THE MECHANISM OF STEROID GLYCOSIDE FORMATION

by

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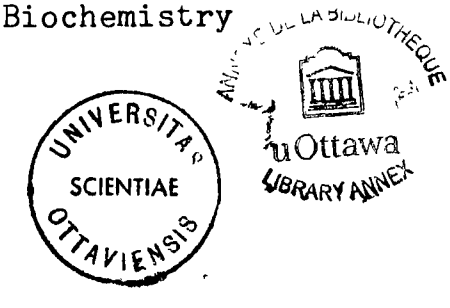

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SUMMARY

Previous work has shown that rabbit liver microsomes transfer glucose from UDP-glucose to the α-hydroxyl group of estrone, 17α-estradiol or 17β-estradiol. These estrogen-3-glucosides are also formed when rabbit liver microsomes are incubated in the absence of exogenous UDP-glucose. These results suggested the existence of two mechanisms of steroid glucosylation and prompted a detailed investigation of the two possible pathways.

17α-Estradiol was used as the substrate in the present study.

The rabbit liver microsomes, when depleted of all endogenous nucleotide sugar donors by repeated washing of the microsomal preparation, retained the ability to effect the formation of small but significant amounts of 17α-estradiol-3-glucoside and 3-galactoside but were unable to form steriod-17-glucosides or N-acetylglucosaminides. The enzymes UDP-glucose dehydrogenase and UDP-glucose pyrophosphorylase inhibited the synthesis of 17α-estradiol-3-glucoside in microsomes incubated in the presence of UDP-glucose, but had no effect on 3-glucoside synthesis in microsomal preparations unfortified with UDP-glucose. These results confirmed that the synthesis of 17α-estradiol-3-glucoside could take place by two different pathways in rabbit liver. One of these, the "UDP-glucose dependent" pathway, involved

the direct transfer of glucose from UDP-glucose to the steroid while the other, the "UDP-glucose independent" pathway did not require the direct involvement of UDP-glucose. The indirect involvement of UDP-glucose was also ruled out since the rabbit liver homogenates and microsomes, when incubated with UDP-glucose, were unable to synthesize any intermediate capable of acting as a sugar donor in the "UDP-glucose independent" pathway. Further evidence against an indirect role for UDP-glucose in the "UDP-glucose independent" pathway was provided by the fact that the microsomes, when incubated with UTP or with UTP and glucose-1-phosphate, were unable to synthesize UDP-glucose. Glucose, glucose-1-phosphate, glucose-6-phosphate and glycogen also proved unable to act as sugar donors in the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside.

Both the "UDP-glucose independent" and the "UDP-glucose dependent" 3-glucosyltransferase activities showed a pH optimum of 7.0, a temperature optimum of 37°C and allowed optimum product formation when incubated for 30 minutes with 5 milligrams of microsomal protein per 3 millilitre assay. The two transferase activities had similar specificities towards seventeen different substrates and their Km towards 17α-estradiol was identical. They both showed the same sensitivity to inhibition by a number of different compounds and lost activity on storage at -4°C at approximately the same rate .

However, several differences were observed in the properties of the "UDP-glucose dependent" and "UDP-glucose independent" transferase systems. Treatment with divalent metal ions, while causing almost complete inhibition of the "UDP-glucose dependent" 3-glucosyltransferase, stimulated the synthesis of 17∝-estradiol-3-glucoside by the "UDP-glucose independent" pathway. The "UDP-glucose independent" 3-glucosyltransferase was more heat labile and more sensitive to inhibition by detergents than was the "UDP-glucose dependent" 3-glucosyltransferase activity. The "UDP-glucose dependent" activity was partially solubilized by treatment with Triton X-100 whereas treatment with snake venom or with phospholipase C and D caused some solubilization of the "UDP-glucose independent" activity. Sonication caused a slight increase in glucoside formation by the "UDP-glucose independent" pathway, but innibited synthesis by the "UDP-glucose dependent" pathway. These results provided further evidence for the existence of two distinct mechanisms of steroid glucosylation.

Crude lipid extracts of rabbit liver microsomes and of pig liver were able to partially restore the "UDP-glucose independent" systhesis of 17∝-estradiol-3-glucoside in microsomal preparations where this pathway had been inhibited due to the presence of Triton X-100. Attempts at purification of these organic extracts by DEAE-cellulose and thin layer chromatography abolished their restorative capa-

city in the Triton X-100 treated microsomal preparations. Other lipids were also examined for their ability to participate in the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside. These studies showed that the restoration of synthetic activity in the Triton X-100 treated microsomal preparations was not due to a non specific lipid effect since synthetic or soyabean lecithin were ineffective at restoring glucoside synthesis when examined under the same conditions used for the studies of the crude organic extracts.

Isoprenoids have been known to act as intermediates in many sugar transfer reactions, and the possible involvement of some pure isoprenoid and related compounds in the synthesis of 17α-estradiol-3-glucoside was therefore examined. Farnesol, geranylgeraniol and vitamin E were unable to stimulate 17α-estradiol-3-glucoside synthesis. However dolichol and retinol did cause an increase in the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside in both the untreated microsomes and in those microsomal preparations where 17α-estradiol-3-glucoside synthesis had decreased due to treatment with Triton X-100. These compounds did not influence the synthesis of 17α-estradiol-3-glucoside by the "UDP-glucose dependent" pathway. Of the compounds examined, dolichol was best able to stimulate the "UDP-glucose independent"synthesis of 17α-estradiol-3-glucoside.

The studies confirmed the involvement of a water insoluble compound in the "UDP-glucose independent" pathway leading to 17α-estradiol-3-glucoside synthesis. This lipid intermediate was unstable but present in an "active" form in the crude organic extracts from either rabbit liver microsomes or pig liver and it appeared to be a compound of similar properties to dolichol.

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GLOSSARY

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

Trivial Name	Compound
Biochanin A	5,7-dihydroxy-4'-methoxyisoflavone
DEAE - cellulose	Diethylaminoethylcellulose
Daidzein	4',7-dihydroxyisoflavone
Diethylstilbestrol	3,4-Bis-(p-hydroxyphenyl)-3-hexene-ethyl
Epitestosterone	17α-Hydroxyandrost-4-en-3-one
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-3-glucuronide	17α-Hydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronic acid
17α-Estradiol-3-glucoside	17α-Hydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranoside
17α-Estradiol-3-xyloside	17α-Hydroxyestra-1,3,5(10)-trien-3-yl-β-D-xylopyranoside
17α-Estradiol-17-galactoside	3-Hydroxyestra-1,3,5(10)-trien-17α-yl-β-D-galactopyranoside

17α-Estradiol-17-glucoside	3-Hydroxyestra-1,3,5(10)-trien-17α-yl-β-D-glucopyranoside
7α-Estradiol-17-N-acetyl-glucosaminide	3-Hydroxyestra-1,3,5(10)-trien-17α-yl-2'-acetamide-2'-deoxy-β-D-glucopyranoside
17α-Estradiol-3-glucuronide-17-N-acetylglucosaminide	Estra-1,3,5(10)-trien-3,17α-diyl-3-β-D-glucopyranosiduronic acid-17-2'-acetamide-2'-deoxy-β-D-glucopyranoside
17α-Estradiol-3-glucuronide-17-N-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
Estrone	3-Hydroxyestra-1,3,5(10)-trien-17-one
Estrone glucoside	17-Oxoestra-1,3,5(10)-trienyl-β-D-glucopyranoside
Estriol	Estra-1,3,5(10)-trien-3,16α,17β-triol
EDTA	Ethylenediaminetetraacetic acid
Formononetin	7-hydroxy-4'-methoxyisoflavone
Genisteim	4',5,7-trihydroxy-isoflavone
17α-Hydroxypregnenolone	3β,17α-Dihydroxy-pregn-5-ene-20-one
KCl	Potassium Chloride
NAD	Nicotinamide adenine dinucleotide
p-Nitrophenylglucoside	p-Nitrophenyl-β-D-glucopyranoside
Retinol	2 methyl-3-phytyl-1,4-naphthoquinone

Testosterone	17β-Hydroxyandrost-4-en-3-one
Tetrahydrocortisol	3α,11β,17α,21-Tetrahydroxy-5β-pregnan-20-one
veen 20	Polyoxyethylene sorbitan monolaureate
Tris	2-amino-2(hydroxymethyl)-1,3-propanediol
Vitamin E (D-α-tocopherol)	2,5,7,8-Tetramethyl-2(4',8',12'-trimethyl-tridecyl)-6-chromanol
UDP-galactose	Uridine-5'-diphosphogalactopyranoside
UDP-glucose	Uridine-5'-diphosphoglucopyranoside
UDP-xylose	Uridine-5'-diphosphoxylopyranoside
ATP	Adenosine-5'-triphosphate
ADP	Adenosine-5'-diphosphate
AMP	Adenosine-5'-monophosphoric acid
UTP	Uridine-5'-triphosphate
UDP	Uridine-5'-diphosphate
UMP	Uridine-5'-monophosphoric acid
GTP	Guanosine-5'-triphosphate
GDP	Guanosine-5'-diphosphate
GMP	Guanosine-5'-monophosphoric acid
CTP	Cytidine-5'-triphosphate
CDP	Cytidine-5'-diphosphate
TTP	Deoxythymidine-5'-triphosphate
TDP	Deoxythymidine-5'-diphosphate

TMP	Deoxythymidine-5'-monophosphoric acid

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

Trivial Name	Enzyme (Enzyme Number)
Almond emulsin (βglucosidase)	β-D-Glucosidase glucohydrolase (EC. 3.2.1.21)
Chymotrypsin	Peptide peptidohydrolase (EC. 3.4.4.5)
α-Glucosidase	α-D-Glucosidase glucohydrolase (EC. 3.2.1.20)
Ketodase (β-glucuronidase)	β-D-Glucuronide glucuronohydrolase (EC. 3.2.1.31)
Lysozyme	(EC. 3.2.1.17)
Neuraminidase	Mucopolysaccharide-N-acetylneuraminylhydrolase (EC. 3.2.1.18)
Papain	Peptide peptidohydrolase (EC. 3.4.4.10)
Phosphodiesterase	Orthophosphoric diester phosphohydrolase (EC. 3.1.4.1)
Phospholipase C	Lecithinase C; Phosphatidylcholine cholinephosphohydrolase (EC. 3.1.4.3)
Phospholipase D	Lecithinase D; Phosphatidyl choline phosphatidohydrolase (EC. 3.1.4.4)
17β-Estradiol dehydrogenase	Estradiol: NAD 17-β-oxidoreductase (EC. 1.1.1.i)
Trypsin	Peptide peptidohydrolase (EC. 3.4.4.4)

UDP-glucose dehydrogenase	UDP-glucose: NAD oxido reductase (EC. 1.1.1.22)
UDP-glucose pyrophosphorylase	UTP:α-D-glucose-1-phosphate uridyltransferase (EC. 2.7.7.9)
UDP-galactose-4-epimerase	(EC. 5.1.3.2)

CHAPTER I INTRODUCTION

A) General Introduction

Conjugation, a process which involves the coupling of two molecules by means of a chemical bond, is generally regarded as a detoxication mechanism. Much of the information available in the past concerning this process was derived from the study of the fate of foreign organic compounds in the body (1). The main conjugation reactions which occur in laboratory animals involve glucuronic acid, sulphate, glycine, cysteine, methyl groups and acetyl groups.

Steroids were the first compounds of endogenous origin shown to undergo conjugation and the isolation of glucuronide, and later, sulphate conjugates of these physiologically important compounds, suggested that conjugation might have metabolic implications in animals wider than those of detoxication (2-4). A number of glycosides of hormonal steroids, other than glucuronides, have now been isolated from animal tissues and excreta, and work on these has contributed to the study of the *in vivo* metabolism of conjugates. The formation and metabolism of steroid glycosides has been most extensively studied in the case of the estrogens, which are eighteen carbon steroids with an aromatic ring A and a phenolic hydroxyl group at position 3.

Estrogen metabolism in the rabbit is relatively simple. Estrone or 17β-estradiol injected into the animal is conver-

ted to 17α-estradiol, and is excreted almost entirely as a double conjugate containing glucuronic acid at the 3-position and N-acetylglucosamine at the 17-position of 17α-estradiol (5). Much smaller amounts of another double conjugate are also found in the urine of rabbits injected with estrone or 17β-estradiol. This conjugate differs from that described above only in that it contains glucose in place of N-acetyl-glucosamine at the 17-position. These novel metabolic conjugates represent the first example of the attachment of a sugar, other than glucuronic acid, to a non sugar receptor in animal tissues (5-7). Rabbit tissues are also capable of effecting the in vitro formation of mono-conjugates of estrone, 17β-estradiol and 17α-estradiol which contain either glucose or galactose at the 3-position of the steroid. The absence of these 3-monoconjugates from urine and bile, and their ready hydrolysis by tissue glycosidases suggests that conjugation in this instance may have some function other than leading to excretion (8-10). This function may be the production of compounds which are not mere end products of metabolism but active intermediates in estrogen metabolism. In the absence of any added nucleotide sugar donors, rabbit liver microsomal preparations are still capable of synthesizing significant amounts of the 3-monoglucosides and 3-monogalactosides of estrone and of both the estradiol epimers, but are unable to effect the formation of any of the double conjugates containing glucu-

ronic acid, N-acetylglucosamine or glucose (11, 12). Therefore 3-monoglycoside synthesis may take place by two different mechanisms in rabbit liver microsomes.

This thesis is concerned with the further investigation of both the "nucleotide independent" and "nucleotide dependent" synthesis of the estrogen 3-monoglycosides in rabbit liver. The specific aims were:

(i) to establish whether or not rabbit liver microsomes could synthesize steroid glucosides in the absence of exogenously added UDP-glucose,

(ii) to establish whether UDP-glucose was present in or synthesized by the microsomes, and then was a donor for the transfer of glucose to the steroids,

(iii) to investigate the specificity of the UDP-glucose independent reaction and its characteristics and requirements for co-factors,

(iv) to test the hypothesis that glucose was transferred to a lipid soluble intermediate, in microsomes, and thence to the steroid, and

(v) to obtain evidence on the nature of such an intermediate.

Estrogen metabolism in mammalian systems, especially estrogen glucoside formation, as well as the role of lipid intermediates in several well documented sugar transfer reactions will be discussed in some detail below. This review will help to provide the background of this area of

lipid research, and will illustrate the reasons for undertaking the study of 3-monoglycoside formation, and should also provide the rationale for the approach taken in this investigation.

B) Estrogen Metabolism in Mammalian Systems

Knowledge of estrogen metabolism has advanced considerably in the last fifteen years. Glucuronidation, sulphatation, and suspected phosphorylation had been the conjugation reactions associated with estrogens and were thought to inactivate these hormones and to facilitate their excretion (13). However, a large number of novel estrogen conjugates have now been isolated from many different animal tissues and excreta. Not all of these conjugates have been found in the urine or bile, which suggests that they might have a role as metabolic intermediates. In fact, the site of conjugation or the nature of the conjugate formed has seemed in many instances to influence the further metabolism of the steroid, either by directing it to reactions which prepare it for rapid excretion or by steps favouring hydrolysis of the conjugate and regeneration of the hormonally active steroid. Attempts at purification of many of the enzymes involved in estrogen metabolism, namely the glycosyltransferases, glycosidases and estrogen dehydrogenases, have been undertaken and the properties of these enzymes shed some light on the mechanisms of estrogen

metabolism.

