STEROID β-D-GLUCOSIDASE ACTIVITY IN RABBIT TISSUES

by

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SUMMARY

Previous work has shown that when rabbits are injected with estrone or 17α-estradiol, one of the products excreted in their urine is a double conjugate of 17α-estradiol with glucuronic acid at position 3 and glucose at position 17. Incubation of 17α-estradiol-3-glucuronide with rabbit liver microsomes and UDP-glucose leads to the formation of this double conjugate. Microsomes from rabbit tissues will also transfer glucose, from UDP-glucose, to the 3-hydroxyl of estrone or 17α- or 17β-estradiol to form 3-glucosides.

These 3-glucosides have not, however, been detected in urine and when a 3-glucoside is injected in vivo, the glucose is entirely removed, in contrast to the situation with the corresponding 3-glucuronides, which are excreted as the double conjugate without removal of the glucuronic acid. Because of these results, it became of interest to study the ability of various rabbit tissues to remove glucose from the steroid and to investigate and purify the steroid glucosidase activity.

Three main substrates were used in the present study; 17α-estradiol-3-glucoside, 17α-estradiol-17-glucoside, and p-nitrophenylglucoside.

Preliminary experiments, using liver homogenates, showed pH optima of 5.5 for the β-glucosidase activity towards both steroid substrates. However, the activity towards the 3-glucoside was approximately 100-fold greater than that towards the 17-glucoside. A tissue distribution study showed that the kidney

and small intestine also exhibited this preference for the 3-glucoside, whereas the ratio of the glucosidase activities towards the 3- and the 17-glucosides of estradiol in the large intestine, spleen, ovaries, uterus, whole blood and plasma was much lower, approximately equal for both substrates.

Subcellular fractionation of the liver enzyme showed that the β-glucosidase activity appeared to be located in the unsedimentable fraction of the cell. Further experiments showed that the enzyme was definitely not associated with the lysosomes and that it was indeed soluble. This soluble enzyme has been purified over 1000-fold using ammonium sulphate precipitation, gel filtration on Sephadex G-150, and ion exchange chromatography on CM-cellulose. The purified enzyme hydrolyses a number of steroid and synthetic glucosides but shows no activity towards other steroid or synthetic glycosides. It does not show disaccharidase activity, nor does it exhibit transglycosylase activity.

Kinetic data for five different substrates have been obtained, K_m's ranging from 5×10^{-10} M for 3-glucoside of 17α-estradiol to 2.9×10^{-4} M for deoxycorticosterone glucoside. The enzyme has a pI of 4.78. A common Ki of 2.3×10^{-4} M was obtained with gluconolactone for the liver β-glucosidase activity towards 17- and 3-glucosides of 17α-estradiol and towards p-nitrophenylglucoside. Similarly with galactonolactone, a common Ki of 1.8×10^{-3} was obtained for the liver β-glucosidase activity toward the same substrates. Double substrate

reactions show an apparent competitive inhibition when these three substrates are assayed in the presence of each other. These results indicate a single active catalytic center for the hydrolysis of both glucosides and galactosides.

Acrylamide disc electrophoresis of the purified liver enzyme on analytical gels at pH 8.3 and on SDS gels yielded a single major protein with minor impurities. The molecular weight as determined by gel filtration and by SDS electrophoresis was 51,000 and 58,000 respectively.

Subcellular fractionation of other tissues has shown that the enzyme appears to be soluble in those tissues where 3/17 ratio is high and only partially soluble in the other tissues. Further purification of the small intestine β-glucosidase showed that its properties closely resembled those of the liver enzyme in that it was soluble, precipitated with the same concentration of ammonium sulphate and co-chromatographed on Sephadex G-150 and CM-cellulose columns. On the other hand, the large intestine and spleen β-glucosidase activity was largely sedimented with the mitochondria and microsomes. The heterogeneity of the distribution indicated that in these tissues the intracellular distribution could be lysosomal. The large intestine enzyme could be solubilized from the combined mitochondrial-microsomal pellet by the detergent Triton X-100. The "soluble" enzyme, however, did not behave in the same manner as the liver or small intestine enzyme and appeared to be much larger in size, being eluted in the void volume of a Sephadex

G-200 column (exclusion limited 800,000).

It was concluded from these results, that in the liver both the 3- and the 17-glucosides of 17α-estradiol as well as a number of other steroid and synthetic glucosides were hydrolysed by a single enzyme. The experiments with the small intestine indicated that the β-glucosidase of this tissue closely resembled that of the liver. Both these tissues showed a high 3/17 ratio of activity towards the two mono-glucosides of 17α-estradiol as compared to that of the large intestine and spleen. Since in these latter tissues the intracellular distribution appeared to be lysosomal, the difference in specificity among the tissues may be due mainly to a difference in intracellular location of the glucosidase. It is also possible, however, that a significant difference in the structure of the isoenzymes of the two types of tissues may be observed when further attempts are made to purify them.

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GLOSSARY

The following compounds have been referred to in this thesis by their trivial names

Trivial Name	Compound
Dehydroisoandrosterone	3β-Hydroxyandrost-5-en-17-one
Dehydroisoandrosterone glucoside	17-Oxoandrost-5-en-3β-yl-β-D-glucopyranoside
Deoxycorticosterone	21-Hydroxypregn-4-en-3,20-dione
Deoxycorticosterone glucoside	3,20-Dioxopregn-4-en-21-yl-β-D-glucopyranoside
Estrone	3-Hydroxyestra-1,3,5(10)-trien-17-one
Estrone glucoside	17-Oxoestra-1,3,5(10)-trien-3-yl-β-D-glucopyranoside
17α-Estradiol	Estra-1,3,5(10)-trien-3,17α-diol
17α-Estradiol-3-galactoside	17α-Hydroxyestra-1,3,5(10)-trien-3-yl-β-D-galactopyranoside
17α-Estradiol-17-galactoside	3-Hydroxyestra-1,3,5(10)-trien-17α-yl-β-D-galactopyranoside
17α-Estradiol-3-glucoside	17α-Hydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranoside
17α-Estradiol-17-glucoside	3-Hydroxyestra-1,3,5(10)-trien-17α-yl-β-D-glucopyranoside

17α-Estradiol-3-glucuronide	17α-Hydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronic acid
17α-Estradiol-17-N-acetylglucosaminide	3-Hydroxyestra-1,3,5(10)-trien-17α-yl-2'-acetamido-2'-deoxy-β-D-glucopyranoside
17α-Estradiol-3-glucuronide-17-N-acetylglucosaminide	Estra-1,3,5(10)-trien-3,17α-di-yl-3-β-D-glucopyranosiduronic acid-17-2'-acetamido-2'-deoxy-β-D-glucopyranoside
17α-Estradiol-3-glucuronide-17-glucoside	Estra-1,3,5(10)-trien-3,17α-di-yl-3-β-D-glucopyranosiduronic acid-17-β-D-glucopyranoside
17β-Estradiol	Estra-1,3,5(10)-trien-3,17β-diol
17β-Estradiol-17-glucoside	3-Hydroxyestra-1,3,5(10)-trien-17β-yl-β-D-glucopyranoside
p-Nitrophenylgalactoside	p-Nitrophenyl-β-D-galactopyranoside
p-Nitrophenylglucoside	p-Nitrophenyl-β-D-glucopyranoside
p-Nitrophenylglucuronide	p-Nitrophenyl-β-D-glucopyranosiduronic acid
p-Nitrophenyl-N-acetylglucosaminide	p-Nitrophenyl-2'-acetamido-2'-deoxy-β-D-glucopyranoside
BIS	N,N'-methylenebisacrylamide
CM-cellulose	Carboxymethylcellulose
DEAE-cellulose	Diethylaminoethylcellulose
EDTA	Ethylenediaminetetraacetic acid
PPO	2,5'-diphenyloxazole
SDS	sodium dodecyl sulphate

sp. ac.	specific activity
TEMED	N,N,N',N'-tetramethylene-diamine
TRIS	2-amino-2(hydroxymethyl)-1,3-propanediol
UDP-Galactose	Uridine-5'-diphosphogalactopyranoside
UDP-Glucose	Uridine-5'-diphosphoglucopyranoside

The following enzymes have also been referred to by their trivial names.

Trivial Name	Enzyme (Enzyme Number)
α-Amylase	α-1,4-Glucan 4-glucanohydrolase (EC. 3.2.1.1)
β-Amylase	α-1,4-Glucan maltohydrolase (EC. 3.2.1.2)
Arylsulphatase A	Aryl-Sulphate sulphohydrolase (EC. 3.1.6.1)
β-Fucosidase	β-D-Fucoside fucohydrolase (EC. 3.2.1.38)
α-Galactosidase	α-D-Galactoside galactohydrolase (EC. 3.2.1.22)
β-Galactosidase Lactase	β-D-Galactoside galactohydrolase (EC. 3.2.1.23)
Glucose oxidase	β-D-Glucose: oxygen oxidoreductase (EC. 1.1.3.4)
α-Glucosidase	α-D-Glucosidase glucohydrolase (EC. 3.2.1.20)
β-Glucosidase Cellobiase	β-D-Glucoside glucohydrolase (EC. 3.2.1.21)

β-Glucuronidase	β-D-Glucuronide glucurono-hydrolase (EC. 3.2.1.31)
β-Glycerophosphatase (Acid Phos-phatase)	Orthophosphoric monoester phosphohydrolase (EC. 3.1. 3.2)
Lysozyme	(EC. 3.2.1.17)
α-Mannosidase	α-D-Mannoside mannohydrolase (EC. 3.2.1.24)
β-N-Acetylglucosaminidase	β-2-Acetamido-2-deoxy-D-glu-coside acetamidodeoxy-glucohydrolase (EC. 3.2.1. 30)
Neuraminidase	Mucopolysaccharide-N-acetyl-neuraminylhydrolase (EC. 3.2.1.18)
Peroxidase	Donor : hydrogen-peroxide oxidoreductase (EC. 1.11. 1.7)
Steroid Glucosyltransferase	UDP-Glucose:steroid glucosyl-transferase
Steroid Glucuronyltransferase	UDP-Glucuronic acid:steroid glucuronyltransferase
Sucrase (β-fructofuranosidase)	β-D-Fructofuranoside fructo-hydrolase (EC. 3.2.1.26)
β-Xylosidase	β-D-Xyloside xylohydrolase
Almond Emulsin	β-Glucosidase
Ketodase	Bovine β-glucuronidase

CHAPTER I INTRODUCTION

A) General Introduction

Small water-insoluble molecules are usually excreted by animals as "conjugates", which are compounds formed by the coupling, by means of a chemical bond, of the compound with another molecule. These "conjugating" molecules are usually acids and the conjugate is much more water soluble than the parent compound. The most common molecules employed by vertebrates in the formation of conjugates are glucuronic acid, sulphate, or one of several amino acids, and the resulting conjugates are strongly acidic. Until quite recently, glucuronic acid was the only carbohydrate known to be employed in conjugation reactions, with the single exception of glucose, conjugates of which were known to occur in insects. The attachment of the sugar to the non-sugar molecule, or aglycone, is by means of a glycosidic bond involving C-1 of the sugar and a hydroxyl group, either phenolic or aliphatic, on the aglycone. The discovery, early history and mechanism of conjugation reactions have been the subject of many reviews, the most definitive being that of Williams (1959).

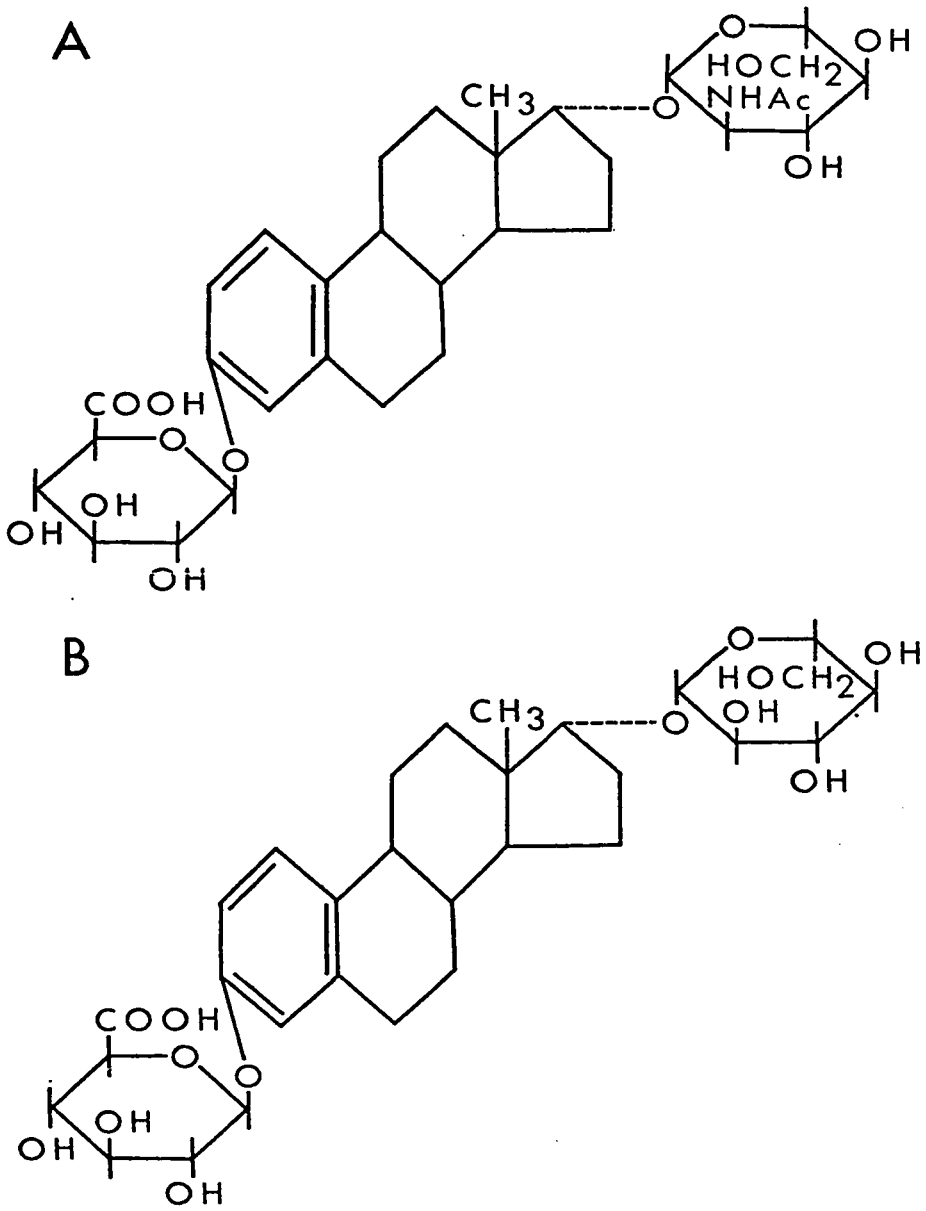
For a long time conjugation was a process which was studied exclusively with, and was thought to be limited to,"foreign" or exogenous materials absorbed or ingested by the animal. The first materials of endogenous origin which were

shown to undergo conjugation were steroids. In 1936 Cohen and Marrian isolated and characterized a glucuronide of estriol from human pregnancy urine, and Schachter and Marrian (1938) showed that estrone sulphate occurred in the urine of pregnant mares. The discovery of these conjugates of naturally occurring and physiologically important compounds caused some speculation, even at that date, as to the possibility that conjugation processes might have a wider metabolic role in animals than the well-established one of detoxication.

In 1964, Layne, Sheth, and Kirdani showed that the major excretion product of administered estrone or 17β-estradiol in the rabbit was a double glycoside of 17α-estradiol, in which glucuronic acid was attached to the phenolic 3-hydroxyl and N-acetylglucosamine to the 17-hydroxyl of the steroid. (FIGURE 1A). This was the first demonstration in animals of a conjugation reaction, for steroids or any other compound, which involved the formation of a glycoside other than a glucuronide. The observation was extended by Layne and his colleagues and also by workers in other laboratories, and the occurrence of steroid N-acetylglucosaminides in rabbits and humans is now well established. This work has been summarized in a recent review (Layne, 1970). Finally, Williamson et al (1969) were able to identify small amounts of a double conjugate in rabbit urine as the 3-glucuronide-17-glucoside of

FIGURE 1

Double Glycosides of 17α-Estradiol Excreted in Rabbit Urine



A. The 17-β-D-N-acetylglucosaminide of 17α-estradiol-3-glucuronide
B. The 17-β-D-glucoside of 17α-estradiol-3-glucuronide

17α-estradiol (FIGURE 1B), thus demonstrating for the first time in mammals the occurrence of conjugation with glucose.

The mechanism of the formation of glucuronic acid conjugates, or glucuronides, was shown by Dutton and Storey (1954) to involve the transfer of glucuronic acid from uridine diphosphate-glucuronic acid (UDP-glucuronic acid) to the aglycone in the presence of appropriate glucuronyl transferases. These enzymes are present in many animal tissues (Dutton, 1966) although liver is usually their best source. Homogenates or microsomal preparations of animal tissues can effect the formation of glucuronides in vitro if they are fortified with UDP-glucuronic acid. Layne and his co-workers (Jirku and Layne, 1965; Collins, Jirku, and Layne, 1968; Collins, Williamson, and Layne, 1970) have shown that rabbit liver homogenates can form steroid N-acetylglucosaminides and glucosides if the uridine nucleotide of these respective sugars is substituted for UDP-glucuronic acid in the incubation medium. Williamson, Polakova, and Layne (1971) found that in addition to effecting the transfer of glucose from UDP-glucose to the 3-glucuronide of 17α-estradiol to form the double glycoside found in small amounts in rabbit urine, rabbit liver microsomes could also effect the formation of the 3-monoglucosides of estrone, 17α-estradiol and 17β-estradiol. Careful searches, however, failed to reveal any trace of these phenolic monoglucosides in rabbit excreta. The 3-galactosides

of the phenolic estrogens can also be formed *in vitro* by rabbit liver microsomes fortified with UDP-galactose (Williamson *et al*, 1971), and human liver and kidney homogenates can, in the presence of the appropriate nucleotide, effect the formation of the 17-glucoside or the 17-galactoside of 17α-estradiol, although these glycosides have not been found in human excreta (Williamson, Layne and Collins, 1972). The presence of significant amounts of 17α-estradiol in human excreta, in either free or conjugated form is in fact open to question (Williamson and Layne, 1971).

The above work indicated that both rabbit and human tissues could form glucosides and galactosides of the steroid hormones, with a high degree of specificity for the particular steroid aglycone and the particular hydroxyl group thereon but that, if such glycosides were formed *in vivo*, their function was not as excretory products. The question therefore arose as to whether these steroid glycosides might be hydrolysed by the glycosidases which are of widespread occurrence in animal tissues and might in fact, as naturally occurring substrates, provide information as to the function of some of these glycosidases. This thesis is concerned with an investigation, primarily in rabbit tissues, of the activity of β-glucosidase towards estrogen and other steroid glucosides, the relative affinity and the specificity of the enzyme towards steroid substrates and the purification and properties of β-glucosidase from rabbit liver. This work has involved consideration of the

nature of the glycosidic bond, the distribution of glycosidases in animal tissues, methods previously used for the purification of glycosidases and the nature and properties of the enzymes. These subjects are reviewed below. The emphasis is on β-glucosidase, but it is not possible to adequately consider this enzyme outside the context of the other glycosidases which have been studied in animal tissues.

B) Historical Background

Enzymes which are capable of hydrolysing natural glycosides are found in many biological materials. These carbohydrases can be divided into two main groups; the glycosidases and the polysaccharidases. The glycosidases hydrolyze heteroglycosides, compounds in which the sugar is attached to a non-carbohydrate aglycone by a glycosidic bond, and oligosaccharides, polymeric carbohydrates consisting of relatively few monosaccharide units which, on hydrolysis, yield only simple sugars. The polysaccharidases hydrolyse polysaccharides of relatively high molecular weight, to yield fragments consisting of units of varying chain length as well as free monosaccharide units. This division of the carbohydrases is, however, arbitrary, since members of one group may exhibit low activity towards the substrates of the other group.

Historically, the glycosidases have been of interest since 1820, when Planche first showed their presence in extracts of certain plants. Robiquet and Boutron-Chalard (1830) then showed

that extracts of bitter almonds could hydrolyse the plant glycoside amygdalin (D-levo-mandelonitrile-β-D-gentiobioside). The active principle of the bitter almond was further investigated by Liebig and Wöhler (1837) and Robiquet (1838) and was named almond emulsin. Several other carbohydrases were discovered before the first of the proteases (pepsin) was reported by Schwann in 1936.

However, in spite of the long history of interest in these enzymes, less is known about their molecular properties and mechanism of action than of those of many other enzymes. A prime reason for this is a purification problem caused by the fact that many glycosidases exist in close association with polysaccharide molecules which confer remarkable stability on the enzymes. Elimination of the polysaccharide in the course of purification often yields an unstable enzyme which cannot withstand further purification. The enzymes may also exist in several forms, each with activity toward the same substrates, and these forms can be separated from each other only with difficulty.

Genetic studies have shown that multiple isoenzymes may occur in many organisms. Lindegren and Lindegren (1953, 1956) have described two genes in Saccharomyces cerevisiae, one producing an enzyme capable of hydrolysing both maltose and turanose, the other producing a single enzyme which hydrolysed maltose, sucrose, α-methylglucoside, turanose and melezitose. There are also cases where the specificity of two enzymes is

the same although structural differences exist between them. This is true of the two β-glucosidases from pig kidney reported by Abrahams and Robinson (1969). Two forms of the enzyme were separated by electrophoresis, isoelectrofocusing and DEAE-cellulose chromatography. They appeared to have the same substrate specificity and similar molecular weights. Robinson and Sterling (1968) have also reported the existence of two β-N-acetylglucosaminidases in human spleen. Both forms of the enzyme have been shown to exist in the lysosomes but the A form can also be found in the soluble fraction. The A form contains a number of sialic acid residues and removal of these by neuraminidase converts this acidic A form to the Basic B form. The specificity of both forms is identical, although the B form appears to be more stable to pH change and to denaturation by heat.

C) General Properties

The glycosidases in general are acidic proteins, lysosyme being a notable exception. The optimum pH of action generally lies between pH 4.5 and 7.0. There is still little known about the structural groups involved in the catalytic action of the enzymes, and so far no stable enzyme-substrate complex or covalently bound enzyme-inhibitor combination has been obtained which would give direct evidence as to the structure of the active site. The hydrolysis of the glycosidic bond by some glycosidases, such as β-amylases, proceeds with inversion of

the stereochemical configuration of the bond, while with other glycosidases, such as α-amylase, the original configuration is retained. The latter case is the more common one. The differences can be explained by proposing two mechanisms by which the hydrolysis could take place. The mechanism of the β-amylases is probably a single displacement one. Koshland (1954) proposed that it involved a direct reaction of two substrates Y and BX (in this case water and amylose respectively) on the surface of the enzyme. He proposed that the Y-B bond starts to form simultaneously with or before the B-X bond has started to break and that the attacking group Y approaches at an angle of 180° to B-X. The role of the enzyme in this mechanism, in addition to the juxtaposition of the substrates, is to polarize the electrons in the substrate molecules so as to increase their tendency to react. Because the Y-B bond is formed at an angle of 180° from the B-X bond cleaved there is an inversion of the configuration. In the case of α-amylase, a double displacement mechanisms is most likely. This mechanism is discussed further below.

Isotope studies, using O^{18}, have established the position of the bond cleaved in these reactions (Cohn, 1949). From experiments with invertase (Koshland and Stein, 1954), α and β-glucosidases (Bunton et al, 1954; Springhorn and Koshland, 1955) and α and β-amylases (Mayer and Larner, 1959; Halpern and Leibowitz, 1959) it is clear that in all the reactions studied the glucosyl- or fructosyl-O bond is split. These

reactions can be expressed by the general equation:

$$D\text{-}OR + H\text{-}OA \rightleftharpoons DOA + ROH$$

where D = carbohydrate residue donor

A = acceptor

R and A = carbohydrate or non-carbohydrate residues

This may be considered in terms of partial reactions for a double displacement mechanism:

$$D\text{-}OR + EH \rightleftharpoons E\text{-}D + ROH$$

$$E\text{-}D + H\text{-}OA \rightleftharpoons EH + DOA$$

E = enzyme

As two inversions of configuration take place, one at each step, the final product has the same configuration as the substrate. In many cases the acceptor molecule will be water and the net accomplishment is hydrolysis of the substrate. However, with an acceptor other than water, the enzyme acts as a transferase or transglycosidase. Some glycosidases possess both hydrolytic and transglycosidic activity while others are solely hydrolytic in function.

This thesis is concerned with glycosidases rather than polysaccharidases and the former enzymes are discussed further below, with special emphasis on β-glucosidase, which was the enzyme studied in this work.

D) Occurrence in Nature

The glycosidases are of widespread occurrence having been found in bacteria [e.g. Escherichia coli (Karström, 1931); Lactobacillus delbruckii, (Hofmann, 1935)], fungi [e.g. Aspergillus niger (Bourquelot, 1893), Myrothecium verrucaria (Hash and King, 1958a)]; and in a wide variety of plants, including almonds, the kernels of apricots, cherries and hips, all plants of the Rosaceae type, as well as soybeans and alfalfa seeds. They also occur in many tissues of warm and cold blood animals. Neuberg and Hofmann, (1935) reported a β-glucosidase in the kidney and liver of horses and cattle, while Steensholt and Veibel (1943) examined the mucosa of the small intestine of pigs and found β-glucosidase activity.

The assay procedures for glucosidases, and indeed for all glycosidases, have traditionally depended on the measurement of the amount of aglycone liberated from a synthetic heteroglycoside by a tissue homogenate or a partially purified enzyme preparation. The glycosides most commonly used for the assay have been those of o-nitrophenol, p-nitrophenol and phenolphthalein, with colorimetric measurement of the free aglycone produced. The more recent use of glycosides of umbelliferone has permitted fluorimetric determination of the aglycone with increased sensitivity over the colorimetric methods.

Comparisons between the studies of early workers are often difficult because of the lack of a standard means of expressing enzyme activity. Many of the assays used did not provide a

means of measurement that was sensitive enough to measure the relatively low activities in some mammalian tissues. Hence, much of the early work was done on plants and fungi which were found to have active glycosidases. The most commonly used were extracts of almonds, the well known "almond emulsin". A number of glycosidases were shown to be present in almond emulsin: β-glucosidase, β- and α-galactosidases, α-mannosidase, β-glucuronidase and β-N-acetylglucosaminidase. Other glycosides, notably the pentosides, could also be hydrolysed. The possibility that the same enzyme might be responsible for the hydrolysis of glycosides of the same homorphous series was recognized by Helferich and his co-workers (1932) and has been discussed by Baumann and Pigman (1957).

A comparison of β-glucosidase activities from a number of plant and fungal sources was made by Miwa et al (1937) and by Kobayashi (1944, 1947). However, it was not until the early 1950's that a comprehensive investigation of animal tissue sources was made. Using an assay for β-glucosidase based on the colorimetric estimation of 6-bromo-2-naphthol liberated from its β-D-glycoside, Cohen et al (1952) compared the enzyme activity in a number of organs from five species, including the human. In the case of the rat, thyroid and small intestine contained the greatest β-glucosidase activity, while the pancreas of the guinea pig had a large content of the enzyme. Dog and human tissues showed low enzyme activity, which was considered negligible in many cases.

Robinson (1956) used a much more sensitive fluorimetric assay to survey the β-glucosidase activity in a number of insect and mammalian tissues. Rabbit tissues were somewhat higher in activity than those of the other warm blooded animals examined.

Conchie, Findlay, and Levvy (1959a) examined the distribution of β- and α-glucosidase, galactosidase, mannosidase, N-acetylglucosaminidase, β-xylosidase and β-glucuronidase in a number of species. They found that in all tissues examined, the enzymes which displayed the greatest activities were α-mannosidase, β-galactosidase and β-N-acetylglucosaminidase. The distribution of these enzymes, along with that of β-glucuronidase, was further investigated in the mouse and rat. The epididymis was by far the best source for all the enzymes in the adult animal, with two exceptions. In the male mouse, the spleen values were somewhat higher than those for the epididymis and in the female rat the preputial gland was the richest source of β-glucuronidase activity. Conchie and his colleagues (Conchie and Mann, 1957; Conchie, Findlay and Levvy, 1959b) have also studied the levels of several glycosidases in mammalian sperm and seminal plasma, and in the testes and epididymis of various farm animals.

Whittemore and Layne (1965) showed that the distribution of β-glucuronidase and β-N-acetylgluconaminidase in the rabbit, as measured by their activity towards their respective steroid glycosides, was much the same as that found for these enzymes

when synthetic substrates were used. Female rabbit liver showed much higher glucuronidase activity towards the steroid substrate than any other tissue. The activity was nearly three times as high as that of the male liver. In the rabbit the epididymis also had a very high level of β-N-acetylglucosaminidase activity.

E) Endocrine Control of Glycosidase Activity

A number of workers have shown that the glycosidases of animal tissues are under endocrine control. Conchie et al (1959a) found that the levels of activity of various glycosidases increased during early life, before diminishing to adult levels. A notable exception, however, was seen in the epididymis. In both the rat and the mouse values for epididymal α-mannosidase, β-galactosidase, β-N-acetylglucosaminidase and β-glucuronidase activities were low in infant animals and increased with age, reaching a maximum at approximately 12 to 14 weeks after birth. This was coincident with the attainment of sexual maturity. Castration of adult male animals led to a large decrease in the activity of these enzymes after four weeks but this trend could be reversed by the administration of testosterone. To a lesser extent, the enzyme levels of the kidney and liver reacted the same way to castration and also to subsequent administration of testosterone.

Ovariectomy caused a considerable decrease in the uterine content of the same four enzymes and the activity could be restored by the administration of estrone. In the female,

however, there was little or no effect of castration on the glycosidase level of other tissues.

Hsia (1966) showed that the level of intestinal β-glucuronidase and β-galactosidase activity in rats was higher in the organs of infants than in those of adults. β-galactosidase, measured with either lactose or 6-bromo-2-naphthyl-β-D-galactopyranoside as substrate, was 20 times higher than the adult level during the first 15 days of life. The glucuronidase activity was also elevated during this period, but not to such a great extent. In contrast, the activity of α-disaccharidases was low or absent at birth and rose to adult levels between 15 and 20 days of life. Doell and Kretchmer (1964) have demonstrated that the increase in the activity of sucrase and other α-disaccharidases is also under hormonal control, although injections of hydrocortisone failed to alter the level of lactase activity.

F) Specificity of the Glycosidases

One of the early problems in the study of the glycosidases was to show whether or not a specific enzyme was responsible for the hydrolysis of a particular type of glycoside, or whether a single enzyme might catalyze the hydrolysis of, for instance, glucosides, galactosides and glucuronides.

