
12	Calibration curve for the determination of molecular weight by gel filtration on Sephadex G-200 superfine	112
13	DEAE-Cellulose anion exchange chromatography of the Large Molecular Weight enzyme fraction from the Sephadex G-200 superfine eluate	115

<u>Figure</u>	<u>Title</u>	<u>Page</u>
14	Scan at 540m $\mu$ of protein-stained polyacrylamide gel of Large Molecular Weight enzyme fraction eluted from DEAE-Cellulose with 0.02 M phosphate pH 6.0	117
15	DEAE-Cellulose anion exchange chromatography of the Small Molecular Weight enzyme fraction from the Sephadex G-200 superfine eluate	119
16	Scan at 540m $\mu$ of protein-stained polyacrylamide gel of Small Molecular Weight enzyme fraction eluted from DEAE-Cellulose with 0.045 M - 0.075 M phosphate pH 7.4	120
17	Susceptibility to denaturation by heat: Large Molecular Weight enzyme forms	135
18	Susceptibility to denaturation by heat: Small Molecular Weight enzyme forms	136
19	A freeze-thaw experiment	138
20	SDS gel electrophoresis	142
21	Calibration curve for the determination of molecular weight on SDS-polyacrylamide gels	145
22	Scan at 540m $\mu$ of protein-stained polyacrylamide gel of SDS-treated Large Molecular Weight enzyme forms	146
23	Scan at 540m $\mu$ of protein-stained polyacrylamide gel of SDS-treated Small Molecular Weight enzyme forms	147
24	Lineweaver-Burke plot to determine Km and Vmax for $\beta$ -hexosaminidase activity toward p-nitrophenyl- $\beta$ -N-acetylglucosaminide	183
25	Lineweaver-Burke plot to determine Km and Vmax for $\beta$ -hexosaminidase activity toward p-nitrophenyl- $\beta$ -N-acetylgalactosaminide	184

<u>Figure</u>	<u>Title</u>	<u>Page</u>
26	Lineweaver-Burke plot to determine $K_m$ and $V_{max}$ for $\beta$ -hexosaminidase activity toward $17\alpha$ -estradiol $17\text{-}\beta\text{-N}$ -acetylglucosaminide	185
27	Dixon plot: N-acetylglucosamino-lactone inhibition of $\beta$ -hexosaminidase activity toward p-nitrophenyl- $\beta\text{-N}$ -acetylglucosaminide	189
28	Dixon plot: N-acetylglucosamino-lactone inhibition of $\beta$ -hexosaminidase activity toward p-nitrophenyl- $\beta\text{-N}$ -acetylgalactosaminide	190
29	Dixon plot: N-acetylglucosamino-lactone inhibition of $\beta$ -hexosaminidase activity toward $17\alpha$ -estradiol $17\text{-}\beta\text{-N}$ -acetylglucosaminide	191
30	Dixon plot: N-acetylgalactosamino-lactone inhibition of $\beta$ -hexosaminidase activity toward p-nitrophenyl- $\beta\text{-N}$ -acetylglucosaminide	192
31	Dixon plot: N-acetylgalactosamino-lactone inhibition of $\beta$ -hexosaminidase activity toward p-nitrophenyl- $\beta\text{-N}$ -acetylgalactosaminide	193
32	Dixon plot: N-acetylgalactosamino-lactone inhibition of $\beta$ -hexosaminidase activity toward $17\alpha$ -estradiol $17\text{-}\beta\text{-N}$ -acetylglucosaminide	194
33	Inhibition of steroid $\beta$ -hexosaminidase activity by free and N-acetylated glucosamine	198
34	Separation of reaction products in the trans-glycosylase assay by gel filtration	204

LIST OF TABLES

<u>Table</u>	<u>Title</u>	<u>Page</u>
I	p-Nitrophenyl glycoside and sulfate assays	40
II	Enzyme recovery with decreasing pH	64
III	Partial purification of $\beta$ -hexosaminidase: enzyme and protein recoveries	66
IV	Affinity chromatography experiments	81
V	Comparison of steroid and p-nitrophenyl $\beta$ - hexosaminidase activity of enzyme species in fractions during purification	108
VI	Comparison of steroid and p-nitrophenyl $\beta$ - hexosaminidase activity of enzyme species in the High Speed Supernatant	110
VII	Purification of rabbit liver $\beta$ -hexosaminidases: 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide as substrate	122
VIII	Purification of rabbit liver $\beta$ -hexosaminidases: p-nitrophenyl- $\beta$ -N-acetylglucosaminide as sub- strate	123
IX	Comparison of the hydrolytic activity of Large Molecular Weight and Small Molecular Weight enzyme forms toward E <sub>2</sub> $\alpha$ -17NAcglc and PNP-NAcglc on a per molecule and a per weight enzyme basis	126
X	Purification of rabbit liver $\beta$ -hexosaminidases: based on the activity of corresponding enzyme forms in the High Speed Supernatant	128
XI	$\beta$ -N-Acetylglucosaminidase and $\beta$ -N-acetylgalacto- saminidase activity: recovery and ratio of the two activities at each step in the purification procedure	130

<u>Table</u>	<u>Title</u>	<u>Page</u>
XII	Comparison of $\beta$ -N-acetylglucosaminidase and $\beta$ -N-acetylgalactosaminidase activity of enzyme forms B to I in the High Speed Supernatant	132
XIII	Glycosidase and phenolsulfatase activity in fractions at various stages during the purification procedure	133
XIV	SDS-treated Large Molecular Weight and Small Molecular Weight enzyme forms: molecular weight and staining intensity of protein fragments	144
XV	Range of substrate concentrations used for the determination of enzyme activity with steroid $\beta$ -N-acetylglucosaminidase	176
XVI	Assay conditions for transglycosylase determinations	181
XVII	$K_m$ and $V_{max}$ values determined for Large and Small Molecular Weight enzyme forms	186
XVIII	N-Acetylhexosamino-lactone inhibition of $\beta$ -hexosaminidase activity: $K_i$ values and type of inhibition	188
XIX	Inhibition of $\beta$ -hexosaminidase activity by lactones	197
XX	Activity of Large and Small Molecular Weight enzyme forms towards steroid $\beta$ -N-acetylglucosaminide substrates	200
XXI	Effect of unconjugated estrogens on the steroid $\beta$ -hexosaminidase activity of Large and Small Molecular Weight enzyme forms	202

TABLE OF CONTENTS

	<u>Page</u>
ACKNOWLEDGEMENTS	i
SUMMARY	ii
GLOSSARY	x
LIST OF FIGURES	xvii
LIST OF TABLES	xx
CHAPTER 1: INTRODUCTION	
A) Introductory Comments	1
B) Classification of N-Acetylhexosaminidases	2
C) Exo- $\beta$ -N-acetylhexosaminidases	3
1) Distribution in nature and common properties	3
2) Natural substrates of $\beta$ -hexosaminidase	8
3) Multiple forms of $\beta$ -hexosaminidase - Occurrence in nature; genetic and physical relation- ship	11
4) Multiple forms of $\beta$ -hexosaminidase - Physiolo- gical significance	15
5) $\beta$ -Hexosaminidase and pathological conditions	17
6) $\beta$ -Hexosaminidase - Variation with age and hor- monal status	22
D) Conjugation of Steroids with N-Acetylglucosamine	24
1) Occurrence and synthesis of steroid N-acetylglu- cosaminide conjugates in nature	24
2) Hydrolysis of steroid $\beta$ -N-acetylglucosaminide conjugates by $\beta$ -hexosaminidase <u>in vivo</u> and <u>in vitro</u>	28
CHAPTER 2: MATERIALS AND GENERAL METHODS	
A) Materials	30
B) General Methods	33

	<u>Page</u>
1) Preparation of radioactive steroid substrates	33
a) Introductory comments	33
b) $17\alpha$ -(6,7- $^3\text{H}_2$ )-Estradiol 3- $\beta$ -glucuronyl-17- $\beta$ -N-acetylglucosaminide	33
c) $17\alpha$ -(6,7- $^3\text{H}_2$ )-Estradiol 17- $\beta$ -N-acetylglucosaminide	34
d) $17\alpha$ -(6,7- $^3\text{H}_2$ )-Estradiol 3- $\beta$ -glucuronide	36
e) $17\alpha$ -(6,7- $^3\text{H}_2$ )-Estradiol 3-sulfate-17- $\beta$ -N-acetylglucosaminide	36
2) Preparation of non-radioactive steroid substrates	38
3) Enzyme assays	38
a) Introductory comments	38
b) Assays with p-nitrophenyl compounds	39
c) Assays with steroid glycosides	41
I) General features	41
II) $17\alpha$ -(6,7- $^3\text{H}_2$ )-Estradiol 17- $\beta$ -N-acetylglucosaminide	42
III) $17\alpha$ -(6,7- $^3\text{H}_2$ )-Estradiol 3- $\beta$ -glucuronide	43
IV) $17\alpha$ -(6,7- $^3\text{H}_2$ )-Estradiol 3- $\beta$ -glucuronyl-17- $\beta$ -N-acetylglucosaminide and $17\alpha$ -(6,7- $^3\text{H}_2$ )-estradiol 3-sulfate-17- $\beta$ -N-acetylglucosaminide	43
V) Estrone 3- $\beta$ -N-acetylglucosaminide and 17 $\beta$ -estradiol 3- $\beta$ -N-acetylglucosaminide	45
4) Radioactivity measurements	45
5) Preparative techniques	46
a) Gel filtration	46
b) Anion exchange chromatography	47
c) Group affinity chromatography	47
6) Analytical techniques	48
a) Polyacrylamide disc gel electrophoresis	48
b) Sodium dodecyl sulfate gel electrophoresis	50
c) Isoelectric focussing	51
7) Protein determinations	51

### CHAPTER 3: PRELIMINARY INVESTIGATIONS

A) Introduction	53
B) Methods	55
1) Preparation of tissue homogenates and high speed supernatant	55
2) Investigation of assays with PNP-NAcglc, PNP-NAcgal and $\text{E}_2\alpha$ -17NAcglc	55

	<u>Page</u>
3) Partial purification of $\beta$ -hexosaminidase	55
a) Ammonium sulfate fractionation and acid precipitation of enzyme activity from the high speed supernatant	55
b) Preparation of partially purified enzyme fraction	56
c) Detergent enzyme recovery studies	57
4) Investigation of multiple enzyme forms	57
a) DEAE-Cellulose chromatography	57
b) Gel filtration	58
c) Isoelectric focussing	58
C) Results	60
D) Discussion	77
 CHAPTER 4: PURIFICATION, ENZYMATIC PROPERTIES AND PHYSICAL PROPERTIES OF RABBIT LIVER $\beta$ -HEXOSAMINIDASES	
A) Introduction	85
B) Methods	88
1) Purification procedure	88
a) General comments	88
b) High speed supernatant	88
c) Acid precipitation	88
d) Ammonium sulfate fractionation	89
e) Concanavalin A Sepharose 4B affinity chromatography	89
f) Gel filtration	90
g) DEAE-Cellulose chromatography of large molecular weight enzyme population	90
h) DEAE-Cellulose chromatography of small molecular weight enzyme population	91
2) Monitoring the purification of $\beta$ -hexosaminidase	92
3) Physical properties of $\beta$ -hexosaminidase isomers	93
a) Stability to increasing temperature	93
b) Estimation of molecular weight	94
c) Subunit composition - SDS gel electrophoresis	95
d) Subunit composition - Interconversion of enzyme forms	96
C) Results	98
1) Purification and substrate specificity of enzyme forms	98
2) Physical studies on highly purified enzyme forms	134
D) Discussion	150
Appendix 1: Derivation of data presented in Table IX	163

	<u>Page</u>
Appendix 2: Calculations to determine the amount, in milligrams, of large and small molecular weight enzyme forms in the high speed supernatant	165
CHAPTER 5: FURTHER ENZYMATIC PROPERTIES OF RABBIT LIVER $\beta$ -HEXOSAMINIDASES	
A) Introduction	167
B) Methods	172
1) Determination of $K_m$ and $V_{max}$	172
2) Inhibition of $\beta$ -hexosaminidase activity by lactones	172
3) The effect of N-acetylglucosamine, glucosamine and free steroids on steroid $\beta$ -hexosaminidase activity	173
4) Preference of the two enzyme populations for steroid substrates	175
5) Determination of transglycosylase activity	175
a) Synthesis of the glycosyl donor	177
b) Preparation of tetrasaccharides of hyaluronic acid and chondroitin sulfate	177
I) Analytical methods	177
II) Digestion of polysaccharides	178
III) Fractionation of mucopolysaccharide digest	178
IV) Identification of isolated carbohydrate	179
c) Transglycosylase assay	180
C) Results	182
1) Kinetic parameters characterizing substrate hydrolysis	182
2) Lactone inhibition of $\beta$ -hexosaminidase activity	187
3) Effect of N-acetylglucosamine and glucosamine on enzyme activity	196
4) Hydrolysis of estrogen conjugates	199
5) Determination of transglycosylase activity	203
D) Discussion	205
CHAPTER 6: GENERAL DISCUSSION	209
BIBLIOGRAPHY	217

CHAPTER 1

INTRODUCTION

A) INTRODUCTORY COMMENTS

In 1964, Layne, Sheth and Kirdani showed that rabbits excrete  $17\alpha$ -estradiol as a conjugate with glucuronic acid at position 3 and N-acetylglucosamine at position 17. This was the first demonstration of the coupling of a naturally-occurring non-sugar aglycone with N-acetylglucosamine, and was followed by the demonstration in other laboratories of the excretion of steroid  $\beta$ -N-acetylglucosaminides by the human (Arcos and Lieberman, 1967; Jirku and Levitz, 1969).

Work in this laboratory over the past twelve years has yielded information on the reactions involved in the transfer of N-acetylglucosamine to the steroid estrogens (Collins, Jirku and Layne, 1968) and led to speculation as to the in vivo role of these conjugates. The possibility that the steroid  $\beta$ -N-acetylglucosaminides might be substrates for hydrolytic action by the  $\beta$ -N-acetylhexosaminidases of animal tissues led to the work reported in the present thesis. This work has involved a detailed study of the nature and purification of the multiple forms of the  $\beta$ -N-acetylhexosaminidase of rabbit liver and an exploration of their activity towards steroid  $\beta$ -N-acetylglucosaminides as compared to that towards other substrates.

The remainder of this introduction reviews present knowledge on the occurrence in nature, physical properties and enzymatic characteristics of  $\beta$ -N-acetylhexosaminidases together with some pathological manifestations of these enzymes. Also included is a review on the occurrence in nature, the in vivo synthesis and the interaction with  $\beta$ -N-acetylgluco-

saminidase of steroid  $\beta$ -N-acetylglucosaminide conjugates.

B) CLASSIFICATION OF N-ACETYLHEXOSAMINIDASES

Compounds containing N-acetylhexosamines covalently linked by glycosidic bonds to other substituents are known to be essential components of almost all living organisms. Enzymes which are capable of cleaving this bond are also widely distributed throughout the living world. These enzymes, generally termed N-acetylhexosaminidases, primarily participate in the degradation of amino sugar-containing compounds (hydrolytic activity), but occasionally also function in the transfer of amino sugars from one compound to another (transglycosyltic activity). In this way the organism is enabled to retrieve amino sugars and reutilize them according to the needs of the moment.

N-Acetylhexosaminidases are often classified according to the anomeric configuration of the glycosidic bond which is cleaved, and the position of the amino sugar residue within the compound. In a sense, such a classification scheme is arbitrary since the mechanism of bond cleavage may well be the same between different "types" of N-acetylhexosaminidases. Nevertheless, such a scheme is convenient and useful for categorizing this diverse group of enzymes.

$\alpha$ -N-Acetylhexosaminidases are so named for their ability to specifically cleave the amino sugar from  $\alpha$ -glycosidic bonds while  $\beta$ -N-acetylhexosaminidases perform an analogous function with  $\beta$ -glycosidically linked amino sugars. The  $\beta$ -N-acetylhexosaminidases have been studied to a greater degree than the  $\alpha$ -enzymes. However, the  $\alpha$ -enzymes occur widely, being found in microorganisms (Watkins, 1959), invertebrates (Zechmeister, Toth and Vajda, 1939; Findlay, Levy and Marsh, 1958) and higher animals

(Weissmann et al, 1967; Werries et al, 1969). The  $\beta$ -N-acetylhexosaminidases are usually further subdivided on the basis of exo- or endo- glycosidase activity. The exo-enzymes catalyse bond cleavage when the amino sugar is present in the compound as a terminally positioned, non-reducing residue, while enzymes of the endo type effect the hydrolysis of internally located residues of N-acetylhexosamine. The endo- $\beta$ -N-acetylhexosaminidases constitute an important class of enzymes with hyaluronidase and chitinase as two well known examples. Lysozyme, while generally considered to function in vivo as an endo- $\beta$ -N-acetylmuraminidase (E.C.3.2.1.17), is also capable of considerable endo- $\beta$ -N-acetylhexosaminidase activity in vitro (Rupley, 1967). Within the last few years an increasing number of endo- $\beta$ -N-acetylhexosaminidases of rather narrow specificity have been isolated from bacteria (Koide and Muramatsu, 1974; Chien et al, 1975). These enzymes promise to serve as useful tools in structural studies of complex glycoproteins.

The exo- $\beta$ -N-acetylhexosaminidases ((E.C.3.2.1.30),  $\beta$ -2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase) constitute the other class of  $\beta$ -N-acetylhexosaminidases. Since the rabbit liver enzyme examined in the present work is an exo- $\beta$ -N-acetylhexosaminidase, the remainder of this review deals specifically with this enzyme class in order to provide a reference framework for the findings presented later in this thesis.

## C) EXO- $\beta$ -N-ACETYLHEXOSAMINIDASES

### 1) Distribution in Nature and Common Properties

Exo- $\beta$ -N-acetylhexosaminidase (hereafter abbreviated  $\beta$ -hexosaminidase) activity within biological systems was first noted in the 1930's (Helferich and Iloff, 1933; Watanabe, 1936). It is now known that  $\beta$ -hexosaminidase is ubiquitously distributed throughout the living world, being found in bacteria (Hughes and Jeanloz, 1964; Berkeley et al, 1973), plants

(Li and Li, 1970), molds (Every and Ashworth, 1973), fungi (Molodstov et al, 1974), invertebrates (Molodstov and Vafina, 1972; Phizackerley and Bannister, 1974) and vertebrates (Pugh, Leaback and Walker, 1957; Bullock and Winchester, 1973). It is generally accepted that most, if not all, mammalian tissues possess  $\beta$ -hexosaminidase activity. The highest activity is usually seen in the adult male epididymis with that of the mature pig being the richest known mammalian source of this enzyme (Conchie, Findlay and Levvy, 1959).  $\beta$ -Hexosaminidase has been obtained in highly purified form from several sources including Aspergillus oryzae (Mega, Ikenaka and Matsushima, 1970), the slime mold Dictyostelium discoideum (Every and Ashworth, 1973), Jack Bean meal (Li and Li, 1970), hen oviduct (Tarentino and Maley, 1971), human kidney (Srivastava et al, 1974) and human placenta (Lee and Yoshida, 1976).

Despite their presence in such widely differing organisms,  $\beta$ -hexosaminidases when viewed as a group possess several common characteristics. With one exception the enzyme is strictly specific towards the stereochemical configuration of the glycosidic bond, cleaving only linkages of the  $\beta$  type. The sole exception is an enzyme from the Limpet, Patella vulgata, which hydrolyses both  $\alpha$  and  $\beta$  N-acetylhexosaminides (Phizackerley and Bannister, 1974). Another parameter which is common to these enzymes is their absolute specificity towards the hexosamine sugar. Only  $\beta$ -glycosides of N-acetylglucosamine or N-acetylgalactosamine, or their synthetic acyl and halogen analogues, are successfully cleaved. Moreover, in the vast majority of cases,  $\beta$ -hexosaminidases possess activity towards both of the above

amino sugars, with  $\beta$ -glycosides of the former sugar generally being hydrolysed more rapidly than corresponding glycosides of the latter. Occasionally, however,  $\beta$ -hexosaminidases have been found which discriminate between the two sugars and are specific for only one configuration of the fourth carbon of the sugar ring. Such enzymes have been described in bacteria (Berkeley *et al.*, 1973), human and calf brain (Hooghwinkel *et al.*, 1972a; Frohwein and Gatt, 1967) and other human tissues (Poenaru and Dreyfus, 1973). As a general rule, the aglycone moiety, especially of simple alkyl and aryl  $\beta$ -N-acetylhexosaminides, is not a major determinant in influencing the course of  $\beta$ -hexosaminidase action, the specificity being dictated by the presence of the amino sugar.

$\beta$ -Hexosaminidases of higher animals are primarily of lysosomal origin, as are most of the acid glycosidases, the family of enzymes to which  $\beta$ -hexosaminidase belongs. By sequestering these potent, lytic enzymes within specialized organelles, the cell is spared from a generalized autolytic destruction. This lysosomal localization of  $\beta$ -hexosaminidase was originally established by the subcellular fractionation work of de Duve and his colleagues with rat liver (Sellinger *et al.*, 1960) and subsequently extended and confirmed by other workers in a variety of tissues, notably rabbit liver (Mellor *et al.*, 1973), human spleen (Robinson and Stirling, 1968), rat kidney (Price and Dance, 1967) and rat nerve cell (Sellinger *et al.*, 1973). In all these tissues,  $\beta$ -hexosaminidase displays the latent activity which is a prominent characteristic of enzymes encapsulated by lysosomes. Occasionally, deviations from the distribution pattern of acid phosphatase, the archetypal lysosomal enzyme, are seen.

These may arise from the heterogeneity of the lysosomal population itself or from the differing degrees of latency exhibited by various lysosomal enzymes. Milson and Wynn (1973) have obtained evidence of lysosomal heterogeneity using a homogeneous population of cultured hamster fibroblasts. They found two populations of lysosomes with different densities after centrifuging a disrupted preparation of cultured cells. In addition, the acid hydrolase profiles varied between the two lysosomal populations. Baccino and Zuretti (1971) have shown that rat liver  $\beta$ -hexosaminidase binds non-specifically, but tenaciously to particulate matter from disrupted cells and that its distribution pattern after centrifugation can vary considerably with the conditions used to disrupt the cells. Despite instances of atypical intracellular distribution, it is well established that the major intracellular population of  $\beta$ -hexosaminidase resides within the lysosome, although there is room for speculation that minor, non-lysosomal populations may also be present within the cell. With bacteria (procaryotes) such considerations do not apply and in many cases  $\beta$ -hexosaminidase is actually extruded into the surrounding environment, probably to function as a digestive enzyme.

A common characteristic of  $\beta$ -hexosaminidases is their susceptibility to inhibition by the aldonolactones of N-acetylglucosamine and N-acetylgalactosamine. This is really a specific case of the more general observation that all  $\beta$ -glycosidases are specifically and competitively inhibited by aldonolactones (and in some cases saccharolactones) corresponding in configuration to the sugar which is normally hydrolysed. In the case of  $\beta$ -hexosaminidase Couling and Goodey (1970) have ascertained that it

is the 1-5 rather than the 1-4 lactones of N-acetylhexosaminic acid which function as the inhibitory agents. These lactones have been useful not only in unravelling the mechanism by which  $\beta$ -hexosaminidase effects cleavage of the  $\beta$ -glycosidic bond, but also in investigations to determine whether the hydrolytic activity of an enzyme preparation towards both  $\beta$ -N-acetylglucosaminides and  $\beta$ -N-acetylgalactosaminides is due to one or two enzymes. If both activities are diminished to the same extent by either of the lactones, then one enzyme would be implicated; a different effect on the two activities would be evidence for the presence of two enzymes.

$\beta$ -Hexosaminidases are also generally inhibited by free N-acetylhexosamine, one product of the enzyme reaction, and by acetamide and acetic acid. Often inhibition is seen with heavy metals and the sulfhydryl reagent, p-chloromercuribenzoate. Lai and Axelrod (1973) have suggested that exo-glycosidases may be universally inhibited by the 1-amino glycosides of their specific sugars and Koshy, Robinson and Stirling (1975) have produced evidence for such an inhibition with  $\beta$ -hexosaminidase. In a recent investigation, Shulman, Lakhtina and Khorlin (1977) have obtained specific and irreversible inhibition of human and boar  $\beta$ -hexosaminidases with 2-acetamido-2-deoxy- $\beta$ -D-glucopyranosyl isothiocyanate and it appears likely that this compound will also inhibit the enzyme from other sources.

It is believed that all  $\beta$ -hexosaminidases have a common reaction mechanism. In all probability fission of the  $\beta$ -glycosidic linkage occurs between the glycosyl-oxygen bond, as is the case with other exoglycosidases (Levy and Snaith, 1972), and the endo-N-acetylhexosaminidase lysozyme (Rupley, 1967). The reaction proceeds by way of retention of the configuration at carbon-1 (Leback and Walker, 1967). Studies with lactone

inhibitors have suggested that the pyranose ring of the amino sugar is distorted into a half-chair configuration as it enters the transition state in the enzyme-substrate complex (Leaback, 1968). The extremely potent and competitive inhibition displayed by lactones is felt to arise in large measure from the mimicry of their energetically favoured, half-chair configuration for the transition state configuration of the amino sugar. Further considerations of lactone inhibition along with observations on the effect of pH upon kinetic parameters and the activity of  $\beta$ -hexosaminidase with a number of synthetic analogues of simple alkyl and aryl  $\beta$ -N-acetylhexosaminides (Yamamoto, 1973, 1974b; Mega *et al*, 1973) have pointed to a reaction sequence which is remarkably similar to that of lysozyme. The pyranoside linkage is hydrolysed by a concerted acid - nucleophile catalytic mechanism proceeding via a glycosyl carbonium ion. Both the carbonyl and nitrogen of the acetamide group interact to stabilize the transient carbonium ion with an oxygen nucleophile (Yamamoto, 1974a, 1974b).

## 2) Natural Substrates of $\beta$ -Hexosaminidase

For the most part,  $\beta$ -hexosaminidase activity has been identified and monitored with chemically synthesized  $\beta$ -N-acetylhexosaminides of chromogenic (p-nitrophenol) or fluorogenic (4-methyl umbelliferone) aglycones which do not occur naturally in animals. While convenient, the use of such compounds has contributed little to knowledge about the natural substrates of the enzyme and therefore, of the physiological role that  $\beta$ -hexosaminidase might play *in vivo*. However, it is well known that many naturally occurring compounds do contain N-acetylhexosamine residues bound in  $\beta$ -glycosidic linkage, and it is to these compounds that investi-

gators have turned in their attempts to delineate the impact this enzyme exerts within the living organism. These compounds include mucopolysaccharides or glycosaminoglycans, glycolipids, glycopeptides, and blood group substances and other glycoproteins. It is not difficult to understand why, in most cases, these compounds have not been favoured for the routine assay of  $\beta$ -hexosaminidase. They are often difficult to obtain in purified form and in the majority of instances must be partially degraded to expose a terminal N-acetylhexosamine residue. Furthermore, these compounds are often hydrolysed by  $\beta$ -hexosaminidase at a lower rate than the simple artificial substrates. Despite these difficulties, in vitro studies with these compounds have suggested that  $\beta$ -hexosaminidase, in conjunction with other glycosidases, does participate in their degradation in vivo.

Evidence of such action has been obtained with chitin, a linear polymer of  $\beta$ -1,4 linked N-acetylglucosamine residues, which is second only to cellulose in being the most abundant polysaccharide in nature. Several authors have noted that chitinase, an endo-N-acetylhexosaminidase which attacks and randomly cleaves the long chitin chain into smaller fragments, is invariably found associated with  $\beta$ -hexosaminidase (Powning and Irzykiewicz, 1964). Since the end products of chitinase action are the di-, tri- and tetrasaccharides of N-acetylglucosamine, it is felt that  $\beta$ -hexosaminidase hydrolyses these compounds to monosaccharides and thus plays a role in the final stages of chitin degradation. In vitro studies lend some support to this assumption. Thus Reyes and Byrde (1973) were able to demonstrate  $\beta$ -hexosaminidase hydrolytic activity with N,N'-diacetylchitobiose using a fungal preparation of the enzyme, while other workers have reported similar findings with the enzyme from other sources (Powning and Irzykiewicz,

wicz, 1964; Tarentino and Maley, 1971). Berkeley et al (1973) have reported the hydrolysis of tri- to pentasaccharide breakdown products of chitin by a bacterial  $\beta$ -hexosaminidase.

$\beta$ -Hexosaminidase is believed to function in the final stages of the degradation of mucopolysaccharides such as hyaluronic acid and chondroitin sulfate in much the same manner as seen in the chitinase system. Once again, the presence of endoglycosidases such as hyaluronidase suggests that  $\beta$ -hexosaminidase exerts its action on the smaller breakdown fragments. Since these mucopolysaccharides are heteropolymers composed of more than one type of sugar,  $\beta$ -hexosaminidase must act in concert with other exo-glycosidases to effect any significant decrease in chain length. Linker, Meyer and Weissmann (1955) were the first to demonstrate this using oligosaccharide breakdown products of hyaluronic acid produced by hyaluronidase action. They showed that these oligosaccharides were susceptible to further hydrolysis by the combined action of  $\beta$ -hexosaminidase and  $\beta$ -glucuronidase. Since then,  $\beta$ -hexosaminidase has specifically been shown to cleave N-acetylglucosamine from trisaccharides of hyaluronic acid (Weissmann, Hadjuanonous and Tornheim, 1964; Werries, Neue and Buddecke, 1975) and to act on oligosaccharides derived from chondroitin 4-sulfate (Buddecke and Werries, 1964; Thompson et al, 1973).

Turning to other types of compounds such as glycoproteins, glycopeptides and glycolipids, it is likely that  $\beta$ -hexosaminidase also participates in the turnover and degradation of their complex saccharide chains. In general, these compounds possess highly individualistic arrays of sugar residues which are fashioned by glycosyl

transferases of narrow specificity. N-Acetylhexosamine residues, when present, are often buried deep within the saccharide chain and either endo-glycosidases or other exo-glycosidases must first act to expose them for  $\beta$ -hexosaminidase attack. Such is the case for the  $\alpha_1$ -acidic glycoprotein of human plasma;  $\beta$ -hexosaminidase releases N-acetylhexosamine only after sialic acid and galactose residues are removed (Hughes and Jeanloz, 1964).  $\beta$ -Hexosaminidase has also been shown to act on ovomucoid (Caygill and Jevons, 1964), IgG glycopeptides (Koide and Muramatsu, 1974) and an asparaginyl-glycosyl fragment of ovalbumin (Tarentino and Maley, 1971; Li and Li, 1970), as well as on several complex oligosaccharides from milk (Yamashita, Tachibana and Kobata, 1976).  $\beta$ -Hexosaminidase is definitely known to play a role in the degradation of sphingolipids in vivo (see section C.5. on Pathological Conditions and  $\beta$ -Hexosaminidase).

What becomes increasingly clear from the above studies is that any compound with an N-acetylhexosamine residue in  $\beta$ -glycosidic linkage can serve as a potential substrate for  $\beta$ -hexosaminidase. The question which remains largely unanswered, however, is the actual extent of the participation of this enzyme in the degradation of these compounds in vivo.

### 3) Multiple Forms of $\beta$ -Hexosaminidase - Occurrence in Nature; Genetic and Physical Relationship

During the early work on  $\beta$ -hexosaminidase, there was little indication that the enzyme might be present in multiple forms in tissues of higher animals. Research was generally directed at determining the total  $\beta$ -hexosaminidase activity of a tissue, its

variation in various hormonal and clinical states or its subcellular distribution. More recent efforts, however, have been concerned with the purification of the enzyme from its various sources. To this end methods which resolve proteins on the basis of size and charge have been employed and through these techniques it has become apparent that  $\beta$ -hexosaminidase is invariably present in the tissues of higher animals in several isoenzyme or multiple forms.

Robinson and Stirling (1968) were the first to isolate, from human spleen, two electrophoretically distinct forms of  $\beta$ -hexosaminidase which differed in charge. Since then, multiple enzyme forms have been observed in other human (Ikonne and Ellis, 1973; Sandhoff and Waessle, 1971; Dance et al, 1969) and animal (Yoshikawa, Koide and Yamanaka, 1972; Verpoorte, 1972; Bullock and Winchester, 1973) tissues. Starch gel and polyacrylamide gel electrophoreses as well as isoelectric focussing have been used to separate the enzyme forms. Generally  $\beta$ -hexosaminidase components have grouped into two main fractions, an acidic fraction with relatively acidic isoelectric points (pH 4.0-5.5) and a more basic fraction with isoelectric points in the neutral or slightly basic region. High resolution techniques have revealed that several closely related enzyme forms are often present within each of the two main populations (Hayase, Reisher and Miller, 1972, 1973; Vikha, Kaverneva and Khorlin, 1971), and that the electrophoretic pattern often varies slightly from organ to organ (Winchester, 1971; Srivastava et al, 1974; Harzer and Sandhoff, 1971). Several minor forms of the enzyme have also been found to exist alongside the major forms. A significantly more acidic form of the enzyme (since termed Hex S) has been described in human tissue

(Beutler, Kuhl and Comings, 1975; Ikonne, Rattazzi and Desnick, 1975), while a form of  $\beta$ -hexosaminidase (Hex C) with a different pH optimum, size and substrate specificity from other forms has been found in several mammals (Poenaru and Dreyfus, 1973; Overdijk et al, 1975), particularly in brain (Hooghwinkel et al, 1972).

Except for Hex C which is immunologically distinct (Braidman et al, 1974) and under separate genetic control, the multiple forms of  $\beta$ -hexosaminidase are genetically related and display many similar properties. They are of similar size and show remarkably similar specificities and kinetic properties with simple alkyl and aryl  $\beta$ -hexosaminide substrates (Carroll and Robinson, 1972; Sandhoff and Waessle, 1971; Robinson and Stirling, 1968; Verpoorte, 1972). Basic enzyme forms, however, are stable over a wider pH range than are acidic forms, and are less easily denatured by heat (Dance, Price and Robinson, 1970; O'Brien et al, 1970a; Robinson and Stirling, 1968). Immunological studies with antibodies specifically raised against acidic and basic enzyme forms have revealed that the basic species (Hex B) contain all the antigenic determinants of the acidic species (Hex A). Hex A, however, only partially cross-reacts with anti-Hex B antibodies and, therefore, contains some unique antigenic determinants (Srivastava and Beutler, 1972; Bartholomew and Rattazzi, 1974). Enzyme forms have also been shown to be closely related by physical studies. Hex A has been observed to convert to more basic enzyme species upon storage, rechromatography on DEAE-Cellulose and controlled heating (Price and Dance, 1972; Hayes and Kritchevsky, 1973; Tallman et al, 1974; Khawaja and Sellinger, 1976).

At first it appeared that the basic enzyme species might be derived from Hex A by the partial or complete removal of sialic acid since crude extracts of bacterial neuraminidase were observed to cause Hex A to shift to more basic enzyme forms (Robinson and Stirling, 1968; Dance et al, 1969). However, further work with highly purified neuraminidase did not reveal any Hex A to Hex B shift (Kint and Huys, 1973; Carmody and Rattazzi, 1974) while amino acid analysis indicated that multiple forms differed in their polypeptide composition (Verpoorte, 1972; Lee and Yoshida, 1976). This last finding pointed to the inadequacy of a precursor-product mechanism in explaining the interconversion of enzyme forms.

The genetic and physical basis behind the proliferation of multiple enzyme forms has recently been worked out for the human enzyme. Heterokaryon somatic cell hybrid experiments (Lally, Rattazzi and Shows, 1974; Chern et al, 1976) have shown that two genes code for  $\beta$ -hexosaminidase. Hex B is exclusively coded by one gene on chromosome 5 while Hex A requires this same gene and an additional one on chromosome 15 in order to be expressed. Two polypeptide chains, therefore, are implicated in Hex A genesis, and one polypeptide chain, also common to Hex A, in Hex B formation. This 'unique-common' subunit arrangement has been confirmed by experiments which have explored the interconversion of Hex A and Hex B. Beutler and Kuhl (1975) have shown that under a variety of disaggregating conditions Hex B never rearranges to form Hex A. Hex A, however, is fully capable of rearranging to produce not only Hex B (exclusively composed of one type of Hex A subunit) but also the minor enzyme form Hex S (composed solely of the other type of Hex A subunit). As would be expected, Hex S is immunologically distinct from Hex B (Beutler et al, 1975). The

number and size of the subunits present in Hex A, Hex B and Hex S have also been studied. Although some confusion still persists as to whether human enzyme forms possess a tetrameric (Beutler et al, 1976; Geiger and Arnon, 1976) or hexameric structure (Srivastava, Wiktorowicz and Awasthi, 1976), the bulk of the evidence indicates that they are tetramers. If the two types of subunits are designated  $\alpha$  and  $\beta$ , then Hex B most likely has a  $\beta_4$  structure, Hex S an  $\alpha_4$  arrangement and Hex A a hybrid  $\alpha_2\beta_2$  composition (Beutler et al, 1976; Geiger and Arnon, 1976).

#### 4) Multiple Forms of $\beta$ -Hexosaminidase - Physiological Significance

The physiological significance of individual enzyme isomers is, to a large extent, still unknown. In general, absolute differences in substrate specificity have not been found among enzyme forms in vitro, and this has made it difficult to assess the relative impact which individual enzyme species exert on N-acetylhexosamine metabolism in vivo.

Both Thompson et al (1973) and Werries et al (1975) have investigated the hydrolytic activity of Hex A and Hex B with mucopolysaccharide breakdown products in vitro. Using short oligosaccharide chains derived from hyaluronic acid and chondroitin 4-sulfate, they found that the terminal N-acetylhexosamine residue was cleaved much more rapidly by Hex A than by Hex B. While this has suggested that Hex A may play the major role in mucopolysaccharide catabolism, other studies have pointed to the importance of Hex B in the degradation of these compounds in vivo. Cantz, O'Brien and Kresse (1975) have shown that while mucopolysaccharides are excessively accumulated in fibroblasts of patients afflicted with

Sandhoffs disease (Hex A and Hex B absent), mucopolysaccharide storage in fibroblasts is normal in patients stricken with Tay-Sachs disease (Hex A absent, Hex B present). Hex B, therefore, would appear to play at least some role in the degradation of mucopolysaccharides in vivo. Neither Hex A nor Hex B, however, is absolutely essential for the in vivo catabolism of mucopolysaccharides containing N-acetylhexosamine outside of the fibroblast. Several studies have shown that mucopolysaccharides are not accumulated in either the liver or spleen, or excessively excreted in the urine of patients where Hex A or Hex A and Hex B are absent (Applegarth and Bozoian, 1972; Strecker and Montreuil, 1971; O'Brien et al, 1970b).

Wenger, Okada and O'Brien (1972) have suggested on the basis of in vitro studies that Hex B may be primarily responsible for the hydrolysis of globoside in vivo. They found that Hex B hydrolysed globoside with a significantly lower Michaelis constant than did Hex A. They also noted that globoside accumulates in the viscera only in Sandhoffs disease and not in Tay-Sachs disease. A similar disease-related accumulation has been found for several related oligosaccharides containing N-acetylglucosamine and mannose in the liver and brain (Ng Ying Kin and Wolfe, 1974; Tsay and Dawson, 1976), suggesting that Hex B alone is fully capable of effecting the catabolism of these sugar chains in vivo.

Hex A is the only form of  $\beta$ -hexosaminidase which is capable of hydrolysing the terminal N-acetylgalactosamine moiety from Tay-Sachs ganglioside (GM<sub>2</sub>). This has been demonstrated in vitro in several laboratories (Sandhoff, 1970; Li et al, 1973; Bach and Suzuki, 1975) and in vivo by the massive accumulation of GM<sub>2</sub> in Tay-Sachs brain where Hex A, but not

Hex B, is absent. It is the buildup of this compound which leads to the lethal symptoms associated with this disorder. Further degradation of the ganglioside by other exo-glycosidases is prevented by the presence of N-acetylgalactosamine at the non-reducing terminal. Several studies have shown that a relatively small (36,000MW), heat stable, protein ''activating'' factor is required by Hex A for maximum GM<sub>2</sub> ganglioside hydrolase activity in vitro (Hechtman, 1977; Li et al, 1974).

Except for the studies cited above on Tay-Sachs ganglioside, neither Hex A nor Hex B has as yet been conclusively shown to act to the mutual exclusion of the other form in the catabolism of naturally-occurring substrates. It is highly unlikely, however, that several isoenzyme forms would have evolved in higher animals unless each was involved in distinct tasks in vivo. Much of the problem in delineating precise roles for Hex A and Hex B in vivo has stemmed from the fact that Hex A is never present in the body when Hex B is absent; thus it has been easy to show that the presence of Hex B is sufficient for the in vivo catabolism of a compound, but extremely difficult to show that the presence of Hex B is necessary for the same catabolism. It is to be expected that future research will increasingly concentrate on determining the in vivo turnover rates of naturally-occurring substrates of  $\beta$ -hexosaminidase in healthy and  $\beta$ -hexosaminidase-deficient people in order to surmount this problem.

##### 5) $\beta$ -Hexosaminidase and Pathological Conditions

Several clinical disorders are characterized by altered  $\beta$ -hexosaminidase isoenzyme patterns and properties, as well as changes in the

total activity of this enzyme. Since these deviations from the norm are usually found to differ characteristically with each disorder, they serve a valuable diagnostic function, both in the original identification of the disease and in the subsequent monitoring of the pathological symptoms. In some cases, investigations of the altered state of  $\beta$ -hexosaminidase have led to a greater understanding of the metabolic defects associated with these disorders and have pointed to possible routes for treatment.

Undoubtedly, the best examples of an appreciation of the underlying  $\beta$ -hexosaminidase defects resulting in clinical applications are represented by the two related disorders, Tay-Sachs syndrome and Sandhoff's disease. Both of these diseases are classed as sphingolipidoses and spring from inborn errors of sphingolipid (ganglioside) metabolism. The primary defect in Tay-Sachs disease is attributed to a point mutation in the structural gene coding for the  $\alpha$  subunits of  $\beta$ -hexosaminidase. Thus Hex A and Hex S are absent throughout the body (Sandhoff, 1969; Okada and O'Brien, 1969). With the related disorder, Sandhoff's disease, the structural gene coding for the  $\beta$  subunit is defective, leading to the total absence of both Hex A and Hex B (Sandhoff, Andreae and Jatzkewitz, 1968; Suzuki et al, 1971), although residual amounts of Hex S and Hex C are still present throughout the tissues of the body. Both of these diseases are manifested as progressive, fatal disorders of the central nervous system characterized clinically by progressive mental and motor deterioration, blindness, paralysis and dementia. Symptoms appear within the first six months after birth with death occurring no later than three years of age (O'Brien, 1973a). Biochemically, Tay-Sachs disease is characterized by a massive accumulation of GM<sub>2</sub> gang-

lioside (GalNAc- $\beta$ -1,4 - (NeuNAc- $\beta$ -2,3) - Gal- $\beta$ -1,4-Glc- $\beta$ -1,1 - (2-N-acyl) sphingosine) in neural tissue (the brain) (Svennerholm, 1962; Ledeen and Salzman, 1965). It is the accumulation of this ganglioside which results in the disruption of neural function and leads to the fatal symptoms. Even greater amounts of GM<sub>2</sub> ganglioside along with lesser amounts of its asialo derivative are accumulated in the brain of Sandhoff's disease patients. Visceral storage of asialo GM<sub>2</sub> as well as globoside (GalNAc- $\beta$ -1,3-Gal- $\beta$ -1,4-Gal- $\beta$ -1,4-Glc- $\beta$ -1,1- (2-N-acyl) sphingosine) is also characteristic as is some demyelination of axonal membranes (Suzuki et al, 1971; Snyder, Krivit and Sweeley, 1972).

Tay-Sachs disease is inherited as an autosomal, recessive genetic trait. Because of this, the disease is fatal only in homozygous carriers of the genetic defect; heterozygotes function normally, although the levels of Hex A relative to Hex B are reduced throughout the body. Several quick and inexpensive screening tests have been developed to identify heterozygotes who carry the defective  $\alpha$ -structural gene. A small blood sample is taken and the relative amounts of Hex A and Hex B determined by exploiting the different thermostability of the two isoenzymes or by taking advantage of their different ionic properties (O'Brien et al, 1970a; Suzuki, Berman and Suzuki, 1971; Dance et al, 1970). Since the Tay-Sachs defect is known to be concentrated in certain high risk populations, namely Jews of Ashkenazic origin, the screening tests have been applied most extensively to this group. Couples who both carry the genetic defect have been identified prior to having offspring and apprised by genetic counselling of the risk of having defective children (1 in 4). The

fetus of an at risk couple can be monitored throughout pregnancy for the presence of Hex A by the sampling of amniotic fluid which is of fetal origin.

At present, no specific therapy is known for victims of Tay-Sachs and Sandhoffs disease. Enzyme replacement therapy has been attempted, but is still in a rudimentary stage of development. Hex A when administered intravenously to Sandhoffs patients is cleared from the circulation with a half-life of about 7.5 minutes and is primarily concentrated in the liver (Brady, Pentchev and Gal, 1975; see also Stahl, Rodman and Schlesinger, 1976). Although accumulated ganglioside in the viscera is degraded to some extent by such injections, the enzyme is unable to cross the blood-brain barrier to effect hydrolysis of the large accumulation of GM<sub>2</sub> ganglioside stored within neural cells. Attempts to infuse Hex A into the circulation of children suffering from Tay-Sachs disease have also been unsuccessful in reducing GM<sub>2</sub> levels in the brain (O'Brien, 1973b). Brady et al (1975) have suggested several methods by which the enzyme might be delivered to the brain, but these ideas still require experimental work. Geiger, Von Specht and Arnon (1977) have recently shown that the covalent attachment of poly (N-vinylpyrrolidone) to Hex A considerably lengthens the half-life of exogenously administered Hex A in the circulation.

Increased serum levels of  $\beta$ -hexosaminidase have been found to occur in humans afflicted with diabetes mellitus (Woplen and Turner, 1965; O'Brien et al, 1970a). Working with streptozotocin-induced diabetic rats, Fushima and Torui (1974) have reported that raised levels of  $\beta$ -hexosaminidase in the serum occur in concert with de-

creased levels in the kidney. Furthermore, both levels return to normal upon injection of insulin. It is interesting to note in passing that free N-acetylglucosamine (the end product of  $\beta$ -hexosaminidase action) elicits a rapid rise in the plasma insulin concentration when injected into living rats and that, in vitro, the biosynthesis of pro-insulin from isolated islets of Langerhans is stimulated by this sugar (Ashcroft, Crossley and Crossley, 1976). The decreased levels of  $\beta$ -hexosaminidase in the kidney are of particular significance since diabetes in man is often accompanied and complicated by kidney microangiopathy, a disorder of the small blood vessels resulting from the deposition of glycoproteins and the thickening of the basement membrane. The decreased levels of  $\beta$ -hexosaminidase along with other glycosidases are assumed to lead to a reduced level of glycosaminoglycan and glycoprotein catabolism, and hence are viewed as contributing factors in the pathogenesis of microangiopathy.

With atherosclerosis, as in diabetes, enhanced levels of serum  $\beta$ -hexosaminidase activity are found. Belfiore et al (1974) have speculated that this altered level may be a manifestation of an increased extracellular secretion of  $\beta$ -hexosaminidase by the cells of the arterial walls, which occurs in response to the accumulation of mucopolysaccharides that takes place in the early stages of the disease. Serum  $\beta$ -hexosaminidase levels are also enhanced in various inflammatory disorders of muscle, connective tissue and joints such as polymyositis, systemic lupus erythematosus and rheumatoid arthritis (Kar and Pearson, 1972, 1973). In this last condition, it is also known that the amount of  $\beta$ -hexosaminidase is increased in the synovial membrane and fluid which surround the affected joints

(Hendry and Carr, 1963; Caygill and Pitkeathly, 1966). Although the exact role of  $\beta$ -hexosaminidase (and other lysosomal enzymes) in the etiology of these inflammatory disorders is unknown, it is suspected that increased extracellular levels of these enzymes are involved in the pathogenesis of these diseases and may result from an abnormal fragility of lysosomes present in the inflamed tissues.