1) Nature of the Conjugates Found:

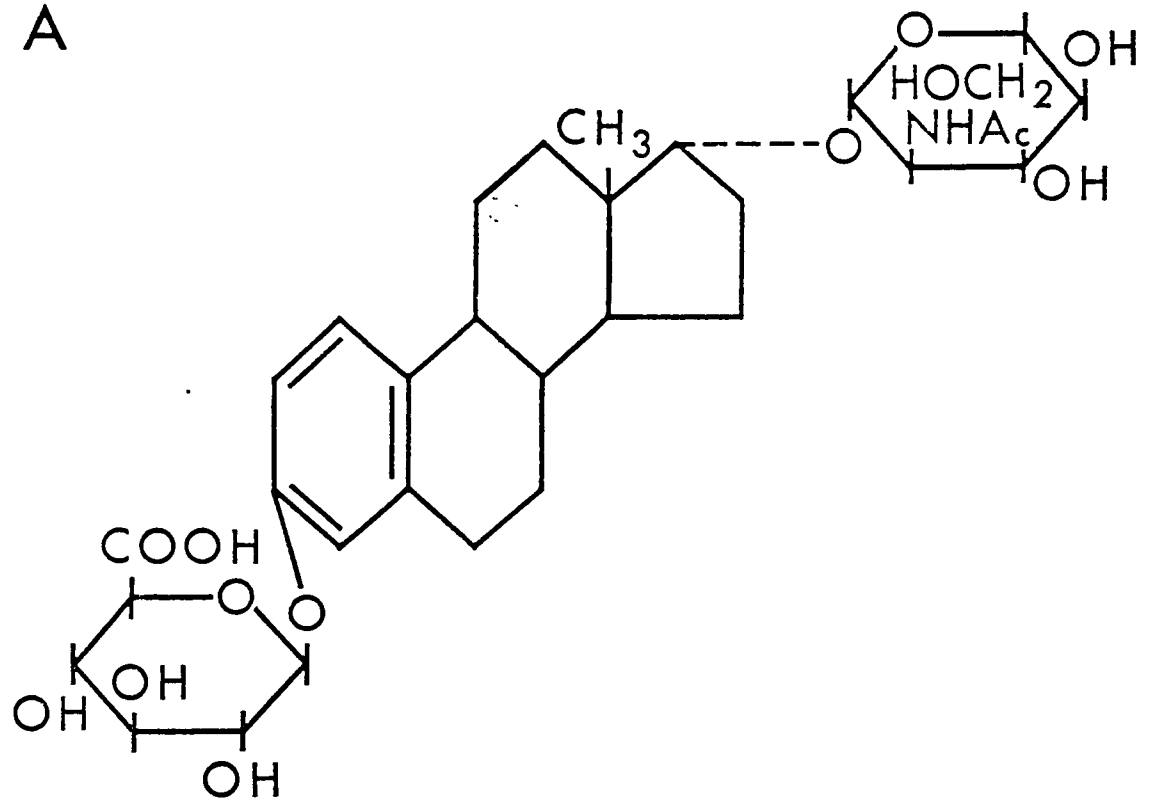
(a) Glucuronides

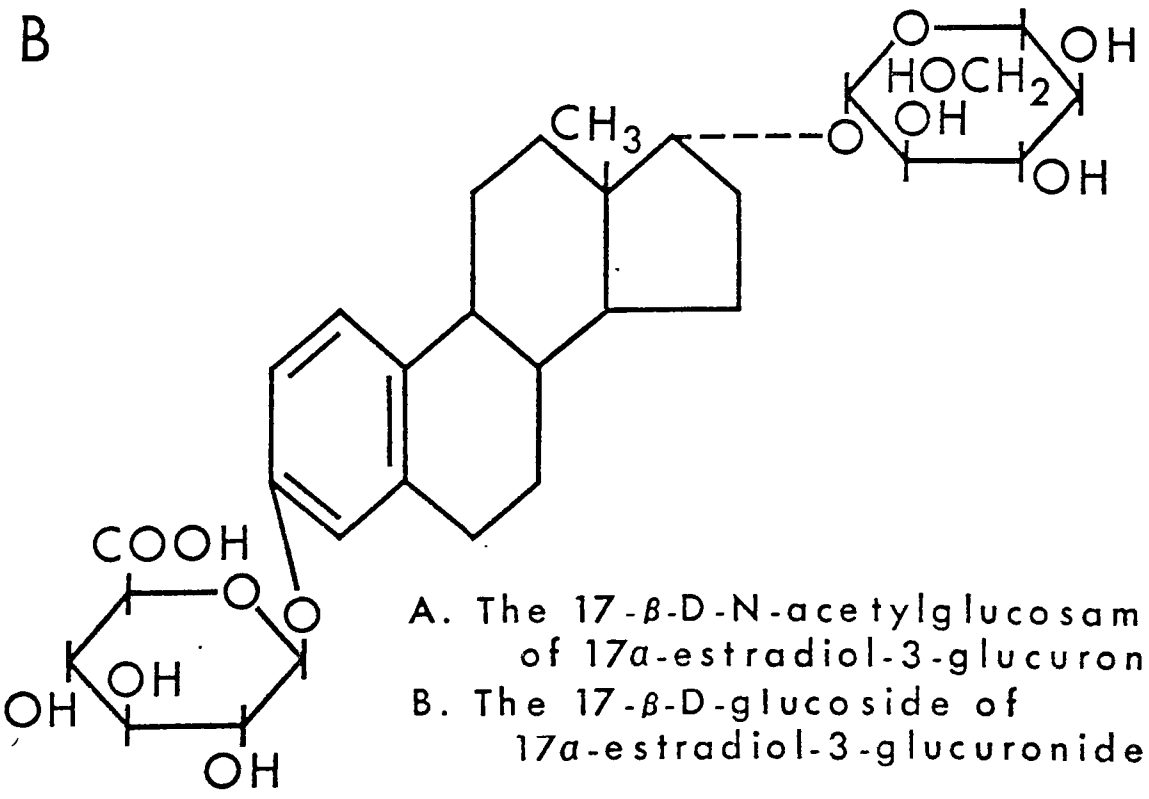
During the last thirty years a large number of estrogen glucuronides have been found in the blood, urine or bile of a variety of animals (14-16). In human pregnancy urine mono- and diglucuronides of estriol have been detected (3, 17, 18). The double conjugates contained either two molecules of glucuronic acid or else one molecule each of glucuronic acid and of sulphate (15). Rabbit urine was also shown to contain double conjugates of 17α-estradiol containing one molecule of glucuronic acid and one molecule of either N-acetylglucosamine or glucose (5-7). In all the above examples of double conjugate formation there appeared to be an ordered sequence for the addition of the "conjugating" acid or sugar molecules. This will be discussed in greater detail in later sections on specificity. The in vitro synthesis of estrogen glucuronide conjugates has also been demonstrated in liver homogenates from rabbit, human, guinea pig and mouse and in kidney homogenates from the human (14, 19-29).

(b) N-Acetylglucosaminides:

Whereas a wide variety of phenolic, alcoholic, carboxylic, amino and mercapto compounds of endogenous or exogenous origin have been shown to form conjugates with glucuronic acid in vertebrates (13), the capacity of the rabbit and human for the excretion of steroids as N-acetylglucosamine conjugates represents a completely novel conjugation reaction. The N-acetylglucosamine conjugate of 17α-estradiol isolated from rabbit urine by Layne, Sheth and Kirdani in 1964 was the first example in animals of a steroid glycoside other than a glucuronide and also the first demonstration of the attachment of N-acetylglucosamine to compounds other than saccharides and nucleotides (30, 5, 6). The conjugate in question was the major excretion product in rabbits injected with estrone, 17β-estradiol or 17α-estradiol. It was shown to be a double glycoside of 17α-estradiol containing glucuronic acid attached to the phenolic hydroxyl group and N-acetylglucosamine attached to the 17α-hydroxyl group. Its structure was defined and both sugars were shown to be attached to the steroid through β glycosidic linkages, (Figure 1). Other N-acetylglucosaminides have been found in human bile and urine, (Table 1) (15). Further study showed that microsomes from a number of rabbit tissues could effect the transfer of N-acetylglucosamine from its uridine nucleotide to the 17α-hydroxyl of the monoglucuronide or monosulphate

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

FIGURE 1

ESTROGEN CONJUGATES WITH N-ACETYLGLUCOSAMINE

Steroid aglycone + other conjugating group	Compound administered	Steroid hydroxyl to which N-acetyl-glucosamine attached	Source of conjugate	Reference
17α-Estradiol-3-glucosiduronate	Estrone or	17α	Rabbit urine	5
	17β-estradiol	17α	Rabbit bile	181
17-Epiestriol-3-glucosiduronate	Estriol	17α	Rabbit urine	182
16,17-Epiestriol-3-glucosiduronate	Estriol	17α	Rabbit urine	182
17α-Estradiol-3-sulfate	Estrone-3-sulfate	17α	Rabbit urine	65
15α-Hydroxyestrone-3-sulfate	Estrone-3-sulfate	15α	Human bile	55
15α-Hydroxyestradiol-3-sulfate	Estrone-3-sulfate	15α	Human bile	55
D-Homoestradiol-17aα-3-glucosiduronate	17α-Ethynyl-estradiol-17β	17aα	Rabbit urine	64

TABLE 1

estrogen conjugates. In the case of the human, adult and fetal kidney, homogenates transferred N-acetylglucosamine to the 15α-hydroxyl of the free steroid as well (15, 16, 30-33).

(c) Glucosides, Galactosides and Xylosides:

Glucoside formation from UDP-glucose is a well known reaction in plants, bacteria, insects and molluscs. In the past it was viewed as a mechanism for the detoxication of exogenous compounds in invertebrates and lower organisms (1, 34-38). Gessner and Vollmer in 1969 reported the glucosylation of p-nitrophenol by mouse liver microsomes and since then the formation of glucosides of some endogenous compounds by other mammalian systems has been reported (39, 40). These findings suggest that formation of glycosides with non-acidic sugars may be a pathway of widespread occurrence and perhaps of considerable significance in mammals (39, 40). The discovery of bilirubin glucoside conjugates in dog and rat bile and also in human post-obstructive bile and of UDP-glucosyltransferase activity in rat liver microsomal preparations, brought into question the concept that the glucuronide was the predominant excretory product of bilirubin. These results, and those of Gessner and Vollmer, suggest that ordinarily glucosylation in vivo is a minor pathway of detoxication,

but that under conditions of UDP-glucuronic acid depletion, UDP-glucose excess, or of saturation of the pathway causing glucuronide formation, glycosylation may gain in importance as an alternate pathway of detoxication (41-46). Van Heyningen in 1971 isolated a glucoside of L-3-hydroxy-kynurenine from the human lens and suggested that changes in the rate of synthesis or degradation of this glycoside may contribute to some of the pathological changes seen in individuals with certain types of cataracts (47). Another example of the possible significance of glycosides in mammalian physiology was the demonstration of retinol glycoside formation on incubation of whole homogenates from rat thyroid with tne nucleotide sugars UDP-glucose, UDP-galactose and UDP-mannose (48). These findings may be of considerable significance in understanding thyroid physiology and with further investigation may help to establish the detailed molecular function of Vitamin A other than its known role in the visual process (48).

Steroid glycoside formation has now been shown in a number of mammalian systems and will be discussed in some detail, since these conjugates are among the most studied of the mammalian glycosides and have made a fundamental contribution to the understanding of the <u>in vivo</u> metabolism of conjugates. As pointed out earlier, microsomal preparations effect the transfer of N-acetylglucosamine from UDP-N-acetylglucosamine to the 17α-hydroxyl group of

17α-estradiol-3-glucuronide (19). In further experiments with rabbit liver microsomes, Williamson and his colleagues found that substitution of UDP-glucose for UDP-N-acetylglucosamine led to the formation of another novel double conjugate with glucose in place of N-acetylglucosamine at position 17 (7). On enzymatic removal of the glucuronic acid at C-3 of the steroid the remaining monoconjugate was shown to be separable from 17α-estradiol-17-N-acetylglucosaminide by thin layer chromatography. The in vitro yield of this double conjugate was low but sufficient amounts were detected in the urine of rabbits injected with estrone or 17β-estradiol to allow for its further characterization by chromatographic and spectroscopic techniques. These studies unequivocally established the structure of this double conjugate. Monoglycoside formation was observed when estrone, 17α-estradiol or 17β-estradiol were incubated with rabbit liver microsomes in the presence of UDP-glucose or UDP-galactose (9, 12) and comparision with authentic compounds established that the glucose was attached as a β glycoside to the 3-position, (Figure 2). This was the first demonstration of the formation of steroid galactosides by an animal tissue. The formation of all these monoconjugates was of particular interest since despite careful searches in both the urine and the bile of rabbits which had been injected with radioactive estrone or 17β-estradiol none of these mono-

3-MONO-GLUCOSIDE OF 17α-ESTRADIOL

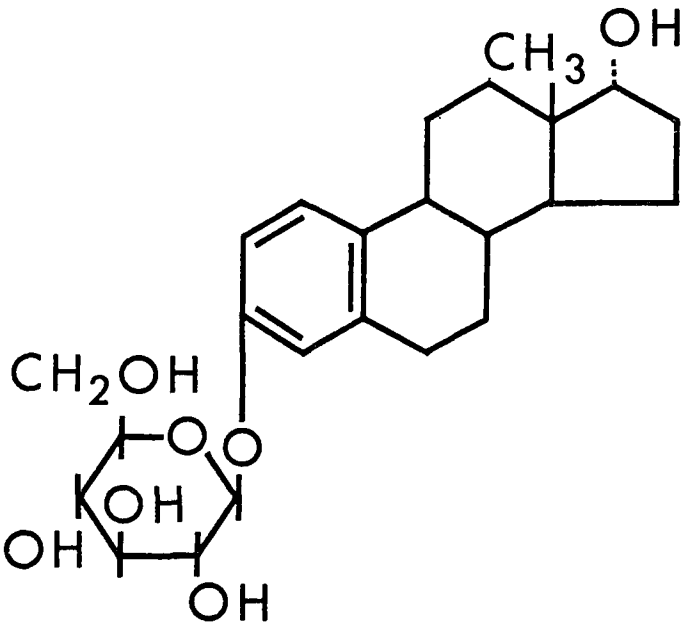


FIGURE 2

STRUCTURE OF DOLICHOL

$$H-[CH_2-C(CH_3)=CH-CH_2]-CH_2-CH(CH_3)-CH_2-CH_2OH$$

16-22

FIGURE 3

conjugates were detected (9, 10, 15). As shown in (Table 2), a number of estrogen glucosides have been formed by the *in vitro* incubation of different mammalian tissue microsomal preparations in the presence of UDP-glucose. In the rabbit, human and chimpanzee galactose can be transferred, but to a lesser degree, when its uridine nucleotide sugar donor is substituted for UDP-glucose (12, 49, 50). Recent studies with rabbit liver homogenates have shown that they can also effect the transfer of xylose from UDP-xylose to the phenolic 3-hydroxyl group of estrone, 17α-estradiol or 17β-estradiol under the same conditions used for the formation of monoglucosides and monogalactosides. The estrone xyloside has been characterized and represents the first record of a steroid pentoside (51). This capacity for steroid monoglycoside formation appears to be of widespread occurrence in mammalian systems, however, the only steroid glucoside, galactoside or xyloside that has so far been identified in excreta is 17α-estradiol-3-glucuronide-17-glucoside which is found in rabbit urine. The predominant conjugate in rabbit urine is, however, the corresponding double conjugate containing N-acetylglucosamine, the ratio of N-acetylglucosaminide to glucoside being about 100:1.