Studies on almond emulsin (Baumann and Pigman, 1957) showed that, on purification, the activity towards β-glucosides and β-galactosides increased but that towards α-

galactosides decreased. This indicated that there was an α-galactosidase which was distinct from β-glucosidase. Heat treatment and exposure to ultraviolet light did not affect the activity of emulsin towards α-mannosides, but the ability to hydrolyse β-glucosides was greatly diminished. Hence, the existence of an α-mannosidase was postulated. Although most preparations of β-glucosidase also hydrolysed β-galactosides, the possibility of the existence of separate β-galactosidases was demonstrated by Hofmann (1933). He found that extracts from the kernels of Rosa canina could effect the hydrolysis of β-galactosides, but only hydrolysed β-glucosides very slowly. He later showed (1934a, 1934b) that preparations from certain strains of bacteria and of soybeans would hydrolyse galactosides but not glucosides.

Much of the uncertainty as to the specificity of various enzyme preparations has been eliminated by the discovery that aldonolactones of the appropriate configuration are competitive inhibitors of glycosidases (Conchie and Levvy, 1957). In general, the requirements for efficient inhibition by an aldonolactone of hydrolysis of a glycoside were found to be identical with respect to the grouping at C-6 and the substituents and conformations at C-2 to C-5. The lactone ring could be either 5- or 6-membered. Thus saccharo-1,4-lactone, (D-glucosaccharo-1,4-lactone), is an inhibitor of β-glucuronidase but has no effect on the activity of a number of other glycosidases. Several closely related aldonolactones are

without effect on β-glucuronidase. The lack of specificity for a five or six membered ring of the lactone is shown by the fact that both the 1,4- and 1,5-lactones of gluconic acid are equally effective as inhibitors of β-glucosidase (Conchie, 1954; Conchie and Levvy, 1957). With the aid of this specific inhibition by appropriate lactones, many investigators have been able to demonstrate the presence of two or more distinct enzymes in crude preparations.

The glycosidases have so far shown high specificity with respect to the sugar portion of the substrate molecule and very little with respect to the aglycone. The precise requirements may differ for enzymes from different sources. Thus for β-glucosidases from Stachybotryx atra, a C-3 polar group with the glucose configuration is required, and the C-4 OH and the C-5 CH_2OH are needed, although the configuration of these groups is not important (Jermyn, 1958). In contrast, for the enzymes from Saccharomyces cerevisiae the requirements for C-1, C-3 and C-4 appear to be absolute while those at C-2 and C-6 are unimportant (Duerksen and Halvorson, 1958). In many cases pentosides and heptosides of the same homorphous series are also substrates for the enzyme which hydrolyses the hexosides. Thus α- and β-L-arabinosides, β-D-xylosides and α-D-lyxosides are hydrolysed by α- and β-galactosidase, β-glucosidase and α-mannosidase respectively (Pigman, 1940). Robinson and Abrahams (1967) as well as Patel and Tappel (1969) have shown that β-glucosidase and β-xylosidase activity are exhibited by a

single enzyme in both the pig and the rat. On the other hand, Loontiens and de Bruyne (1963) separated a β-D-xylosidase from a β-D-glucosidase present in commercial hemicellulase by means of column electrophoresis on Sephadex G-25. One requirement of all glycosidases is, however, the specificity of C-1. β-Glycosidases are without action on α-glycosidases and visa versa (Veibel, 1950).

The aglycone specificity is much less rigid. Aryl glycosides are, in general, better substrates for glycosidases and the enzymes have higher affinities for these substrates than for alkyl glycosides. With alkyl substrates there is an increasing rate of hydrolysis with increasing chain length up to n-decyl (Larner, 1960). The degree of branching has a rough inverse relationship to the affinity of the enzyme for the substrate. Substitution in the aromatic ring of phenyl-glycosides tends to increase the rate of hydrolysis. An exception to this is the substitution of an amino group, which decreases the rate (Veibel, 1950). The substituent group probably exerts an influence on the glycosidic linkage undergoing hydrolysis as well as on the formation and stability of the enzyme-substrate complex. The inhibiting effect of the amino group might be due to its ionic charge or to the formation of a bond with the enzyme that is so stable that the dissociation of the products of hydrolysis from the enzyme is inhibited.

Helferich and Goerdeler (1940) showed that the natural plant glucosides salicin and glucovanillin were much better

substrates for almond emulsin and snail emulsin than the synthetic phenyl glucosides. However, the ease with which the released aglycones could be determined made the synthetic substrates the preferred ones for assay of the enzyme. More recently, the use of natural substrates has become of greater importance, especially in the light of the role that the glycosidases play in sphingolipid diseases. Bosmann and Hemsworth (1971) have shown that, in general, the glycoprotein substrates are more reactive than the nitrophenyl glycosides.

With the natural oligoglycoside and disaccharide substrates, the situation with regard to glycosidase specificity is less well defined. This is illustrated by a comparison of the β-glucosidases from three fungal sources. Jermyn (1955a; 1955c) and Hash and King (1958b) isolated an aryl-B-glucosidase from Stachybotryx atra and from Myrothecium verrucaria respectively which had no activity towards cellobiose, while Bucht and Eriksson (1969) reported the isolation of a cellobiase from Stereum sanguinolentium which showed little aryl-β-glucosidase activity. However, Wood (1971) has shown that Fusarium solani nitrophenyl-β-glucosidase and cellobiase activity appear to be present in the same enzyme protein. The source of the glycosidase is therefore important in determining whether or not natural carbohydrates are substrates for it.

Some glycosidases have been shown to be inhibited by the corresponding free sugar while others are not. Horikoshi

(1942) tried to classify the enzymes into two forms; the "Taka" type which is only inhibited by its specific sugar or sugar acid lactone and the "Emulsin" type which shows cross inhibition by similar glycosides and by the acid lactones of these glycosides but not by the free sugars. Examples of the Emulsin type are β-glucosidases or β-galactosidases which hydrolyse both types of glycosides. They will be inhibited by both glucosides and galactosides in the presence of substrate and by both glucono- and galactono-lactones, but are unaffected by glucose or galactose.

Many cases of the occurrence of β-glucosidase and β-galactosidase in close association have been reported. In 1966 Price and Robinson compared these two activities in eleven different sources and showed that these almost all consisted of a number of closely related isoenzymes, a very few of which showed absolute specificity for one or other of the glycosides. Only yeast and pig kidney yielded a β-galactosidase component which appeared free of β-glucosidase activity, while Helix pomatia had a small amount of β-glucosidase activity which was not inhibited by galactonolactone. Price and Robinson concluded that Horikoshi's "Taka" and "Emulsin" types were not physically distinct enzymes but the two extremes of a scale made up of mixtures of isoenzymes. They also showed the possibility that both "Taka" and "Emulsin" types occurred in the same preparation.

G. Intracellular Location of β-Glycosidases

The glycosidases are acid hydrolases and as such have been traditionally assumed to occur in the cytoplasmic granules called lysosomes. Novikoff, Podber and Ryan (1953) demonstrated that careful differential centrifugation of rat liver homogenates led to the separation of the acid phosphatase activity from that of succinoxidase and cytochrome oxidase. The authors believed that these results simply reflected a physical heterogeneity among the mitochondria and microsomes. Shortly before this, Walker and Levvy (1951) and Walker (1952) showed that β-glucuronidase of mouse liver behaved similarly when subjected to differential centrifugation. Further studies by Gianetto and de Duve (1955) and Appelmans and de Duve (1955) showed that several rat liver acid hydrolases were enclosed in a separate cytoplasmic structure and only displayed a small part of their total activity when the integrity of the granule was maintained.

Further work on this problem finally led de Duve and his colleagues to call these granules lysosomes and to develop the theory of lysosomes (1955). At first it was considered that lysosomes formed a single population of enzymatically homogeneous granules, within which the hydrolytic enzymes displayed structure-linked latency, becoming active only on the death of the cell. However, a number of reservations have since become necessary, as de Duve himself admitted in 1963, as follows: "individual lysosomal particles may differ quite

widely from each other in a number of properties such as size, structure, relative enzyme equipment, density in various media and sedimentation coefficient."

De Duve et al (1955) and Sellinger et al (1960) showed that other glycosidases, β-N-acetylglucosaminidase and β-galactosidase were also lysosomal in distribution in rat liver, and Beck and Tappel (1968) added β-glucosidase and β-xylosidase to this list.

As the lysosomal theory was based almost entirely on results obtained with a single tissue, namely rat liver, Conchie and his coworkers (Conchie, Hay, and Levvy, 1961; Conchie and Hay, 1963; Conchie and Levvy, 1963) studied the distribution of a number of glycosidases in the liver, kidney, spleen and T2146 tumor of the mouse. Distribution between the mitochondrial, microsomal and soluble fractions varied, not only from enzyme to enzyme but also, for a particular enzyme, from tissue to tissue. In the liver and kidney all the enzymes behaved like β-glucuronidase, in that they distributed themselves between the mitochondrial and microsomal fractions. This general resemblance did not extend to mouse spleen or cancer tissues. Whereas a large part of the β-glucuronidase activity was found in the soluble fraction of these tissues, β-N-acetylglucosaminidase was essentially microsomal in both tissues with little activity in the supernatant. α-Mannosidase and β-galactosidase more closely resembled β-glucuronidase in that they exhibited high activity in the supernatant.

The question then arose as to whether these apparently soluble enzymes were in fact soluble, or simply found in the supernatant fraction due to leakage from the lysosome during isolation. Many authors have shown that lysosomes are fragile, and that the methods for their isolation led to fairly low recoveries of the intact organelles. Patel and Tappel (1969) found that β-glucosidase and β-xylosidase activities in rat liver were definitely associated with the lysosomes and were strongly bound to the lysosomal membrane. Freezing and thawing only affected a solubilization of 17% of the β-glucosidase or the β-xylosidase activities. Only after treatment with deoxycholate were the enzymes solubilized to any great extent. Bosmann and Hemsworth (1971) have reported that α- and β-fucosidases, α-mannosidase and β-xylosidase are bound to the lysosomal membrane in rat brain. β-Glucosidase and β-galactosidase are also insoluble in rat brain as well as in calf brain (Gatt and Rapport, 1966). In contrast, Robinson and his coworkers found that β-glucosidase in pig and in human kidney was soluble (Abrahams and Robinson, 1969; Dance et al, 1969). The enzyme from both sources was recovered completely in the high speed supernatant and did not exhibit latency. Brady, Kanfer and Shapiro (1965a) reported that the β-glucosidase which attacked glucocerebrosides was soluble. Hsia (1966) demonstrated that newborn rats had two β-galactosidase activities, the one which hydrolysed lactose

was particulate, whereas the one which hydrolysed the synthetic substrate 6-bromo-2-naphthol-β-D-galactoside was soluble.

The question as to the location of the glycosidases is then not simply answered by terming them "lysosomal". In some mammalian tissues, especially those of the rat, all the acid hydrolases appear to be located in the lysosomes. However, when the tissues of other animals were tested, some of the enzymes appeared to be soluble while the rest exhibited the typical properties of lysosomal enzymes. Abrahams and Robinson (1969) have shown that the β-glucosidase of pig kidney was soluble, while β-galactosidase and β-N-acetylglucosaminidase were lysosomal. Dance et al (1969) showed that in human kidney, these three enzymes followed the same pattern of distribution.

H) Transglycosylation

In the scheme for glycosidase action mentioned earlier (Section C) it was stated that water can be replaced by another acceptor molecule, thereby making the enzyme act as a transferase. Dedonder (1961), in his review on glycosidases and transglycosidation, classified transosylases arbitrarily into four classes:

(a) phosphotransosylases - the normal donor is a glycosylphosphate

(b) true transosylases - the donor is a glycoside and the enzyme functions in the synthesis of polysaccharides

(c) transosylase-glycosidases - the donor is a glycoside but the main action of the enzyme is hydrolytic

(d) nucleotide-transosylases - the donor is a nucleotide-ose

The enzymes of category (d) are more generally known as transferases, and those of category (c) are the ones of most interest with respect to the work in this thesis.

Although mainly regarded as hydrolytic, the glycosidases have been known to function as transosylases since 1937 when Rabaté observed the transfer of D-glucose residues from various glycosides to such acceptors as ethanol by preparations from the leaves of a number of plants. Since then, numerous instances of transosylation by glycosyl hydrolases have been demonstrated. Bacon and Edelman (1950) and Blanchard and Abin (1950) showed the formation of some di- and trisaccharides during the hydrolysis of sucrose by yeast invertase. A number of enzymes acting on maltose can produce tri- and tetrasaccharides. Pan et al (1951a, 1951b) using an extract from Aspergillus niger isolated a trisaccharide, panose (O-α-D-glucopyranosyl-(1- 6)-O-α-D-glucospyranosyl-(1- 4)-D-glucopyranose). Pazur and French (1952) used maltase from Aspergillus oryzae, and obtained one disacharide (isomaltose), two trisaccharides, (panose and dextrantriose) and one tetrasaccharide, (α-dextrantriosyl-D-glucose).

The trans-β-glucosidases are found chiefly in microorganisms. Cellulolytic molds frequently secrete more than one β-glucosidase, depending upon the strain and the conditions of growth. Crook and Stone (1957) have shown that purified cellobiase from Aspergillus niger, a crude filtrate from Myrothecium verrucaria and a preparation from Helix pomatia acted on cellobiose to produce a series of oligosaccharides of the cellodextrin type. Transfer of a β-glucosyl residue to hydroxyl groups at C-6, C-3, or C-2 of glucose or to C-4, C-6, or C-3 of the non-reducing residue of cellobiose has been shown (Manners, 1960). The cellobiase of Cladophora rupestris transferred xylose and produced 3-O-β-D-glucopyranosyl-D-xylose. However, not all cellobiases are transferases. Duerksen and Halvorson (1958) purified an enzyme from Saccharomyces cerevisiae which acted on aryl glucosides and cellobiose but which did not exhibit transferase activity. The properties of the aryl-β-glucosidases from Myrothecium verrucaria (Hash and King, 1958) and from Stachybotryx atra (Jermyn, 1955) have been extensively studied. With p-nitrophenylglucoside as a donor, primary alcohols but not glycerol, acted as acceptors in the M. verrucaria system. In fact the latter system had a marked preference for alcohol rather than water as an acceptor. The β-glucosidase from S. atra, on the other hand, transferred β-glucosyl units to glycerol.

More recently, the formation of riboflavin glycosides by transosylation, catalysed by enzymes from various sources has

been reported by Suzuki and Uchida (1970a, 1970b). They isolated and characterized both α- and β-glucosides and galactosides of riboflavin. Cellobiose and lactose were used respectively as the donor substrates.

Pan (1970) demonstrated the formation of estriol-16α(α-D-glucoside) by transglucosylation from maltose using Aspergillus niger as the enzyme source. He also found glucoside formation in this system with 17α-epiestriol and 16α-hydroxytestosterone as the acceptor molecules.

There has been only one report of transglycosylation in mammalian systems. This is the work of Fishman and Green (1957) which showed the transfer of β-glucuronyl residues from aryl and from aliphatic glucuronides to aliphatic alcohols and glycols by enzymes from a number of sources, including the liver of mice, lambs, cattle and calves, as well as the tissues of snails and bacteria. However, very high concentrations (2 to 3M) of the acceptor were required.

I) Medical Significance of the Glycosidases

Recent studies have established that a number of diseases of sphingolipid metabolism are the result of deficiencies of certain enzymes. The sphingolipidoses comprise a group of inherited metabolic diseases which are characterized by the accumulation of excessive quantitites of certain glycolipids or phospholipids in various tissues of the body. The accumulating lipids have a common basic structure called ceramide, comprised

of the long chain amino alcohol sphingosine acylated on the nitrogen with a fatty acid.

It had been shown (Trams and Brady, 1960; Crocker and Mays, 1961) that the synthesis of two sphingolipids, glucocerebroside and sphingomyelin, that respectively accumulate in the various tissues in patients with Gaucher's disease and Niemann-Pick disease, which are two of the more common lipidoses, occur at normal rates. It therefore seemed logical to examine the catabolism of the lipids involved. Procedures for introducing radioactive tracers into the lipid molecules were established, and investigations with these labelled materials provided, in rapid succession, identification of the enzyme defects of Gaucher's disease, Niemann-Pick disease, Fabry's disease and Tay-Sachs disease.

In Gaucher's disease excessive quantities of glucocerebroside (ceramide-glucose) accumulated in the peripheral tissues. The characteristics of "glucocerebrosidase" were determined and the activity of the enzyme was measured in specimens from normal human tissues and from those of patients with the disease. The mean activity of the enzyme from patients with the adult form of Gaucher's disease decreased to 15% of that of the control series (Brady, Kanfer and Shapiro, 1965b). The glucocerebrosidase activity was virtually absent in tissues from patients with the infantile form of the disease (Brady et al, 1965). The major source of this glucocerebroside which accumulates in the peripheral tissues is believed to be cellular

lipids from senescent leucocytes (Kattlove et al, 1969).

The ceramide trihexoside, galactosyl-galactosyl-glucosyl-ceramide, accumulates in various tissues of patients with Fabry's disease. With labelled substrates Brady et al (1967a) showed that intestinal biopsy specimens from patients with the disease had no hydrolytic activity towards the trihexoside. There is an enzyme which is specific for the trihexoside and it has been demonstrated in, and partially purified from, rat intestinal tissues (Brady et al, 1967b). It catalyses the removal of only the terminal galactose of the ceramide-trihexoside. Lack of this enzyme is an inborn error of metabolism transmitted by the X-chromosome and therefore females are carriers while only males show severe clinical manifestations of the disease. It is interesting, therefore, that heterozygous female carriers of the Fabry trait had a level of ceramide-trihexosidase activity in their tissues intermediate between that of normal subjects and patients with Fabry's disease.

Another disease in which a galactosidase appears to be missing is gargoylism. Öckerman and Hultberg (1968) separated, by gel filtration of the high speed supernatants of liver biopsy specimens from both control subjects and patients, three distinct peaks of activity towards the synthetic substrate 4-methyl-umbelliferyl-β-galactoside. They designated these Peaks I, II, and III. The maximum β-galactosidase activity was obtained at approximately pH 4.0 - 4.2 for Peak I, pH 4.4 - 4.7 for Peak II and pH 5.0 - 5.5 for the third peak. In the control, Peak II

was larger than Peaks I and III. In the patients with gargoylism a different pattern was seen, involving a marked decrease in Peak II, a slight decrease in Peak I and no significant change in Peak III. The findings seem to indicate the selective decrease in the liver of patients with the disease of one or more, but not all, of the enzymes with β-galactosidase activity.

A glycolipid called Tay-Sachs ganglioside (N-acetylgalactosaminyl-(N-acetylneuraminyl)-galactosylglucosylceramide) accumulates in the tissues of patients with Tay-Sachs disease. The enzymatic hydrolysis of the N-acetylneuraminyl moiety of Tay-Sachs ganglioside in tissue specimens from patients with Tay-Sachs disease was found to be equal to that in the control tissue samples (Kolodny et al, 1969). However, these authors subsequently found that there was a complete absence of the hexosaminidase that catalyses the cleavage of the N-acetylgalactosaminyl residue of Tay-Sachs ganglioside in tissues of patients with the disease (Kolodny, Brady and Volk, 1969). At the same time Okada and O'Brien (1969) showed, by the use of the artificial substrates p-nitrophenyl-β-D-N-acetylgalactosaminide and 4-methylumbelliferyl-β-D-N-acetylgalactosaminide, that there was a missing hexosaminidase component in extracts of tissues obtained from Tay-Sachs patients.

In Niemann-Pick disease there is an accumulation of sphingomyelin (ceramide-phosphorylcholine). The cause is similar to that of the diseases in which there is a lack of a particular

glycosidase in that sphingomyelinase is missing from the tissues of patients with the disease. Recently, Wilson (1972) has shown that the mean β-glucuronidase level was statistically lower in patients with cystic fibrosis. This fact seems to correlate with the increase of uronic acid-containing macromolecules in the skin of patients with this disease.

Other mucopolysaccharidoses such as Hurler's and Sanfilippo syndromes exhibit a more generalized change in the levels of glycosidases. Gorden and Feleki (1970) reported increases in β-N-acetylglucosaminidase, β-glucuronidase and arylsulfatase A in the serum of patients with either disease while the serum acid phosphatase activity was depressed in Hurler's syndrome. The activities of α-mannosidase, α-fucosidase and hyaluronidase are also increased in the livers of patients with either disease and those with Sanfilippo syndrome also have higher arylsulfatase A activity. Brady et al (1970) have shown that the degree of enzymatic hydrolysis of non-sialic acid-containing sphingolipid in brain tissue of patients with generalized gangliosidoses is greater than that in control subjects.

J) Development of the Studies Reported in this Thesis

As stated on page 4, this work was initiated in order to investigate the extent to which steroid glucosides could be hydrolysed by the glucosidases of rabbit liver. It became apparent from the earliest experiments that, not only were the glucosides of the phenolic 3-hydroxyl of the steroid estrogens

very much better substrates for the liver glucosidase than were the glucosides of the alcoholic 17-hydroxyl, but the 3-glucosides were in fact hydrolysed by the enzyme very rapidly indeed. When tissues other than liver were used, the ratio between the rates of hydrolysis of the 3- and the 17-glucosides of 17α-estradiol was found to vary. Because of this, and because these steroid glucosides were the first naturally occurring substrates of the hetero-glycoside category to be found in animals, the purification and further examination of the steroid glucosidase activity was undertaken.

CHAPTER 2

MATERIALS AND GENERAL METHODS

A) Materials

Chemicals were obtained from the following companies as indicated.

Amersham/Searle Corporation (Arlington Heights, Illinois)
Tritiated dehydroisoandrosterone, 17β-estradiol and estrone.

Beckman Scientific Instruments Division (Montreal, Quebec)
Biosolv, BBS-3.

Bio-Rad Laboratories (Richmond, California)
Bio-Gel P-150.

Canadian Laboratory Supplies Ltd. (Ottawa, Ontario)
Silica Gel N (standard grade), Sucrose (Baker Analyzed).

Eastman Kodak Co. (Rochester, New York)
Acrylamide, BIS-acrylamide, 2-mercaptoethanol, Pyronin Y, TEMED.

Fisher Scientific Co., Ltd. (Montreal, Quebec)
Ampholytes (LKB) ammonium persulphate, EDTA, glucose, galactose, trichloroacetic acid, all buffer salts and solvents.

Frazer Medical Supplies Ltd. (Vancouver, British Columbia)
PPO.

General Biochemicals (Chagrin Falls, Ohio)

Ammonium sulphate (enzyme grade)

Mandel Scientific Co. Ltd. (Montreal, Quebec)

Carboxymethylcellulose (Whatman)

Pharmacia Fine Chemicals AB. (Montreal, Quebec)

Blue Dextran 2000, Sephadex G-150, G-200.

Pierce Chemical Co. (Rockford, Illinois)

Sodium dodecyl sulphate.

Rohn and Haas (Philadelphia, Pennsylvania)

Triton-X-100, Triton-WR-1339.

Schwarz/Mann (Orangeburg, New York)

Deoxycorticosterone glucoside.

Sigma Chemical Co. (St. Louis, Missouri)

Almond Emulsin, 2-aminobiphenyl, bovine serum albumin, Bromphenol Blue, cellobiose, Coomasie Brilliant Blue, cytochrome C, dehydroisoandrosterone (radioinert), o-dianisidine, 17α-estradiol (radioinert), 17β-estradiol (radioinert), estrone (radioinert), galactonolactone, gentiobiose, gluconolactone, glucose oxidase, lactose, myoglobin, p-nitrophenylgalactose, p-nitrophenylglucose, p-nitrophenylglucuronic acid, p-nitrophenyl-N-acetylglucosamine, ovalbumin, peroxidase, UDP-galactose, UDP-glucose.

Warner Chilcott (Toronto, Ontario)

Ketodase.

17β-estradiol-17-glucoside was a gift of Drs. H. Machleidt and W. Eberlein of Karl Thomae Gmbh, Biberach-an-der-Riss, Germany.

B) General Methods

1) Preparation of Substrates

Williamson et al (1971) showed that rabbit liver microsomes could transfer glucose from UDP-glucose to the 3-hydroxyl of 17α-estradiol-3-glucuronide to form the double glycoside, 17α-estradiol-3-glucuronide-17-glucoside, and could also effect the formation of 3-monoglucosides of estrone, 17α-estradiol and 17β-estradiol. Glucosides of these estrogens were therefore prepared following the method described by these authors using partially purified glucosyl transferases. These were obtained from rabbit liver homogenates in the following manner:

> Microsomes were prepared from the homogenates by differential centrifugation. Nuclei, cell debris and mitochondria were removed by centrifuging the homogenates, prepared in isotonic KCl (1:4, wt/vol), at 11,700 xg for 40 minutes. Microsomes were precipitated by centrifuging the supernatant at 105,000 xg for 1 hour. The microsomes were washed twice with KCl and finally suspended in a volume of KCl equal to the weight of the liver.
>
> The microsomal suspension was diluted with 4 volumes of 0.15 M Tris-HCl buffer, pH 8.0. A 2% solution of Triton X-100 prepared in 0.15 M Tris-HCl buffer, pH 8.0 was slowly added to the diluted microsomal suspension until the final concentration

of Triton X-100 was 0.1%. After standing in ice for 30 minutes, the suspension was centrifuged at 105,000 x g for 60 minutes, the supernatant was decanted off and was then concentrated in an Amicon ultrafiltration cell to the original volume of the microsomal suspension.

To prepare the 3-monoglucosides, approximately 3 x 10^6 dpm of either [6,7-^{3}H] estrone (sp. ac. = 5.6 c/mMole), 17α-[6,7-^{3}H] estradiol (sp. ac. = 5.6 c/mMole), or 17β-[6,7-^{3}H] estradiol (sp. ac. = 5.6 c/mMole) were added to conical centrifuge tubes, which were fitted with ground glass stoppers, in methanol which was subsequently evaporated under a stream of nitrogen. Aliquots of the concentrated Triton supernatant in 0.15 M Tris-HCl buffer, pH 8.0, (2 ml) were added to each tube and the reaction was initiated by the addition of 2 μmoles of UDP-glucose in 1 ml of isotonic KCl. The tubes were incubated for 1 hour at 37°C in a continuous shaking water bath. At the end of this time period, the reaction was stopped by the addition of 5 ml of benzene followed by immediate shaking. This was followed by extraction with a second 5 ml aliquot of benzene. Thus the unreacted free estrogen was removed from the reaction mixture. The tubes were then extracted 3 times with 5 ml of ethyl acetate to remove the formed 3-glucosides. The monoconjugates were purified by thin layer chromatography

on Silica Gel N (standard grade). The solvent system used was chloroform:ethanol, 4:1 (vol/vol).

The 17-glucoside of 17α-[6,7-^{3}H] estradiol was prepared in a similar manner. The 3-glucuronide of 17α-[6,7-^{3}H] estradiol (sp. ac. = 4.7 c/mMole) was used as substrate for the glucosyl transferase. It was incubated with the Triton supernatant and UDP-glucose as described above for the 3-glucosides. The reaction was stopped by the addition of 0.2 ml of 1 N HCl to lower the pH of the solution to pH 2 followed by two extractions with 5 ml of ethyl acetate to remove the unreacted monoglucuronide. The aqueous solution, which contained the double conjugate, 17α-estradiol-3-glucuronide-17-glucoside, was made 70% in ethanol to precipitate the protein which was removed by centrifugation. The supernatant was taken to dryness under vacuum on a rotary evaporator. The sediment was dissolved in 9.5 ml of 0.1 M acetate buffer, pH 5.0 and 0.5 ml of Ketodase solution (β-glucuronidase) was added. The reaction mixture was incubated in an oven at 37^{o}C for 18 hours to remove the glucuronic acid moiety from position 3 of the double conjugate. Gluconolactone (20 mg) was added to the reaction mixture to help prevent the removal of glucose from the 17-position of the steroid by the small amount of β-glucosidase present in the Ketodase solution (Williamson and Layne, 1970). The reaction was stopped by extraction with benzene to remove any released free estradiol and was then extracted with ethyl acetate to remove the mono-17-glucoside. The 17α-[6,7-^{3}H] estradiol-

17-glucoside was purified by thin layer chromatography as described above for the 3-glucosides.

The 3-glucoside of 17α-[6,7-^{3}H] estradiol was also prepared chemically by reduction of the methyl ester of 17α-[6,7-^{3}H] estradiol-3-glucuronide (sp. ac. = 4.7 c/mMole) with sodium borohydride (Wolfrom and Anno, 1952; Brown and Rapoport, 1963). A benzene solution of diazomethane was added dropwise to a cold solution of the glucuronide in ethanol (2 ml) until the yellow colour persisted. This was stirred for one hour after which the excess diazomethane was destroyed by the addition of 2 drops of glacial acetic acid and the ethanol was then evaporated under a stream of nitrogen. The residue was dissolved in 4 ml of methanol and this solution was added to a flask containing 100 mg of sodium borohydride. The reaction mixture was stirred at room temperature for 30 minutes and then evaporated to dryness under a stream of nitrogen. The residue was taken up in 10 ml of water and was extracted 3 times with 10 ml of benzene followed by 3, 10 ml extractions with ethyl acetate. The ethyl acetate extracts were evaporated and the 3-glucoside was purified by thin layer chromatography as described above.

17α-[6,7-^{3}H] Estradiol-3-galactoside (sp. ac. = 5.6 c/mMole) was prepared in the same manner as the corresponding 3-glucoside with UDP-glucose being replaced by UDP-galactose in the incubation medium (Williamson et al, 1971). [7-^{3}H] Dehydroisoandrosterone-3-glucoside (sp. ac. = 12.4 x 10^{-6} c/mM) was prepared

by incubation of the steroid with surviving slices of potato tuber (Schneider, 1970). 17α-[6,7-^{3}H] Estradiol-17-N-acetyl-glucosaminide (sp. ac. = 4.7 c/mM) and 17α-[6,7-^{3}H] estradiol-3-glucuronide (sp. ac. = 4.7 c/mM) were prepared as described by Layne (1965) and Whittemore and Layne (1965). The specific activities of the labelled glycosides were adjusted as required by the addition of unlabelled synthetic material (Williamson et al, 1969; Williamson et al, 1971).