6)  $\beta$ -Hexosaminidase - Variation with Age and Hormonal Status

Serum levels of  $\beta$ -hexosaminidase are known to rise progressively in women along with  $\beta$ -glucuronidase during pregnancy, and fall rapidly after parturition to normal values (Walker, Woolen and Pugh, 1960; Himaya et al, 1971). Similar findings have been reported in other mammals (Braidman and Robinson, 1973). This rise is derived in part from an enhanced extracellular secretion of Hex A into the blood but is also due, in large measure, to the presence of an entirely new, electrophoretically distinct form of  $\beta$ -hexosaminidase (Hex P) which is unique to the pregnant state (Stirling, 1972). A form with similar but not identical properties is seen in non-pregnant serum and in patients with tissue necroses such as hepatitis. Immunological evidence (Jones, Williams and Prochazka, 1975) has indicated that Hex P is of placental origin, and is derived from placental Hex B. At present, it is uncertain what role Hex P plays during the developmental process of pregnancy. While the overall increase of  $\beta$ -hexosaminidase in the serum in concert with  $\beta$ -glucuronidase might suggest an adaptation for increased mucopolysaccharide catabolism, the need for a new form of  $\beta$ -hexosaminidase in this process is obscure. Layne and co-workers (personal communication)

have investigated whether pregnancy serum, due to its content of Hex P, might preferentially hydrolyse steroid  $\beta$ -hexosaminides, but were unable to identify any preferential activity.

In addition to the placenta,  $\beta$ -hexosaminidase activity is found to be under the control of sex hormones in a number of organs such as liver, epididymis and uterus (Conchie and Findlay, 1959). In all cases, these organs respond to either estrogens or androgens by undergoing cell proliferation. With an adequate supply of hormones,  $\beta$ -hexosaminidase activity is high, but following gonadectomy the levels of this enzyme are decreased. It thus appears that the activity of  $\beta$ -hexosaminidase is related to the degree of cell proliferation in progress in these organs. Similar results have been obtained with cancerous cells (Rampini, Buré and Mazière, 1975).

$\beta$ -Hexosaminidase also exhibits age-dependent variations. For instance, in rabbits in the first few weeks after birth,  $\beta$ -hexosaminidase follows a complex pattern of development which differs from organ to organ. In liver, for example,  $\beta$ -hexosaminidase activity, which is higher in newborn animals than in adults, continues to increase for the first two weeks after which it falls to adult levels. In the spleen  $\beta$ -hexosaminidase activity rises steadily until adult levels are attained by five weeks of age. The relative amount of each isoenzyme form in the tissues also varies during this developmental period. Braidman and Robinson (1973) have suggested that these changes are associated with developmental processes in early life, such as myelination in the brain and a change from a haemopoietic to a haemolytic function in the spleen. In addition, they noted that the drop of serum  $\beta$ -hexosaminidase activity coincides with the switchover of the young rabbit

from mother's milk to solid food, and suggested that  $\beta$ -hexosaminidase might be associated with the metabolism of oligosaccharides derived from maternal milk.

Several studies have also indicated age-dependent variations of  $\beta$ -hexosaminidase activity and isoenzyme forms in humans. Woolen and Turner (1965) have shown that the levels of  $\beta$ -hexosaminidase in the blood slowly increase with advancing age, while the relative activity of Hex C is greater in embryonic and infant tissues than in adult tissues (Poenaru and Dreyfus, 1973). Finally, Harzer and Sandhoff (1971) have described age-dependent variations of  $\beta$ -hexosaminidase in human brain.

D) CONJUGATION OF STEROIDS WITH N-ACETYLGLUCOSAMINE

1) Occurrence and Synthesis of Steroid N-Acetylglucosaminide Conjugates in Nature

Steroid  $\beta$ -N-acetylglucosaminides are not distributed widely in nature. To date, they have been isolated and identified in only two mammals, the rabbit and human. In all but one of the conjugates examined, estrogens have comprised the aglycone moiety.

In the human, the in vivo synthesis of five steroid  $\beta$ -N-acetylglucosaminide conjugates has been reported. Arcos and Lieberman (1967) showed that pregnenolone is partially excreted in the urine as the 3-sulfate-20N-acetylglucosaminide of 5-pregnene- $3\beta$ ,  $20\alpha$ -diol. The amino sugar is attached in  $\beta$ -glycosidic linkage to the 20-hydroxyl (Matsui and Fukushima, 1969). Levitz and co-workers working with  $15\alpha$ -hydroxyestrone and  $15\alpha$ -hydroxyestradiol- $17\beta$  found that both are excreted to a significant extent in the bile and urine as their 3-sulfo- $15\alpha$ -N-acetylglucosaminide double conjugates as well as their

15 $\alpha$ -N-acetylglucosaminide monoconjugates (Jirku and Levitz, 1969; Frey, Jirku and Levitz, 1971).

The conjugation of estrogens with N-acetylglucosamine is quantitatively far more important in the rabbit than in the human. In fact, estrogens are virtually entirely excreted into the urine as a double conjugate of 17 $\alpha$ -estradiol with N-acetylglucosamine attached in  $\beta$ -glycosidic linkage to the 17 $\alpha$ -hydroxyl and glucuronic acid joined through a  $\beta$ -glycosidic bond to the phenolic 3-hydroxyl. Layne, Sheth and Kirdani (1964) were the first to isolate and tentatively identify this compound in the urine of rabbits injected with estrone. Their finding generated considerable interest since this was the first demonstration of the coupling of a naturally occurring non-sugar aglycone with N-acetylglucosamine. Subsequent work established an identical mode of elimination for exogenously administered 17 $\beta$ -estradiol (Collins, Williams and Layne, 1967), and led to an unequivocal identification of the structure of the double conjugate (Layne, 1965; Guillam et al, 1974).

Two transferase activities are required for the synthesis of this double conjugate in the rabbit, an N-acetylglucosaminyl transferase activity and a glucuronyl transferase activity. Although both activities are bound to the microsomal membrane and have not as yet been physically separated, Labow, Williamson and Layne (1971, 1973) have shown that the two activities are affected differently by detergents, solvents, inhibitors and degradative enzymes, thereby indicating that each is a distinct enzyme. Both transferases are concentrated in the liver with only minor amounts being found in the kidney and intestine (Collins et al, 1968). The liver, therefore, appears to be the princi-

pal site of formation of the double conjugate in vivo.

Studies with rabbit liver homogenates and washed microsomal preparations have established that the double conjugate is synthesized in vivo by the transfer of glucuronic acid and N-acetylglucosamine in an ordered, sequential manner (Jirku and Layne, 1965; Collins et al, 1968; Collins, Williamson and Layne, 1970). Glucuronic acid is first transferred from uridine diphosphate glucuronic acid to the phenolic hydroxyl of the free steroid, and then N-acetylglucosamine is transferred from its uridine diphosphate compound to the 17 $\alpha$ -hydroxyl group. This ordered mechanism is a consequence of the specificities of the two transferase enzymes. The glucuronyl transferase requires an unconjugated 17-hydroxyl group in order to effect transfer of glucuronic acid to the phenolic 3-hydroxyl of estrogens while the N-acetylglucosaminyl transferase will only transfer N-acetylglucosamine to estrogens which have undergone prior conjugation at the 3-hydroxyl with either glucuronic acid or sulfate. In the rabbit conjugation of phenolic steroids with sulfate is not normally found in vivo and the onus falls on the glucuronyl transferase to prepare the estrogen for subsequent conjugation with N-acetylglucosamine. It is only when preformed estrogen-3-sulfates are exogenously administered to the living rabbit or added to liver preparations in vitro (Collins and Layne, 1969) that sulfate can substitute for glucuronic acid in preparing the estrogen for double conjugate formation.

The N-acetylglucosaminyl transferase is also highly specific in its requirement for a 17 $\alpha$ -hydroxyl group to which it transfers N-acetylglucosamine. Because of this, estrogens must first be converted in

vivo to 17 $\alpha$ -hydroxyl derivatives prior to conjugation with N-acetylglucosamine. Hasnain and Williamson (1974, 1975, 1977) have shown that this is accomplished by 17 $\alpha$  and 17 $\beta$  steroid dehydrogenases present in the cytosol of rabbit liver. They found that these dehydrogenases are capable of converting both estrone and 17 $\beta$ -estradiol, either before or after glucuronidation of the phenolic 3-hydroxyl, to 17 $\alpha$ -estradiol.

Besides acting on 17 $\alpha$ -estradiol to form the 3-glucuronyl-17 $\alpha$ - $\beta$ -N-acetylglucosaminyl double conjugate, the transferases in rabbit liver have also been shown to act on a number of other estrogen metabolites not normally synthesized in vivo. Collins et al (1968) working with rabbit liver homogenates fortified with uridine diphosphate N-acetylglucosamine and uridine diphosphate glucuronic acid demonstrated that 17 $\beta$ -methyl-17 $\alpha$ -estradiol, 17-epiestriol and 16, 17-epiestriol are all converted to double conjugates with glucuronic acid at the 3 position and N-acetylglucosamine at the 17 $\alpha$  position. Double conjugates of both of the triols have also been recovered in the urine of rabbits injected intravenously with estriol (Collins and Layne, 1968). Collins and Layne (1969), using washed liver microsomal preparations, showed that 15 $\alpha$ -hydroxyestrone-3-sulfate is also a substrate for the N-acetylglucosaminyl transferase. With this compound, however, N-acetylglucosamine is transferred to the 15 $\alpha$ -hydroxyl rather than to the more usual 17 $\alpha$ -hydroxyl. In fact, this is the only recorded instance where the rabbit liver N-acetylglucosaminyl transferase has ever transferred N-acetylglucosamine to a hydroxyl group other than 17 $\alpha$ . 15 $\alpha$ -Hydroxyestrone-3-sulfate would not be excreted as its 15 $\alpha$ -N-acetylglucosaminyl double conjugate in vivo, however, since the steroid dehydrogenases in rab-

bit liver would act to convert the monosulfated diol to the 17 $\alpha$ -triol. The close proximity of the two  $\alpha$ -hydroxyl groups sterically prevents the transfer of N-acetylglucosamine to either group (Polakova et al, 1971).

2) Hydrolysis of Steroid  $\beta$ -N-Acetylglucosaminide Conjugates by  $\beta$ -Hexosaminidase in vivo and in vitro

The in vivo synthesis of estrogen  $\beta$ -N-acetylglucosaminides in the rabbit and human has led to speculation about the physiological significance of these conjugates. They undoubtedly serve as excretory forms of estrogen metabolites since they are found in the urine and bile. This does not preclude, however, the possibility that these conjugates might also function as transport forms of estrogens, to be taken up by tissues and deconjugated by  $\beta$ -hexosaminidase and other exoglycosidases or sulfatases to produce the free steroid. This would represent a potentially important physiological pathway for the distribution of estrogens to target tissues throughout the body.

Evidence exists from in vitro studies that  $\beta$ -hexosaminidase from several sources, including rabbit (Whittemore and Layne, 1965), human (Tomasi, Fukushima and Kolodny, 1974) and jack bean meal (Li, Li and Fukushima, 1971), can hydrolyse steroid  $\beta$ -N-acetylglucosaminide conjugates. Some evidence also exists that  $\beta$ -hexosaminidase hydrolyses these conjugates in vivo as well. Fukushima et al (1972) showed that testosterone 17- $\beta$ -N-acetylglucosaminide when administered orally to humans is metabolized and excreted in the urine in a form essentially free of N-acetylglucosamine. Similarly, Jirku, Kadner and Levitz (1974) reported that N-acetylglucosamine is cleaved from the 3-sulfo-15 $\alpha$ - $\beta$ -N-acetylglucosaminide double conjugate of 15 $\alpha$ -hydroxyestrone

after this compound was administered intraduodenally to humans. Preliminary work in this laboratory has shown that, in rabbits,  $\beta$ -hexosaminidase cleaves the amino sugar from  $17\alpha$ -estradiol  $17\beta$ -N-acetylglucosaminide when this compound is administered intravenously (Labow and co-workers, personal communication). In addition, the 3-glucuronyl moiety of  $17\alpha$ -estradiol is also partially cleaved in vivo when  $17\alpha$ -estradiol 3-glucuronide is injected intravenously (Williamson and Layne, 1970). Both sugars of the  $17\alpha$ -estradiol double conjugate are, therefore, susceptible to hydrolysis by exoglycosidases in vivo in the rabbit.

The work presented in this thesis is primarily involved with an examination of the hydrolytic activity of rabbit liver  $\beta$ -hexosaminidases in vitro with estrogen  $\beta$ -N-acetylglucosaminide conjugates. The multiple forms of the liver enzyme were purified to near homogeneity and the two main classes of enzyme isomers were characterized according to their physical properties, and their activity towards steroid  $\beta$ -N-acetylhexosaminides as compared to that towards other substrates.

CHAPTER 2

MATERIALS AND GENERAL METHODS

A) MATERIALS

Chemicals were obtained from the following companies as indicated:

Amersham/Searle Corporation (Arlington Heights, Illinois)

Tritiated estrone, estrone 3-sulfate (potassium salt) and D-glucosamine hydrochloride.

Analabs Inc., New England Nuclear (North Haven, Connecticut)

Silica Gel H.

British Drug House, Canada, Ltd. (Toronto, Ontario)

Amberlite XAD-2 resin, acetic anhydride.

Calbiochem (San Diego, California)

Glucosamine hydrochloride (A Grade), saccharo-1,4-lactone (A Grade).

Canadian Laboratory Supplies Ltd. (Canlab) (Ottawa, Ontario)

Silica Gel N, Silica Gel G, methanol (Baker-Spectranalyzed), silver carbonate, platinum oxide.

Central Oxygen Co. (Ottawa, Ontario)

Nitrogen

Eastman Kodak Co. (Rochester, New York)

Acrylamide, BIS-acrylamide, TEMED, 2-mercaptoethanol.

Fisher Scientific Co., Ltd. (Cornwall, Ontario)

Ferric ammonium sulfate, potassium cyanide, potassium ferricyanide, potassium tartrate, ammonium persulfate, calcium chloride, phosphorus pentoxide, magnesium sulfate (anhydrous), sodium tetraborate, cupric sulfate pentahydrate, sodium chloride, bromine, sodium metal, hydroquinone, glycine, p-nitrophenol, benzidine dihydrochloride, sodium hydroxide, trichloroacetic acid, sulfuric acid (37N and technical grade), glacial acetic acid, formic acid, hydrochloric acid, ethylene glycol monomethyl ether (Cellosolve), 1,4-dioxane, N,N-dimethylformamide, Bromphenol Blue, Biuret Reagent, Phenol Reagent (2N), decolorizing carbon (Norit, neutral), ampholytes (LKB Ampholine), all buffer salts and solvents not otherwise listed.

ICN/K and K Laboratories, Inc. (Plainview, New York)

m-phenylphenol (3-hydroxy-diphenyl)

Johns-Manville Products Corporation (Lompoc, California)

Celite Filter-Aid

Kent Laboratories Ltd. (Vancouver, British Columbia)

2,5-diphenyloxazole (scintillation grade).

Koch-Light Laboratories Ltd. (Colnbrook, England)

D-Glucurono-lactone, N-acetylglucosamino-lactone, N-acetylgalactosamino-lactone, p-nitrophenyl- $\alpha$ -N-acetylglucosaminide.

Mandel Scientific Co., Ltd. (Montreal, Quebec)

DE-52 DEAE-Cellulose (Whatman).

Matheson of Canada (Whitby, Ontario)

Hydrogen sulfide, hydrogen chloride.

Miles Laboratories Inc., Research Products Division (Elkhart, Indiana)

Horse spleen apoferritin.

New England Nuclear, Canada (Lachine, Quebec)

Aquasol-2.

Pharmacia Fine Chemicals AB (Montreal, Quebec)

All Sephadex gels, AH-Sepharose 4B, activated CH-Sepharose 4B, CNBr-activated Sepharose 4B, Concanavalin A Sepharose 4B, Blue Dextran 2000.

Pierce Chemical Co. (Rockford, Illinois)

Sodium dodecyl sulfate (SDS) (sequanal grade).

Rohm and Haas (Philadelphia, Pennsylvania)

Triton-X-100.

Schwarz/Mann (Orangeburg, New York)

Ammonium sulfate (ultra pure).

Sigma Chemical Co. (St. Louis, Missouri)

Glucuronic acid (Grade number 1), N-acetyl-D-glucosamine, N-acetyl-

D-galactosamine, D-glucono-1,5-lactone, D-galactono-1,4-lactone,  $\alpha$ -methyl-D-glucoside,  $\alpha$ -methyl-D-mannoside, sodium lauryl sulfate, 2-aminoethanol, bovine serum albumin, ovalbumin, riboflavin, 1-ethyl-3 (3-dimethyl-aminopropyl) - carbodiimide HCl, sucrose, TRIS, sodium deoxycholate, DEAE-Sephadex (A-25), estrone, estrone 3-sulfate (potassium salt), 17 $\alpha$ -estradiol, 17 $\beta$ -estradiol, estriol, Coomassie Brilliant Blue R, naphthol AS-BI-N-acetyl- $\beta$ -D-glucosaminide, naphthol AS-BI- $\beta$ -D-glucuronide, Fast Garnet GBC salt, chondroitin sulfate (mixed isomers), hyaluronic acid (Grade number 1), testicular hyaluronidase (type III), bovine liver  $\beta$ -glucuronidase (type B-1), phenolsulfatase (type V, Limpet), aldolase, chymotrypsin A, ribonuclease A, all p-nitrophenyl glycoside and sulfate compounds with the exception of the  $\alpha$ -N-acetylglucosaminide.

Steraloids, Inc. (Pawling, New York)

17-epiestriol, 16,17-epiestriol.

B) GENERAL METHODS

1) Preparation of Radioactive Steroid Substrates

a) Introductory Comments

Radioactively labelled diconjugates of  $17\alpha$ -estradiol with N-acetylglucosamine attached to the  $17\alpha$ -hydroxyl and glucuronic acid or sulfate attached to the phenolic 3-hydroxyl were obtained from the urine of rabbits previously injected with tritiated estrone or estrone 3-sulfate. These latter two compounds were available commercially. Monoconjugates were derived from diconjugates by selective hydrolysis of the unwanted sugar by an appropriate glycosidase. Further purification of the conjugates in the crude urinary preparation was achieved by chromatography on columns of Amberlite XAD-2 resin and Sephadex A-25, thin layer chromatography on silica gel and solvent extraction from aqueous media. The methods by which each of the radioactive steroids used in this work were isolated and purified are described in greater detail below.

b)  $17\alpha$ -(6,7- $^3\text{H}_2$ )-Estradiol 3- $\beta$ -glucuronyl-17- $\beta$ -N-acetylglucosaminide

The procedure employed in the preparation of this compound was broadly derived from that of Guillam et al (1974). About 5 mCi (specific activity 40-45 Ci per millimole) of (6,7- $^3\text{H}_2$ ) - estrone was injected into a virgin female New Zealand white rabbit via the marginal ear vein. The urine was collected for three days, combined and then processed on an Amberlite XAD-2 column as described by Bradlow (1968). The methanol eluate contained the desired radioactive double conjugate. The methanol was evaporated, the residue taken up in several ml of water and applied to a 2.5 cm x 32 cm column of Sephadex A-25. Following a 500 ml wash with aqueous 0.075 M NaCl, radioactivity was eluted over a 200 ml, linear NaCl gradient of 0.075 M to 0.3 M (Hobkirk and Nilsen, 1970). Fractions of 16 ml were

collected. The fractions containing the major peak of eluted radioactivity were pooled and the water evaporated by means of a flash evaporator. The residue was taken up in a small volume of methanol and centrifuged. The residue in the methanol supernatant was essentially free of NaCl and was evaporated to dryness. The residue was redissolved in 5 ml of water and reapplied to a smaller 1.5 cm x 25 cm column of Sephadex A-25. The column was washed with 200 ml of 0.05 M aqueous NaCl and the radioactivity was then eluted over a 700 ml gradient of 0.05 - 0.20 M NaCl. Fractions of 5 ml were collected. Fractions which contained the main radioactive peak were pooled and processed as described earlier for the first anion exchange column. The radioactive double conjugate in the final methanol extract was adjusted to a specific activity of 10,000 DPM per picomole by the addition of radioinert double conjugate. The radioactive compound was judged to be radiochemically pure by TLC against an authentic sample of double conjugate on thin layered plates of silica gel G in a chloroform: isopropyl alcohol: formic acid (5:3:1 by volume) solvent system. This system was known to effectively separate estrogen diglycosides from monoconjugates (Collins, Jirku and Layne, 1968). The radioinert standard was visualized by heating the plate to 130°C after spraying with 20% H<sub>2</sub>SO<sub>4</sub> in ethanol.

c) 17 $\alpha$ -(6,7-<sup>3</sup>H<sub>2</sub>)-Estradiol 17- $\beta$ -N-acetylglucosaminide

The initial steps in the preparation of this compound, i.e. injection of the rabbit with radioactive estrone, urine collection and purification on Amberlite XAD-2 resin, were performed as described previously for the double conjugate. Since the methanol eluant from the XAD column contain-

ed primarily the double conjugate, it was necessary to remove the glucuronyl moiety by treatment with  $\beta$ -glucuronidase. Thus the methanol fraction was evaporated to dryness, the residue redissolved in 0.1 M acetate buffer pH 5.0 and incubated overnight at 37°C with approximately 6000 Fishman units of bovine  $\beta$ -glucuronidase (Sigma) (Whittemore and Layne, 1965). At the end of this time period, the aqueous solution was first washed three times with equal volumes of benzene to remove any free steroid which might have been formed during the reaction, and then treated in an analogous manner with ethyl acetate to extract the desired monoconjugate. Intact double conjugate remained in the aqueous phase. The 17-N-acetylglucosaminyl monoconjugate in the ethyl acetate fraction was further purified by preparative thin layer chromatography. Thus ethyl acetate was evaporated, the residue taken up in a few drops of methanol and subsequently chromatographed on silica gel H in a 7:3 (V/V) chloroform: ethanol system (system A) (Collins, Jirku and Layne, 1968). This solvent system was particularly effective at separating 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide from residual amounts of contaminating 17 $\alpha$ -estradiol 3-glucuronide, 17 $\alpha$ -estradiol 17-glucoside and free estradiol, as well as from brown material present in the ethyl acetate fraction. The bulk of the radioactivity migrated with an Rf corresponding to that of authentic 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide. This compound was scraped from the plates and eluted from the silica gel with methanol. The methanol fraction was evaporated to dryness, the residue redissolved in several ml of water and the monoconjugate extracted into ethyl acetate in order to separate it from any residual silica gel which remained in the aqueous phase. The ethyl acetate was then evaporated and the residue taken up in metha-

nol. The specific activity of the purified compound was adjusted to 10,000 DPM per picomole by the addition of radioinert  $17\alpha$ -estradiol  $17\beta$ -N-acetylglucosaminide.

d)  $17\alpha$ -(6,7- $^3\text{H}_2$ )- Estradiol 3- $\beta$ -glucuronide

$17\alpha$ -(6,7- $^3\text{H}_2$ )- Estradiol 3-glucuronide was prepared by treating the radioactive double conjugate,  $17\alpha$ -estradiol 3-glucuronyl- $17\beta$ -N-acetylglucosaminide, present in the methanol extract from the XAD column with hyaluronidase to remove the N-acetylglucosamine (Layne, 1965). The glucuronyl monoconjugate was then extracted into ethyl acetate pH 2.0 and purified by TLC in methyl ethyl ketone: ethanol: benzene: water (3:3:3:1 by volume) (system B). The specific activity of the purified monoconjugate was adjusted to 10,000 DPM per picomole by the addition of radioinert  $17\alpha$ -estradiol 3-glucuronide.

e)  $17\alpha$ -(6,7- $^3\text{H}_2$ )- Estradiol 3-sulfate- $17\beta$ -N-acetylglucosaminide

This compound was prepared according to the procedure of Collins and Layne (1969). 250  $\mu\text{Ci}$  of the potassium salt of (6,7- $^3\text{H}_2$ )-estrone 3-sulfate (specific activity 1.1 Ci per millimole) was injected into the marginal ear vein of an immature female, New Zealand white rabbit. Radioactivity was collected in the urine and processed on XAD as described previously for the 3-glucuronyl double conjugate. The methanol extract from the XAD column contained primarily the 3-glucuronyl  $17\beta$ -N-acetylglucosaminyl double conjugate of  $17\alpha$ -(6,7- $^3\text{H}_2$ )-estradiol in conjunction with lesser amounts of  $17\alpha$ -(6,7- $^3\text{H}_2$ )-estradiol 3-sulfate- $17\beta$ -N-acetylglucosaminide. This fraction was evaporated to dryness and the residue dissolved in several ml of 0.1 M citrate buffer pH 4.5. Following washes with benzene and ethyl acetate pH 2.0, the aqueous phase was evaporated to dryness. The residue

was taken up in a small volume of methanol and spotted on a preparative thin layer plate of silica gel N. The plate was run in system B. The radioactivity which migrated with an Rf between 0.25 and 0.45 was scraped from the plate, eluted from the silica gel with methanol and chromatographed for a second time in system B. The final product was judged to be free from other radioactive compounds but was still contaminated with a small amount of brown coloured material.

Because there was no reference standard, the identity of the radioactive product could not be established directly as being  $17\alpha$ -estradiol 3-sulfate- $17\beta$ -N-acetylglucosaminide by comparison of Rf values obtained with the standard and unknown compound following TLC. Instead, an indirect identification was made by selectively treating aliquots of the purified compound with either phenolsulfatase or  $\beta$ -hexosaminidase, and then identifying the steroid products of the reaction with authentic standards. TLC in system A revealed that phenolsulfatase action produced a radioactive compound which co-migrated with  $17\alpha$ -estradiol- $17\beta$ -N-acetylglucosaminide, while incubation of the purified compound with  $\beta$ -hexosaminidase resulted in a radioactive compound ( $17\alpha$ -estradiol 3-sulfate) with an Rf which was very similar to that of estrone 3-sulfate in system B. As a result of these identifications, it could be accepted with confidence that the purified material was, in fact,  $17\alpha$ -estradiol 3-sulfate- $17\beta$ -N-acetylglucosaminide.

The phenolic 3-sulfate group of this compound was known to be relatively labile (Quamme, Layne and Williamson, 1971). Because of this, the double conjugate was always checked for loss of the sulfate group by TLC in system B before use in enzyme assays. Loss of the sulfate moiety over a period of several months was negligible when the compound was stored in methanol at  $-20^{\circ}\text{C}$ .

## 2) Preparation of Non-Radioactive Steroid Substrates

Estrone 3- $\beta$ -N-acetylglucosaminide and 17 $\beta$ -estradiol 3- $\beta$ -N-acetylglucosaminide were synthesized in this laboratory by the improved Koenigs-Knorr procedure of Conrow and Bernstein (1971) (see also Layne et al, 1976).

## 3) Enzyme Assays

### a) Introductory Comments

The enzymatic activities of several glycosidases were monitored during the course of the work presented in this thesis. The greatest attention was focussed on  $\beta$ -hexosaminidase. Three substrates, 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide tritiated in the steroid moiety, p-nitrophenyl- $\beta$ -N-acetylglucosaminide and p-nitrophenyl- $\beta$ -N-acetylgalactosaminide, were routinely employed to assay  $\beta$ -hexosaminidase activity in crude and purified preparations of the enzyme. In all cases, the assay comprised measurement of the amount of free aglycone released by enzyme action. Furthermore, these three substrates, in conjunction with four additional estrogen conjugates, 17 $\alpha$ -(6,7- $^3\text{H}_2$ )-estradiol 3-glucuronyl-17- $\beta$ -N-acetylglucosaminide, 17 $\alpha$ -(6,7- $^3\text{H}_2$ )-estradiol 3-sulfate-17- $\beta$ -N-acetylglucosaminide, 17 $\beta$ -estradiol 3- $\beta$ -N-acetylglucosaminide and estrone 3- $\beta$ -N-acetylglucosaminide, were utilized to obtain a profile of the enzymatic characteristics of the various purified isoenzyme forms of  $\beta$ -hexosaminidase.  $\beta$ -Glucuronidase was assayed with two substrates -- p-nitrophenyl- $\beta$ -D-glucuronide and the glucuronyl monoconjugate, 17 $\alpha$ -(6,7- $^3\text{H}_2$ )-estradiol 3- $\beta$ -glucuronide. The presence of other glycosidases and phenolsulfatase at various stages during the purification of  $\beta$ -hexosaminidase was measured with the appropriate p-nitrophenyl glycoside or sulfate substrate.

b) Assays with p-Nitrophenyl Compounds

The activity of glycosidases has traditionally been monitored with p-nitrophenyl glycosides through a colorimetric procedure which measures the amount of liberated p-nitrophenol. In the present work, glycosidases as well as phenolsulfatase were assayed in 1 ml of aqueous buffer containing the appropriate p-nitrophenyl glycoside or sulfate. The buffer used for each enzyme assay was chosen from the literature on the basis of frequency of use. The pH and molarity of each buffer were adjusted to maximize enzyme activity in aliquots of the 100,000 xg supernatant of rabbit liver. Bovine serum albumin at a concentration of 0.01% was added to the assays for  $\beta$ -hexosaminidase to enhance enzyme activity (Verpoorte, 1972). Table I details the enzymes which were assayed with p-nitrophenyl compounds and specifies the composition of the reaction media.

The assay mixtures were routinely incubated for 1 hour at 37°C, at which time 1.5 ml of borate-NaOH buffer pH 10.5 (0.3 M in borate) were added to terminate the reaction and to impart basic conditions. Under these conditions (a pH of approximately 9.5), free p-nitrophenol (liberated from the parent glycoside or sulfate by both enzymatic and non-enzymatic hydrolysis) was present in its anion form and displayed a characteristic yellow colour. The amount of yellow colour present in the assay after the addition of base was quantitatively measured in a split beam Cary 15 spectrophotometer at 410 m $\mu$  with distilled water in the reference cell. Yellow colour due to non-enzymatic hydrolysis of the substrate and to coloured pigments present in the enzyme aliquot was determined with blank incubations lacking enzyme and substrate, respectively. Absorption values at 410 m $\mu$  which were obtained with the two blank incubations were then subtracted from the absorption value obtained with the complete assay to give a true

ENZYME	ASSAY BUFFER*	SUBSTRATE**
Phenolsulfatase	0.1 M Acetate pH 6.0	5 mM PNP-SO <sub>4</sub>
α-Glucosidase	0.1 M Acetate pH 4.5	2 mM PNP-α-glc
α-Galactosidase	0.1 M Acetate pH 4.5	2 mM PNP-α-gal
α-Mannosidase	Phosphate-citrate pH 4.5	2 mM PNP-α-man
α-N-Acetylglucosaminidase	0.05 M Citrate pH 5.0	2 mM PNP-α-NAcglc
β-Glucosidase	Phosphate-citrate pH 5.5	2 mM PNP-β-glc
β-Galactosidase	Phosphate-citrate pH 5.5	2 mM PNP-β-gal
β-Glucuronidase	0.2 M Acetate pH 4.4	2 mM PNP-β-gluc
β-Hexosaminidase	0.1 M Citrate pH 4.4	2.5 mM PNP-β-NAcglc
	0.1 M Citrate pH 4.1	2.0 mM PNP-β-NAcgal

\*Phosphate-citrate buffer was prepared according to McIlvaine (1921).

\*\*See Glossary for name of compound corresponding to abbreviated form written below.

Table I. p-Nitrophenyl Glycoside and Sulfate Assays

measure of the yellow colour due to enzyme action. Nanomoles of liberated p-nitrophenol were estimated from the corrected absorbance by comparison with a p-nitrophenol standard curve. With crude enzyme fractions, the acid pH of the reaction mixture resulted in the precipitation of protein which was not redissolved by the addition of base. In these cases, the yellow colour present in the assay was determined spectrophotometrically after removal of insoluble protein by centrifugation.

With all p-nitrophenyl assays, enzyme aliquots were adjusted to give less than 15% hydrolysis of the parent compound. The accuracy of assays with p-nitrophenyl- $\beta$ -N-acetylglucosaminide and p-nitrophenyl- $\beta$ -N-acetylgalactosaminide were tested through multiple analyses and estimated to be within the range of  $\pm 5\%$ . Assays on enzyme fractions with all substrates were performed in triplicate.

c) Assays with Steroid Glycosides

I) General Features

The measurement of the extent of hydrolysis of monoconjugates of estrone and estradiol by  $\beta$ -hexosaminidase and  $\beta$ -glucuronidase was based on the fact that intact monoconjugates were not extracted from aqueous media by benzene while free estrone and estradiol were partitioned nearly completely into benzene (Collins et al, 1968) under the same conditions. With diconjugates of estradiol, measurement of the production of monoconjugates from diconjugates by enzyme action was based on the fact that the former but not the latter were extracted by ethyl acetate pH 2.0 from aqueous media. Where estrogen glycosides were available with tritium in the steroid moiety, assays consisted of measuring the amount of radioactivity, originally added to the aqueous assay medium in the form of the intact glycoside, which was extracted into the organic phase (either benzene or ethyl acetate) from the aqueous assay medium following enzyme action. Organic extracts were

periodically checked to ensure that the radioactivity being measured corresponded to the appropriate free estrogen or monoconjugate. With non-radioactive estrogen glycosides, steroid compounds resulting from enzyme action were extracted into organic solvent as described above for the radioactive steroid assays, and then the amount of extracted steroid was quantitatively determined by a colorimetric procedure. Blank incubations without added enzyme were run with all radioactive and non-radioactive steroid assays.

Steroid glycosides were routinely added to the aqueous assay medium in 10  $\lambda$  to 20  $\lambda$  of methanol. Due to the limited quantity and low solubility of these steroid conjugates, assays were performed with levels of substrate less than those required to saturate the enzyme. Enzyme aliquots were adjusted to give less than 10% hydrolysis of the parent compound with radioactive monoconjugates, 15% with 17 $\alpha$ -estradiol 3-sulfate-17- $\beta$ -N-acetylglucosaminide, 20% with radioinert monoconjugates and 25% with 17 $\alpha$ -estradiol 3-glucuronyl-17- $\beta$ -N-acetylglucosaminide. Under these conditions, initial velocity levels were maintained for the duration of the assay. Assays with these substrates were performed in triplicate or quadruplicate when measuring hydrolytic activity in enzyme preparations. Single assay determinations were sufficient when monitoring enzyme activity in column effluent fractions.

The following sections describe in greater detail the conditions under which enzyme activity was assayed with steroid substrates.

## II) 17 $\alpha$ -(6,7-<sup>3</sup>H<sub>2</sub>)-Estradiol 17- $\beta$ -N-acetylglucosaminide

In routine assays of  $\beta$ -hexosaminidase with this substrate, 40 picomoles of 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide (specific activity 10,000 DPM/picomole; 398,000 DPM) were added in 20  $\lambda$  of methanol to 2.0 ml

of 0.1 M citrate buffer pH 4.4 containing 0.01% BSA. BSA was included since it acted to stimulate enzyme activity, especially that from purified enzyme preparations. The reaction was initiated by the addition of an appropriate aliquot of enzyme preparation. Incubations were carried out at 37°C for two hours with continuous shaking in a water bath, after which enzyme action was terminated by the addition of 2 ml of benzene to the aqueous assay medium with immediate shaking. The resulting protein-benzene emulsion was broken by centrifugation. Benzene (1 ml) was placed in an appropriate liquid scintillation cocktail and counted for radioactivity.

In kinetic and inhibition experiments, the concentration of 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide was varied as required by the addition of varying amount of radioinert compound to 40 picomoles of radioactive compound (specific activity 10,000 DPM per picomole).

III) 17 $\alpha$ -(6,7-<sup>3</sup>H<sub>2</sub>)-Estradiol 3- $\beta$ -Glucuronide

$\beta$ -Glucuronidase was assayed in 2 ml of 0.1 M acetate buffer pH 5.0 containing 0.01% BSA and 32 picomoles of 17 $\alpha$ -estradiol 3-glucuronide for 1 hour at 37°C by a similar procedure as described previously for the routine assay of  $\beta$ -hexosaminidase with 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide.

IV) 17 $\alpha$ -(6,7-<sup>3</sup>H<sub>2</sub>)-Estradiol 3- $\beta$ -glucuronyl-17- $\beta$ -N-acetylglucosaminide and 17 $\alpha$ -(6,7-<sup>3</sup>H<sub>2</sub>)-Estradiol 3-sulfate-17- $\beta$ -N-acetylglucosaminide

Assays with these substrates were performed solely with highly purified preparations of the isoenzyme forms of  $\beta$ -hexosaminidase. Both assays consisted of incubating the appropriate aliquot of enzyme fraction in 2 ml of 0.1 M citrate buffer pH 4.4 containing 200  $\mu$ g of BSA. In the case of the

3-glucuronyl double conjugate, substrate was varied from 9 nM to 50  $\mu$ M as required and assays were incubated at 37°C in a shaking water bath for 1 hour. With the 3-sulfo double conjugate, assays were performed with 40 picomoles (77,000 DPM) of substrate for 2 hours at 37°C. Following the time period allotted for incubation, the pH of the reaction media was lowered to 2.0 by the addition of 1 N HCl and the aqueous phase extracted with ethyl acetate. One ml of the 2 ml ethyl acetate extract was counted for liberated monoconjugate in assays with the 3-glucuronyl double conjugate and 2.5 ml of 3.0 ml counted in assays with the 3-sulfo compound.

Although the enzyme preparations incubated with these double conjugates were highly purified, they were, nevertheless, checked for  $\beta$ -glucuronidase and phenolsulfatase activity in order to confirm that the liberated monoconjugate extracted into the ethyl acetate phase in these assays resulted solely from  $\beta$ -hexosaminidase action and not, in part, from enzymatic removal of the three-phenolic group.  $\beta$ -Glucuronidase activity in these enzyme preparations was determined with 17 $\alpha$ -estradiol 3-glucuronide as described previously. Phenolsulfatase activity was monitored in the 3-sulfo double conjugate assay after first totally eliminating  $\beta$ -hexosaminidase activity (>99% as measured with identical aliquots of enzyme fraction in the routine assay with 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide) by the addition of suitable amounts of N-acetylgalactosamino-lactone to the assay medium. The presence of phenolsulfatase would be indicated by an increase of the extracted radioactivity over that obtained from a blank assay without enzyme fraction. No evidence was found to indicate the presence of either of these enzymes in the purified enzyme preparations.

Examination of the reaction products of the 3-sulfo diconjugate assay by TLC in system B revealed the presence of 17 $\alpha$ -estradiol 17- $\beta$ -N-acetyl-

glucosaminide in the organic phase of blank incubation extracts and 17 $\alpha$ -estradiol in those of the complete assay. Both of these compounds arose from the non-enzymatic removal of the sulfate group from the double conjugate and 17 $\alpha$ -estradiol 3-sulfate, respectively, under the conditions of the assay.

V) Estrone 3- $\beta$ -N-acetylglucosaminide and 17 $\beta$ -Estradiol 3- $\beta$ -N-acetylglucosaminide

As with the two radioactive double conjugate substrates, both of these radioinert substrates were employed solely to investigate the enzymatic properties of highly purified enzyme forms of  $\beta$ -hexosaminidase. Assays were performed in 2 ml of 0.1 M citrate buffer pH 4.4 containing 200 $\mu$ g of BSA and 24 to 121 nanomoles of substrate. Incubation was continued for 1 to 2 hours at which time the free steroid liberated from its 3-monoconjugate was extracted from the incubation medium into benzene. Two ml of the 2.5 ml benzene extract were evaporated to dryness and the amount of free steroid determined by the Kober reaction (Osawa and Slaunwhite Jr., 1970). The optical density of the final, coloured product was read at 530 m $\mu$  in a split beam Cary 15 Spectrophotometer with ethylene dichloride in the reference cell. Blank incubations without added enzyme were included with each set of assays. Nanomoles of liberated steroid were estimated (from the corrected absorbance at 530 m $\mu$ ) from standard curves of estrone and 17 $\beta$ -estradiol.

4) Radioactivity Measurements

Radioactivity was measured by liquid scintillation counting in a Mark III Nuclear Chicago spectrophotometer. Variable quench modes of counting were employed throughout. For samples labelled with either tritium or carbon-14, efficiency was determined by the channels ratio

method and quench correction curves were obtained with tritium and carbon-14 quench standards. For samples dually labelled with tritium and carbon-14, efficiency was determined by the external standard method. Non-polar samples were counted in 10 ml of a scintillation mixture containing 10 ml toluene, 0.4 mg 2,5-diphenyloxazole (PPO), while hydrophilic samples were counted in 10 ml of pure aquasol or 10 ml of aquasol diluted 1:1 with xylene.

## 5) Preparative Techniques

### a) Gel Filtration

Sephadex gels of the G-15, G-25, G-200 fine and G-200 superfine series were used in this work to separate substances of different size. Sephadex gels were swollen according to the manufacturer's instructions (Pharmacia Fine Chemicals). When preparing columns, the gel suspension was allowed to pack by gravity feed at hydrostatic pressures well within the suggested limits. Columns were preequilibrated by running through several column volumes of the same buffer to be used in eluting the sample from the gel. Whenever possible, samples were applied to columns through a flow adaptor. Care was taken to avoid overloading columns with sample. Downward gravity elution was used throughout. With columns of the more loosely crosslinked gel, Sephadex G-200, a constant pressure head was maintained throughout the run. This was achieved by using large buffer reservoirs and a slow rate of buffer flow through the column. This arrangement ensured that the difference in height between the top of the reservoir buffer and the outflow tube decreased by only a fraction of a centimeter over a 24 hour period and permitted a constant pressure differential to be maintained by adjustment of the height of the buffer reservoir every 24 hours. Effluent fractions of a predetermined size were collected

by employing an LKB Ultrorac fraction collector. Gel columns were packed and subsequently run at a temperature of 4°C.

b) Anion Exchange Chromatography

Preswollen DE-52 diethylaminoethylcellulose (DEAE-Cellulose) was purchased in the Cl<sup>-</sup> form. Since columns of this gel were to be eluted with phosphate buffer with phosphate as the counter ion, it was necessary to first change the gel to its phosphate form. This was done according to the manufacturer's instructions (Whatman Chemical Co.). Suspensions of the gel were degassed prior to packing into columns under 4 psi of nitrogen. Gel beds were preequilibrated with at least 5 column volumes of the phosphate buffer which was to be used to initially develop the column following sample application. Samples were dialysed against this same buffer before they were applied to the column and then, following application, were eluted from the gel with phosphate buffer of increasing molarity but constant pH. Linear gradients of phosphate buffer were delivered to the gel surface by means of a flow adaptor. Effluent fractions were collected with an LKB Ultrorac fraction collector.

DEAE-Cellulose anion exchange chromatography was performed at 4°C.

c) Group Affinity Chromatography

A gel with Concanavalin A, a plant lectin, coupled to Sepharose 4B was purchased from Pharmacia Fine Chemicals, and employed to separate glycoproteins from other substances. All steps in this technique were performed at room temperature. The preswollen gel was suspended in 0.1 M citrate buffer pH 4.0, degassed and packed into columns by gravity feed. Following preequilibration of the column with several column volumes of the suspending citrate buffer, the sample was run onto the gel and the column washed with the same citrate buffer used previously until protein as detected by absorbance at 280 m $\mu$  was no longer eluted. Glycoproteins

which had bound to the gel matrix were then dislodged and eluted from the column by the addition of 0.3 M methyl- $\alpha$ -D-glucose and 0.075 M methyl- $\alpha$ -D-mannose to the eluting citrate buffer.

6) Analytical Techniques

a) Polyacrylamide Disc Gel Electrophoresis

Separation and spacer gels were prepared and polymerized in round cylindrical glass support tubes as described by Davis (1964) except that the total polyacrylamide concentration in the separation gel equalled 5.5% (1% in BIS, 4.5% in acrylamide). Following polymerization, gel tubes were snugly fitted into the grommets of a double chambered electrophoresis apparatus (E.C. Chemical Corporation). Samples were prepared for electrophoresis in the following manner. In cases where the enzyme was dissolved in buffer of low molarity ( $<0.03$ ), an appropriate aliquot of the enzyme fraction was mixed in 1 ml test tubes with 1 $\lambda$  of 0.01% Bromphenol Blue and 1/8 to 1/4 volume of 8X to 4X concentrated spacer gel buffer (minus TEMED) containing 40% to 20% sucrose. Where the enzyme was present in buffers of higher molarity, the enzyme fraction was first dialysed against spacer gel buffer (minus TEMED) prior to the addition of sucrose to 5% and 1 $\lambda$  of 0.01% Bromphenol Blue to appropriate aliquots. The entire sample was then layered on top of the spacer gel and carefully covered with a layer of running buffer (3.0 g TRIS, 21.6 g glycine in 1500 mls, pH 8.8 at 4°C) which reached to the top of the gel tube. Running buffer was also added to both reservoir chambers to adequately cover the electrodes. The polarity of the electrodes was adjusted so that the anode was in the bottom chamber. Electrophoresis at 4°C was initially performed at 2 milliamperes per gel until the tracking dye, Bromphenol Blue, had entered the separation gel, and then continued

at 3 milliamperes per gel until the tracking dye had migrated the length of the separation gel. Following electrophoresis, gels were extruded from the supporting glass tubes by rimming them with a thin needle under water, and stained to identify either protein,  $\beta$ -hexosaminidase enzyme activity or  $\beta$ -glucuronidase enzyme activity.

Protein bands were visualized according to a published procedure (Bulletin AN 32, Ortec Inc. (1970)). Gels were fixed with trichloroacetic acid (12.5%), stained with Coomassie Brilliant Blue R-250 (0.2%), and destained with an aqueous solution of 25% ethanol-10% acetic acid followed by 10% acetic acid. All of these steps were performed at 60°C. Gels were stored at room temperature in 10% acetic acid.

Areas of the gel containing  $\beta$ -hexosaminidase were permanently stained a deep violet colour by a modification of the histochemical staining procedure of Hayashi (1965). The method consisted of first reacting  $\beta$ -hexosaminidase with the  $\beta$ -N-acetylglucosaminide of 7-bromo-3-hydroxy-2-naphth-o-anisidide (naphthol AS-BI-NAcglc) and then coupling the liberated aglycone with a diazo dye to produce an insoluble, coloured complex. To do this, extruded gels were rinsed in 0.1 M citrate buffer pH 4.5, and then incubated with the same buffer containing 0.028% naphthol AS-BI-NAcglc and 4% cellosolve (used to deliver the relatively insoluble substrate to the aqueous buffer) at room temperature until white bands were discerned encircling the gel where enzyme was located. Gels were then transferred to a 0.1% aqueous solution of fast Garnet GBC (diazotized o-aminotoluene) and allowed to sit at room temperature until the faint white bands were stained violet. Stained gels were stored in 10% acetic acid.

$\beta$ -Glucuronidase was located on polyacrylamide gels by a procedure which was closely related to that described above to stain  $\beta$ -hexosamini-

dase. Gels were first incubated at 37°C in 0.1 M acetate buffer pH 5.0 containing 0.01% naphthol AS-BI- $\beta$ -glucuronide and then treated at room temperature with a 0.1% aqueous solution of Fast Garnet GBC. Stained gels were stored in 10% acetic acid at room temperature.

b) Sodium Dodecyl Sulfate (SDS) Gel Electrophoresis

The standard procedure of Weber, Pringle and Osborn (1972) was followed both for preparing and polymerizing 10% polyacrylamide-SDS gels in cylindrical glass tubes and for dissociating quaternary protein structure; spacer gels were not used. The enzyme fraction which was to be examined was first dialysed against 0.01 M sodium phosphate buffer pH 7.0 and then lyophilized. Suitable aliquots (20-80 $\mu$ g protein) were dissolved in 0.01 M sodium phosphate buffer pH 7.0 containing 1% SDS and 1% 2-mercaptoethanol and heated in a boiling water bath for 3-7 minutes. Protein standards were subjected to dissociation by a similar procedure. After heating, enzyme samples and protein standards were cooled to room temperature, and aliquots containing 10 $\mu$ g of standard or 10-20 $\mu$ g of enzyme transferred by lambda pipette into 1 ml test tubes and diluted with 1 volume of 0.01 M sodium phosphate buffer pH 7.0 containing 10% sucrose and 3 $\lambda$  of 0.01% Bromphenol Blue. Processed samples, in a volume of less than 80 $\lambda$ , were then transferred to the surface of 10% polymerized electrophoresis gels and layered with enough running buffer (0.1 M sodium phosphate pH 7.2 containing 0.1% SDS) to completely fill the remaining space above the gel to the top of the supporting glass tube. Both reservoirs of the electrophoresis apparatus (the same as used for polyacrylamide disc gel electrophoresis) were filled with running buffer to adequately cover the electrodes. Electrophoresis was performed at 4-5 milliamperes per gel at room temp-

erature until the tracking dye had migrated the length of the gel. Gels were then extruded from the supporting glass tubes and stained for protein as described previously.