ESTROGEN GLUCOSIDES, GALACTOSIDES AND XYLOSIDES FORMED IN VITRO BY TISSUES FORTIFIED WITH UDP-GLUCOSE UDP-GALACTOSE OR UDP-XYLOSE

Tissue	Steroid	Glucoside Formed	Galactoside Formed	Xyloside Formed	Reference
Rabbit Liver	Estrone	+	+	+	
	17α-Estradiol	+	+	+	
	17β-Estradiol	+	+	+	
	Estriol	–	–	–	
	17α-Estradiol-3-glucuronide	+	–	–	(7,9,12,45)
Human Liver or Kidney	Estrone	–	–	–	
	17α-Estradiol	+	+		
	17β-Estradiol	–	–		
	Estriol	+	–		(43,70)
	17α-Estradiol-3-glucuronide	–	–		
Sheep Liver	Estrone	–	–		
	17α-Estradiol	+	–		
	17β-Estradiol	–	–		(62)
	Estriol	–	–		
	17α-Estradiol-3-glucuronide	+	–		
Mouse Liver	Estrone	–	–		
	17α-Estradiol	+	–		
	17β-Estradiol	+	–		
	Estriol	–	–		(63)
	17α-Estradiol-3-glucuronide	+	–		
Chimpanzee Liver	Estrone	+	not tested		
	17α-Estradiol	+	+		
	17β-Estradiol	–	–		
	Estriol	+	not tested		(44)
	17α-Estradiol-3-glucuronide	+	–		

TABLE 2

(d) Sulphates, Phosphates and other Conjugates:

Many active endocrine tissues have been shown to synthesize estrogen sulphates (52-54). As discussed in section 1(a) the *in vivo* and *in vitro* synthesis of double conjugates of estriol containing both sulphate and glucuronic acid has been reported (55, 56). These studies suggested that the conjugates may play a specific role as metabolic intermediates.

There has been speculation that estrogen phosphates may be formed in some animals *in vivo* but the existence of these conjugates has not been established in animal tissues or excreta (15). Methoxy derivatives of estrogens have been found in man and some other animals, though not in rabbits. It is thought that methylation may have a regulatory role in estrogen metabolism. These and some other relatively minor steroid conjugation reactions have been discussed in a review by Layne in 1970 (15).

2) Specificity of the Estrogen Glycosyl Transferases:

The estrogen glycosyltransferases have a specific requirement for the appropriate uridine nucleotide which acts as the source of the sugar which is transferred to the steroid. Collins and his colleagues in 1970 examined a number of nucleotides containing glucose for their ability to donate their sugar moiety to the 17α-hydroxyl group

of 17α-estradiol-3-glucuronide and found that UDP-glucose could not be replaced by any of those tested (9). In all the estrogen transferase reactions studied the products formed were β-glycosides.

The transferases also show high specificity with regard to their steroid substrates. The steroid glucuronyltransferases have been detected in a number of animal species, but all exhibit a high substrate specificity and show interesting variations from one species to another (16, 57-62). The enzyme involved in the transfer of the neutral sugar, N-acetylglucosamine to steroids in both humans and rabbits shows an even more rigid specificity (33, 55, 63, 64). In the rabbit the enzyme transfers N-acetylglucosamine to the 17α-hydroxyl group of 17α-estradiol-3-glucuronide. There is no evidence for the synthesis of 3 or 17-mono-N-acetylglucosamine conjugates or of double conjugates containing glucuronic acid at position 17 and N-acetylglucosamine at position 3. The enzyme appears to have an absolute requirement for steroids containing a 17-hydroxyl group of α orientation and a phenolic A ring linked at the 3 hydroxyl to an ionizable conjugating group, preferably glucuronic acid. The human N-acetylglucosaminyltransferase shows a definite requirement for the 15α-hydroxyl group of estrogens and glycosylates both the free steroid or its 3-monosulphate at this position (15, 33, 55).

Steroid glucosyltransferase activity has now been found in a number of different mammalian species namely the rabbit, human, sheep, mouse and chimpanzee. In all species studied, these transferases exhibit a strong preference, and in some instances an absolute requirement, for a steroid containing the phenolic A ring characteristic of mammalian steroid estrogens. In vitro studies have shown a glucosyltransferase in rabbit liver, kidney and intestine which will transfer glucose from UDP-glucose to the 17α-hydroxyl group of 17α-estradiol-3-glucuronide to form a double conjugate. This is analogous to the reaction catalyzed by the N-acetylglucosaminyltransferase and this enzyme is known as the 17-glucosyltransferase, being specific for the 17α-hydroxyl group of the steroid. When UDP-galactose is substituted for UDP-glucose in the above in vitro studies, no galactose transfer to the 17α-hydroxyl group of the estrogen glucuronide can be demonstrated. A microsomal preparation from rabbit liver, small or large intestine, but not kidney, is also able to effect the transfer of glucose from UDP-glucose to the 3-hydroxyl group of estrone, of either of the estradiol epimers, p-nitrophenol and diethylstilbestrol to form monoglucosides. Monogalactosides and monoxylosides of estrone and both estradiols are also formed, although to a lesser extent, by rabbit liver microsomal preparations (9, 51). Estriol, however which differs from the phenolic estrogens only by the

presence of a 16α-hydroxyl group, does not form a monoglycoside under similar conditions. Many other phenolic and nonphenolic steroids have been examined for their ability to act as substrates for the rabbit liver glucosyltransferase. Many of these compounds are unable to form glycoside conjugates, but the rabbit liver enzyme transfers glucose from UDP-glucose to some biologically interesting phenols and alcohols (9, 66, 67). Substrate specificity studies suggest the presence of two distinct glucosyltransferases in the rabbit. One of these is responsible for the transfer of glucose to the 17α-hydroxyl position of preformed estrogen monoglucuronides and is similar in many of its properties to the 17-N-acetylglucosaminyltransferase. The other 3-glucosyltransferase is similar in some ways to the 3-glucuronyltransferase and is responsible for 3-monoglucoside formation (68).

Human liver and kidney homogenates can also form estrogen glucosides, and the transferases involved show some interesting differences in their distribution and specificity when compared to those of the rabbit. The human tissue homogenates, while capable of transferring glucose, and to a lesser extent galactose, to the 17α-hydroxyl group of 17α-estradiol and glucose to the 16α-hydroxyl group of estriol, are unable to transfer glucose to the 17-hydroxyl group of 17β-estradiol or to the 3 hydroxyl group of the phenolic estrogens. This suggests that the enzyme has a

high specificity towards the 17α-and the 16α-hydroxyl groups. It differs from the enzyme involved in monoglycoside formation in the rabbit in that the rabbit enzyme is detected only in the liver, is specific for the 3 phenolic hydroxyl group and does not form estriol glucosides or 17-monogalactosides of any of the estrogens. The human glucosyltransferase also differs from the 17 glucosyltransferase found in rabbit liver, kidney and large intestine in that it can not form double conjugates. The presence of glucuronic acid at position 3 completely inhibits the transfer of glucose from UDP-glucose to the 17 position of the steroid. Preliminary studies suggested that human kidney tissue was more active in glycoside formation than the liver which is yet another point of variance with the results obtained with both the 3 and the 17 glucosyltransferases of rabbit tissues.

Studies on glucoside formation in the sheep, mouse and chimpanzee have given more information on species differences and similarities among the steroid glucosyltransferases (50, 68, 69). The sheep liver enzyme resembles both the rabbit and the human glucosyltransferases in some of its properties. Both the rabbit and the sheep liver 17-glucosyltransferases show a high specificity for the 17α-hydroxyl group, their preferred substrate being the monoglucuronide of 17α-estradiol and both enzymes have certain common properties which are quite distinct from

those of the 3-glucuronyltransferase. The sheep liver glucosyltransferase resembles the human enzyme in its capacity to form the 17-glucoside of the free steroid, 17α-estradiol and small amounts of the 16α-glucoside of estriol, as well as its inability to effect 3 monoglycoside formation with any of the phenolic estrogens. However, the sheep enzyme lacks 17 galactosyltransferase activity which is found in the human but not in any of the other species examined. The mouse and the rabbit glucosyltransferases are similar in that both form glucosides with p-nitrophenol, 17α-estradiol, 17β-estradiol, 17α-estradiol-3-glucuronide and diethylstilbestrol but not with estriol, 17α-estradiol-17β-3-glucuronide or with a variety of non-phenolic steroids (69). However, the mouse glucosyltransferase differs from the rabbit liver 3-glucosyltransferase in that it will use 17α-estradiol-3-glucuronide as a substrate and also in that p-nitrophenol is the preferred substrate (39, 40). The lack of N-acetylglucosaminyl or galactosyl transferase activities, as well as their specificity for 17α-estradiol-3-glucuronide as a substrate are some common properties of the mouse and the sheep glycosyltransferases. The chimpanzee liver can effect the transfer of glucose to the 17α-hydroxyl group of both 17α-estradiol and 17α-estradiol-3-glucuronide and to the phenolic hydroxyl group of estrone. It does not form 3 monoglycosides with 17β-estradiol or 17α-estradiol, or monoconjugates with p-nitro-

phenol or diethylstilbestrol. From these facts it would appear that the chimpanzee liver glucosyltransferase differs in specificity from the transferases in all the other species examined, (Table 2). These limited data presently available suggest that the glucosyltransferases vary in their specificity from species to species, but a phenolic Ring A as found in the steroid estrogens is an important requirement.

3) Purification of the Estrogen Glycosyltransferases:

The glycosyltransferase activities which have been widely studied in mammalian tissues appear to be due to a group of enzymes specific as to species and substrate. Apart from recent studies with the natural substrates bilirubin, retinol and the steroid hormones, much of the information concerning these enzymes has been derived from studies with synthetic substrates (9, 13, 50, 51, 67-72). For the most part they appear to be microsomal enzymes and in the rabbit all three sugar transferases, glucuronyl, N-acetylglucosaminyl and glucosyl, are closely associated. Attempts at their separation and purification have met with only limited success.

(a) Glucuronyltransferases:

The rabbit, sheep and human liver glucuronyltransferases have been partially solubilized by sonication, by

treatment with trypsin or with Triton X-100. They appear to be contained in particles of relatively high molecular weight. Lipid reconstitution studies and the effect of treatment with phospholipase C and D and of treatment with snake venom, suggested that the estrogen glucuronyltransferase activity in the rabbit and the human depended on the presence of phospholipid (60, 68, 72). The glucuronyltransferase activity could not be physically separated from that of the N-acetylglucosaminyltransferase in rabbit liver nor from that of the glucosyltransferase activity in sheep liver. However, their different responses to treatment with various enzymes and detergents, as well as to physical treatments, have made it possible to destroy one activity, allowing for the study of the other one in its absence.

(b) N-Acetylglucosaminyltransferases:

As yet there are only two known hetero N-acetylglucosaminyltransferases. The human enzyme has been found in the fetal and adult kidney and in adult liver, but has not been studied in detail. The rabbit N-acetylglucosaminyltransferase has been mainly studied in the liver, since this is the best source of the enzyme, but it is also found in the kidney and intestine. As mentioned earlier it is closely associated with both the glucuronyl and glucosyltransferases of microsomes, although the N-acetylglucosaminyl and the glucuronyltransferases do show different tissue

distributions, substrate specificities and different pH activity curves. They also differ in their sensitivity to sulphydryl inhibitors, solubilizing agents, treatment with snake venom and phospholipases and to temperature. This suggests that the activities may reside in separate proteins, but physical separation, which would confirm this, has not yet been achieved (9, 15, 71, 72).

(c) Glucosyltransferases:

These enzymes in the human, mouse and chimpanzee liver have not been studied in any detail. Initial work with the sheep enzyme showed it to be microsomal, and it appeared to be distinct from the glucuronyltransferase as illustrated by differences in its response to sonication and to treatment with Triton X-100, cetyltrimethylammonium bromide and with trypsin.

The 17-glucosyltransferase, first shown to occur in rabbit liver by Williamson and his colleagues in 1969 (7), transfers glucose from UDP-glucose to the 17α-hydroxyl group of 17α-estradiol-3-glucuronide. Further study showed it to resemble the rabbit N-acetylglucosaminyltransferase in its tissue distribution and intracellular location and in its sensitivity to temperature, various inhibitors, phospholipases, protease, snake venom and the detergent Triton X-100 as well as in its pH activity curve (9, 71, 72).

A physical separation of these two microsomal activities has not been achieved, so that as yet there is no concrete evidence that two separate proteins are responsible for the two enzyme activities.

The rabbit liver 3-glycosyltransferase resembles the 3-glucuronyltransferase in some of its properties and differs from both the 17 N-acetylglucosaminyltransferase and 17-glucosyltransferase in its response to treatment with phospholipases and proteases (72). Its pH optimum is 7.0 as compared to 8.0 for the N-acetylglucosaminyltransferase and the 17-glucosyltransferase. Little work has been done on the purification and properties of the 3-glycosyltransferase, but the reaction seems to require a water insoluble intermediate. This thesis records the investigation of this and of other properties of the 3 glucose transfer reaction in greater detail.

4) Significance of the Estrogen Conjugates:

The widespread occurrence of glucuronic acid conjugation may be attributed to the ease with which UDP-glucuronic acid can be produced in the body from carbohydrate sources and with which the acid can be transferred enzymically to various chemical groups. Conjugation with glucuronic acid, and to a lesser extent with N-acetylglucosamine and glucose, converts a weakly acidic or nonacidic organic compound into

a more polar compound which is much more water soluble and thus more easily excreted than the unconjugated compound. The synthesis of glucuronic acid monoconjugates of the estrogens, as described in earlier sections, may be important in directing estrogen metabolism towards the formation of the elimination products of these compounds. 17α and 17β-Steroid dehydrogenase activities have been found in rabbit liver and when presented with these steroids in their unconjugated form, these enzymes catalyze the interconversion of 17β-estradiol and estrone which are thought to be the two hormonally active estrogens. However, glucuronidation at the 3-position of the estrogen causes a shift towards inactivation by 17α- reduction and directs metabolism towards their excretion as the double conjugate, 17α-estradiol-3-glucuronide-17-N-acetylglucosaminide (73-75). An insufficient number of N-acetylglucosaminide conjugates are known to permit generalization about their role.

The only steroid glucoside conjugate which has been detected in excreta is the double conjugate 17α-estradiol-3-glucuronide-17-glucoside (7). This raises the question as to whether the monoglucosides are indeed formed *in vivo* and, if so, whether they act as intermediates in the metabolism of steroids. Many tissues have been shown to synthesize estrogen monoglycosides (7, 9, 49, 50, 68, 69, 76). Their absence from the urine and bile is not surprising, since they are poorly soluble in water and readily hydrolyzed by

the active β - D -glucosidases shown to exist in the cytosol of a number of rabbit and human tissues (8, 77). Preliminary experiments showed that the estrogen-3-monoglucosides were much more readily hydrolyzed by these glucosidases than were the estrogen-17-glucosides. In vivo results obtained in the human and the rabbit were similar, and showed that 17β-estradiol-3-glucoside was almost completely hydrolyzed, whereas 17β-estradiol-3-glucuronide was excreted largely unchanged (78, 79). This susceptibility of the steroid monoglucosides to hydrolysis by the tissue β-glycosidases suggests a possible role as metabolic intermediates. Plant glucosides have been shown to act as intermediates in the synthesis of coumarin in clover leaf and this role seems worth investigating in the case of the mammalian monoglycosides (80).

A recent experiment by Williamson and Layne suggests that conjugation with glucose may facilitate transport of the steroid across membranes. Passage through the liver cell was found to occur more readily with 17β-estradiol-3-glucoside than with the free steroid (81). It has previously been shown that glucuronic acid itself does not cross membranes easily nor does 17α-estradiol-3-glucuronide (81, 82,). Further study is, however, needed to establish a definite role for the estrogen glucosides in transport. These pieces of evidence on possible roles for the estrogen monoglycosides made their formation in animal tissues of interest for

further investigation.