2) Enzyme Assays

a) Radioactive Steroid Glycosides

The measurement of the extent of hydrolysis of steroid glycosides by tissue enzymes in vitro was based on the fact that intact glycosides cannot be extracted from aqueous solution by benzene, whereas the free steroids examined in this study partition practically completely into benzene under the conditions used (Collins et al, 1968). Since the glycosides of the phenolic steroids and dehydroisoandrosterone were available with tritium in the steroid, the assay consisted of measuring the amount of radioactivity, added to the incubation medium in the form of the glycoside, which was extracted into benzene from the buffer following incubation. Blank incubations, in which no enzyme was added, were carried in each set of assays and occasional examination of the benzene extracts by thin layer chromatography was done to ensure that the

radioactivity being measured was in the form of the appropriate steroid aglycone.

b) *Deoxycorticosterone Glucoside*

Since deoxycorticosterone is a Δ^4-3-ketosteroid, it exhibits a strong absorbance at 240 mμ (Dorfman, 1953). The method of assay for the activity towards deoxycorticosterone glucoside was therefore based on this fact. After incubation the released steroid was extracted from the buffer with benzene. The benzene extract was dried over anhydrous sodium sulphate, evaporated to dryness under a stream of nitrogen and redissolved in ethanol. The released steroid was then assayed by measurement of the absorbance at 240 mμ.

c) *17β-Estradiol-17-β-Glucoside*

The 17β-estradiol liberated from its 17-glucoside was extracted from the incubation medium by benzene and assayed by the Kober reaction (Osawa and Slaunwhite, 1970).

d) *p-Nitrophenylglycosides*

The hydrolysis of these substrates was measured by colorimetric determination of the p-nitrophenol liberated in the aqueous buffer. The technique used was based on that described by Patel and Tappel (1969). Protein was removed from the incubation medium by addition of an equal volume of 10% trichloro-

acetic acid at the end of the incubation period. An aliquot of the supernatant was taken to pH 10.7 by the addition of 1 N NaOH. The characteristic yellow colour developed with p-nitrophenol under these conditions was measured at 400 mμ.

e) *General Procedures Common to All Assays*

Assays for β-glucosidase and β-galactosidase were carried out in McIlvaine's sodium phosphate-citrate buffer, pH 5.5 (McIlvaine, 1921). β-Glucuronidase was assayed in 0.1 M sodium acetate buffer, pH 5.0 and β-N-acetylglucosaminidase in 0.1 M citrate buffer, pH 4.3. Due to the limited quantity of some of the substrates, especially the estrogen glycosides, routine assays were not made at saturation levels of substrate. For the estrogen glycosides 0.50 pmoles per assay (2.3×10^{-3} μC) were regularly used. Care was taken to ensure that initial velocity conditions were maintained. The activity towards the other substrates was assayed under maximum velocity conditions or as near as possible to these as substrate solubility in the aqueous buffer would allow. Assays were routinely carried out in a total reaction volume of 2 ml. Conical 15 ml centrifuge tubes with fitted ground glass stoppers were used as the reaction vessels. The glycosides were added first in methanol solution, the methanol then being evaporated under a stream of nitrogen. The appropriate buffer was then added and the reaction was initiated by the addition of the appropriate dilution of the enzyme preparation. Incubations were carried out at 37°C

for 1 hour in a continuous shaking water bath. Air was the gaseous phase. Reactions were terminated by the addition of 2 ml of benzene (with the exception of the assays with p-nitrophenylglycosides) with immediate shaking. Emulsions, if formed, were broken by centrifuging and an aliquot of the extract was taken for counting in the case of the radioactive substrates, or processed as described above in the case of the assays with deoxycorticosterone and 17β-estradiol glucosides. The p-nitrophenylglycoside assays were stopped by the addition of 2 ml of trichloroacetic acid and then processed as described above.

For experiments on substrate specificity and in the kinetic experiments, a 1 ml total reaction volume was used. The specificity experiments were carried out at saturation concentrations of the substrates and those determining Km values at concentrations ranging between 1/5 the Km value and saturation concentrations of each substrate.

3) Radioactivity Measurements

Radioactivity was measured by liquid scintillation counting in a Nuclear Chicago Unilux II spectrophotometer. Efficiency was determined by the channels ratio method and ^{3}H quench correction curves were determined using Nuclear Chicago standards. Non polar samples were counted in 10 ml of a scintillation medium containing 10 ml toluene, 0.4 mg 2,5-diphenyl-

oxazole (PPO) while hydrophilic samples were counted in a medium of 9.0 ml toluene, 40 mg PPO and 1.0 ml 'Biosolv, BBS-3' (Beckman Instruments Inc.).

4) Preparation of Tissue Homogenates

Female, virgin New Zealand white rabbits, used in this study, were killed by cervical dislocation. Tissues were excised immediately following death, stripped of all adhering connective tissue, minced with scissors and homogenized at top speed for one minute with 4 volumes of 0.25 M sucrose in a Sorvall Omnimixer tissue blender (Collins et al, 1968). Homogenates were maintained in an ice bath at all times and used immediately following preparation.

5) Gel Filtration Techniques

a) Preparation and Packing of Gels

Sephadex G-150 and G-200 were prepared as directed by the manufacturer (Pharmacia Fine Chemicals) by swelling the beads in 0.01 M sodium phosphate buffer, pH 7.0, 1 mM in 2-mercaptoethanol for 3 days at room temperature, or for 5 hours in a boiling water bath. Bio-Gel P-150 polyacrylamide gel beads were swollen in the same buffer for 24 hours at room temperature. The gel slurry was then allowed to cool at 4°C. The column to be used was mounted vertically and precooled to 4°C. The bed support was covered to a depth

of 4-6 cm with the suspension buffer and air bubbles were removed from the bed support by forcing liquid back and forth through the net with a syringe attached to the outlet tube. A column extending device was fitted and sufficient gel slurry to form the complete bed was poured carefully into the column with the aid of a glass rod to prevent air bubbles being trapped in the gel bed. The gel was allowed to pack by gravity feed and when packed was stabilized by running 2 to 3 column volumes of eluting buffer through the column under constant pressure, which was maintained by use of a Mariotte Flask. A pressure differential of 30 cm was used.

b) *Sample Application*

The solvent in the column was allowed to run out until the upper bed surface was exposed. The sample was then applied carefully to the top of the bed with a Pasteur pipette. Care was taken not to disturb the surface of the bed. The sample was allowed to run into the bed and was washed in with several ml of eluting buffer. The bed surface was carefully covered with buffer, the column filled to its original level and the system made airtight. The sample was allowed to percolate through the column without further interruption.

6) Preparation of Carboxymethylcellulose for Ion Exchange Chromatography

The carboxymethylcellulose (CM-cellulose) was washed before use in the following manner. The dry material was allowed to sink in 0.5 N NaOH and was kept in this reagent, with occasional gentle stirring, for 1 hour. The suspension was then filtered and washed with distilled water until the filtrate was free of alkali. The fine particles were removed by suspending the cellulose in water and allowing the fibers to settle for 15 minutes. The water and the fine material remaining in suspension were decanted and the process was repeated until all the fibers settled within a 15 minute period. The cellulose was stored in water suspension at $4^{o}C$ until used. A few drops of toluene were added to prevent bacterial growth.

7) Electrophoresis Techniques

a) Alkaline Gels

Polyacrylamide disc electrophoresis was performed at pH 8.3 according to the method described by Davis (1964). The following stock solutions were used:

A

1 N HCl	48 ml
Tris*	36.6 gm
TEMED*	0.23 ml
Water to 100 ml	
(pH 8.9)	

B

1 N HCl	approximately 48 ml (volume determined by titrating to pH 6.7)
Tris	5.98 gm
TEMED	0.46 ml
Water to 100 ml	
(pH 6.7)	

C

Acrylamide	28 gm
BIS*	0.735 gm
Water to 100 ml	

D

Acrylamide	10 gm
BIS	2.5 gm
Water to 100 ml	

E

Ammonium per-sulphate	0.14 gm
Water to 100 ml	
(made fresh daily)	

F

Sucrose	40 gm
Water to 100 ml	

Stock Buffer

Tris	6.0 gm
Glycine	28.8 gm
Water to 1 litre	
(pH 8.3)	

* Tris = 2 amino-2(hydroxymethyl)-1,3-propanediol
TEMED = N,N,N',N',-tetramethylenediamine
BIS = N,N',-methylenebisacrylamide

All solutions were filtered through millipore filters, deaerated under vacuum and stored in brown glass bottles at 4°C.

A 7% small pore gel solution was made by mixing 1 part A, 2 parts C, 1 part distilled water and 4 parts E. The small pore gels were poured first using a disposable syringe to ensure an even flow of acrylamide solution down the side of each gel tube. This prevented air bubbles forming in the gels. A water layer was placed on top of each gel solution as this eliminated the meniscus and gave a flat gel surface. This step must be carried out very carefully so as not to disturb the surface of the gel solution. Mixing of the water with the gel solution tends to give an irregular surface and pore size at the top of the gel and can cause distortion of the protein band during electrophoresis.

After applying the water layer, the gels were allowed to polymerize (approximately 20 minutes). When polymerization was complete, the tubes were inverted and the water was allowed to drain.

The small pore gels were then covered with 1-2 ml of large pore gel solution which in turn was covered with a layer of water and polymerized as described above. The composition of the large pore gel, a 3% acrylamide solution, was 1 part B, 2.5 parts D, 3.5 parts F containing ammonium persulphate in a concentration

of 0.14 mg/100 mls and 1 part water. After polymerization of the large pore gel (approximately 20 minutes) the water was removed and a few µl of 0.01% Bromphenol Blue solution was placed on top of each gel. An aliquot of the protein solution to be examined, containing 5-20 µg of protein, to which a few drops of glycerol had been added, was pipetted onto the top of each gel in not more than 100 µl of solution. The apparatus was assembled and the samples were covered carefully with the running buffer, which was a 1:1 dilution of the stock buffer. Electrophoresis was performed at 3 milliamps per gel for approximately one hour at 4°C. The anode was in the bottom chamber. The run was stopped when the tracking dye, Bromphenol Blue, was within a half a centimeter of the end of each tube.

The gels were removed from the supporting glass tubes by rimming with a thin needle under water. The protein in each gel was fixed in 12.5% trichloroacetic acid at 65°C for 30 minutes, then stained in a 0.2% solution of Coomasie Brilliant Blue in 45% ethanol, 10% acetic acid for 30 minutes at the same temperature. Destaining was carried out at the same temperature in the following manner:

> Gels were washed free of excess dye and allowed to stand at 65°C for 20 minutes in the destaining solution which consisted of 25% ethanol-10% acetic acid. The solution was then decanted and the

process was repeated twice, each time for 30 minutes. The destaining solution was then replaced by a 10% acetic acid solution and the process repeated through several changes at 65°C. The gels were then allowed to stand at room temperature overnight in 10% acetic acid. The staining-destaining procedure was that described by the Ortec company in their Bulletin (AN 32,1970).

b) Acid Gels

The procedure for electrophoresis at pH 4.5 was that described by Karavolas, Baedecker and Engel (1970). The general manipulations were the same as those described above for the alkaline gels.

Stock solutions were made as follows: Solution A-24 ml of 1 N KOH, 8.6 ml of glacial acetic acid, 2 mls of TEMED and sufficient water to make 100 ml; Solution B- 30 gm acrylamide, 0.4 gm BIS dissolved in water and made up to 100 ml; Solution C- 0.28 gm of ammonium persulphate in 100 ml of water (made fresh before use). The small pore gel solution was made by mixing 1 part A with 1 part B, and 2 parts C. The large pore gel solution was that used for the alkaline gels. The electrolyte buffer containing 3.12 gm of β-alanine and 0.8 ml of glacial acetic acid per liter. Pyronin Y (0.1% aqueous solution) was used to mark the front. The same current, 3 milliamps per gel was used but the electrodes were

reversed during electrophoresis. Gels were stained and destained as described above.

c) *SDS Gels*

The method of Weber and Osborn (1969) was followed for disc gel electrophoresis with sodium dodecyl sulphate (SDS) acrylamide gels. The following solutions were used:

A Sample Buffer A 1% SDS, 1% 2-mercaptoethanol (BME) solution in 0.02 M sodium phosphate buffer pH 7.0.

B Dialysis Buffer 0.1% SDS, 0.1% BME in 0.02 M sodium phosphate buffer, pH 7.0.

C Gel Buffer 8.82 gm $NaH_2PO_4.2H_2O$ plus 20.45 gm Na_2HPO_4 plus 2 gm SDS in 1 liter of water.

D Acrylamide Solution 22.2 gm acrylamide plus 0.6 gm BIS in water and made up to 100 ml.

E Ammonium Persulphate 15 mg/ml ammonium persulphate in water (made fresh).

The protein samples were dissolved in, or diluted 1:1 with, the sample buffer A and incubated at 37°C for 2 hours in a shaking water bath. The samples were then dialyzed overnight against the dialysis buffer B.

The gel solution was made by mixing 15 ml of gel buffer, which had been previously deaerated, 13.5 ml of acrylamide solution, 50 µl of TEMED and 1.5 ml of ammonium persulphate. The gel tubes were filled to within several centimeters from

the top, covered with a layer of water and allowed to polymerize. An aliquot of 10 to 50 µl of the dialyzed sample, to which a few drops of glycerol had been added, equivalent of not more than 20 µg of protein, was pipetted onto the top of the gel. Bromphenol Blue was used as the tracking dye and electrophoresis was performed at 8 milliamps per gel at room temperature. The electrolyte buffer used was a 1:1 dilution of the gel buffer. A typical run took 4.5 hours. Staining and destaining procedures were the same as those described above.

8) Protein Determinations

Protein was determined by three methods. Protein contents of column eluents were regularly monitored by reading the absorbance of the fractions at 280 mµ. For more accurate determinations, the protein content of fractions which were high in protein (e.g. homogenates and high speed supernatants) was measured by the method of Lowry et al (1951) while for those fractions with lower amounts of protein, the content was determined spectrophotometrically by the method of Warburg and Christian (1942).

CHAPTER 3 PRELIMINARY EXPERIMENTS

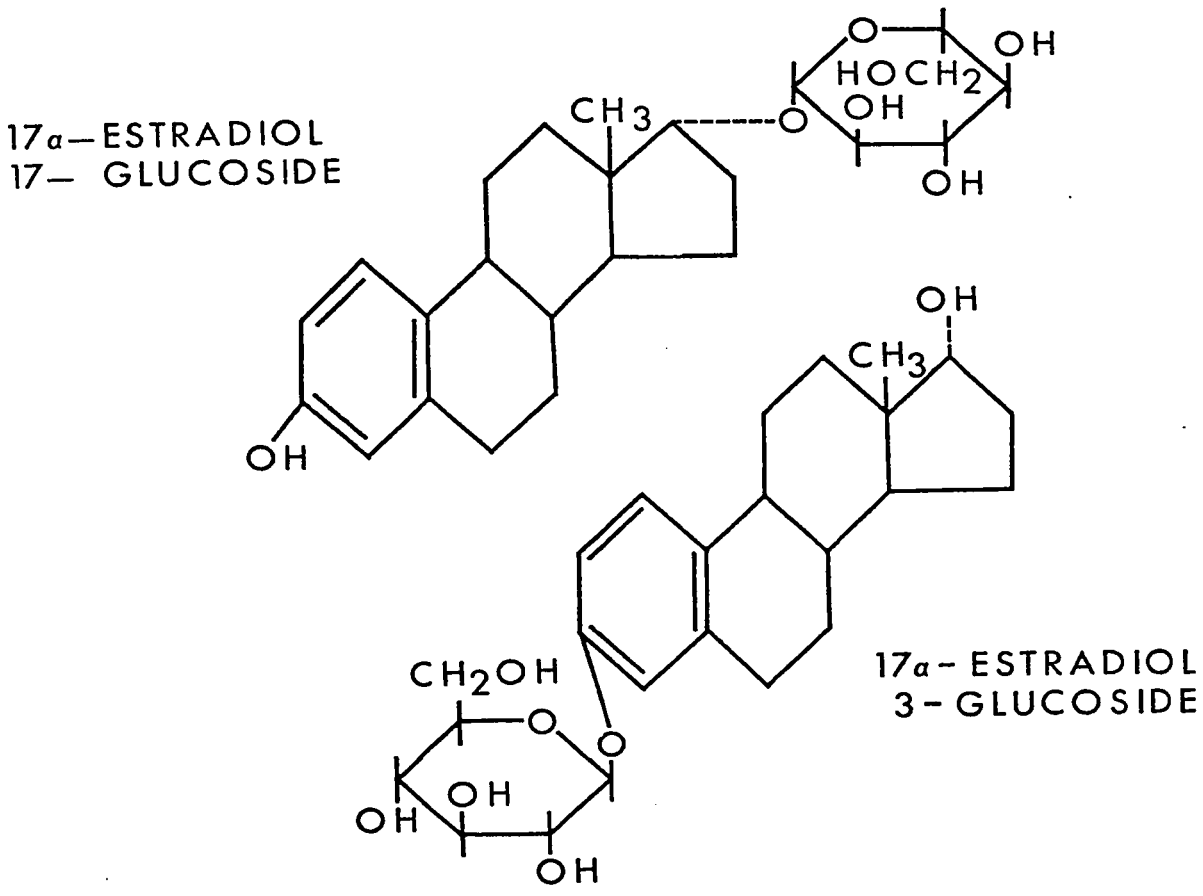
A) Introduction

As described in the introduction, rabbit tissue preparations are capable of forming β-glucosides of 17α-estradiol. The possibility that rabbit tissues might contain an active β-glucosidase which would hydrolyse these glucosides has therefore been examined and is the basis of this thesis. Two main substrates were used in this study. The first was the 17-β-D-glucoside of 17α-estradiol, formed from the double conjugate shown in Figure 1B by enzymatic removal of the glucuronic acid moiety from position 3. The second substrate was the 3-β-D-glucoside of 17α-estradiol formed by direct transfer of glucose from UDP-glucose to the phenolic hydroxyl (see section 2B1). These two substrates are shown in Figure 2.

Preliminary experiments showed that these glucosides were indeed hydrolysed by homogenates of rabbit tissues and the appropriate dilution of the homogenates which was required to obtain optimal assay conditions for each substrate was determined. Since liver was the tissue which had shown the highest glucosyl transferase activity (Collins et al, 1970), it was used for the preliminary experiments on the glucosidase. Kidney tissue was also examined.

FIGURE 2

Mono-glucosides of 17α-Estradiol



B) Methods

1) Progress Curves

To determine the time period over which the velocity of the reaction for a given substrate was linear, a series of assays were set up. The degree of hydrolysis of the 17-glucoside and the 3-glucoside of 17α-estradiol by both liver and kidney homogenates was determined over a time interval of 0 to 22 hours.

2) pH Activity Curves

Sodium phosphate-citrate buffers of 0.15 molarity and ranging in pH from pH 4.0-7.0 were used to determine the optimum pH for the assay of β-glucosidase activity towards the 17- and the 3-glucosides of 17α-estradiol. Increments of 0.5 pH units were used in preliminary experiments to find the approximate range. In final experiments increments of 0.25 pH units were used except for the extreme ends of the range.

3) Tissue Distribution of Steroid Glucosidase Activity

A comparison of the β-glucosidase activity towards the two 17α-estradiol glucosides in a number of rabbit tissues was undertaken. Appropriate enzyme concentrations were determined for each tissue and the linearity of the enzyme velocity was assured. The following tissues were tested: liver, kidney, small and large intestines, spleen, ovaries, uterus, whole blood and plasma. Homogenates were prepared as previously described (section 2B4). Blood was obtained by heart puncture immediately after

death and was diluted in a 1:1 ratio with 0.15 M phosphate-citrate buffer to prevent clotting. Plasma was obtained by centrifuging an aliquot of the blood at 12,000 x g for 15 minutes.

C) Results

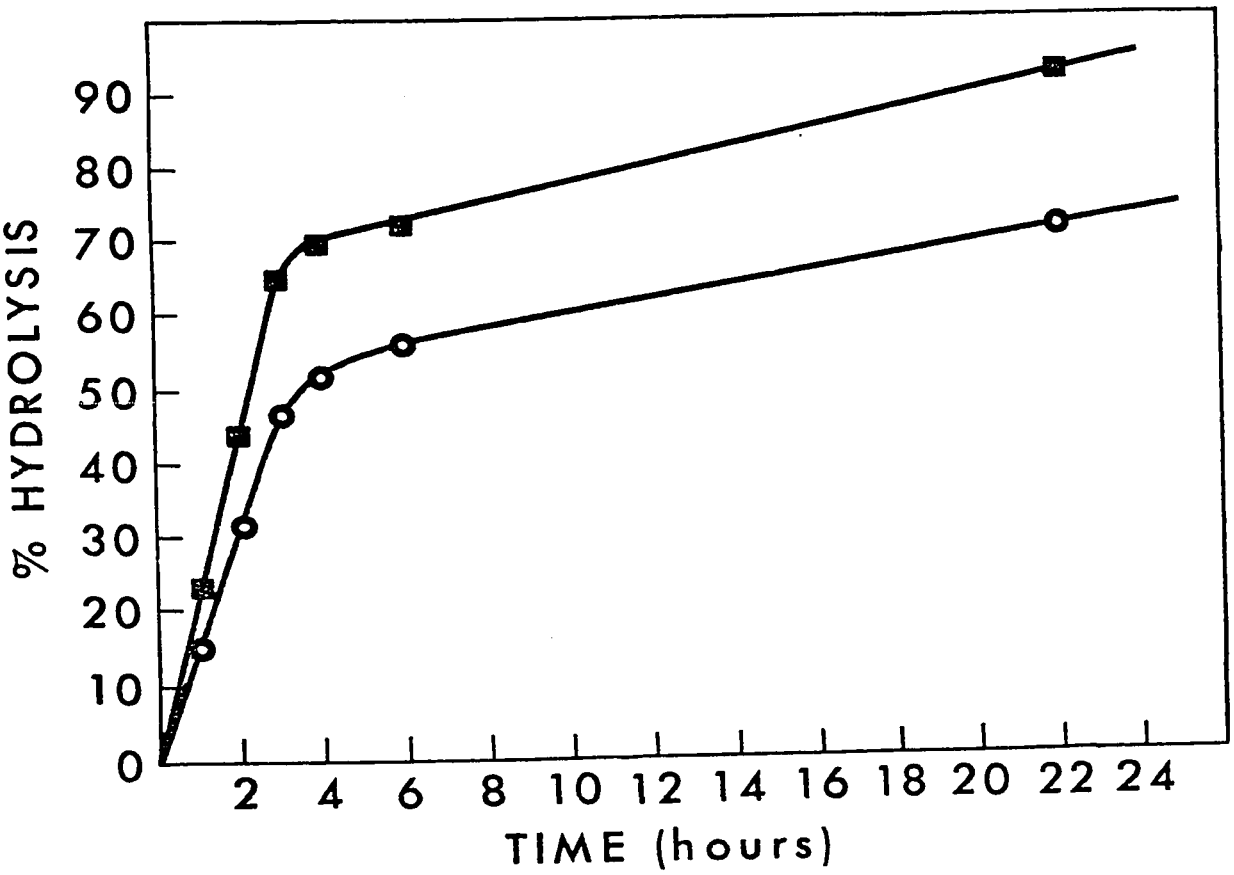
A 100 fold dilution of the liver homogenate was required before the activity towards the 3-glucoside could be measured accurately. In general 0.1 ml of homogenate was required to assay the activity towards the 17-glucoside and 1 µl was required for the 3-glucoside.

Figure 3 shows the progress curves for the β-glucosidase activity of liver homogenate towards the two substrates. Assays were performed at 1,2,3,4,6, and 22 hours. The activity towards both substrates was linear up to 3 hours at which point 45% hydrolysis of the 17-glucoside and 60% hydrolysis of the 3-glucoside had been achieved. Similar results were obtained with kidney homogenates which displayed linearity over a time interval of up to 3 hours with both substrates.

The pH activity curves for liver homogenates in 0.15 M phosphate-citrate buffer are shown in Figure 4. No significant difference in the pH relationship was observed when determinations were made in 0.1 M sodium acetate buffer. Activity towards both glucosides showed a single pH optimum in the region of pH 5.5 to 5.7. The relative activity towards the 3-glucoside did not appear to be as greatly affected by pH change as was that towards the 17-glucoside. However, as the optimum pH for the activity towards the two substrates only differed by 0.20 pH units, all further assays were carried out in McIlvaine's buffer at pH 5.5. Kidney homogenates showed the same pattern as liver. The optimum pH for the activity was also pH 5.5.

FIGURE 3

Progress Curves for the Hydrolysis of 17α-Estradiol-3-Glucoside and 17α-Estradiol-17-Glucoside by Rabbit Liver Homogenate

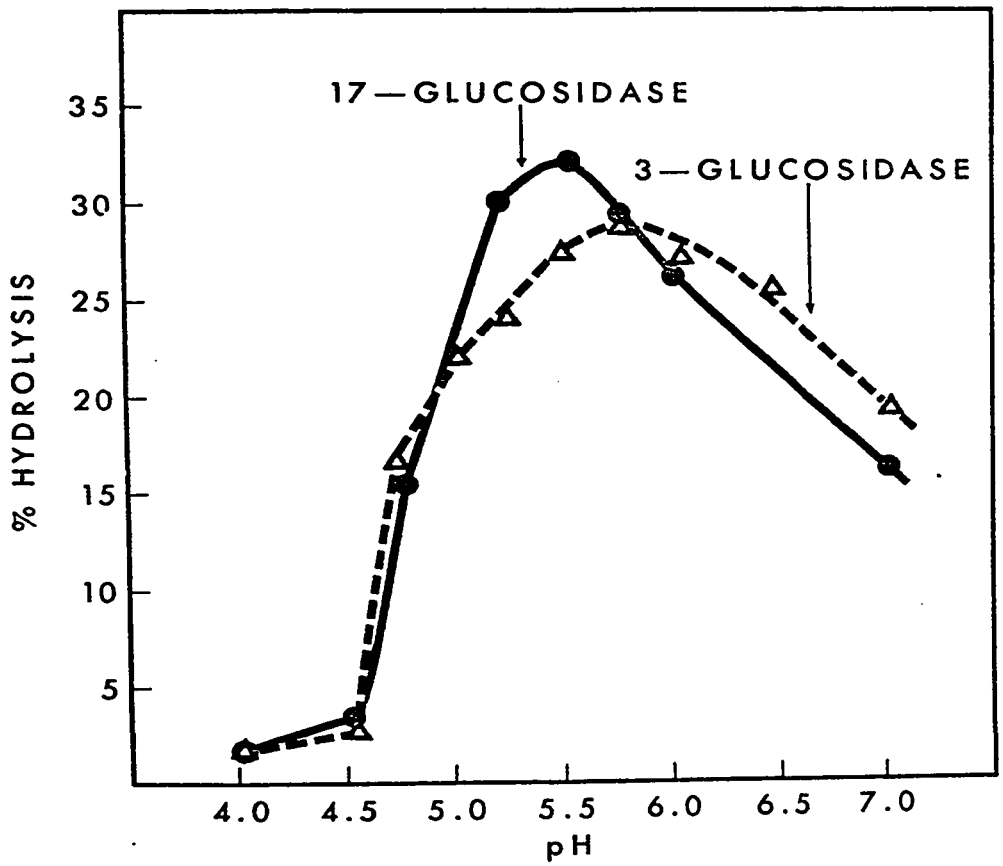


■ = Activity with 17α-estradiol-3-glucoside

o = Activity with 17α-estradiol-17-glucoside

FIGURE 4

pH Activity Curves for the Hydrolysis of the 3- and the 17-Glucosides of 17α-Estradiol by Rabbit Liver Homogenate



Curves were obtained in 0.15 M sodium phosphate-citrate buffer.

TABLE 1

Distribution in Rabbit Tissues of β-Glucosidase Activity Towards the 3- and 17-Glucosides of 17α-Estradiol

Results are expressed as pmoles of substrate hydrolysed per ml of homogenate per hour.

TISSUE	SUBSTRATE HYDROLYSED		
	17-glucoside	3-glucoside	Ratio of 3- to 17-glucoside
Liver	1.1	148	131
Kidney	1.8	65	37
Small intestine	1.9	112	59
Large intestine	0.3	0.9	3
Spleen	0.3	0.8	3

The results of the distribution study are given in Table 1. Liver, kidney and small intestine showed a very high hydrolytic activity towards the 3- as compared to the 17-glucoside, while with the spleen and large intestine both activities were lower and more nearly equal. In the other tissues tested, ovaries, uterus, whole blood and plasma, the results were very similar to those of spleen and large intestine in that both glucosides were hydrolysed poorly and at about the same rate.

D) Discussion

The results indicate that rabbit tissues readily hydrolyse the β-D-glucosides of 17α-estradiol. Although the enzyme velocity was linear over a long period of time and up to a point at which 45% or more of the substrate had been hydrolysed, experiments were routinely carried out with an incubation period of 1 hour in order to ensure that all measurements were comparable. Because of the rarity of the substrates, most assays had to be carried out at a substrate concentration less than that required for saturation. The per cent hydrolysis was therefore always kept as low as possible without sacrificing accuracy of the assays. In general, this meant working with a 20 to 30% hydrolysis in the one hour incubation period. If the per cent hydrolysis exceeded 40% in a given experiment, the reaction was repeated at a lower enzyme concentration.

The pH optimum exhibited for the hydrolysis of the two substrates correlates well with that found by other authors who used synthetic substrates (Fischer and Stein, 1960). Robinson (1956) showed that pH optima for β-glucosidase in animal tissues lie between the range of pH 5.1 to 5.7. Robinson, Price and Dance (1967) have shown little difference in pH activity curves estimated in either McIlvaine's phosphate-citrate buffer or 0.1 M acetate buffer.

The results of the tissue distribution study are very interesting. The great variation among the tissues in the ratios between the glucosidase activities towards the 3- and 17-

glucosides, while they do not rule out the possibility that one glucosidase may be responsible for the hydrolysis of the two substrates, suggest that different forms of the enzyme may exist.

Attempts were therefore made to isolate and purify the enzyme from the liver. This tissue was chosen as a representative of those which exhibited a high affinity for the phenolic glucoside of 17α-estradiol as compared to that towards the 17-glucoside.

CHAPTER 4

PURIFICATION OF β-GLUCOSIDASE FROM RABBIT LIVER

A) Methods

All steps in the purification of the enzyme were carried out at 4°C.

1) Intracellular Location of β-Glucosidase Activity

Homogenates of liver tissue, prepared as previously described, (section 2B4) were subjected to differential centrifugation. Nuclei and cell debris were removed by centrifugation at 1020 xg for 15 minutes and discarded. The supernatant was then centrifuged at 11,700 xg for 40 minutes to remove the mitochondria, and the microsomal fraction was then sedimented by centrifuging at 105,000 xg for 60 minutes (Beckman L2-65B ultracentrifuge, type 30 rotar at 30,000 rpm). The mitochondrial and microsomal fractions were taken up and homogenized in 0.25 M sucrose and these fractions, as well as the high speed supernatant, were assayed for hydrolytic activity towards the 17- and 3-glucosides of 17α-estradiol.

2) Acid Precipitation

The high speed supernatant was slowly titrated to pH 5.0 with 0.1 M acetic acid. The solution was gently stirred during this operation and was allowed to remain in an ice bath

with constant stirring for 30 minutes. The precipitate formed was removed by centrifugation at 20,200 xg for 15 minutes and was discarded.

3) Ammonium Sulphate Fractionation

The acidified supernatant was subjected to fractionation with ammonium sulphate. Solid ammonium sulphate was slowly added to the solution until an amount equal to 30% of the saturation value had been added. This amount was calculated by the method of di Jeso (1968). The solution was allowed to stand in ice, with occasional stirring, for 15 minutes. The precipitate formed was removed by centrifugation at 20,200 xg for 15 minutes. Further amounts of ammonium sulphate were added to the supernatant to bring the solution to 45, 55 and 65% of the saturation value respectively. The precipitates which were sedimented between saturation values of 0-45% and 45-65% were dissolved in 0.01 M sodium phosphate buffer, pH 7.0, 1 mM in 2-mercaptoethanol. These fractions were assayed for β-glucuronidase and β-N-acetylglucosaminidase as well as for glucosidase activity using the respective glycosides of 17α-estradiol as substrates.