### c) Isoelectric Focussing

Sucrose density gradient column isoelectric focussing was performed at 4°C according to the procedure described in the LKB 8100 Ampholine instruction manual. A linear gradient of sucrose from 50% to 2%, including enzyme fraction, 0.1% triton X-100 and ampholytes (2% pH 3-10 or 1.9% pH 4-8 buffered with 0.1% pH 3-10), was formed manually in an LKB 8102 isoelectrofocussing column of 440 ml capacity. Enzyme fractions were dialysed against 1% glycine-NaOH buffer pH 7.0 containing 0.1% triton X-100 before suitable aliquots were incorporated into the sucrose gradient. The gradient was layered over the anode solution (60% sucrose, 1% H<sub>2</sub>SO<sub>4</sub>) and finally the cathode solution (2% ethanolamine) was layered over the gradient. Power was held at 2 Watts for the first 24 hours of the run and then increased to 4 Watts. Over the course of the run, the current progressively decreased until eventually a constant value was obtained after about 4-5 days. At this time, the column contents were allowed to drain by gravity flow, care being taken to avoid unduly disturbing the sucrose gradient containing the resolved proteins with too rapid a flow rate. Effluent fractions of appropriate size were collected and the pH of every third or fourth determined at 4°C. Fractions were also assayed for enzyme activity.

### 7) Protein Determinations

Protein was determined by one of three methods. When it was necessary to quantitatively determine the amount of protein present in an enzyme fraction, either the biuret method of Gornall, Bardawill and David (1949)

or a modification (Schacterle and Pollack, 1973) of the earlier Lowry procedure (Lowry et al., 1951) was used. When an approximation of the protein content was sufficient, as when monitoring column effluent fractions, protein was read at 280  $m\mu$  in a Cary 15 split beam spectrophotometer against the appropriate buffer in the reference cell.

## CHAPTER 3

### PRELIMINARY INVESTIGATIONS

#### A) INTRODUCTION

Previous studies had established that the  $\beta$ -hexosaminidase present in rabbit liver could effectively hydrolyse steroid  $\beta$ -N-acetylglucosaminides (Whittemore and Layne, 1965), and that the enzyme was predominantly or entirely of lysosomal origin (Mellor *et al.*, 1973). The latter group had also demonstrated, however, that  $\beta$ -hexosaminidase was not firmly attached to the lysosome in rabbit liver and that much of the enzyme activity was found in the cytosol following even gentle tissue homogenization. For this reason, attempts were not made in the present work to extract the enzyme from lysosomal preparations. Instead, intact liver tissue was vigorously homogenized in a tissue blender and the enzyme isolated in the high speed supernatant following centrifugation of homogenates at 10,000 xg and then at 100,000 xg.

Starting with the high speed supernatant, studies were undertaken to provide information which would be useful in the eventual purification and characterization of rabbit liver  $\beta$ -hexosaminidase. These studies are described in this chapter.

It was first necessary to develop assays which would reliably indicate the levels of  $\beta$ -hexosaminidase activity present in enzyme preparations. Accordingly, assays were devised with three substrates, namely p-nitrophenyl- $\beta$ -N-acetylglucosaminide (PNP-NAcglc), p-nitrophenyl- $\beta$ -N-acetylgalactosaminide (PNP-NAcgal) and 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide (E<sub>2</sub> $\alpha$ -17NAcglc).

The high speed supernatant was subjected to ammonium sulfate frac-

tionation and low pH in order to determine whether such techniques were suitable for the initial purification of rabbit liver  $\beta$ -hexosaminidase. Favourable results were obtained with both techniques, which were then combined to produce a partially purified enzyme preparation. Detergent was also added to the homogenizing buffer before liver tissue was homogenized in the tissue blender to determine whether this would increase the yield of  $\beta$ -hexosaminidase in the partially purified enzyme preparation.

Studies were then undertaken to determine whether  $\beta$ -hexosaminidase was present in rabbit liver as a single entity or in multiple forms as several enzyme isomers. Anion exchange chromatography, gel filtration and isoelectric focussing were employed in these investigations; the latter technique with the high speed supernatant fraction and the former two with the partially purified enzyme preparation. The first two techniques were not suitable for use with the high speed supernatant because the low enzyme to protein ratio in this fraction necessitated the application of quantities which caused serious overloading of gel columns and which consequently led to reduced resolution of enzyme forms in column filtrates.

B) METHODS

1) Preparation of Tissue Homogenates and High Speed Supernatant

One to two virgin female New Zealand white rabbits between 2 and 3 months of age were killed by cervical dislocation. Livers were excised immediately and, after removal of the gall bladder, were minced with scissors and homogenized at top speed for 1 minute in 9 volumes of isotonic 0.15 M KCl in a Sorval Omnimixer tissue blender. Ice surrounded the mixing chamber throughout homogenization. Homogenates were centrifuged at 4°C at 10,000 xg for 45 minutes to remove cell debris and then centrifuged at 100,000 xg for 1 1/2 hours at 4°C to obtain high speed supernatant. High speed supernatant was either stored at 4°C or frozen at -20°C until needed.

2) Investigation of Assays with PNP-NAcglc, PNP-NAcgal and E<sub>2</sub>α-17NACglc

Assays with all three substrates were performed as outlined previously under General Methods. The effect of pH on enzyme activity in aliquots of high speed supernatant was tested in 0.1 M citrate buffer. With all three substrates increments of 0.2 pH units were used in the range of the pH optimum while larger increments sufficed for determinations outside this area. Progress curves were derived at the pH optimum by incubating enzyme assays for varying lengths of time at 37°C and monitoring the release of liberated aglycone against time. Enzyme aliquots were adjusted for both investigations so that hydrolysis did not exceed 10% with the steroid substrate and 15% with the p-nitrophenyl compounds.

3) Partial Purification of β-Hexosaminidase

a) Ammonium Sulfate Fractionation and Acid Precipitation of Enzyme Activity from the High Speed Supernatant

Increments of solid ammonium sulfate were sequentially added to the same aliquot of high speed supernatant to give concentrations of salt which equalled 25%, 35%, 45%, 55%, and 65% of the saturation value. The nomograph of di Jeso (1968) was used to calculate the requisite amounts of salt. After each addition, the resulting mixture was gently stirred at 4°C for 20 minutes and then centrifuged to remove precipitated protein. Enzyme activity was monitored in the supernatant with 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide at each salt concentration to determine the percentage of high speed supernatant  $\beta$ -hexosaminidase activity which still remained in solution.

The pH of the high speed supernatant was reduced in stages by sequentially adding increments of solid citric acid to the same aliquot. After each addition of acid, the solution was stirred at 4°C for 20 minutes. Precipitated protein was then removed by centrifugation and the supernatant assayed with E<sub>2</sub> $\alpha$ -17NAcglc and 17 $\alpha$ -estradiol 3-glucuronide to determine the percentage of high speed supernatant  $\beta$ -hexosaminidase and  $\beta$ -glucuronidase activity, respectively, which was still present in solution.

#### b) Preparation of Partially Purified Enzyme Fraction

Liver was homogenized in 9 volumes of 0.15 M isotonic KCl, and high speed supernatant obtained as described previously. Solid ammonium sulfate was added to the high speed supernatant to a concentration of 30% maximum solubility. The resulting mixture was stirred for 20 minutes at 4°C and then centrifuged at 10,000 xg for 20 minutes. Precipitate was discarded and the supernatant subjected to 60% ammonium sulfate fractionation. The solution was stirred and centrifuged as described above. The resulting precipitate was dissolved in a volume of isotonic KCl which was

equal to 1/10 the volume of the high speed supernatant. The redissolved precipitate was placed in a dialysis bag and dialysed against several liters of 0.1 M citrate buffer pH 4.0 for 72 hours, with several changes. Following dialysis, precipitated protein was removed from the enzyme preparation by centrifugation at 10,000 xg for 20 minutes to give a clear yellow supernatant which contained partially purified  $\beta$ -hexosaminidase.

Throughout the purification procedure,  $\beta$ -hexosaminidase activity was monitored with  $E_2\alpha$ -17NAcglc and protein was quantitatively determined by the biuret method.

c) Detergent - Enzyme Recovery Study

Liver tissue was homogenized in 9 volumes of 0.15 M isotonic KCl - 0.3% sodium deoxycholate. Partially purified enzyme preparations were prepared, and  $\beta$ -hexosaminidase activity and protein monitored throughout, as described in the previous section. Enzyme and protein recoveries were compared at each stage in the purification procedure between non-detergent and detergent homogenized preparations.

4) Investigation of Multiple Enzyme Forms

a) DEAE-Cellulose Chromatography

Anion exchange chromatography was performed at 4°C with 0.9 cm x 15 cm columns of DEAE-cellulose preequilibrated with either 0.01 M phosphate buffer pH 6.0 or pH 7.9. A suitable aliquot of partially purified enzyme preparation was dialysed against preequilibrating buffer and applied to the appropriate column. Enzyme activity was eluted stepwise from the column by employing phosphate buffer of increasing molarity but constant pH. Effluent fractions were assayed for enzyme activity with 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide.

b) Gel Filtration

Gel filtration was performed with a 2.5 cm x 95 cm column of Sephadex G-200 fine in 0.06 M phosphate buffer pH 7.0 at 4°C. A suitable aliquot of partially purified enzyme fraction (900 mg protein) was dialysed against this same buffer and concentrated to 5 ml in a Diaflow apparatus (Amicon Corporation) fitted with an XM-50 membrane. Sample was applied to the column through a flow adaptor. Columns were run with a 12 cm pressure head which resulted in a flow rate of 12 ml per hour. Five ml fractions were collected, and assayed for enzyme activity with the steroid substrate, E<sub>2</sub>α-17NACglc, and for protein by absorbance at 280 mμ. Distinct peaks of enzyme activity were pooled, concentrated and suitably prepared for electrophoresis on 5.5% polyacrylamide gels. Following electrophoresis, gels were stained to visualize enzyme activity with naphthol AS-BI-NACglc as described previously.

c) Isoelectric Focussing

Isoelectric focussing was performed in either 2% pH 3-10 or 2% pH 4-8 ampholyte gradients as described in General Methods. High speed supernatant (47 ml) was dialysed against 1% glycine-NaOH buffer pH 7.0 containing 0.1% triton X-100 before application to the column. Following focussing, effluent fractions were monitored for enzyme activity with 17α-estradiol 17-β-N-acetylglucosaminide and p-nitrophenyl-β-N-acetylglucosaminide. Distinct peaks of enzyme activity were dialysed against 0.02 M phosphate pH 7.2, and then centrifuged to remove precipitated protein. Enzyme fractions were concentrated in a diaflow apparatus and suitable aliquots applied to 5.5% polyacrylamide gels. Following electrophoresis, gels were stained to visualize enzyme activity

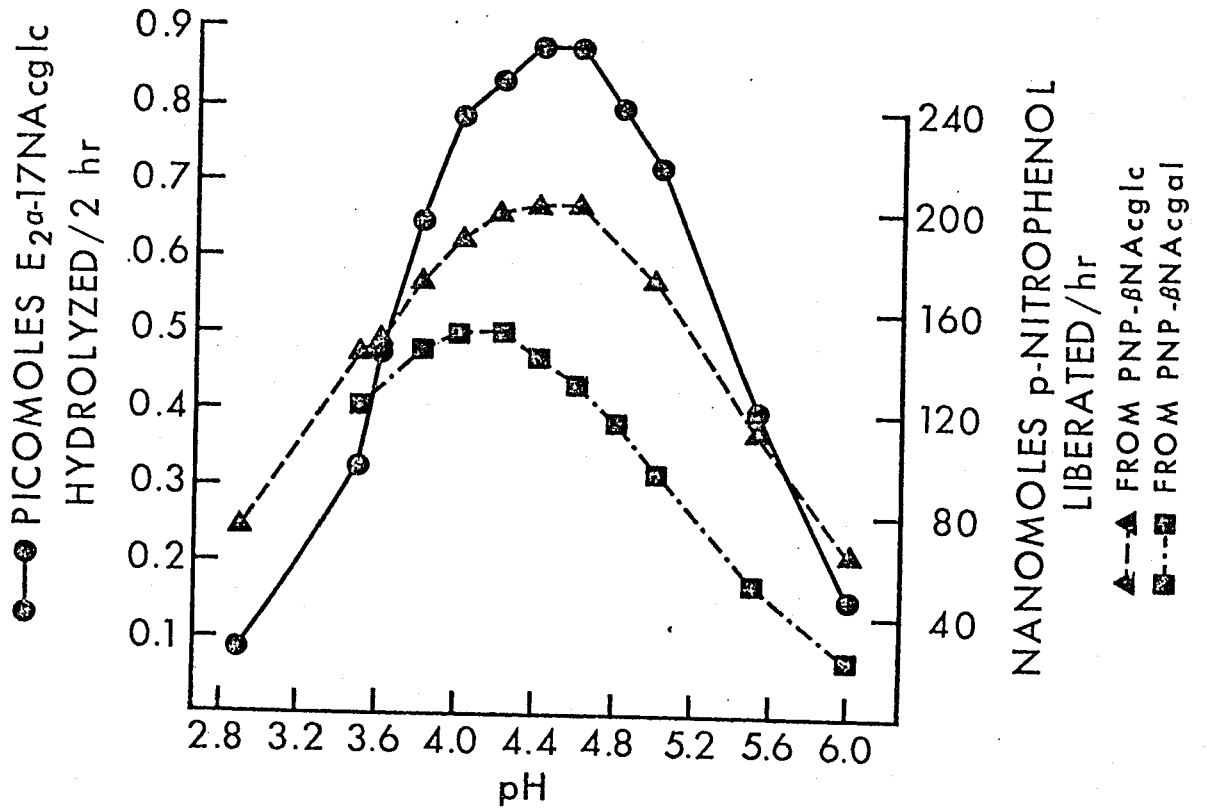
with naphthol AS-BI-NAcglc.

C) RESULTS

The activity of  $\beta$ -hexosaminidase with three different substrates in 0.1 M citrate buffer of varying pH is shown in Figure 1. A pH optimum centered around pH 4.4 was obtained with both the radioactive steroid substrate and p-nitrophenyl- $\beta$ -N-acetylglucosaminide. The pH optimum was shifted to pH 4.1 with p-nitrophenyl- $\beta$ -N-acetylgalactosaminide.

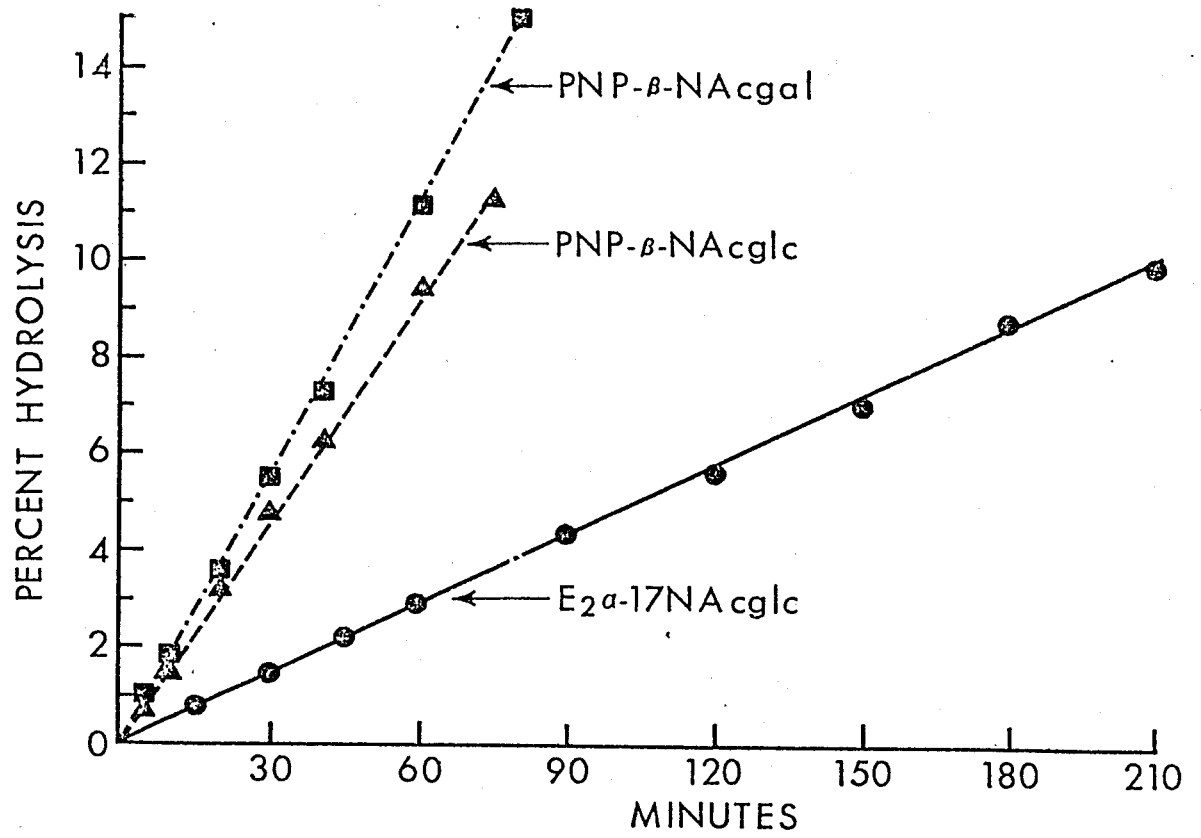
Progress curves of assays performed at the optimum pH with these three substrates are illustrated in Figure 2. Under conditions of the assays,  $\beta$ -hexosaminidase was stable to temperatures of 37°C for at least 3 1/2 hours. The enzyme liberated free aglycone from the p-nitrophenyl compounds at a constant rate for at least 75 minutes at which time hydrolysis of the parent compound equalled approximately 12-15%. With the steroid compound, initial velocity levels were maintained for 3 1/2 hours at which time 10% of the parent compound had undergone hydrolysis. In this work, enzyme assays with the former two substrates were routinely performed for 1 hour while those with 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide covered a time span of 2 hours.

The suitability of ammonium sulfate fractionation and low pH for the purification of  $\beta$ -hexosaminidase was examined with the enzyme from high speed supernatant by adding increasing concentrations of ammonium sulfate and citric acid, respectively. Figure 3 details the results from the salt experiment. Significant quantities of enzyme precipitated from solution between concentrations of 25% and 65% ammonium sulfate. Essentially all of the enzyme was removed from solution by 65% ammonium sulfate. Table II shows the susceptibility of  $\beta$ -hexosaminidase and a related enzyme,  $\beta$ -glucuronidase, to denaturation by increasing concentrations of citric acid and, hence, decreasing pH.  $\beta$ -Hexosaminidase was observed to progressively lose activity as the pH was reduced. The loss



For each curve enzyme aliquots of constant volume were taken and activity determined at different pH values in 0.1 M citrate buffer.

Figure 1. pH Activity Curves



Enzyme activity was determined under routine assay conditions towards all three substrates.

Figure 2. Progress Curves

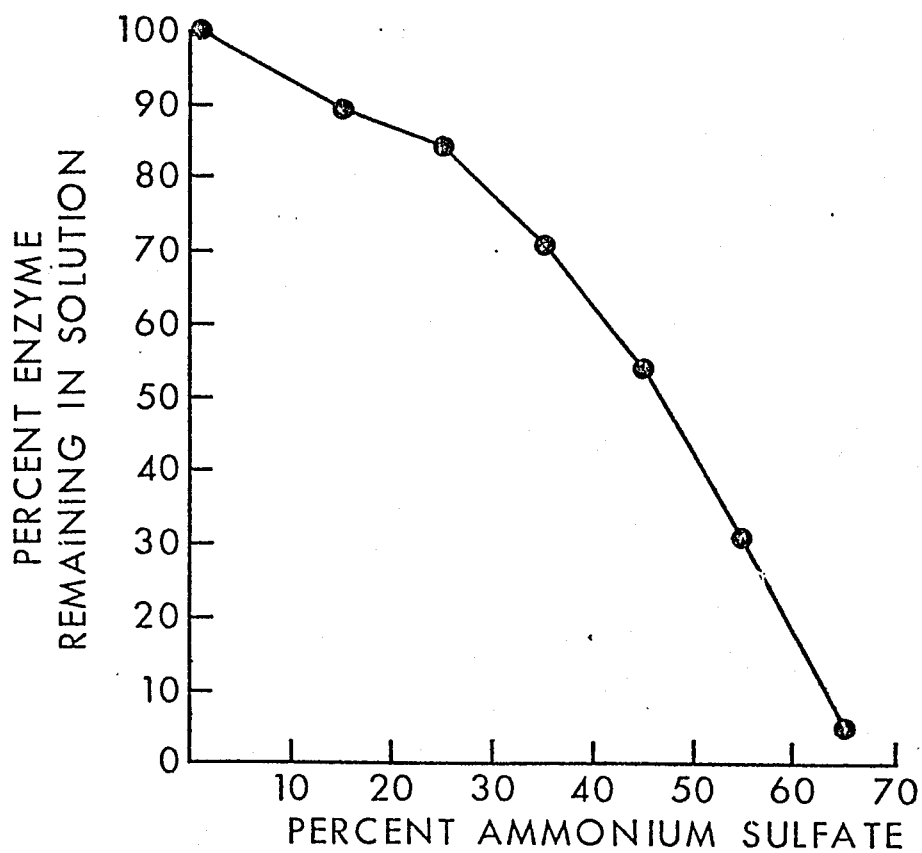


Figure 3. Ammonium Sulfate Fractionation of  $\beta$ -Hexosaminidase in High Speed Supernatant

pH	Percent Recovery	
	$\beta$ -Hexosaminidase	$\beta$ -Glucuronidase
High Speed Supernatant	100	100
pH 5.5	90	94
pH 4.6	84	78
pH 4.0	76	-
pH 3.5	72	75
pH 2.85	65	0

$\beta$ -Hexosaminidase was assayed with  $17\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide.  $\beta$ -Glucuronidase was assayed with  $17\alpha$ -estradiol 3- $\beta$ -glucuronide.

Table II. Enzyme Recovery with Decreasing pH

of activity was not particularly severe, however, with 65% of the original enzyme activity remaining at pH 2.85.  $\beta$ -Glucuronidase was far more sensitive to pH values below 3.5, with complete elimination of activity at pH 2.85. The significance of this complete loss of  $\beta$ -glucuronidase activity at low pH is discussed in the next chapter.

Partially purified enzyme preparations were obtained by employing both ammonium sulfate fractionation and acid precipitation. Enzyme from the high speed supernatant was first treated with 30% to 60% ammonium sulfate, and then the resulting precipitate was dissolved in isotonic KCl and dialysed against 0.1 M citrate buffer pH 4.0. Most of the enzyme was not denatured under these acidic conditions. Table III outlines the recovery of protein and enzyme at each stage of the purification procedure. This table also shows the differences in enzyme and protein recoveries which were obtained between livers homogenized in isotonic KCl and those homogenized in isotonic KCl containing 0.3% deoxycholate. Enzyme and protein values of non-detergent homogenized liver preparations are expressed as a percentage of the values obtained with the 10% homogenate, while those for detergent homogenized liver preparations are expressed as a percentage of the corresponding non-deoxycholate value for the same stage of purification. Approximately 24% of the enzyme activity and 3.6% of the protein originally present in the 10% homogenate were recovered in the final partially purified enzyme preparation when livers were homogenized without detergent. The presence of 0.3% deoxycholate in the homogenizing buffer significantly increased enzyme recovery in the 100,000 xg supernatant, and after ammonium sulfate fractionation and acid precipitation. Except for the high speed supernatant fraction, protein values were not increased in parallel with enzyme activity by deoxycholate. In the final two stages of purification, protein levels actually dropped slightly below those obtained

Fraction	Percent Enzyme Recovery		Percent Protein Recovery	
	* Without Deoxycholate	↑ With Deoxycholate	* Without Deoxycholate	↑ With Deoxycholate
10% Homogenate	100	102	100	99
High Speed Supernatant	55	132	48	133
30%-60% Ammonium Sulfate	33	155	26	91
Acid Supernatant	24	155	3.6	87

\* EXPRESSED AS A PERCENTAGE OF THE 10% HOMOGENATE VALUE.

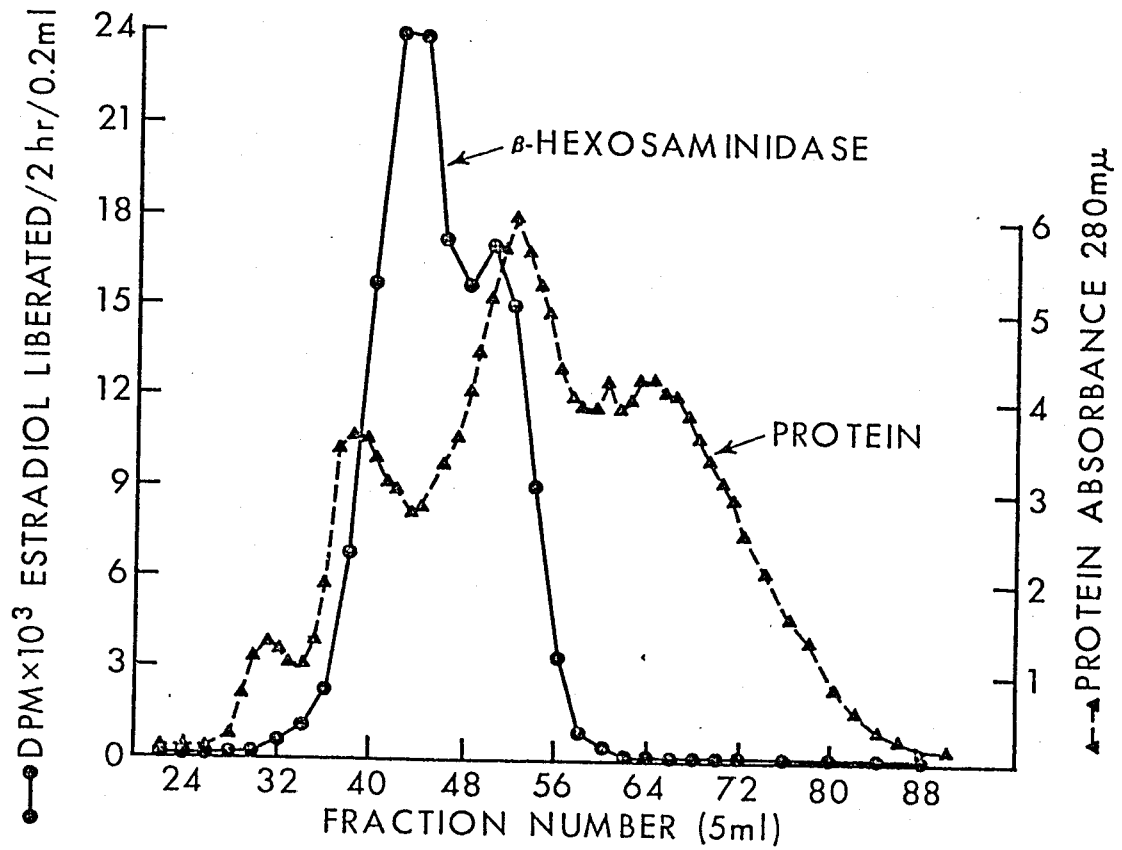
↑ EXPRESSED AS A PERCENTAGE OF THE CORRESPONDING NON-DEOXYCHOLATE VALUE

Table III. Partial Purification of  $\beta$ -Hexosaminidase: Enzyme and Protein Recoveries

when livers were homogenized in the absence of detergent. These results indicated that the inclusion of deoxycholate in the homogenizing medium was advantageous in that it led to an enhanced specific activity of the enzyme in the final partially purified preparation.

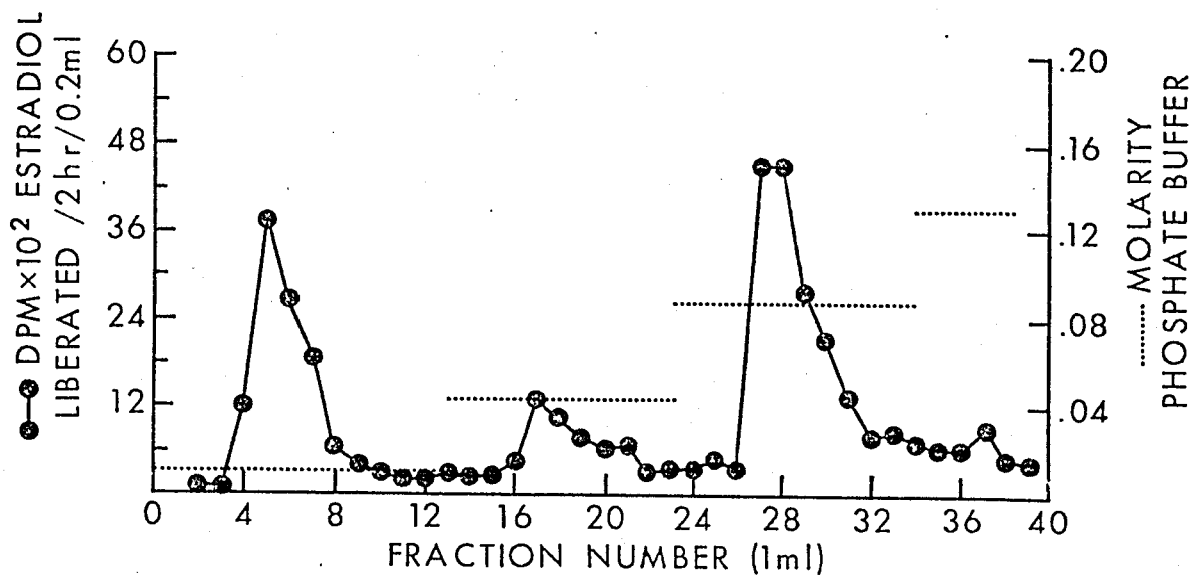
Suitable aliquots of the partially purified enzyme preparation were examined by gel filtration and anion exchange chromatography in order to ascertain whether  $\beta$ -hexosaminidase was present in rabbit liver in the form of several populations characterized by different physical properties. Gel filtration was employed to test for the presence of differently sized enzyme populations. The results from such an experiment with a 2.5 cm x 95 cm column of Sephadex G-200 fine are shown in Figure 4.  $\beta$ -Hexosaminidase was, first of all, retained by the gel and was not eluted in the void volume. Of more interest, however, was the elution pattern itself which showed that the enzyme was eluted as two partially resolved, distinct peaks of activity, thereby indicating the presence of two differently sized populations of the enzyme. Electrophoresis in 5.5% polyacrylamide gels was performed with each of the two enzyme populations separated by the Sephadex G-200 fine column. Gels were stained for enzyme activity. The relative position of each enzyme population with respect to the dye front is shown schematically in the two left hand columns of Figure 8. The smaller sized population migrated more rapidly down the gel than did the larger sized population. Rf values of 0.14 to 0.24 and 0.29 to 0.42 were obtained for large and small molecular weight forms respectively.

Analysis of the partially purified enzyme preparation by anion exchange chromatography revealed that differently charged forms of the enzyme were also present. Figures 5A and 5B depict the results which were obtained when enzyme was applied to columns of DEAE-Cellulose in



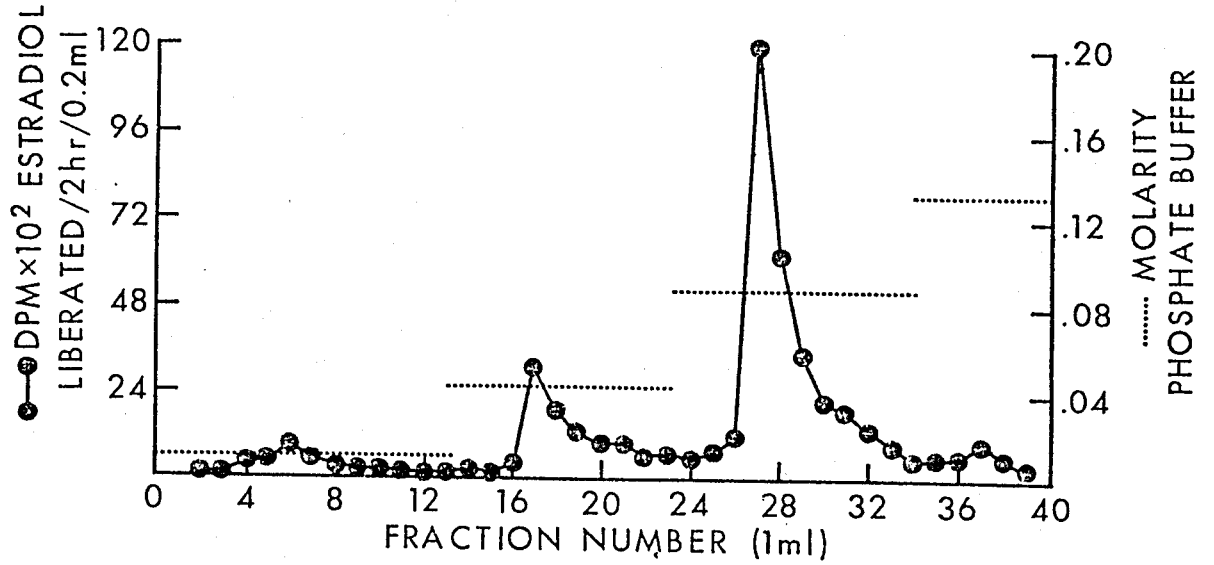
Enzyme activity was determined with  $17\alpha$ -estradiol  $17\beta$ -N-acetylglucosaminide under routine assay conditions.

Figure 4. Sephadex G-200 Fine Gel Filtration of Partially purified Enzyme Preparation



Enzyme activity was determined with 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide under routine assay conditions.

Figure 5a. DEAE-Cellulose Chromatography pH 6.0

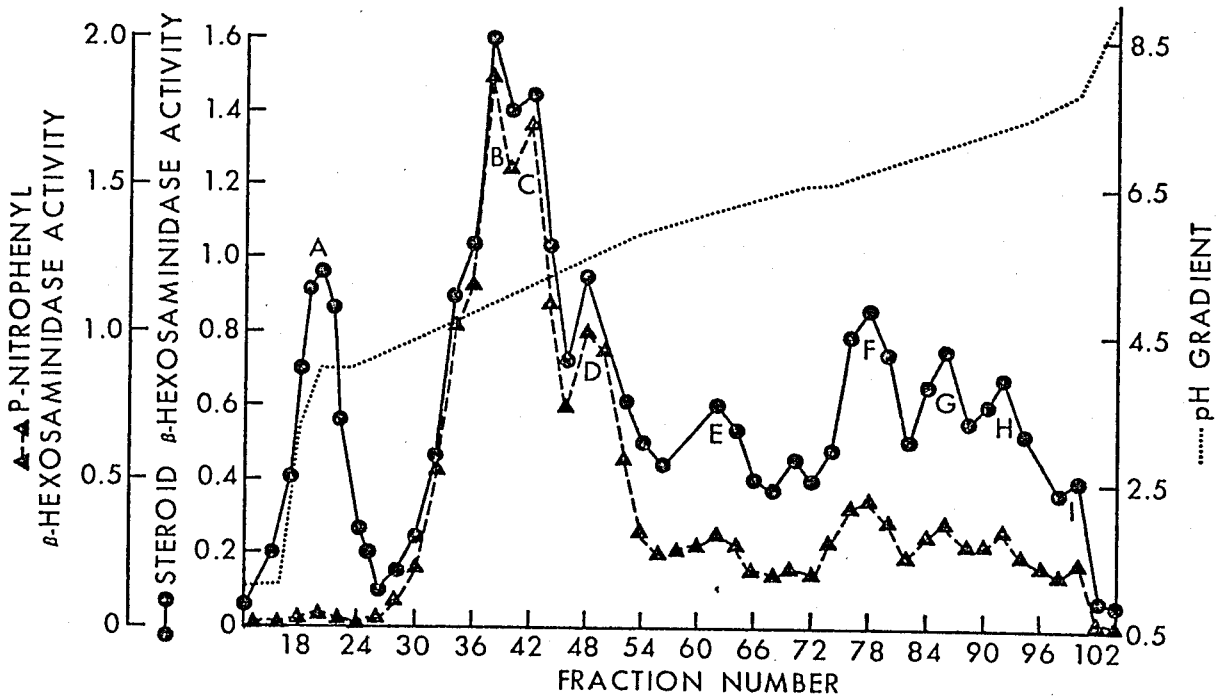


Enzyme activity was determined with  $17\alpha$ -estradiol  $17\beta$ -N-acetylglucosaminide under routine assay conditions.

Figure 5b. DEAE-Cellulose Chromatography pH 7.9

0.01 M phosphate buffer, and then eluted with phosphate buffer of increasing molarity. In Figure 5A, pH 6.0 phosphate buffer was used to generate the elution profile while pH 7.9 phosphate buffer was employed in Figure 5B. At pH 6.0, a significant percentage of the enzyme was eluted from the gel with 0.01 M phosphate buffer. However, at least two additional forms of the enzyme were bound to the gel and were only dislodged with phosphate buffer of higher molarity. At pH 7.9, three populations of the enzyme which bound with varying tenacity to the gel were also seen. Significantly more enzyme was bound to the gel at this pH and was not eluted with 0.01 M phosphate buffer suggesting that the surface charge of at least one form of the enzyme had changed from an overall positive to negative value when the pH was changed from 6.0 to 7.9.

The DEAE-cellulose experiments indicated that rabbit liver  $\beta$ -hexosaminidase existed as several differently charged isomers. Further information as to the number and characteristics of these charged species was obtained upon examining the enzyme in the high speed supernatant by isoelectric focussing. The use of the high speed supernatant in this study, rather than a more highly purified enzyme preparation, ensured the examination of all forms of the enzyme originally present in the liver extract. Preliminary experiments in ampholyte gradients of pH 2-10 established that precipitation of protein from the crude enzyme fraction during the isoelectric focussing run did not adversely affect the linearity of the pH gradient, and that the enzyme forms focussed between pH 4.0 and pH 8.0. Experiments were then performed in pH 4-8 ampholyte gradients to enhance the separation of the charged forms of the enzyme. The results from such an experiment are shown in Figure 6. Nine distinct forms of the enzyme were resolved on this column. The most acidic form focussed at the extreme acid end of the gradient at pH 4.0. Three forms of progressively less

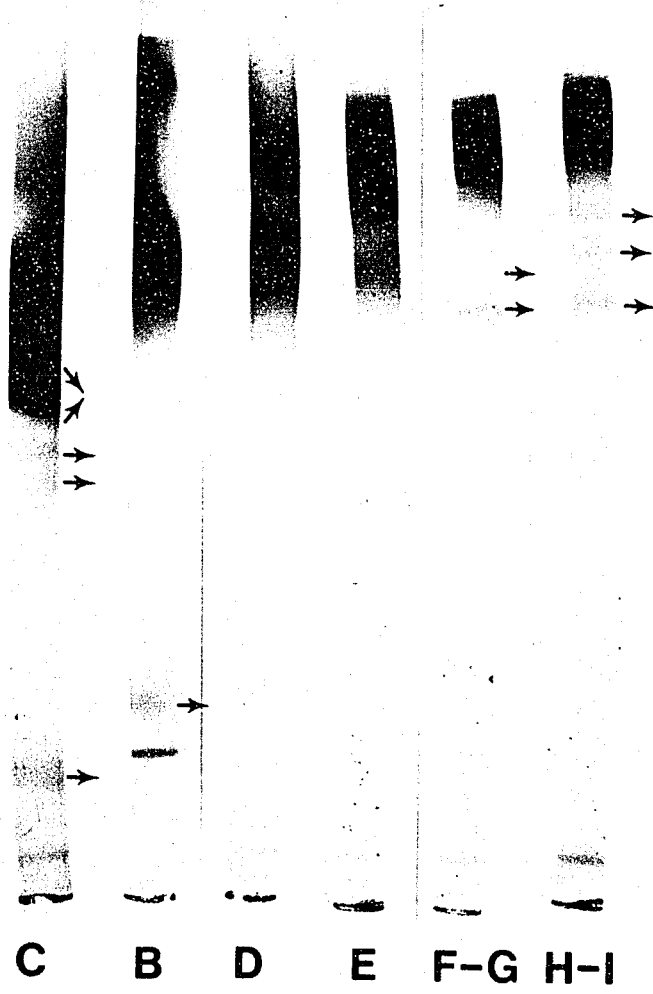


p-Nitrophenyl  $\beta$ -hexosaminidase activity is expressed as nanomoles  $\times 10^2$  PNP-NAcglc hydrolysed per hour per 0.1 ml enzyme fraction under routine assay conditions. Steroid  $\beta$ -hexosaminidase activity is expressed as picomoles  $E_2\alpha$ -17NAcglc hydrolysed per 2 hours per 0.5 ml enzyme fraction. Assays with  $E_2\alpha$ -17NAcglc were carried out under routine assay conditions using twice the normal concentration of steroid substrate.

Figure 6. Isoelectric Focussing pH 4-8: High Speed Supernatant

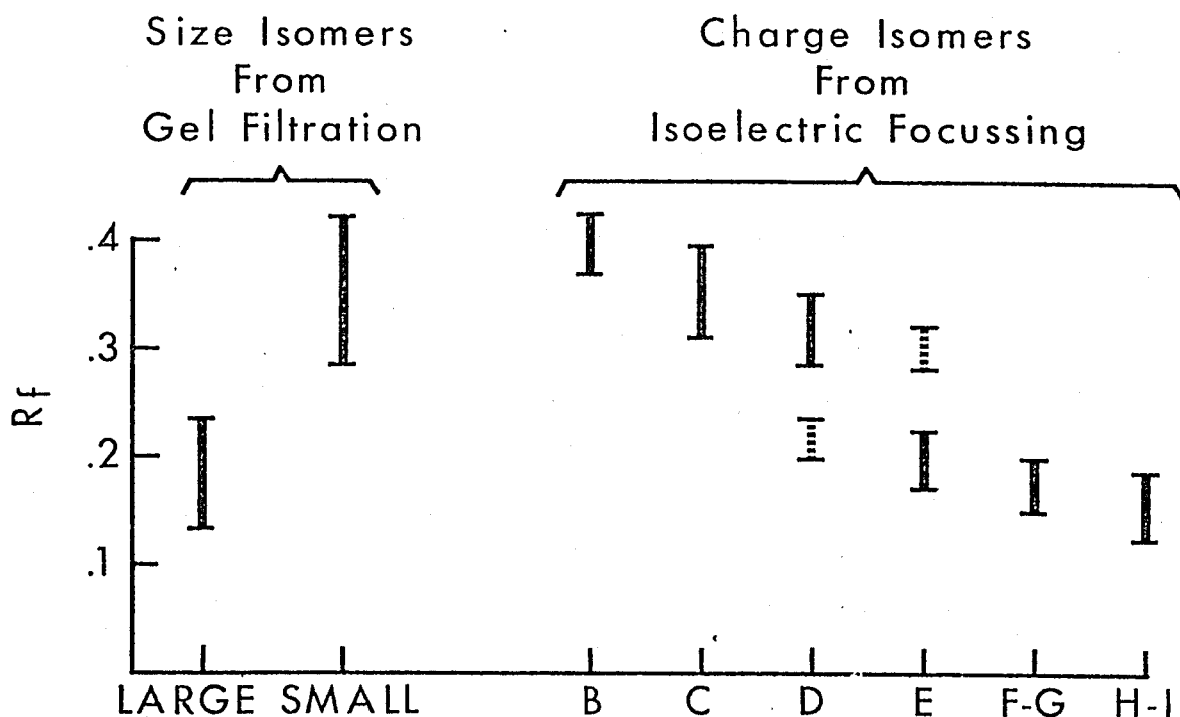
acidic overall change came to rest between pH 4.75 and pH 5.75, while five forms of a still more basic character migrated to positions along the pH gradient which ranged from pH 6.0 to pH 8.25. Both freshly prepared high speed supernatant and high speed supernatant stored for several days at 4°C showed the same complement of multiple forms upon isoelectric focussing. All of the resolved forms of the enzyme possessed activity towards both p-nitrophenyl- $\beta$ -N-acetylglucosaminide and 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide, although with the most acidic form (A), activity toward the p-nitrophenyl compound was minimal. With forms B through to I, the more acidic species (B, C, D) displayed relatively less activity than did the basic species (E, F, G, H, I) toward the steroid substrate as compared to the p-nitrophenyl substrate.

Each of the charge isomers of the enzyme from the isoelectric focusing gradient (forms F and G combined, forms H and I combined) were concentrated in a Diaflow apparatus and suitably prepared for electrophoresis. Following electrophoresis on 5.5% polyacrylamide electrophoresis gels, gels were stained to visualize enzyme activity. A picture of the stained gels is shown in Figure 7 while Figure 8 shows schematically the R<sub>f</sub> values obtained with the variously charged enzyme isomers (and also the R<sub>f</sub> values obtained with the two differently sized enzyme isomers from the Sephadex G-200 fine gel filtration experiment). The gel with form A is not shown in Figure 7 since this form did not successfully hydrolyse naphthol-AS-BI-NAcglc to produce a coloured band. As expected, the isoelectric pH of each of the enzyme forms was roughly reflected in the distance each migrated on the gels towards the anode (bottom of the gel). In other words, as the enzyme forms became increasingly basic in overall charge, going from form B through to form I, the distance of migration decreased. The charged forms of the enzyme tended to congre-



Suitably processed preparations of the enzyme forms separated by isoelectric focussing (Figure 6) were applied to polyacrylamide gels. Following electrophoresis, gels were stained to visualize  $\beta$ -hexosaminidase activity. The sharply defined band with the greatest  $R_f$  value on the gels corresponds to the tracking dye, Bromphenol Blue. Coloured bands marked with arrows arise from pigments which were present in preparations along with enzyme forms whereas those not so marked are a manifestation of  $\beta$ -hexosaminidase activity. Enzyme forms applied to gels are indicated by the letters below each gel.

Figure 7. Electrophoresis of Charge Isomers of  $\beta$ -Hexosaminidase Separated by Isoelectric Focussing



Solid vertical lines denote intense staining due to enzyme activity. Broken vertical lines denote weakly stained enzyme bands.

Figure 8. Schematic Representation of the Relative Distance of Migration with Respect to the Dye Front on 5.5% Polyacrylamide Gels of the Multiple Forms of Rabbit Liver  $\beta$ -Hexosaminidase

gate together in two groups on the gels following electrophoresis, the more basic forms (E to I) in the upper reaches of the gel and the more acidic forms (B, C, D) in an area further down towards the anode. A relatively large gap separated the two groups.

By correlating the Rf values obtained with the differently sized isomers from the gel filtration experiment with those obtained with the charged forms from the isoelectric focussing experiment, it was possible to establish which of the charged forms of the enzyme corresponded to which of the size isomers. The results from Figure 8 indicate that the smaller molecular weight population of  $\beta$ -hexosaminidase from rabbit liver was composed of forms B, C, and D, while the larger molecular weight population was a mixture of forms E through to I.

D) DISCUSSION

The pH optima obtained for rabbit liver  $\beta$ -hexosaminidase in vitro in citrate buffer with all three substrates correlate well with those which have been obtained with the enzyme from other mammalian and lower animal sources (Murphy, 1972; Carroll and Robinson, 1973; Robinson and Stirling, 1968; Li et al, 1973; Aruna and Basu, 1976). These enzymes have been found to function as acid hydrolases with pH optima usually ranging from 3.5 to 5.2 depending on the source of the enzyme and the substrate being hydrolysed; pH optima of 4.0 - 4.4 have been most frequently observed. The enzyme has usually been assayed in citrate or citrate-phosphate buffer. When acetate buffer has been used, pH optima have been higher than in citrate buffer in order to compensate for the inhibitory effect of acetic acid (but not acetate ion) (Pugh et al, 1957; Robinson and Stirling, 1968; Murphy, 1972).

Widely differing pH optima have not generally been encountered when various substrates have been reacted with the enzyme from a single source. For instance, p-nitrophenyl- $\beta$ -N-acetylglucosaminide and p-nitrophenyl- $\beta$ -N-acetylgalactosaminide are both hydrolysed most efficiently by human urinary  $\beta$ -hexosaminidase at pH 4.4 (Banerjee and Basu, 1975), while purified preparations of beef liver enzyme display maximum activity at pH 4.5 and 4.2 with p-nitrophenyl- $\beta$ -N-acetylglucosaminide and a trisaccharide breakdown product of hyaluronic acid containing N-acetylglucosamine at the reducing terminal, respectively (Weissmann et al, 1964). Notwithstanding one of the examples cited above, small differences in pH optima have often been seen between corresponding  $\beta$ -N-acetylglucosaminide and  $\beta$ -N-acetylgalactosaminide substrates, with pH optima for the former generally being higher than for the latter (Every and Ashworth, 1973; Li and Li, 1970; Mega et al, 1970; Tarentino and Maley, 1971;

Bullock and Winchester, 1973; Hayase et al, 1973). Figure 1 shows that the  $\beta$ -hexosaminidase from rabbit liver follows this pattern with the two p-nitrophenyl compounds. The enzyme displays a similar pH optimum with p-nitrophenyl- $\beta$ -N-acetylglucosaminide and 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide.