A further interesting finding with respect to the in vitro synthesis of the 3-monoconjugates was that in the absence of added nucleotide sugar donors, rabbit liver microsomal preparations still retained a significant 3-glucosyl and 3-galactosyltransferase activity while the 17-glucosyl, 17-N-acetylglucosaminyl and 3-glucuronyltransferase activities were greatly decreased (12). These results suggested the existence of two glucosylation mechanisms for 3-monoglucoside formation in rabbit liver. One of these was apparently independent of exogenous nucleotide sugars while the other was dependent on UDP-glucose as its sugar donor. The suggestion that a water insoluble intermediate might play a role in steroid glycosylation merited further investigation since polyprenol lipids have been shown to act as intermediates in sugar transfer reactions in a number of systems. This will be discussed in Section C.

C) Lipid Intermediates in Sugar Transfer Reactions

Most of the di-, oligo- and polysaccharides which occur either free or attached to proteins and lipids are synthesized by transfer of sugars from nucleotide sugars.(83). The involvement of lipid intermediates in many of these sugar transfer reactions is now being investigated in bacterial, yeast, fungal, plant, protozoan, insect and mammalian systems. Mass and nuclear magnetic resonance spectroscopy have been

used to identify these lipids as polyisoprenoid compounds whose terminal hydroxyl group is joined to the sugar by way of a phosphate or pyrophosphate bridge. These lipids, although their overall properties are quite similar, can vary in chain length, number of cis or trans double bonds, degree of saturation, number of phosphate groups attached and in the sugar being carried.

1) Bacterial Systems:

The involvement of lipid intermediates in bacterial glycan biosynthesis has been discussed in a number of review articles (83-91). In the gram positive bacteria Staphylococcus aureus and Micrococcus lysodeikticus, membrane bound undecaprenol phosphate, a polyisoprenoid containing eleven isoprene units, each with a double bond, accepts N-acetyl-O-muramyl pentapeptide phosphate and N-acetyl-D-glucosamine from their cytoplasmic nucleotides. The disaccharide is then transferred from the lipid to form a glycosidic bond with the polysaccharide acceptor and is ready for further reaction leading to peptidoglycan formation (92, 93). A similar pathway involving an undecaprenol phosphate intermediate occurs in the synthesis of the O-antigen portion of lipopolysaccharides in Salmonella typhimurium and Salmonella newington. However in this pathway a polymer of about sixteen to twenty repeating units is built up on the lipid intermediate before transfer to the core lipopolysaccharide

(88, 94, 95). Some strains of Salmonella utilize polyisoprenoid intermediates to modify this O-antigen side chain of the lipopolysaccharide. The glycosyl lipid involved in this pathway differs from the peptidoglycan and O-antigen intermediates in that it contains a phosphodiester rather than a pyrophosphoryl bridge between the lipid and the sugar (88). Teichoic acid biosynthesis in gram positive bacteria also proceeds from nucleotide precursors through an undecaprenol phosphate intermediate to the final wall polymer. The undecaprenol phosphate is actually shared by both the peptidoglycan and teichoic acid biosynthesis which facilitates a certain amount of order in the composition of the wall polymers. Here again, as in the pathway of O-antigen modification in Salmonella, the lipid intermediates are phosphodiesters rather than the pyrophosphates (88, 90, 96). Yet another example of lipid involvement in bacterial polymer biosynthesis is seen in some strains of Aerobacter where an undecaprenolpyrophosphate intermediate has been shown to participate in the biosynthesis of capsular polysaccharide (88, 97). Evidence that a lipid intermediate plays a role in mannan biosynthesis in Micrococcus lysodeikticus and the yeasts Saccharomyces cerevisiae and Hansenula holstii is now fairly conclusive (98-106). The undecaprenol phosphate, joined through a phosphodiester bridge to mannose, acts as the donor of mannose to the non reducing termini of the endogenous mannan in M. lysodeikticus. In the yeast S. cerevi-

siae the mannan is a glycoprotein containing mannosyl residues attached to the protein by both alkaline-labile and alkaline-stable bonds suggesting possible attachment of the sugars to serine and threonine or to asparagine residues (107, 108). It has now been shown that a polyprenol phosphate mannose acts as the donor of the first mannose to serine or threonine residues of the protein, but that GDP-mannose is the donor of all the other mannose residues in these particular carbohydrate side chains in both S. cerevisiae and H. holstii (109-112). However, recent studies have shown the synthesis of polyprenol pyrophosphoryl - oligosaccharides by cell free preparations from S. cerevisiae and also by particulate fractions of Baker's yeast. These oligosaccharide side chains were identical to those found attached through an alkaline-stable N-glycosidic linkage to the asparagine residue in many glycoproteins. This suggested that the lipid may indeed play an intermediary role in the synthesis of some of the carbohydrate side chains of the yeast mannans (106, 109-111, 113, 114). The above studies also suggested that, whilst undecaprenols play a major role in the synthesis of bacterial cell wall, the lipid intermediate in yeast mannan biosynthesis is a mannosyldolicholphosphate. The dolichols of yeast cells are usually three to four isoprene units shorter than the mammalian dolichols, but both are α- saturated polyprenols, whereas the bacterial lipids are fully unsaturated (103,

105, 106, 110, 113-116). Poly - cis - polyprenols are also found in Aspergilli and the cell walls of these fungi contain mannose in polymer form (117, 118). Initial studies by Barr and Hemming demonstrated the formation of a mannolipid by particulate enzyme preparations from the thallus of Aspergillus niger, but the role of this lipid in mannan biosynthesis has yet to be established (104, 105, 119).

2) Plant, Protozoan and Insect Lipids:

Since polyprenols are also present in plants their involvement in analogous sugar transfer reactions would not be unexpected. The formation of polyprenol - linked sugars in mung bean shoots, soya bean, cotton fibres, Phaseolus aureus, and pea seedlings have been reported, and their possible role as intermediates in glycoprotein biosynthesis is being investigated (68, 91, 120-130). Kauss, in 1969, demonstrated the formation of a polyprenolphosphorylmannose from GDP-mannose and an endogenous lipid by a particulate preparation from mung bean shoots.(120). However, this mannose - containing lipid proved unable to donate significant amounts of mannose to the polysaccharide under the conditions examined, so that its function as an intermediate remains to be conclusively established. Preliminary studies with particulate preparations of Phaseolus aureus suggested the involvement of a mannolipid as an intermediate in glycoprotein biosynthesis. There is evidence that

phytanol phosphate, a fully saturated polyprenol phosphate containing four isoprene units, acts as the mannose acceptor, but its intermediary role remains to be confirmed (120-125). Forsee and Elbein, working with cotton fibres and Brett and Leloir, working with soya bean and pea seedlings have demonstrated the formation of lipid - linked oligosaccharides similar to the corresponding derivatives in mammalian systems (126-128, 130). In mammals these compounds, dolicholpyrophosphoryl-(N-acetylglucosamine)-(mannose)$_n$, are intermediates in the biosynthesis of the core regions of the oligosaccharide side chains of those glycoproteins containing the alkaline-stable asparagine-N-acetylglucosamine linkage. Most of the plant glycoproteins studied to-date contain this alkaline-stable linkage and also a core region containing N-acetylglucosamine and mannose (131). The transfer of both N-acetylglucosamine and mannose from their respective nucleotide sugars to the glycoprotein has been reported but direct transfer of the oligosaccharide chain or of the individual sugars from the lipid-sugars to the protein could not be demonstrated (122, 127, 129, 130). These preliminary studies indicate a role in glycoprotein biosynthesis analogous to the intermediary role of the lipid in bacterial cell wall and mannan biosynthesis, but direct evidence to conclusively establish this remains to be uncovered (126-130). The studies with soya bean and pea seedlings also indicated that the lipid acceptors were α-

saturated polyprenols similar to the dolichols which occur in both yeast and mammalian systems (128, 130).

Cell free particulate preparations from the protozoan ciliate Tetrahymena pyriformis are also capable of effecting the synthesis of glucosyl phosphorylpolyprenol or mannosyl-phosphorylpolyprenol from UDP-glucose or UDP-mannose and an endogenous acceptor lipid (132). These preparations were later shown to transfer glucose from an exogenously supplied glycosylpnosphorylpolyprenol to glycoprotein. The nature or tne function of the glycosylated protein synthesized has not yet been investigated but the studies undertaken do provide direct evidence for tne involvement of a dolichol type lipid linked intermediate in glycoprotein biosynthesis in tnis protozoan (133). Quesada Allué and his colleagues have now shown tne presence of α- saturated polyprenol-phosphate-sugar type compounds in the insects Triatoma infestans and Ceratitis capitata and are currently investigating tne possibility of their involvement in insect glycoprotein syntnesis (134, 135). Tnis was the first evidence of the existence of isoprenolphosphates in insects and makes tne study of these lipid intermediates even more interesting since their widespread occurrence in many prokaryotic and eukaryotic systems lends support to tne idea of their general involvement in cell wall polymer and glycoprotein biosynthesis.

3) Mammalian Systems:

The involvement of polyprenols in mammalian sugar transfer reactions has been the subject of a number of recent review articles (83-88, 119, 136-140). The endogenous family of mammalian polyprenols are the dolichols, (Figure 3, p.12), (141-144). They are distinguished from undecaprenol, an allylic alcohol, because they have an α- saturated isoprene unit and also their hydrocarbon chain is longer. The functional form is dolichol monophosphate but its exact mode of formation, whether by dephosphorylation of dolichol pyrophosphate or by phosphorylation of dolichol, is not clear. Both the polyprenol phosphatase and kinase enzymes are present in bacteria but to-date only the phosphatase has been described for mammalian tissues (145). In 1969 Caccam and his coworkers and Zatz and Barondes reported the formation of mannolipids by several animal tissues on incubation with GDP-mannose. These mannolipids were chemically and chromatographically similar to the mannosylphosphorylundecaprenols observed in bacteria (146, 147). Evidence then appeared for the synthesis of a glucosylphosphoryldolicnol by rat liver microsomal preparations and its synthesis was stimulated by the addition of exogenous dolichol phosphate (148). In the seven years since these initial studies, preparations from a variety of mammalian tissues have been shown to catalyze the transfer of various sugars from their nucleotide donors to endogenous acceptor lipids such as dolichol. These are summarized

in(Table 3). The sugars most studied are mannose, glucose and N-acetylglucosamine. N-acetylglucosaminyl phosphate and N-acetylmannosaminyl phosphate are transferred from their nucleotide donors to dolichol phosphate and give rise to a product now thought to be a glycosylpyrophosphoryldolichol. All the other sugars studied are transferred to the acceptor lipids in the free form, rather than as the sugar phosphates. Various studies confirmed this and allowed the postulation of a general reaction whereby polyprenol - linked monosaccharides are synthesized, (Figure 4) (119, 148, 149-151).

Many of these enzymatically formed glycolipids were only partially characterized since the fact that their chemical and chromatographic properties were similar to the bacterial lipid intermediates was often used as an experimental basis for their tentative identification. Similarly, the ability of exogenous dolichol phosphate or other polyprenol phosphates to stimulate glycolipid biosynthesis was taken as evidence for the involvement of similar or identical endogenous polyprenols in the biosynthetic reaction (123, 149, 152-157). There is considerably more evidence available on the structure of mannosylphosphoryldolichol. Sufficient quantities of this glycolipid have been obtained to allow for the elucidation of its structure by nuclear magnetic, infra-red and mass spectroscopy (157-159). Another valuable aid in the structural studies of these glycolipids has been the chemical synthesis of some polyprenol phosphates and their deri-

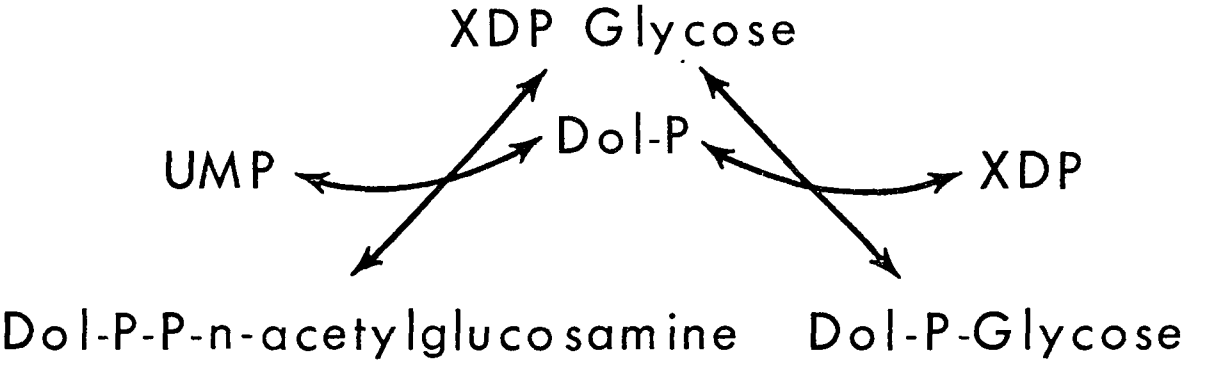
POLYISOPRENOL-LINKED MONOSACCHARIDES FORMED BY MEMBRANE PREPARATIONS FROM ANIMAL TISSUES

Monosaccharide-Linked to Polyprenol Phosphate	Tissue and Reference
Mannose	Rat liver (149,186,189) brain (123), parotid (187); bovine liver (158,179), thyroid (152), aorta (153) and adrenal medulla (187); Calf pancreas (150, 154, 166,190,191), and brain (155); mouse myeloma (158); rabbit (143,193,194) and pig (159,169,192) liver; hen oviducts (152); human lymphocytes (156) and embryonic chick brain (185).
Glucose:	Rat liver (148,149,186) and brain (185,186); hen oviduct (183); human lymphocytes (156) and embryonic chick brain (185); calf pancreas (151).
N-acetylglucosamine*	Rat (149) and pig (151) liver; calf pancreas (184); and human lymphocytes (156).
Xylose:	Hen oviduct (183).
N-acetylmannosamine*	Pig liver (151).

* Linked to the polyprenol via a pyrophosphate bridge.

TABLE 3

REACTIONS OF DOLICHOL PHOSPHATE LEADING TO FORMATION OF MONOSACCHARIDE DERIVATIVES



XDP represents nucleoside diphosphate
Dol-P represents dolichol phosphate
Dol-P-P represents dolichol pyrophosphate

FIGURE 4

ASSEMBLY OF AN OLIGOSACCHARIDE-LIPID

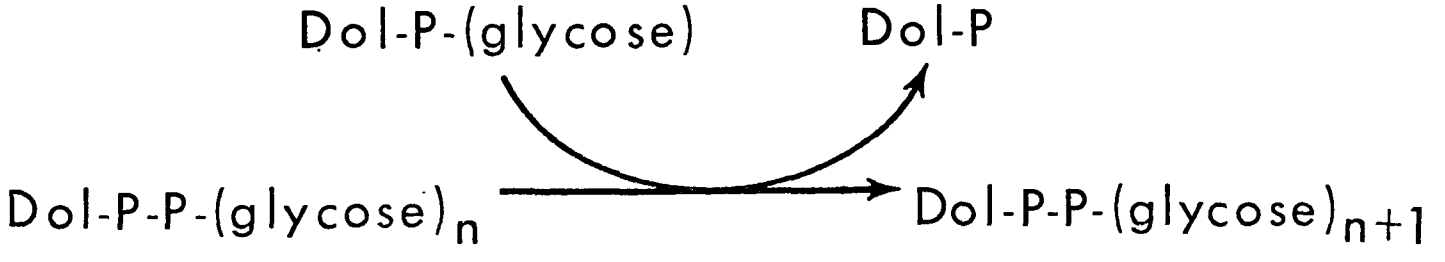


FIGURE 5

vatives. These compounds provide useful standards for chemical and chromatographic comparison (160-164). The sensitivity of the polyprenol - linked monosaccharides to mild acid hydrolysis suggested that the anomeric carbon of the sugar is glycosidically linked to the polyprenol phosphate. Various groups have examined this anomeric linkage in different glycosylphosphoryldolichols and their studies suggested it to be of the β - configuration (148, 165-167).