4) Gel filtration of Ammonium Sulphate 45-65% Fraction

The 45-65% fraction obtained by the ammonium sulphate fractionation was applied to a 2.5/45 cm column of Sephadex G-150 (prepared as described in section 2B5), and eluted with 0.01 M sodium phosphate-1 mM 2-mercaptoethanol buffer. In

this case fine grade Sephadex (particle diameter 40-120μ) was used. Fractions of 2.5 mls were collected and assayed for β-glucosidase and β-N-acetylglucosaminidase activity. Protein content was monitored by measuring the absorbance of the eluate at 280 mμ.

5) Carboxymethylcellulose Chromatography of Sephadex Filtrate

Before use, the suspension of CM-cellulose in water, prepared as described in section 2B6 was filtered and the cellulose was resuspended in 2 to 3 volumes of the starting buffer. The suspension was allowed to stand with constant gentle stirring for 1 hour. It was then filtered and resuspended in the buffer. The process was repeated until the pH of the filtrate was that of the starting buffer. The column was packed in the same manner as described for the gel filtration chromatography, section 2B5. In these experiments, the starting buffer was 0.01 M sodium phosphate at 5.5 which again was made 1 mM in 2-mercaptoethanol. The pooled fractions from the gel filtration column, which contained the β-glucosidase activity, were concentrated in an Amicon ultrafiltration cell to approximately 5 ml, dialyzed overnight against 2 litres of 0.01 M sodium phosphate-1 mM 2-mercaptoethanol buffer, pH 5.5, and applied to the column. The column was eluted with the same buffer, and protein was estimated in the collected fractions by measurement of the absorbance at 280 mμ. The fractions containing protein were

assayed for β-glucosidase activity.

6) Disc Gel Electrophoresis of CM-cellulose Fraction

Analytical electrophoresis at pH 8.3 was performed as described previously using 5-10 µl (20-50 µg) of the CM-cellulose filtrate. Gels were stained for protein with Coomasie Blue and destained as described (see section 2B7a).

7) Preparative Acrylamide Gel Electrophoresis

A Shandon Preparative Polyacrylamide Electrophoresis apparatus was set up according to the instructions of the manufacturer using the Davis alkaline gel system. The small pore gel was 6 to 7 cm in height (24 mls) and, after polymerization, it was covered with 0.5 cm of large pore gel (3-4 mls) solution which was then allowed to polymerize. In each case the gel solution was covered with a water layer during polymerization. The apparatus was then assembled and checked to make sure that there were no leaks in the system. An aliquot of the CM-cellulose filtrate was concentrated in an Amicon ultrafiltration cell to a volume of approximately 2 mls. Glycerol was added to both the tracking dye and the sample to make them denser than the electrolyte buffer. The dye solution was then taken up in a Pasteur pipette and introduced to the surface of the gel by passing the pipette through the buffer. The sample was then applied in the same manner. The sample contained not more than 50 mg protein per run. Once the sample had been

applied, electrophoresis was started using a current of 40 ma. The protein was eluted with 0.1 M sodium phosphate buffer, pH 7.0 at a rate of 5 mls per hour. Fractions of 1 ml were collected and monitored for protein by reading the absorbance at 280 mμ. Fractions containing protein were assayed for enzyme activity towards both monoglucosides of 17α-estradiol. Elution of enzyme activity generally took 9-10 hours.

8) Analytical Gel Electrophoresis of Enzyme Fraction from Preparative Electrophoresis

Analytical gels (pH 8.3) were run to determine the degree of purity of the enzyme fraction obtained by preparative electrophoresis (see Figure 6). To determine whether one or both protein bands contained enzymatic activity the gels were incubated with various substrates in the following manner. After removal from the supporting glass tube on completion of the electrophoresis run, the gel was aligned with a parallel gel which had been stained, and was cut in two between the two bands. Each half was preincubated for 10 minutes in sodium citrate buffer, pH 4.3, and was then transferred to 2 mls of 0.15 M citrate-phosphate buffer, pH 5.5. The gels were crushed with a glass rod, the reaction initiated by addition of substrate, and then incubation was allowed to proceed overnight at 37° C. The steroid substrates were added in 50 μl of methanol, while the p-nitrophenylglucoside was added in buffer solution. The 17α-estradiol-3-glucoside, -17-glucoside, and

dehydroisoandrosterone glucoside, as well as p-nitrophenyl-glucoside were used. In the case of the 17-glucoside of 17α-estradiol, the amount of protein applied to one gel was not enough to measure its activity towards the substrate. Therefore 10 gels were pooled and assayed together.

As further confirmation, whole gels were assayed for activity towards p-nitrophenylglucoside by the method of Maley (1971) where gels were preincubated at pH 4.3 for 10 minutes followed by incubation in the presence of 5 mM p-nitrophenyl-glucoside at pH 5.5. After approximately four hours a yellow band was visible in the gel. The gels were aligned with a stained gel and the position of the yellow band was compared with that of the protein stain.

9) Gel Filtration of CM-Cellulose Filtrate

Superfine grade Sephadex G-150 (particle diameter, 10-40μ), was used for this gel filtration step. 1.5 x 90 column was poured and eluted as previously described (section 4A4). The CM-cellulose filtrate was concentrated by ultrafiltration to 0.5 ml and applied to the column. Enzyme activity was detected as described above. The fractions containing enzyme activity were monitored by analytical gel electrophoresis and those judged to be purest were pooled, concentrated, and reapplied to the same column. Again the enzyme fractions were monitored by electrophoresis and those fractions containing only one major protein band were pooled. Acid analytical gels at pH 4.3 were also run with this fraction.

10) Assay of Crude and of Purified Enzyme for Activity Towards a Number of Substrates

Liver homogenate and the purified enzyme preparation were assayed for activity towards the following substrates: 17α-estradiol-3-glucoside, 17α-estradiol-17-glucoside, 17β-estradiol-3-glucoside, 17-β-estradiol-17-glucoside, estrone-3-glucoside, dehydroisoandrosterone glucoside, deoxycorticosterone-21-glucoside and p-nitrophenylglucoside. All assays were carried out at conditions of substrate saturation. The activities toward a number of these substrates were assayed after each step in the purification procedure. These assays were again carried out under substrate saturation conditions.

11) S D.S. Gel Electrophoresis

Disc electrophoresis on S.D.S. gels were used to determine the molecular weight of the purified enzyme. Gels were set up as described in general methods (2B7c). Standard protein solutions of bovine serum albumin (molecular weight 68,000) ovalbumin (43,000) myogloblin (17,200) and cytochrome C (11,700) were made up to concentrations of 1 mg/ml (wt/vol). The standard proteins and the enzyme sample were processed as previously described. After the gels were removed from the supporting glass tubes, each was measured and the distance that the tracking dye had migrated was recorded. The gels were then fixed, stained and destained in the usual manner. The gels were then remeasured and the distances to which

protein bands had migrated were recorded. The second measurement of gel length is necessary since the gels swell slightly during the staining procedure. The mobility of each protein band was then calculated according to the following formula:

$$\text{mobility} = \frac{\text{distance of protein migration}}{\text{length after staining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

The logarithm of the molecular weight of each standard protein was plotted against its respective mobility.

12) Molecular Weight Determination by Gel Filtration

The 1.5 x 90 cm column of Sephadex G-150 superfine gel used above was calibrated for molecular weight determination using the same standard proteins as those used for S.D.S. electrophoresis. An aliquot of each standard (5 mg/ml) was applied to the column and was eluted by gravity feed under constant pressure. The protein was detected by reading the absorbance at 280 mμ and the elution volume for each standard was determined. The void volume was determined using one ml of a 0.2% Blue Dextran 2000 solution. The Blue Dextran was run twice, once before and once after the running of the standard proteins to make sure that the gel bed had remained stable. The Kav of each protein was then determined by the formula: Kav = (Ve-Vo)/(Vt-Vo) where Ve is the elution volume of the protein, Vo the void volume as determined by the elution volume of Blue Dextran 2000 and Vt, the total bed volume (Fischer, 1969). These values

were plotted against the logarithm of the corresponding molecular weights (Determann 1967, Fischer 1969). Molecular weight of the enzyme was determined for its Kav value from the graph. The molecular weight of the enzyme was also determined in the same manner using Bio-Gel P-150 polyacrylamide gel beads (particle size 50 - 150 mesh).

13) Determination of the Isoelectric Point by Isoelectrofocusing

a) General Method

An LKB 8100 Ampholine electrofocusing column (volume 110 ml) was set up as directed by the manufacturers. The following solutions were used:

Cathode Solution	0.1 gm of NaOH dissolved in 10 ml of distilled water
Anode Solution	0.2 ml of phosphoric acid 12 gms sucrose in 14 ml distilled H_2O
Dense Solution	2 ml of ampholine solution 40 ml of distilled water 28 gms of sucrose
Light Solution	6.5 ml of ampholine solution enzyme solution to be used diluted to 60 ml with distilled water

A discontinuous sucrose density gradient of 24 successive steps was established by mixing the light and dense solutions and pouring these manually into the column. After the dense anode solution was poured into the inner electrode chamber, each step was introduced by pouring it down the side of the focusing chamber at a rate which caused little mixing of the layers, approximately 4 ml per minute. The top layer was covered with the cathode solution, the power supply was connected and the focusing commenced. A current of 3 mA was applied and as the resistance increased along the column during the run, the current was readjusted periodically to maintain it between 2-3 mA. When the current had remained constant for at least 12 hours, the power supply was disconnected, the inner electrode chamber sealed off and the column emptied by gravity feed at the rate of 1-2 ml per minute. Approximately 1.3 ml fractions were collected. The pH of each fraction was read immediately.

b) *Preliminary Focusing Experiment pH 3-10*

The CM-cellulose filtrate was used for these experiments. An aliquot containing approximately 25 mg of protein was dialyzed against distilled water to remove the salts which interfere with the focusing. A 1% ampholine solution ranging in pH from 3-10 was used. The experiment was set up as described above. Every third fraction was assayed for activity towards 17- and 3-glucosides of 17α-estradiol and towards p-nitrophenyl glucoside.

c) *Focusing over pH range 4-6*

The enzyme activity was found to be within the pH range of 4-6 in the first experiment and the focusing was therefore repeated over this narrower range. The apparatus was set up as before using a 1% solution of ampholines of pH 4-6 containing 0.1% of pH 3-10 ampholines. The fractions from the previous run which contained the enzyme activity were pooled and used without further treatment.

d) *Fine Range Focusing pH 4.4 - 5.2*

Ampholine solutions of a pH range of 2 units are the narrowest range provided by the manufacturers, and narrower ranges must therefore be obtained through electrofocusing. A 6% solution of pH 4-6 ampholines containing 0.6% of pH 3-10 ampholines were focused in the usual manner without the addition of the enzyme sample. The column was drained and the pH of the fractions was measured. The fractions between pH 4.4 and 5.2 were pooled and used as the stock ampholine solution for the run with enzyme present. The pooled enzyme fractions from the previous run of pH 4-6 were used as the enzyme source. Fractions were assayed for glucosidase activity and the pI was calculated from a plot of pH vs. fraction number.

B) Results

The intracellular distribution study showed that the bulk of the β-glucosidase activity towards both substrates was recovered in the high speed supernatant (Table 2). The fraction was used as the enzyme source for further purification. No activity was lost on bringing the supernatant fraction to pH 5.0 while a 2 fold purification was achieved. Both these supernatants had considerable β-glucuronidase and β-N-acetylglucosaminidase activity as measured by their ability to hydrolyse the respective glycosides of 17α-estradiol. However, fractionation with ammonium sulphate separated the β-glucuronidase activity completely from the β-glucosidase activity while the β-N-acetylglucosaminidase activity was distributed almost equally between the two fractions (Table 3). The β-glucuronidase activity precipitated between 0-45% of saturation concentrations of ammonium sulphate and the β-glucosidase activity between the 45-65% fraction. The pattern of precipitation did not change with the pH of the solution used, since both the high speed supernatant (at pH 6.7) and the acidified supernatant (pH 5.0) gave exactly the same results. However, it was found that sharper separation of these two enzyme activities and better recovery of β-glucosidase could be achieved if the fractionation was made in four steps rather than two (see section 4B3). Purification over this step was approximately 5 fold.

TABLE 2

Intracellular Distribution of β-Glucosidase Activity in Rabbit Liver

Results are expressed as total pmoles of substrate hydrolyzed per hour per 300 mls of homogenate.

Tissue Fraction	Substrate Hydrolyzed			
	17α-Estradiol-17-glucoside		17α-Estradiol-3-glucoside	
	pmoles/hr	% Recovery	pmoles/hr	%Recovery
Homogenate	351	100	19,269	100
Mitochondria	19	6	741	4
Microsomes	30	7	710	4
High Speed Supernatant	298	85	17,048	88

TABLE 3

Distribution of β-Glucosidase, β-Glucuronidase and β-N-acetylglucosaminidase between Fractions Precipitated by Ammonium Sulphate

Results are expressed as pmoles of substrate hydrolysed per hour per ml of fraction.

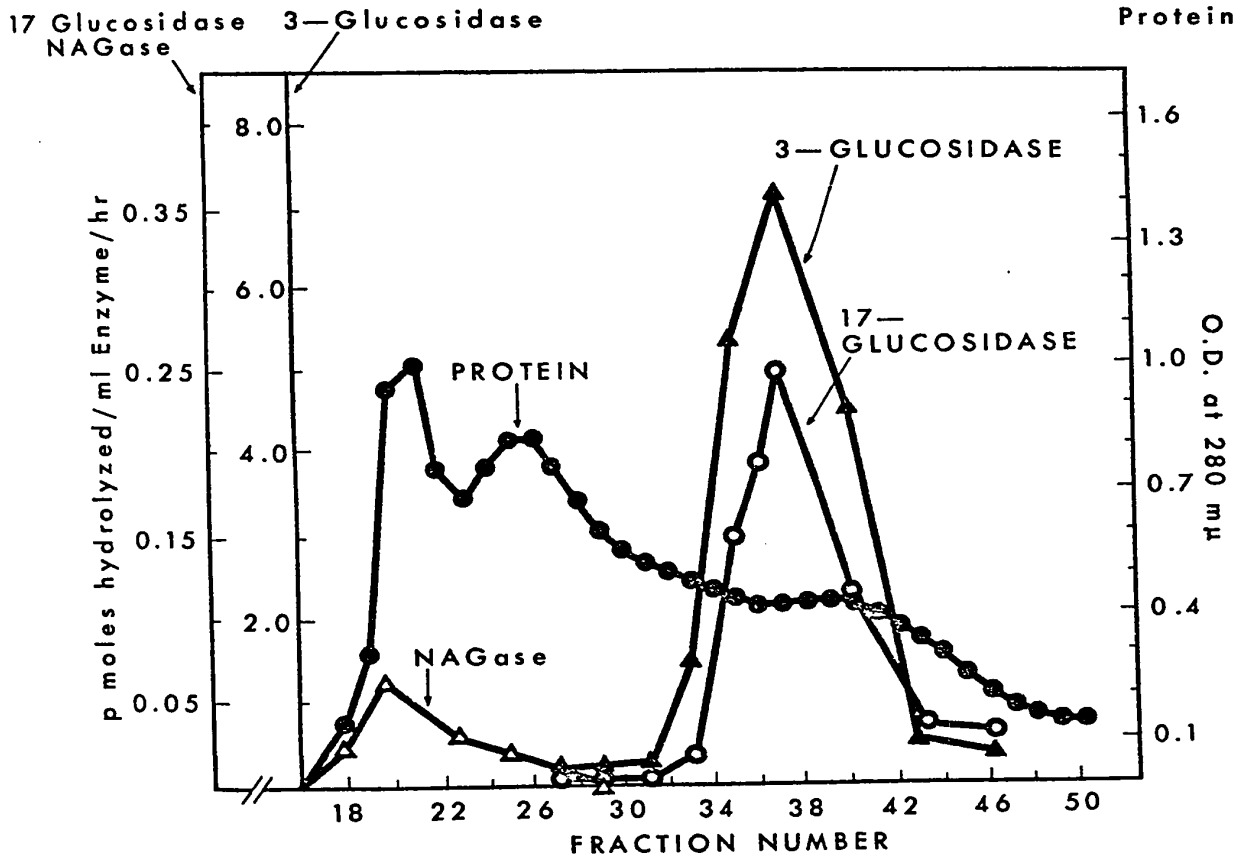
Fraction	Substrate Hydrolysed			
	17α-estradiol-17-glucoside	17α-estradiol 3-glucoside	17α-estradiol-3-glucuronide	17α-estradiol 17-N-acetylglucosamine
High Speed Supernatant	1.7	199.0	6.0	0.3
0-45% $(NH_4)_2SO_4$	0.3	1.3	5.5	0.2
45-65% $(NH_4)_2SO_4$	1.7	125.0	0.1	0.1

Figure 5 shows the elution profile of the 45-65% ammonium sulphate fraction on the Sephadex G-150 column. The remaining N-acetylglucosaminidase activity was eluted in the void volume of the column and was completely separated from the β-glucosidase activity. The activity toward both estradiol glucosides was eluted together. This step gave a 1.5 to 2 fold purification but was more important for its removal of β-N-acetylglucosaminidase from the preparation. Chromatography on the cation exchanger, carboxymethyl cellulose gave a 4 to 5 fold purification over the Sephadex filtrate (Filtrate I). The enzyme was not adsorbed to the cellulose but was eluted with the starting buffer. This indicated that the enzyme still had an overall negative charge at this pH (5.5). The enzyme remained stable at this pH, that of its optimum activity, for several weeks at 4°C.

Analytical gel electrophoresis of the CM-cellulose filtrate is shown in Figure 6A. Five major protein bands which are well differentiated were present in the CM-cellulose filtrate. Because of the good separation of these bands it was decided to attempt preparative electrophoresis as a method of purification. Figure 7 shows the elution profile of the enzyme obtained with this larger gel while Figure 6B shows that two main proteins still remained in the enzyme fractions after the procedure. Since these two bands were separated by more than a centimeter on the analytical gels, it was possible to cut the gels in two between these bands with little fear of

FIGURE 5

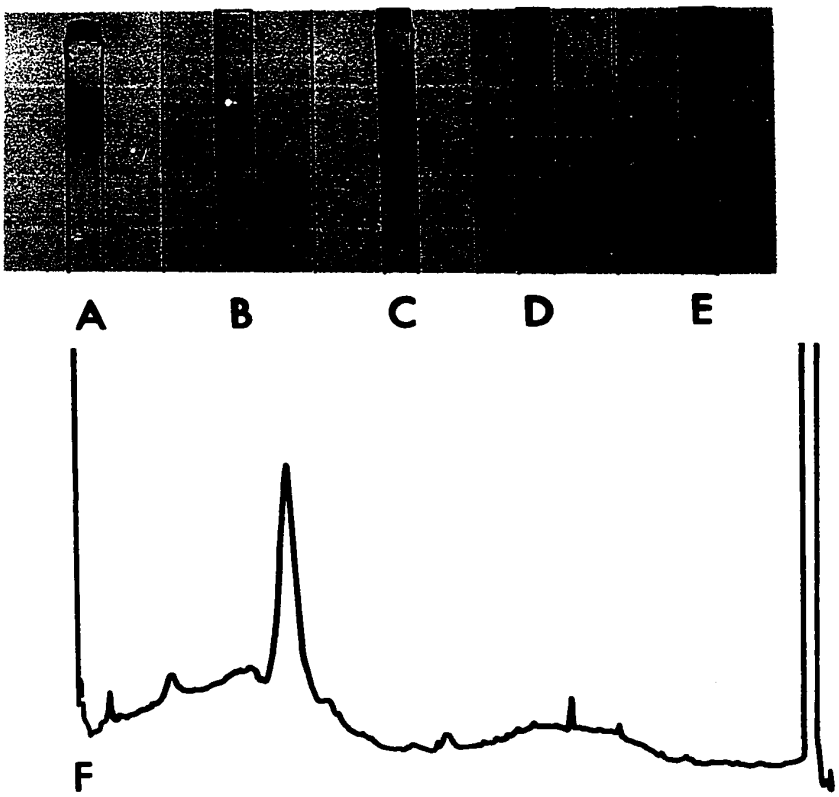
Elution Profile of the Ammonium Sulphate 45-65% Fraction on a 2.5 x 45 cm Sephadex G-150 Column



● = Protein
o = Activity toward 17α-estradiol-17-glucoside
▲ = Activity toward 17α-estradiol-3-glucoside
Δ = Activity toward 17α-estradiol-17-N-acetylglucosaminide

FIGURE 6

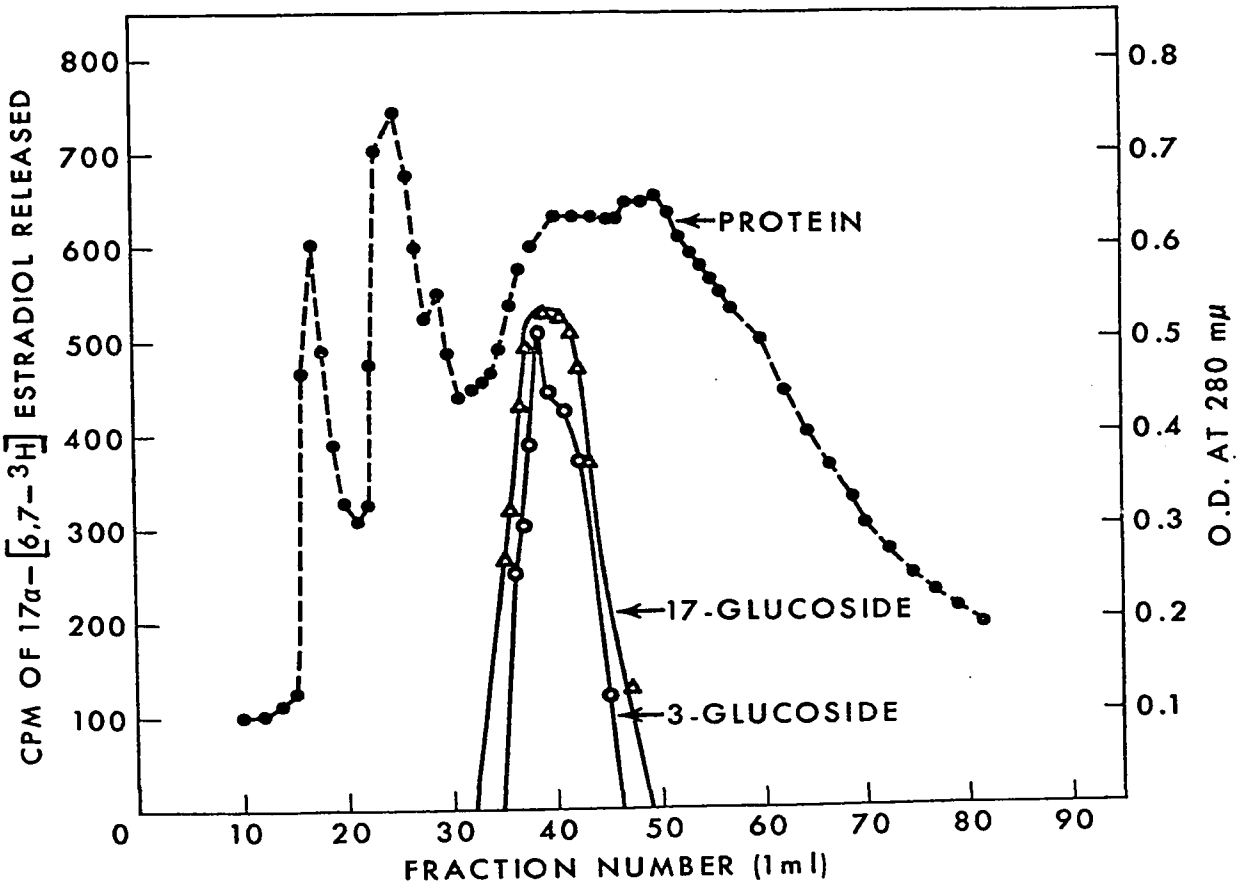
Analytical Electrophoresis Gels of Various Rabbit Liver β-Glucosidase Preparations



A Davis Gel, pH 8.3 - CM-cellulose Filtrate
B Davis Gel, pH 8.3 - Preparative Electrophoresis Fraction
C Davis Gel, pH 8.3 - Sephadex Filtrate II
D Davis Gel, pH 8.3 - Sephadex Filtrate III (Purified Enzyme Preparation)
E S D S Gel, pH 7.0 - Sephadex Filtrate III
F Scan of Gel D at 570 mμ

FIGURE 7

Elution Profile of the CM-cellulose Filtrate on a Preparative Electrophoresis Davis Gel, pH 8.3



● = Protein

Δ = Activity towards 17α-estradiol-17-glucoside

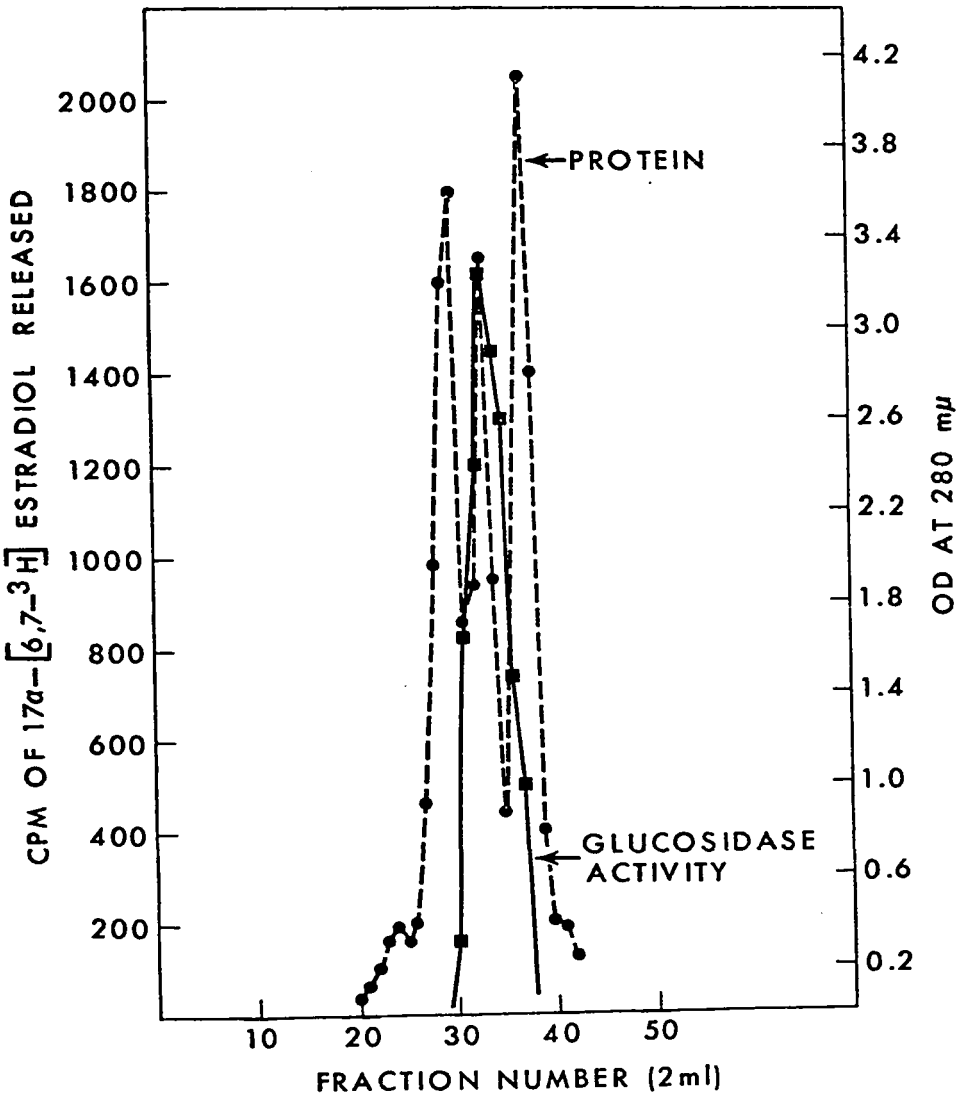
o = Activity towards 17α-estradiol-3-glucoside

cross contamination of one band with the other. The results of incubating these two proteins with the two glucosides of 17α-estradiol, dehydroisoandrosterone glucoside and p-nitrophenyl-glucoside showed that the activity towards all the substrates resided in the upper band. This was verified by incubation of the whole gels with p-nitrophenylglucoside. A bright yellow band appeared in the gel after 3 to 4 hours and corresponded exactly to the upper band when aligned with a stained gel. However, the preparative electrophoresis technique did not prove to be a valuable one for purification because, although a 10 fold purification in terms of protein was achieved, only 1% of the glucosidase activity was recovered. Therefore, an alternative method was attempted.