Both ammonium sulfate fractionation and acid precipitation have previously been employed by other workers in the purification of  $\beta$ -hexosaminidase from tissue extracts (Li and Li, 1970; Verpoorte, 1972; Tallman et al, 1974; Srivastava et al, 1974). In the present work, rabbit liver  $\beta$ -hexosaminidase was salted out of solution over a rather broad spread of ammonium sulfate concentrations ranging from 25% to 65% of the saturation value. It was never ascertained whether the presence of multiple forms of the enzyme (charge and size isomers) contributed to the need for such a large spread of salt concentrations to completely precipitate the enzyme from solution. It is entirely possible that the various isoenzyme forms could have possessed different solubility characteristics which would cause them to be affected to differing degrees by salt-water interactions. The evidence for this in the literature is not clear cut. For instance, Srivastava et al (1974) have demonstrated that the  $\beta$ -hexosaminidase from crude extracts of human placenta which is present as two forms of differing charge requires a 25% to 65% ammonium sulfate cut to be completely precipitated from solution (82% recovery). On the other hand, Bullock and Winchester (1973) have shown that a rather narrow range of ammonium sulfate from 30% to 40% serves to completely remove ram testes and epididymis  $\beta$ -hexosaminidase from solution, even though each is present in at least 3 to 4 species of differing overall charge. Rabbit liver  $\beta$ -hexosaminidase was also remarkably stable to acid pH down to 2.85. Both ammonium sulfate fractionation (30-60%) and

acid precipitation (pH 4.0) result in the loss of considerable protein from the enzyme fraction, with low pH being particularly noteworthy in this respect.

Considerably more enzyme was dissolved along with protein when deoxycholate was added to the homogenizing buffer. Mellor et al (1973) have previously demonstrated that rabbit liver  $\beta$ -hexosaminidase exhibits latency in carefully prepared lysosomal preparations. A decrease in enzyme activity (Table III) was not seen in the 10% homogenate when detergent was absent because, in all probability, the lysosomal structure had been partially damaged by the vigorous methods employed to homogenize the intact liver, and the population of the enzyme which was still loosely bound to the organelle had free access to the substrate. Upon centrifugation, however, much of the loosely bound enzyme activity was lost from the high speed supernatant. The inclusion of deoxycholate in the homogenizing buffer did not lead to an increase in protein in the latter stages of purification following ammonium sulfate fractionation and treatment with acid at pH 4.0.

$\beta$ -Hexosaminidase is generally present in mammalian tissues and in those of many lower animals in a number of differently charged forms (see Section C.3. Chapter 1). Anion exchange chromatography (Robinson, Jordan and Horsburgh, 1972; Ikonne and Ellis, 1973), electrophoresis (Price and Dance, 1972; Yoshikawa et al, 1972) and isoelectric focussing (Sandhoff, 1969; Hayase and Kritchevsky, 1973) have been used to resolve the enzyme forms. It is not unexpected, therefore, that the rabbit liver enzyme should follow a similar pattern and exist as a number of differently

charged isomers. Somewhat surprising, however, are the results from the gel filtration experiment which show that this enzyme is also present as isomers which differ significantly in size. Only the following few reports have appeared in the literature describing multiple forms of  $\beta$ -hexosaminidase which differ significantly in size. Tarentino and Maley (1971) have isolated two  $\beta$ -hexosaminidase isoenzymes from hen oviduct with molecular weights of 118,000 daltons and 158,000 daltons, respectively, while Seyama and Yamakawa (1974a, 1974b) have isolated and purified from equine kidney three charge isomers of  $\beta$ -hexosaminidase, one of which at 250,000 daltons is twice the size of the other two and is a complex of the two smaller molecular weight forms. In addition, an isoenzyme of  $\beta$ -hexosaminidase, Hex C, has been extracted from human tissue which differs significantly in size (as well as in substrate specificity and genetic origin) from the three other similarly sized forms of  $\beta$ -hexosaminidase (Hex A, Hex B and Hex S) which are present (Penton, Poenaru and Dreyfus, 1975; Reuser and Galjaard, 1976). Even though the enzyme species described above show differences in size, they differ markedly from those in rabbit liver when two factors are considered in combination: (1) the correlation between size and charge and (2) the magnitude of the differences in size. Since only very small differences in size, if any, have been shown between enzyme forms in all other reports in the literature, it is concluded that rabbit liver  $\beta$ -hexosaminidase is considerably different from any other previously studied  $\beta$ -hexosaminidase in the interplay between size and charge in its isomer forms.

Several attempts were made to further purify the enzyme from the partially purified preparation by means of affinity chromatography on gels complexed with 'enzyme-specific' ligands. Table IV lists some of the gels

AFFINITY GEL *	METHOD GEL ** SYNTHESISED	COMMENTS ***
AH-Sepharose 4B 17 $\alpha$ -Estradiol 3- $\beta$ -Glucuronide	1	Enzyme did not bind to gel.
CNBr-Sepharose 4B Benzidine 1,4-N-Acetylgalactosamino-lactone	2, 3, 4	Enzyme did not bind to gel.
CH-Sepharose 4B p-Aminophenyl- $\beta$ -N-acetylglucosaminide	5	Enzyme eluted with 0.4 M NaCl in 0.05 M acetate pH 5.0 or with 0.1 M citrate pH 4.5. Gel capacity was low. A small fraction of the total enzyme bound to the gel.
CH-Sepharose 4B p-Aminophenyl- $\beta$ -glucuronide	5	Enzyme eluted with 0.2 M NaCl or 0.5 M glucuronic acid in 0.05 M acetate pH 5.0, with 0.1 M acetate pH 5.0 or with 0.1 M citrate pH 4.5. Enzyme binding varied with each gel preparation. With some syntheses, a major fraction of the total enzyme bound to the gel.

NOTE: Legend on next page.

Table IV. Affinity Chromatography Experiments

\*CNBr Sepharose 4B refers to cyanogen bromide activated Sepharose 4B.

AH - Sepharose 4B refers to diaminodipropylamino Sepharose 4B.

CH - Sepharose 4B refers to succinylated AH - Sepharose 4B.

\*\*The numbers below refer to the following references:

- (1) Cuatrecasas and Anfinsen (1971)
- (2) Pravdic and Fletcher Jr. (1971)
- (3) Kanfer, Petrovitch and Mumford (1973)
- (4) Pokorny and Glaudemans (1975)
- (5) Junowicz and Paris (1973)

\*\*\*Enzyme was initially applied in 0.05 M acetate buffer pH 5.0 to gels.

Table IV. Affinity Chromatography Experiments

which were synthesized, and the conditions under which the enzyme was applied to and eluted from the gel columns. Because negative or inconsistent results were obtained in experiments with these gels, affinity chromatography with 'enzyme-specific' ligands could not be used in the eventual purification of the enzyme. The loss of this technique was particularly unfortunate since it represented the sole available means by which to effect further purification of  $\beta$ -hexosaminidase on the basis of enzymatic properties. This meant that further purification of  $\beta$ -hexosaminidase would depend entirely on techniques which were responsive to physical properties of the enzyme species. This increased the probability that complete purification of the enzyme species would not be achieved on the basis of common physical properties and that enzyme species would not be purified intact as one unit, since physical properties between enzyme species had already been found to differ widely.

The preliminary work served to clarify the following points:

- (1) rabbit liver  $\beta$ -hexosaminidase is present in multiple forms;
- (2) these forms differ in size and charge;
- (3) the mix of size and charge among the enzyme species is significantly different from that found among the multiple forms of any other  $\beta$ -hexosaminidase previously reported;
- (4) large and small molecular weight enzyme forms, as well as the most acidic form, isomer A, differ from each other in their ability to hydrolyse steroid and p-nitrophenyl  $\beta$ -N-acetylglucosaminide substrates;
- (5) these differences in enzymatic specificity could mean that different species are involved in different physiological roles in the rabbit in vivo;
- (6) considerable enhancement of the specific activity of the enzyme could be achieved through fractionation with 30-60% ammonium sulfate

- followed by treatment with citric acid at pH 4.0;
- (7) specific activity is further enhanced by including 0.3% deoxycholate in the homogenizing buffer;
  - (8) the partially purified enzyme preparation contains both large MW (forms E to I) and small MW (forms B-D) enzyme species.

The preliminary work suggested that further purification of rabbit liver  $\beta$ -hexosaminidase would be a fruitful avenue for ongoing research into the physical and enzymatic properties of the enzyme species. Accordingly, a purification procedure was devised to isolate and purify at least some of the enzyme species of  $\beta$ -hexosaminidase relatively free from contaminating protein. Highly purified enzyme species were then examined to determine the physical basis which underlay the proliferation of nine or more enzyme forms and to delineate in greater detail differences in enzyme specificity. These studies are presented in the next two chapters.

CHAPTER 4

PURIFICATION, ENZYMATIC PROPERTIES AND PHYSICAL PROPERTIES OF  
RABBIT LIVER  $\beta$ -HEXOSAMINIDASES

A) INTRODUCTION

Selected enzyme forms of rabbit liver  $\beta$ -hexosaminidase were purified to near homogeneity by a combination of techniques which included acid precipitation, ammonium sulfate fractionation, Concanavalin A Sepharose affinity chromatography, gel filtration and anion exchange chromatography. Each of these techniques made use of a different physical property of the enzyme. The techniques used in the early stages of the purification procedure not only eliminated much of the contaminating protein from the enzyme preparation, but also kept the various forms of the enzyme intact as one unit. In this way, considerable enhancement of the specific activity of the enzyme was achieved without having to simultaneously purify several different enzyme fractions. Unfortunately, additional techniques were not available to continue this approach and remove the remainder of the contaminating protein from the enzyme preparation with all forms of the enzyme still intact as one unit. Instead, gel filtration was used to separate the enzyme species into two differently sized populations, and then selected components of these populations were purified to near homogeneity by DEAE-Cellulose anion exchange chromatography.

Preliminary studies (see Figure 6, isoelectric focussing experiment) had shown that the various species of rabbit liver  $\beta$ -hexosaminidase present in crude enzyme preparations differed in their ability to hydrolyse  $\beta$ -N-acetylglucosaminides of  $17\alpha$ -estradiol and p-nitrophenol. These

differences in hydrolytic specificity were further examined in the present work by measuring enzyme activity with  $17\alpha$ -estradiol  $17\text{-}\beta$ -N-acetylglucosaminide, p-nitrophenyl- $\beta$ -N-acetylglucosaminide and p-nitrophenyl- $\beta$ -N-acetylgalactosaminide in each enzyme fraction obtained from the purification procedure. Within an enzyme fraction, the ability of enzyme species to hydrolyse different substrates (under standard assay conditions) was determined by comparing the total enzyme activity obtained with each substrate. Between fractions, the ability of different enzyme species to hydrolyse the same substrate was determined by comparing the enzyme activity given by equivalent numbers of enzyme molecules.

In the latter stages of the purification procedure, highly purified preparations of large and small MW enzyme species were examined to determine a number of their physical properties. The parameters chosen for study included size, subunit composition and susceptibility to denaturation with increasing temperature. Studies were also designed to determine whether the more acidic (negatively charged) forms of rabbit liver  $\beta$ -hexosaminidase displayed a similar propensity as those of the enzyme from other mammalian sources in converting to more basic (positively charged) forms when frozen and thawed in solutions of high salt concentration (Beutler and Kuhl, 1975). In addition, the converse possibility was examined, namely the elimination and rearrangement of subunits within the more basic enzyme forms to produce isomers of a more negative overall charge. These studies on the interconversion of enzyme forms, as well as those which investigated the subunit composition of enzyme isomers by SDS-polyacrylamide gel electrophoresis and their molecular weights by

gel filtration, were undertaken with a view to uncovering the structural basis behind the proliferation of nine or more species of the enzyme.

B) METHODS

1) Purification Procedure

a) General Comments

All steps in the purification procedure were carried out at 4°C with two exceptions; 37°C for acid precipitation at pH 3.5, and room temperature for Concanavalin A Sepharose affinity chromatography.

In a typical enzyme preparation, ten rabbit livers were used.

b) High Speed Supernatant (HSS)

Ten immature female New Zealand white rabbits between 2 and 3 months of age were killed by cervical dislocation. Livers were excised immediately and homogenized in isotonic 0.15 M KCl - 0.3% sodium deoxycholate. Eighteen percent homogenates were prepared as described previously for 10% homogenates (Chapter 3). The enzyme was obtained in the HSS following centrifugation of tissue homogenates at 10,000 xg for 45 minutes at 4°C and then at 100,000 xg for 90 minutes at 4°C.

c) Acid Precipitation

The HSS was warmed to 37°C and then the pH was lowered to 3.5 by the slow addition of solid citric acid with gentle stirring. Small amounts of solid sodium citrate were added along with the citric acid to ensure that the pH did not drop too rapidly. The solution was maintained at 37°C for an additional 30 minutes during which time it was stirred occasionally. Following centrifugation at 10,000 xg for 20 minutes to remove denatured matter, the solution was cooled to 4°C. Solid citric acid was added to lower the pH of the solution to 2.85. The solution was maintained at 4°C for 30 minutes with occasional stirring and then centrifuged at 10,000 xg for 20 minutes to bring down matter which was denatured under the conditions of increased acidity. This material was discarded. The pH

of the acidified supernatant was raised to 3.5 by the addition of solid sodium citrate.

d) Ammonium Sulfate Fractionation

Solid ammonium sulfate was slowly added over 10 minutes to the acidified supernatant to give a salt concentration equal to 60% of the saturation value. The required amount of salt was calculated by the method of diJeso (1968). The mixture was gently stirred at 4°C for 60 minutes and then centrifuged at 10,000 xg. The supernatant was discarded, and the precipitate resuspended in a volume of 0.1 M citrate buffer pH 4.0 equal to 1/10 - 1/7 the volume of the acidified supernatant. Material which did not redissolve in the citrate buffer was removed by centrifugation at 10,000 xg for 20 minutes.

e) Concanavalin A Sepharose 4B (Con A Sepharose) Affinity Chromatography

The 0-60% ammonium sulfate fraction was allowed to warm to room temperature and then applied to a column of 100 ml of Con A Sepharose previously equilibrated with several column volumes of 0.1 M citrate buffer pH 4.0. Several hundred ml of this same buffer were run through the gel bed until protein as measured by absorption at 280 m $\mu$  could no longer be detected in the filtrate. This filtrate was discarded. Glycoproteins which had bound to the gel matrix were then eluted from the gel column with 0.1 M citrate buffer containing 0.3 M methyl- $\alpha$ -D-glucoside and 0.075 M methyl- $\alpha$ -D-mannoside. Elution was continued (several hundred ml) until  $\beta$ -hexosaminidase activity could no longer be detected in the eluate fractions with the routine steroid assay (17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide as substrate). Eluate fractions containing  $\beta$ -hexosaminidase activity were pooled, concentrated to a volume of less than 50 ml in an Amicon Diaflow

apparatus fitted with an XM-50 membrane, and then dialysed in the same apparatus against 1 to 2 litres of 0.06 M potassium phosphate buffer pH 7.0 - 0.1 M NaCl to remove sugar from the enzyme preparation and to change the buffer in preparation for gel filtration.

f) Gel Filtration

The enzyme preparation from the Con A Sepharose step was concentrated to a volume of approximately 2 ml in a small Amicon cell, allowed to sit for 24 hours at 4°C at atmospheric pressure to eliminate dissolved gas, and then applied through a flow adaptor to the surface to a 2.5 x 95 cm column of Sephadex G-200 superfine. Gel columns were prepared and preequilibrated as described previously (General Methods). Columns were run in 0.06 M potassium phosphate buffer pH 7.0 - 0.1 M NaCl at a hydrostatic pressure of 10-12 cm which yielded a flow of 35-45 ml of buffer every 24 hours. Two ml fractions were collected and aliquots assayed for enzyme activity with the routine steroid assay and for protein by absorption at 280 m $\mu$ . Distinct peaks of enzyme activity were pooled and suitably prepared for DEAE-Cellulose anion exchange chromatography.

Following gel filtration, the loss of  $\beta$ -hexosaminidase activity from all enzyme fractions became increasingly pronounced when preparations were stirred or otherwise agitated. For this reason all manipulations which involved agitation of highly purified enzyme preparations (for example concentration and dialysis in an Amicon Diaflow apparatus) were performed as gently as possible.

g) DEAE-Cellulose Chromatography of the Large Molecular Weight Population of  $\beta$ -Hexosaminidase from the Sephadex G-200 Eluate

Eluate fractions containing the first peak of enzyme activity which eluted from the Sephadex column were pooled, concentrated to a volume of

approximately 5 ml in an Amicon Diaflow apparatus, and then dialysed in the same apparatus against a sufficient volume of 0.02 M potassium phosphate buffer pH 6.0 to completely replace the gel filtration buffer in the enzyme preparation. The dialysed enzyme preparation was applied to a 2.5 cm x 14 cm column of DEAE-Cellulose prepared and preequilibrated as described previously (General Methods). Columns were developed first with 250 ml of 0.02 M potassium phosphate buffer pH 6.0 and then with 250 ml of 0.10 M potassium phosphate buffer pH 6.0. Fractions of 8 ml were collected and aliquots monitored for enzyme activity with the usual steroid substrate. Fractions containing distinct peaks of enzyme activity were pooled, dialysed against 0.06 M potassium phosphate buffer pH 7.0 and concentrated to a volume of approximately 2 ml in an Amicon cell. Purified preparations were stored at 4°C or frozen at -20°C until needed.

h) DEAE-Cellulose Chromatography of the Small Molecular Weight Population of  $\beta$ -Hexosaminidase from the Sephadex G-200 Eluate

The small molecular weight population of the enzyme from the second peak of enzyme activity which was eluted from the Sephadex column was prepared for DEAE-Cellulose anion exchange chromatography as described above for the large molecular weight population of the enzyme with the single exception that the enzyme preparation was dialysed against 0.045 M potassium phosphate buffer pH 7.4 instead of 0.02 M potassium phosphate buffer pH 6.0. Columns of DEAE-Cellulose (2.5 cm x 12 cm) were preequilibrated with this same buffer. The enzyme preparation was applied to the anion exchange column through a flow adaptor and the column was washed with 250 ml of preequilibrating buffer. The column was further developed with a succession of pH 7.4 potassium phosphate buffers of increasing

molarity; 250 ml of a linear gradient of 0.045 - 0.075 M phosphate buffer, 130 ml of 0.075 M phosphate buffer and 130 ml of 0.15 M phosphate buffer. Eluate fractions of 8 ml were collected and aliquots were assayed for enzyme activity with 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide. The fractions containing the major peak of enzyme activity in the column filtrate were pooled and concentrated to a volume of approximately 2 ml in a Amicon cell. The concentrated enzyme preparation was stored as described above until needed.

## 2) Monitoring The Purification of $\beta$ -Hexosaminidase

The recovery of  $\beta$ -hexosaminidase activity at each stage of the purification procedure was quantitatively determined with three substrates--17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide, p-nitrophenyl- $\beta$ -N-acetylglucosaminide and p-nitrophenyl- $\beta$ -N-acetylgalactosaminide. The presence of  $\beta$ -glucuronidase was monitored with both 17 $\alpha$ -estradiol 3- $\beta$ -glucuronide and p-nitrophenyl- $\beta$ -glucuronide, while other glycosidases and phenolsulfatase were assayed with their respective p-nitrophenyl glycoside or sulfate substrates. All assays were performed as outlined previously under General Methods.

Protein was quantitatively determined in the enzyme preparation at each stage of purification by the Lowry method (Schacterle and Pollack, 1973) with bovine serum albumin as the protein standard.

Isoelectric focussing of partially purified enzyme preparations in pH 4-8 ampholyte gradients was performed as outlined previously under General Methods. This technique was used to determine the complement of charge isomers of  $\beta$ -hexosaminidase which was present in enzyme fractions.

The extent of contamination of  $\beta$ -hexosaminidase by other protein in more highly purified enzyme preparations was determined by analytical

polyacrylamide gel electrophoresis. Two aliquots from each enzyme fraction examined were suitably prepared for electrophoresis and applied to polyacrylamide gels (see General Methods for details). Following electrophoresis, separate gels were stained for protein and for  $\beta$ -hexosaminidase activity in the usual manner with Coomassie Blue and naphthol-AS-BI-NAcglc, respectively. Protein and enzyme stained gels were then aligned and visually examined to determine the amount of contaminating protein still present in the enzyme fraction, and also to determine the nature of the contaminating protein with respect to  $\beta$ -hexosaminidase (for example, whether more positively or negatively charged). Accurate determinations of the percentage of contaminating protein were made by scanning protein stained gels at 540  $m\mu$ . This procedure involved placing the gels in a Gilford Linear Transport unit which was fitted to a Gilford 2400-2 Spectrophotometer. The gels were then mechanically fed at a predetermined rate past the light source (set at 540  $m\mu$ ) of the spectrophotometer and a trace was obtained of the absorption profile of the stained protein bands on a chart recorder (hooked up to the spectrophotometer) using previously calibrated chart paper. Peaks on the chart paper were then correlated with protein bands on the gel and the areas under the peaks from the recorded trace were calculated to determine the percentage of protein of non- $\beta$ -hexosaminidase origin still remaining in the enzyme preparation.

### 3) Physical Properties of $\beta$ -Hexosaminidase Isomers

#### a) Stability To Increasing Temperature

The susceptibility of  $\beta$ -hexosaminidase isomers to denaturation with increased temperature was determined by heating highly purified preparations of the enzyme forms for varying periods of time to different temperatures, and then measuring the enzyme activity which remained in

each case. Thirty aliquots of constant volume were taken from each enzyme fraction examined and transferred by pipette into test tubes containing 2.0 ml of 0.1 M citrate buffer pH 4.4 and 200  $\mu$ g of BSA. The contents of two of the test tubes were warmed to 37°C and maintained at this temperature until all other incubations at higher temperatures were completed. These two samples represented "unheated" enzyme preparation and hence were representative of the total enzyme activity present in samples which were heated to higher temperatures at zero incubation time. The remaining twenty-eight test tubes were divided into sets of 7 and each set incubated with gentle shaking in one of four water baths set at either 45°C, 50°C, 55°C or 62°C. Tubes were withdrawn from each of the four water baths after incubation for 5, 10, 15, 20, 30, 45 and 60 minutes and cooled to 37°C. Upon completion of all incubations at the various temperatures, 40 picomoles of radioactive 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide (specific activity 10,000 DPM per picomole) were added to the contents of each test tube and enzyme activity was determined as described previously under General Methods for the routine steroid assay. Graphs of enzyme activity against time of incubation were plotted for each of the four temperatures.

#### b) Estimation of Molecular Weight

The molecular weight of the two differently sized populations of the enzyme was determined by the method of Andrews (1965) on calibrated columns of Sephadex G-200 superfine. Two to three mg of the following highly purified protein standards of known molecular weight were used to calibrate the gel column: horse spleen apoferritin (460,000-490,000 Daltons), aldolase (150,000 Daltons), bovine serum albumin (monomer 68,000 Daltons, dimer 136,000 Daltons) and ovalbumin (43,000 Daltons). Columns of

Sephadex G-200 superfine (2.5 cm x 95 cm) were set up as previously described and the gel bed stabilized with several column volumes of 0.06 M potassium phosphate buffer pH 7.0 - 0.1 M NaCl. Enzyme samples or protein standards in a volume of less than 2 ml were delivered to the surface of the gel bed through a flow adaptor. Several runs with the same column were necessary to encompass all the protein standards and enzyme fractions which were to be examined since enzyme fractions were not run in concert with protein standards, and protein standards with similar molecular weights were not sieved together on the same gel run. Columns were run at a hydrostatic pressure of 12 cm. Fractions of 2 ml were collected, and aliquots were either assayed with the routine steroid assay to detect  $\beta$ -hexosaminidase activity or read at 230  $m\mu$  to detect protein (standards). The elution volumes of the protein standards were calculated from the absorption peaks of their respective elution profiles in the column filtrate, and a standard curve plotted of elution volume against logarithm of the corresponding molecular weight. The elution volume of each of the two differently sized populations of  $\beta$ -hexosaminidase in the column filtrate was then determined and the molecular weight of each estimated from the standard curve.

c) Subunit Composition - SDS Gel Electrophoresis

Electrophoresis on SDS - polyacrylamide gels was used to examine the subunit structure of the two different molecular weight forms of the enzyme. SDS-polyacrylamide gels were prepared, and protein samples disaggregated under the influence of SDS and processed for electrophoresis, as described previously under General Methods. The following highly purified protein standards of known molecular weight were run alongside enzyme fractions on separate SDS-polyacrylamide gels each time electro-

phoresis was performed in order to calibrate Rf values in terms of protein size: bovine serum albumin (monomer 68,000 Daltons), ovalbumin (43,000 Daltons), chymotrypsin A (25,000 Daltons) and ribonuclease A (13,700 Daltons). At the conclusion of the electrophoresis run, a small piece of copper wire was inserted through the gels to mark the dye front (Bromphenol Blue), and then gels were stained to visualize protein in the usual manner with Coomassie Blue. Gel protein bands from enzyme fractions were examined in two ways: for size and for relative intensity of staining. In order to determine the former, standard curves were first plotted of the Rf values obtained with the protein standards against the logarithm of the corresponding molecular weight (Weber, Pringle and Osborn, 1972). Rf values of gel protein bands from enzyme preparations were then calculated and the molecular weight of each protein fragment estimated from the standard curve. The relative intensity of staining of protein bands was quantitatively determined by scanning gels at 540 m $\mu$  with a Gilford Spectrophotometer as described previously. Assessments were made of the subunit composition of the two differently sized populations of the enzyme by combining data on their molecular weight (gel filtration experiment) with data on the size and relative number of their subunits (SDS-polyacrylamide gel electrophoresis experiment).

#### d) Subunit Composition - Interconversion of Enzyme Forms

The subunit composition of the large and small molecular weight forms of the enzyme was further investigated by studying the conversion of  $\beta$ -hexosaminidase isomers of one size and charge to other isomers of a different size and charge. The method of Beutler and Kuhl (1975) was used to induce conversion of enzyme forms. The procedure consisted of

disrupting the quaternary protein structure of enzyme isomers by freezing and thawing enzyme preparations in the presence of high concentrations of salt, and then determining by polyacrylamide gel electrophoresis whether reaggregation of enzyme subunits resulted in the presence of new forms of the enzyme or whether the same forms of the enzyme which were originally present in the enzyme preparation reappeared. The procedure was as follows. Three aliquots were taken from each highly purified enzyme fraction which was to be examined. One aliquot (control) was held at 4°C while the other two, with 3M NaCl added to one of them, were frozen and thawed four times in a dry ice - acetone bath. Following freezing and thawing, each aliquot was prepared for disc gel electrophoresis as described previously under General Methods, and then subjected to electrophoresis. At the conclusion of the electrophoresis run, gels were stained to visualize enzyme activity and the migration pattern of the enzyme bands on the gels for each of the three aliquots from the same original enzyme fraction were compared. Conclusions were drawn as to the subunit composition of the large and small MW forms of the enzyme on the basis of the migration characteristics of enzyme species on the gels.

C) RESULTS

1) Purification and Substrate Specificity of Enzyme Forms

Batches of ten rabbit livers were homogenized in five volumes (approximately 5500 ml) of 0.15 M isotonic KCl-0.3% sodium deoxycholate to obtain 18% homogenates of liver tissue.  $\beta$ -Hexosaminidase was recovered in the HSS following centrifugation of homogenates, and subsequently purified. Enzyme and protein recoveries were gauged from the HSS after each step in the purification procedure.

The HSS was treated with citric acid to lower the pH to 3.5, and then to 2.85. Eighty-five percent of the protein present in the HSS was lost from the resultant acidified supernatant. Almost all the protein which was denatured came out of solution as the pH was lowered to 3.5; very little additional protein was lost from solution as the pH was dropped from 3.5 to 2.85. Although lowering the pH from 3.5 to 2.85 was rather ineffective in eliminating large amounts of contaminating protein from the enzyme preparation, it was extremely effective in totally eliminating all  $\beta$ -glucuronidase activity, albeit at the expense of 13% of the  $\beta$ -hexosaminidase activity present in the HSS (Table II, Chapter 3). Some  $\beta$ -hexosaminidase activity was thus sacrificed in order to completely denature  $\beta$ -glucuronidase.

The removal of  $\beta$ -glucuronidase from the enzyme preparation at this stage in the purification procedure was extremely important in terms of the ultimate purity which could be achieved for  $\beta$ -hexosaminidase. In cases where  $\beta$ -glucuronidase was not removed by low pH, significant quantities of this enzyme were found to co-purify with  $\beta$ -hexosaminidase throughout the rest of the purification procedure, especially with the large MW forms of the enzyme. Small MW enzyme forms were also contaminated by  $\beta$ -glucuronidase in highly purified preparations, although to

a lesser degree. In terms of protein, this contamination was rather severe. When highly purified preparations of large and small MW enzyme forms were subjected to electrophoresis and then polyacrylamide gels were stained to visualize protein,  $\beta$ -hexosaminidase activity and  $\beta$ -glucuronidase activity, at least 25%, and usually more, of the total protein was found to co-migrate with  $\beta$ -glucuronidase activity. It was thus imperative to completely eliminate  $\beta$ -glucuronidase from the HSS with low pH, even at the expense of 13% of the  $\beta$ -hexosaminidase activity, in order to eventually achieve purified preparations of  $\beta$ -hexosaminidase which would be free from contamination by  $\beta$ -glucuronidase.

$\beta$ -Hexosaminidase was precipitated from the acid supernatant with 60% ammonium sulfate and redissolved in several hundred ml of 0.1 M citrate buffer pH 4.0 (1/10-1/7 of the volume of the acidified supernatant). Following this step, the specific activity of  $\beta$ -hexosaminidase was increased about 4 fold over that found in the acidified supernatant if p-nitrophenyl- $\beta$ -N-acetylglucosaminide (PNP-NAcglc) was used to measure enzyme activity in both preparations (Table VIII) and about 6 fold if the steroid substrate, 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide (E<sub>2</sub> $\alpha$ -17NAcglc), was used (Table VII). This large difference in fold purification of the enzyme with the two substrates did not result from significantly different measurement of the percentage of enzyme activity recovered in the ammonium sulfate fraction (as gauged from the HSS), but rather from different measurement of the percentage of enzyme activity recovered in the acidified supernatant. This was the only instance in the first three stages of purification where large differences in enzyme yield were consistently encountered with the two different substrates. It never became clear what caused this large discrepancy; perhaps it was due to the unmasking of an inhibitor or activator by low pH which

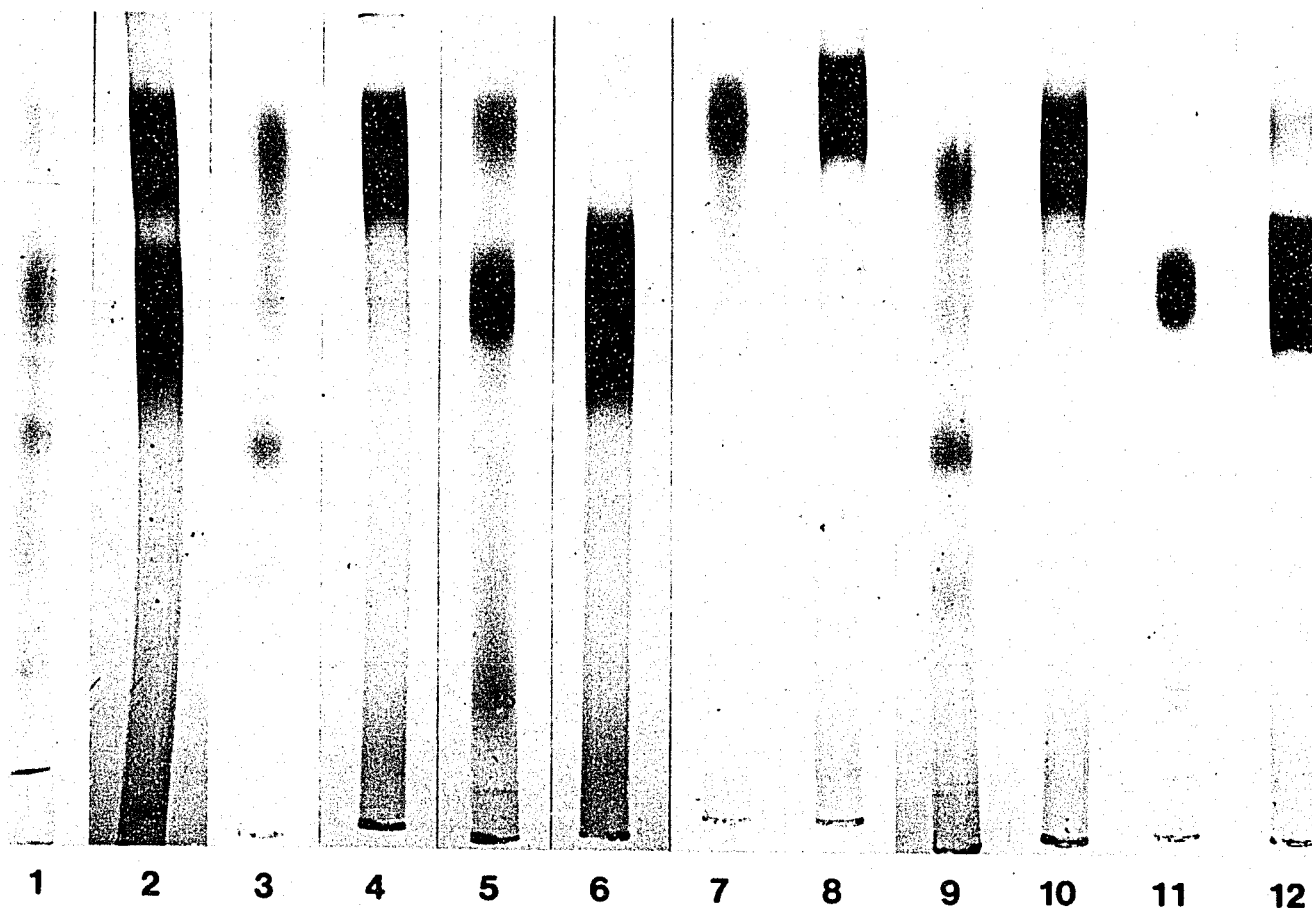
affected enzyme activity with the two substrates in different ways or perhaps the answer lay in the mechanics of the assays themselves. With the ammonium sulfate fraction, however, both substrates gave nearly equivalent measurement of enzyme yield with recovery exceeding 100% when gauged from the acidified supernatant. Besides significantly increasing the specific activity of the enzyme, the ammonium sulfate fractionation step also served to decrease the volume of the enzyme preparation from several thousand ml to a more manageable volume of several hundred ml, thus facilitating further purification of the enzyme.

The largest single increase in the specific activity of the enzyme for any one step in the purification procedure was obtained following chromatography of the ammonium sulfate fraction on columns of Con A Sepharose. The bulk of the protein from the ammonium sulfate fraction (greater than 98%) was eluted from the gel column with 0.1 M citrate buffer pH 4.0.  $\beta$ -Hexosaminidase, along with a minor fraction of the applied protein, was bound to the gel under these conditions, and was subsequently dislodged from the gel matrix and eluted from the column with 0.1 M citrate buffer pH 4.0 containing 0.3 M methyl- $\alpha$ -D-glucoside and 0.075 M methyl- $\alpha$ -D-mannoside. The enzyme was present in the Con A Sepharose filtrate fractions as a single peak of activity with approximately 75% of the enzyme activity originally applied to the gel being recovered in this peak (as measured with either PNP-NAcglc or E<sub>2</sub> $\alpha$ -17NAcglc). The eluate fractions containing  $\beta$ -hexosaminidase activity were pooled, concentrated to a volume of less than 50 ml in a Diaflow apparatus and subsequently dialysed against 0.06 M phosphate buffer pH 7.0-0.1 M NaCl to remove free sugar. The enzyme in this partially purified preparation could be stored at 4°C for several weeks or frozen at -20°C for one month with less than 10% loss of activity.

The behaviour of  $\beta$ -hexosaminidase on columns of Con A Sepharose gel strongly indicated that the enzyme was composed in part of sugar residues. The presence of protein-bound carbohydrate is a sine qua non for the formation of an enzyme-lectin complex since carbohydrate is specifically required by Con A for complex formation (Agrawal and Goldstein, 1968; Svensson, Hammarstrom and Kabat, 1970; Kennedy and Rosevear, 1973). These complexes can be disrupted and broken in several ways. In this work, dissociation of the  $\beta$ -hexosaminidase-lectin complex was accomplished by the addition of relatively large amounts of free sugar which competed with enzyme sugar residues for the binding sites on the lectin molecule and which eventually succeeded in displacing them from the complex.

The protein species and  $\beta$ -hexosaminidase isomers present in the Con A fraction were visualized, following electrophoresis of suitably prepared aliquots, on polyacrylamide gels stained for protein (Figure 9-1) and enzyme activity (Figure 9-2), respectively. Less than 50% of the protein in this fraction co-migrated on polyacrylamide gels with  $\beta$ -hexosaminidase activity. Further examination of enzyme stained gels revealed that the enzyme did not migrate as a single entity under the electrophoresis conditions used, but rather as two distinct fractions which came to rest in different positions on the gels following electrophoresis. Previous studies (Chapter 3) on enzyme fractions from less highly purified preparations (prepared from HSS by 30-60% ammonium sulfate fractionation, titration to pH 4.0 with citric acid and Sephadex G-200 fine gel filtration) had indicated the presence of two differently sized forms of  $\beta$ -hexosaminidase in rabbit liver (Figure 4). When enzyme stained polyacrylamide gels of these two differently sized populations of the enzyme were aligned with enzyme stained polyacrylamide gels of

## MONITORING ENZYME PURIFICATION BY POLYACRYLAMIDE GEL ELECTROPHORESIS



Oddly numbered gels were stained to visualize protein whereas gels with even numbers were stained to visualize  $\beta$ -hexosaminidase activity.

Gels 1 and 2: Con A Sepharose fraction

Gels 3 and 4: Large MW G-200 superfine fraction

Gels 5 and 6: Small MW G-200 superfine fraction

Gels 7 and 8: Large MW DEAE-Cellulose pH 6.0 fraction (eluted with  
0.02 M phosphate buffer)

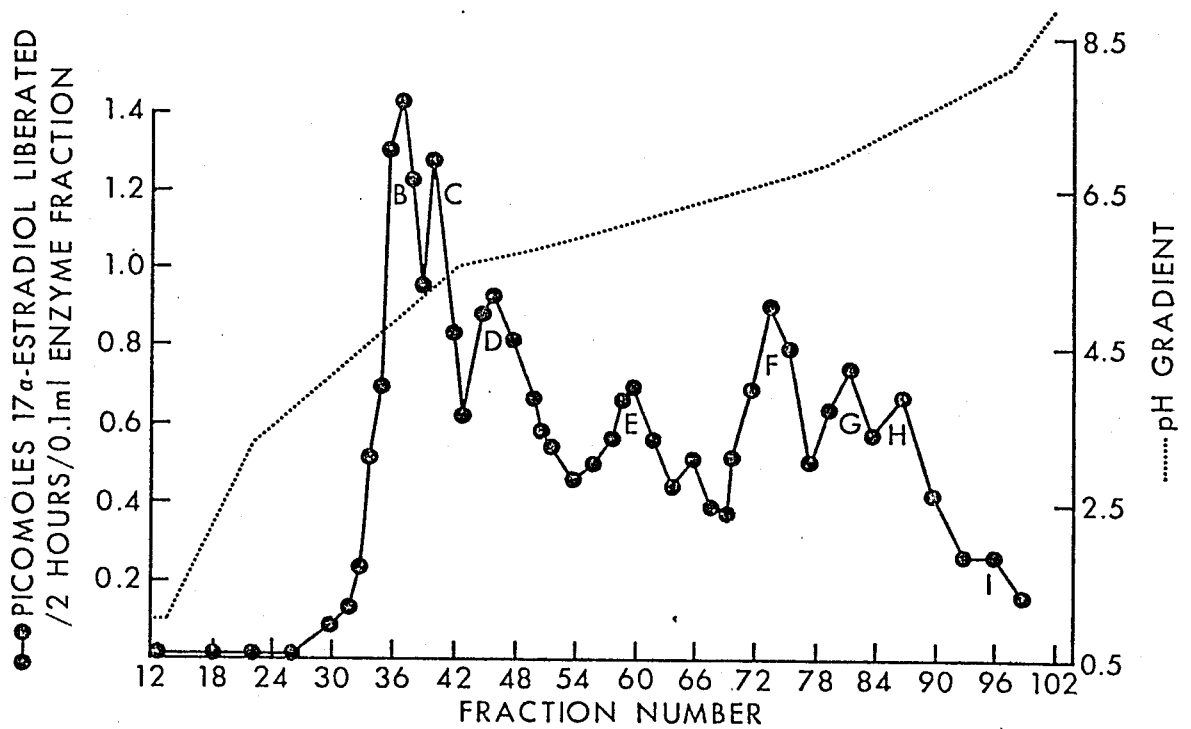
Gels 9 and 10: Large MW DEAE-Cellulose pH 6.0 fraction (eluted with  
0.10 M phosphate buffer)

Gels 11 and 12: Small MW DEAE-Cellulose pH 7.4 fraction

Figure 9. Monitoring Enzyme Purification by Polyacrylamide Gel  
Electrophoresis

the Con A Sepharose preparation, the more slowly migrating fraction of the enzyme present in the Con A Sepharose filtrate was found to correspond to large molecular weight forms of the enzymes and the more rapidly migrating fraction to small molecular weight forms. There could be no doubt that significant percentages of both differently sized enzyme populations had survived the first three purification steps and were present together in the Con A Sepharose filtrate.

Each of these two enzyme populations was known from preliminary studies with crude enzyme preparations (HSS, Chapter 3) to be composed of a number of enzyme species of different overall charge. Since the entire complement of  $\beta$ -hexosaminidase charge isomers had already been established in the initial liver extract (Figure 6), it was of interest to subject the Con A Sepharose enzyme preparation to isoelectric focussing in pH 4-8 ampholyte gradients in order to determine if any of the charge isomers in the HSS had been lost from the enzyme preparation following the first three purification steps. The results from the isoelectric focussing experiment are shown in Figure 10. Eight distinct forms of the enzyme, corresponding to forms B to I from the HSS (Figure 6), were still present in the Con A Sepharose preparation. Only the most negatively charged form of the enzyme, form A, was absent and additional isoelectric focussing experiments pointed to its loss after the acid precipitation pH 2.85 step. The similarity of the relative amounts of the eight most positively charged forms of the enzyme in the Con A Sepharose preparation and the HSS indicated that forms B-I were roughly equal in their stability to low pH (down to 2.85), to precipitation by 60% ammonium sulfate and to Con A Sepharose chromatography. Equally as important, the lack of any new enzyme forms in the Con A Sepharose preparation indicated that generation of enzyme artifacts had not occurred



Enzyme activity was measured under routine assay conditions with  $E_2\alpha$ -17NAcglc. The designation of enzyme forms is the same as that used in Figure 6.

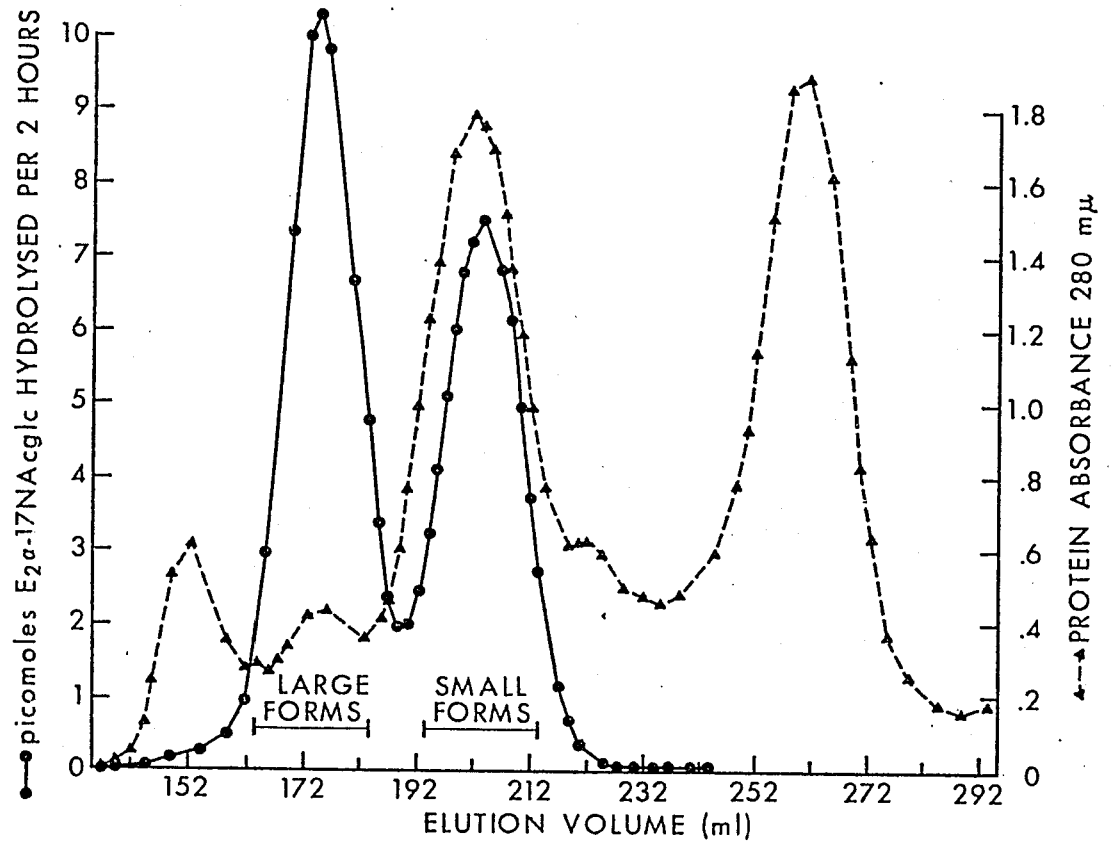
Figure 10. Isoelectric Focussing pH 4-8: Con A Sepharose Fraction

throughout the first three steps in the purification procedure.

The Con A Sepharose step was the last stage in the purification procedure where enzyme forms were purified as a unit. The removal of most of the rest of the contaminating protein was accomplished by techniques which also fractionated the enzyme into its various component forms.

The Con A Sepharose filtrate was subjected to gel filtration on 2.5 cm x 95 cm columns of Sephadex G-200 superfine. Two distinct populations of the enzyme were observed in the column filtrate (Figure 11); the larger molecular weight population was eluted first followed by the smaller population which was sieved to a greater extent by the stationary gel beads and consequently required a greater volume of running buffer to pass through the gel column. The separation of the two differently sized populations of the enzyme was noticeably superior under the conditions which were used to sieve the Con A Sepharose preparation in these studies than under those which had been used to fractionate the enzyme from relatively crude preparations on 2.5 cm x 95 cm columns of Sephadex G-200 fine (Figure 4). In the present studies, improved resolution of the two enzyme populations was achieved by decreasing the volume of the enzyme sample applied to the column from 5 to 2 ml, by using an enzyme preparation which contained less total protein, by packing the column with a Sephadex gel of superior sieving qualities, and by collecting smaller fractions of column filtrate to mitigate against overlap of the two enzyme forms. With these modifications of the earlier procedure, the two sized forms of the enzyme were almost entirely separated from each other in the column filtrate.

The protein profile in Figure 11 shows that well over 50% of the protein in the Con A Sepharose preparation was either eluted before or



1.8 ml fractions were collected. Enzyme activity was determined under standard assay conditions with E<sub>2</sub>α-17NAcglc using 30λ of filtrate.

Figure 11. Sephadex G-200 Superfine Filtration of Con A Sepharose Fraction

after the two enzyme fractions in the Sephadex G-200 column filtrate. Of the protein which did elute with the enzyme species, the bulk co-migrated through the column with the small molecular weight forms while only a small fraction was found in eluate fractions which contained the large molecular weight forms. Eluate fractions containing each population of the enzyme were separately pooled and concentrated to a volume of 10-20 ml in an Amicon cell. The large molecular weight (MW) fraction contained approximately 5 mg protein while the small MW fraction contained approximately 20 mg. The enzyme from either of these fractions was relatively stable to storage at 4°C or to freezing at -20°C in 0.06 M potassium phosphate buffers of various pH values ranging from 6.0 to 7.9 (with or without the inclusion of 0.1 M NaCl in the buffer); less than 10% enzyme activity was lost over a period of several weeks.