What then is the role of these dolichol derivatives? There is now good support for the hypothesis that dolichol-linked monosaccharides are not end products but that they serve as intermediates in the synthesis of some glycoproteins and also in the assembly of oligosaccharide phospholipids, another class of intermediates. Their involvement in glycoprotein biosynthesis was first suggested by Behrens and Leloir (148) but on further investigation they found that the major glucosylated endogenous acceptor was not a glycoprotein but a lipid - linked oligosaccharide (168). Since glucose is not commonly found in glycoproteins these workers examined the possible involvement of dolicholphosphorylglucose in the glucosylation of certain compounds known to contain glucose, namely, collagen, glycogen andcerebroside. However, the evidence argued against such involvement in these sugar transfer reactions (149). Similar studies were carried out in other mammalian systems, and indicated the involvement of the dolichol-linked monosaccharides in the assembly of the oligo-

saccharide phospholipids, (Figure 5) (152, 169-172). These oligosaccharide phospholipids have now been detected in a number of animal tissues, (Table 4). They were difficult to obtain in amounts sufficient to allow detailed structural studies and as a result much of the information currently available has been deduced from indirect evidence. The glucose - containing oligosaccharide phospholipid derivatives, formed by rat liver preparations on addition of exogenous glucosylphosphoryl dolichol, had the unusual property of being insoluble in water or chloroform : methanol (2:1) but were readily soluble in chloroform : methanol : water (1:1:0.3), (168, 173). Chemical studies suggested that the oligosaccharide chain contained approximately twenty glycose residues consisting of both glucose and N-acetylglucosamine. The linkage between the oligosaccharide chain and the lipid appears to be a pyrophosphate one but additional evidence is needed to confirm this. The lipid moiety is almost certainly dolichol, since di-N-acetylchitobiosyl pyrophosphoryl dolichol serves as a precursor for the oligosaccharide phospholipid. This conclusion is also supported by the fact that a mild acid hydrolysate of a rat liver fraction containing the oligosaccharide phospholipid resembles dolichol phosphate in its ability to stimulate the incorporation of glucose from UDP-glucose into glucosyl phosphoryl dolichol. Molecular weight studies also suggested the lipid component to be dolichol (168, 173).

LIPID-LINKED OLIGOSACCHARIDES FORMED
BY MEMBRANE PREPARATIONS FROM ANIMAL TISSUES

Sugar(s) Linked to Lipid	Number of Glycose Units in the Oligosaccharide Chain	Tissue and Reference
Glucose, hexosamine	20	Rat liver (168,188)
Glucose	20	Rat brain and kidney Human lymphocytes Pig thyroid (188)
Mannose, N-acetylglucosamine	7	Mouse myeloma (170)
Mannose, N-acetylglucosamine	7-9	Hen oviduct (174)
Mannose, N-acetylglucosamine	3-16	Rat liver (186)
Mannose, N-acetylglucosamine	5-15	Bovine aorta (175)
Mannose	-	Bovine adrenal medulla (187) Rat parotid (187) and Calf brain (155)

TABLE 4

Many of the oligosaccharide lipids found in other systems do not contain glucose. Their oligosaccharide chains contain both mannose and N-acetylglucosamine and are especially interesting since the structure of the oligosaccharide chain in some instances appears identical to the core oligosaccharide chain of many glycoproteins. These oligosaccharide lipids have been intensively studied and the intermediates in the hen oviduct and mouse myeloma appear to have similar, if not identical structures (170, 174, 175). In the mouse myeloma the structure was proposed to be

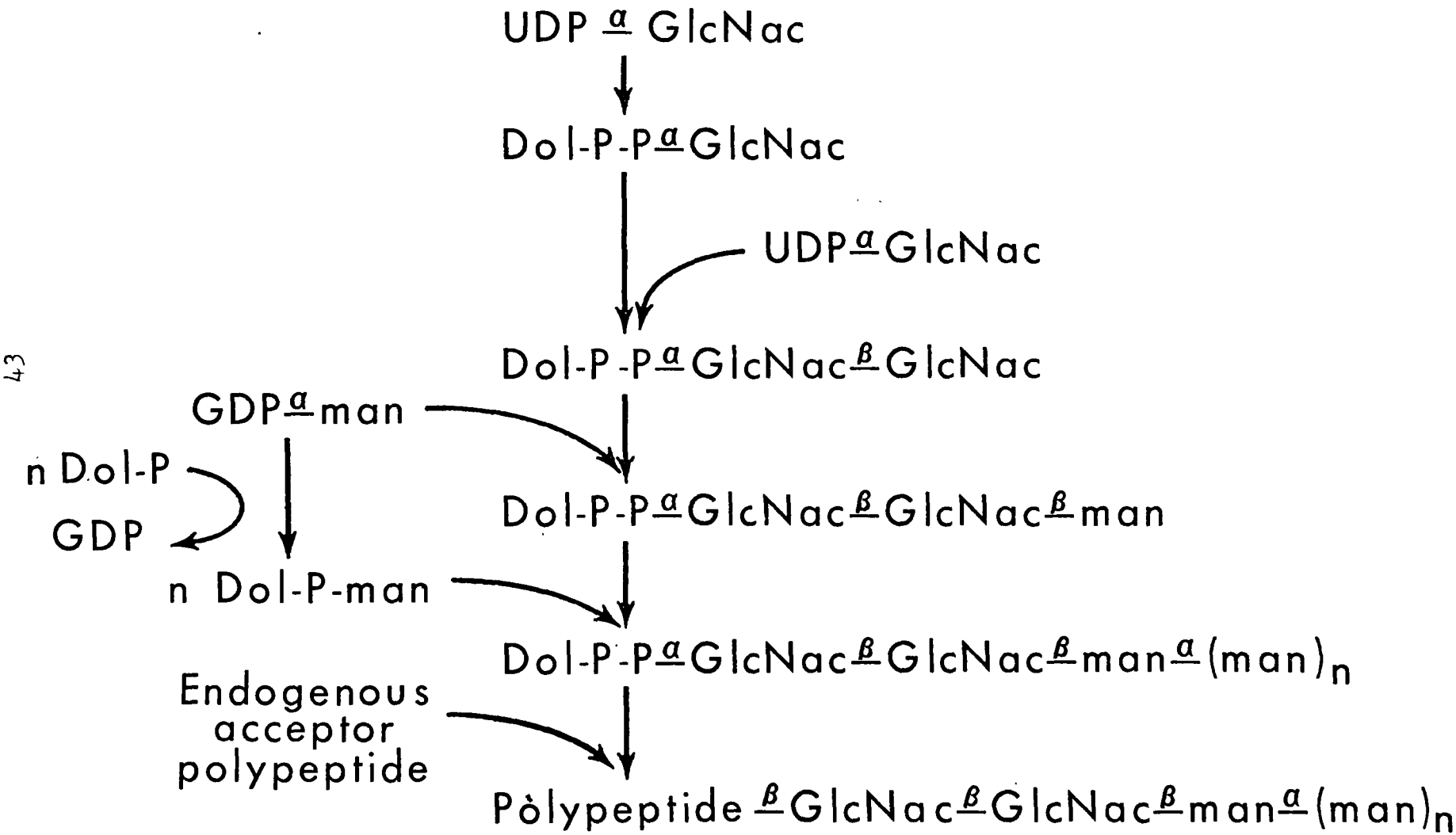
$$(Man)_5 - (GlcN\text{-}Ac) - P - P - \text{dolichol}$$

and the microsomal preparations were shown to effect the transfer of the oligosaccharide chain from its lipid carrier to an acceptor protein. The oligosaccharide degradation products of the glycoprotein and those of the oligosaccharide - lipid were chromatographically identical in these preparations. These and other studies undertaken by Lucas and his co-workers, (174), provided evidence for the en bloc transfer of the oligosaccharide to the protein. Little is at present known about the exact nature of the proteins glycosylated by the dolichol derivatives, but those studied in the rat liver, mouse myeloma and hen oviduct appear to be tightly associated with the membrane preparations (170, 174, 176, 177). Recent studies provide the first evidence for the involvement of an oligosaccharide lipid in the synthesis of a soluble secretory glycoprotein, a kappa-type light chain immunoglobulin in

myeloma cells (178). These immunoglobulins also contain the mannose N-acetylglucosamine oligosaccharide core common to many glycoproteins and oligosaccharide lipids. This raises the question as to whether these lipid intermediates are involved in the biosynthesis of all the glycoproteins containing this common core structure. Based on the information available from studies on glycoprotein biosynthesis in a number of different systems a scheme for their biosynthesis has been proposed and is illustrated in (Figure 6), (162, 170, 172, 174, 176, 179, 180). Much still remains to be clarified concerning this pathway. None of the enzymes involved have yet been purified nor has their subcellular distribution been well documented. No one tissue has yet been shown to be capable of effecting all steps in the biosynthetic sequence and as mentioned earlier the exact nature and eventual role of the glycoprotein has yet to be explored. However, it is interesting to note that the overall scheme is remarkably similar to those proposed to illustrate the biosyntnesis of complex glycans in bacteria (88) and since undecaprenol phosphate is suggested to be rate limiting for complex glycan biosynthesis in the bacterial systems it is not unreasonable to assume that dolichol phosphate might exert a similar regulatory function in glycoprotein biosynthesis.

FIGURE 6

A POSTULATED SEQUENCE IN THE ASSEMBLY OF A GLYCOPROTEIN



4) A Possible Role in Steroid Glycoside Formation:

The involvement of polyprenol type lipids as intermediates in the many different sugar transfer reactions discussed above prompted the investigation of the involvement of polyprenol type lipids in estrogen-3-glycoside biosynthesis in rabbit liver. This study was also of special interest since to-date lipid intermediates have only been implicated in sugar transfer reactions leading to the synthesis of macromolecules. Their involvement in the synthesis of a glycoside of a small molecule such as a steroid hormone would be completely novel and might have far reaching and ex citing implications in steroid metabolism and its regulation.

CHAPTER 2

MATERIALS AND GENERAL METHODS

A) Materials

Chemicals were obtained from the following companies as indicated.

Amersham/Searle Corporation (Arlington Heights, Illinois)

($1,2-{}^{3}H_2$)epitestosterone, ($6,7-{}^{3}H_2$)estrone, ($6,7-{}^{3}H_2$)17β-estradiol, ($2,4,6,9-{}^{3}H_4$)estriol, ($1,2-{}^{3}H_2$)testosterone and UDP-D-($6-{}^{3}H$)glucose.

(^{14}C) 17α-estradiol, (^{14}C)retinol, and UDP-D-(U-^{14}C)glucose.

(^{32}P)UTP

NCS and PCS tissue solubilizers

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

Silica Gel H

J.T. Baker Chemical Company (Phillipsburg, N.J.)

Ammonium molybdate

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

Glucose

Canadian Laboratory Supplies Ltd. (Ottawa, Ont.)

Silica Gel N, G.

Eastmen Kodak Co. (Rochester, New York)

Anisaldehyde, Eugenol, 2-mercaptoethanol

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

Biuret reagent, calcium chloride, cholesterol, Cutscum, ethylene diamine tetraacetic acid, Fiske - Subbarow reagent, manganous chloride, p-nitrophenol, all buffer salts, acids and solvents, Redi/Plate precoated with Silica Gel G.

General Biochemicals (Chagrin Falls, Ohio)

p-chloromercuribenzoate

Chemical And Radioisotope Division, International Chemical And Nuclear Corp. (Irvine, California)

(^{14}C)p-nitrophenol

Mandel Scientific Co. Ltd. (Montreal, Quebec)

Chromatography paper and Diethylaminoethylcellulose (Whatman)

Merck

phenolphthalein

New England Nuclear, Canada, (Lachine, Quebec)

Aquasol, (7-^{3}H)17α-hydroxypregnenolone and (1,2-$^{3}H_2$)tetrahydrocortisol, (U-^{14}C)glucose-1-phosphate and (^{14}C)diethylstilbestrol

Nutritional Biochemicals Corp. (Cleveland, Ohio)

Soybean lecithin

Pharmacia Fine Chemicals AB. (Montreal, Quebec)

Sephadex G10 and Sepharose 2B

Rohm and Haas (Philadelphia, Pennsylvania)

Triton X-100, Amberlite XAD-2 Resin

Sigma Chemical Co. (St. Louis, Missouri)

Almond emulsin, bovine serum albumin, chymotrypsin, cetyltrimethylammonium bromide, dolichol, dolichol phosphate, digitonin, deoxycholate glycogen, glucose-1-phosphate, glucose-6-phosphate, α-glucosidase, lysozyme, magnesium chloride, neuraminidase, nicotinamide adenine dinucleotide, p-nitrophenyl, papain, protease, phosphodiesterase, pyrophosphate, phospholipase C, phospholipase D, polyoxyethylene sorbitan monolaureate, retinol, snake venom (Trimeresurus flavoviridis) synthetic lecithin, trypsin, trypsin inhibitor, glucose, UDP-glucose, UDP-galac tose, UDP-xylose, UDP-glucose pyrophosphorylase, UDP-glucose dehydrogenase, UDP-galacotse epimerase, all the mononucleotides, and all the non-radioactive steroids.

Warner Chilcott (Toronto, Ontario)

Ketodase

Calf brain dolichol phosphate was a gift from Dr. L.S. Wolfe, Donner Laboratory of Experimental Neurochemistry, Montreal Neurological Institute, McGill University, Montreal, Quebec. Geranylgeraniol and farnesol were gifts from Dr. S. Kushwaha, Department of Biochemistry, University of Ottawa.

B) General Methods

1) Preparation of Substrate; (6,7-$^3H_2$) 17α-Estradiol

A non pregnant female New Zealand rabbit was injected intravenously with 5 millicuries of (6,7-$^3H_2$) estrone. The urine was collected for two days and then the combined collections were applied to an Amberlite XAD-2 column. The column was washed with water and then the double conjugate, estradiol-3-β-glucuronide-17α-β-N-acetylglucosaminide was eluted with methanol (195). This eluate on treatment overnight with hyaluronidase in 0.1M citrate buffer (pH 4.0) at 37°C yielded the monoconjugate 17α-estradiol-3-β-D-glucuronide. The monoconjugate was then treated with β-glucuronidase (Ketodase) for two successive 24 hour periods at 37°. The benzene extracts from these two incubations were pooled and an aliquot chromatographed against an authentic standard of 17α-estradiol to assess its purity (4, 5, 196).

2) Preparation of Microsomes

a) Rabbit

Virgin female New Zealand rabbits were killed by cervical dislocation. The liver was excised immediately and homogenized with 4 volumes of ice cold 0.15M KCL in a Sorvall Omnimixer homogenizer for 1 minute. The homogenate was centrifuged at 10,000 X g for 30 minutes. The supernatant was then centrifuged in a Beckman L2-65B ultracentrifuge at

approximately 105,000 X g (30,000 r.p.m., 30k rotor) for 90 minutes. The supernatant was decanted and the pellet was re-suspended in 0.15M KCL and centrifuged at 105,000 X g (40,000 r.p.m., 40K rotor) for 60 minutes. The microsomal pellet was re-suspended in 0.15M KCL to a volume, in millilitres, equal to the original wet weight of tissue, in grams. The preparations were stored at -10°C until used.

b) Rat (197)

A male rat was fasted for 18 hours before being killed by decapitation. The liver was excised immediately after death and homogenized in 0.88M sucrose in a Sorvall Omni-mixer homogenizer for 1 minute to obtain a 10 per cent homogenate (w/v). All procedures were carried out at +4°C. The homogenate was centrifuged at 24,000 X g for 20 minutes (17,5000 r.p.m., 40K rotor) in a Beckman L2-65B ultracentrifuge. The supernatant was removed and the pellet was re-suspended in 0.88M sucrose and centrifuged at 105,000 X g (40,000 r.p.m., 40K rotor) for 3 hours. The pellet contained both the rough and smooth microsomes and after removal of the supernatant, was re-suspended in 0.25M sucrose in 0.01M glycylglycine to a volume, in millilitres equal to the original wet weight of liver, in grams. The preparations were stored at -10°C until used.