Preliminary experiments using a 1.5 x 40 column showed that some separation of the five proteins of the CM-cellulose filtrate was achieved by gel filtration on Sephadex G-150 although there was considerable overlap of the protein bands. Therefore a longer column was poured. Figure 8 shows the elution profile of the first filtration step (Sephadex filtrate II) on this column while Figure 9 shows the results of the second (Sephadex filtrate III). Glucosidase activity was monitored by using the 3-glucoside of 17α-estradiol, since the preparative electrophoresis had shown that the activity towards all the substrates was due to one protein. Analytical gel electrophoresis at pH 8.3 was used to monitor the glucosidase peak and showed that the Sephadex filtrate II contained 2 main

FIGURE 8

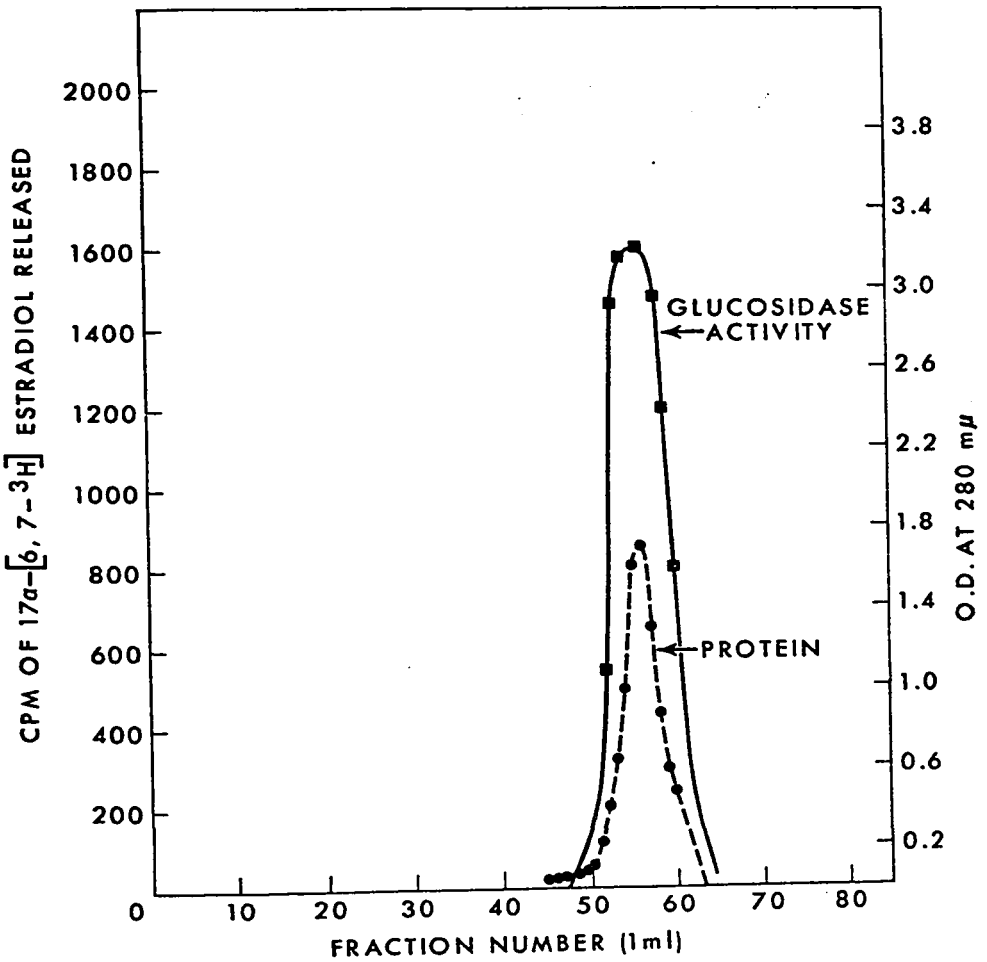
Elution Profile of the CM-cellulose Filtrate on a 1.5 x 90 column of Sephadex G-150, Superfine



● = Protein
■ = Activity toward 17α-estradiol-3-glucoside

FIGURE 9

Elution Profile of the Sephadex Filtrate II on a 1.5 x 90 Column of Sephadex G-150, Superfine



● = Protein

■ = Activity towards 17α-estradiol-3-glucoside

protein bands with some minor contaminants (Figure 6C). By rechromatographing this filtrate and by selecting only those fractions which contained one major band of protein (as monitored by the analytical gels) a relatively pure enzyme preparation was obtained (Figure 6D). An analytical gel of this purified preparation was scanned in a Gilford spectrophotometer equipped with a model 2410 linear transporter at 570 mμ and the percent contamination was calculated to be 17.5% (see Figure 6F). When the purified enzyme preparation was run on acid gels at pH 4.3, no protein could be detected.

After each step in the purification scheme, the activity towards a number of substrates was measured. Table 4 gives the total units and specific activities of each fraction while Table 5 shows the fold purification and the percent recovery of β-glucosidase activity as measured with 17α-estradiol-3-glucoside, deoxycorticosterone-21-glucoside and p-nitrophenyl-glucoside. The degree of purification was basically the same regardless of which substrate was used. 17α-estradiol-17-glucoside gave similar results.

The homogenate and purified enzyme preparation were assayed for activity towards eight different glucosides (Table 6). The ratio of the activity towards each substrate to the activity towards 17α-estradiol-3-glucoside remained fairly constant during purification with one exception. The activity towards dehydroisoandrosterone glucoside increased. However, since it was impossible to saturate the enzyme with this substrate before

TABLE 4

Purification of Rabbit Liver β-Glucosidase

Total Units and Specific Activity as Measured by 3 Substrates for Each Step in the Purification Procedure

Total units are expressed as μmoles of substrate hydrolysed per hour and specific activity as total units per mg protein.

Fraction	Total Protein (mg)	Total Units			Specific Activity		
		17α-estradiol-3-glucoside	p-nitrophenyl-glucoside	Deoxycorticosterone glucoside	17α-estradiol-3-glucoside	p-nitrophenyl-glucoside	Deoxycorticosterone glucoside
					($\times 10^{-6}$)		
Homogenate	29,281	0.18	10395	517	6.09	0.36	0.02
High Speed Supernatant	8182	0.15	9450	469	18.45	1.16	0.05
Acid Supernatant	4049	0.17	10394	557	40.67	2.57	0.14
45-65% $(NH_4)_2SO_4$	623	0.14	8280	373	223.60	13.29	0.60
Sephadex Filtrate I	329	0.11	7560	375	348.00	23.12	1.15
CM-Cellulose Filtrate	60	0.08	6003	237	1296.00	100.00	3.95
Sephadex Filtrate II	26	0.06	4303	229	2330.00	165.00	8.80
Sephadex Filtrate III	6	0.04	2459	123	7306.00	417.00	20.80

TABLE 5

Purification of Rabbit Liver β-Glucosidase Activity as Measured with Three Different Substrates

Specific activities were measured as micromoles of substrate hydrolysed per mg protein per hour. R is the ratio of the specific activity in the fraction to that in the homogenate.

Fraction	17α-estradiol-3-glucoside		Deoxycorticosterone 21-glucoside		p-nitrophenyl-glucoside	
	R	Recovery	R	Recovery	R	Recovery
Supernatant (105,000 xg)	3.0	84.7	3.2	91.0	3.51	91.0
Acidified Supernatant	6.7	92.4	7.8	108.0	7.7	100.0
$(NH_4)_2SO_4$ (45-65%)	36.7	78.2	33.8	72.0	39.7	80.0
Sephadex Filtrate I	57.2	63.8	64.8	72.6	69.0	72.7
CM-cellulose Filtrate	213.0	43.6	304.0	62.5	298.0	57.7
Sephadex Filtrate II	383.0	34.0	498.0	44.2	466.0	41.4
Sephadex Filtrate III (Purified Enzyme)	1198.0	24.2	1177.0	23.8	1174.0	23.6

TABLE 6

A Comparison of β-Glucosidase Activity towards Eight Substrates with Homogenate and Purified Enzyme Preparations

Results are expressed as the ratio of the specific activity of the enzyme towards each substrate compared to the specific activity of the enzyme towards 17α-estradiol-3-glucoside (R).

Substrate	Homogenate		Purified Enzyme	
	SA (μmoles/mg/hr)	R	SA μmoles/mg/hr	R
17α-Estradiol-3-glucoside	2.1×10^{-5}	1	8.6×10^{-3}	1
17β-Estradiol-3-glucoside	1.5×10^{-5}	0.7	1.0×10^{-2}	1.2
Estrone-3-glucoside	3.8×10^{-5}	1.8	1.3×10^{-2}	1.5
17α-Estradiol-17-glucoside	2.9×10^{-3}	138	7.9×10^{-1}	91
17β-Estradiol-17-glucoside	5.0×10^{-3}	239	2.6	301
Deoxycorticosterone-21-glucoside	9.3×10^{-2}	4386	28.7	3316
p-nitrophenylglucoside	4.6×10^{-1}	21,509	126	14,578
Dehydroisoandrosterone glucoside*	15.1	712,736	15,940	1,844,267

* Solubility limit of buffer reached before enzyme saturation.

the limit of solubility of the substrate in the assay buffer was reached, the ratio of substrate concentration to enzyme concentration was probably larger for the purified preparation than for the crude. This could have caused an increase in the velocity of the reaction with the purified preparation as compared to the homogenate and would account for the higher specific activity. Both crude and purified preparations of the enzyme also showed considerable activity towards 17α-estradiol-3- and 17-galactosides and towards p-nitrophenyl-galactoside.

Table 7 gives the Kav values for each standard protein and the enzyme as obtained on both G-150 and P-150 gels. Figure 10 shows the calibration curves for the determination of molecular weight by gel filtration on both Sephadex G-150 and Bio-Gel P-150 columns. Both columns gave a molecular weight for the purified enzyme of 51,300. The calibration curve for molecular weight determination by S.D.S gel electrophoresis is shown in Figure 11. The calculated mobility of each standard and protein bands of the enzyme preparation are given in Table 8. The molecular weight of the enzyme was calculated to 58,000 by this method. A sample gel is shown in Figure 6E.

The results of the fine range pH 4.4-5.2 isoelectrofocusing experiment are shown in Figure 12. From this graph the pI of the enzyme was calculated to be 4.78. Initially the focusing experiments were attempted as a method for purifying the enzyme. The preliminary experiment over a wide pH range

TABLE 7

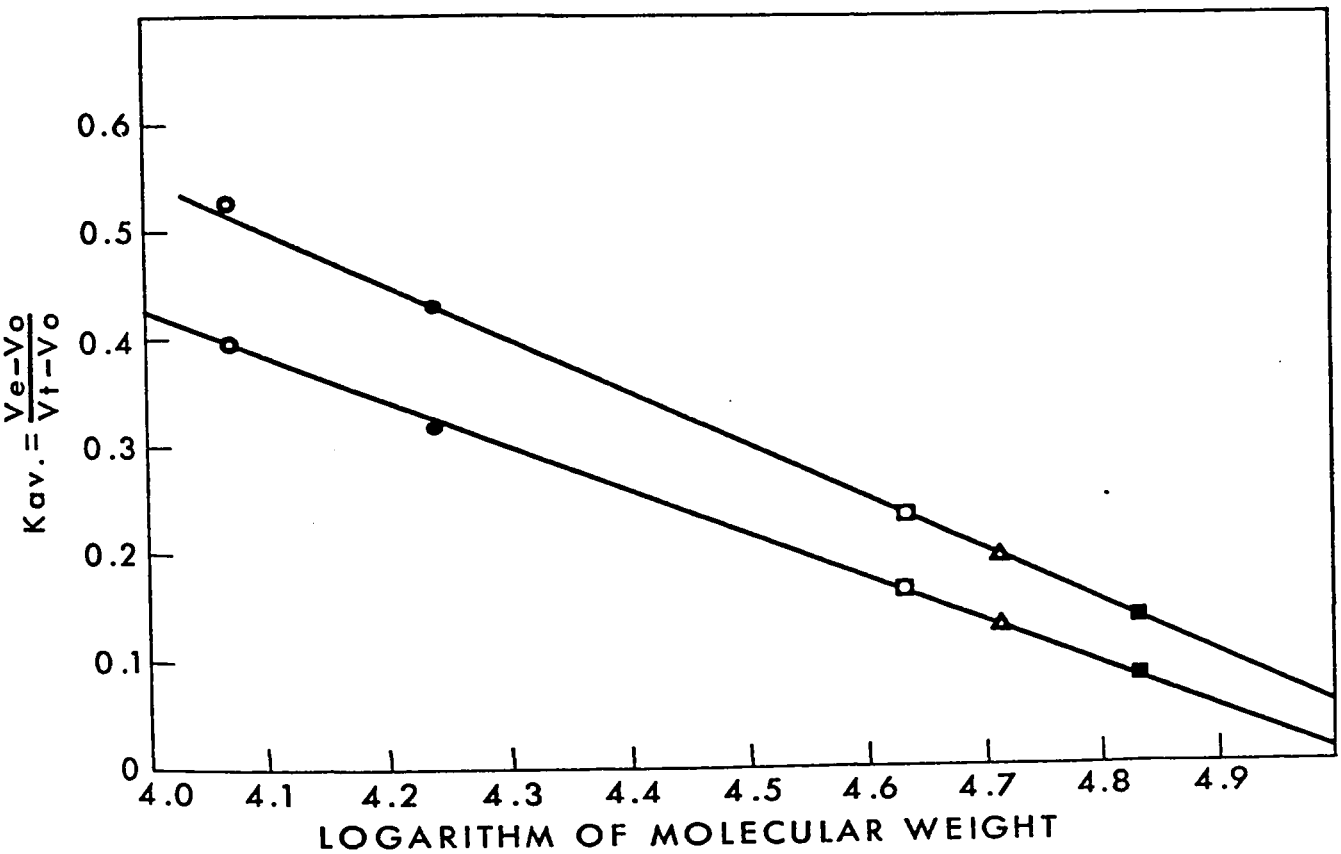
Determination of Molecular Weight of β-Glucosidase by Gel Filtration on Sephadex G-150 and on Bio-Gel P-150

Kav values obtained for each standard protein.

Protein	Kav G-150	Kav P-150	MW	Log MW
Bovine Serum Albumin	0.13	0.08	68,000	4.83
Ovalbumin	0.23	0.17	43,000	4.63
Myoglobin	0.43	0.31	17,200	4.24
Cytochrome C	0.54	0.40	11,700	4.07
Purified Enzyme Preparation	0.19	0.13	51,300	4.71

FIGURE 10

Calibration Curves for the Determination of Molecular Weight of Rabbit Liver β-Glucosidase by Gel Filtration on Sephadex G-150 and on Bio-Gel P-150



o = Cytochrome C
● = Myoglobin
□ = Ovalbumin
Δ = β-glucosidase
■ = Bovine serum albumin
Upper Curve = Sephadex G-150
Lower Curve = Bio-Gel P-150

TABLE 8

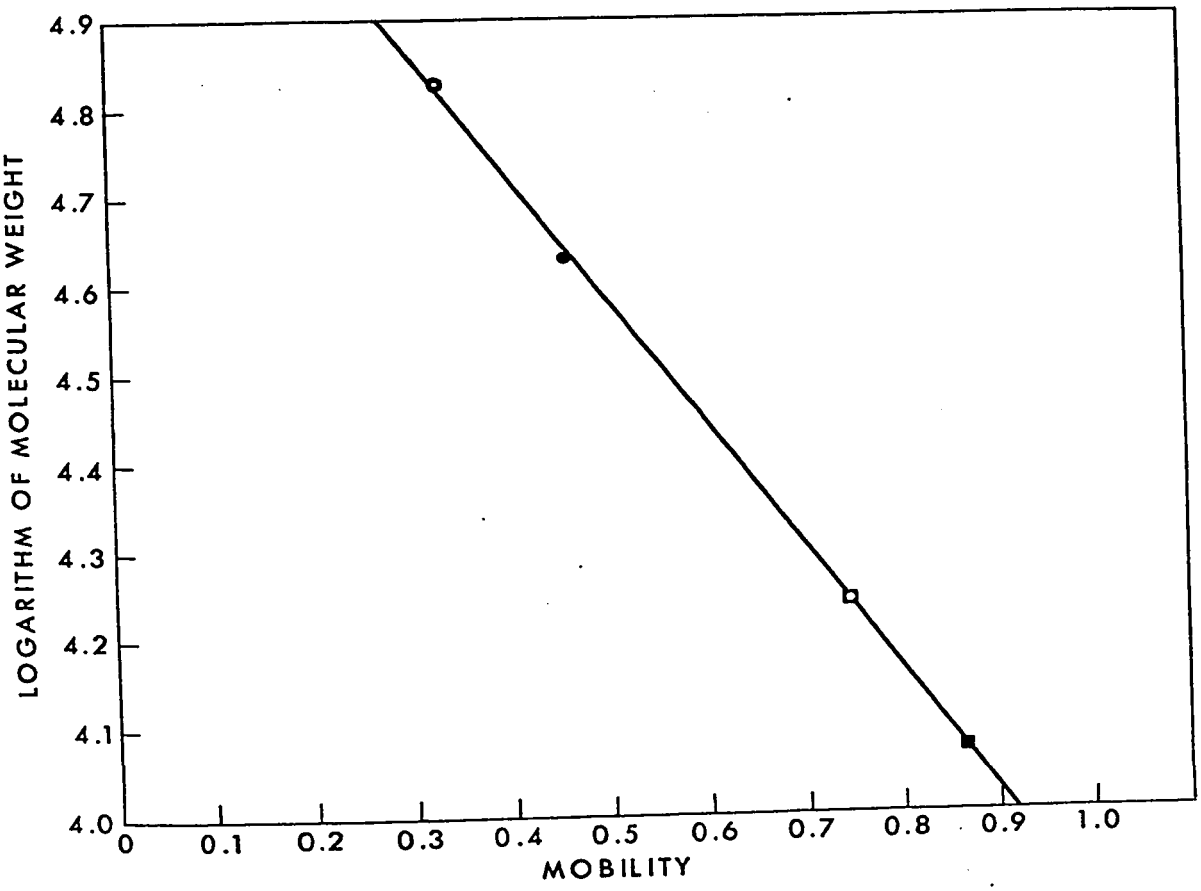
Molecular Weight Determination of β-Glucosidase by S.D.S. Gel Electrophoresis

Mobility on S D S. Gels of Standard Proteins and Enzyme

Protein	Mobility	MW	Log MW
Bovine Serum Albumin	0.327	68,000	4.83
Ovalbumin	0.461	43,000	4.63
Myoglobin	0.744	17,200	4.24
Cytochrome C	0.865	11,700	4.07
Enzyme	0.375	58,000	4.76

FIGURE 11

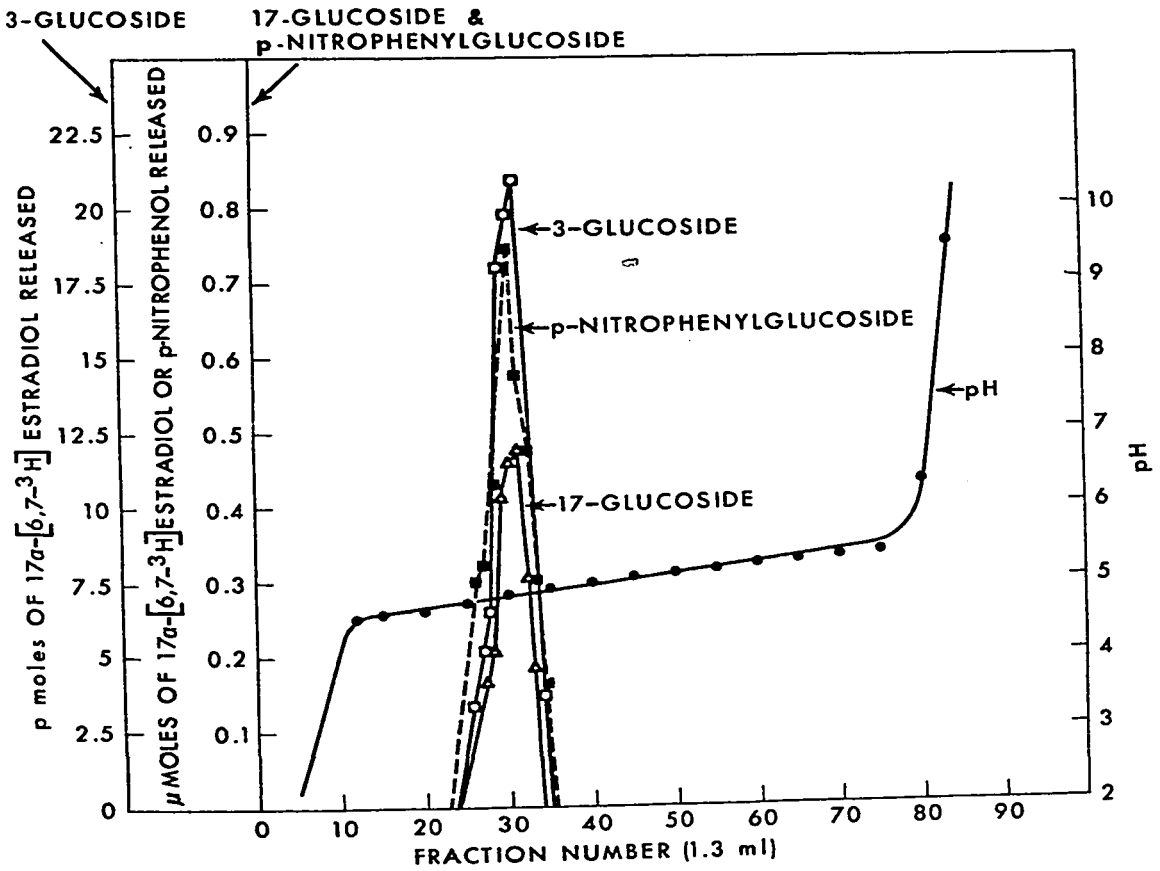
Calibration Curve for the Determination of Molecular Weight of Rabbit Liver β-Glucosidase by S.D.S. Gel Electrophoresis



o = Bovine Serum Albumin
● = Ovalbumin
□ = Myoglobin
■ = Cytochrome C

FIGURE 12

Elution Profile of Rabbit Liver β-Glucosidase from an Isoelectrofocusing Column over a pH Gradient of 4.4-5.2



● = pH gradient
□ = Activity toward 17α-estradiol-3-glucoside
Δ = Activity toward 17α-estradiol-17-glucoside
■ = Activity toward p-nitrophenylglucoside

(3-10) was performed to find the approximate range of the pI of the enzyme. This was found to be between pH 4.5 and 5.0. The enzyme activity, as measured by both 17- and 3-glucosides of 17α-estradiol and p-nitrophenylglucoside, was more wide spread in this column and abundant amounts of precipitate formed at several locations during the focusing period. Approximately 50% of the enzyme activity was lost. This was probably due to precipitation of the enzyme at its isoelectric point. The fractions containing the remaining glucosidase activity were focused over narrower pH ranges to obtain an accurate measure of the isoelectric point. After each focusing step further activity was lost. The enzyme fractions from the final experiment (pH 4.4-5.2) were monitored by analytical gels and showed that two main protein bands remained in the preparation. The technique was not used further as a purification procedure.

C. Discussion

Glycosidases have generally been regarded as lysosomal enzymes although several authors have reported the presence of β-glucosidases in "soluble" form in homogenates of animal tissues. Abrahams and Robinson (1969) and Dance et al (1969) found that the β-glucosidases of pig kidney and of human kidney were soluble and were not associated with the lysosomes. Brady et al (1965) found that, in rat and in human spleen, the enzyme which cleaved the glycosidic bond of glucocerebrosides was present mainly in the 100,000 xg supernatant, obtained by differential centrifugation of a homogenate of the tissue. The rabbit liver enzyme studied in the present work was also found to be located in the high speed supernatant. It is possible, however, that this was due to a leakage of glucosidase from the lysosomes during tissue preparation and centrifugation. The possibility has been investigated further and the results are discussed in Chapter 7.

The enzyme was stable under acid conditions at a pH at least as low as 5.0. This was verified during the chromatography on CM-cellulose. The enzyme preparation at this stage could be stored at 4^{o} C for several weeks with little loss of activity and for months if it had been lyophilized. Fractionation with ammonium sulphate was unaffected by changes in pH as similar patterns of protein precipitation were observed if the technique was carried out at either pH 7 or pH 5. This

step was an especially valuable means of purification, since it separated the β-glucuronidase activity from the β-glucosidase activity. The β-N-acetylglucosaminidase activity was, however, distributed about equally between the fraction containing β-glucuronidase and that containing β-glucosidase. It was also possible to separate the β-glucuronidase activity from the β-glucosidase activity by gel filtration on Sephadex G-150, since the β-glucuronidase was eluted with the β-N-acetylglucosaminidase in the void volume of the column. However, the ammonium sulphate fractionation provided an easy method of concentrating the β-glucosidase as well as separating it from the β-glucuronidase activity and this step was therefore usually included in the purification procedure. In addition, some β-N-acetylglucosaminidase free of β-glucuronidase activity could be obtained by applying the 45-65% ammonium sulphate fraction to the Sephadex column.

The method followed for the gel filtration steps was based on that of Hultberg and Öckerman (1970). Robinson, Price and Dance (1966) used 0.01 M sodium phosphate buffer, pH 7.6, containing 0.4 M NaCl and 0.01 M EDTA as the elution buffer in their gel filtration experiments with β-glucosidase from rat kidney. Hultberg and Öckerman (1970) found that by using this same eluent the β-glucosidase activity in the soluble fraction of human liver homogenates could be fractionated into two peaks. However, they found that by eluting with 0.01 M phosphate buffer, pH 7.0, without EDTA or NaCl, two other small peaks of

activity were seen in addition to the two originally found. Therefore, as attempts were being made in the present work to separate, if possible, two distinct glucosidases with specificities for a glucosidic linkage at either a phenolic or aliphatic hydroxyl group, the column was eluted with 0.01 M phosphate buffer, pH 7.0, without the addition of EDTA or NaCl. It was found, however, that the rabbit liver enzyme did not dissociate into more than one peak, and the activities towards all the substrates tested were present in the same column fractions.

Although the enzyme was not adsorbed to the cation exchanger CM-cellulose, chromatography on this material provided a 4-5 fold purification. While the preparative electrophoresis step was not useful as a means of purification, one important fact was established by this experiment. When the pooled glucosidase fractions eluted from the preparative gel were tested for purity by analytical gel electrophoresis, two bands of protein were evident (Figure 6B). Incubation of these analytical gels with four substrates indicated that only one of these protein bands, the upper one, had β-glucosidase activity. This then was the first evidence that indicated that one protein may be responsible for the hydrolysis of all substrates tested and especially both the estradiol glucosides. Thus the difference in specificity observed among the tissues (Table I) may be due to some other factor rather than due to the presence of two

types of glucosidases each responsible for the hydrolysis of one of the estradiol glucosides.

The two final gel filtration steps carried out on the CM-cellulose filtrate removed practically all contaminating proteins. The superfine grade Sephadex gave much sharper elution profiles and the longer column gave better resolution. The second filtration step at this stage was needed because of the overlap of the peaks (see Figures 8 and 6C).

The fact (Table 5) that the degree of purification over each step in the procedure was the same regardless of which substrate was used to test the activity is further evidence that one enzyme was responsible for the hydrolysis of all the glucosides tested. This was also indicated by the finding that the purified enzyme retained its activity towards all the substrates that were hydrolyzed by the homogenate and that the ratio of activities remained constant throughout the purification procedure. The enzyme does not appear to be specific for one type of substrate since the synthetic substrate p-nitrophenylglucoside as well as various types of steroid glucosides were hydrolysed. The activity towards p-nitrophenyl-glucoside was followed throughout most of this work because of its widespread use as a substrate for β-glucosidase by other workers. The fact that the enzyme also readily hydrolysed the galactoside substrates suggests that it falls into the "Emulsin" category (see section 1F). The activity toward the galactoside was invariably less than that toward the corresponding glucoside,

in the ratio of approximately 1:3.

The molecular weight of the enzyme as determined by three methods is in reasonable agreement (see Tables 7 and 8). The S.D.S polyacrylamide gel electrophoresis method has an accuracy of ±5% of the molecular weight (Weber and Osborn, 1969). The error of the gel filtration technique is larger, and is about ±10% (Andrews, 1970).

Flodin (1962) and Abrahams and Robinson (1969) have shown that some carbohydrase enzymes show a degree of affinity for dextran and are thus unduly retarded by columns of this material. The latter authors found that a somewhat higher molecular weight was indicated for pig kidney β-glucosidase when determined on Bio-Gel P-150. In the case of the rabbit liver enzyme this effect was not seen, and the Sephadex and polyacrylamide columns gave identical values for the molecular weight of the enzyme.

Isoelectricfocusing did not prove to be a useful method of purification, mainly because of the large losses of enzyme activity which ensued from it. Preliminary studies showed the enzyme to be stable in high density sucrose solutions at 4^{o} C for the period of time needed to focus the enzyme (four-five days). Therefore the loss of activity must have been due to precipitation of the protein at its isoelectric point. Abrahams and Robinson (1969) found two forms of the pig kidney enzyme which could be separated by DEAE-cellulose chromatography and which exhibited different isoelectric points. Their

specificities were identical and because they could not be separated by gel filtration, the distinction between the two forms appeared to be one of charge rather than molecular size. The rabbit liver enzyme did not exhibit these properties. Only one enzyme peak was found after focusing, although this did contain more than one type of protein, as evidenced by analytical gel electrophoresis of the material in the peak obtained by isoelectric focusing. Although analytical gel electrophoresis of the purified enzyme showed that there were some minor contaminants, only one major protein was present and this was responsible for the hydrolysis of all glucosides tested. Acid gels showed that, even though the enzyme did not migrate, no very basic protein, which would not migrate at pH 8.3 was present in the preparation.

The results therefore indicate that rabbit liver contains only one β-glucosidase, responsible for the hydrolysis of a number of steroid glucosides as well as p-nitrophenylglucoside. Among these substrates, however, the enzyme shows a marked preference for glucosides of the phenolic 3-hydroxyl of the steroid estrogens. Kinetic experiments which demonstrate this are presented in the next chapter. The enzyme has an isoelectric point of 4.78, and a molecular weight of 50,000-60,000.

CHAPTER 5

KINETIC PROPERTIES OF β-GLUCOSIDASE

A) Introduction

Two enzyme preparations were used in these kinetic experiments. Km values were determined with both a partially purified preparation, the Sephadex filtrate I (see Table 4), and with the purified enzyme preparation. The inhibitor studies with gluconolactone and galactonolactone as well as those showing the effect of EDTA were carried out with the partially purified Sephadex filtrate I. All other experiments were carried out using the purified enzyme.

B) Methods

1) Determination of Km Values Towards Six Substrates

The Sephadex filtrate I, prepared as described in section 4A4 was used to determine the Km values of the glucosidase activity toward the following substrates: 17α-estradiol-17-glucoside, 17α-estradiol-3-glucoside, deoxycorticosterone glucoside, dehydroisoandrosterone glucoside, 17β-estradiol-17-glucoside and p-nitrophenylglucoside. Reactions were carried out in a volume of 1 ml and the activity was determined as described previously. Table 9 gives the range of concentrations of each substrate used for the determinations. Km values were calculated from conventional Lineweaver-Burk plots (Lineweaver and Burk, 1934). The Km values for the 17- and the 3-glucosides of 17α-estradiol and for p-nitrophenylglucoside were redetermined using the purified enzyme preparation.

2) Inhibition by Gluconolactone and Galactonolactone

The extent of inhibition of the β-glucosidase activity towards three substrates by glucono-1,5-lactone and galactono-1,5-lactone was studied. Values for Ki were determined by plotting 1/V against I (where V is the velocity expressed as picomoles of 17α-estradiol released per hour from either the 3- or 17-glucoside or as micromoles of p-nitrophenol released from its glucoside per hour and I is the inhibitor concentration in moles per liter) at 2 or 3 different substrate

TABLE 9

Range of Concentrations Used for the Determination of Km Values

Substrate	Concentration
17α-Estradiol-3-glucoside	10^{-10} to 1.6×10^{-8} M
17α-Estradiol-17-glucoside	5.2×10^{-5} to 5.2×10^{-4} M
17β-Estradiol-17-glucoside	3×10^{-5} to 3×10^{-4} M
p-Nitrophenylglucoside	5×10^{-4} to 5×10^{-3} M
Deoxycorticosterone glucoside	2×10^{-4} to 1.2×10^{-3} M
Dehydroisoandrosterone glucoside	5×10^{-5} to 5×10^{-4} M

concentrations according to the method of Dixon (1953). The concentration of gluconolactone ranged from 5×10^{-5} to 5×10^{-4} M. Assays were performed at 4×10^{-10}, 8×10^{-10}, and 1.2×10^{-9} M concentrations of 17α-estradiol-17-glucoside, 3×10^{-10}, 9×10^{-10}, and 1.5×10^{-9} M concentrations of 17α-estradiol-3-glucoside, and 5×10^{-5} and 10^{-4} M concentrations of p-nitrophenylglucoside.