Approximately 75% of the enzyme activity present in the Con A Sepharose preparation was recovered in the column filtrate following Sephadex G-200 gel filtration when enzyme activity was measured with either E<sub>2</sub>α-17NAcglc or PNP-NAcglc. These two substrates, however, gave consistently different values for the percentage of enzyme activity which was distributed between the two differently sized populations of the enzyme. With E<sub>2</sub>α-17NAcglc (Table VII), approximately 60% of the recovered activity was found to reside with the large MW forms of the enzyme and 40% with the small MW forms. These percentages were reversed when PNP-NAcglc was used (Table VIII). The different values of relative enzyme activity which were obtained with these two substrates indicated that large and small molecular weight forms of the enzyme exhibited different specificities toward PNP-NAcglc and E<sub>2</sub>α-17NAcglc. A quantitative measure of these differences in enzyme specificity is presented in Table V which shows the ratios of enzyme activities which

Fraction	Activity Ratio	
	$\frac{\text{Micromoles PNP-NAcglc hydrolysed/hr/fraction}}{\text{Picomoles E}_2 \alpha\text{-17NAcglc hydrolysed/2 hr/fraction}}$	
Ammonium Sulfate	1.99	} 2.06
Con A Sepharose	2.12	
Sephadex Filtrate Large MW Forms	0.97	
Sephadex Filtrate Small MW Forms	3.62	

ASSAYS WITH EACH SUBSTRATE WERE CARRIED OUT UNDER ROUTINE CONDITIONS.

Table V. Comparison of Steroid and p-Nitrophenyl  $\beta$ -Hexosaminidase Activity of Enzyme Species in Fractions during Purification

were obtained with the two substrates for a number of enzyme fractions under standard assay conditions. The enzyme in the ammonium sulfate and Con A Sepharose preparations, where large and small molecular weight forms of the enzyme were present together, gave an activity ratio of approximately 2. The ratio was lowered to 0.97 with the large MW enzyme fraction from the Sephadex G-200 filtrate and raised to 3.62 with the small MW population of the enzyme. These ratios indicated that the large MW population of  $\beta$ -hexosaminidase displayed relatively greater activity than the small MW population towards the steroid substrate as compared to the p-nitrophenyl substrate.

PNP-NAcglc/ $E_2\alpha$ -17NAcglc activity ratios were also separately determined for forms A to I of the enzyme (Figure 6) following isoelectric focussing of HSS in pH 4-8 ampholyte gradients. Under the conditions of the focussing experiment, enzyme species which comprised each of the two differently sized populations of the enzyme in the Sephadex G-200 superfine column filtrate were separated from one another in the ampholyte-sucrose focussing gradient. Because of this enhanced resolution, it was possible to use activity ratios of forms B to I to determine whether individual enzyme isomers of the same size displayed similar hydrolytic properties with PNP-NAcglc and  $E_2\alpha$ -17NAcglc. As Table VI shows, activity ratios were very similar for different enzyme species of constant size. Forms E to I, the large MW enzyme forms, gave ratios ranging from 2.18 to 2.39 while ratios ranging from 3.72 to 3.98 were obtained for the small MW forms B to D. Unexpectedly, activity ratios of large and small MW enzyme forms following isoelectric focussing of HSS were not identical to those obtained for analogous forms of the enzyme in the Sephadex G-200 filtrate (Table V). Minor differences were seen between the activity ratios of small MW enzyme forms in the two preparations, while major

Enzyme Form	Activity Ratio
	$\frac{\text{Micromoles PNP-NAcglc hydrolysed/hr/form}}{\text{Picomoles E}_2 \alpha\text{-17NAcglc hydrolysed/2 hr/form}}$
A	0.51
B	3.98
C	3.86
D	3.72
E	2.18
F	2.28
G	2.21
H	2.26
I	2.39

ENZYME SPECIES IN THE HIGH SPEED SUPERNATANT WERE SEPARATED FROM ONE ANOTHER BY ISOELECTRIC FOCUSING IN A pH 4-8 AMPHOLYTE GRADIENT. ASSAYS WITH EACH SUBSTRATE WERE CARRIED OUT UNDER ROUTINE ASSAY CONDITIONS.

Table VI. Comparison of Steroid and p-Nitrophenyl  $\beta$ -Hexosaminidase Activity of Enzyme Species in the High Speed Supernatant

differences were found to occur between the values obtained for analogous large MW forms. Differences in activity ratios between large and small MW enzyme forms in each preparation were therefore, not comparable. In both preparations, however, large MW enzyme forms displayed a significantly lower activity ratio with the two substrates than did small MW enzyme forms indicating that forms E to I when compared to forms B to D preferred to hydrolyse  $E_2\alpha$ -17Nacglc over PNP-Nacglc. The exact magnitude of this preference, however, appeared to vary between enzyme preparations which had previously undergone different treatment.

A far lower activity ratio was obtained with form A than with any of the other enzyme forms in the isoelectric focussing gradient. Form A, therefore, exhibited the greatest preference of any of the enzyme species for the steroid over the p-nitrophenyl substrate. Form A was not found in more highly purified enzyme preparations since it was denatured in the first step of the purification procedure, namely treatment of HSS with citric acid pH 2.85.

The molecular weight of the two differently sized enzyme populations in the Sephadex G-200 column filtrate was determined by gel filtration on a 2.5 cm x 95 cm column of Sephadex G-200 superfine which was calibrated, in terms of elution volume, with proteins of known molecular weight. Figure 12 shows the standard curve obtained by plotting the logarithm of the molecular weight of protein standards against the respective elution volumes. The molecular weight of both differently sized populations of the enzyme was estimated from the standard curve on the basis of elution volumes in the Sephadex G-200 column filtrate. The large MW enzyme forms were calculated to be 270,000 Daltons while the molecular weight of the small enzyme forms was estimated at 150,000 Daltons.

The protein species and enzyme isomers which were present in the two

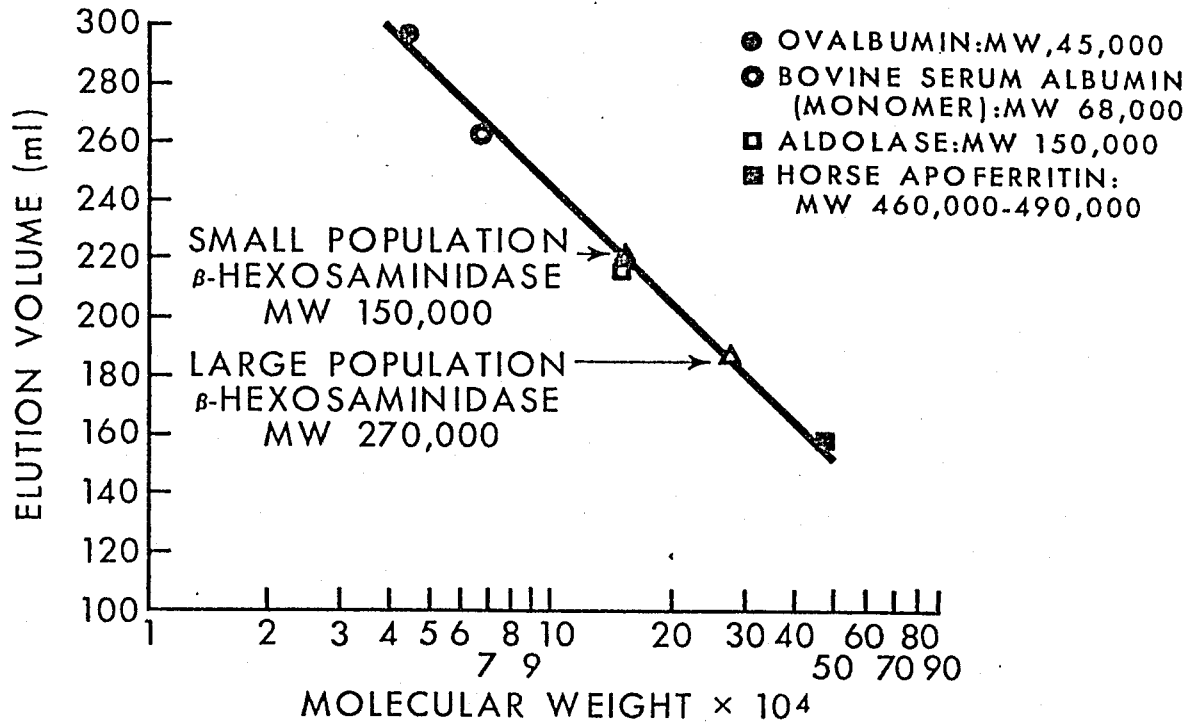


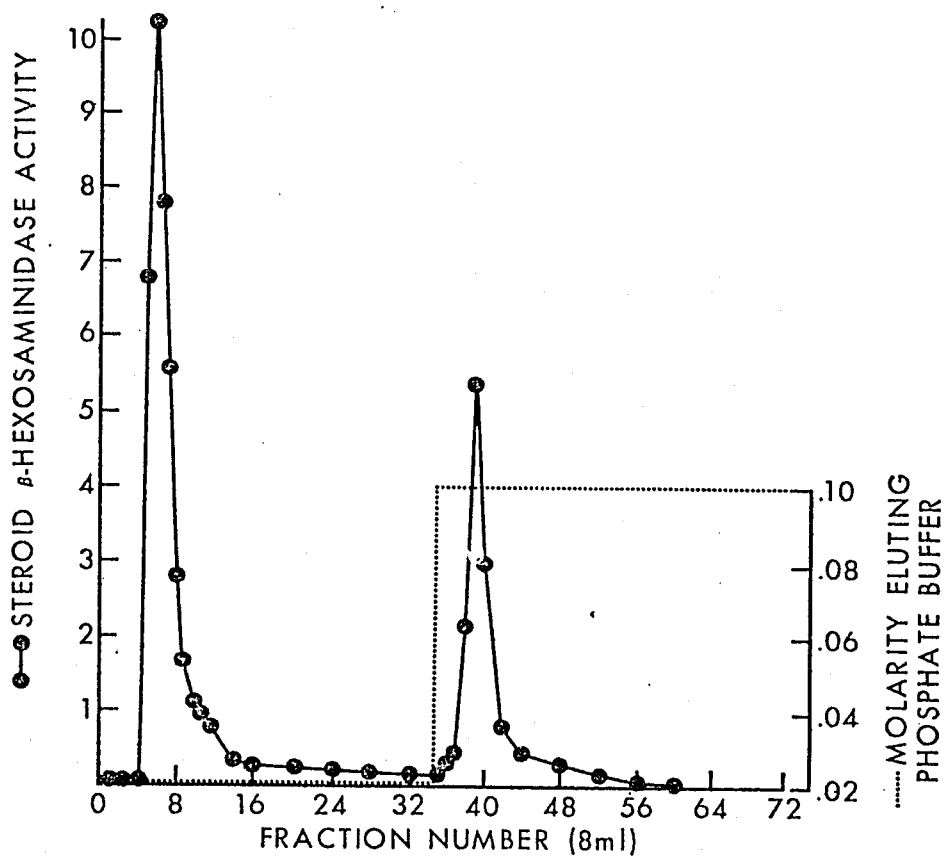
Figure 12. Calibration Curve for the Determination of Molecular Weight by Gel Filtration on Sephadex G-200 Superfine

MW fractions in the Sephadex G-200 column filtrate were examined by applying suitably prepared aliquots of each fraction to polyacrylamide gels and, following electrophoresis, staining gels for protein and  $\beta$ -hexosaminidase activity, respectively. These gels are shown in Figures 9-3 and 9-4. (large MW fraction) and Figures 9-5 and 9-6 (small MW fraction). As expected, the gels which were stained for enzyme activity showed only very minor contamination of one molecular weight form of the enzyme by the other. Scanning of the protein stained gels at 540  $m\mu$  as described previously indicated that the enzyme comprised 40% of the total protein in the large MW fraction and 48% in the small MW fraction. The distribution on the protein stained gels of protein species which were of non- $\beta$ -hexosaminidase origin was of interest in indicating how this protein might be separated from the enzyme in the final step of the purification procedure, namely DEAE-Cellulose anion exchange chromatography. With the large MW fraction, all contaminating protein was observed to migrate more rapidly down the gel than the large MW forms of the enzyme, under the electrophoresis conditions which were used. Since all protein species in this fraction were of approximately the same size, having eluted together from the gel filtration column, the contaminating protein was, therefore, more negatively charged than the enzyme protein. This fact suggested that the former could be removed from the latter on columns of anion exchange resin by adjusting conditions in such a way as to select for only the more positively charged large MW species of the enzyme. With the small MW enzyme population, a different strategy was needed for further purification of the enzyme isomers since contaminating protein in this fraction was both more negatively and more positively charged than the small MW forms of the enzyme. In this case, therefore, elimination of contaminating protein depended upon selecting

for those small MW forms of the enzyme which were neither the most positively nor the most negatively charged, or in other words, selecting for the moderately charged forms of the small MW enzyme population.

Figure 13 shows the results which were obtained when the large MW forms of the enzyme from the Sephadex G-200 filtrate were subjected to DEAE-Cellulose anion exchange chromatography in potassium phosphate buffer pH 6.0. More than 50% of the enzyme activity which was recovered in the column filtrate was eluted from the anion exchange resin with 0.02 M phosphate buffer pH 6.0. This lack of retention on the gel indicated that many of the large molecular weight enzyme species possessed an overall positive surface charge at pH 6.0 in phosphate buffer. A significant percentage of the large MW enzyme forms were, however, negatively charged at this pH and were bound to the column. Bound enzyme species were eluted from the column with 0.1 M phosphate buffer pH 6.0. It might have been expected that all the large molecular weight forms of the enzyme would have been eluted from the anion exchange resin with 0.02 M phosphate buffer pH 6.0 since, as Figure 10 shows, all these enzyme species (forms E-I) possessed an isoelectric pH which was well in excess of pH 6.0. These isoelectric pH values, however, were a measure of the total charge of the enzyme while with anion exchange chromatography, a different physical parameter was being measured, namely the surface charge of the enzyme species. Since the more acidic large MW enzyme species were retained by the DEAE-Cellulose resin at pH 6.0, the surface and total overall charges of these forms were not equivalent.

While specific experiments were not done to confirm which charge isomers of the enzyme were present in each of the two distinct enzyme fractions eluted from the DEAE-Cellulose pH 6.0 column, it was considered likely that the enzyme fraction eluted with 0.02 M phosphate buffer from



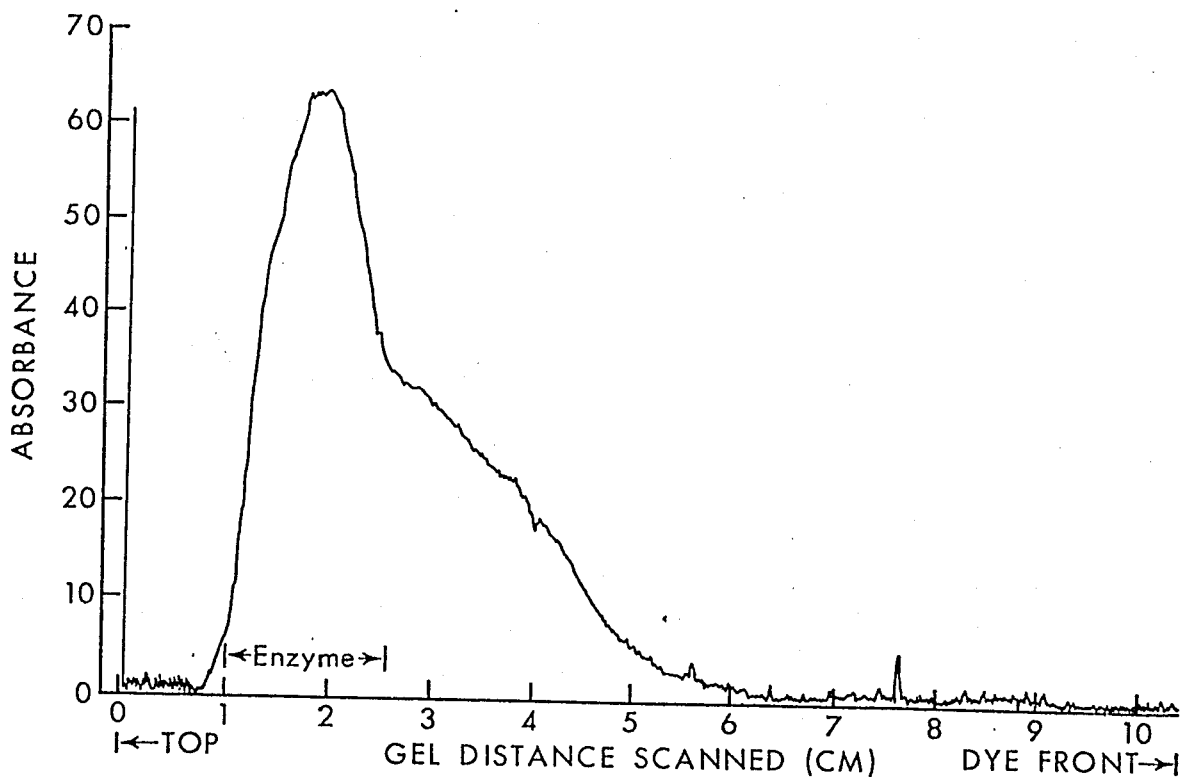
Steroid  $\beta$ -hexosaminidase activity is expressed as picomoles  $E_2\alpha$ -17NAcglc hydrolysed per 0.2 ml eluate per hour under standard assay conditions. Enzyme forms were eluted from the gel with phosphate buffer pH 6.0.

Figure 13. DEAE-Cellulose Anion Exchange Chromatography of the Large Molecular Weight Enzyme Fraction from the Sephadex G-200 Superfine Eluate

the anion exchange resin was composed of isomer forms I, H, G (and perhaps a little of form F), while the 0.1 M phosphate buffer eluted forms E and F. This reasoning was based on the isoelectric pH's of the large MW enzyme forms previously determined in less highly purified preparations (Figures 6 and 10), and on the similarity of Rf values obtained on polyacrylamide electrophoresis gels between enzyme isomers I, H and G from the HSS and enzyme species in the 0.02 M phosphate eluate, and between isomers E and F from the HSS and enzyme species in the 0.1 M phosphate eluate.

Both enzyme fractions from the DEAE pH 6.0 column filtrate were separately concentrated to a volume of approximately 2 ml in an Amicon cell. Protein in the more basic enzyme fraction was equal to approximately 0.5 mg and that in the more acid fraction equalled approximately 0.7 mg (Table VII). Protein stained electrophoresis gels of these two fractions (Figures 9-7 and 9-9, respectively) when compared to enzyme stained gels (Figures 9-8 and 9-10, respectively) indicated that some non- $\beta$ -hexosaminidase protein still contaminated the more basic enzyme isomers and that a considerable fraction of the total protein in the more acid enzyme fraction did not co-migrate with  $\beta$ -hexosaminidase activity.  $\beta$ -Hexosaminidase accounted for approximately 59% of the enzyme protein in the more basic enzyme fraction (Figure 14). This fraction represented the most highly purified preparation which was achieved with the large molecular weight forms of  $\beta$ -hexosaminidase.

Approximately 6.5% of the enzyme activity present in the HSS was recovered in the column filtrate following anion exchange chromatography pH 6.0 when enzyme activity was measured with the steroid substrate. As expected, the recovery of enzyme activity was lowered (to 3.2%) when PNP-NAcglc was used to assay enzyme activity. With both substrates,



The gel was placed in a Linear Transport Unit fitted to a Gilford 2400-2 Spectrophotometer and scanned at 1 cm/min at a wavelength of 540  $m\mu$  with an auxiliary slit width of 1.41 mm. Chart speed was 1 inch/min and sensitivity of the chart recorder to absorbance was set at the 0.2 level. Absorbance is expressed as a percentage of the full scale deflection of the pen on the chart recorder under these conditions.

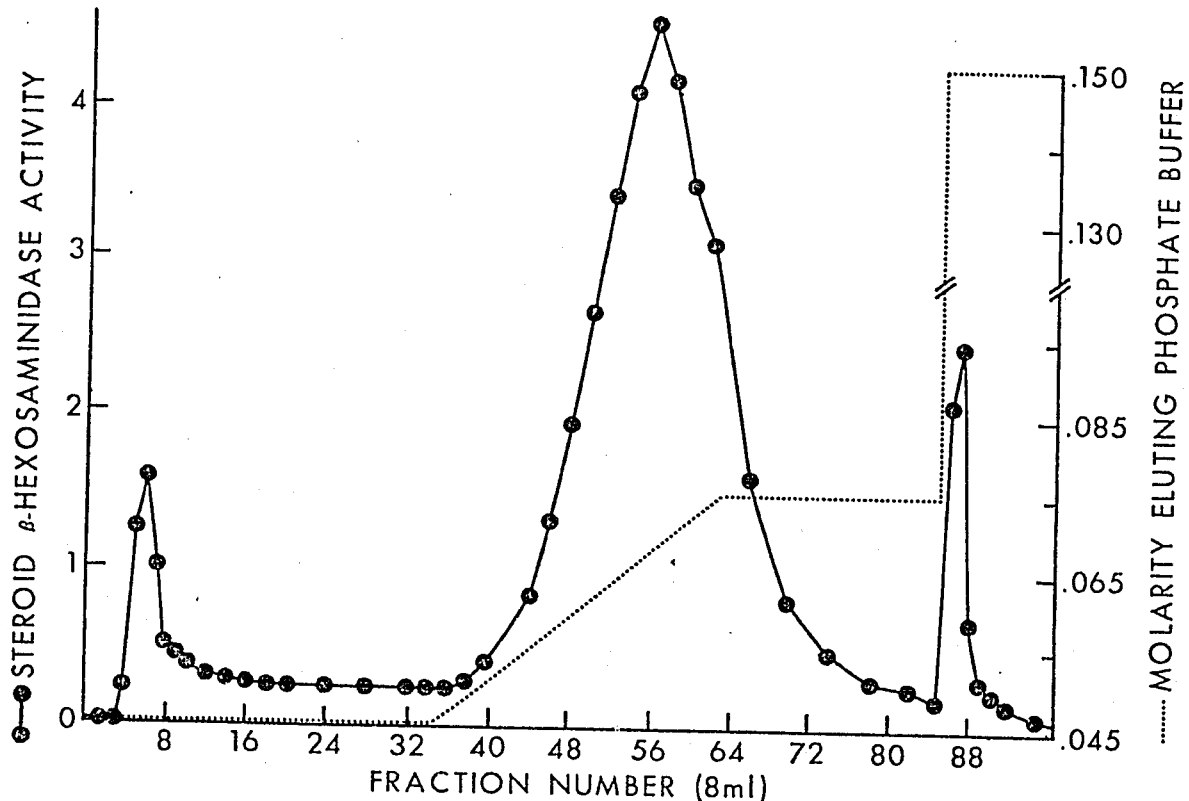
Figure 14. Scan at 540  $m\mu$  of Protein-stained Polyacrylamide Gel of Large Molecular Weight Enzyme Fraction Eluted from DEAE-Cellulose with 0.02 M phosphate pH 6.0

approximately 37% of the enzyme activity originally applied to the column was recovered in the DEAE pH 6.0 column filtrate.

Further purification of the small molecular weight forms of the enzyme from the Sephadex G-200 filtrate was achieved by DEAE-Cellulose anion exchange chromatography in phosphate buffer pH 7.4.

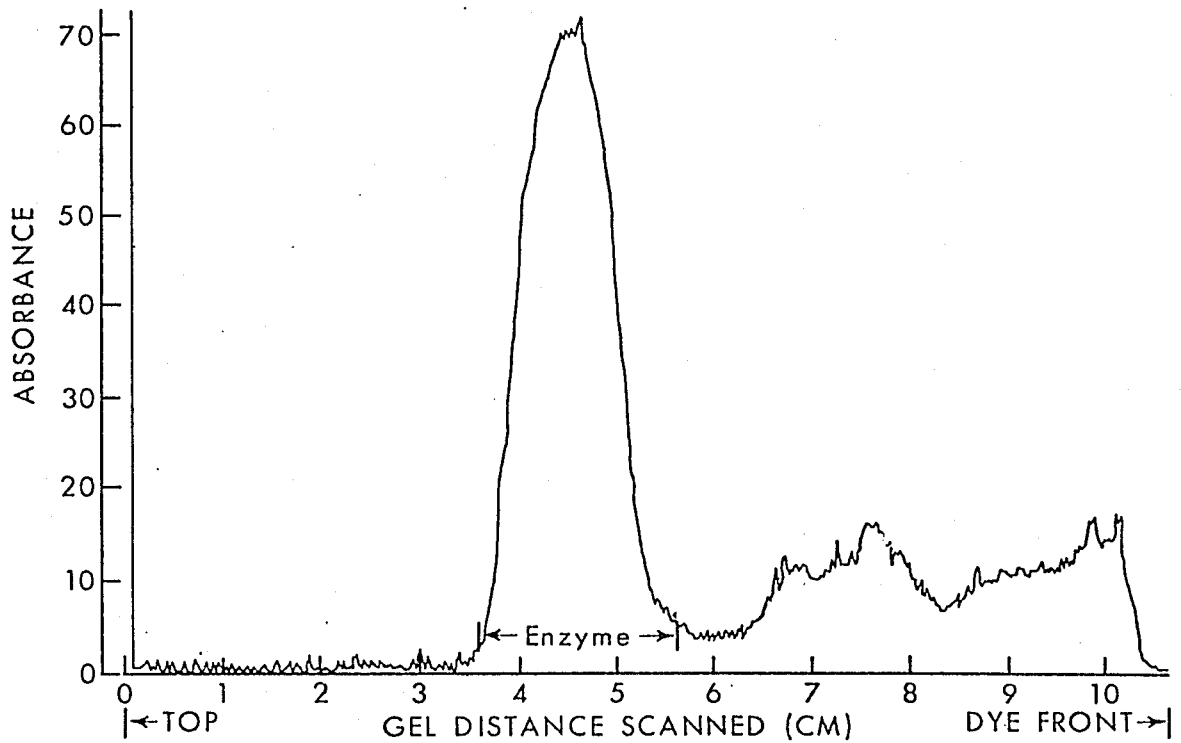
Figure 15 shows the enzyme profile which was obtained in the column filtrate following anion exchange chromatography. A small fraction of the total enzyme activity recovered in the column filtrate was eluted from the gel with 0.045 M phosphate buffer. This fraction contained the most basic isomers of the small MW enzyme population. More acid forms were retained on the gel under these conditions and were almost completely recovered in the column filtrate as the molarity of the eluting phosphate buffer was raised from 0.045 to 0.075. A residual amount of enzyme activity which was still bound to the gel at this point was eluted from the column with 0.15 M phosphate buffer.

The eluate fractions which contained the moderately charged small MW forms of the enzyme (eluted from the gel as the molarity of the phosphate buffer was raised from 0.045 to 0.075) were pooled and concentrated to a volume of about 2 ml in an Amicon cell. Approximately 7% (1.2 mg) of the protein originally applied to the column was recovered in this fraction. Protein and enzyme stained electrophoresis gels (Figures 9-11 and 9-12, respectively) showed that 64% of this protein (as determined by scanning protein stained gels at  $540\text{ m}\mu$ , Figure 16) migrated with the same  $R_f$  as  $\beta$ -hexosaminidase. Approximately 1.7% of the enzyme activity originally present in the HSS was recovered in this fraction when  $E_2\alpha$ -17NAcglc was used to measure enzyme activity. As expected, this value was increased (to approximately 2.9%) when PNP-NAcglc was used to assay enzyme activity.



Steroid  $\beta$ -hexosaminidase activity is expressed as picomoles  $E_2\alpha$ -17NAcglc hydrolysed per 0.2 ml eluate per 2 hours under standard assay conditions. Enzyme forms were eluted from the gel with phosphate buffer 7.4.

Figure 15. DEAE-Cellulose Anion Exchange Chromatography of the Small Molecular Weight Enzyme Fraction from the Sephadex G-200 Superfine Eluate



Scanning speed, auxiliary slit width and chart recorder sensitivity were the same as shown in Figure 14.

Figure 16. Scan at 540 m $\mu$  of Protein-stained Polyacrylamide Gel of Small Molecular Weight Enzyme Fraction Eluted from DEAE-Cellulose with 0.045 M - 0.075 M phosphate pH 7.4.

An overall summary of the purification procedure is presented in Tables VII and VIII. The data in these tables quantitatively describe the total units of enzyme activity present in preparations at each stage of purification as well as the percent recovery and fold purification of enzyme species. In both Tables, enzyme recovery and hence fold purification are gauged from the total activity of all enzyme species in the HSS. A different substrate is used to monitor enzyme activity in each table;  $17\alpha E_2$ -NACglc in Table VII and PNP-NACglc in Table VIII.

Following the first three steps of purification, protein was reduced in the enzyme preparation from approximately 150 grams in the HSS to 107 mg in the Con A Sepharose column filtrate. Approximately 36% of the HSS enzyme activity was recovered in the latter fraction with about a 500 fold increase in the specific activity of enzyme species. Enzyme species (with the exception of form A) remained intact as one unit throughout the first three steps of purification. Enzyme species were then split into two populations of different size by gel filtration. From this point on in the purification procedure, the fold purification which was computed for the large and small MW forms of  $\beta$ -hexosaminidase in enzyme fractions varied significantly with the substrate which was used to assay enzyme activity. As pointed out previously, these enzyme forms differed in their relative hydrolytic specificities toward  $E_2\alpha$ - $17$ NACglc and PNP-NACglc. A fold purification of approximately 2600 was obtained with the large MW enzyme forms following chromatography on columns of Sephadex G-200 superfine when enzyme activity was measured with the PNP compound. The fold purification was raised to 5400 when the steroid substrate was used. Following DEAE-Cellulose chromatography pH 6.0, there was very little increase in the specific activity (as measured with either substrate) of the more negatively charged forms of

Fraction	Total Protein mg	Activity		Specific Activity* picomoles/hr/mg protein	Purification*
		Total picomoles/hr	Yield* %		
High Speed Supernatant	147,750	38,146	100	0.258	1
Acidified Supernatant	25,410	12,562	32.9	0.494	1.9
0-60% Ammonium Sulfate	6,234	18,222	47.8	2.92	11.3
Con A Sepharose	108	13,694	35.9	126.8	491
<u>Large MW Forms</u>					
Sephadex G-200	5.03	6,996	18.3	1390	5386
DEAE-Cellulose pH 6.0 (0.02 M Phosphate)	0.47	1,489	3.9	3144	12,177
DEAE-Cellulose pH 6.0 (0.10 m Phosphate)	0.68	1,013	2.6	1499	5,807
<u>Small MW Forms</u>					
Sephadex G-200	17.2	3,929	10.3	228	886
DEAE-Cellulose pH 7.4	1.2	643	1.7	530	2054

\* CALCULATED FROM TOTAL  $\beta$ -HEXOSAMINIDASE ACTIVITY IN THE HIGH SPEED SUPERNATANT.

Table VII. Purification of Rabbit Liver  $\beta$ -Hexosaminidases: 17 $\alpha$ -Estradiol 17- $\beta$ -N-acetylglucosaminide as Substrate

Fraction	Total Protein	Activity		Specific Activity*	Purification*
		Total	Yield*		
	mg	micromoles/hr	%	micromoles/hr/mg protein	-fold
High Speed Supernatant	147,750	153,450	100	1.039	1
Acidified Supernatant	25,410	68,952	44.9	2.71	2.6
0-60% Ammonium Sulfate	6,234	72,485	47.2	11.6	11.2
Con A Sepharose	108	58,094	37.8	538	518
<u>Large MW Forms</u>					
Sephadex G-200	5.03	13,515	8.8	2686	2586
DEAE-Cellulose pH 6.0 (0.02 M Phosphate)	0.47	2,975	1.9	6330	6092
DEAE-Cellulose pH 6.0 (0.10 M Phosphate)	0.68	2,020	1.3	2970	2859
<u>Small MW Forms</u>					
Sephadex G-200	17.2	28,448	18.5	1657	1595
DEAE-Cellulose pH 7.4	1.2	4,392	2.9	3623	3489

\* CALCULATED FROM TOTAL  $\beta$ -HEXOSAMINIDASE ACTIVITY IN THE HIGH SPEED SUPERNATANT.

Table VIII. Purification of Rabbit Liver  $\beta$ -Hexosaminidases: p-Nitrophenyl- $\beta$ -N-acetylglucosaminide as Substrate

the large MW enzyme population. However, with the more basic large MW enzyme species, which were eluted from the anion exchange resin with 0.02 M phosphate buffer pH 6.0, the fold purification of enzyme isomers was increased about 2 1/4 times over that found in the Sephadex G-200 filtrate to 6092 (p-nitrophenyl compound) and to 12,177 (steroid substrate). With the small MW forms of the enzyme, the fold purification was always lower when E<sub>2</sub>α-17NAcglc was used to measure enzyme activity than when PNP-NAcglc was used. Thus while the specific activity of these forms following gel filtration was only enhanced by a factor of 1.8 over that from the Con A Sepharose filtrate with the former substrate, a factor of approximately 3 was obtained with the latter substrate. The specific activity was further increased by a factor of approximately 2 1/4 following DEAE-Cellulose chromatography pH 7.4. The fold purification of the moderately charged small MW forms of the enzyme was calculated to be 2054 with the steroid substrate and 3490 with PNP-NAcglc.

Throughout the latter stages of the purification procedure, higher and lower specific activities were obtained for large MW and small MW enzyme forms, respectively, when E<sub>2</sub>α-17NAcglc instead of PNP-NAcglc was used to measure enzyme activity. The differences in specific activity indicated that large MW enzyme species exhibited relatively greater activity than did small MW enzyme species towards the steroid substrate as compared to the p-nitrophenyl compound. A precise measure of the different abilities of large and small MW enzyme forms to hydrolyse each of these two substrates under standard assay conditions was obtained by comparing enzyme activity between the two differently sized enzyme populations on a per molecule and on a per weight basis. To do this, the amount of enzyme protein in milligrams in each of the two

enzyme fractions from the Sephadex G-200 filtrate was calculated, and then hydrolytic activity per mg enzyme protein was computed. Since the molecular weight of forms E to I was approximately 1.8 times that of forms B to D, the number of enzyme molecules of the latter species present in 1 mg enzyme protein would be 1.8 times that of the former. Accordingly, enzyme activity per mg enzyme protein obtained with the large MW forms was multiplied by a factor of 1.8 in order to permit direct comparison of enzyme activity between equal numbers of large and small MW enzyme molecules. Calculations are presented in greater detail in Appendix 1 at the end of this chapter. As Table IX shows, on a per molecule enzyme basis, large MW forms displayed 13.21 times the hydrolytic activity of small MW forms with the steroid compound and 3.52 times the hydrolytic activity with the p-nitrophenol compound. Even when enzyme activity was compared on a straight weight basis, large MW isomers hydrolysed both substrates more rapidly than did small MW isomers (7.34 times more rapidly with E<sub>2</sub>α-17NAcglc, 1.96 times more rapidly with PNP-NAcglc). The difference in substrate turnover between large and small MW forms was more pronounced with E<sub>2</sub>α-17NAcglc than with PNP-NAcglc.

The activity per mg enzyme protein calculated for large and small sized enzyme species in the Sephadex G-200 column filtrate with E<sub>2</sub>α-17-NAcglc and PNP-NAcglc was used in concert with the total enzyme activity found in the high speed supernatant (HSS) with both substrates to estimate the amount of each differently sized enzyme population, in milligrams, present at the start of the purification procedure in the liver extract. Calculations are shown in Appendix 2. The HSS, derived from 10 rabbit livers, was estimated to contain 5.72 mg of the large MW enzyme species and 33.26 mg of the small MW enzyme species. Therefore,

Basis of Comparison of Activity	Substrate	Ratio Enzyme Activity Large Forms:Small Forms
Molecular Equivalents	E <sub>2</sub> α-17NAcglc	13.21:1
	PNP-NAcglc	3.52:1
Equivalent Weight	E <sub>2</sub> α-17NAcglc	7.34:1
	PNP-NAcglc	1.96:1

Enzyme activity with both substrates was determined under routine assay conditions. A detailed derivation of the above data is presented in Appendix 1.

Table IX. Comparison of the Hydrolytic Activity of Large Molecular Weight and Small Molecular Weight Enzyme Forms toward E<sub>2</sub>α-17NAcglc and PNP-NAcglc on a per Molecule and a per Weight Enzyme Basis

each rabbit liver (approximately 120 grams in weight) contributed an average of 0.572 mg of large MW enzyme forms and 3.326 mg of small MW enzyme forms to the liver extract. The concentration by weight of large MW enzyme forms in the HSS was only about 1/5 - 1/6 of the small MW enzyme forms but, as stated earlier, the former exhibited appreciably greater hydrolytic activity with both the steroid substrate and the p-nitrophenyl substrate.

In the summaries of the purification procedure presented earlier in Tables VII and VIII, enzyme recoveries were calculated as a percentage of the total enzyme activity in the HSS. Table X shows the recoveries of enzyme species and their fold purification in highly purified fractions when the former parameter is calculated as a percentage of the total activity of only similarly sized forms in the HSS. The total activity of each of the two differently sized enzyme populations in the HSS was determined by multiplying the total amount of each by the enzyme activity per mg enzyme protein. With this method, the percent recovery and fold purification of enzyme species were independent of the substrate used to measure enzyme activity. Table X shows that large MW enzyme forms in the 0.02 M phosphate eluate from the DEAE-Cellulose pH 6.0 column were enriched approximately 23,800 times over those in the HSS while small MW enzyme forms following anion exchange chromatography were enriched approximately 4850 times over corresponding forms in the HSS. Approximately 35% of the large MW population from the HSS and 25% of the small MW enzyme population were recovered in the Sephadex G-200 filtrate. Both enzyme populations, therefore, were roughly equal in their stability to treatments throughout the first four steps in the purification procedure.

Fraction	Total Protein	17 $\alpha$ -Estradiol 17- $\beta$ -N-Acetyl-glucosaminide			p-Nitrophenyl- $\beta$ -N-Acetyl-glucosaminide		
		Total Activity	Yield	Purification	Total Activity	Yield	Purification
	mg	picomoles/hr	%	-fold	picomoles/hr	%	-fold
<u>Large MW Forms</u>							
High Speed Supernatant	147,750	20,008	100	1	38,650	100	1
Sephadex G-200	5.03	6,996	35	10,270	13,515	35	10,271
DEAE-Cellulose pH 6.0 (0.02 M phosphate)	0.47	1,489	7.44	23,395	2,975	7.69	24,197
<u>Small MW Forms</u>							
High Speed Supernatant	147,750	15,848	100	1	114,813	100	1
Sephadex G-200	17.2	3,929	25	2,125	28,448	25	2,128
DEAE-Cellulose pH 7.4	1.2	643	4.06	4,985	4,392	3.83	4,710

Table X. Purification of Rabbit Liver  $\beta$ -Hexosaminidases: Based on the Activity of Corresponding Enzyme Forms in the High Speed Supernatant

Preliminary investigations with crude preparations of the enzyme (Chapter 3) had indicated that rabbit liver  $\beta$ -hexosaminidase possessed  $\beta$ -N-acetylgalactosaminidase activity along with  $\beta$ -N-acetylglucosaminidase activity. In order to determine whether these two activities underwent the same percentage recovery throughout the purification of  $\beta$ -hexosaminidase, enzyme activity was measured separately with p-nitrophenyl- $\beta$ -N-acetylgalactosaminide (PNP-NAcgal) and PNP-NAcglc in enzyme preparations after each stage of purification. Ratios of the two activities were also calculated for enzyme fractions throughout the purification procedure to determine whether different forms of the enzyme exhibited the same relative hydrolytic specificity toward the two p-nitrophenyl compounds. PNP-NAcgal and PNP-NAcglc were particularly well suited for the comparison of  $\beta$ -N-acetylgalactosaminidase and  $\beta$ -N-acetylglucosaminidase activity since, with their common aglycone moiety, only the effect of the two different sugar residues on the catalytic mechanism of  $\beta$ -hexosaminidase was being measured. The results which were obtained with these two substrates are shown in Table XI. With both substrates, similar percentages of enzyme activity were recovered following each stage of purification indicating that neither  $\beta$ -N-acetylgalactosaminidase nor  $\beta$ -N-acetylglucosaminidase activity purified independently of the other throughout the purification procedure. The ratios of the two activities showed that large and small MW forms of the enzyme in all preparations which were tested displayed a marked but similar preference for the  $\beta$ -N-acetylglucosaminide compound over the  $\beta$ -N-acetylgalactosaminide compound. About 5.45 (5.21-5.63) times more p-nitrophenol was liberated by enzyme action from the former compound than from the latter under standard assay conditions.

The ratio of enzyme activities with these two substrates was also

Fraction	Percent Activity Recovered †		Activity Ratio *
	$\beta$ -N-Acetylglucosaminidase	$\beta$ -N-Acetylgalactosaminidase	
High Speed Supernatant	100	100	5.42
Acidified Supernatant	-	-	-
Ammonium Sulfate	47.2	48.5	5.28
Con A Sepharose	37.9	38.7	5.30
<u>Large MW Forms</u>			
Sephadex G-200	8.8	8.7	5.43
DEAE-Cellulose pH 6.0 (0.02 M Phosphate)	1.9	2.0	5.21
<u>Small MW Forms</u>			
Sephadex G-200	18.5	17.8	5.63
DEAE-Cellulose pH 7.4	2.9	2.9	5.32

\* ACTIVITY RATIOS ARE EXPRESSED AS  $\frac{\text{MICROMOLES PNP-NAcglc hydrolysed/hr/fraction}}{\text{MICROMOLES PNP-NAcgal hydrolysed/hr/fraction}}$   
 ENZYME ASSAYS WITH BOTH SUBSTRATES WERE CARRIED OUT UNDER ROUTINE ASSAY CONDITIONS.

† BASED ON TOTAL ACTIVITY IN THE HSS.

Table XI.  $\beta$ -N-Acetylglucosaminidase and  $\beta$ -N-Acetylgalactosaminidase Activity: Recovery and Ratio of the Two Activities at Each Step in the Purification Procedure

determined for charge isomers of the enzyme, forms B through to I, following isoelectric focussing of HSS in pH 4-8 ampholyte gradients (Figure 6). Ratios of  $\beta$ -N-acetylglucosaminidase to  $\beta$ -N-acetylgalactosaminidase activity for these enzyme forms are shown in Table XII. Slight differences in activity ratios were observed among the various enzyme forms, but on the whole values were fairly constant suggesting that large and small MW charge isomers of the enzyme possessed the same relative ability to hydrolyse sugar residues from PNP-NAcglc and PNP-NAcgal. This is exactly what was found in enzyme fractions throughout the purification procedure (Table XI). Activity ratios with enzyme forms from the isoelectric focussing experiment were, for reasons not readily evident, slightly higher than those which were obtained with enzyme species throughout the purification procedure.

In addition to monitoring  $\beta$ -hexosaminidase activity with a number of substrates in enzyme preparations as purification progressed, activities of several other glycosidases and phenolsulfatase were measured with their respective p-nitrophenyl glycoside or sulfate substrates (and also with  $17\alpha$ -estradiol 3- $\beta$ -glucuronide in the case of  $\beta$ -glucuronidase) in order to follow the progressive loss of these enzymes from  $\beta$ -hexosaminidase fractions with increasing purification, and also to provide an additional yardstick to judge the effectiveness of each step in the purification procedure in removing contaminating protein from  $\beta$ -hexosaminidase. The results from this study are shown in Table XIII. None of the enzymes which were monitored could be found in the most highly purified preparations of the large and small MW forms of  $\beta$ -hexosaminidase. The absence of  $\alpha$ -N-acetylglucosaminidase activity indicated that rabbit liver  $\beta$ -hexosaminidase specifically cleaved only

Enzyme Form	Activity Ratio
	$\frac{\text{Micromoles PNP-NAcglc hydrolysed/hr/form}}{\text{Micromoles PNP-NAcgal hydrolysed/hr/form}}$
B	5.64
C	6.18
D	5.73
E	5.95
F	6.12
G	6.05
H	6.18
I	6.02

ENZYME SPECIES IN HIGH SPEED SUPERNATANT WERE SEPARATED FROM ONE ANOTHER BY ISOELECTRIC FOCUSING IN A pH 4-8 AMPHOLYTE GRADIENT. ASSAYS WITH EACH SUBSTRATE WERE CARRIED OUT UNDER ROUTINE ASSAY CONDITIONS.

Table XII. Comparison of  $\beta$ -N-Acetylglucosaminidase and  $\beta$ -N-Acetylgalactosaminidase Activity of Enzyme Forms B to I in the High Speed Supernatant

Fraction	Enzyme Activity*							
	$\alpha$ -N-acetyl-glucosaminidase	phenol-sulfatase	$\alpha$ -mannosidase	$\alpha$ -glucosidase	$\alpha$ -galactosidase	$\beta$ -glucosidase	$\beta$ -galactosidase	$\beta$ -glucuronidase
High Speed Supernatant	756	272	12,600	7,056	N.D.†	441,000	240,600	340,700 (7.7x10 <sup>6</sup> )†
0-60% Ammonium Sulfate	24	49	24	49	32	74	254	0 (22)
Con A Sepharose	2	8	2	30	23	2	163	0 (18)
<u>Large MW Fractions</u> Sephadex G-200	0	0	0	0	6	0	23	0 (16)
DEAE-Cellulose pH 6.0	0	0	0	0	0	0	0	0 (0)
<u>Small MW Fractions</u> Sephadex G-200	0	2	0	0	10	0	8	0 (0)
DEAAE-Cellulose pH 7.4	0	0	0	0	0	0	0	0 (0)

\* EXPRESSED AS MICROMOLES OF THE APPROPRIATE P-NITROPHENYL GLYCOSIDE OR SULFATE (Section B.3.II, Chapter 2) HYDROLYSED PER HOUR. ACTIVITY WAS DETERMINED AS DESCRIBED UNDER GENERAL METHODS.

‡ NON-DETECTABLE

† DATA IN BRACKETS REFERS TO ACTIVITY TOWARDS 17  $\alpha$ -ESTRADIOL 3- $\beta$ -GLUCURONIDE AND IS EXPRESSED AS PICOMOLES HYDROLYSED PER HOUR USING THE ASSAY CONDITIONS DESCRIBED UNDER GENERAL METHODS.

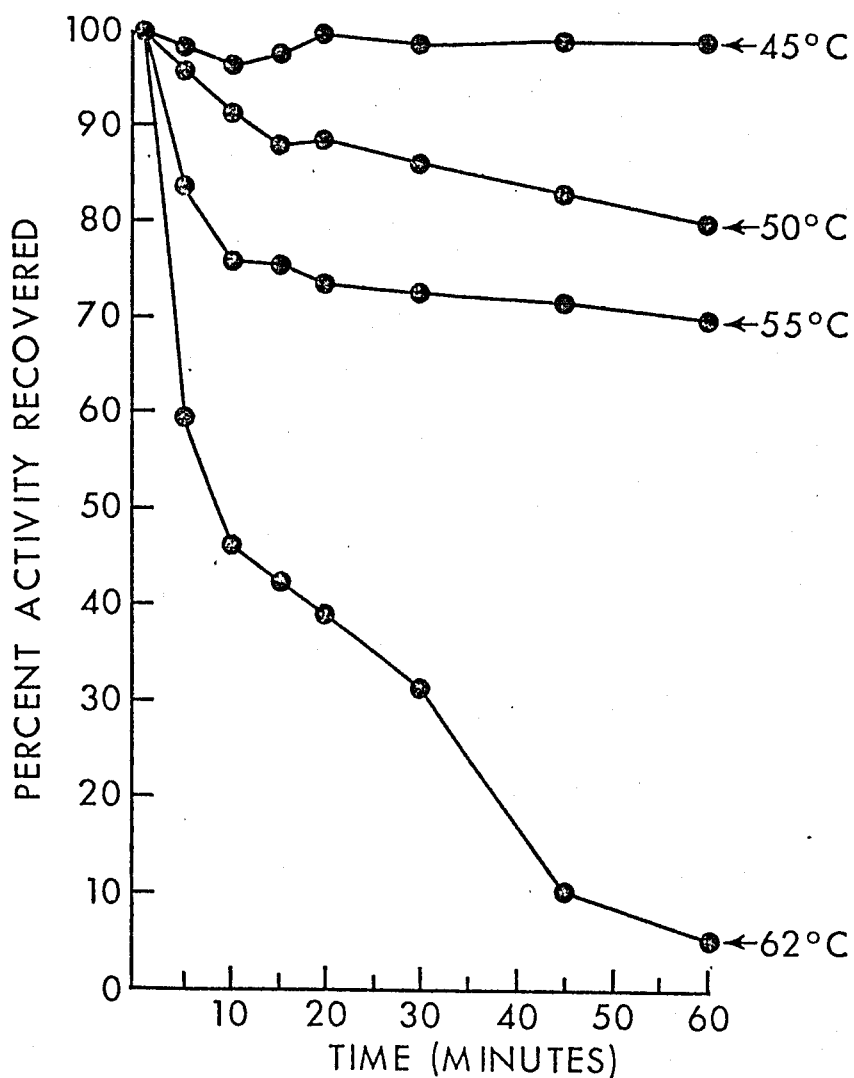
Table XIII. Glycosidase and Phenosulfatase Activity in Fractions at Various Stages during the Purification Procedure

$\beta$ -glycosidically linked N-acetylhexosamine.