3) Incubation Methods, Extraction and Assay

Incubations were carried out in buffer prepared by mixing solutions of Tris (hydroxymethyl) aminomethane and HCl in proportions which gave a molarity of 0.2 and a pH of 7.0. The usual incubation medium contained 2 millilitres of buffer, 0.5 millilitres of the microsomal preparation and 0.5 millilitres of 0.15M KCl. The steroids were added in methanol solution to dry 15 millilitre centrifuge tubes fitted with ground glass stoppers. The methanol was removed under a nitrogen stream before addition of the buffer. In the nucleotide dependent assays the uridine nucleotides were dissolved in the buffer to give a final concentration of 0.06mM. Each assay contained 550,000 d.p.m. of steroid (39 curies/millimole). Incubations were carried out by shaking the unstoppered tubes in a water bath at 37°C for 30 minutes.

Following the incubation the medium was extracted twice with 5 millilitres of benzene directly in the incubation tube (8). The procedure quantitatively removed all the free steroid present, but none of the steroid conjugate (67). The conjugate was removed by two extractions with 5 millilitres of ethyl acetate. This extract contained the steroid-3-glucosides and usually a smaller amount of 17α-estradiol-3-galactoside (12). An aliquot of 0.5 millilitres of the ethyl acetate was counted in 10 millilitres of a scintillation mixture consisting of equal parts of xylene and of Aquasol. The amount of steroid conjugate formed was

calculated from the counts in the ethyl acetate and reported in the results as the per cent conversion of 17α-estradiol to 17α-estradiol-3-glucoside. When the 3 millilitre assay contained 550,000 d.p.m. of the substrate 17α-estradiol (39 curies per millimole) 20 per cent conversion of the 17α-estradiol to 17α-estradiol-3-glucoside represented the formation of 5.1 picomoles of conjugate per gram of liver per hour. The radioactive material in the benzene and ethyl acetate extracts was occasionally examined by thin layer chromatography on silica gel N against authentic standards in order to ensure that the assay was operating satisfactorily. The thin layer chromatographic systems were benzene : ethyl acetate (7:3) for the free steroids and chloroform:ethanol (4:1) for steroid glucosides.

4) Preparations of Lipid Fractions

a) Rabbit Liver Lipids

(i) Total Lipids

Fresh rabbit liver was extracted with 3 volumes of chloroform : methanol (2:1) to give a fraction containing the total liver lipids.

(ii) Neutral Lipids

The phospholipid fraction was precipitated from the

total lipid fraction by treatment with ice cold acetone and 5 per cent magnesium chloride in ethanol according to the procedure of Artom (198).

(iii) Phospholipids

A chloroform : methanol fraction containing phospholipids was obtained by first treating the liver with 2 volumes of acetone to extract out the neutral lipids and then treating with 3 volumes of chloroform : methanol (2:1).

(iv) Acceptor Lipid

The chloroform : methanol extract containing the phospholipids was made 0.1N in NaOH, incubated for 15 minutes at 37° and then made 0.1N in HCl. This mixture was refluxed for 15 minutes and then the chloroform layer was washed according to the procedure of Folch and his co-workers (199). The extract was then poured into a DEAE-cellulose column in the acetate form, prepared as described in Section 5 of this chapter. The column was washed with chloroform : methanol (2:1) and then eluted with 0.1M ammonium acetate in chloroform :methanol (2:1). The ammonium acetate fractions were pooled, washed free of ammonium acetate and concentrated (200). Further purification was achieved using silica gel G thin layer chromatography with solvent systems chloroform: methanol : ammonia : water (80:30:0.5:3) and chloroform : methanol : formic acid : water (70:18.5:8:0.5). The silica

gel was treated with 5% per cent HCl in ethanol, filtered and washed with ethanol before preparation of 0.75mm plates. The lipids were detected with either phosphomolybdic acid or anisaldehyde spray reagents and eluted from the chromatograms with 0.6N HCl in chloroform : methanol (2:1) (148,201, 202).

b) Rabbit Liver Microsomal Lipids

(i) Phospholipids

The microsomes were extracted with 2 volumes of N-butanol and then washed with 1 volume of water. This extract was allowed to stand for several hours and then concentrated to dryness. The residue was re-suspended in butanol : benzene : methanol (1:1:1) and further purified by preparative chromatography on Silica Gel G plates in the solvent system chloroform : methanol : water (50:50:10) (203). As mentioned in Section (a) (iv) above the silica gel was pre-treated with 5 per cent HCl in ethanol before use. The lipids were detected on the chromatograms with either phosphomolybdic acid or Rosenbergs spray reagent (204) and eluted with 0.6N HCl in chloroform : methanol (2:1).

Phospholipids were also prepared by treating the lyophilized microsomes with 2 volumes of acetone and then extracting the residue with 3 volumes of chloroform : methanol (2:1).

(ii) Microsomal Acceptor Lipid

The procedure was a modification of that of Behrens and Leloir (148) and is illustrated in (Figure 7.)

Crude organic extracts were also prepared by treating 100 milligrams of lyophilized rabbit liver microsomes with 20 millilitres of either chloroform : methanol (2:1) or chloroform : methanol : water (1:1:0.3).

c) Pig Liver Lipids

(i) Neutral Lipids

The ground pig liver was treated with 2 volumes of acetone.

(ii) Phospholipids

The residue left after acetone treatment was extracted with 3 volumes of chloroform : methanol (2:1).

(iii) Acceptor Lipid

This was again a modification of the procedure of Behrens and Leloir (148) as illustrated in (Figure 7.)

Phosphate determinations of the lipid extracts were carried out according to the procedure of Bartlett (205) as described in Section 10 of this Chapter.

PREPARATION OF RABBIT LIVER MICROSOMAL
OR PIG LIVER ACCEPTOR LIPID

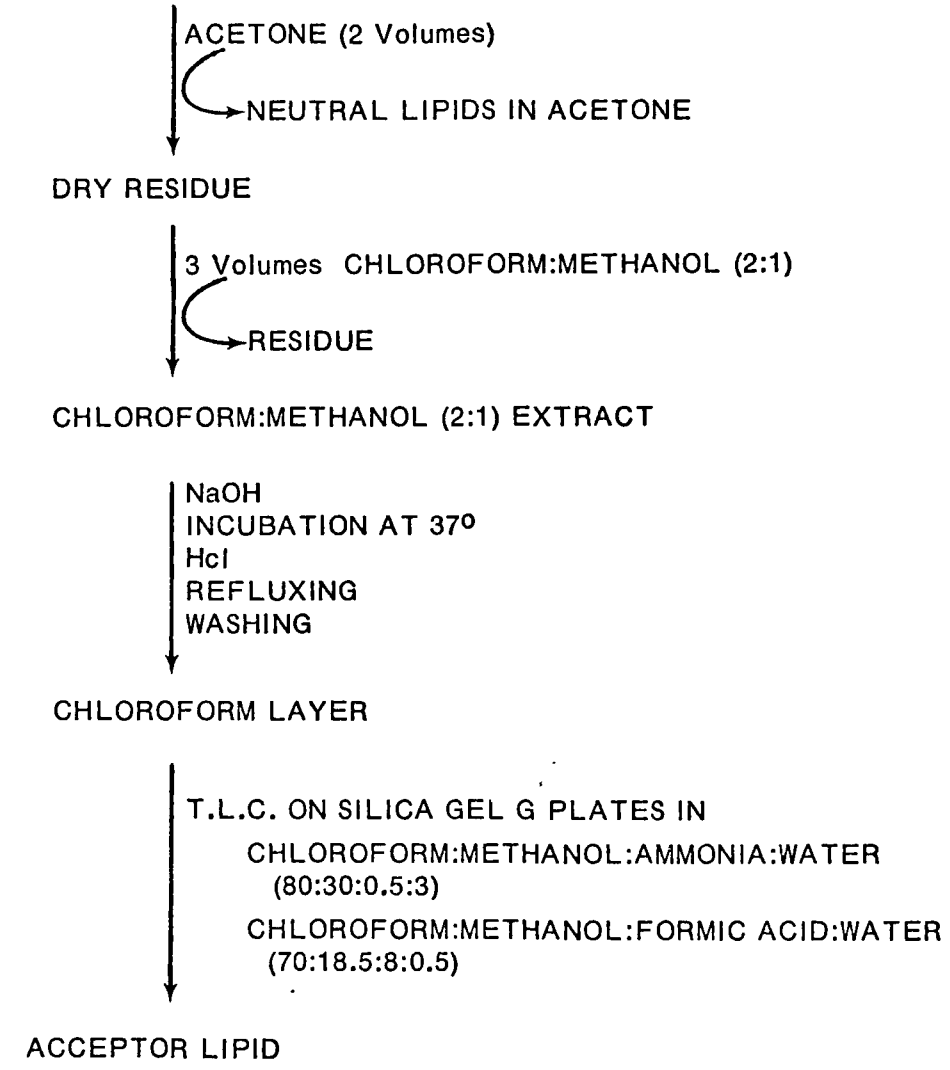


FIGURE 7

5) Preparation of DEAE for Ion Exchange Chromatography

The DEAE-cellulose was treated in the following manner to remove various impurities:

The dry ion exchangerwas stirred into 15 volumes of 0.5N HCl and allowed to stand in the acid for at least 30 minutes. The suspension was then filtered through a Buchner funnel under gentle suction from a water aspirator and washed with distilled water until free of acid. The ion-exchanger was then stirred into 15 volumes of 0.5N NaOH and again allowed to stand for at least 30 minutes before washing with distilled water until the filtered effluent was nearly neutral. The material was washed alternately three times each with acid and base. After the final wash the DEAE was converted to the acetate form by passage of 3 bed volumes of re-distilled glacial acetic acid through the material. The excess acid was removed by washing the bed with 3 volumes of methanol. The ion exchanger resin was then removed from the funnel and air-dried in a clean fume free atmosphere. It was dried to constant weight in a vacuum desiccator over KOH pellets. This throughly dried material was then weighed and left to stand overnight in glacial acetic acid which broke up any aggregates present and permitted more uniform packing of the column. A 30 x 2.5 centimetre double-thickness Pyrex glass column fitted with a Teflon stopcock and a solvent reservoir was used. The column was packed and tested for

uniformity of packing according to the procedure of Rouser and his co-workers (206).

6) Thin Layer Chromatography

Silica gel N without binder was used for the separation of the steroids and silica gel G with $CaSO_4 \cdot \frac{1}{2}H_2O$ binder for the lipid studies.

The preparative thick plates and those being used in the lipid identification work were pre-run in the chromatographic chambers in chloroform : methanol (1:1) to remove any traces of lipids. All plates were dried in air at room temperature and then activated at 120°C (30 minutes for thin plates, overnight for thick plates) before the sample was applied. The lipid samples thought to contain highly unsaturated lipids were applied to the plates in a special plexi-glass box with an inlet for flushing with nitrogen (Brinkman Instruments Inc.). In all cases the lipids were chromatographed immediately after the application of the sample. All-glass tanks with ground glass covers and lined with filter paper to aid in saturating the tank with solvent vapour were used. Freshly made up solvent systems were used for each run and the chambers were always sealed with masking tape in an effort to obtain reproducible results (202, 207, 208).

7) Preparation of Sepharose 2 B Columns

50 millilitres of the agarose gel Sepharose was diluted with about 50 millilitres of 0.05M Tris buffer, pH 7.6 and was de-aerated with a vacuum pump. A 2.5 x 30 centimetre column was used and partially filled with buffer. The column was then filled with the de-aerated sepharose slurry by pouring down a glass rod. All the slurry required to produce the whole bed was poured into the column at one time. The slurry was gently mixed with the glass rod to remove air bubbles and was allowed to settle until the bed height was stable at about 20 centimetres.

8) Preparation of Dowex 1 x 8 (200-400) Ion Exchange Columns

30 grams of Dowex ion exchange resin 1 x 8 (200-400) was mixed with distilled water and allowed to settle. The excess water was drawn off with a vacuum pump. This washing procedure was repeated several times to get rid of any contaminants and air bubbles. The resin was packed as recommended in the Dow Chemical Company booklet (209). A 100 millilitre burette was used as the column. To convert the column into the formate form a 3M sodium formate solution was run through until no chloride could be detected in the eluate. About 400 millilitres of 50 per cent formic acid was then run through and finally the column was washed several times with

water until no formate was detectable in the eluent. The column was then ready for use (210).

9) Preparation of the Amberlite XAD-2 Column (195)

The neutral cross linked polystyrene polymer Amberlite XAD-2 slurried in water was poured into a glass column containing a coarse fitted disc layered with about 1 centimetre of sand and half filled with water. The water was allowed to slowly run through the column in order to remove the fines. When all the water had drained from the column 3 volumes of methanol were run through and then a further 5 volumes of water. The column was then ready for use.

10) Phosphorus Determination (205)

Aliquots of a stock solution containing 1, 2, 3, 4 and 5 micrograms of any phosphate salt were added to graduated test tubes in volumes ranging from 0.2 to 1 millilitres. All volumes were adjusted to 2 millilitres with water. The lipid extracts were evaporated to dryness under a stream of nitrogen in graduated test tubes and 2 millilitres of water were added to each tube. 0.5 millilitres of concentrated sulphuric acid was added to all tubes which were heated for 3 hours at 160^{o}C. The tubes were cooled in ice prior to the addition to each of 2 drops of 30 per cent hydrogen peroxide . Heating at 160^{o}C was continued for a further 1½ hours. The tubes were then

again cooled in ice and all volumes were adjusted to 1 millilitre with water. 5.3 millilitres of 0.22 per cent ammonium molybdate and 0.3 millilitre of Fiske-Subbarow reagent were added to all tubes, mixed well and heated in a boiling water bath for 7 minutes. The optical densities were read at 830mμ in a Gilford model 2400-2 spectrophotometer and the amount of inorganic phosphate in the lipid fractions was calculated from the optical density by comparison with a standard curve.

11) Protein Determinations

Protein was determined by the method of Gornall, Bardawill and David (211) or Lowry, Rosebrough, Farr and Randall (212).

CHAPTER 3

THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE BY RABBIT LIVER MICROSOMES

A) Introduction

As discussed in Chapter 1 rabbit liver microsomes have been shown to effect the transfer of glucose or of galactose from their respective uridine nucleotides to the 3α-hydroxyl group of estrone, 17α-estradiol and 17β-estradiol (9). Further experiments showed that, in the absence of added UDP-glucose or UDP-galactose, the microsomes retained the ability to form small amounts of the estrogen-3-glucosides and galactosides (12). The preliminary experiments discussed in this chapter investigate in detail the capacity of the rabbit liver microsomes to synthesize these and other estrogen glycosides in the absence of exogenously added uridine nucleotide sugars and when endogenous nucleotide sugars have been removed by washing or by treatment with enzymes specific for these nucleotide sugars. These studies suggested the existence of a "UDP-glucose independent" and a "UDP-glucose dependent" pathway of estrogen-3-glucoside synthesis. Any indirect involvement of UDP-glucose in the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside is also examined. This involved (i) a study of the capacity of the rabbit liver homogenate to effect the synthesis, from exogenous UDP-glucose, of an inter-

mediate sugar donor capable of participating in the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside, (ii) an examination of the ability of the microsomal fraction to incorporate glucose from UDP-D-(6-^{3}H)glucose and to transfer any incorporated glucose to the steroid and (iii) an examination of the capacity of the microsomes to effect the synthesis of UDP-glucose from UTP and glucose-1-phosphate or from UTP and any endogenous glucose donor.