Similar assays were performed in the presence of galactonolactone. The concentration of this inhibitor ranged from 1×10^{-3} to 1×10^{-2} M. Assays were performed at 6×10^{-10} and 1.6×10^{-9} M concentrations of 17α-estradiol-17-glucoside, 1.8×10^{-9} and 4.5×10^{-9} M concentrations of 17α-estradiol-3-glucoside and 5×10^{-5}, 10^{-4} and 1.5×10^{-4} M concentrations of p-nitrophenylglucoside.

3) Competition Between Substrates

The hydrolysis of tritiated 17α-estradiol-3-glucoside was assayed in the presence of radioinert 17α-estradiol-17-glucoside. The radioinert 17-glucoside, 5×10^{-2} μmoles, was added to each reaction tube in methanol and the methanol was evaporated under a stream of nitrogen. Radioactive 17α-estradiol-3-glucoside was added in similar fashion. Concentrations of 1 to 16 picomoles of 3-glucoside per ml of reaction mixture were used. These were the same concentrations used to determine the Km value. A Lineweaver-Burk plot was used to determine the apparent type of inhibition by the 17-glucoside. The

3-glucoside was also assayed in the presence of 0.1 μmoles of p-nitrophenylglucoside and the apparent type of inhibition was determined.

In a similar manner, the activity towards p-nitrophenylglucoside was assayed in the presence of either 17- or the 3-glucoside of 17α-estradiol. The concentration of the 17-glucoside present was 5×10^{-2} μmoles and of the 3-glucoside, 1.1×10^{-2} μmoles per ml of reaction mixture. The range of concentrations of p-nitrophenylglucoside were the same as those used for the Km determination. Likewise 17α-estradiol-17-glucoside was assayed in the presence of p-nitrophenylglucoside (1 μmole per reaction). The apparent type of inhibition was then determined.

4) Heat Stability Studies

Aliquots of phosphate-citrate buffer, pH 5.5 were brought to various temperatures by heating in a water bath. Appropriate aliquots of the purified enzyme preparation were then added and the mixtures were heated for 10 minutes. The mixtures were then rapidly cooled, substrates were added and the mixtures were incubated for 1 hour at 37°C. They were then processed as usual. Three substrates were assayed in this manner, 17α-estradiol-17-glucoside, 17α-estradiol-3-glucoside and p-nitrophenylglucoside. The temperatures used for preincubation were 37°, 40°, 45°, 50°, and 55°C. The activity of each preincubated sample was compared to that of an untreated one.

5) The Effect of EDTA and of Metal Ions on β-Glucosidase

Separate aliquots of the Sephadex filtrate I were brought to concentrations of EDTA of 0.01, 0.025, and 0.05 M respectively. The solutions were allowed to remain at $4^{o}C$ for 1 hour and then were assayed for activity towards each of the glucosides of 17α-estradiol. The aliquot which was 0.025 M in EDTA was divided into smaller fractions and these were made either 0.025 M or 0.05 M in $MgCl_2$, $MnCl_2$, or $CaCl_2$. Each fraction was then assayed for glucosidase activity.

C) Results

The Km values as determined with the partially purified Sephadex filtrate I are listed in Table 10. It was not possible to determine the Km value for dehydroisoandrosterone glucoside. Double reciprocal plots 1/V versus 1/[S] repeatedly gave straight lines which appeared to pass through the origin. No value for 1/Km could be obtained which was significantly different from zero. The enzyme also could not be saturated with this substrate before the limit of its solubility in the buffer was reached.

The Km and Vmax values of the three substrates which are of most importance in this study, 17α-estradiol 17- and 3-glucosides and p-nitrophenylglucoside, were redetermined with the purified enzyme preparation. The results are shown in Table 11. Figure 13 shows a typical Lineweaver-Burk plot for the enzyme activity towards the 3-glucoside of 17α-estradiol. There was good correlation between the values obtained for the 17-glucoside of 17α-estradiol and for p-nitrophenylglucoside with both the crude and purified enzyme preparations. The discrepancy was a little larger for 17α-estradiol-3-glucoside but the results indicated that this substrate was by far the preferred one.

Figure 14 shows the Dixon plot for the determination of the Ki for gluconolactone in the presence of 17α-estradiol-17-glucoside. Figure 15 shows a similar plot for the determination

TABLE 10

Km Values for Five Substrates as Determined with the Sephadex Filtrate I

Km values were determined by means of the conventional Lineweaver-Burk double reciprocal plots.

Substrate	Km M
17α-Estradiol-3-glucoside	5×10^{-10}
17α-Estradiol-17-glucoside	3.9×10^{-5}
Deoxycorticosterone glucoside	2.9×10^{-4}
p-Nitrophenylglucoside	2.1×10^{-3}
17β-Estradiol-17-glucoside	1.3×10^{-3}
Dehydroisoandrosterone glucoside	—

TABLE 11

Km Values and Vmax Values as Determined with the Purified Enzyme

Vmax is expressed as μmoles of substrate hydrolysed per hour at 37°C.

Substrate	Km M	Vmax
17α-Estradiol-3-glucoside	4.3×10^{-9}	1.25×10^{-6}
17α-Estradiol-17-glucoside	3.0×10^{-5}	2.0×10^{-2}
p-Nitrophenylglucoside	7.0×10^{-4}	1.33×10^{-1}

FIGURE 13

Lineweaver-Burk Plot for the Determination of the Km for the β-glucosidase Activity Toward 17α-Estradiol-3-glucoside

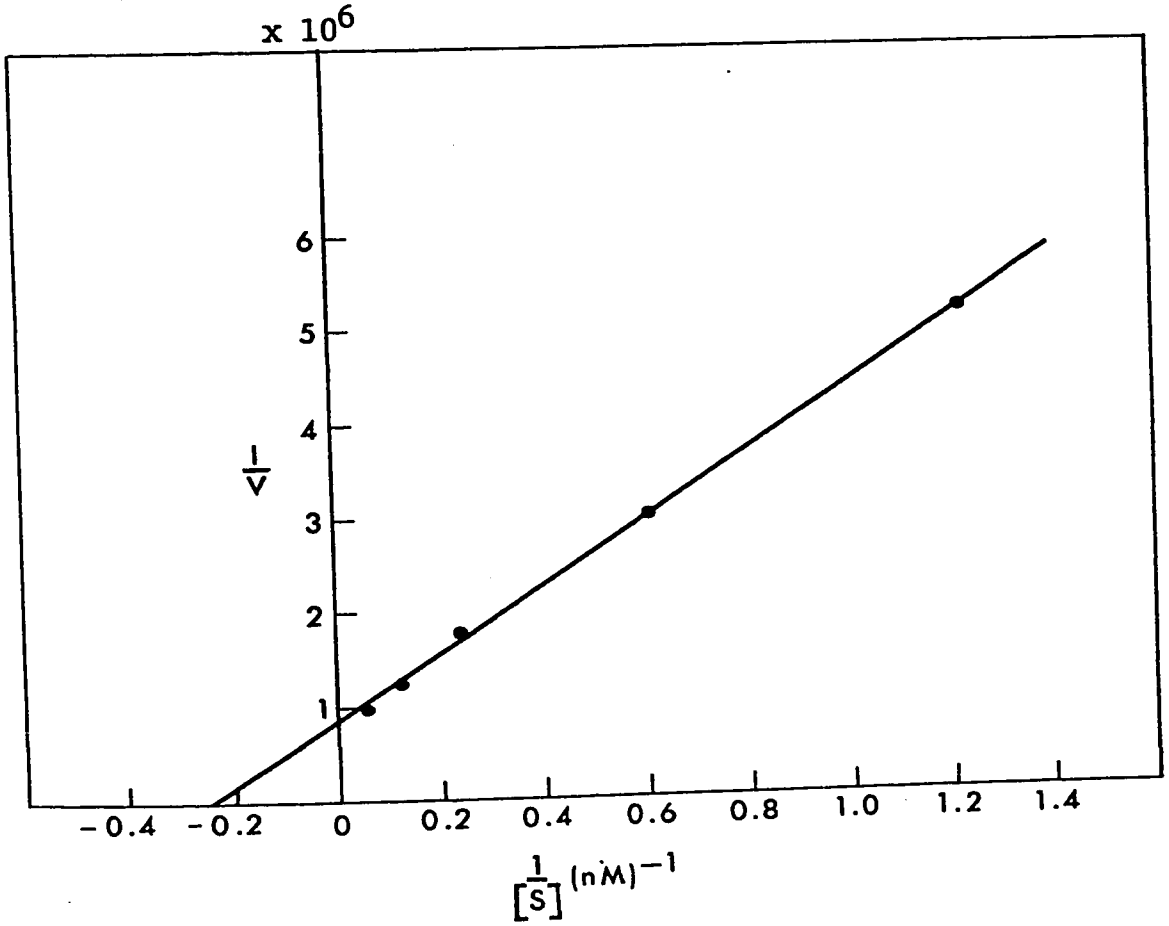


FIGURE 14

Dixon Plot for the Determination of the Ki for Gluconolactone for the β-Glucosidase Activity toward 17α-Estradiol-17-Glucoside

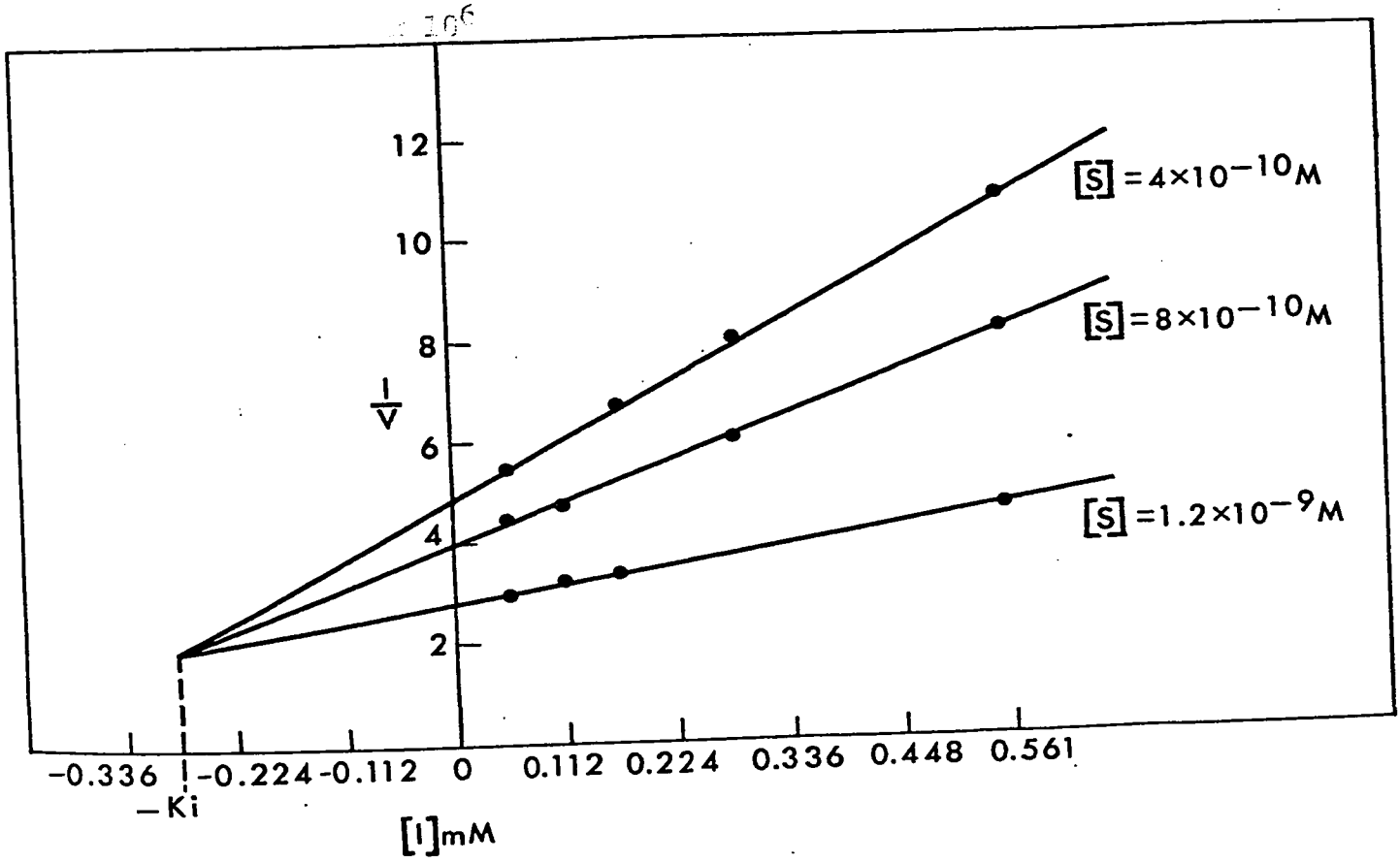
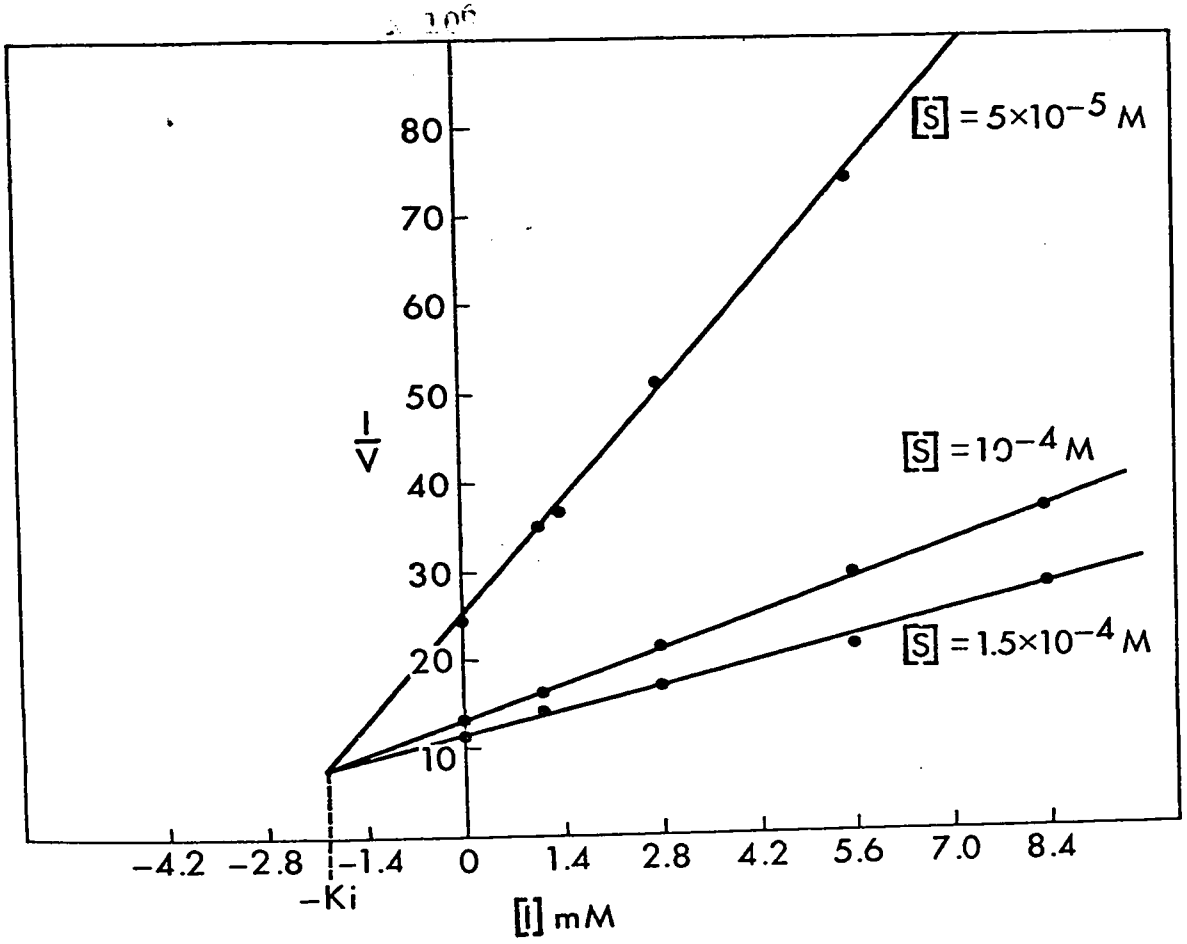


FIGURE 15

Dixon Plot for the Determination of the Ki for Galactonolactone for the β-Glucosidase Activity Toward p-Nitrophenylglucoside



of the Ki for galactonolactone in the presence of p-nitrophenylglucoside. Table 12 gives the Ki values obtained with 17α-estradiol-17- and 3-glucoside and p-nitrophenylglucoside for both these inhibitors. The type of inhibition was competitive and in both cases common Ki values were obtained with all three substrates.

Figures 16 and 17 show the effect of assaying the activity for the 3-glucoside of 17α-estradiol in the presence of 17α-estradiol-17-glucoside and of p-nitrophenylglucoside and of assaying for p-nitrophenylglucoside in the presence of both the 17- and the 3-glucosides of 17α-estradiol respectively. The 17-glucoside acted as an apparent competitive inhibitor of the activity towards the 3-glucoside. Both 17α-estradiol glucosides acted as apparent competitive inhibitors of the activity towards p-nitrophenylglucoside. On the other hand, a mixed type of inhibition was observed when the 3-glucoside of 17α-estradiol was assayed in the presence of p-nitrophenylglucoside. A similar result was found when the 17-glucoside was assayed in the presence of this synthetic substrate.

The stability of the enzyme to heat is shown in Figure 18. The effect of heating at various fixed temperatures between 37° and 55°C caused the same proportionate activation or loss activity towards all three substrates tested.

A concentration of 0.025 M EDTA completely inhibited the glucosidase activity towards both estradiol glucosides while a

TABLE 12

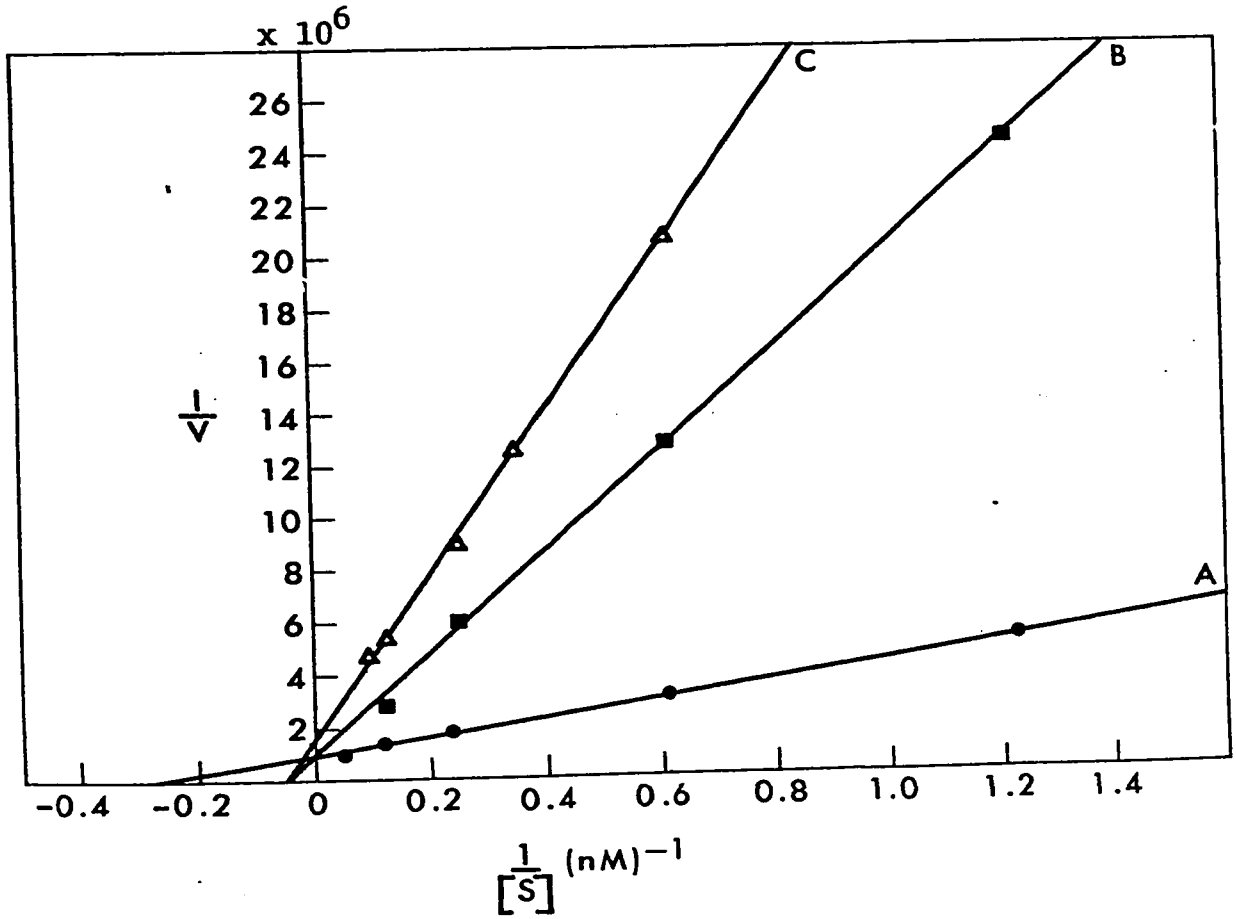
Ki Values of Glucono-1,5-Lactone and of Galactono-1,5-Lactone for the β-Glucosidase Activity Towards Three Substrates

Substrate	Ki Gluconolactone M	Ki Galactonolactone M
17α-estradiol-3-glucoside	2.2×10^{-4}	1.54×10^{-3}
17α-estradiol-17-glucoside	2.8×10^{-4}	2.35×10^{-3}
p-nitrophenylglucoside	2.3×10^{-4}	1.85×10^{-3}

FIGURE 16

Competition Between Substrates

The effect of assaying for the β-glucosidase activity toward 17α-estradiol-3-glucoside in the presence of 17α-estradiol-17-glucoside and in the presence of p-nitrophenylglucoside

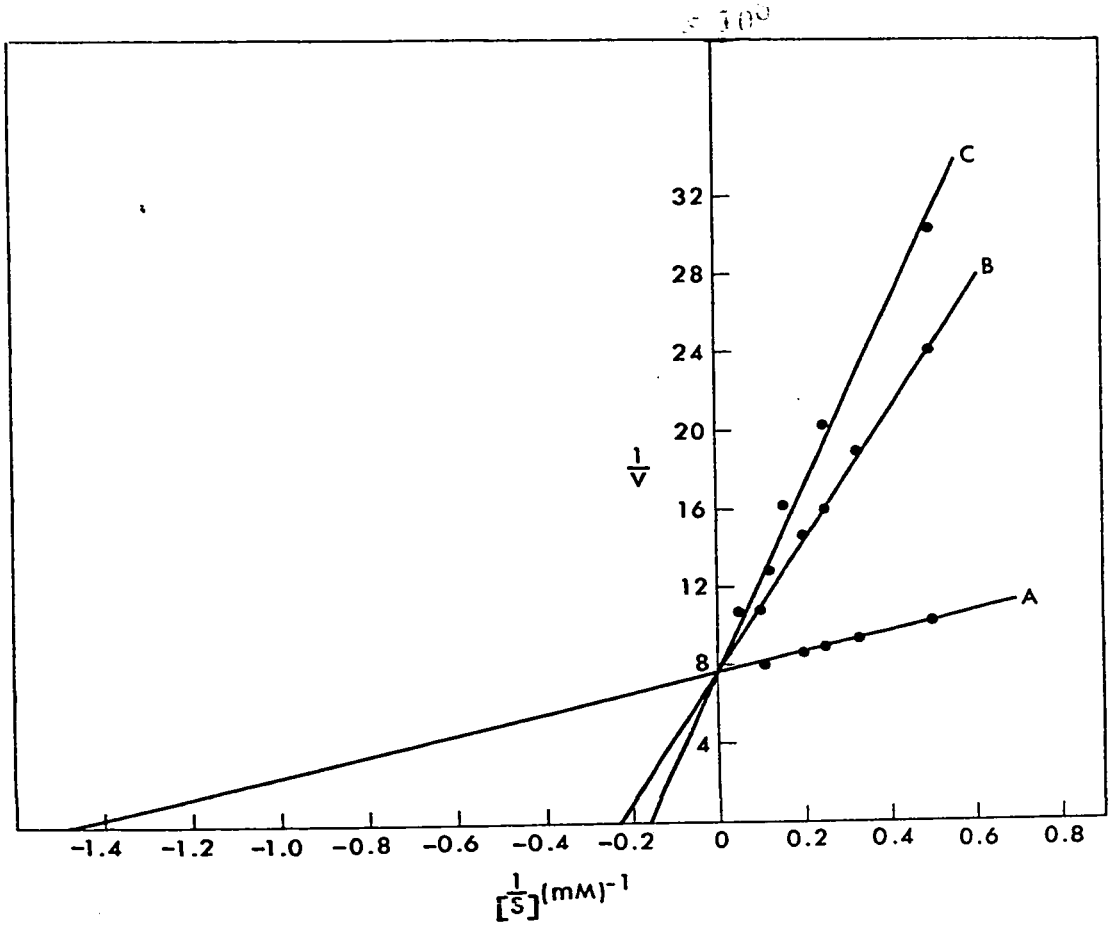


Curve A = Km curve for the 3-glucoside
Curve B = 3-glucoside assayed in the presence of 17-glucoside
Curve C = 3-glucoside assayed in the presence of p-nitrophenylglucoside

FIGURE 17

Competition Between Substrates

The effect of assaying for the β-glucosidase activity toward p-nitrophenylglucoside in the presence of the 17- and the 3-glucosides of 17α-estradiol.



Curve A = Km curve for p-nitrophenylglucoside

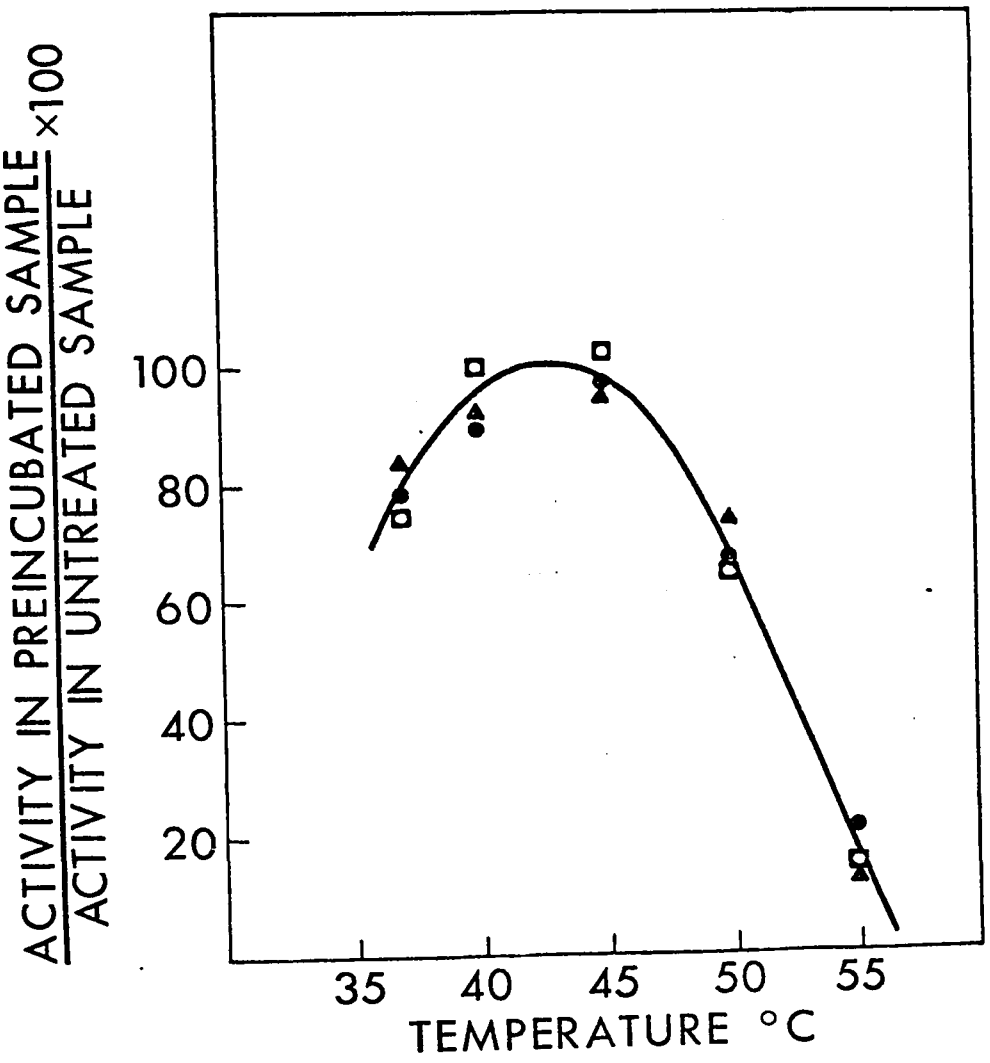
Curve B = p-nitrophenylglucoside assayed in the presence of the 3-glucoside

Curve C = p-nitrophenylglucoside assayed in the presence of the 17-glucoside

FIGURE 18

Heat Stability Studies

The effect of preincubating the enzyme at various temperatures followed by assay of the β-glucosidase activity toward 3 substrates



□ = activity toward 17α-estradiol-3-glucoside
▲ = activity toward 17α-estradiol-17-glucoside
● = activity toward p-nitrophenylglucoside

TABLE 13

Assay of β-Glucosidase in the Presence of EDTA

Activity towards the 17- and the 3-glucosides of 17α-estradiol was assayed with the Sephadex filtrate I. Results are expressed in pmoles of substrate hydrolysed per ml of enzyme per hour.

Concentration of EDTA M	17-glucoside	3-glucoside
	pmoles	pmoles
0	2.35	234
0.01	2.37	214
0.025	0	0
0.05	0	0
0.025+0.05M Ca^{++}	0	0
0.025+0.05M Mg^{++}	0	0
0.025+0.05M Mn^{++}	0	0

concentration of 0.01 M had little effect (Table 13). When metal ions were added to the inactivated glucosidase at the same or double the concentration of EDTA present no activity was restored regardless of whether calcium, magnesium or manganese were used.

D) Discussion

The results in Chapter 3 have shown that several rabbit tissues, especially liver, hydrolyse the phenolic glucosides of estrogens more readily than the aliphatic ones. The kinetic experiments reported in the present chapter show that the Km value for 17α-estradiol-3-glucoside is much lower than that towards any other substrate. The liver enzyme, therefore, has a high affinity for this substrate. It seems likely that the fact that the 3-glucosides of estrogens are absent from rabbit excreta, in spite of their demonstrated formation by rabbit tissues in vitro (Collins, Williamson, and Layne, 1970; Williamson, Polakova, and Layne, 1971) may be explained by their ready hydrolysis by tissue glucosidases. In both rabbit (Williamson and Layne, 1970) and man (Hobkirk et al, 1971) the rapid in vivo hydrolysis of injected estrogen-3-glucosides contrasts with the much less effective hydrolysis of the corresponding glucuronides.