2) Physical Studies on Highly Purified Enzyme Forms

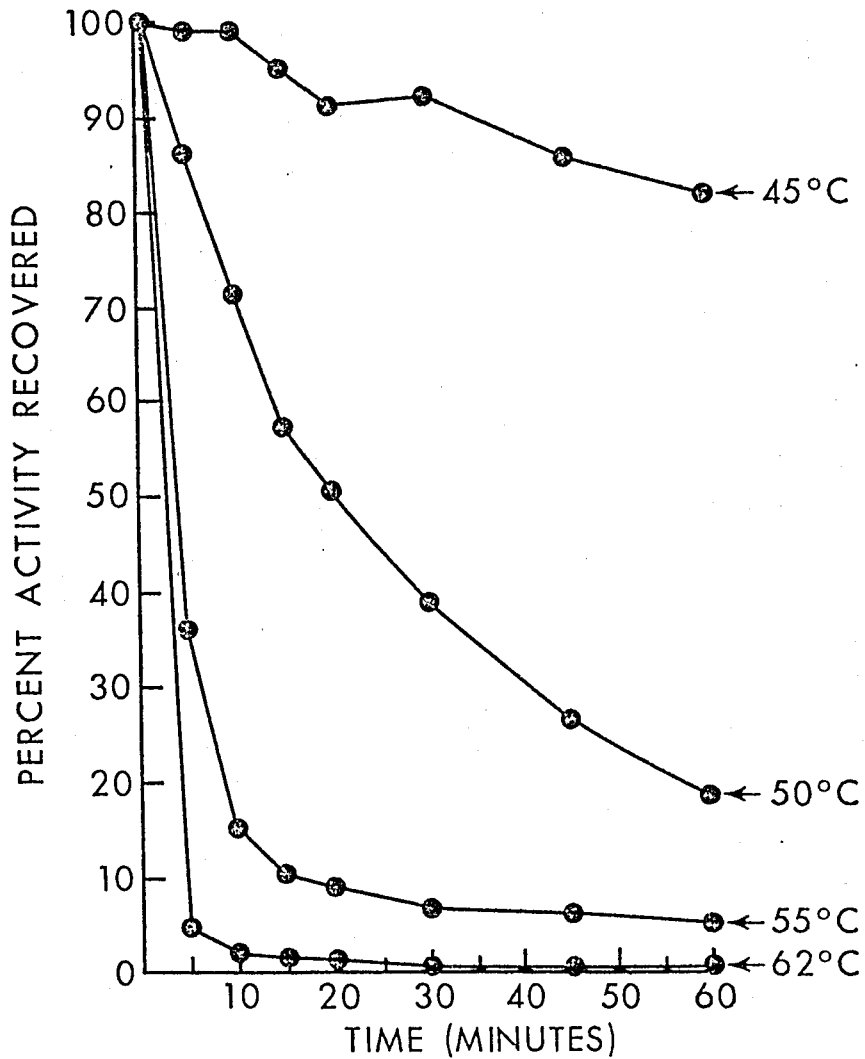
The stability of large and small MW enzyme forms in the Sephadex G-200 column filtrate to increasing temperature was determined by incubating aliquots of each enzyme fraction for varying lengths of time in 0.1 M citrate buffer pH 4.4 - 0.01% BSA at four different temperatures. Temperature stability curves for large and small MW enzyme species at 45°C, 50°C, 55°C and 62°C are shown in Figure 17 and Figure 18 respectively. At all temperatures examined, a greater percentage of the starting enzyme activity was recovered with time for large MW as opposed to small MW enzyme forms. Both differently sized enzyme populations were relatively stable to incubation at 45°C for 1 hour. Forms E to I, large MW enzyme species, were also fairly stable at 50°C and 55°C losing only 15% and 25% enzyme activity, respectively, over 1 hour. Small MW enzyme species, forms B to D, however, were far more susceptible to denaturation at 50°C and 55°C. Approximately 80% of the enzyme activity of these forms was lost after 1 hour at 50°C while at 55°C, 90% enzyme activity was lost after the first 15 minutes of incubation and another 5% over the next 45 minutes. Small MW forms were essentially totally denatured after 5 minutes at 62°C while large MW forms were denatured more slowly with approximately 95% enzyme activity being lost after 60 minutes at 62°C. The biphasic temperature stability curve which was obtained with the large MW enzyme population at 62°C suggested that individual enzyme species were affected to differing degrees by the temperature.

Enzyme species in three different preparations - the Con A Sepharose column filtrate, and the large and small MW fractions from the Sephadex G-200 superfine column filtrate - were examined by the freezing and



Large MW forms from the Sephadex G-200 eluate were heated to four different temperatures for varying lengths of time and then assayed for activity. (See text for further details).

Figure 17. Susceptibility to Denaturation by Heat:  
Large Molecular Weight Enzyme Forms



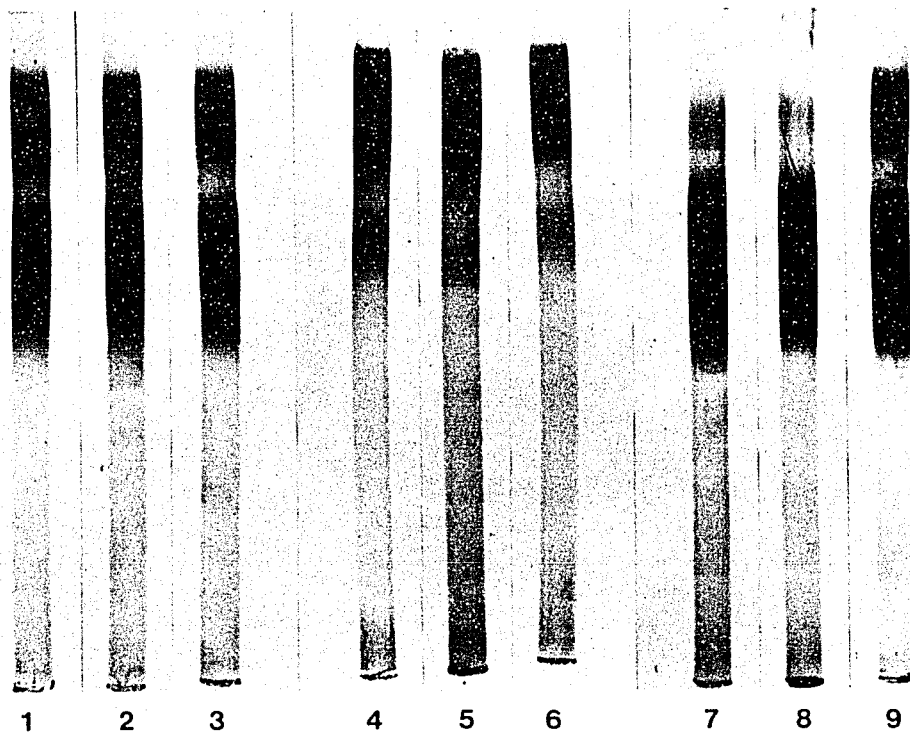
Small MW forms from the Sephadex G-200 eluate were heated to four different temperatures for varying lengths of time and then assayed for activity. (See text for further details).

Figure 18. Susceptibility to Denaturation by Heat: Small Molecular Weight Enzyme Forms

thawing method of Beutler and Kuhl (1975) to test for interconversion between large and small MW enzyme forms. Separate aliquots from each of the three preparations were either left at 4°C, frozen and thawed 4 times in a dry ice-acetone bath in the absence of salt or frozen and thawed 4 times in a dry ice-acetone bath in the presence of 3M NaCl. There was no loss of enzyme activity in any of the three preparations following freezing and thawing in the absence of added salt. In 3M NaCl, however, the large MW forms from the Sephadex G-200 filtrate lost about 5% activity when frozen and thawed, while activity losses for the two other preparations, comprised in part or entirely of small MW forms, ranged from 20% to 25%.

Treated aliquots from all three preparations were suitably processed for polyacrylamide disc gel electrophoresis and applied to gels. Following electrophoresis, gels were stained to visualize enzyme activity (Figure 19). As expected, interconversion of large and small MW enzyme forms was not seen with any of the three differently treated aliquots (gels 1, 2, 3) of the Con A Sepharose preparation since enzyme forms of both sizes were already present in this preparation to mask any additional partial conversion of one sized form to the other. With the large MW enzyme species from the Sephadex G-200 column filtrate (gels 4, 5, 6), there was no shift in staining from the large to the small MW "region" on the gels, indicating that forms E to I were not converted to new enzyme forms under the experimental conditions. In the same vein, small MW forms of the enzyme were not converted to large MW forms following incubation at 4°C or freezing and thawing in the absence of salt. This was concluded when gels 7 and 8 failed to show staining in regions where the large MW enzyme forms would be expected to migrate. Treatment with 3 M NaCl together with

## A FREEZE-THAW EXPERIMENT



Con A Sepharose fraction -- gels 1, 2, 3.  
Sephadex G-200 Large MW Enzyme fraction --  
gels 4, 5, 6.  
Sephadex G-200 Small MW Enzyme fraction --  
gels 7, 8, 9.

Fractions on gels 1, 4 and 7 were left at 4°C prior to electrophoresis; those on gels 2, 5 and 8 were frozen and thawed four times in 0.06 M potassium phosphate pH 7.0 prior to electrophoresis; and those on gels 3, 6, and 9 were frozen and thawed four times in 0.06 M potassium phosphate pH 7.0 - 3 M NaCl prior to electrophoresis.

Figure 19. A Freeze-Thaw Experiment

freezing and thawing, however, did definitely induce conversion of small MW forms to enzyme species which migrated with the same  $R_f$  on polyacrylamide gels following electrophoresis as forms E to I, the large MW enzyme forms. Whether these newly formed enzyme species were actually forms E to I was never directly established. It seemed unlikely, however, with  $R_f$  values on polyacrylamide gels being a function of both size (sieving by the gel) and charge (anodal attraction) that enzyme forms other than forms E to I would co-migrate with the latter. Furthermore, previous work by Beutler and Kuhl (1975) with human placental  $\beta$ -hexosaminidase had established that converted forms of the enzyme corresponded to pre-existing enzyme species. In all likelihood then, newly formed enzyme species in the present work with rabbit liver  $\beta$ -hexosaminidase corresponded to forms E to I.

There could be little doubt from the experimental data that small MW forms of the enzyme contained all the different types of subunits which comprised large MW enzyme forms, since reaggregation of subunits of the former gave enzyme forms which appeared to be identical with the latter. What could not be established conclusively from the experimental data, however, was whether the reverse was also true, namely whether large MW enzyme species were also composed of all the types of subunits present in the small MW enzyme species. This ambiguity remained because two different modes of subunit rearrangement could be postulated to account for the formation of large MW forms from small MW forms of the enzyme. On the one hand, if large MW enzyme species were formed from small MW forms by the complete reaggregation of dissociated subunits from two molecules of the latter (molecular weights of large and small MW forms were in the approximate ratio of 2:1, see Figure 12), then this would mean that both

differently sized enzyme species shared the same types of subunits. Conversely, if formation of large MW enzyme species was the result of the re-aggregation of only certain types of subunits within small MW enzyme forms (from two, three or more enzyme molecules), then this would mean that isomer forms E to I lacked one or more of the polypeptide(-carbohydrate) chains present within isomer forms B to D. The experimental data seemed to point to the latter interpretation. If large and small MW weight enzyme forms shared the same subunits, it seemed likely that the conversion of the latter enzyme forms to the former would be mimicked by the conversion of the former forms to the latter under the same experimental conditions. Yet the experimental data conclusively showed that this was not the case. However, if large MW forms of the enzyme lacked subunits present in the small MW forms, then it would be expected that conversion of the former forms to the latter would not occur under any experimental conditions, including those used in the present study.

In an attempt to obtain a more complete understanding of the subunit composition of the enzyme forms, large and small MW enzyme species were treated with SDS to disrupt quaternary protein structure and the dissociated subunits were examined directly on SDS-polyacrylamide gels following electrophoresis. Only the most highly purified fractions of large and small MW enzyme species were used for this work, namely the basic enzyme fraction from the DEAE-Cellulose pH 6.0 column filtrate in the case of large MW enzyme forms and the moderately charged enzyme fraction from the DEAE-Cellulose pH 7.4 column filtrate in the case of small MW enzyme forms.

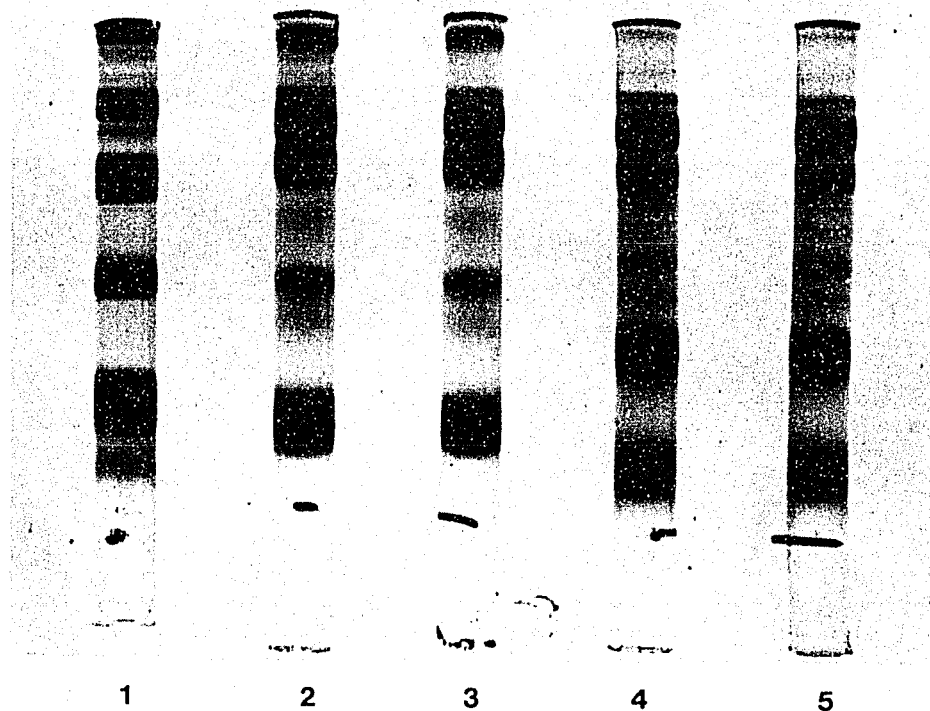
An examination of gels 7-8 and 11-12 in Figure 9 shows that enzyme species in these highly purified preparations were still contaminated by some non-enzyme protein. However, contaminating protein in these pre-

parations (especially in the case of the large MW enzyme fraction where contamination was more severe) stained in a fairly diffuse manner on polyacrylamide gels. This indicated that residual amounts of many non-enzyme species rather than major amounts of one or two were present in the enzyme fractions. Because of this, it was considered likely that contaminating protein components would not obscure the staining pattern of enzyme protein components to any significant degree on SDS-polyacrylamide gels following electrophoresis.

Aliquots of the two highly purified enzyme preparations were incubated in 1% SDS-0.02 M sodium phosphate buffer pH 7.0 at 100°C for either 2 or 7 minutes in order to disrupt quaternary protein structure and to dissociate enzyme subunits. Four highly purified protein standards of known molecular weight were similarly treated for 2 minutes. Aliquots of these heated preparations were suitably processed for SDS-polyacrylamide gel electrophoresis and applied to 10% polyacrylamide gels. Following electrophoresis, protein bands were visualized by staining gels with Coomassie Blue. Gels are shown in Figure 20. The similarity of protein staining on SDS gels between the 2 and 7 minute heated enzyme preparations (gels 2 and 3, gels 4 and 5) indicated that non-enzymatic hydrolysis of peptide bonds did not occur during the dissociation of enzyme samples in 1% SDS at 100°C. Any increase in the intensity of staining of low MW protein bands in 7 minute as opposed to 2 minute heated enzyme preparations would have indicated the generation of spurious protein fragments which were artifacts of the heating step at 100°C (Weber, Pringle and Osborn, 1972).

The protein-stained gels showed that both large and small MW enzyme forms were fragmented by SDS to give a very large sized protein fragment

## SDS GEL ELECTROPHORESIS



Protein standards were run on gel 1; large MW enzyme species heated for 2 and 7 minutes in 1% SDS at 100°C on gels 2 and 3 respectively; and small MW enzyme species heated for 2 and 7 minutes in 1% SDS at 100°C on gels 4 and 5 respectively. Pins inserted into the gel represent the dye front. Electrophoresis was carried out in 10% acrylamide gels.

Figure 20. SDS Gel Electrophoresis

weighing well over 100,000 Daltons. This protein fragment was stained more prominently in preparations of dissociated large MW as opposed to small MW enzyme species. Both enzyme preparations when dissociated by SDS gave five additional protein fragments which were prominently stained on SDS gels. Of these, the three largest fragments from preparations of both large and small MW enzyme forms migrated with the same Rf values. The two smaller protein fragments, however, exhibited different migration characteristics on SDS gels with each preparation. The molecular weight and relative intensity of staining of these six major protein fragments from preparations of the large and small MW enzyme species are shown in Table XIV.

The molecular weight of the enzyme protein fragments was estimated from a standard curve (Figure 21) derived by plotting Rf values obtained on gel 1 (Figure 20) with the four protein standards of known molecular weight against the logarithm of their respective molecular weights. Staining intensity of protein fragments was determined by scanning gels at  $540\text{ m}\mu$  in a Gilford Spectrophotometer as described previously. Absorption profiles at  $540\text{ m}\mu$  of protein bands from dissociated large and small MW enzyme preparations are shown in Figure 22 and Figure 23 respectively. Absorbance values of individual protein bands were assumed to follow Beer's Law and to vary linearly with the intensity of staining. The relative amount of each protein fragment in each enzyme preparation was determined by calculating the area under the corresponding peak in the absorption profile and then normalizing values with respect to the 59,000 Dalton protein fragment.

The 270,000 Dalton large MW enzyme species gave 3 major protein fragments weighing 59,000 Daltons, 45,500 Daltons and 10,000 Daltons which

Population	R <sub>f</sub> of band	Molecular Weight	Relative Intensity of Staining
Large Forms	0.05	>100,000	-
	0.167	69,000	-
	0.219	59,250	1
	0.302	45,500	0.94
	0.526	24,250	0.086
	0.833	10,000	0.509
Small Forms	0.05	>100,000	-
	0.167	69,000	-
	0.228	58,500	1
	0.307	45,500	0.245
	0.649	17,000	0.625
	0.886	<10,000	0.30

R<sub>f</sub> of fragments from Figure 20. Molecular weight of fragments from Figure 21. Relative intensity of staining of fragments from Figures 22 and 23.

Table XIV. SDS-treated Large Molecular Weight and Small Molecular Weight Enzyme Forms: Molecular Weight and Staining Intensity of Protein Fragments

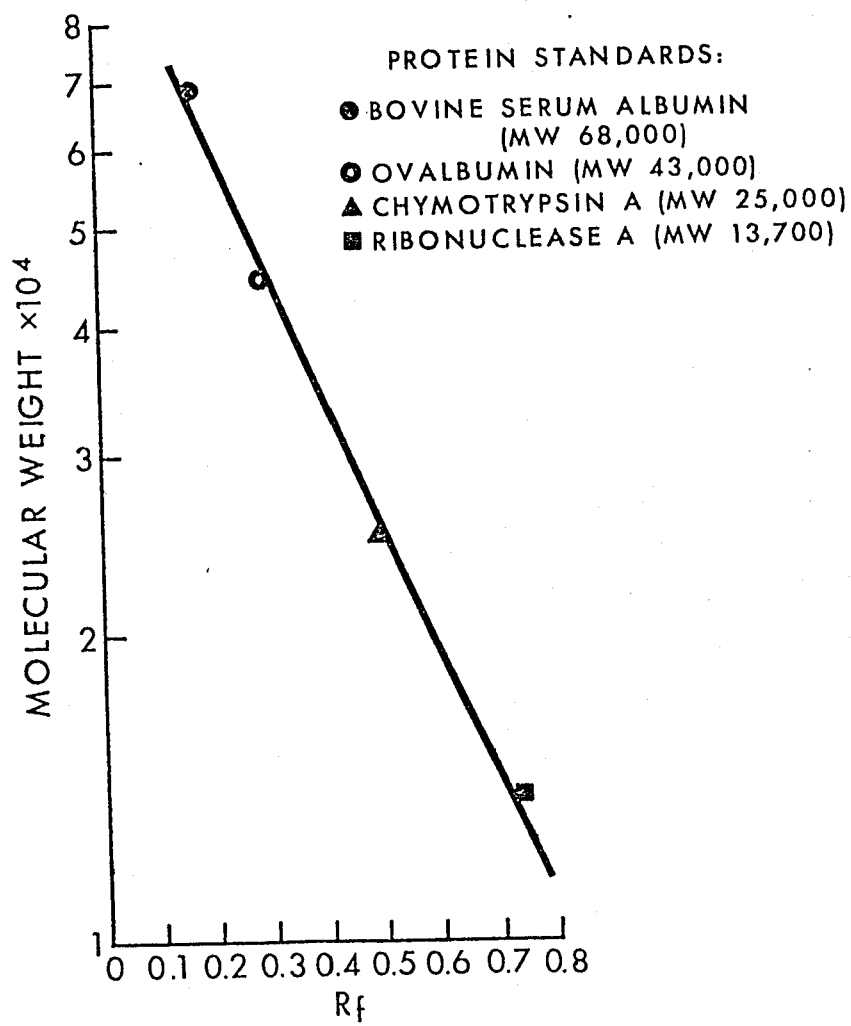
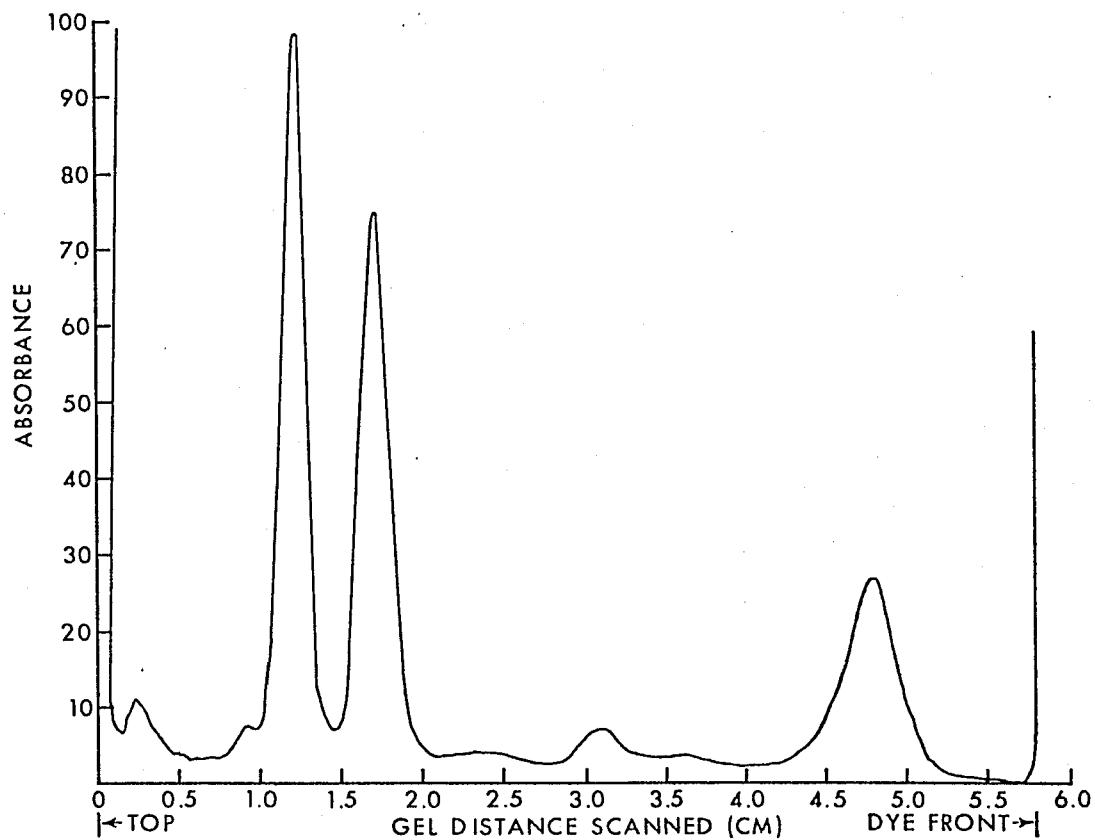
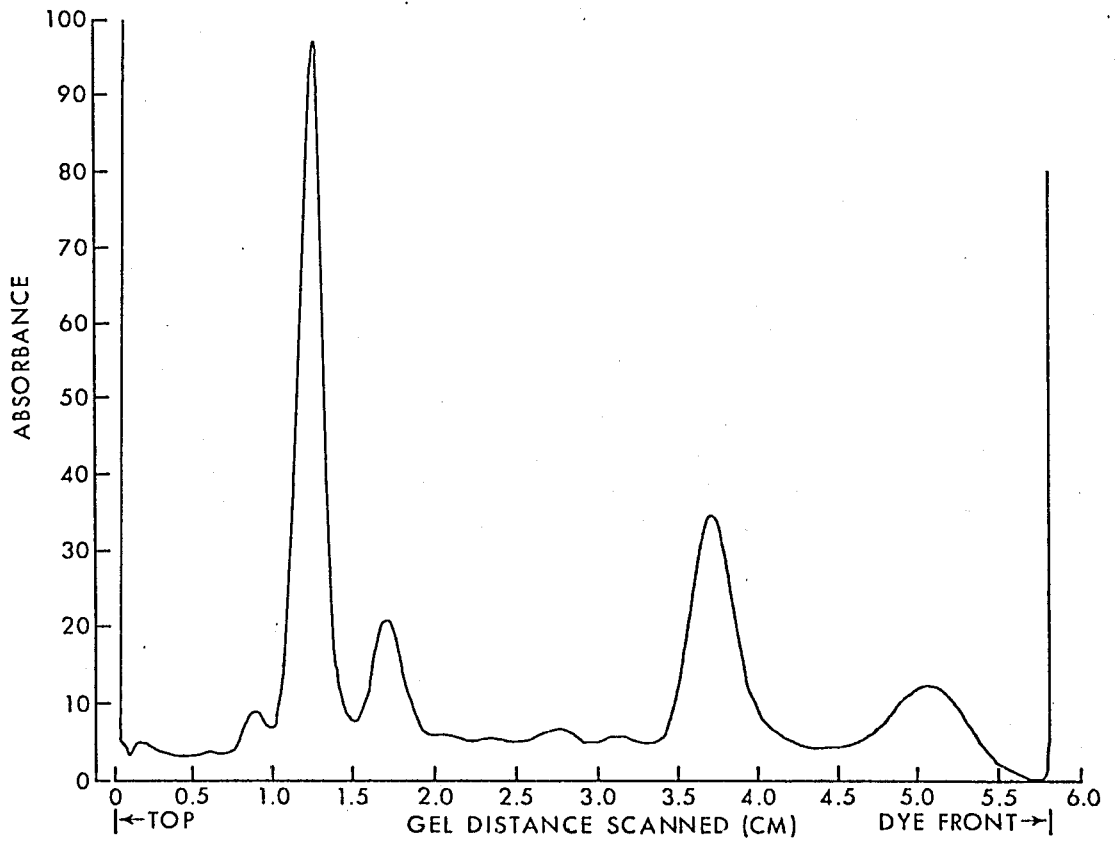


Figure 21. Calibration Curve for the Determination of Molecular Weight on SDS-polyacrylamide Gels



Gel #2 from Figure 20 was placed in a Linear Transport Unit fitted to a Gilford 2400-2 Spectrophotometer and scanned at 0.5 cm/min at a wavelength of 540  $m\mu$  with an auxiliary slit width of 1.38 mm. Chart speed was 1 inch/min and sensitivity of the chart recorder to absorbance was set at the 1.7 level. Absorbance is expressed as a percentage of the full scale deflection of the pen on the chart recorder under these conditions.

Figure 22. Scan at 540  $m\mu$  of Protein-stained Polyacrylamide Gel of SDS-treated Large Molecular Weight Enzyme Forms



The above scan is of gel #4 Figure 20. Scanning speed, auxiliary slit width and chart recorder sensitivity were the same as shown in Figure 22.

Figure 23. Scan at 540 m $\mu$  of Protein-stained Polyacrylamide Gel of SDS-treated Small Molecular Weight Enzyme Forms

were present in an approximate ratio of 1:1:0.5, respectively. The very large MW protein fragment ( $>100,000$  Daltons) as well as the two fragments which weighed 69,000 Daltons and 24,000 Daltons were present in far lower amounts than the three major fragments. Disregarding these minor protein fragments, a subunit composition of two 59,000 MW subunits, two 45,000 MW subunits and one 10,000 MW subunit was tentatively postulated for the large MW enzyme forms giving a total molecular weight of 219,000 Daltons. While this value was considerably lower than the 270,000 MW calculated for the large MW enzyme species from the calibrated Sephadex G-200 superfine column, there were reasons to suspect that the molecular weight as determined by gel filtration was inflated. These reasons are presented in the Discussion Section later in this chapter.

Following disruption by SDS of the quaternary protein structure of small MW enzyme forms, four major protein fragments weighing 59,000 Daltons, 45,500 Daltons, 17,000 Daltons and slightly less than 10,000 Daltons were obtained on SDS gels in the approximate ratio of 1:0.25:0.625:0.30, respectively. The two smallest major protein fragments, in order of decreasing size, were present in the approximate ratio of 2:1 which suggested, in combination with their molecular weights of 17,000 Daltons and  $<10,000$  Daltons respectively, that they arose from the fragmentation of the 45,500 Dalton protein fragment into three sub-fragments, two of which weighed 17,000 Daltons and one of which weighed  $<10,000$  Daltons. In all likelihood then, the 59,000 Dalton and 45,500 Dalton (including sub-fragments) subunits were present in equal amounts within the small MW enzyme species and the enzyme species were composed of a dimer structure. Such a dimer structure gave a total molecular weight of 104,500 Daltons which was considerably lower than the 150,000 Dalton molecular weight estimated for these forms from gel filtration data. As for the large MW enzyme forms,

however, the molecular weight obtained for the small MW enzyme forms from the calibrated Sephadex column was probably inflated.

Although the fragmentation pattern of enzyme species following treatment with SDS was sufficiently complex and difficult to interpret as to preclude any unequivocal assessment of subunit structure, the subunit structure mentioned above for the large and small MW enzyme forms were considered to offer the most plausible explanation of the experimental results from the SDS studies. In all likelihood, however, the pentamer and dimer structures postulated for the large and small MW enzyme forms, respectively, did not truly represent their subunit composition since they failed to adequately explain the conversion of small to large MW enzyme forms in the freezing and thawing - salt experiment. Their most serious failing stemmed from the indication of a subunit or protein fragment (10,000 MW) which was present exclusively in the large MW enzyme forms. This meant, of-course, that subunits from small MW enzyme species could not rearrange under any experimental conditions to form large MW enzyme species. Yet it is exactly this rearrangement which was found in the salt-conversion experiment. Because of the lack of correlation between SDS and salt conversion studies, a final assessment could not be made in this work of the subunit structure of large and small MW enzyme species of rabbit liver  $\beta$ -hexosaminidase.

D) DISCUSSION

Considerably more liver tissue was used in each preparation of the enzyme in the main experimental work (10 livers) than in the preliminary investigations (1-2 livers) detailed in Chapter 3. In part, this was due to the need for more highly purified enzyme preparations; with increasing purification, less and less of the starting HSS enzyme activity was recovered. In addition, the enzyme components were split into several different fractions during purification, thereby reducing the total amount of enzyme activity in any one fraction. Finally, the studies themselves were more numerous and required greater amounts of enzyme (especially those described in the next chapter). For these reasons, considerably more liver tissue was needed from which to extract and purify the enzyme in order to permit sustained study.

The first three steps in the purification procedure served three purposes:

- 1) the bulk of the contaminating protein originally present in the HSS was eliminated;
- 2) large and small MW forms of the enzyme were kept intact as one unit, thus facilitating purification;
- 3) the specific activity of enzyme forms were enhanced approximately 500 times over that found in the HSS.

Following Con A Sepharose affinity chromatography, total protein in the enzyme preparation was reduced sufficiently to permit the application without overloading of the entire enzyme preparation onto a single 2.5 cm x 90 cm column of Sephadex G-200 superfine. Gel filtration served not only to significantly increase the specific activity of enzyme species but also to effectively separate enzyme species into two distinct populations dif-

fering in size. This meant that large and small MW enzyme forms in a highly purified state could be separately tested for similarities and differences in their enzymatic and physical properties. Even more highly purified preparations of large and small MW enzyme forms were obtained following DEAE-Cellulose anion exchange chromatography, albeit at the expense of considerable enzyme activity and the elimination of some enzyme species. The relatively low percentage of contaminating protein in these preparations permitted SDS-dissociation studies to be undertaken to directly examine the subunit structure of large and small MW enzyme forms.

Both large and small MW enzyme forms of rabbit liver were roughly similar in their stability to treatments during the first four purification steps. This was shown in three separate ways:

- 1) by isoelectric focussing. Large and small MW enzyme forms were present in approximately equal proportions in HSS and Con A Sepharose preparations (Figure 6 and Figure 10);
- 2) by PNP-NAcglc/E<sub>2</sub>α-17NAcglc activity ratios. Closely similar ratios were obtained for ammonium sulfate and Con A Sepharose preparations (Table V);
- 3) by percent recovery of enzyme activity. Both large and small MW enzyme forms were recovered to roughly the same extent in the Sephadex G-200 column filtrate when recovery was gauged from the activity of corresponding enzyme forms in the HSS (Table X).

Large and small MW enzyme forms differed slightly in their stability to DEAE-Cellulose chromatography at pH 6.0 and pH 7.4 respectively. A greater percentage of the applied enzyme activity was recovered in the column filtrate with large MW enzyme forms than with small MW enzyme forms.

It is unlikely that any of the enzyme species examined in the present work were artifacts generated by the purification procedure. In the first place, similar patterns of enzyme species were obtained when either freshly prepared HSS or HSS which had been stored for 2-3 days at 4°C were used for isoelectric focussing. Secondly, isoelectric focussing profiles of enzyme species in the Con A Sepharose filtrate failed to reveal any new enzyme forms. Finally, Rf values on polyacrylamide gels following electrophoresis of enzyme species in the most highly purified preparations were similar to those of enzyme species present in the Con A Sepharose fraction. For these reasons it was concluded that rabbit liver  $\beta$ -hexosaminidase exists in at least 9 different forms in vivo.

In the present work two different methods were used to calculate the percent recovery of large and small MW enzyme forms in the latter stages of the purification procedure. In one method, the total enzyme activity present in the HSS was designated as 100% and then the recoveries of large and small MW enzyme forms were estimated as a percentage of the total starting activity (Table VII and Table VIII). In the second method, the total HSS activity of large MW enzyme forms and of small MW enzyme forms was calculated first. Then the recoveries of large and small MW enzyme species were estimated as a percentage of the corresponding HSS enzyme activity (Table X). Each of these two methods had its advantages and disadvantages. The first method was particularly useful when comparing the fold purification achieved for rabbit liver  $\beta$ -hexosaminidase with that reported by other workers using  $\beta$ -hexosaminidases from other sources. In general, the purification of  $\beta$ -hexosaminidases has been based on total starting activity, even when a number of enzyme species have been present in the crude extract and have been eventually separated into different fractions during the purification procedure. However, the significantly

different abilities of both large and small MW enzyme forms from rabbit liver to hydrolyse  $\beta$ -N-acetylglucosaminide derivatives of  $17\alpha$ -estradiol and p-nitrophenol (Tables V, VI and IX) meant that the first method gave widely differing measurement of the fold purification of differently sized enzyme species when the two substrates were used to monitor enzyme recovery. This was confusing since a consistent measure of the increase in specific activity of enzyme species could not be obtained. With the second method, however, the increase in the specific activity of enzyme forms was independent of the substrate used, and a consistent measure of fold purification was achieved.

Table VII shows that when fold purification was calculated by the first method with  $E_2\alpha$ -17NAcglc, large MW enzyme forms (basic species) were purified 12,177 times and small MW enzyme forms 2054 times from the HSS following DEAE-Cellulose anion exchange chromatography. When enzyme recovery was calculated on the basis of PNP-NAcglc activity using the first method, the overall fold purification of large MW enzyme forms (basic species) was lowered to 6092 and that of small MW enzyme forms raised to 3490 (Table VIII). When the second method was used to calculate fold purification (Table X), large MW enzyme forms (basic species) and small MW enzyme forms were enriched approximately 23,800 times and 4850 times, respectively, from the HSS following purification.

While complete purification of large and small MW enzyme forms of rabbit liver  $\beta$ -hexosaminidase could not be attained, the overall purification of enzyme species was either roughly equal to or greater than that which has been described in other work where mammalian, lower animal and plant  $\beta$ -hexosaminidases have been purified to homogeneity. Some of the largest increases in specific activity with respect to crude enzyme ex-

tracts have been reported with the enzyme from human placenta - Hex A 5800 times, Hex B 5100 times (Geiger and Arnon, 1976), jack bean meal - 3060 times (Li and Li, 1970), the slime mold Dictyostelium discoideum - 5000 times (Every and Ashworth, 1973), monkey brain - Hex B 2240 times (Aruna and Basu, 1976) and human liver - Hex A 3000 times, Hex B 1000 times (Sandhoff, 1970). A considerably lower enhancement of specific activity has been described for purified beef spleen enzyme forms - Hex A 310 times, Hex B 260 times (Verpoorte, 1972), and the predominant purified enzyme form from human blood - 195 times (Verpoorte, 1974).

$\beta$ -Hexosaminidases from fungi, lower animals and mammals have been examined by either Con A Sepharose affinity chromatography or direct carbohydrate analyses to establish if bound carbohydrate was incorporated in the enzyme. Beutler, Guinto and Kuhl (1975) found that both Hex A and Hex B from human placenta were retained on columns of Con A Sepharose and that both were eluted together when sugar was added to the eluting buffer. Direct analysis of the carbohydrate composition of Hex A and Hex B (Lee and Yoshida, 1976) revealed that the former contained 2.5% N-acetylglucosamine and 2.5% neutral sugar and that the latter was composed of 3.5% N-acetylglucosamine and 2.5% neutral sugar. Sialic acid was not found in either isoenzyme. However, Geiger and Arnon (1976) detected 1.65 moles of sialic acid per mole of Hex A. Aruna and Basu (1976) working with Hex A and Hex B from monkey brain reported that the latter contained 19% neutral sugar (glucose, galactose, mannose), 5.3% amino sugar and 0.34% sialic acid while the former contained a considerably higher percentage of neutral sugar and sialic acid but only one-half the percentage of amino sugar. Both Hex A and Hex B were retained on Con A Sepharose in the absence of exogenously added sugar. Banerjee and Basu (1975) established

the glycoprotein nature of Hex A from human urine through its affinity for Con A Sepharose and through analyses which showed the presence of bound sialic acid, galactose, glucose, mannose and hexosamine. Others have obtained evidence of bound carbohydrate in  $\beta$ -hexosaminidases from (the limpet) Patella vulgata (Phizackerley and Bannister, 1974), (the fungus) Aspergillus oryzae (Mega et al, 1970), beef spleen - Hex A and Hex B (Verpoorte, 1972) and human plasma - predominant  $\beta$ -hexosaminidase isomer (Verpoorte, 1974). In the present work, the glycoprotein nature of large and small MW rabbit liver enzyme forms was established through their retention on columns of Con A Sepharose. It seems likely, since  $\beta$ -hexosaminidases from such widely differing organisms have been found to be glycoproteins, that bound carbohydrate may be a common characteristic of eucaryotic  $\beta$ -hexosaminidases.

Several workers have reported that the MW of  $\beta$ -hexosaminidases when estimated from gel filtration data is often in excess of the MW when estimated by other methods. Srivastava et al (1974a) using Sephadex G-200 gel filtration estimated the molecular weight of both Hex A and Hex B from human placenta at 140,000 Daltons but found that sedimentation-equilibrium centrifugation gave a molecular weight of 100,000 Daltons for both isoenzymes. Additional studies were carried out and through analytical ultracentrifugation and subunit analysis it was concluded that the molecular weight of Hex A and Hex B was, in fact, 100,000 Daltons (Beutler et al, 1976). Every and Ashworth (1973) used two methods to calculate the molecular weight of the  $\beta$ -hexosaminidase from the cellular slime mold, Dictyostelium discoideum. They found that Sephadex G-200 gel filtration gave a molecular weight of 168,000 Daltons but that analytical ultracentrifugation indicated a molecular

weight of 115,000 Daltons. The latter value agreed very closely with the total molecular weight of the enzyme subunits estimated from SDS-polyacrylamide gel electrophoresis. In yet another study, Overdijk et al (1975) reported that gel filtration on "freshly prepared" columns of Sephadex G-200 gave a higher estimate of the molecular weight of bovine brain Hex A, Hex B and Hex D than did gel filtration on "aged" columns of Sephadex G-200 or on columns of Bio-Gel P-200. In all of the above studies, the  $\beta$ -hexosaminidases examined were also shown to contain bound carbohydrate. Andrews (1965) has reported that glycoproteins often behave anomalously on Sephadex columns.

In the present work, gel filtration on calibrated columns of Sephadex G-200 was used for molecular weight determinations. The large enzyme forms were estimated to weigh 270,000 Daltons and the small ones 150,000 Daltons. These results could have been too high for the following three reasons:

- 1) both large and small MW enzyme forms contained bound carbohydrate;
- 2) other workers have demonstrated that  $\beta$ -hexosaminidases which contain sugar residues give anomalously high molecular weight estimates with Sephadex gel filtration;
- 3) the most plausible subunit structure postulated from the SDS-polyacrylamide experiments gave molecular weights for large and small MW enzyme forms which were lower than those estimated from gel filtration.

It would have been desirable to use a second method based on a different principle to check the molecular weight estimates obtained by gel filtration. Three commonly used methods might have been tried, namely sedimentation-equilibrium centrifugation, analytical ultracentrifugation and polyacrylamide gels of differing acrylamide concentration (Hedrick and

Smith, 1968). All of these methods were considered unsuitable for use with the enzyme preparations obtained in the present work; the former two because enzyme species even in the most highly purified preparations were still contaminated by considerable amounts of non- $\beta$ -hexosaminidase protein, and the latter because enzyme staining (with either naphthol-AS-BI-NAcglc or Coomassie Blue) on polyacrylamide gels was sufficiently diffuse as to preclude the accurate measure of  $R_f$  values which were needed to calculate molecular weight. For these reasons confirmatory experiments were not undertaken.

Even if the molecular weight estimates are considered to be somewhat high, they still suggest that rabbit liver small MW enzyme forms are comparable in size with most other  $\beta$ -hexosaminidases which have been studied while large MW enzyme forms are considerably larger. Previous work has shown that  $\beta$ -hexosaminidases usually vary in size between 100,000 Daltons and 160,000 Daltons throughout the animal and plant kingdoms (see for example Edwards, Thomas and Westwood, 1975; Phizackerley and Bannister, 1974; Li and Li, 1970; Tarentino and Maley, 1971; Verpoorte, 1972; Geiger and Arnon, 1976). In only two cases have  $\beta$ -hexosaminidases been found with molecular weights in excess of 200,000 Daltons. Overdijk et al (1975) showed that human Hex C weighed more than 200,000 Daltons while Seyama and Yamakawa (1974a) reported a molecular weight of 250,000 Daltons for one of their  $\beta$ -hexosaminidase forms from equine kidney. As pointed out previously in Chapter 3, the multiple forms of rabbit liver  $\beta$ -hexosaminidase differ sharply from those of all other  $\beta$ -hexosaminidases previously reported in their size and charge relationships.

In two important respects, however, rabbit liver multiple forms of

$\beta$ -hexosaminidase are similar to those of other mammalian  $\beta$ -hexosaminidases, namely in their susceptibility to physical inactivation by heat and in their behaviour when frozen and thawed. The present work showed that:

- 1) acidic or small MW enzyme forms lost activity more rapidly than basic or large MW enzyme forms when heated to temperatures above 45°C (Figure 17 and Figure 18); and
- 2) acidic or small MW enzyme forms were converted to basic or large MW enzyme forms, but the reverse did not occur, when enzyme species were frozen and thawed in 3 M NaCl (Figure 19).

The greater susceptibility of acidic enzyme forms to physical inactivation by heat appears to be a general property of mammalian  $\beta$ -hexosaminidases since enzyme species from a number of sources including human serum, liver, brain, placenta and spleen (Price and Dance, 1972; Ben-Yoseph, Geiger and Arnon, 1975; Robinson and Stirling, 1968), monkey brain (Aruna and Basu, 1976), and equine kidney (Seyama and Yamakawa, 1974) have been shown to follow this pattern. The difference in temperature stability between human Hex A and Hex B has proven to be so distinctive that it is the basis for one of the principal screening methods now in use for diagnosing heterozygous and homozygous carriers of Tay Sach's disease (Berry, 1975; O'Brien et al., 1970a). In addition, Geiger and Arnon (1976) have reported that the heat inactivation of human Hex A and Hex B is amplified by mild reducing conditions, while Ben-Yoseph et al. (1975) have demonstrated that Hex A and Hex B are protected against thermal inactivation when complexed with anti-Hex A-B antibodies. Somewhat analogous to the present findings, Beutler and Kuhl (1975) showed that repeated freezing and thawing of human placenta  $\beta$ -hexosaminidase in 3 M NaCl caused the partial conversion of Hex A to Hex B and Hex S. However, Hex B only converted

to Hex A when frozen and thawed in the presence of Hex S; Hex B alone did not change to Hex A.

In the present work, the freezing and thawing experiment showed that small MW enzyme forms (acidic species) contained all the types of subunits present in large MW enzyme forms (basic species). In addition, the same experiment strongly suggested that acidic enzyme species contained subunits not present in basic enzyme species. In these respects the rabbit liver multiple forms are similar to correspondingly charged enzyme forms in the human (Hex A - forms B-D; Hex B - forms E-I). It is likely, however, that further research will reveal, if not significant, then at least subtle differences in the subunit structures of rabbit and other mammalian  $\beta$ -hexosaminidase multiple forms. This suggestion is based on two findings in the present work:

- 1) rabbit liver  $\beta$ -hexosaminidase species did not behave identically to those from human placenta when frozen and thawed. In particular, acidic or small MW enzyme forms did not convert to still more acidic forms as had Hex A from human placenta. The possibility exists, however, that forms B-D were converted to very acidic species, but that these species were not revealed as coloured bands on polyacrylamide gels because of their limited ability or complete inability to hydrolyse naphthol AS-BI-NAcglc. In the preliminary investigations form A was observed to behave in this way.
- 2) the interplay between size and charge in rabbit liver multiple forms was significantly different from that found in any other mammalian  $\beta$ -hexosaminidase.

For reasons which never became clear, the SDS-dissociation studies in the present work led to a subunit structure of enzyme species incompatible with their freezing and thawing behaviour. The SDS experiments could

not, therefore, serve as the basis for establishing the subunit structure of rabbit liver  $\beta$ -hexosaminidase multiple forms. Judging from the experience of others who have studied the subunit composition of the enzyme forms from human placenta (Srivastava et al, 1974; Tallman et al, 1974; Beutler et al, 1976; Geiger and Arnon, 1976), a complete understanding of the physical composition of rabbit liver enzyme species will require studies consisting of treatment with a number of dissociating agents followed by examination of the disaggregated subunits by a number of techniques to determine their physical, chemical and immunological properties.

Large and small MW enzyme forms were found in the present work to differ sharply in their enzymatic properties. PNP-NAcglc/ $E_2\alpha$ -17NAcglc activity ratios clearly showed that forms E to I were more specific than forms B to D for the steroid as opposed to the p-nitrophenyl substrate. This was confirmed by comparison, on a per molecule enzyme basis, of the steroid and p-nitrophenyl  $\beta$ -hexosaminidase activity of large and small MW forms. These latter studies also revealed that large forms were definitely superior to an equivalent molecular amount of small forms in hydrolysing both  $E_2\alpha$ -17NAcglc and PNP-NAcglc; the steroid substrate was hydrolysed 13.21 times more rapidly and the p-nitrophenyl compound 3.52 times more rapidly. Differences in activity between the large and small MW populations were more pronounced with the steroid substrate than with the p-nitrophenyl substrate.