B) Methods

1) An Examination of the Capacity of Rabbit Liver Microsomes, Washed and Unwashed, to Effect the Synthesis of 17α-Estradiol Mono- and Double Conjugates when Incubated in the Absence of Uridine Nucleotide Sugars.

The microsomes were prepared as described in the general methods Chapter 2 Section 2(a), and were washed up to 4 times. Each wash consisted of the re-suspension of the microsomal pellet in a few millilitres of 0.15M KCl and centrifugation for 60 minutes at 105,000 X g (40,000 r.p.m. 40K rotor) in a Beckman L2-65B ultracentrifuge. After washing, the volume of the microsomal preparations was adjusted with 0.15M KCl so that 1 millilitre of suspension was equivalent to 1 gram wet weight of liver tissue. One millilitre of microsomal suspension contained approximately 10 milligrams of protein as determined by the method of Lowry (9, 212).

The incubation mixtures, extraction and assay procedures for the "nucleotide independent" synthesis of 17α-estradiol-3-glucuronide or 3-glucoside were exactly as described in Chapter 2, Section 3 except that each assay contained 1.47 x 10^6 d.p.m. of the substrate, 17α-estradiol (88.9 d.p.m. x 10^3/picomole or 40.4 curie/millimole). The ethyl acetate fraction contained any 17α-estradiol-3-glucosides or 17α-estradiol-3-galactosides formed but the 3-glucuronide formed was not

extracted into either benzene or ethyl acetate and the amount formed was measured by counting a 0.? millilitre aliquot of the water layer.

When examining for the "nucleotide independent" synthesis of 17-N-acetylglucosaminide or 17-glucosyl double conjugates the incubation methods were again as described in Chapter2, Section 3 except that each assay contained 1.13 $x10^6$ d.p.m. of the steroid substrate which in this case was 17α-estradiol-3-glucuronide (68.9 d.p.m./picomole, or 31.3 microcurie/millimole). Following the 30 minute incubation at 37°C 0.25 millilitres of 1.0N HCl was added to each 3 millilitre assay to lower the pH to 2.0. The mixture was then extracted three times with 5 millilitres of ethyl acetate which quantitatively removes the unreacted substrate, 17α- estradiol-?-glucuronide. A 0.3 millilitre aliquot of the aqueous layer was counted since any double conjugates formed would remain in this layer.

To determine whether uridine nucleotide sugars present in the microsomal suspension survived washing the microsomes, were pre-incubated with UDP-D-(U ^{14}C)glucose (0.15 micromole, 660,000 d.p.m./micromole) or UDP-D-(U ^{14}C)glucuronic acid (0.15 micromole, 420,000 d.p.m./micromole) and then subjected to repeated washing as described previously. An aliquot of the washed microsomes was counted for ^{14}C after each wash to obtain an estimate of the amount of nucleotide remaining.

2) The Effect of Treatment with UDP-Glucose Dehydrogenase, UDP-Glucose Pyrophosphorylase and UDP-Galactose-4-Epimerase on the Synthesis of the 17α- Estradiol-3-Glucoside

Microsomal preparations were pre-incubated with UDP-glucose dehydrogenase, UDP-glucose pyrophosphorylase and UDP-galactose-4-epimerase and then assessed for their ability to form glycosides of 17α-estradiol both in the presence and absence of UDP-glucose. The enzymes were added to the incubation mixture in 0.2M Tris pH 7.0. Amounts of UDP-glucose dehydrogenase were added which gave a final concentration of 1 unit of enzyme activity per 3 millilitre assay, UDP-glucose pyrophosphorylase in amounts which gave a final concentration of 2 units per assay and UDP-galactose-4-epimerase in amounts which gave a final concentration of 0.05 units per assay.

3) Other Studies to Examine the Involvement, Direct or Indirect, of UDP-Glucose in the "UDP-Glucose Independent" Synthesis of 17α-Estradiol-3-Glucoside

a) The capacity of the homogenate for the synthesis of an intermediate sugar donor when incubated with UDP-glucose.

Rabbit liver homogenates were pre-incubated with and without UDP-glucose. The microsomes, prepared from these homoge-

nates as described in Chapter 2, Section 2, were then assessed for their capacity to effect the "UDP-glucose independent" synthesis of 17∝-estradiol-3-glucoside, (Table 5).

b) Glucose Incorporation from UDP-D-(6-^{3}H)glucose into the microsomes

The flow chart, seen in (Table 6), demonstrates the procedure followed in the initial tritium incorporation studies. The effect of the presence of magnesium chloride on the incorporation of tritiated glucose from UDP-D-(6-^{3}H)glucose into the microsomes and into 17∝-estradiol-3-glucoside was examined. Three methods were used to separate the microsomal fraction from the reaction mixture, namely centrifugation, filtration and chromatography. Where centrifugation was used the incubation mixtures were centrifuged in a Beckman L2-65B ultracentrifuge at approximately 105,000 X g (40,000 r.p.m., 40K rotor) for 60 minutes. The supernatant was discarded and the pellet resuspended in KCl and centrifuged again using the same conditions. The supernatants would be expected to contain any unreacted UDP-glucose. These incubations were carried out in duplicate and one pellet was solubilized by treatment, with 1 millilitre of the tissue solubilizer NCS and 0.01 millilitre of water at 37°C for 10 minutes. NCS had a tendency to produce chemiluminescence and in order to overcome this the solubilized mixtures were cooled and 1 drop of ascorbic acid and

THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE IN MICROSOMES PREPARED FROM RABBIT LIVER HOMOGENATES PRETREATED WITH UDP-GLUCOSE

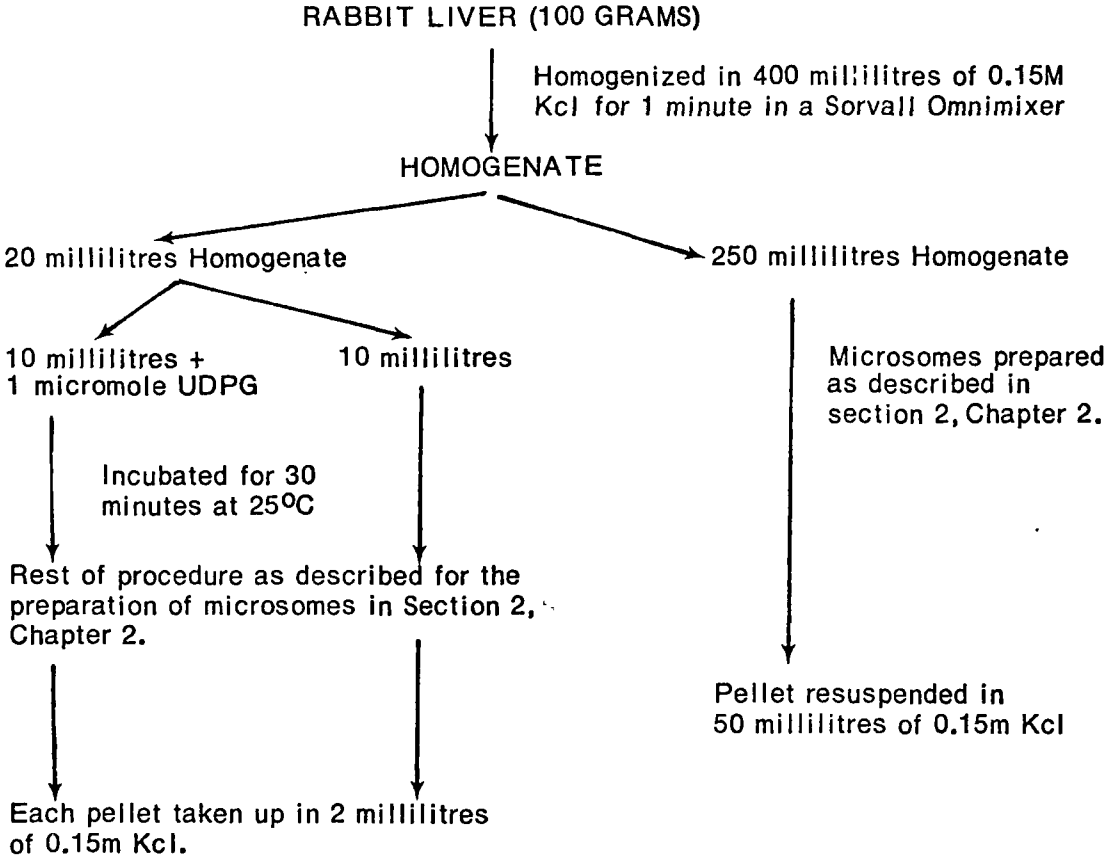


TABLE 5

FLOW SHEET FOR STUDIES OF THE INCORPORATION OF [^{3}H]UDP-GLUCOSE INTO MICROSOMAL PREPARATIONS

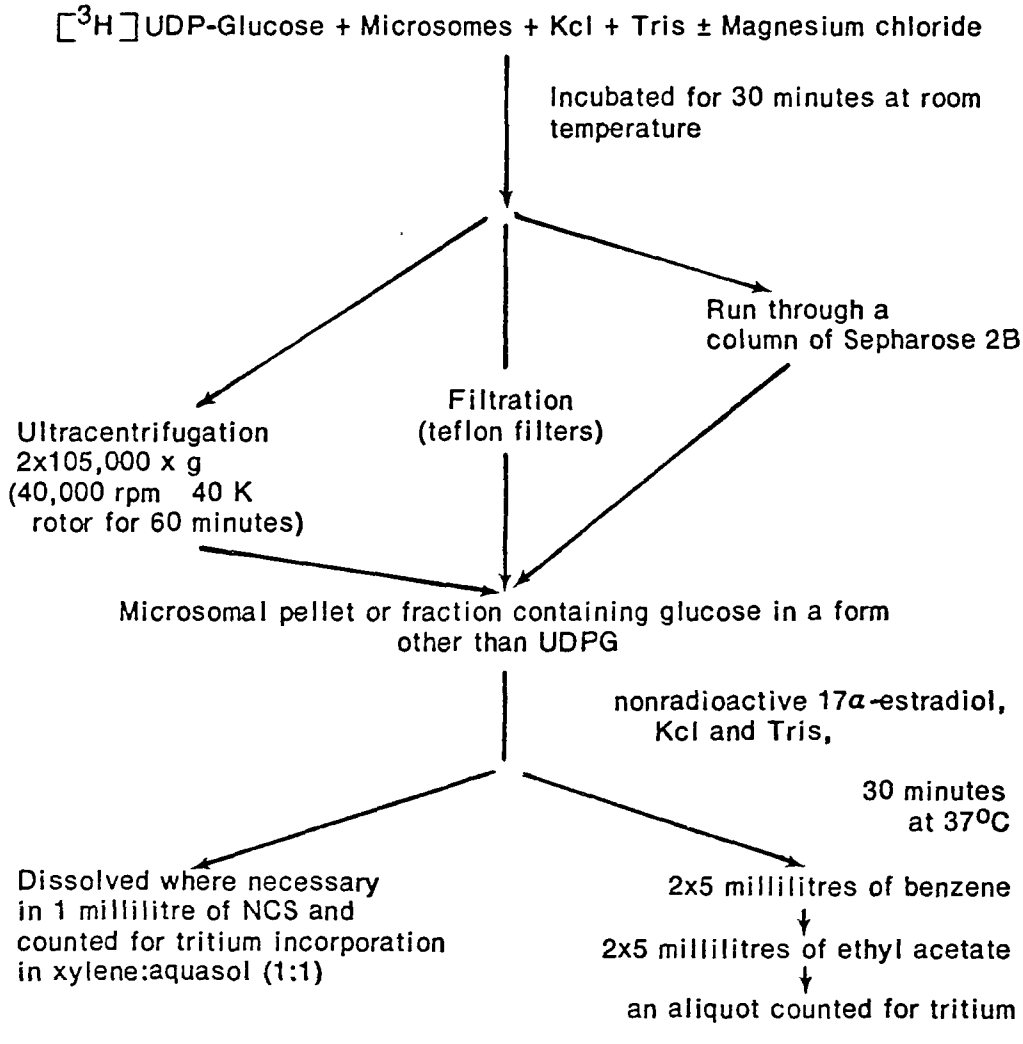


TABLE 6

0.034 millilitres of glacial acetic acid were added before counting for incorporated tritium in a mixture of aquasol : xylene (1:1). The other pellet was re-incubated with cold 17α-estradiol and other reagents of the standard assay and the procedure for the assay and extractions were exactly as described in the general methods in Chapter 2.

When filtration through teflon filters was the method used for separating the unreacted UDP-glucose from microsomes, the microsomal fraction, retained on the teflon paper, was either solubilized with NCS as described above or assessed for its ability to effect the synthesis of 17α-estradiol-3-glucoside.

When chromatography was used, one millilitre fractions from the sepharose 2B column were counted to locate the fractions containing tritium. UDP-D-(U-^{14}C)glucose was co-chromatographed to facilitate the detection of free UDP-D-(6-^{3}H) glucose. Those fractions thought to contain tritiated glucose incorporated into the microsomes were pooled, concentrated down and assessed for their ability to effect the synthesis of 17α-estradiol-3-glucoside.

After it had been established that a very small percentage of the initial tritium in UDP-D-(6-^{3}H)glucose could be incorporated into the microsomal fraction, studies were undertaken to determine where this tritium was located, and whether it could be easily washed out of the microsomes. The washing involved re-suspension of the pellet in KCl and centrifugation at 105,000 X g (40,000 r.p.m., 40 K rotor) for 60 minutes as

described previously.

To discover which microsomal fraction contained the tritium, different lipids were extracted and analyzed for any evidence of tritiated glucose. Total, neutral and phospholipids were prepared as described in Section 4, Chapter 2. Various groups have reported the presence of nucleotide pyrophosphatases in microsomal preparations (179, 213, 214). Inhibitors of these enzymes were included in the incubation mixture to ensure that the UDP-D-(6-^{3}H)glucose remained intact and available for reaction with components in the microsomal preparation. In one experiment ATP, at a concentration of 0.1mM per 0.54 millilitre assay, was used as the inhibitor. The method used was that of Zatta et al and the time course of tritium incorporation from UDP-D-(6-^{3}H)glucose (5.5nanomoles/0.54 millilitre assay) into two different lipids in the microsomes was studied. This reaction was studied in both the presence and absence of manganese chloride or magnesium chloride (5mM/ 0.54 millilitre assay). These same studies were later repeated, but the assay scaled up twenty fold, using 1 and 4mM AMP per assay as the nucleotide pyrophosphatase inhibitor and tritium incorporation into the lipids at 37°C and at 25°C, both in the presence and the absence of Triton X-100 and Cutscum was examined. The isolated lipids were chromatographed against dolichol monophosphate from calf brain (supplied by Dr. L.S. Wolfe) in chloroform: methanol : water (60:35:6), sprayed with anisaldehyde which gives a specific reaction with

isoprenoids (201) and then centimetre sections of the silica gel were scraped into scintillation vials to which was added 0.04 millilitres of methanol and 10 millilitres of aquasol : xylene before counting for tritium.

c) The ability of rabbit liver microsomes to synthesize UDP-glucose from UTP and glucose-1-phosphate or from UTP and any endogenous sugar donor was examined.