The Km value found for the activity towards 17α-estradiol-17-glucoside, 3×10^{-5} M, is quite similar to that found for pig kidney β-glucosidase using 4-methylumbelliferyl-β-D-glucoside, 3.3×10^{-5} M (Abrahams and Robinson, 1969). The Km value for the activity of the rabbit liver enzyme towards p-nitrophenylglucoside, 7×10^{-4} M, is also reasonably close to that found for the β-glucosidase activity of Saccharomyces cerevisiae, 8×10^{-5} M (Duerksen and Halvorson, 1958) and of

Stachybotryx atra, 5×10^{-5} M (Jermyn, 1955b) towards the same substrate.

Purification of the rabbit liver glucosidase (Chapter 4) has indicated that one protein is responsible for the hydrolysis of both the 3- and the 17-glucosides of 17α-estradiol as well as a number of steroid and synthetic substrates. The fact that the Ki for gluconolactone (Table 12) has the same value when measured with three of these substrates indicates that one active site is responsible for the hydrolysis of these three substrates. It is probable that all the other substrates are hydrolysed at this site as well. The Ki values obtained with galactonolactone (Table 12) also indicate that one site is responsible, both for the hydrolysis of all the glucosides tested and for the galactosides. This is also further evidence that the rabbit liver β-glucosidase is more like an "Emulsin" type of glycosidase. Horikoshi's (1942) definition calls for cross inhibition by the acid lactones of the glycosides that are hydrolyzed by the enzyme. It also states that the enzyme is not inhibited by the free sugars. It is obvious from the double reciprocal plots that significant product inhibition does not occur and even when the enzyme was assayed in the presence of great excesses of either glucose or galactose (at least 10 times the concentration that would be released on 100% hydrolysis) no inhibition was seen.

The competition between substrates (Figures 16 and 17) is further evidence for the conclusion that one site is responsible for the hydrolysis of all the glucosides tested. The two estradiol

glucosides are mutually competitive and both are competitive with p-nitrophenylglucoside. When the activity toward the 17α-estradiol glucosides was measured in the presence of p-nitrophenylglucoside, the inhibition appeared to be mixed rather than competitive. This result is difficult to explain. p-Nitrophenylglucoside is a smaller molecule than any of the steroid substrates and has a reactive nitro group which could react with a functional group in or near the catalytic center of the enzyme, thus causing a non-competitive effect in addition to the competitive one it exerts. Levvy, McAllan, and Marsh (1958) showed that anomalously high Ki values for mammalian β-glucuronidase were obtained with both boiled saccharate and boiled mucate when o-nitrophenylgalacturonide was used as substrate. They have attributed these results to a reaction between the nitro group and the protein. Due to the much higher affinity of the enzyme for 17α-estradiol-3-glucoside, it was not possible to obtain quantitative data on the hydrolysis of the 17-glucoside of this steroid in the presence of the 3-glucoside.

The heat stability studies also endorses the idea that one enzyme is responsible for the hydrolysis of the substrates tested. The effect of heating the enzyme at various temperatures was the same regardless of which substrate was assayed. The enzyme appears to be slightly unstable at 37°C when no substrate is present. Progress curves, as assayed with the homogenate, have shown that the initial velocity was linear for 3 hours (Figure 3). After each step in the purification

of the enzyme the purer preparation was again tested to ensure that the reaction was still linear. Therefore, it would seem that the substrates must stabilize the enzyme so that it is less affected by a temperature of 37°C. The fact that the enzyme is inactivated less at 40 or 45°C may be due to the conversion of the enzyme to a more active configuration at these higher temperatures. The loss of activity at temperatues higher than this was most likely due to denaturation of the protein.

From the studies with EDTA, it was concluded that high concentrations irreversibly inactivate the enzyme. No activity could be restored by the addition of metals. The enzyme itself had no metal ion requirement since dialysis caused no loss of activity.

CHAPTER 6

TRANSGLUCOSYLATION: ABILITY OF RABBIT LIVER β-GLUCOSIDASE TO ACT AS A TRANSOSYLASE AND ITS ACTION ON DISACCHARIDE SUBSTRATES

A) Introduction

The ability of glycosidases to catalyze transosylase reactions has been described previously in section 1H. In many of these cases, disaccharides were the donor substrates in the transglycosylation reactions although synthetic glycosides of p-nitrophenol have also been used. (Hash and King, 1958; Jermyn, 1955). Although the work of Fishman and Green (1957) with β-glucuronidase is the only reported transglycosylation involving mammalian systems, it was possible that the rabbit liver β-glucosidase could be responsible, *in vivo*, for some of the synthesis of the steroid glucosides.

Therefore, the liver β-glucosidase was first tested, with both crude and purified preparations, for its ability to hydrolyse several disaccharide substrates and secondly, to see whether it could act as a transosylase to form glucosides of estradiol. Since almond emulsin was known to hydrolyse these substrates and has been shown to be capable of transferring glucose to primary and secondary alcohols (Courtois and Leclerc, 1956), it was used in these experiments as a control.

B) Methods

1) Incubation Procedure and Detection of Sugars by Paper Chromatography

The incubations were carried out with 1 ml of a solution of almond emulsin (0.05 mg/ml; sp. ac. = 4.6 µmoles of glucose released from Salicin per mg protein per minute) and with 1 ml each of rabbit liver homogenate, high speed supernatant and purified enzyme. They were set up essentially according to the procedure of Abrahams and Robinson (1969). The sugars used were cellobiose, gentiobiose and lactose while deoxycorticosterone glucoside and estrone glucoside were also assayed. The respective incubation tubes contained either 20 mg of disaccharide, 4 mg of estrone glucoside or 5 mg of deoxycorticosterone glucoside. Each substrate was dissolved in 1 ml of enzyme solution, either almond emulsin or one of the rabbit liver preparations, which had been previously dialyzed against 0.015 M citrate-phosphate buffer at pH 5.5. The reactions were carried out in conical centrifuge tubes which were fitted with ground glass stoppers. Each tube was stoppered and incubated for 24 hours in an oven at 37°C. At the end of this time period, 20 µl of each reaction mixture was applied to a Whatman number 1 filter paper and the chromatogram was developed by descending chromatography in a butanol:pyridine:0.1N HCl (5:3:2 by vol.) system for 18 hours (Li and Shetler, 1964). Standards were run at the same time. The sugars were detected by spraying with

2-aminobiphenyl reagent as described by Gordon, Thornburg and Werum (1956). Steroid glucosides and free steroids could be detected under ultraviolet light. Blank incubations, where buffered enzyme solution was replaced by buffer alone were also carried through the procedure.

2) Incubation of Disaccharides and Detection of Glucose by the Glucose Oxidase Method

The disaccharides were incubated overnight with the purified enzyme preparation as described above. The reaction was stopped by placing each tube in a boiling water bath for 2 minutes. The denatured protein was removed by centrifugation and 0.5 ml of the supernatant was assayed for glucose by the glucose oxidase method of Hugget and Nixon (1957) using o-dianisidine as the oxygen acceptor.

3) Attempts to Demonstrate Transglucosylation by Almond Emulsin

A reaction mixture containing 500 µg of radioinert 17β-estradiol, approximately 50,000 dpm of tritiated 17β-estradiol sp. ac. = 5.6 c/mM), 20 mg of cellobiose and 1 mg of powdered almond emulsin in 1 ml of 0.15 M phosphate-citrate buffer, pH 5.5, was incubated at 37°C for 18 hours. The reaction mixture was then extracted several times with 1 ml of benzene to remove the unreacted estradiol, followed by extraction with three 1 ml volumes of ethyl acetate to remove any estradiol glucoside which might have been formed. The combined benzene and the

combined ethyl acetate extracts were separately evaporated under a stream of nitrogen, and each was applied to a thin layer plate of Silica Gel N. The plate was developed in chloroform:ethanol, 4:1 (by vol.). Standards of 17β-estradiol and 17β-estradiol-3-glucoside were run in parallel lanes. The products were detected by spraying with 2.0% sulphuric acid in ethanol followed by heating at 110°C for 15 minutes. The plate was then marked into 1 cm strips and each section was scraped into a counting vial, scintillation fluid was added and the radioactivity in each vial was assayed.

4) Attempts to Demonstrate Transglucosylation with Rabbit Liver β-Glucosidase

A similar experiment to that described above was set up using 1 ml purified rabbit liver enzyme solution which had been previously dialyzed against 0.15 M citrate-phosphate buffer, pH 5.5. The substrate used as the glucose donor was p-nitrophenylglucoside and as the acceptor, 17β-estradiol. The incubation system again consisted of radioinert 17β-estradiol, 50,000 dpm of tritiated estradiol (sp. ac. = 5.6 c/mM) and 4 μmoles of p-nitrophenylglucoside. These were added to 1 ml of the dialyzed enzyme solution and the mixture was incubated for 18 hours and processed as described above for the experiment with almond emulsin.

C) Results

The paper chromatography system consisting of butanol:pyridine:0.1N HCl (5:3:2, by vol.) gave good separation of glucose from the disaccharides used. The liberated steroids, estrone and deoxycorticosterone moved with the solvent front while the intact steroid glucosides trailed closely behind the front. Table 14 gives the Rf values for the various substrates used and their hydrolysis products. Table 15 shows the results of the incubations with the various enzyme preparations. Typical chromatograms, those obtained with the homogenate and the high speed supernatant, are shown in Figures 19 and 20. Almond emulsin hydrolysed both steroid glucosides and disaccharide substrates. The crude rabbit liver preparations hydrolysed lactose but not cellobiose or gentiobiose. The purified β-glucosidase had, however, no lactase activity left. The limit of detection of sugars by the 2-aminobiphenyl spray is 2 µg and thus it can be concluded that there was less than 0.5% hydrolysis of the disaccharide substrates. The blank incubations gave negative results, which indicated that no chemical hydrolysis was taking place in the reaction mixture.

Several difficulties were encountered in the measurement of the released glucose by the glucose oxidase method. Cellobiose gave a highly positive reaction with the glucose oxidase test. This was thought to be due either to glucose being present in the cellobiose or to a glucosidase in the glucose oxidase enzyme solution. The cellobiose was purified by column

TABLE 14

Rf Values for Chromatography of Sugars and Steroid Glucosides on Whatman Number 1 Paper

Chromatograms were developed by descending chromatography with n-butanol:pyridine:0.1N HCl (5:3:2 by vol.)

COMPOUND	Rf
Cellobiose	0.13
Gentiobiose	0.11
Lactose	0.09
Estrone glucoside	0.83
Deoxycorticosterone glucoside	0.87
Glucose	0.29
Galactose	0.24
Estrone	1
Deoxycorticosterone	1

TABLE 15

Hydrolysis of Steroid Glucosides and Disaccharides as Determined by Paper Chromatography

A + indicates hydrolysis was observed and glucose detected on the chromatograms.

Enzyme Preparation	Estrone-Glucoside	Deoxycorti-Costerone-Glucoside	Cellobiose	Gentiobiose	Lactose
Blank	-	-	-	-	-
Almond Emulsin	+	+	+	+	+
Rabbit Liver Homogenate	+	+	-	-	+
High Speed Supernatant	+	+	-	-	+
Purified Enzyme	+	+	-	-	-

FIGURE 19

Separation of Mono- and Disaccharides by Paper Chromatography after Incubation of Cellobiose, Gentiobiose and Lactose with Rabbit Liver Homogenate

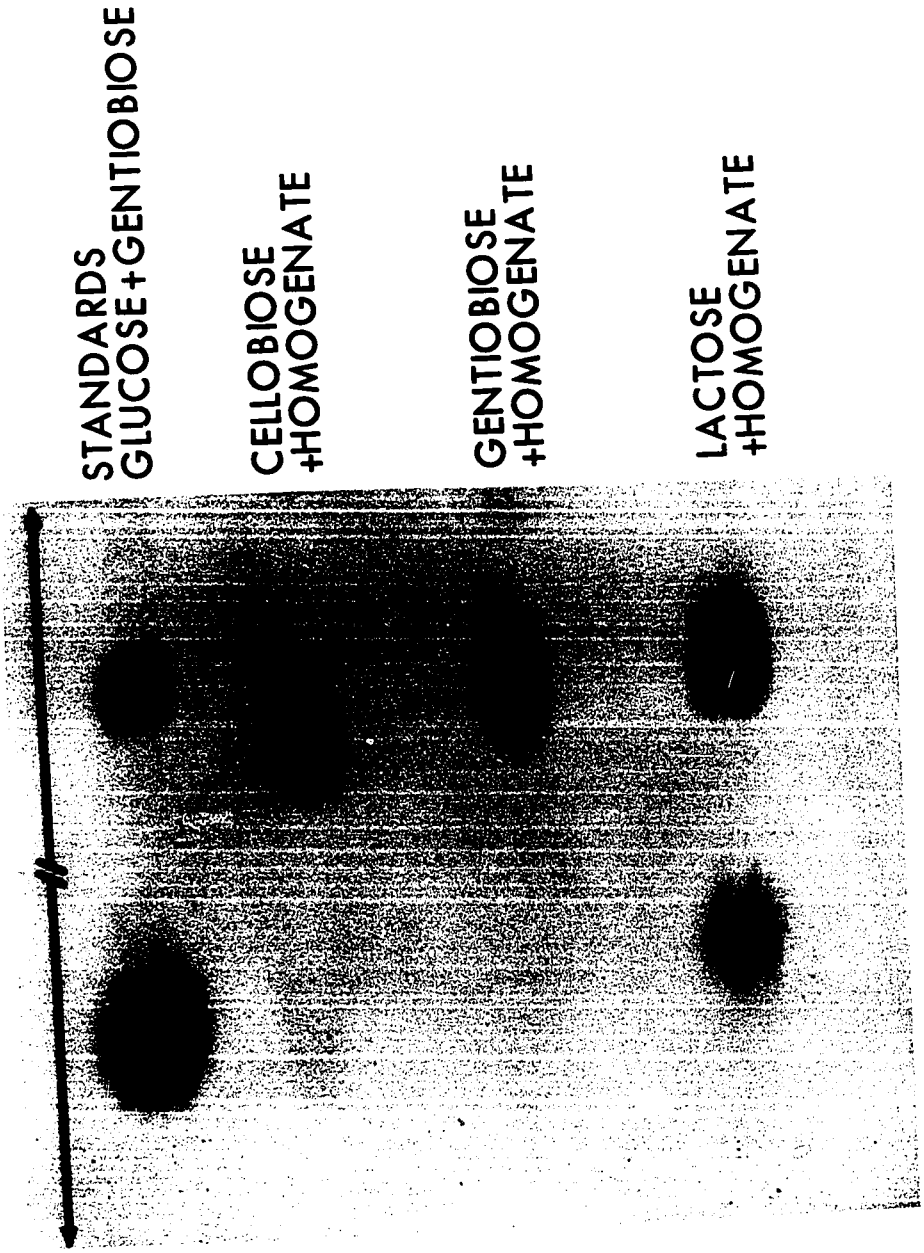
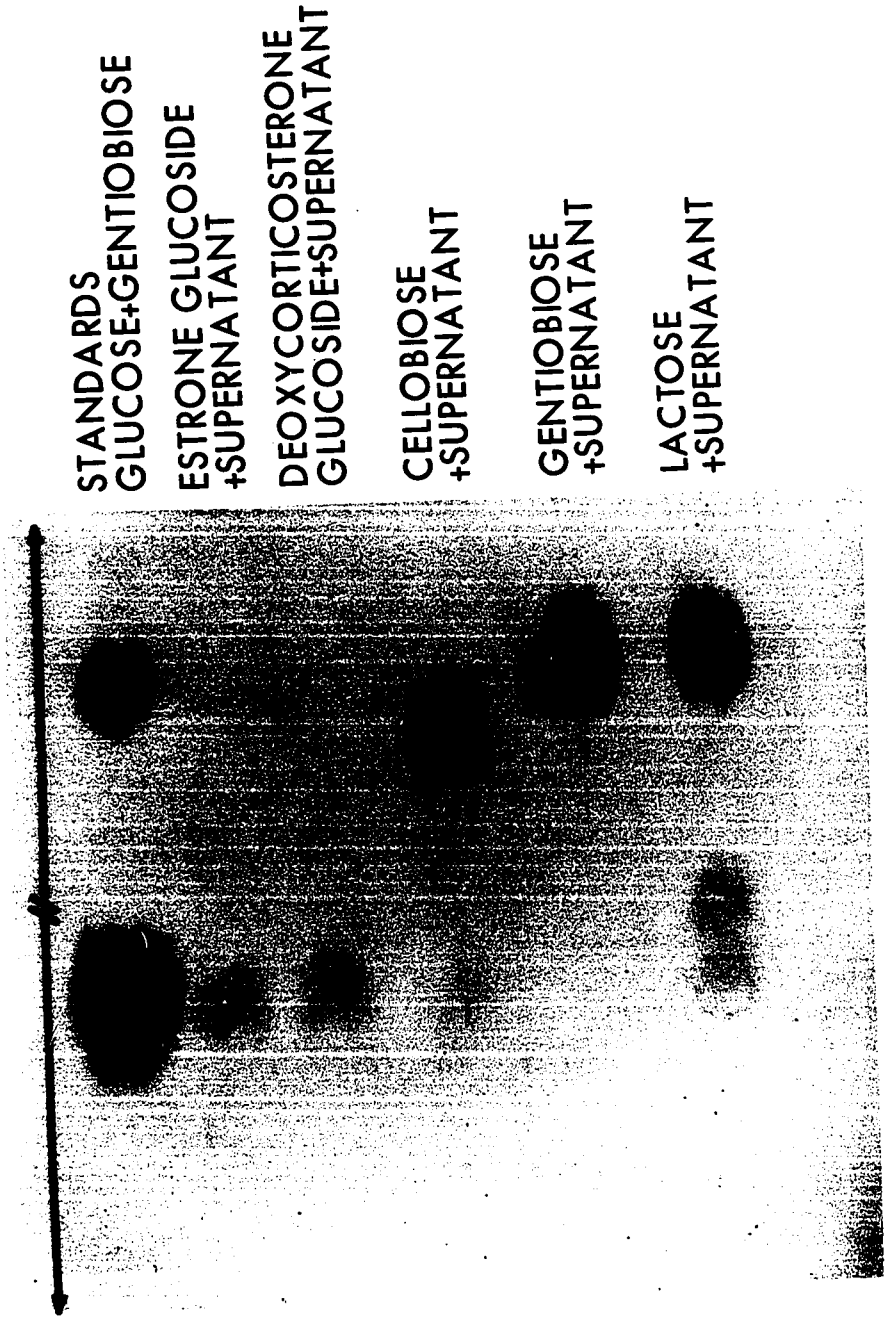


FIGURE 20

Separation of Mono- and Disaccharides and Steroid Glucosides by Paper Chromatography after Incubation of Estrone Glucoside, Deoxycorticosterone Glucoside, Cellobiose, and Lactose with Rabbit Liver High Speed Supernatant



chromatography on Sephadex G-10 but the purified solution still gave a positive reaction. High purity glucose oxidase and peroxidase enzyme solutions were obtained but even with these, there was still a positive reaction. Lactose and gentiobiose gave much smaller blank reactions. (Table 16)

A second problem was encountered in preparing the standard curves for the assay of glucose by the glucose oxidase method. To mimic the conditions of the samples to be tested, standard glucose solutions were diluted 1:1 (vol/vol) with the purified enzyme preparation and the solutions were then heated for 2 minutes in a boiling water bath and centrifuged to remove the denatured protein. The supernatants were then assayed for glucose and were found to contain approximately 1/3 to 1/2 the expected concentration. The amount of glucose removed from the solution with the protein varied from assay to assay (Table 16). Different deproteinization agents were tried but the results were always the same. As a result it was not possible to assay for liberated glucose by this method.

Both attempts to form glucosides by transglucosylation failed. Although almond emulsin readily hydrolysed cellobiose, it did not transfer glucose to estradiol under the conditions used. Both benzene and ethyl acetate extracts showed plainly visible spots which co-chromatographed with 17β-estradiol. No 17β-estradiol glucoside was detected and when the plate was scraped and counted, radioactivity was only found in those sections which corresponded in mobility to reference estradiol.

TABLE 16

Determination of Glucose by Glucose Oxidase Method

Values Obtained with Disaccharide Substrate Blank Incubations and Removal of Glucose from Incubation Mixture during Deproteinization Procedures

10 mg of either cellobiose, gentiobiose or lactose were taken up in 1 ml of water and 3 ml of glucose oxidase reagent - (Hugget and Nixon, 1957) was added. Tubes were incubated for 1 hour at 37°C and then the absorbance at 420 mμ was read.

Two, 50 μg aliquots of standard glucose solution in 0.5 ml water were diluted with 0.5 mls of the purified enzyme preparation and placed in a boiling water bath for 2 minutes. The denatured protein was removed by centrifugation and 0.5 mls of each supernatant (which should have contained 25 μg glucose) were diluted to 1 ml with water and assayed for glucose.

Sample	OD 420	μg Glucose
St. 1	0.045	10
St. 2	0.11	25
St. 3	0.22	50
St. 4	0.45	100
Cellobiose Blank	0.41	93
Gentiobiose Blank	0.08	18
Lactose Blank	0.08	18
Glucose Deproteinized 1	0.06	14
(equivalent to 25 μg) 2	0.042	10

Since rabbit liver glucosidase did not hydrolyse the disaccharide substrates, p-nitrophenylglucoside was used as the donor substrate at a concentration of 4 mM. The yellow colour of the liberated p-nitrophenol was clearly visible at the end of the incubation period. However, as in the case of almond emulsin, no conjugate of estradiol could be detected.

D) Discussion

All hydrolase reactions can be considered to be transferase reactions in which the acceptor molecule is usually water. However, when water is replaced by another acceptor molecule, quite often an alcohol or sugar, the result is the formation of a new glycoside. Frequently the acceptor molecule is also the donor. β-Glucosidase from several fungi will transfer glucose from one cellobiose molecule to another (Crook and Stone, 1957). Most reported cases of transglucosylation reactions have involved nonmammalian systems. In the one reported evidence of transglycosylation by mammalian systems with β-glucuronidase, Fishman and Green (1957) found that very high concentrations of the acceptor molecule (2-3M) were needed.

In the present experiments no transferase activity could be detected with either almond emulsin or the rabbit liver β-glucosidase under the conditions used. Even though cellobiose was a substrate for almond emulsin and p-nitrophenylglucoside for the rabbit enzyme, no transfer of glucose to estradiol from these substrates was observed. This may have been due to the presence of too low a concentration of the acceptor compound in the incubation medium. Courtois and Leclerc (1954) have demonstrated the formation of glucosides of primary and secondary alcohols with almond emulsin using phenylglucoside and p-nitrophenylglucoside as donors. They needed molar

concentrations of acceptor for the reaction to proceed, and, as stated above, Fishman and Green (1957) also found that high concentrations of acceptor were needed. The relative insolubility of estradiol in aqueous solutions precludes higher concentrations than 2 mM of this acceptor being used. It therefore seems unlikely that in vivo the rabbit liver glucosidase is responsible for the synthesis of estrogen glucosides.

The results do show that disaccharides are not natural substrates for the rabbit liver enzyme. Since it has been well established that both the steroid substrates used can be hydrolysed by the rabbit enzyme, their inclusion in this experiment confirms the validity of the method for the detection of the released glucose. Abrahams and Robinson (1969), using pig kidney glucosidase, were also unable to show hydrolysis of disaccharide substrates.

The crude preparations of the rabbit liver enzyme exhibit some lactase activity which is no longer detectable in the purified preparation. The lactase has been separated from the β-glucosidase activity during the purification procedure. In fact this separation took place during the first gel filtration step. When the Sephadex filtrate I was used as the enzyme source for this experiment, it was not possible to detect glucose or galactose on the chromatograms.

Rabbit liver therefore contains an enzyme which will hydrolyse natural steroid glucosides and synthetic p-nitrophenyl-glucoside but will not hydrolyse naturally occurring sugar

glucosides. Doell and Kretchmer (1962) have also found that some glycosidases with activity toward disaccharides were different from those which hydrolysed aryl glucosides. Jermyn (1955) and Hash and King (1958) have purified β-glucosidases from Stachybotryx atra and Myrothecium verrecuria respectively which did not hydrolyse cellobiose or catalyze transfer reactions. Duerksen and Halvorson (1958) found that the β-glucosidase from Saccharomyces cerevisiae did hydrolyse aryl and alkyl glucosides as well as cellobiose but exhibited no transferase activity.

CHAPTER 7

FURTHER STUDIES ON THE CHARACTERISTICS OF β-GLUCOSIDASE IN RABBIT LIVER, INTESTINES AND SPLEEN

A) Introduction

In Chapter 3, the tissue distribution study showed that the β-glucosidase of liver, kidney and small intestine had a high preference for the 3-glucoside of 17α-estradiol as compared to the 17-glucoside. On the other hand, the enzymes of spleen, large intestine and several other tissues did not exhibit this preference for the phenolic glucoside (see Table I). The studies on the liver enzyme and its purification have all indicated that only one enzyme is responsible for the hydrolysis of these two glucosides, so that the difference in the rates of hydrolysis of the glucosides in the two types of tissues was not apparently due to the presence of varying amounts of two enzymes. To investigate this problem further, homogenates of spleen, large intestine and small intestine were subjected to differential centrifugation to compare the intracellular distribution of the β-glucosidase in the two types of tissues.

The small and large intestine were taken as representatives of the two types of tissues and their β-glucosidases were further characterized. Attempts were made to purify the small intestine enzyme according to the same procedure used for the liver enzyme. As the intracellular distribution for the large intestine enzyme was different from that found in liver (see

below), attempts were made first to solubilize it and then to partially characterize it.

The final set of experiments in this chapter were carried out with the liver enzyme. β-Glucosidase, like all acid hydrolases, has been considered to be lysosomal in intracellular location. In section (4C) it was proposed that the high recovery of the β-glucosidase activity in the high speed supernatant might be due to leakage of the enzyme from the lysosomes during the differential centrifugation purification step. Therefore, to establish whether this was the case and the enzyme is, in fact, lysosomal, or whether it is indeed "soluble", rabbit liver lysosomes were carefully prepared and assayed for β-glucosidase activity. Several other acid hydrolases were also assayed for purposes of comparison. These "lysosomal" enzymes were also tested for latency, a characteristic of lysosomal enzymes.

B) Methods

1) Intracellular Distribution

All tissues were homogenized as described in general methods (section 2B4) and the homogenates were subjected to differential centrifugation as described for the liver homogenate in section (4A1). The whole homogenate, mitochondrial and microsomal fractions and the high speed supernatant were assayed for activity towards the 17- and 3-glucosides of 17α-estradiol.

2) Ammonium Sulphate Fractionation of Small Intestine High Speed Supernatant

The high speed supernatant of the homogenate of the small intestine was subjected to fractionation with ammonium sulphate in the same manner as described for the liver high speed supernatant (section 4A3).

3) Gel Filtration of the 45-65% Ammonium Sulphate Fraction of the Small Intestine High Speed Supernatant

The 45-65% ammonium sulphate fraction of the high speed supernatant was applied to a 2.5 by 40 cm. column of Sephadex G-150 and eluted as described for the liver preparation in section (4A4). Protein content of the collected fractions was monitored by measuring the absorbance at 280 mμ. Fractions were assayed for activity towards the 17- and 3-glucosides of 17α-estradiol.

4) Chromatography of the Sephadex Filtrate on Carboxymethylcellulose

Those fractions from the Sephadex column which contained glucosidase activity were concentrated in an Amicon ultrafiltration cell and dialyzed against 0.01 M sodium phosphate buffer, pH 5.5. The dialyzed filtrate was then applied to a column of CM-cellulose equilibrated with the same buffer and the activity was eluted as described for the liver preparation in section (4A5).

5) Solubilization of Large Intestine β-Glucosidase from the Combined Mitochondrial-Microsomal Pellet

The homogenate of the large intestine was centrifuged at 1020 x g for 15 minutes to remove nuclei and cell debris and the resultant supernatant was then centrifuged at 105,000 x g for 1 hour to obtain the combined mitochondrial-microsomal pellet. The pellet was resuspended in 0.25 M sucrose and diluted to the original volume of the homogenate.

Aliquots of 5 ml of the mitochondrial-microsomal suspension were subjected to sonication for 30, 60, 120 and 240 seconds respectively. Additional 5 ml aliquots were brought to concentrations of 0.01, 0.05, 0.1 and 0.2% with Triton X-100. Each aliquot was allowed to stand in ice with occasional stirring for 30 minutes. After this time, all fractions were centrifuged at 105,000 x g for 1 hour. The supernatants were decanted and the pellets were resuspended in 5 ml of 0.25 M sucrose. Both super-

natant and pellet fractions were then assayed for activity towards the 17- and 3-glucosides of 17α-estradiol.

6) Time Study of the Solubilization of the Large Intestine β-Glucosidase by Triton X-100

Following the above procedure, 5 ml aliquots of the mitochondrial-microsomal suspension were brought to a concentration of 0.2% with Triton X-100 and were allowed to stand in ice for 30, 60 and 120 minutes before they were centrifuged at 105,000 x g for 1 hour. The supernatants and pellet were assayed for β-glucosidase activity as described above.