The most acidic enzyme isomer, form A, was found by far to be the most steroid specific of all the enzyme forms of rabbit liver  $\beta$ -hexosaminidase (Table VI). Form A also differed from other enzyme species in its greater susceptibility to denaturation by low pH. Unfortunately, this meant that form A was lost from the enzyme preparation at such an

early stage in the purification procedure that its hydrolytic activity with PNP-NAcglc and E<sub>2</sub>α-17NAcglc could not be compared on a per molecule enzyme basis with that of the other enzyme species.

The rabbit liver enzyme forms differ significantly in their steroid β-N-acetylglucosaminidase activity. Very few other β-hexosaminidase have been tested for steroid β-N-acetylglucosaminidase activity primarily because of the unavailability of steroid N-acetylglucosaminides as substrates. In the human, as in the rabbit, these conjugates have been found to occur in vivo (Arcos and Lieberman, 1967; Jirku and Levitz, 1969; Frey et al, 1971). In the human, the in vivo hydrolysis of one of these conjugates, 15α-hydroxyestrone 3-sulfate-15-β-N-acetylglucosaminide, and of a synthetically prepared steroid conjugate, testosterone 17-β-N-acetylglucosaminide, has been reported (Jirku et al, 1974; Fukushima et al, 1972). Two groups have examined the multiple forms of human β-hexosaminidase in vitro to determine whether their hydrolytic activity with steroid β-N-acetylglucosaminides paralleled their hydrolytic activity with non naturally-occurring fluorometric or colorimetric substrates. Layne and co-workers (personal communication) obtained similar PNP-NAcglc/E<sub>2</sub>α-17NAcglc activity ratios with both pregnancy and non-pregnancy human serum which indicated that Hex P did not differ from Hex A in steroid specificity. Tomasi et al (1974) tested human liver Hex A and Hex B with three substrates, namely dehydroepiandrosterone 3-β-N-acetylglucosaminide, testosterone 17-β-N-acetylglucosaminide and 4-methylumbelliferyl β-N-acetylglucosaminide, and found that the steroid β-N-acetylglucosaminide activity of each was directly proportional to the 4-methylumbelliferyl β-N-acetylglucosaminidase activity. The multiple forms of the human enzyme, therefore, are unlike those of the rabbit liver enzyme in that

they are similar in their specificity for steroid substrates.

The present work with rabbit liver  $\beta$ -hexosaminidase constitutes the only report in which the multiple forms of a  $\beta$ -hexosaminidase have been shown to vary in their ability to hydrolyse steroid  $\beta$ -N-acetylglucosaminide conjugates.

A number of more detailed investigations on the enzymatic properties of large and small MW enzyme forms are presented in the next chapter.

APPENDIX 1

Derivation of Data Presented in Table IX

- 1) Total milligrams of enzyme forms in Sephadex G-200 fractions:

Table VII shows that the large MW fraction contains 5.03 mg protein while that in the small MW fraction equals 17.17 mg.

But only 40% and 48% of the total protein in the large and small MW fractions, respectively, corresponds to enzyme (as determined from scans at 540  $m\mu$  of protein-stained electrophoresis gels).

Therefore, 2.00 mg and 8.24 mg of large and small forms, respectively, are present in these fractions.

- 2) Hydrolytic activity per milligram enzyme protein:

Tables VII and VIII show that under standard assay conditions:

2.00 mg large MW forms hydrolyse 6996 picomoles  $E_2\alpha$ -17NAcglc/hr  
and 13515 micromoles PNP-NAcglc/hr

Therefore, 1 mg will hydrolyse 3998 picomoles  $E_2\alpha$ -17NAcglc/hr (1)

and 6757 micromoles PNP-NAcglc/hr (2)

similarly

8.24 mg small MW forms hydrolyse 3929 picomoles  $E_2\alpha$ -17NAcglc/hr  
and 28448 micromoles PNP-NAcglc/hr

Therefore, 1 mg will hydrolyse 476 picomoles  $E_2\alpha$ -17NAcglc/hr (3)

and 3452 micromoles PNP-NAcglc/hr (4)

- 3) Comparison of hydrolytic activity by weight:

(1) and (3) show that 7.34 times more  $E_2\alpha$ -17NAcglc is hydrolysed

by 1 mg of large forms than by an equivalent weight of small forms

similarly

(2) and (4) show that the corresponding ratio with PNP-NAcglc is 1.96:1.

4) Comparison of hydrolytic activity on a per molecule enzyme basis:

The molecular weight of large forms is 1.8 times that of small forms. Therefore, there are 1.8 times more small MW than large MW enzyme molecules in 1 mg.

If 'P' and 'Q' are defined as the activity ratios given by equivalent molecular amounts of large forms over small forms for E<sub>2</sub>α-17NAcglc and PNP-NAcglc, respectively, then:

$$1/1.8 P = 7.34$$

$$\therefore P = 13.21$$

and

$$1/1.8 Q = 1.96$$

$$\therefore Q = 3.52$$

APPENDIX 2

Calculations To Determine the Amount, in Milligrams,  
of Large MW and Small MW Enzyme Forms in the High  
Speed Supernatant

- 1) From Appendix 1:

Activity per mg large MW forms= 3998 picomoles/hr  $E_2\alpha$ -17NAcglc hydrolysed  
= 6757 micromoles/hr PNP-NAcglc hydrolysed  
similarly

Activity per mg small MW forms= 476 picomoles/hr  $E_2\alpha$ -17NAcglc hydrolysed  
= 3452 micromoles/hr PNP-NAcglc hydrolysed

- 2) Now Table VII and Table VIII show that total enzyme activity in the liver extract is 153,450 micromoles PNP-NAcglc hydrolysed per hour and 38,146 picomoles  $E_2\alpha$ -17NAcglc hydrolysed per hour. The contribution of form A to the total activity towards PNP-NAcglc is negligible and need not be considered. However, approximately 6% of the hydrolysis of  $E_2\alpha$ -17NAcglc is due to form A. Therefore, to a first approximation, large and small forms in the high speed supernatant hydrolyse 35,857 picomoles  $E_2\alpha$ -17NAcglc per hour.

- 3) If 'Y' is defined as the total mg of large MW forms in the high speed supernatant

and

If 'Z' is defined as the total mg of small MW forms in the high speed supernatant

Then combining data from Section (1) and (2) above:

$$3998 Y + 476 Z = 35,857$$

and

$$6757 Y + 3452 Z = 153,450$$

Solving for Y and Z:

$$Y = 5.72$$

$$Z = 33.26$$

CHAPTER 5

FURTHER ENZYMATIC PROPERTIES OF RABBIT LIVER  $\beta$ -HEXOSAMINIDASES

A) INTRODUCTION

The results presented in Chapters 3 and 4 showed that large and small MW enzyme forms of rabbit liver  $\beta$ -hexosaminidase differed in substrate specificity and hydrolytic activity towards the three substrates routinely used to monitor enzyme activity throughout purification. The studies presented in this chapter were undertaken to provide additional information on the enzymatic properties of enzyme forms B to D and E to I.

The hydrolysis of PNP-NAcglc, PNP-NAcgal and E<sub>2</sub> $\alpha$ -17NAcglc by each enzyme population was further examined by monitoring the effect of substrate concentration on enzyme activity. Km values for these substrates were determined together with the maximal velocity of the reactions at infinite substrate concentration (Vmax). With these two kinetic constants the hydrolytic activities of each enzyme population could be assessed and compared in terms of substrate affinity and rate of cleavage of the  $\beta$ -hexosaminide linkage.

The inhibition of the p-nitrophenyl and steroid  $\beta$ -hexosaminidase activity of large and small MW enzyme forms by N-acetylglucosamino-lactone and N-acetylgalactosamino-lactone was studied. Both of these lactones were known to act as potent, competitive inhibitors of the hydrolytic activity of other  $\beta$ -hexosaminidases (Findlay, Levvy and Marsh, 1958; Conchie et al, 1967; Every and Ashworth, 1973; Kanfer and Spielvogel, 1973; Pokorny et al, 1974; Mian et al, 1975). In the present work, studies were designed to determine whether large and small MW enzyme forms were equally susceptible to lactone inhibition, whether the lactones inhibited enzyme activity towards PNP-NAcglc, PNP-NAcgal

and  $E_2\alpha$ -17NAcglc in a competitive manner and, if so, whether  $K_1$  values indicated that all the enzyme species in each population possessed both N-acetylglucosaminidase and N-acetylgalactosaminidase activity. Other lactones, free and N-acetylated glucosamine, and a number of unconjugated steroids were also tested for their effect on the hydrolytic activity of large and small MW enzyme forms.

Studies presented earlier in this work had shown that large MW enzyme forms exhibited a greater preference for  $E_2\alpha$ -17NAcglc over PNP-NAcglc than did small MW enzyme forms. In order to determine if this might be a general trend with steroid substrates, large and small MW enzyme forms were reacted with a number of estrogen  $\beta$ -N-acetylglucosaminides in which the sugar was attached either to the phenolic three-hydroxyl or to the aliphatic 17-hydroxyl. Steroid  $\beta$ -N-acetylglucosaminidase activity was normalized with respect to p-nitrophenyl  $\beta$ -N-acetylglucosaminidase activity before the amount of steroid aglycone liberated by each enzyme population was compared.

Large and small MW enzyme forms were also tested for their ability to catalyse the transfer of N-acetylhexosamine to compounds other than water; that is, they were tested for transglycosylase activity. In order that these studies might be viewed in their proper perspective, a brief review is presented which summarizes past research into the transglycosylase activity of  $\beta$ -hexosaminidases and which delineates the salient mechanistic features which have been found to characterize this type of reaction among exo-glycosidases.

Although exo-glycosidases have always been regarded primarily as hydrolytic enzymes, studies have shown that these enzymes can occasionally function as transglycosylases as well, with organic alcohols replacing water as the final acceptor of the cleaved sugar.  $\beta$ -Hexosaminidases from

3 sources have been shown to catalyse such reactions. Mega, Ikenaka and Matsushima (1972a) showed that the  $\beta$ -hexosaminidase from Aspergillus oryzae could transfer N-acetylglucosamine from one phenyl- $\beta$ -N-acetylglucosaminide to another acting as an acceptor molecule to form phenyl di- and tri-N-acetylglucosaminides. The enzyme also catalysed transglycosylation reactions when free sugars, glycols and aliphatic C<sub>1</sub> to C<sub>4</sub> alcohols were used as sugar acceptors (Mega, Ikenaka, Matsushima, 1972b). Vikha et al (1971) working with pig epididymis  $\beta$ -hexosaminidase found that the hydrolysis of PNP-NAcglc was accompanied by the synthesis of disaccharides of N-acetylglucosamine. Werries et al (1975) used a somewhat different system to demonstrate the transglycosylase activity of Hex A and Hex B from bovine spleen. In their system, phenyl- $\beta$ -N-acetylglucosaminide acted as the sugar donor and tetrasaccharides derived from hyaluronic acid and chondroitin 4-sulfate functioned as the sugar acceptors. While pentasaccharides were synthesized by both isoenzymes, Hex B exhibited considerably higher transglycosylase activity than did Hex A. Further studies revealed that the transglycosylase activity of each isoenzyme form tended to be inversely proportional to its hydrolytic activity towards a trisaccharide breakdown product of hyaluronic acid (N-acetylglucosamine- $\beta$ (1,4)-glucuronic acid- $\beta$ (1,3)-N-acetylglucosamine).

Studies with  $\beta$ -hexosaminidase and other exo-glycosidases (Mumford, Raghaven and Kanfer, 1976; Carter and Kanfer, 1976; Huber, Kurz and Wallenfels, 1976; Wells, Ray, Kuo and Iritani, 1975; Sinnott and Souchard, 1973; Pan, 1970; Suzuki and Uchida, 1970a, 1970b; Dedonder, 1961) have consistently pointed to the close interrelationship between transglycosylation and hydrolysis reactions. Both reactions are believed to proceed via a glycosylated enzyme intermediate. Mega et al (1972b) have



to synthesize glycosides from free sugars is convincing evidence for the fact that transglycosylation and hydrolysis are not reverse reactions.

In the present work, the large and small MW forms of rabbit liver  $\beta$ -hexosaminidase were tested for transglycosylase activity by determining whether N-acetyl-(6- $^3$ H)-glucosamine was transferred from p-nitrophenyl- $\beta$ -D-N-acetyl-(6- $^3$ H)-glucosaminide to tetrasaccharide breakdown products of hyaluronic acid and chondroitin sulfate with the subsequent formation of tritiated pentasaccharides. The possibility that p-nitrophenyl- $\beta$ -N-acetylglucosaminide might act as both the donor and acceptor of the radioactive amino sugar was also investigated.

## B) METHODS

Large and small MW enzyme forms from the Sephadex G-200 eluate, prepared as described in Section B.1 Chapter 4, were used for all studies with one exception, namely the hydrolysis of the 3-sulfo-17- $\beta$ -N-acetylglucosaminyl double conjugate of 17 $\alpha$ -estradiol. In this study, enzyme fractions from the DEAE-Cellulose column eluates were used in order to ensure that sulfatase activity was absent from the enzyme preparations.

The data from Lineweaver-Burke and Dixon plots were analysed by the method of least squares.

### 1) Determination of Km and Vmax

The two enzyme populations were separately tested with three substrates, namely PNP-NAcglc, PNP-NAcgal and E<sub>2</sub> $\alpha$ -17NAcglc, and the two kinetic parameters Km and Vmax determined. Assays with all three substrates were carried out as described previously under General Methods. The concentration of the tritiated steroid substrate (specific activity 180 DPM/picomole) was varied between  $1.00 \times 10^{-7}$  M and  $1.25 \times 10^{-6}$  M in assays, while that of the p-nitrophenyl compounds ranged from  $5.0 \times 10^{-5}$  M to  $8 \times 10^{-4}$  M. Michaelis constants (Km) and maximum reaction velocities (Vmax) were estimated from reciprocal plots by the method of Lineweaver and Burke (1934).

### 2) Inhibition of $\beta$ -Hexosaminidase Activity by Lactones

The effect of N-acetylglucosamino-lactone and N-acetylgalactosaminolactone on the hydrolytic activity of large and small MW enzyme forms towards three substrates was studied. Lactone solutions were freshly prepared in assay buffer at room temperature one-half hour before use. The type of inhibition as well as values for the inhibition constant (Ki) were

determined by plotting the reciprocal of the reaction velocity against the inhibitor concentration at two different substrate concentrations according to the method of Dixon (1953). Assays were carried out at  $3.22 \times 10^{-7}$  M and  $6.25 \times 10^{-7}$  M concentrations of  $E_2\alpha$ -17NAcglc (specific activity 562 DPM/picomole and 290 DPM/picomole respectively),  $1.0 \times 10^{-3}$  M and  $2 \times 10^{-3}$  M concentrations of PNP-NAcglc, and  $0.5 \times 10^{-3}$  M and either  $1.5 \times 10^{-3}$  M or  $2.0 \times 10^{-3}$  M concentrations of PNP-NAcgal. The concentration of N-acetylglucosamino-lactone ranged from  $1.82 \times 10^{-6}$  M to  $1.14 \times 10^{-5}$  M in the assay medium, while that of N-acetylgalactosamino-lactone ranged from  $1.38 \times 10^{-6}$  M to  $1.37 \times 10^{-5}$  M. Enzyme activity towards the three substrates was determined as described previously under General Methods.

Studies were also undertaken to determine whether lactones other than those derived from 2-N-acetylamino-2-deoxy-alonic acid (N-acetylhexosaminic acid) inhibited the  $\beta$ -hexosaminidase activity of rabbit liver enzyme forms. The following lactones were used: galactono-1, 4-lactone, glucono-1, 4-lactone, saccharo-1, 4-lactone and glucurono-lactone. Assays consisted of incubating large and small MW enzyme forms in 1.0 ml of 0.1 M citrate pH 4.4 containing 200 $\mu$ g bovine serum albumin, 1 $\mu$ mole PNP-NAcglc and 75 nmoles of the appropriate lactone for 1 hour at 37°C. Assays without lactone and with 75 nmoles of N-acetylgalactosamino-lactone were also carried out with each set of incubations. The hydrolytic activity of enzyme forms was quantitatively determined as described previously under General Methods.

3) The Effect of N-Acetylglucosamine, Glucosamine and Free Steroids on Steroid  $\beta$ -Hexosaminidase Activity

Large and small MW enzyme forms were incubated with  $E_2\alpha$ -17NAcglc under

routine assay conditions (General Methods) in the presence of varying concentrations of N-acetylglucosamine and glucosamine. The concentration of the former amino sugar in the incubation medium ranged from  $2.5 \times 10^{-4}$  M to  $1.25 \times 10^{-2}$  M, while that of the latter was varied between  $1.0 \times 10^{-3}$  M and  $1.25 \times 10^{-2}$  M. Graphs were plotted of enzyme activity versus concentration of the amino sugar.

The potential inhibition of steroid  $\beta$ -hexosaminidase activity by free steroids was also examined. The procedure consisted of measuring the hydrolysis of tritiated  $E_2\alpha$ - $17NAcglc$  in the routine assay by large MW and by small MW enzyme forms either in the presence or absence of added free steroid. The following radioinert steroids were used:  $17\alpha$ -estradiol (875 nmoles/2 ml assay), estrone (900 nmoles/2 ml assay),  $17\beta$ -estradiol (750 nmoles/2 ml assay), estriol (750 nmoles/2 ml assay), 17-epiestriol (900 nmoles/2 ml assay) and 16, 17-epiestriol (900 nmoles/2 ml assay). Radioinert steroids were added to dry reaction tubes in methanol and the methanol was evaporated under a stream of nitrogen. Citrate buffer 0.1 M pH 4.4 (2 ml) containing bovine serum albumin at a concentration of 0.01% was then added and the reaction tubes were vigorously shaken to disperse steroid throughout the aqueous medium. The remainder of the assay was carried out as described previously under General Methods.

The free steroids at the concentrations used in this study did not completely dissolve in the aqueous assay medium; a considerable fraction remained in suspension. Only the dissolved fraction, however, could reasonably be expected to play a part in any interaction of the free steroid with the dissolved enzyme forms and in any alteration of enzyme activity. For this reason, the concentration of 'active' free steroid in the assays was considered to be equal to that required to produce a saturated solution under the assay conditions used. The large amount

of free steroid in the assay, both in solution and in suspension, did not adversely affect the solubility of tritiated  $E_2\alpha$ -17NAcglc in the reaction medium or adversely alter the partitioning of  $^3H$ - $E_2\alpha$ -17NAcglc and liberated unconjugated  $^3H$ -17 $\alpha$ -estradiol in the aqueous-benzene two-phase system at the conclusion of the incubation period. Any alteration in benzene extracted tritium then could be attributed solely to the action of free steroid on the hydrolytic mechanism of the  $\beta$ -hexosaminidase enzyme forms.

#### 4) Preference of the Two Enzyme Populations for Steroid Substrates

The steroid specificity of large and small MW enzyme forms towards a number of estrogen  $\beta$ -N-acetylglucosaminide conjugates of 17 $\alpha$ -estradiol, 17 $\beta$ -estradiol and estrone was studied. The following conjugates were used as substrates: 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide ( $E_2\alpha$ -17NAcglc), 17 $\alpha$ -estradiol 3-glucuronyl-17- $\beta$ -N-acetylglucosaminide ( $E_2\alpha$ -3GA-17NAcglc), 17 $\alpha$ -estradiol 3-sulfo-17- $\beta$ -N-acetylglucosaminide ( $E_2\alpha$ -3SO<sub>4</sub>-17NAcglc), 17 $\beta$ -estradiol 3- $\beta$ -N-acetylglucosaminide ( $E_2\beta$ -3NAcglc) and estrone 3- $\beta$ -N-acetylglucosaminide ( $E_1$ -3NAcglc). Assays with all substrates were carried out as described previously under General Methods. The range of concentrations used for each steroid conjugate in the assay are listed in Table XV. At all concentrations of substrate, initial velocity conditions were maintained throughout the incubation period of the assay.

Steroid  $\beta$ -hexosaminidase activity was normalized with respect to p-nitrophenyl  $\beta$ -N-acetylglucosaminidase activity before comparisons between large and small MW enzyme species were undertaken to establish the relative preference of enzyme forms for the steroid conjugates.

#### 5) Determination of Transglycosylase Activity

The transglycosylase activity of large and small MW enzyme forms was

Steroid Conjugate	Concentration
E <sub>2</sub> $\alpha$ -17NAcglc	20 nM - 1.25 $\mu$ M
E <sub>2</sub> $\alpha$ -3GA-17NAcglc	9 nM - 50 $\mu$ M
E <sub>1</sub> -3NAcglc	12 $\mu$ M - 60 $\mu$ M
E <sub>2</sub> $\beta$ -3NAcglc	12 $\mu$ M - 60 $\mu$ M
E <sub>2</sub> $\alpha$ 3S5O <sub>4</sub> -17NAcglc	20nM

Table XV. Range of Substrate Concentrations used for the Determination of Enzyme Activity with Steroid  $\beta$ -N-Acetylglucosaminides

determined with p-nitrophenyl- $\beta$ -N-acetyl-(6- $^3$ H)-glucosaminide as the glycosyl donor and tetrasaccharides derived from hyaluronic acid and chondroitin sulfate as the potential sugar acceptors. The formation of tritiated pentasaccharides was used as an indication of transglycosylase activity. The enzymatic synthesis of the di-N-acetylglucosamide of p-nitrophenyl was also investigated.

a) Synthesis of the Glycosyl Donor

p-Nitrophenyl- $\beta$ -N-acetyl-(6- $^3$ H)-D-glucosaminide was prepared according to the method of Leaback (1963). One millicurie of D-(6- $^3$ H)-glucosamine hydrochloride (specific activity 19 Ci/ mmole) was added to 25 grams of radioinert glucosamine hydrochloride for the synthesis. The final synthesized product was recovered in 11% yield (4.25 grams). Specific activity was 52,885 DPM/mg. The identity of the synthesized compound was established through infrared spectroscopy (KBr wafer, Beckman IR-20 Infrared Spectrophotometer) against an authentic sample of PNP-NAcglc; infrared spectra of the two samples were identical. The synthesized compound was stored at  $-20^{\circ}\text{C}$  in a tightly capped brown bottle until needed.

b) Preparation of Tetrasaccharides of Hyaluronic Acid and Chondroitin Sulfate

I) Analytical Methods

Uronic acid was determined according to the method of Blumenkrantz and Asboe-Hansen (1973) with glucuronic acid as the colour standard. The absorbance of the final coloured product was measured at 520 m $\mu$  in a Cary 15 Split Beam Spectrophotometer against distilled water in the reference cell.

Reducing sugar (as N-acetylglucosamine or N-acetylgalactosamine) was

estimated by the method of Park and Johnson (1949). Carbohydrate samples were diluted to a volume of 2 ml for determinations. Absorbance measurements were made at 690  $\mu$  in a Cary 15 Split Beam Spectrophotometer with distilled water in the reference cell.

## II) Digestion of Polysaccharides

Oligosaccharide mixtures were obtained through the action of testicular hyaluronidase on hyaluronic acid (umbilical cord) and on chondroitin sulfate (whale and shark cartilage, mixed isomers). Mucopolysaccharide digestion was carried out essentially as described by Flodin, Gregory and Roden (1964). Hyaluronic acid (500 mg) was added to 25 ml of 0.1 M sodium acetate pH 5.0 containing 0.15 M NaCl. After 1 hour of stirring, 50 mg of hyaluronidase was added and incubation was started at 37°C under toluene. An additional 50 mg of hyaluronidase was added after 24 hours and 48 hours. The digestion was continued for a total of 72 hours. The toluene was removed by partial concentration in a rotary evaporator and filtration through a small bed of celite. The filtrate was freeze-dried and the residue taken up in 8 ml of water. The sample was divided into two equal portions for subsequent gel filtration.

Chondroitin sulfate (1 gram) was digested under the same conditions except that five additions of 50 mg of hyaluronidase were made, at the start of the incubation and after 1, 3, 4 and 7 days. The incubation was terminated after 9 days and the sample processed as described above. The freeze-dried residue was taken up in 12 ml of 0.5 M NaCl and then divided into three equal portions for subsequent gel filtration.

## III) Fractionation of Mucopolysaccharide Digest

Oligosaccharide breakdown products were separated by gel filtration through 2.2 cm x 200 cm columns of Sephadex G-25 superfine as described

by Flodin et al (1964). Columns were run in 1 M NaCl when fractionating the chondroitin sulfate digest and in 0.15 M NaCl for fractionation of the hyaluronate digest. Effluent fractions of 10 ml were collected and monitored for oligosaccharide by analysis for uronic acid.

Fractions containing the main oligosaccharide peak in the effluent of each gel filtration run were pooled, concentrated to a volume of approximately 10 ml in a rotary evaporator and desalted on a 2.2 cm x 200 cm column of Sephadex G-25 fine. Effluent fractions containing uronic acid were pooled and freeze-dried. Because the main oligosaccharide peak in the initial gel runs was not completely separated from adjacent carbohydrate peaks (due most likely to overloading of the gel columns), freeze-dried desalted oligosaccharides were rechromatographed on 2.2 cm x 200 cm columns of Sephadex G-25 in the appropriate salt eluant. Rechromatography served to eliminate minor contaminating oligosaccharides from the main uronic acid peak. Effluent fractions containing the main oligosaccharide peak were pooled and then desalted as described above. Desalted oligosaccharide fractions from the same original mucopolysaccharide digest were pooled and freeze-dried. Lyophilized sugar chains were stored at 4°C until needed.

#### IV) Identification of Isolated Carbohydrate

The chain length of the isolated oligosaccharides was established through analyses for uronic acid and for reducing sugar. Uronic acid to Park-Johnson reducing sugar molar ratios of 1.79 and 1.85 were found for the hyaluronate and chondroitin sulfate breakdown products respectively. These ratios indicated that both oligosaccharides contained approximately twice as much glucuronic acid as reducing sugar, and that both, therefore, were tetrasaccharides. The end product of hyaluronidase action on muco-

polysaccharides such as hyaluronic acid and chondroitin sulfate is normally the tetrasaccharide (Flodin et al, 1964).

c) Transglycosylase assay

Assays were carried out in 1.5 ml of 0.1 M citrate - 0.01% bovine serum albumin at either pH 4.4 (pH optimum for hydrolysis of PNP-NAcglc) or pH 6.5 (pH optimum of transglycosylase activity of beef spleen Hex A and Hex B (Werries et al, 1975)). Assays contained 18  $\mu$ moles of PNP-(6-<sup>3</sup>H)-NAcglc (326,000 DPM), 12  $\mu$ moles of tetrasaccharide, and enzyme in varying amounts. The mixtures were incubated at 37°C for 1, 4 or 24 hours. The various combinations of enzyme activity, incubation time and buffer pH which were used in the assay are listed in Table XVI.

Reactions were terminated by immersing assay tubes in a boiling water bath for 3 minutes. Mixtures were then fractionated on a Sephadex G-15 column (1.2 cm x 200 cm) with 1 M NaCl as eluant. Fractions of 3 ml were collected. One ml aliquots of fractions were mixed with 10 ml of Aquasol and radioactivity was measured for 10 minutes as described previously under General Methods. Carbohydrate was localized in effluent fractions by analysis for uronic acid.

↑ Trial	Buffer pH	*Milliunits Enzyme Activity	Incubation Time (Hours)	↓Percent Hydrolysis of PNP-NAcglc
1	6.5	140	1	21
2	6.5	140	4	75
3	6.5	140	24	>98
4	6.5	280	1	38
5	6.5	16	24	50
6	4.4	16	8	40

↑ LARGE AND SMALL MW ENZYME FORMS WERE SEPARATELY TESTED FOR TRANSGLYCOSYLASE ACTIVITY WITH THE CHONDROITIN SULFATE BREAKDOWN PRODUCT AND WITH THE HYALURONIC ACID TETRASACCHARIDE UNDER EACH SET OF ASSAY CONDITIONS.

\* ONE UNIT OF ENZYME IS EQUAL TO THAT AMOUNT OF ENZYME CATALYSING THE HYDROLYSIS OF 1.0 MICROMOLE OF PNP-NAcglc PER MINUTE AT pH 4.4 UNDER ROUTINE ASSAY CONDITIONS.

↓ AN APPROXIMATION DETERMINED FROM THE AMOUNT OF TRITIUM WHICH DID NOT MIGRATE WITH PNP-[6-<sup>3</sup>H]-NAcglc FOLLOWING FRACTIONATION OF THE REACTION MIXTURE BY GEL FILTRATION.

Table XVI. Assay Conditions for Transglycosylase Determinations

C) RESULTS

1) Kinetic Parameters Characterizing Substrate Hydrolysis

The apparent Michaelis constants ( $K_m$ ) of large and small MW enzyme forms for PNP-NAcglc, PNP-NAcgal and  $E_2\alpha$ -17NAcglc as well as the maximum velocity ( $V_{max}$ ) of the reactions were calculated from Lineweaver-Burke plots (Figures 24, 25, and 26) and are summarized in Table XVII. Michaelis constants of the two enzyme populations for each substrate were very similar, the greatest difference being found with PNP-NAcglc and the least with  $E_2\alpha$ -17NAcglc. The steroid substrate was hydrolysed with a  $K_m$  which was two to three orders of magnitude lower than those which characterized the hydrolysis of the p-nitrophenyl compounds. Of the two p-nitrophenyl compounds, the  $K_m$  for the galactosaminide was lower than that for the corresponding glucosaminide.

Unlike  $K_m$  values,  $V_{max}$  values differed considerably between the two enzyme populations. In all cases, large MW enzyme forms cleaved the amino sugar from the N-acetylhexosaminide in the enzyme-substrate complex more efficiently. The greatest difference in catalytic rate was found with the steroid substrate, with large MW enzyme forms processing the compound 7.87 times as rapidly as small MW enzyme forms;  $V_{max}$  values were most similar with PNP-NAcglc, the rate of cleavage differing by a factor of only 2.3. Both enzyme populations hydrolysed PNP-NAcglc at 6 to 9 times the rate attained with the corresponding galactosaminide.

It had been concluded from results presented previously for PNP-NAcglc and  $E_2\alpha$ -17NAcglc (Table IX) (and by analogy for PNP-NAcgal from Table XI) that large MW enzyme forms were more active than small MW enzyme forms in hydrolysing these three compounds. The kinetic data revealed that this greater activity stemmed almost exclusively from a more efficient cleavage of the amino sugar in the enzyme-substrate complex and only minimally, if

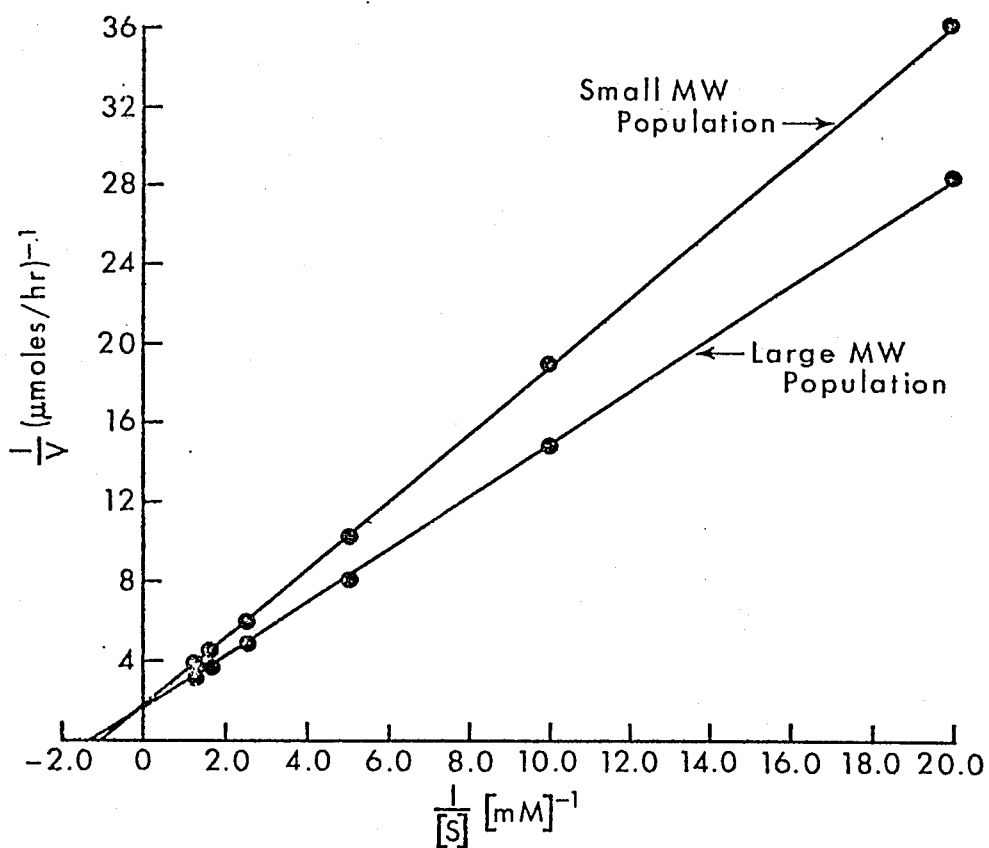


Figure 24. Lineweaver-Burke Plot to Determine  $K_m$  and  $V_{max}$  for  $\beta$ -Hexosaminidase Activity Toward p-Nitrophenyl- $\beta$ -N-acetylglucosaminide

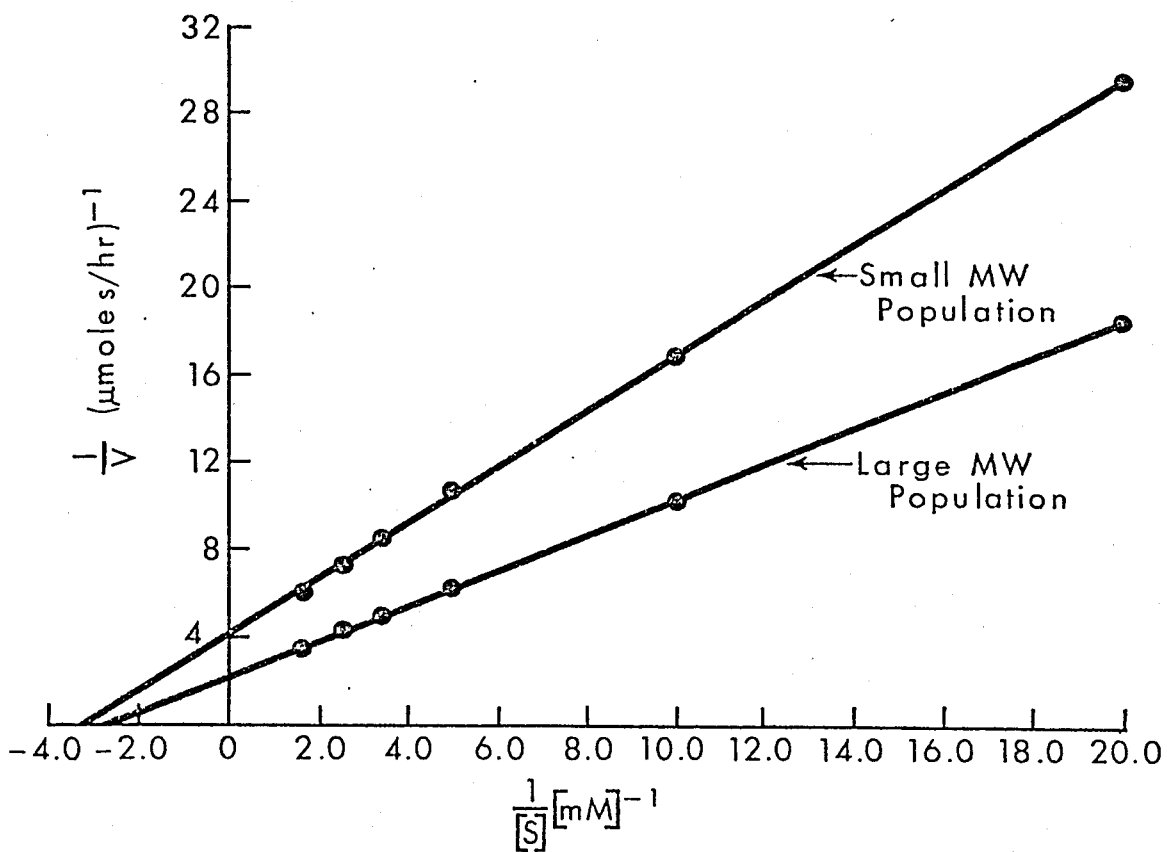


Figure 25. Lineweaver-Burke Plot to Determine  $K_m$  and  $V_{max}$  for  $\beta$ -Hexosaminidase Activity Toward p-Nitrophenyl- $\beta$ -N-acetylgalactosaminide

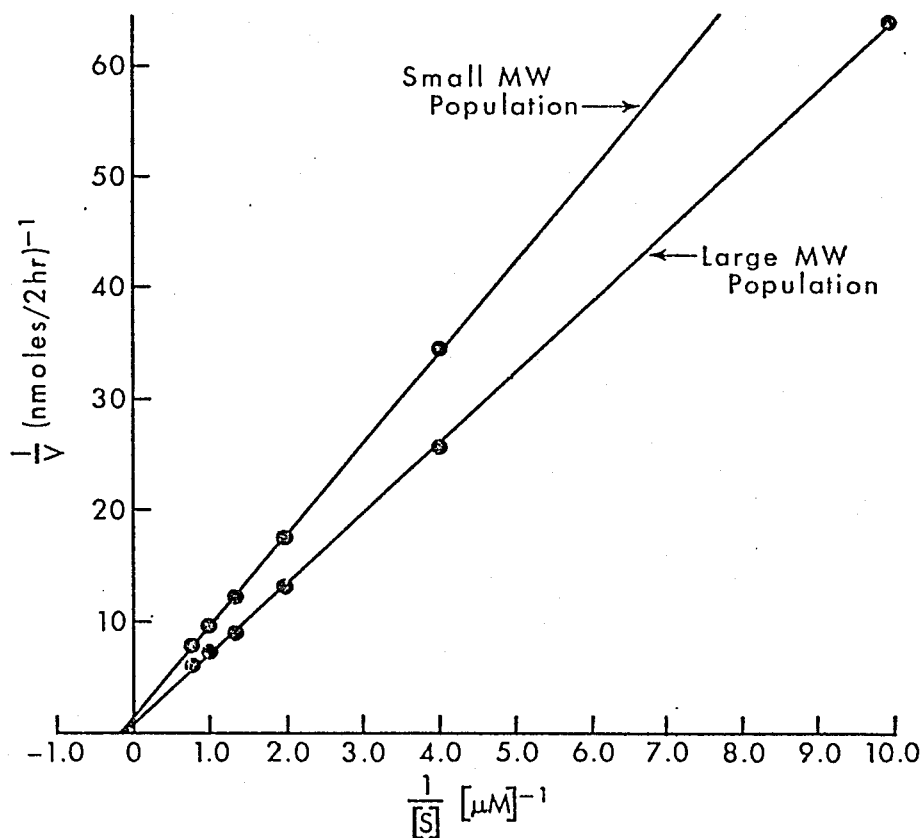


Figure 26. Lineweaver-Burke Plot to Determine  $K_m$  and  $V_{max}$  for  $\beta$ -Hexosaminidase Activity Toward  $17\alpha$ -Estradiol  $17\beta$ -N-acetylglucosaminide

Substrate	Enzyme Population	$K_m$ [M]	$V_{max}$
17 $\alpha$ -Estradiol 17- $\beta$ -N-acetyl- glucosaminide	Large MW	$7.79 \times 10^{-6}$	$25.57 \times 10^{-9}$
	Small MW	$6.33 \times 10^{-6}$	$3.25 \times 10^{-9}$
p-Nitrophenyl- $\beta$ -N-acetyl- galactosaminide	Large MW	$3.86 \times 10^{-4}$	$3.89 \times 10^{-5}$
	Small MW	$3.30 \times 10^{-4}$	$1.03 \times 10^{-5}$
p-Nitrophenyl- $\beta$ -N-acetyl- glucosaminide	Large MW	$0.90 \times 10^{-3}$	$22.2 \times 10^{-5}$
	Small MW	$1.18 \times 10^{-3}$	$9.54 \times 10^{-5}$

$V_{max}$  IS EXPRESSED AS MOLES OF SUBSTRATE HYDROLYSED PER MINUTE AT 37° C PER MILLIGRAM ENZYME PROTEIN.

Table XVII.  $K_m$  and  $V_{max}$  Values Determined for Large and Small Molecular Weight Enzyme Forms

at all, from an enhanced affinity for these substrates. The same earlier work in this thesis had shown that the two enzyme populations differed to a greater extent in their activity towards  $E_2\alpha$ -17NAcglc than in their activity towards PNP-NAcglc. The greater spread in  $V_{max}$  values with the steroid as opposed to the p-nitrophenyl compound indicated that these activity differences were directly related to the catalytic rates of the respective reactions.

Under routine assay conditions both enzyme populations liberated aglycone from PNP-NAcglc more rapidly than from PNP-NAcgal (Table XI). The kinetic data showed that this could be attributed to an increased catalytic rate with the  $\beta$ -N-acetylglucosaminide compound and not to a lower  $K_m$  value. Enzyme species had a lower  $K_m$  for the galactosaminide than for the glucosaminide.

## 2) Lactone Inhibition of $\beta$ -Hexosaminidase Activity

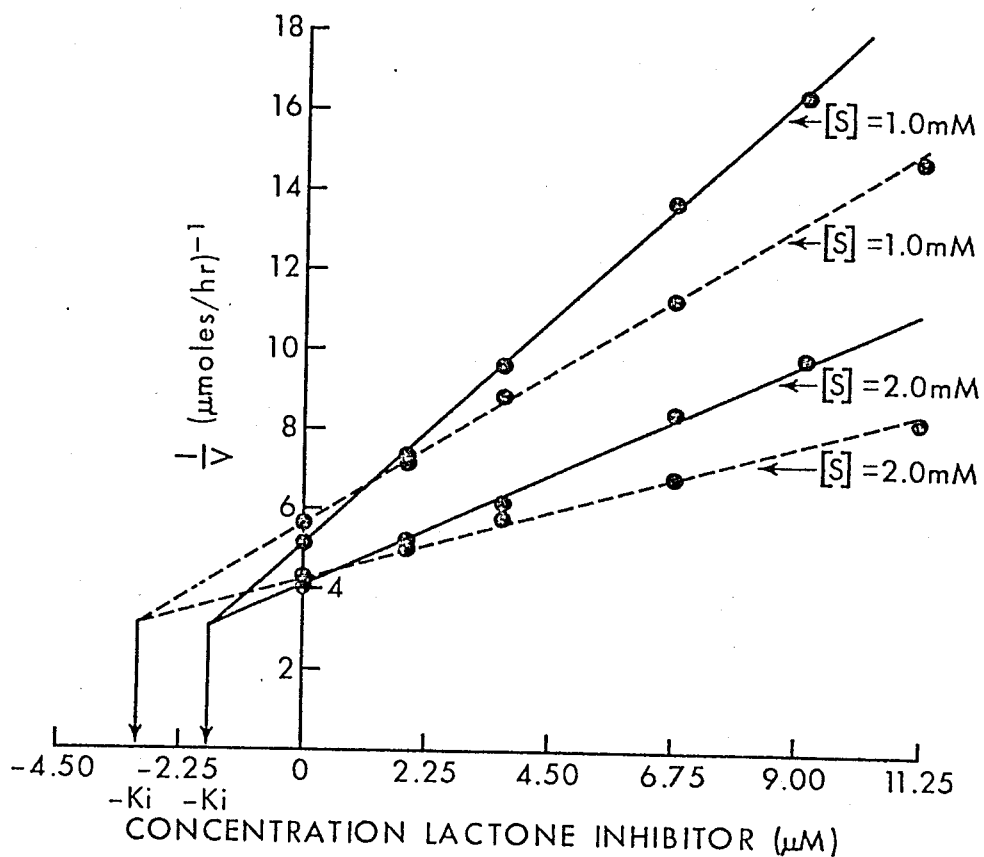
N-acetylglucosamino-lactone and N-acetylgalactosamino-lactone were used to inhibit the  $\beta$ -hexosaminidase activity of large and small MW enzyme forms towards PNP-NAcglc, PNP-NAcgal and  $E_2\alpha$ -17NAcglc. The results summarized in Table XVIII were derived from the Dixon plots shown in Figures 27 to 32.

The Dixon plots indicated that both lactones acted as competitive inhibitors with the p-nitrophenyl substrates, but exhibited apparent non-competitive behaviour when tested with the steroid substrate. However, the experimental conditions used may cast some doubt on the veracity of the latter result since the concentrations of the steroid substrate used were considerably lower than the  $K_m$  value. The pertinent equations for competitive and non-competitive inhibition (Dixon and Webb, p. 318 and p. 322 respectively, 1964) contain both the substrate concentration and the  $K_m$  value in the denominator. As the substrate concentration becomes

Lactone	Substrate	K <sub>i</sub> [M]	
		Large MW Forms	Small MW Forms
N-acetylglucosamino-lactone	p-Nitrophenyl-β-N-acetyl-glucosaminide	1.73x10 <sup>-6</sup> (C)	3.0x10 <sup>-6</sup> (C)
	p-Nitrophenyl-β-N-acetyl-galactosaminide	1.07x10 <sup>-6</sup> (C)	4.97x10 <sup>-6</sup> (C)
	17 α-Estradiol 17-β-N-acetyl-glucosaminide	2.90x10 <sup>-6</sup> (NC)	4.35x10 <sup>-6</sup> (NC)
N-acetylgalactosamino-lactone	p-Nitrophenyl-β-N-acetyl-glucosaminide	0.68x10 <sup>-6</sup> (C)	2.28x10 <sup>-6</sup> (C)
	p-Nitrophenyl-β-N-acetyl-galactosaminide	1.67x10 <sup>-6</sup> (C)	3.12x10 <sup>-6</sup> (C)
	17 α-Estradiol 17-β-N-acetyl-glucosaminide	1.40x10 <sup>-6</sup> (NC)	2.20x10 <sup>-6</sup> (NC)

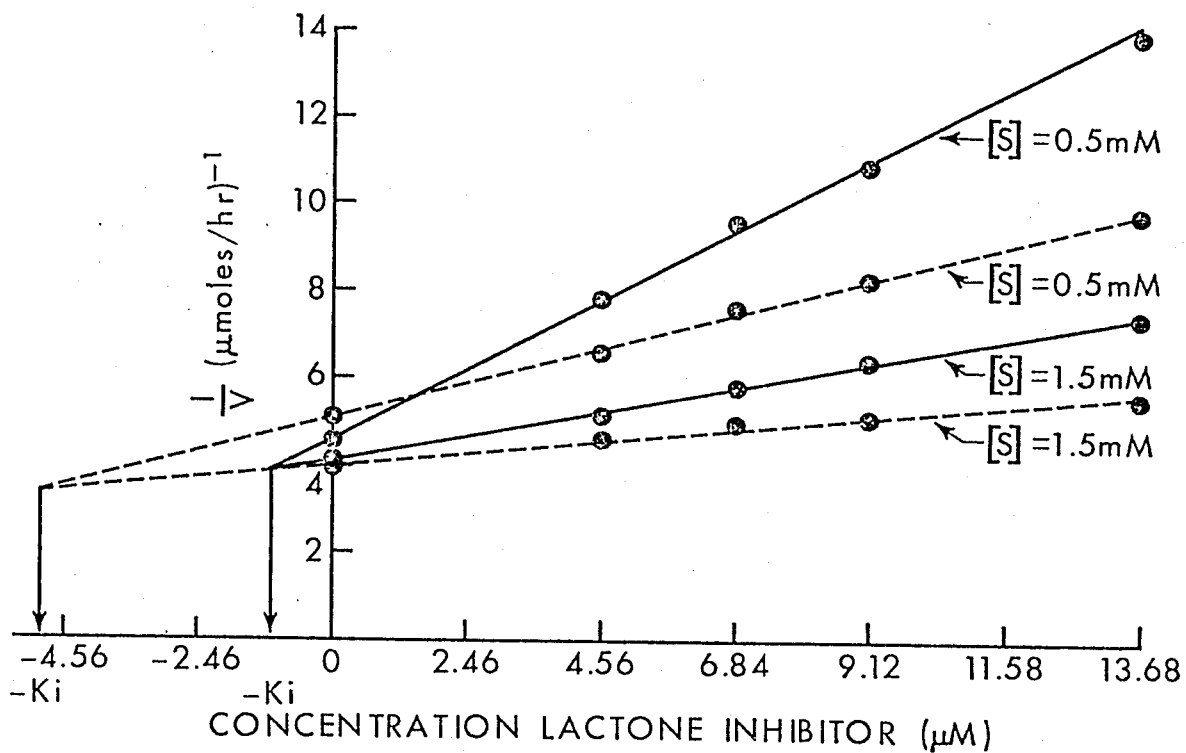
C AND NC REFER TO COMPETITIVE AND NON-COMPETITIVE INHIBITION, RESPECTIVELY.