7) Gel Filtration of the Solubilized Large Intestine β-Glucosidase on Sephadex G-150 and G-200

Both types of Sephadex were prepared in 0.01 M sodium phosphate buffer, pH 7.0, as described earlier (section 2B5a) and 1.5 by 40 cm columns were packed. An aliquot (50 ml) of the mitochondrial-microsomal suspension were brought to a concentration of 0.2% with Triton X-100 and after 30 minutes the Triton solubilized enzyme preparation was obtained by centrifugation at 105,000 x g. This Triton supernatant was concentrated to 2 ml in an Amicon ultrafiltration cell and applied to the Sephadex G-150 column. The column was eluted with the phosphate buffer, protein content of the fractions estimated by reading the absorbance at 280 mμ and the glucosidase activity measured in the usual manner. The fractions which contained glucosidase activity were pooled, reconcentrated and applied

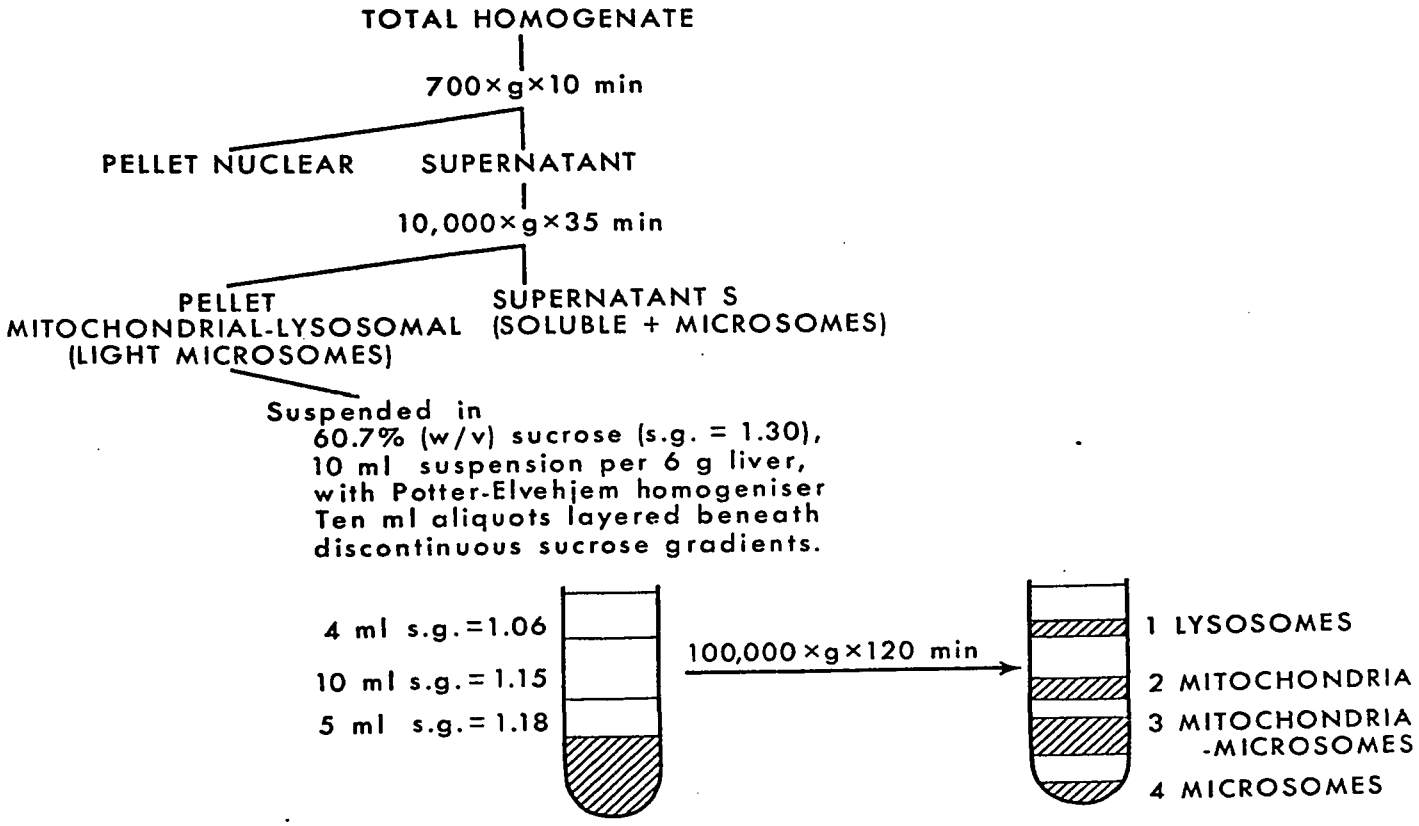
to the G-200 column. The column was eluted in a similar manner and the glucosidase activity was estimated in the collected fractions.

8) Preparation of Rabbit Liver Lysosomes

Lysosomes were prepared from rabbit liver by a modifications of the method of Trouet (1964) devised by Mellors et al (1973). Rabbits were injected intraperitoneally with a sterile solution of 20 ml of 15% Triton WR-1339 in normal saline. The animals were allowed feed for 24 hours and were then starved. They were killed after 48 hours by cervical dislocation. The livers were removed, minced with scissors and homogenized in 8 volumes (vol/vol) of 0.25 M sucrose containing 0.001 M EDTA, pH 7.4 for 20 seconds at top speed in a Waring blender. The homogenate was fractionated according to the scheme represented in Figure 21. Centrifugation was carried out in an International BD-2 centrifuge or in a Beckman Spinco L2-65B ultracentrifuge using rotors SW 27 and Ti 50.1. Four distinct layers were visible at the end of the density gradient centrifugation (Figure 21) and were siphoned off using Pasteur pipettes. These layers and other cell fractions were assayed for β-glycerophosphatase activity by the method of de Duve et al (1955). Assays were also carried out by the methods described in section(2B2) for the following glycosidases: β-glucosidase activity towards the 17- and the 3-glucosides of 17α-estradiol and p-nitrophenyl-

FIGURE 21

Flow Diagram of the Method for Obtaining Rabbit Liver Lysosomes



glucoside, β-glucuronidase activity towards 17α-estradiol-3-glucuronide and p-nitrophenylglucuronide, and β-N-acetylglucosaminidase activity towards 17α-estradiol-17-N-acetylglucosaminide and p-nitrophenyl-N-acetylglucosaminide.

9) The Latency of Rabbit Liver Lysosomal Enzymes as Determined by Various Substrates.

The latency of rabbit liver lysosomal enzymes was determined in vitro as follows:

A rabbit was killed by cervical dislocation and its liver was perfused in situ with ice-cold 0.9% saline. The liver was removed, minced finely with scissors and suspended in 0.25 M sucrose-EDTA (pH 7.4) at a concentration of 10 g liver per 40 ml sucrose-EDTA. The liver was quickly homogenized at 4°C using a motor-driven Teflon Potter-Elvehjem pestle, and at first a loose fitting boiling tube, and secondly a tight fitting glass homogenizer tube (clearance 0.006 in). The homogenate was centrifuged at 700 x g for 10 minutes to remove nuclei and debris as a pellet and the supernatant was centrifuged at 15,000 x g for 20 minutes to yield a lysosomal-mitochondrial pellet which was suspended in 0.25 M sucrose-EDTA and was used for latency assays.

Ten replicate 2 ml aliquots of this suspension were incubated at 37°C for 10 minutes to allow limited release of lysosomal enzymes from the particulate

suspension. Ten replicate controls, containing 0.1% Triton X-100 to facilitate lysis of the lysosomes, were incubated with the test samples. All samples were then centrifuged at 15,000 x g for 10 minutes to sediment cell particles and the resultant supernatants were assayed for β-glycerophosphatase, β-glucosidase, β-glucuronidase and β-N-acetylglucosaminidase, using the same substrates described in the previous section. The activity of the first series was designated as the free enzyme activity and that of the Triton control series as the total enzyme activity. % latency was determined by the formula:

$$\% \text{ Latency} = \frac{\text{Free enzyme activity}}{\text{Total enzyme activity}} \times 100$$

C) Results

Table 17 shows the intracellular distribution of β-glucosidase activity in the small and large intestines and the spleen. With the small intestine, all the activity was recovered in the high speed supernatant. This is one of the tissues which exhibited a high 3/17 ratio of activity towards the 17- and 3-glucosides of 17α-estradiol (Table 1), and like the liver it appears to have a soluble β-glucosidase. Kidney tissue homogenates gave similar results on centrifugation. The large intestine and spleen both have a low 3/17 ratio of activity towards the 17α-estradiol glucosides and in both of these tissues most of the glucosidase activity was distributed between the mitochondrial and microsomal fractions. Only 20% of the large intestine and 25-35% of the spleen activity were found in the high speed supernatant of these tissues. The distribution of the β-glucosidase activity of these tissues is quite different from that of the small intestine and liver.

The small intestine high speed supernatant was fractionated with ammonium sulphate and the results are shown in Table 18. Like the liver preparation, the β-glucosidase of the small intestine was precipitated in the 45-65% fraction. When this fraction was run through a gel filtration column, the small intestine enzyme had a similar elution volume to that of the liver glucosidase. (Figure 22 - see also Figure 5) A liver preparation run through the same column also showed a peak of β-glucosidase activity at fraction 31. The column used for the

TABLE 17

Intracellular Distribution of β-Glucosidase in Several Tissues of the Rabbit

Results are expressed as pmoles of substrate hydrolysed per ml of fraction per hour. Substrates used were 17α-estradiol-17-glucoside and 17α-estradiol-3-glucoside

Fraction	Small Intestine		Large Intestine		Spleen	
	17-glucoside	3-glucoside	17-glucoside	3-glucoside	17-glucoside	3-glucoside
Homogenate	1.37	181	0.41	1.15	0.53	0.75
Mitochondria	0.27	1.1	0.27	0.67	0.34	0.50
Microsomes	0.44	1.2	0.14	0.33	0.17	0.38
Supernatant	2.11	261	0.09	0.23	0.12	0.29

TABLE 18

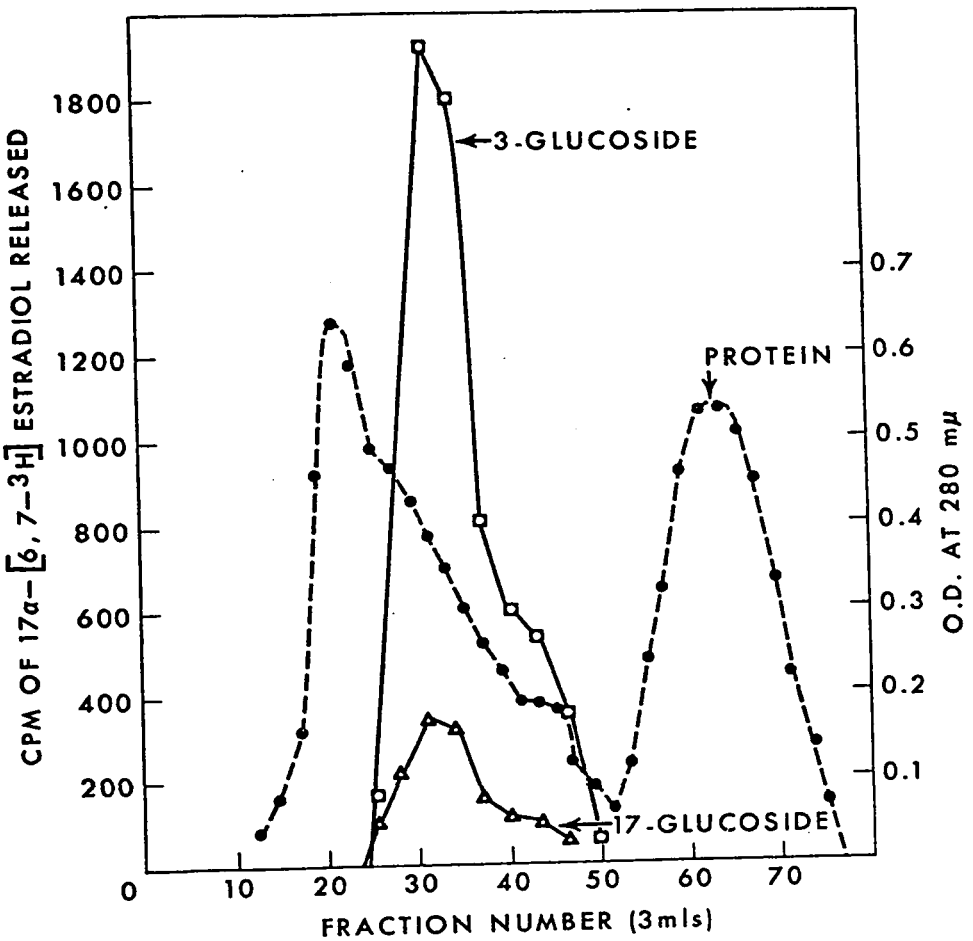
Ammonium Sulphate Fractionation of Small Intestine β-Glucosidase

Results are expressed as pmoles of substrate hydrolysed per ml of fraction per hour. Substrates used were 17α-estradiol-17-glucoside and 17α-estradiol-3-glucoside.

Fraction	Substrate Hydrolysed	
	17-glucoside	3-glucoside
High speed supernatant	2.11	261
0-45% $(NH_4)_2SO_4$	0.20	3.52
45-65% $(NH_4)_2SO_4$	2.80	290

FIGURE 22

Elution Profile of Small Intestine 45-65% Ammonium Sulphate Fraction on a 2.5 x 45 Column of Sephadex G-150



● = Protein
□ = Activity toward 17α-estradiol-3-glucoside
Δ = Activity toward 17α-estradiol-17-glucoside

experiment reported in Figure 5 was slightly longer and the elution volume of the glucosidase obtained with that column was therefore slightly higher. Chromatography of the Sephadex filtrate of the material from small intestine on CM-cellulose also gave similar results to those obtained with the liver enzyme. The activity was not adsorbed to the cellulose and was eluted with the starting buffer.

Attempts to solubilize the β-glucosidase of the large intestine from the combined mitochondrial-microsomal fraction by sonication failed. No activity was released even after 5 minutes of this treatment. The effect of Triton X-100 is shown in Table 19. When the suspension was brought to a concentration of 0.2% in Triton X-100, 30% of the activity toward the 3-glucoside and 50% of the activity toward the 17-glucoside was solubilized. However the amount of solubilization varied greatly from one experiment to another and from one intestinal preparation to the next. Higher concentrations of Triton X-100 did not serve to better the percent solubilization but did inhibit the glucosidase activity towards both substrates.

When the 0.2% Triton mitochondrial-microsomal suspension was allowed to stand for longer periods of time before centrifuging, the percent solubilization was not increased. Table 20 gives the percent solubilization, after 30 minutes, 1 hour, and 2 hours, of the β-glucosidase activity towards both estradiol glucosides.

TABLE 19

Solubilization of Large Intestine β-Glucosidase by Triton X-100

5 ml aliquots of the mitochondrial-microsomal suspension were brought to varying concentrations of Triton X-100. After 30 minutes fractions were centrifuged at 105,000 x g and supernatants and pellets were assayed for activity towards the 17- and 3-glucosides of 17α-estradiol. % solubilization was calculated by the equation:

$$\frac{\text{activity in the supernatant}}{\text{activity of mitochondrial-microsomal suspension}} \times 100$$

Units of activity of the mitochondrial-microsomal suspension were 1.4 pmoles of 17-glucoside and 3.3 pmoles of 3-glucoside hydrolysed per 5 ml of suspension per hour.

% Triton X-100	Supernatant		Pellet		% Solubilization	
	17-glucoside pmoles	3-glucoside pmoles	17-glucoside pmoles	3-glucoside pmoles	17-glucoside	3-glucoside
0	0.24	0.27	1.00	3.17	17	8
0.01	0.26	0.23	0.76	3.09	18	7
0.05	0.35	0.60	0.55	2.23	25	18
0.1	0.47	0.63	0.56	1.89	33	19
0.2	0.74	1.01	0.40	1.93	53	31
0.5	0.31	0.35	0.36	0.39	22	11

TABLE 20

Effect of Time on the Solubilization of Large Intestine β-Glucosidase by Triton X-100

Results are expressed as the % solubilization of β-glucosidase as measured toward both glucosides of 17α-estradiol.

Substrate	30 Minutes	1 Hour	2 Hours
17-Glucoside	70 %	70 %	67 %
3-Glucoside	77 %	75 %	78 %

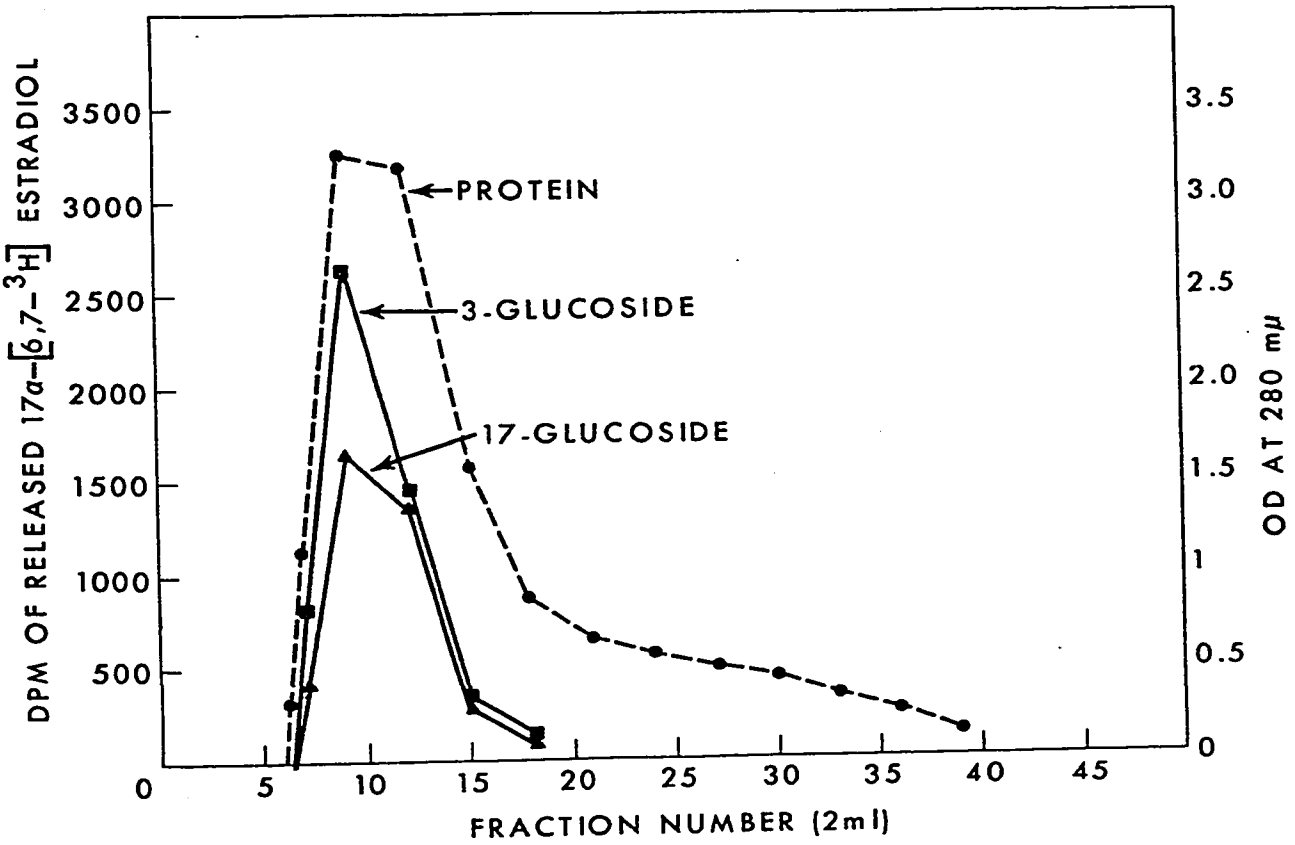
When the Triton supernatant of the large intestine was chromatographed on a Sephadex G-150 column, the activity towards both estradiol glucosides was eluted with the main protein peak in the void volume of the column (Figure 23). When the pooled glucosidase fractions from this column were run on a Sephadex G-200 column, the enzyme activity was again eluted in the void volume.

The results on the subcellular distribution and latency of the β-glucosidase in the rabbit liver, as compared to that of the other glycosidases, is of great interest. β-glycerophosphatase has been firmly established to be a lysosomal enzyme (de Duve et al, 1955) and was therefore used in these studies as a marker enzyme to show which fractions were rich in lysosomes. Table 21 shows that the isolated lysosomes also contained β-glucuronidase and β-N-acetylglucosaminidase which were purified several fold as compared to the specific activities found in the homogenate. Similar purifications were obtained whether the activity of these enzymes was measured towards estradiol glycosides or the equivalent p-nitrophenyl substrates. In contrast, the specific activities of the β-glucosidase towards neither the p-nitrophenyl nor the estradiol glucoside substrates were increased in the isolated lysosomes. This enzyme showed high specific activities in the post lysosomal supernatant (microsomal and soluble fraction).

Table 22 shows that β-glucosidase in rabbit liver does not display latency in the manner of the typical lysosomal enzymes.

FIGURE 23

Elution Profile of the Triton Supernatant Obtained by Treatment of the Combined Mitochondrial-Microsomal Pellet of the Large Intestine with Triton X-100 on a Sephadex G-150 Column



● = Protein
■ = Activity toward 17α-estradiol-3-glucoside
▲ = Activity toward 17α-estradiol-17-glucoside

TABLE 21

The Intracellular Distribution of Steroid Glycosidases and Other Acid Hydrolases in Rabbit Liver

Substrate	Units	Specific activities units per hr. per mg protein									
		Homogenate		Nuclear		Mitochondrial + Lysosomal		Lysosomes		Microsomal + soluble	
β-glycerophosphate	mmoles	0.39	(1.0)*	0.14	(0.4)	0.17	(0.5)	3.01	(7.7)	0.34	(0.9)
					5.2†		5.5		3.6		43.7
17α-Estradiol-3-β-glucuronide	pmoles	2.9	(1.0)	3.1	(1.1)	4.2	(1.4)	13.4	(4.6)	1.9	(0.6)
					15.1		17.2		2.1		32.6
p-Nitrophenyl-β-glucuronide	μmoles	0.26	(1.0)	0.33	(1.3)	0.15	(0.6)	0.87	(3.3)	0.08	(0.3)
					17.9		7.1		1.5		15.3
17α-Estradiol-17-β-N-acetylglucosaminide	pmoles	0.014	(1.0)	0.008	(0.6)	0.020	(1.4)	0.052	(3.7)	0.012	(0.9)
					8.1		17.5		1.7		42.7
p-Nitrophenyl-β-N-acetylglucosaminide	nmoles	3.0	(1.0)	2.5	(0.8)	4.3	(1.4)	51	(16.9)	2.9	(1.0)
					11.8		17.5		7.8		48.1
17α-Estradiol-17-β-glucoside	pmoles	0.061	(1.0)	0.032	(0.5)	0.033	(0.5)	0.058	(1.0)	0.060	(1.0)
					7.4		6.6		0.4		48.9
17α-Estradiol-3-β-glucoside	pmoles	4.9	(1.0)	3.0	(0.6)	1.3	(0.3)	0.4	(0.1)	5.7	(1.2)
					8.7		3.2		0.04		57.9
p-Nitrophenyl-β-glucoside	μmoles	0.52	(1.0)	0.24	(0.5)	0.18	(0.3)	0	(0)	0.52	(1.0)
					6.5		4.2		0		49.8

* Figures in parentheses are purification factors of enzyme compared to the homogenate

† % recoveries compared to the homogenate

TABLE 22

Latency of Glycosidases in Crude Mitochondrial-Lysosomal Pellet from Homogenate of Rabbit Liver

Procedure as described in text. Results show the mean ± standard error of the mean for ten determinations.

Substrate	$\frac{\text{Free enzyme activity}}{\text{Total enzyme activity}} \times 100$
β-glycerophosphate	16.2 ± 3.1
17α-estradiol-3-β-glucuronide	8.3 ± 1.0
17α-estradiol-17-β-N-acetylglucosamine	16.7 ± 1.6
17α-estradiol-17-β-glucoside	116.1 ± 8.4
17α-estradiol-3β-glucoside	92.0 ± 4.1

Treatment of the mitochondrial-lysosomal pellet with 0.1% Triton X-100 did not release more β-glucosidase in the soluble fraction, whereas the lysosomal enzymes, β-glycerophosphatase, β-glucuronidase, and β-N-acetyl-glucosaminidase all exhibited latency as judged by this technique.

D) Discussion

The results show that the intracellular distribution of β-glucosidase in the two types of tissues described in Chapter 3 is markedly different. In those tissues in which the ratio of steroid 3- to 17-glucosidase is high, the enzyme appears to be located in the cytosol, whereas in the tissues with a low ratio, the enzyme is distributed between the mitochondrial and microsomal fractions. This result indicates that the activity in the latter tissues is probably lysosomal, although the activity in the large intestine did not exhibit latency.

The enzyme activity in the small intestine behaved very similarly to that of the liver enzyme in that it was precipitated at the same concentration of ammonium sulphate and co-chromatographed on Sephadex G-150 and CM-cellulose columns. In contrast, the enzyme from large intestine had to be solubilized by the use of the detergent Triton X-100. The "solubilized" enzyme appeared to be contained in a particle of much larger molecular weight than were the "soluble" enzymes. The liver enzyme had a molecular weight of 50-60,000, while it is apparent that the large intestine enzyme must be larger than 800,000 in molecular weight, since this is the exclusion limit of Sephadex G-200, from which the enzyme was eluted in the void volume. It is possible that on solubilization, particles of membrane still remain associated with the enzyme, and thus the apparent molecular weight is much larger than expected. The fact that the

intracellular distribution of β-glucosidase is different for the two types of tissues and that the large intestine enzyme behaves so differently during gel filtration chromatography, indicates that the two types of tissues contain enzymes that are different in their intracellular location, and possibly also might account for the different specificity of the enzymes of these two types of tissues.

Wattiaux, Wibo and Baudhuin (1963) observed that an intraperitoneal injection of the non-ionic detergent Triton WR-1339 was taken up by rat liver lysosomes. This uptake caused a marked decrease in the density of the organelles which enabled their separation from other cell particles by isopycnic density gradient centrifugation. In the method used here, the lysosomes (band 1, Figure 21) floated free of most of the mitochondria, although there are still some lighter mitochondria associated with the fraction (Mellors et al, 1973).

Dance et al (1969) have shown that the β-glucosidase of human kidney was located in the soluble fraction while β-N-acetylglucosaminidase and β-galactosidase were lysosomal in location. The latter two enzymes exhibited the characteristic latency of lysosomal enzymes, but this was not the case with the β-glucosidase. Abrahams and Robinson (1969) found that the same pattern was exhibited by pig kidney glycosidases, in that the β-glucosidase of this organ was soluble and did not exhibit latency. The results of the present work show

clearly that the β-glucosidase of rabbit liver is not associated with the lysosomes, nor does it exhibit the latency characteristic of lysosomal acid hydrolases. The behaviour of β-glucosidase in both these respects is in marked contrast to that of β-glucuronidase and β-N-acetylglucosaminidase. The highest specific activity for β-glucosidase was obtained in the soluble-microsomal fraction. As it has previously been shown that no appreciable glucosidase was associated with rabbit liver microsomes, these results support the conclusion that this enzyme is localized in the unsedimentable fraction of the cell.

The β-N-acetylglucosaminidase activity towards p-nitrophenyl-N-acetylglucosaminide appears to have an anomalously high purification factor. This could be caused by the presence of an endogenous inhibitor in the cruder fractions of high protein content. Such an effect has been described by Weissmann *et al* (1967).

CHAPTER 8 GENERAL DISCUSSION

The results of the work described in this thesis indicate that the glucosidase of rabbit tissues can effectively hydrolyse glucosides of the steroid estrogens, and in the liver, the glucoside of the phenolic 3-hydroxyl of 17α-estradiol is a very good substrate indeed for the tissue β-glucosidase, as judged from the Km and Vmax values shown in Table 11. These steroid glucosides represent the first naturally occurring substrates to be discovered for the mammalian hetero-glucosidases, which, together with other hetero-glycosidases, may justifiably be considered to have been, for many years, enzymes in search of a substrate.

The early results reported in Chapter 3 showed that homogenates of liver, kidney and small intestine had a much greater ability to hydrolyse the 3- than and 17-glucoside of 17α-estradiol, in contrast to the situation in the other tissues (Table 1) where the activity towards these two substrates was lower and more nearly equal. This suggested that two separate enzymes might exist, with specificities for the phenolic and the alcoholic steroid glycosides respectively. However, the results obtained on purification of the liver β-glucosidase activity provide compelling evidence that, at least in this tissue, the steroid 3- and 17-glucosidase activities are contained in a single protein, as shown by the fact that the ratio of the two activities for the pure preparation is very similar to that of

the homogenate (Table 6) and by the presence of both activities in a preparation which yielded a single major band on analytical electrophoretic gels.

The kinetic studies provide further evidence that, in the liver, one enzyme and one active center is responsible for the hydrolysis of at least 3 substrates and most likely all of those tested. Gluconolactone, a specific inhibitor of β-glucosidase, inhibited the activity towards both glucosides of 17α-estradiol and toward p-nitrophenylglucoside to the same extent. A common Ki of 2.3×10^{-4} was obtained with this inhibitor. The double substrate reactions (Figures 15 and 16) also point to the fact that one active center is responsible for the hydrolysis of both the glucosides of 17α-estradiol as well as of p-nitrophenylglucoside. Because of this, further investigationsof the β-glucosidase of other rabbit tissues were carried out to seek the reason for the variation among tissues in affinity for the 3- and the 17-glucosides of 17α-estradiol.

The liver β-glucosidase appeared, in differential centrifugation studies, to be located in the unsedimentable fraction of the cell (Table 2). It is interesting, therefore, that the β-glucosidase of the small intestine and kidney are also located in this fraction while the enzyme of the large intestine and spleen appears to be distributed between the mitochondrial and microsomal fractions of the cell, with only a small portion appearing in the high speed supernatant (Table 17). This

difference in intracellular location could account for the difference in specificity between the two types of tissues but also raises the possibility that the enzymes of the spleen and large intestine are completely different from those of the liver, kidney and small intestine, not only in intracellular location but also in structure and properties. Certainly the small intestine enzyme behaved very similarly to that of the liver during purification. It was precipitated by the same concentration of ammonium sulphate and co-chromatographed on Sephadex G-150 and on carboxymethylcellulose. The large intestine enzyme on the other hand, once solubilized by Triton X-100, appeared to be a much larger molecule than either the liver or small intestinal enzymes in that it was eluted in the void volume of a Sephadex G-200 column (exclusion limit 800,000). Further studies must be carried out to determine the characteristics of the large intestine enzyme before more definite conclusions as to its difference from the enzymes of the liver and small intestine can be drawn.

The acid hydrolases have been traditionally associated with the lysosomes, and the behaviour of the β-glucosidase activity of the spleen and the large intestine, which is distributed between the mitochondrial and microsomal fractions on differential centrifugation, is typical of lysosomal enzymes. The presence of the liver enzyme in the "soluble" fraction is therefore of great interest. Work in this laboratory has shown (Williamson, Polakova and Layne, 1971) that estrogen

glucosides can be formed by rabbit liver homogenates *in vitro*, but are not present in rabbit excreta. The glucosyl transferases which form these glucosides are located in the membrane of the cell. It is tempting to suggest that the steroid glucoside may be formed at the cell membrane for transport into the cell, and may then be hydrolysed in the cytosol by the very active steroid glucosidase, in order that the free steroid may either be attached to the nuclear chromatin to exert its hormonal effects or may be metabolized by microsomal enzymes. The transport of glycosides through membranes is well documented (Segal *et al*, 1973).

The β-glucosidase of rabbit liver exhibits the characteristics of an "Emulsin" type of glycosidase. Its properties closely fit Horikoshi's definition (see section 1F) of an "Emulsin" enzyme. The enzyme hydrolyses both glucosides and galactosides. It is inhibited by both gluconolactone and galactonolactone. It is not inhibited by glucose or galactose. The liver enzyme does not act as a transosylase. It was inactive towards dissaccharide substrates and did not transfer glucose from p-nitrophenylglucoside to 17β-estradiol.

The work which has been described suggests that further study of the characteristics of the β-glucosidase of spleen and large intestine would be of importance. However, it seems probable that the main point of interest, namely the investigation of the significance of the *in vivo* formation and breakdown of steroid glucosides could best be initially pursued by

a model membrane system, together with a comparison of the rates of uptake and localization of the free steroids and of their glucosides by various cell preparations. If for instance, whole cells of liver could be shown to readily take up estrogen glucosides from solution, but that this uptake led to the localization of the corresponding free steroid in the nuclear fraction of the cell, support would be provided for the idea advanced as to the significance of the soluble, or cytosol located, steroid β-glucosidase.

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