Table XVIII. N-Acetylhexosamino-lactone Inhibition of β-Hexosaminidase Activity: K<sub>i</sub> Values and Type of Inhibition



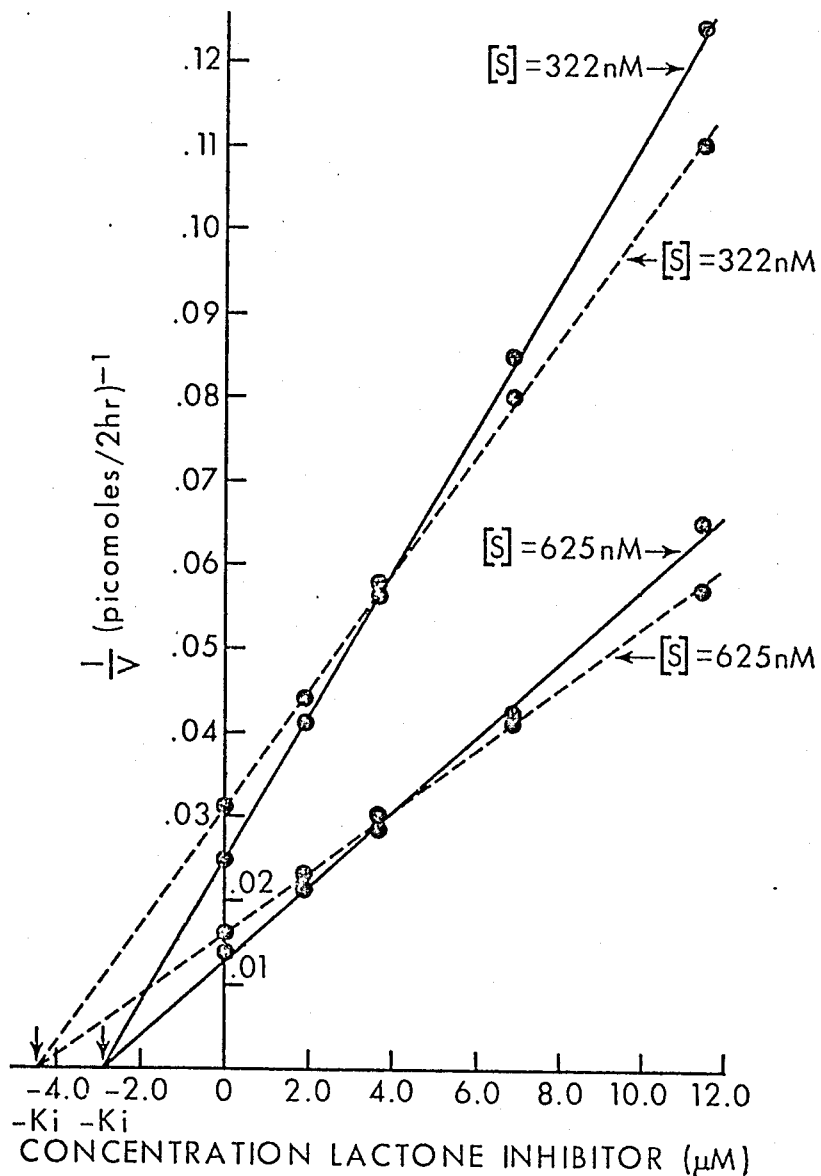
Solid lines refer to the activity of large MW enzyme forms and the broken lines to that of small MW forms.

Figure 27. Dixon Plot: N-Acetylglucosamino-lactone Inhibition of  $\beta$ -Hexosaminidase Activity Toward p-Nitrophenyl- $\beta$ -N-acetylglucosaminide



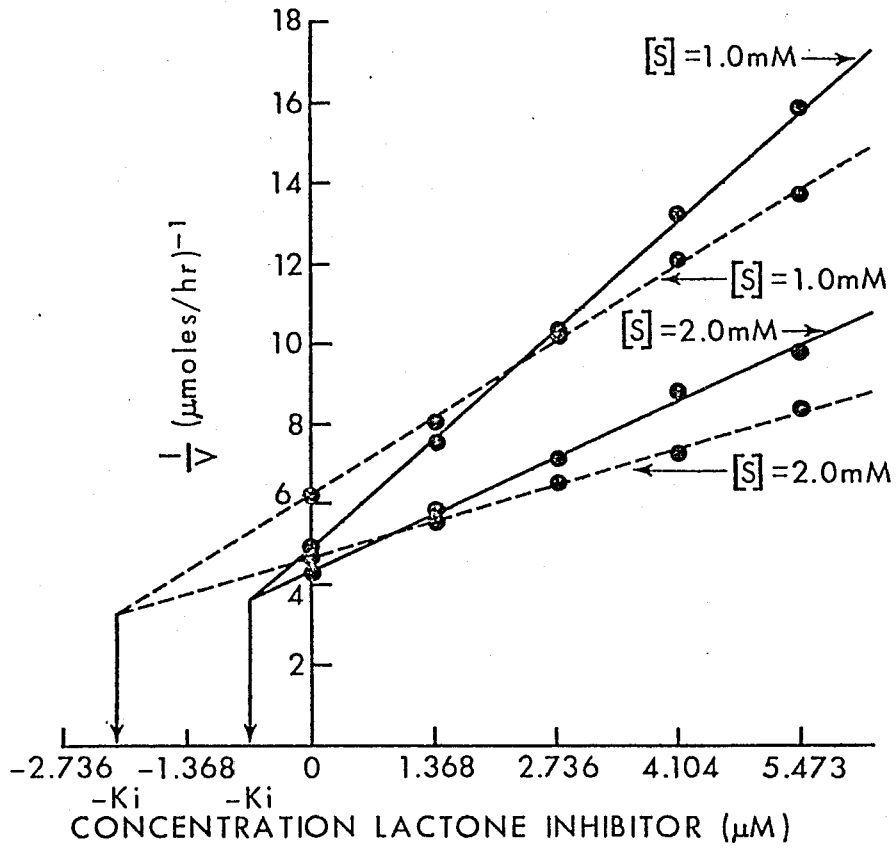
Solid lines refer to the activity of large MW enzyme forms and the broken lines to that of small MW forms.

Figure 28. Dixon Plot: N-Acetylglucosamino-lactone Inhibition of  $\beta$ -Hexosaminidase Activity Toward p-Nitrophenyl- $\beta$ -N-acetylgalactosaminide



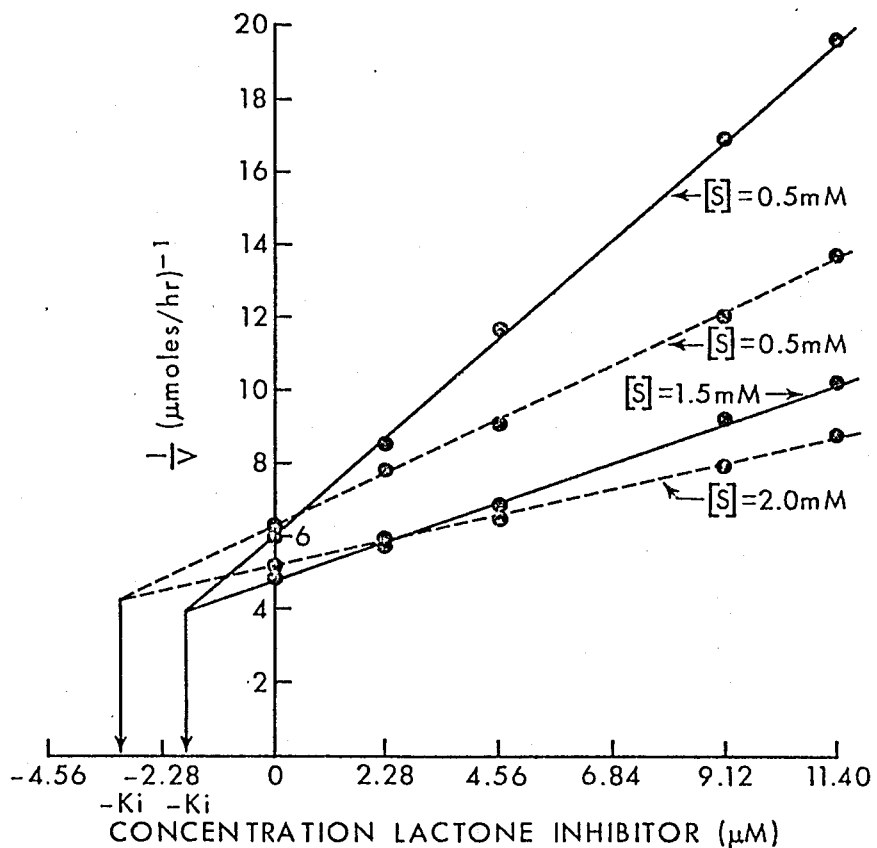
Solid lines refer to the activity of large MW enzyme forms and the broken lines to that of small MW forms.

Figure 29. Dixon Plot: N-Acetylglucosamino-lactone Inhibition of  $\beta$ -Hexosaminidase Activity Toward  $17\alpha$ -Estradiol  $17\beta$ -N-acetylglucosaminide



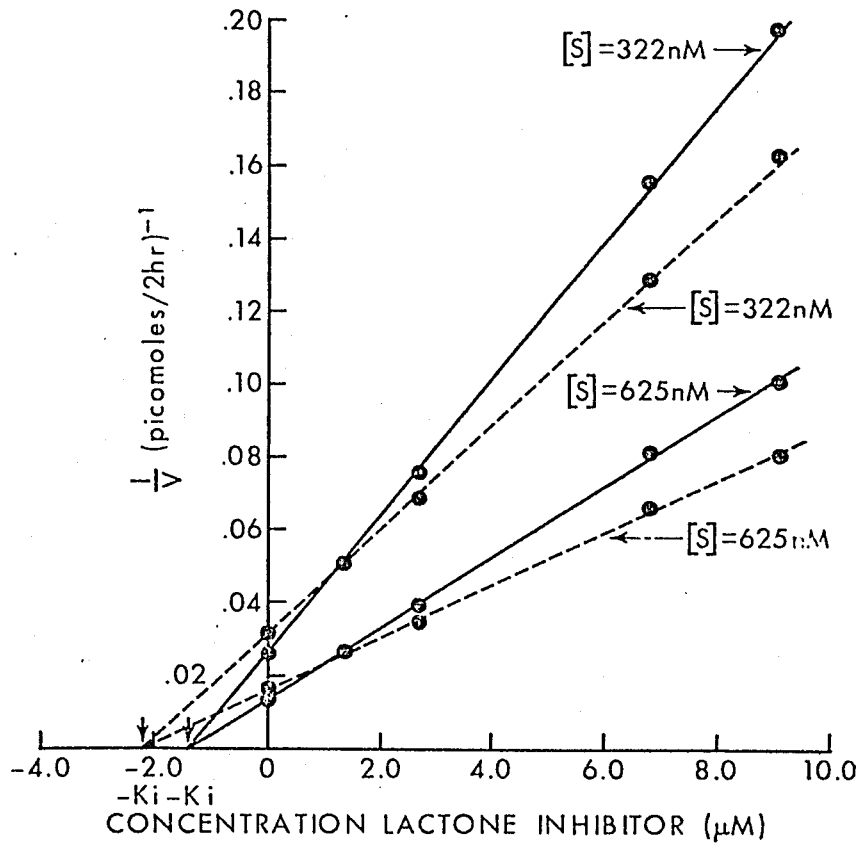
Solid lines refer to the activity of large MW enzyme forms and the broken lines to that of small MW forms.

Figure 30. Dixon Plot: N-Acetylgalactosamino-lactone Inhibition of  $\beta$ -Hexosaminidase Activity Toward p-Nitrophenyl- $\beta$ -N-acetylglucosaminide



Solid lines refer to the activity of large MW enzyme forms and the broken lines to that of small MW forms.

Figure 31. Dixon Plot: N-Acetylgalactosamino-lactone Inhibition of  $\beta$ -Hexosaminidase Activity Toward p-Nitrophenyl- $\beta$ -N-acetylgalactosaminide



Solid lines refer to the activity of large MW enzyme forms and the broken lines to that of small MW forms.

Figure 32. Dixon Plot: N-Acetylgalactosamino-lactone Inhibition of  $\beta$ -Hexosaminidase Activity Toward 17 $\alpha$ -Estradiol 17- $\beta$ -N-acetylglucosaminide

smaller, the sum of the two terms approaches the  $K_m$  value and the two equations tend to become indistinguishable producing Dixon plots which intersect at the abscissa signifying non-competitive inhibition. Nevertheless, the  $K_i$  values obtained from the point of intersection are still valid under these conditions. Since the concentrations of the steroid substrate used in the study were 1/10 and 1/20 of the Michaelis constant, it would be prudent to conduct additional experiments at appreciably higher concentrations of  $E_2\alpha$ -17NAcglc to unequivocally establish the exact nature of the type of lactone inhibition. It should be mentioned that a non-competitive mode of inhibition at higher concentrations of the steroid substrate would be very unusual and, therefore, highly noteworthy since lactones corresponding in configuration to sugars hydrolysed by an exo-glycosidase have invariably been found to inhibit the hydrolytic activity of that exo-glycosidase in a competitive manner (Levy and Snaith, 1972).

Both lactones were extremely potent inhibitors of enzyme activity, with inhibition constants in the range of  $10^{-6}$  M. Of the two enzyme populations, the large MW enzyme forms were slightly more susceptible to inhibition by the lactones. N-acetylglucosamino-lactone gave roughly equivalent  $K_i$  values against all three substrates and the same was true for N-acetylgalactosamino-lactone. According to theory described by Dixon and Webb (p. 203, 1964), if a competitive inhibitor inhibits two enzyme activities with the same  $K_i$  value, then both activities would be catalysed by the same enzyme. Taking into account the instability of the active lactone inhibitor in solution (Findlay *et al*, 1958), the closely similar  $K_i$  values obtained against the two p-nitrophenyl substrates in the present work indicated that each of the  $\beta$ -hexosaminidase species which comprised the large and small MW enzyme populations possessed both N-acetylglucosaminidase and N-acetylgalactosaminidase activity, and that each participated in the hydrolysis of the

two p-nitrophenyl compounds. This lack of specificity for the configuration of the fourth carbon in the amino sugar ring had been demonstrated earlier in this work through the failure of fractionation techniques to separate N-acetylglucosaminidase and N-acetylgalactosaminidase activity throughout the purification procedure (Table XI), and by the presence of both activities in each of the enzyme forms resolved by isoelectric focussing (Table XII).

A number of lactones corresponding in configuration to glucose, galactose and glucuronic acid were also tested for their inhibition of the hydrolytic activity of both enzyme populations. Table XIX shows that these lactones had a negligible inhibitory effect on the hydrolysis of the p-nitrophenyl substrate at a concentration where N-acetylgalactosaminolactone completely inhibited enzyme activity. From this it was concluded that only lactones derived from N-acetylhexosaminic acid could inhibit the hydrolytic activity of rabbit liver  $\beta$ -hexosaminidases. In addition, these results supported earlier data (Table XIII) which had shown that both enzyme populations were strictly specific for the N-acylamino moiety and catalysed hydrolytic reactions exclusively with sugars containing this group when bound in  $\beta$ -glycosidic linkage.

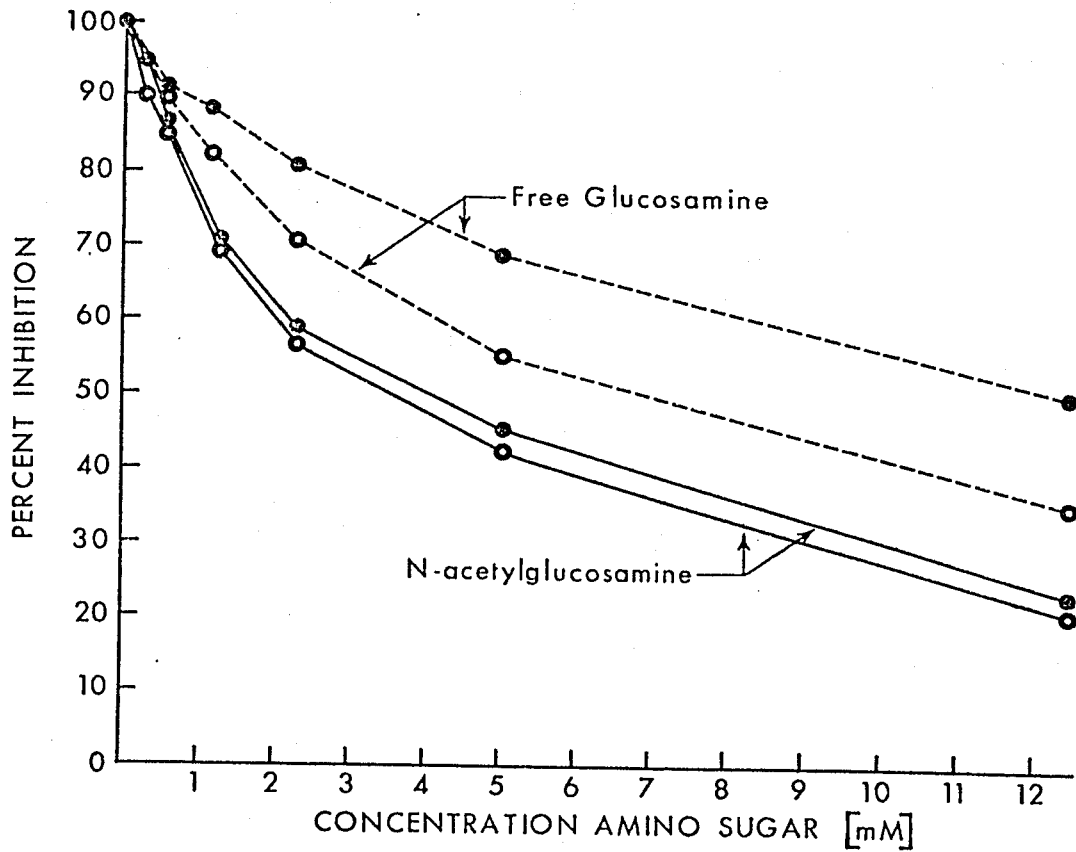
### 3) Effect of N-Acetylglucosamine and Glucosamine on Enzyme Activity

The reduction in steroid  $\beta$ -hexosaminidase activity which resulted from the addition of millimolar amounts of free and N-acetylated glucosamine to the routine  $E_2\alpha$ -17NAcglc assay is shown in Figure 33. The hydrolytic activity of both enzyme populations was inhibited by N-acetylglucosamine more readily than by free glucosamine. Neither amino sugar, however, was as potent an inhibitor as the two lactones derived from N-acetylhexosaminic acid; these latter two compounds produced a comparable degree of inhibition

Lactone	Activity (Percent Control)	
	Small MW Population	Large MW Population
Control (lactone absent)	100	100
Galactono-1,4-lactone	102	95
Glucono-1,5-lactone	99	100
Saccharo-1,4-lactone	103	96
Glucurono-lactone	98	95
N-acetylgalactosamino-lactone	1.5	1.3

Assays were carried out with p-Nitrophenyl- $\beta$ -N-acetylglucosaminide (1 mM) in the presence of 75 nanomoles of lactone.

Table XIX. Inhibition of  $\beta$ -Hexosaminidase Activity by Lactones



Lines with solid circles refer to the percent inhibition of the activity of small MW enzyme forms while those with open circles refer to the percent inhibition of the activity of large MW enzyme forms.

Figure 33. Inhibition of Steroid  $\beta$ -Hexosaminidase Activity by Free and N-Acetylated Glucosamine

when present at one-thousandth the concentration of the amino sugar. The inhibition curves generated by N-acetylglucosamine were essentially the same for both enzyme populations. Glucosamine, on the other hand, was somewhat more active in inhibiting the activity of large MW enzyme forms.

#### 4) Hydrolysis of Estrogen Conjugates

Large and small MW enzyme forms were reacted with a number of estrogen conjugates containing N-acetylglucosamine in  $\beta$ -glycosidic linkage (Table XX). Although all five conjugates tested underwent enzymatic hydrolysis, they were not all hydrolysed at the same rate. The major factor influencing hydrolysis appeared to be the position at which the amino sugar was attached to the steroid nucleus. At equal concentrations, conjugates with N-acetylglucosamine at the three position were hydrolysed at least thirty times more rapidly than those in which N-acetylglucosamine was bound through the  $17\alpha$ -hydroxyl. The enhanced hydrolysis of the C-3 N-acetylglucosaminides may have been due to the presence of an aromatic ring adjacent to the conjugated amino sugar. The hydrolysis of the  $17\alpha$ -N-acetylglucosaminides was influenced to some extent by other hydrophilic substituents conjugated to the steroid nucleus. Hydrolysis was slightly enhanced by substitution of glucuronic acid at the 3-position of the 17-monoconjugate and somewhat decreased when sulfate replaced glucuronic acid at the phenolic 3-hydroxyl. Large MW enzyme forms displayed essentially the same activity towards the two 3-N-acetylglucosaminide conjugates and the same was true for the small MW enzyme forms.

Steroid  $\beta$ -hexosaminidase activity did not parallel p-nitrophenyl  $\beta$ -hexosaminidase activity. For each of the steroids tested, the activity ratios indicated that large MW enzyme forms exhibited a greater preference for the steroid conjugate over the p-nitrophenyl compound than did small

Substrate	Concentration M	Moles Substrate Hydrolysed per Hour by 100 mU Enzyme *		Ratio Substrate Hydrolysed Large Forms/Small Forms
		Small MW Forms x10 <sup>-12</sup>	Large MW Forms x10 <sup>-12</sup>	
17 $\alpha$ -Estradiol 17- $\beta$ -N-acetylglucosaminide	2.0x10 <sup>-8</sup>	0.742	2.118	2.85 2.93 2.75 2.70 } 2.81
	1.0x10 <sup>-7</sup>	4.210	12.331	
	5.0x10 <sup>-7</sup>	21.905	60.208	
	1.25x10 <sup>-6</sup>	48.114	129.789	
17 $\alpha$ -Estradiol 3- $\beta$ -glucuronyl-17- $\beta$ -N-acetylglucosaminide	1.9x10 <sup>-8</sup>	1.079	2.599	2.41 2.22 2.28 2.27 2.28 2.32 } 2.30
	1.8x10 <sup>-7</sup>	7.860	17.422	
	5.6x10 <sup>-7</sup>	22.784	51.867	
	7.5x10 <sup>-6</sup>	296.50	673.08	
	2.5x10 <sup>-5</sup>	948.03	2158.48	
	5.0x10 <sup>-5</sup>	1857.16	4305.74	
17 $\alpha$ -Estradiol 3-sulfo-17- $\beta$ -N-acetylglucosaminide	2.0x10 <sup>-8</sup>	0.279	0.728	2.61
Estrone 3- $\beta$ -N-acetylglucosaminide	1.2x10 <sup>-5</sup>	20.37	35.81	1.76 1.69 1.61 1.61 } 1.67
	2.4x10 <sup>-5</sup>	41.32	69.72	
	4.0x10 <sup>-5</sup>	75.91	122.33	
	6.0x10 <sup>-5</sup>	112.23	180.64	
17 $\beta$ -Estradiol 3- $\beta$ -N-acetylglucosaminide	1.2x10 <sup>-5</sup>	20.18	33.28	1.65 1.70 1.61 1.60 } 1.64
	2.4x10 <sup>-5</sup>	40.93	69.72	
	4.0x10 <sup>-5</sup>	72.64	117.26	
	6.0x10 <sup>-5</sup>	102.24	163.21	

\* 1 UNIT (U) OF ENZYME HYDROLYSES 1 MICROMOLE p-NITROPHENYL- $\beta$ -N-ACETYL-GLUCOSAMINIDE PER MINUTE UNDER ROUTINE ASSAY CONDITIONS. ENZYME ASSAYS WITH EACH STEROID CONJUGATE WERE CARRIED OUT AS DESCRIBED PREVIOUSLY UNDER GENERAL METHODS.

Table XX. Activity of Large and Small Molecular Weight Enzyme forms toward Steroid  $\beta$ -N-acetylglucosaminide Substrates

MW enzyme forms. Deviations from p-nitrophenyl  $\beta$ -hexosaminidase activity were most pronounced with the conjugates in which N-acetylglucosamine was attached to the aliphatic  $17\alpha$ -hydroxyl. Activity ratios ranged from 2.30 to 2.81. Activity differences were less pronounced with the 3-N-acetylglucosaminides as indicated by the lower ratios. This closer equivalence of steroid to p-nitrophenyl  $\beta$ -hexosaminidase activity may have reflected the similar positioning of the amino sugar adjacent to an aromatic ring in both PNP-NAcglc and these latter steroid conjugates.

Table XXI shows that the steroid  $\beta$ -hexosaminidase activity of large and small MW enzyme forms was not reduced when unconjugated estrogens were added to the routine  $E_2\alpha$ - $17N$ Acglc assay in quantities which exceeded their solubility limit in the citrate buffer. The failure of saturated solutions of three of these steroids, namely  $17\alpha$ -estradiol,  $17\beta$ -estradiol and estrone, to inhibit enzyme activity was of particular interest since previous work (Table XX) had established that their N-acetylglucosaminyl monoconjugates were excellent enzyme substrates. The low affinity exhibited by these free steroids for the substrate binding site on the enzyme species contrasted sharply with their ready incorporation into enzyme-substrate complexes when conjugated with N-acetylglucosamine. This dichotomy between the two sets of estrogens could only be interpreted to mean that the amino acids comprising the substrate binding site interacted predominantly with the amino sugar substituent of steroid (and, by extrapolation, perhaps other classes of)  $\beta$ -hexosaminides to form the enzyme-substrate complex. However, it should be stressed that the aglycone (or non amino sugar) moiety did have an effect on enzyme activity. Tables XX and XXI when taken together suggest that the non amino sugar portion of the substrate either played a secondary, but still important, role in promoting or obstructing the formation of the enzyme substrate complex or that it affected enzyme activity by

Unconjugated Steroid Added	Concentration* [ $\mu$ M]	Enzyme Activity (Percent Control)	
		Large MW Forms	Small MW Forms
Control (no free steroid)	-	100	100
17 $\alpha$ -Estradiol	437	100	99
17 $\beta$ -Estradiol	375	99	102
Estrone	450	101	98
Estriol	375	103	101
16-Epiestriol	450	100	99
16, 17-Epiestriol	450	101	102

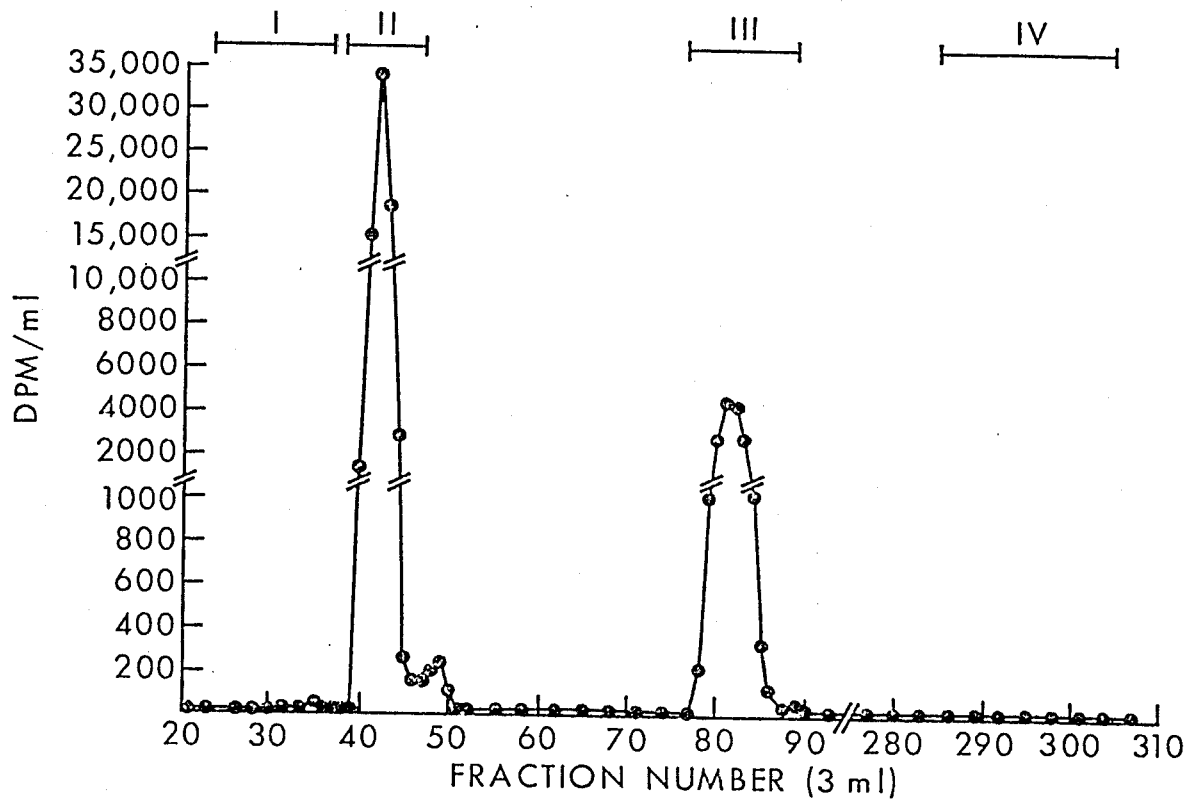
\* A considerable fraction of the added unconjugated steroid remained in suspension and did not dissolve in the assay buffer.

Table XXI. Effect of Unconjugated Estrogens on the Steroid  $\beta$ -Hexosaminidase Activity of Large and Small Molecular Weight Enzyme Forms

influencing the catalytic rate of bond cleavage.

#### 5) Determination of Transglycosylase Activity

The two enzyme populations were tested for transglycosylase activity using a number of different assay conditions. The radioactivity profile shown in Figure 34 is typical of the results which were obtained from the various transglycosylase assays following separation of the reaction components by gel filtration. Greater than 99.9% of the radioactivity eluted in two peaks, one of which corresponded to unreacted PNP-NAcglc and the other of which corresponded to liberated N-acetylglucosamine. Neither enzyme population catalysed the transfer of N-acetylglucosamine from the p-nitrophenyl substrate to either the chondroitin sulfate or hyaluronate tetrasaccharide. A small fraction of the amino sugar, however, was transferred to an acceptor other than water since a small peak of radioactivity consistently appeared in the eluate fractions just after N-acetylglucosamine. On the basis of the elution pattern of PNP-NAcglc and N-acetylglucosamine, it was considered probable that this peak represented trace amounts of the di-N-acetylglucosaminide of p-nitrophenol.



- I: Tetrasaccharide
- II: N-acetylglucosamine
- III: p-Nitrophenyl- $\beta$ -N-acetylglucosaminide
- IV: p-Nitrophenol

Reaction mixture contained large MW enzyme forms (140 mU), p-nitrophenyl- $\beta$ -N-acetyl-[6-<sup>3</sup>H]-glucosaminide (18 micromole, 326,000 DPM) and tetrasaccharide (12 micromole) in 1.5 ml 0.1 M citrate pH 6.6 - 0.01% BSA. Incubation was for 4 hours at 37°C.

Figure 34. Separation of Reaction Products in the Transglycosylase Assay by Gel Filtration

D) DISCUSSION

In most respects, large and small MW enzyme forms are similar in their enzymatic properties. Both enzyme populations:

- 1) give similar  $K_m$  values for substrates;
- 2) have similar affinities for lactones derived from N-acetylhexosaminic acid and are inhibited by them in the same manner;
- 3) undergo very similar percentage losses in activity in the presence of N-acetylglucosamine and quite similar losses in the presence of glucosamine;
- 4) display little affinity both for unconjugated estrogens and for lactones which do not correspond in configuration to N-acetylglucosamine or N-acetylgalactosamine;
- 5) preferentially hydrolyse C-3  $\beta$ -N-acetylglucosaminide conjugates of estrogens over C-17 conjugates;
- 6) are composed of enzyme species which are able to catalyse the hydrolysis of both  $\beta$ -N-acetylglucosaminides and  $\beta$ -N-acetylgalactosaminides;
- 7) hydrolyse PNP-NAcglc more rapidly than PNP-NAcgal;
- 8) fail to show any but trace amounts of transglycosylase activity.

In one important respect, however, the two enzyme populations deviate significantly from each other in their enzymatic properties and that is in their activity towards substrates. Although neither enzyme population was found to exclusively catalyse the hydrolysis of any of the substrates tested, substrates were consistently hydrolysed by large MW enzyme forms more rapidly than by small MW enzyme forms. The kinetic studies showed that this was due to a more efficient cleavage of the  $\beta$ -hexosaminide bond once substrates were bound in the enzyme-substrate complex. Activity differences were most pronounced with substrates in which the amino sugar

was linked to an aliphatic hydroxyl group, and somewhat muted when the two populations were assayed with substrate containing N-acetylglucosamine or N-acetylgalactosamine attached to a phenolic hydroxyl. The two enzyme populations diverged more widely from each other in their steroid  $\beta$ -hexosaminidase activity than in their p-nitrophenyl  $\beta$ -hexosaminidase activity.

In many of their enzymatic properties, the two rabbit liver enzyme populations are quite similar to other  $\beta$ -hexosaminidases.  $K_m$  values for PNP-NAcglc and PNP-NAcgal, for example, are of the same order of magnitude as those given by the enzyme from other sources for the hydrolysis of the corresponding amino sugar derivatives of p-nitrophenol and 4-methylumbelliferone (Tarentino and Maley, 1971; Reyes and Byrde, 1973; Every and Ashworth, 1973; Edwards et al., 1975; Geiger et al., 1977; Banerjee and Basu, 1975). In addition, the rabbit liver enzyme species have a higher  $K_m$  and  $V_{max}$  for PNP-NAcglc than for PNP-NAcgal. With the sole exception of Hex C (Besley and Broadhead, 1976), such behaviour is typical of all other  $\beta$ -hexosaminidases which have been studied (Vikha et al., 1971; Tallman et al., 1974; Hayase et al., 1973; Li and Li, 1970; Winchester, 1971; Edwards et al., 1975). In general when the aglycone moiety is kept constant, rate constants and Michaelis constants are always higher for reactions with the glucosaminide derivative than for those with the galactosaminide derivative. This signifies that while  $\beta$ -hexosaminidases may bind the galactosaminide derivative more tenaciously in an enzyme-substrate complex, bond cleavage always occurs more rapidly with the glucosaminide derivative. The similarity between  $K_m$  values given by the large and small MW enzyme forms for each substrate is also characteristic of the multiple forms of other  $\beta$ -hexosaminidases. Human Hex A, Hex B, Hex P and Hex I<sub>2</sub> differ minimally, and in some cases not at all, from each other in their

Michaelis constants for a variety of substrates including globoside and asialo GM<sub>2</sub> (Wenger et al., 1972), β-N-acetylhexosaminides of p-nitrophenol (Kanfer and Spielvogel, 1973a; Sandhoff and Waessle, 1971) and β-N-acetylhexosaminides of 4-methylumbelliferone (Price and Dance, 1972; Stirling, 1972; Srivastava et al., 1974a). The same is true for the multiple forms of beef spleen and porcine kidney β-hexosaminidases (Verpoorte, 1972; Wetmore and Verpoorte, 1972). Some of the widest differences in Michaelis constants have been reported for multiple forms from equine kidney (Seyama and Yamakawa, 1974) and ram gonad (Bullock and Winchester, 1973), but even in these cases values vary by less than an order of magnitude.

Further evidence of typical behaviour is provided by the lactone inhibition experiments. As is the case with other β-hexosaminidases (Kanfer and Spielvogel, 1973; Mian et al., 1975; Findlay et al., 1958), the two rabbit liver enzyme populations are inhibited in their hydrolytic action by both N-acetylglucosamino-lactone and N-acetylgalactosamino-lactone; in addition, K<sub>i</sub> values are in the range of 10<sup>-6</sup> - 10<sup>-7</sup> M and inhibition is competitive (against the two p-nitrophenyl substrates). For reasons discussed under Results, it would be prudent to consider the non-competitive nature of the lactone inhibition obtained from Dixon plots when E<sub>2</sub>α-17NAcglc was the substrate as inconclusive pro tempore.

Finally the rabbit liver enzyme forms are inhibited in their hydrolytic activity by N-acetylglucosamine more readily than by glucosamine. This is the case with other β-hexosaminidases as well (Kanfer and Spielvogel, 1977; Banerjee and Basu, 1975; Mian et al., 1975); N-acetylation of the free amino sugar (either glucosamine or galactosamine) results in a more potent inhibitor of enzyme activity. It is interesting to note that studies have consistently shown that N-acetylgalactosamine inhibits both

hydrolytic activities of  $\beta$ -hexosaminidases with a lower  $K_i$  than N-acetylglucosamine (Robinson and Stirling, 1968; Edwards et al, 1975; Mian et al, 1975). Inhibition with these sugars may be either competitive or non-competitive (Vikha et al, 1971; Mian et al, 1975; Pugh, Leaback and Walker, 1957; Aruna and Basu, 1976; Frowein and Gatt, 1967).

CHAPTER 6  
GENERAL DISCUSSION

This is the first report not only of the purification of a rabbit  $\beta$ -hexosaminidase but also of a detailed examination of the physical and catalytic properties of the multiple forms of this enzyme from the rabbit. The rabbit enzyme is present in liver extracts in at least nine forms which differ in overall charge. For a number of reasons it is likely that these multiple forms are true intracellular species and not artifacts generated by the extraction procedure. Firstly, artifacts could be expected to be characterized by close similarities in structure and enzymatic properties. However, on the basis of their substrate specificities, their  $V_{max}$  values, their size and their absorption properties on DEAE-Cellulose, the large and small MW populations are distinctly different. In addition, the most negatively charged species, form A, differs markedly from large and small forms in substrate specificity and is uniquely characterized by extreme sensitivity to low pH. Secondly, there is no evidence that the large and small forms of the enzyme tend to interconvert, except possibly to the most minimal extent, throughout the purification procedure; under routine extraction and purification conditions, appreciable aggregation and fragmentation of enzyme species does not occur. Finally, storage of the liver extract for several days at 4°C does not result in an altered focussing profile. It is therefore unlikely that proteases or glycosidases are responsible for the generation of multiplicity within the large and small MW enzyme populations once the integrity of the intracellular structure is destroyed by homogenization. Indirect evidence to support the existence of multiple forms in rabbit liver in vivo comes from a comparison with the situation in other vertebrates; in

tissues, multiple forms are the norm rather than the exception (Sandhoff, 1969; Tarentino and Maley, 1971; Vikha et al, 1971; Robinson, Jordon and Horsburgh, 1972; Bullock and Winchester, 1973; Hayase et al, 1973; Srivastava et al, 1976).

With the exception of form A which is lost in the first stage of purification, the enzyme forms have been purified to near homogeneity. The purified enzyme species were studied as two populations - a large MW population containing the five species with the most basic isoelectric points (forms E-I) which weigh approximately 270,000 Daltons, and a small MW population composed of the three more negatively charged species (forms B-D) with molecular weights estimated at 150,000 Daltons. The decision to consider the species comprising each population as one entity when determining their enzymatic properties is justified on the basis of the preliminary studies with the resolved enzyme species in the isoelectric focussing gradient. The substrate specificities of forms B-D on the one hand and of forms E-I on the other were sufficiently similar to suggest that, with the substrates used in this work, additional information would not be forthcoming from a study of individual enzyme species as opposed to entire populations.

In the study of physical properties, a somewhat different rationale constituted the basis for grouping whole populations according to size as opposed to using individual enzyme species. The similar sizes and substrate specificities of the constituent members of each population suggested that they were closely similar in structure and pointed to the feasibility of determining, as a first approximation, the physical properties of individual members from a study of the whole population. In addition, there was evidence that structural differences between populations were much

greater than those within populations. It was, therefore, decided not to concentrate on the comparatively minor differences in structure within populations but rather to determine the overall structural relationship between large and small enzyme forms. This decision was taken in full recognition of the fact that a complete understanding of the physiological significance of individual enzyme species might well require a rigorous and detailed investigation of these minor differences in structure.

The two main populations of the rabbit liver enzyme differ markedly in size, which is contrary to the situation for multiple forms of  $\beta$ -hexosaminidase in almost all other vertebrates examined. Despite this, however, it would appear that in many respects the rabbit liver enzyme forms possess physical properties which are typical of those of corresponding forms in other higher animals. In particular, this applies to their subunit composition and structural relationship. For example, the two main enzyme populations in the rabbit, namely the large and small forms, exhibit analogous conversion behaviour to human Hex B and Hex A, respectively, when exposed to conditions which disaggregate quaternary structure (Beutler and Kuhl, 1975). A number of common structural subunits characterize the two major enzyme populations of each of these species. This conclusion is based on the fact that, when subjected to freezing and thawing in 3 M NaCl, small forms can convert to large forms (rabbit) and Hex A can convert to Hex B (human). However, large forms and Hex B do not convert to small forms and Hex A, respectively, under these conditions. This can be interpreted to mean that the major acidic populations in both species contain structural elements which are absent from the basic forms. The above interpretation has been experimentally confirmed for the human isoenzymes by direct examination of their dis-

aggregated subunits (Beutler et al, 1976; Geiger and Arnon, 1976).

Studies with the human isoenzymes have definitely established that in Tay-Sachs disease, Hex B is present when Hex A is totally absent from the body as judged by immunological and electrophoretic criteria. Clearly, the synthesis of Hex B is not dependent on the prior synthesis of Hex A. By analogy, it can be postulated that large or basic forms in the rabbit are not generated in vivo from small or acidic forms by a precursor-product type mechanism, even though this can take place in vitro through freezing and thawing in high salt concentration. Since a precursor-product type mechanism must be considered to be highly unlikely in vivo, the logical alternative must be the de novo synthesis of each population, at the same time bearing in mind the possibility that, within a population, enzyme species could be derived from one another through post-translational modification of a common subunit structure.

The synthesis of 17- $\beta$ -N-acetylglucosaminide conjugates of 17 $\alpha$ -estradiol by the rabbit in vivo, most notably 17 $\alpha$ -estradiol 3- $\beta$ -glucuronyl-17- $\beta$ -N-acetylglucosaminide, has led to speculation as to whether these metabolites might be substrates for hydrolytic action by  $\beta$ -hexosaminidase. Previous studies (Whittemore and Layne, 1965; Mellor et al, 1973; Labow, unpublished results) with the rabbit have supported this possibility in that the monoconjugate, 17 $\alpha$ -estradiol 17- $\beta$ -N-acetylglucosaminide, was found to serve as a substrate for  $\beta$ -hexosaminidase action both in vivo and in vitro. The present work shows that the multiple forms of rabbit liver  $\beta$ -hexosaminidase vary considerably in their reactivity towards this substrate. In addition, differences in activities were demonstrated among the enzyme species for four other estrogen  $\beta$ -N-acetylglucosaminides, none of which had been tested previously for its susceptibility to

hydrolysis by rabbit or any other  $\beta$ -hexosaminidase. It was observed that all the rabbit liver enzyme forms have steroid  $\beta$ -hexosaminidase activity, with the large forms being appreciably more active than the small forms. In addition, differences in steroid  $\beta$ -hexosaminidase activity between the two populations are more pronounced for 17-N-acetylglucosaminide conjugates than for 3-N-acetylglucosaminides. With both populations, hydrolysis of the steroid  $\beta$ -N-acetylglucosaminide linkage is not significantly affected by the presence of other, non-amino sugar hydrophilic substituents on the steroid nucleus. The limited tests performed with form A pointed to its extremely high specificity for steroid conjugates as compared to p-nitrophenyl- $\beta$ -N-acetylglucosaminide. While it is not possible to perform parallel tests with individual forms in vivo, it is entirely plausible that different enzyme forms would vary in their reactivities to naturally-occurring estrogen  $\beta$ -N-acetylglucosaminides in vivo as well.

17 $\alpha$ -Estradiol 3- $\beta$ -glucuronyl-17- $\beta$ -N-acetylglucosaminide is quantitatively by far the most important steroid conjugate synthesized by the rabbit, estrogens being almost entirely excreted in this form in the urine (Layne et al, 1964; Layne, 1965). Even more important, it is also the only steroid  $\beta$ -N-acetylglucosaminide which the rabbit is known to produce in the course of normal metabolism (Collins et al, 1968). In the author's opinion, the first priority of future research should be to determine the extent to which this double conjugate is hydrolysed in vivo by rabbit  $\beta$ -hexosaminidases. This is essential for establishing the physiological significance of the steroid  $\beta$ -hexosaminidase activity which was demonstrated in the present study in vitro. Studies with the double conjugate are expressly pinpointed since present knowledge of

the hydrolysis of estrogen  $\beta$ -N-acetylglucosaminides in the rabbit is based solely on studies in which preformed  $17\alpha$ -estradiol  $17\beta$ -N-acetylglucosaminide has been exogenously administered. The weakness in this approach lies in the fact that this monoconjugate could only arise in vivo through hydrolysis of the 3-phenolic glucuronyl moiety of the double conjugate and, as yet, this has not been demonstrated in vivo.

A suggested procedure to determine the extent to which N-acetylglucosamine is cleaved from the double conjugate in vivo would be to inject preformed double conjugate labelled with tritium in the steroid nucleus and carbon-14 in the amino sugar moiety. An altered  $^{14}\text{C}/^3\text{H}$  ratio in the double conjugate excreted in the urine would indicate cleavage of N-acetylglucosamine from the steroid prior to reconstitution and excretion of the double conjugate. The extent of the change in the  $^{14}\text{C}/^3\text{H}$  ratio would bear a direct relationship to the impact that  $\beta$ -hexosaminidases exert on estrogen  $\beta$ -N-acetylglucosaminide metabolism in the rabbit.

Although the rabbit obviously utilizes steroid conjugation, particularly that of estrogens, as a mechanism for excretion, there is strong evidence that conjugates of C-18 steroids may also be formed in vivo which remain localized in tissues. Such conjugation could serve to protect the steroid nucleus from degradative metabolism or to influence intracellular compartmentalization (Williamson and Layne, 1975). Such localization would appear to be the case with the 3-glucoside monoconjugates of  $17\alpha$ -estradiol,  $17\beta$ -estradiol and estrone. In vitro studies with liver microsomes and homogenates have demonstrated the presence of transferases which specifically transfer glucose to the 3-phenolic hydroxyl of unconjugated estrogens to form 3-glucoside monoconjugates (Collins, Williamson and Layne, 1970; Labow, Williamson and Layne, 1973;

Labow and Layne, 1974). Such conjugation is effected both through a UDP-glucose dependent mechanism and through a UDP-glucose independent mechanism possibly involving lipid intermediates to transfer the sugar (Williamson, Polakova and Layne, 1971; A. Comerton, thesis in preparation). Despite rigorous examination, however, these conjugates have not been detected in the excreta of living rabbits (Collins, Williamson and Layne, 1970) or in the circulatory fluid following perfusion of isolated rabbit liver with unconjugated steroids (Layne, Quamme, Labow, Mellor, Polakova and Williamson, 1970; Quamme, Layne and Williamson, 1972). Since 3-glucoside monoconjugates do not accumulate in the liver, the sugar moiety must be cleaved in vivo. It would appear that this is done by the (steroid-)  $\beta$ -glucosidase of rabbit liver which has been demonstrated to be extremely specific for the 3-phenolic glucosidic bond of these monoconjugates in vitro (Mellor and Layne, 1971, 1974). This  $\beta$ -glucosidase is far less active in hydrolysing the glucosidic bond of estrogen 17- $\beta$ -glucoside monoconjugates.

Bearing this in mind, there is a striking parallel between the specificities of large and small MW forms of  $\beta$ -hexosaminidase and the corresponding  $\beta$ -glucosidase in rabbit liver as determined in vitro in that both glycosidases cleave the sugar moiety from 3-phenolic bonds in estrogens more efficiently than from the 17-aliphatic hydroxyl. It is interesting to speculate that this may be more than mere coincidence and may reflect the fact that  $\beta$ -hexosaminidases, like  $\beta$ -glucosidase, are intricately involved in steroid conjugate metabolism in hydrolysing 3-glycosidic conjugates of estrogens in vivo. To establish whether  $\beta$ -hexosaminidases perform such a function in vivo, it will first be necessary to demonstrate experimentally that the rabbit liver can synthesize 3-N-acetylglucosaminide

conjugates of estrogens. If the rabbit should, in fact, have such a capability, it would be of importance towards unravelling a major hydrolytic function of the rabbit  $\beta$ -hexosaminidases in vivo.

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