Three different experiments were undertaken to investigate this : (i) Incubation with non-radioactive UTP and glucose-1-phosphate (ii) incubation with (^{32}P)UTP and non-radioactive glucose-1-phosphate and (iii) incubation with (^{32}P)UTP. The procedure used in (i) was a modification of the standard assay described in Chapter 2 and of that reported in the literature by Berthillier and Got (215). Non-radioactive UTP (2.4 mM), glucose-1-phosphate (0.25mM), mercaptoethanol (4mM) and magnesium chloride (250 mM) were added to a 4 millilitre assay. Incubations were carried out at both room temperature and 37°C. The ethyl acetate extracts were collected and counted for their 17α-estradiol-3-glucosiole content. These reaction were carried out in duplicate and one incubation was stopped by boiling and then centrifuged at 10,000 X g for 2 minutes. The supernatant was then assessed for its UDP-glucose content by re-incubation with approximately 0.05 units of UDP-glucose dehydrogenase and 0.25 micro-

moles of NAD. Changes in absorbance at 340 nm were followed in a Cary spectrophotometer (model 15). This study was designed to demonstrate whether UDP-glucose was actually being synthesized by the microsomal preparations.

A similar experiment was performed using non-radioactive glucose-1-phosphate and (^{32}P)UTP. The reaction was terminated by boiling and the mixture was then centrifuged at 10,000 X g for 2 minutes. The supernatant was applied to a Dowex column which was prepared as described in the general methods (209). The column was first washed with water to remove any free glucose and glucose-1-phosphate. A gradient of ammonium formate, 0.1M to 0.5M was used to elute the UDP-glucose. One millilitre fractions were then collected. UDP-D-(6-^{3}H)glucose was applied to the column together with the reaction mixture so as to help locate any (^{32}P)UDP-glucose. An aliquot of each fraction was counted for ^{32}P and ^{3}H and also chromatographed against a UDP-glucose standard on silica gel G plates in the solvent system butanol : acetone : acetic acid : ammonium hydroxide : water (4.5:1.5:1:1:2). The plates were sprayed with 4 per cent sulphuric acid in 50 per cent ammonium sulphate. Centimetre sections were scraped and counted for ^{3}H and ^{32}P in 0.04 millilitres of methanol and 10 millilitres of aquasol: xylene (1 : 1).

C) Results

1) The Effect of Washing of the Microsomes on Steroid Glycoside Synthesis

(Figure 3)illustrates the effect of washing on the ability of microsomes, unfortified with sugar nucleotides, to form 17α-estradiol-3-glucoside and galactoside as compared to their ability to form 17α-estradiol-3-glucuronide or the 17β-D-glucoside or N-acetylglucosaminide of 17α-estradiol-3-glucuronide. The 3-glucuronyl transferase activity is the lowest of those examined since the un-washed microsomes formed only 1000 picomoles of 17α-estradiol-3-glucuronide per millilitre of microsomal preparation per hour as compared to almost 6,500 picomoles of the double conjugates and 9,300 picomoles of the 17α-estradiol-3-glucosides and galactosides. This 3-glucuronyl transferase activity declined rapidly on washing of the microsomes since with just one wash the amount of steroid-3-glucuronide formed was only twenty per cent of that formed by the unwashed microsomes. The combined 17-glucosyl and N-acetylglucosaminyl transferase activities declined at a slower rate than the 3-glucuronyl transferase on repeated washing of the microsomes. However, after four washes, the microsomes retained only ten per cent of the capacity of the unwashed microsomes for double conjugate synthesis. As mentioned above the 17α-estradiol-3-monoglucosides and galactosides constituted the bulk of the conjugates formed in this

EFFECT OF REPEATED WASHING ON THE ABILITY OF RABBIT LIVER MICROSOMES TO FORM GLYCOSIDES OF 17α-ESTRADIOL

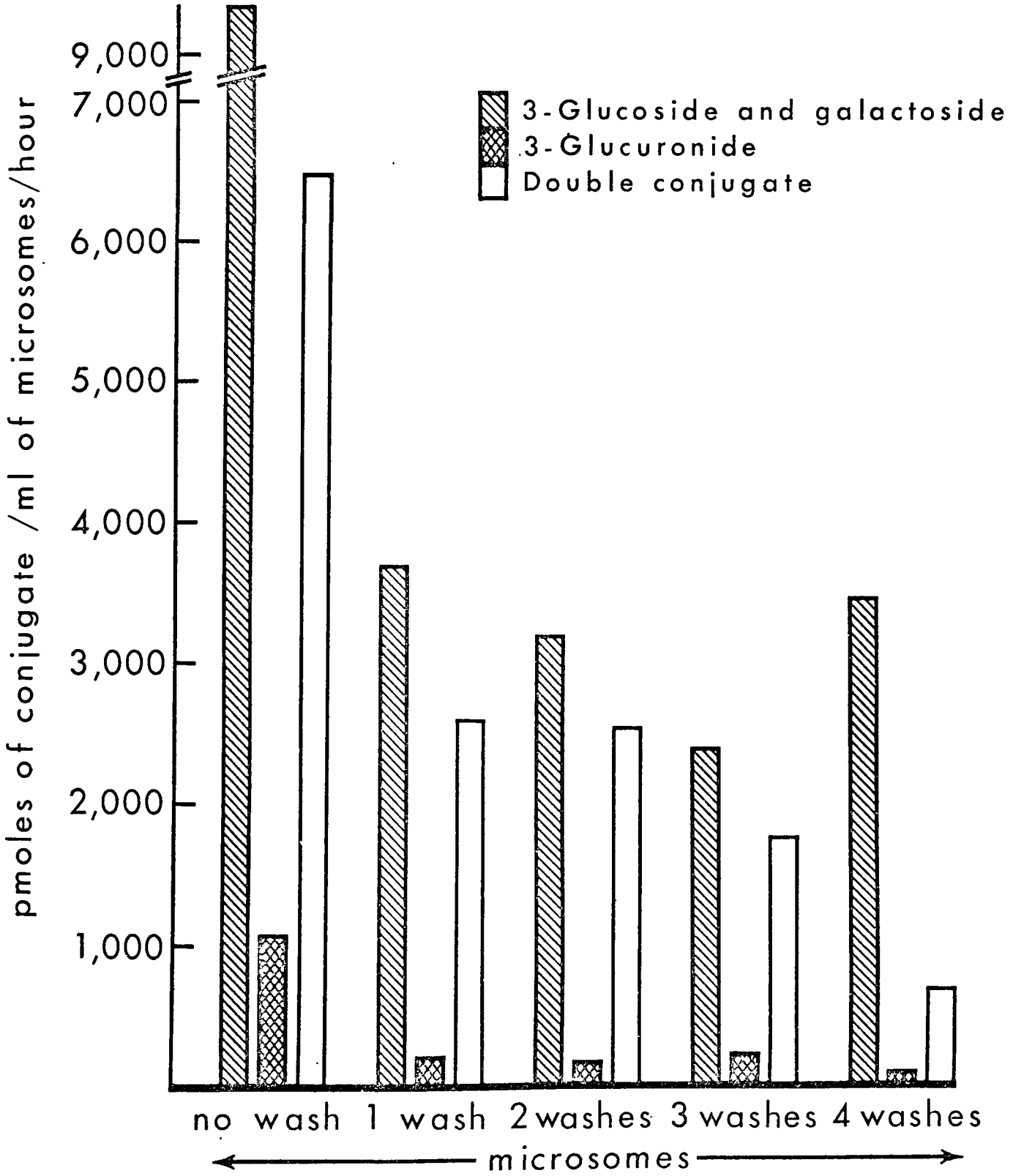


FIGURE 8

study. The synthesis of these monoconjugates was especially interesting due to the fact that, even after four washes, the microsomes still retained almost forty per cent of the capacity of the unwashed microsomes for 3-glycoside synthesis.

(Figure 9) shows the pattern obtained when microsomes, pre-incubated with UDP-D-(U-^{14}C)glucose or UDP-D-(U-^{14}C)glucuronic acid, were subjected to repeated washing. The concentration of the nucleotides declined rapidly on washing of the microsomal suspensions. The UDP-glucuronic acid was removed more easily, being almost completely lost after only one wash.

(Table 7) depicts some of the results obtained when 17α-estradiol-3-glucoside synthesis was studied at different times between 1973 and 1977. The incubation of microsomes with 17α-estradiol in the absence of exogenous UDP-glucose, as described in Chapter 2, allows for the study of the "UDP-glucose independent" glucosyltransferase. However, when UDP-glucose is included in the incubation the amount of 17α-estradiol-3-glucoside formed is due to both the "UDP-glucose independent" and "dependent" glucosyltransferase activities. Since the contribution by the "UDP-glucose dependent" transferase can not be directly measured, in most of the studies undertaken the difference in the amount of the product formed in the presence and absence of UDP-glucose was used as the estimation of the contribution by the "UDP-glucose dependent" glucosyltransferase. As seen in (Table 7) the amount of conjugate synthesized varied from preparation to preparation but a

THE REMOVAL OF NUCLEOTIDE SUGARS FROM THE MICROSOMES ON WASHING

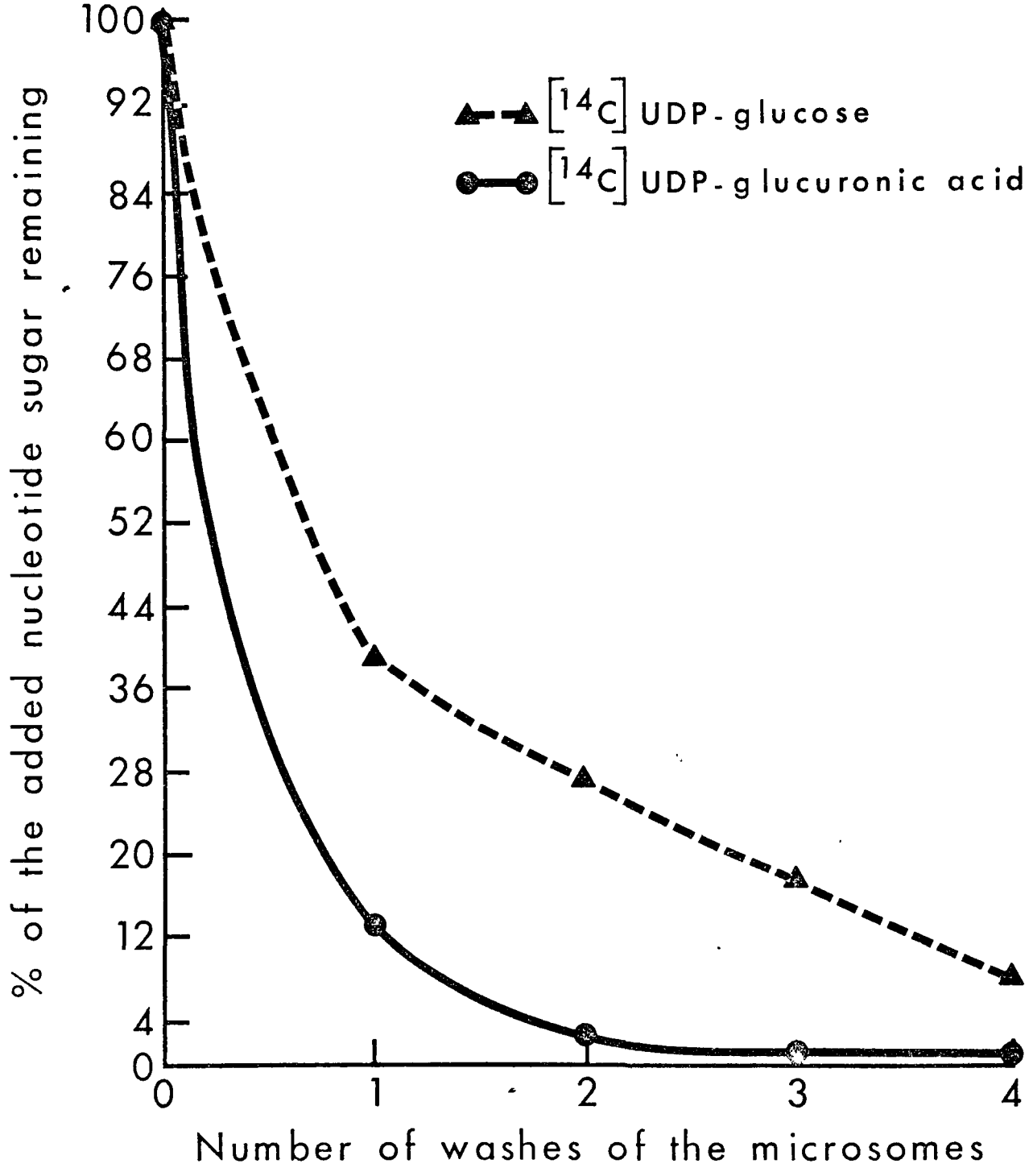


FIGURE 9

17α-ESTRADIOL-3-GLUCOSIDE SYNTHESIS BY RABBIT LIVER MICROSOMES

Date of Study	% Conversion of 17α-Estradiol to 17α-Estradiol-3-Glucoside		
	UDP-Glucose Independent	UDP-Glucose Dependent + Independent	UDP-Glucose Dependent
November 73	37.	46.	9.
December 73	4.1	12.3	8.2
January 74	7.3	18.4	11.1
July 74	7.7	18.5	10.8
January 76	34.6	46.6	12.0
November 76	44.5	69.7	25.2
January 77	40.2	48.4	8.2
February 77	42.4	58.1	15.7
March 77	2.6	6.2	3.6
March 77	15.6	28.04	12.44

TABLE 7

consistent feature of the 3-glucosylation reaction was that when the "UDP-glucose independent" glucosyltransferase activity was high the contribution by the "UDP-glucose dependent" pathway was of minor importance but when the "UDP-glucose independent" glucosyltransferase activity was low the "UDP-glucose dependent" pathway was responsible for most of the conjugate formed. This variation from preparation to preparation and sometimes even from season to season influenced other properties of the 3-glucosyl-transferase(s) and these will be mentioned at relevant points in the following chapters. The conjugates formed either in the presence or absence of UDP-glucose were identical and their identity has been established by crystallization to constant specific activity with authentic samples of the estrogen-3-β-D-glucosides (12).

2) Effect of Enzymes Specific for Nucleotide Sugars on Steroid Glucoside Synthesis

The results obtained when studies with the enzymes specific for UDP-glucose and UDP-galactose were undertaken are shown in (Table 8). The presence of UDP-glucose dehydrogenase and pyrophosphoroylase did not cause any significant change in the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside whereas the "UDP-glucose dependent" pathway was almost completely inhibited. The presence of UDP-galactose did not cause any change in the amount of steroid glycoside formed, however when UDP-galactose-4-epimerase, which converts

THE EFFECT OF UDP-GLUCOSE DEHYDROGENASE, UDP-GLUCOSE PYROPHOSPHORYLASE AND UDP-GALACTOSE-4-EPIMERASE ON THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE BY RABBIT LIVER MICROSOMES

Additions to Standard Incubation	% Conversion of 17α-Estradiol to* 17α-Estradiol-3-Glycoside	
	-UDP-Glucose Independent	+ UDP-Glucose Dependent
None	37	9
+ 2 Units UDP-Glucose Pyrophosphorylase	37	0
+ 1 Unit UDP-Glucose Dehydrogenase	40	2
+ UDP-Galactose (0.5 micromole)	39	-
+ UDP-Galactose + UDP-Galactose-4 -Epimerase	54	-

* Average of 3 experiments

TABLE 8

UDP-galactose to UDP-glucose, was included in the assay there was an increase in the amount of glucoside formed.

?) The Involvement of UDP-Glucose in the "UDP-Glucose Independent" Reaction

Microsomes, prepared from rabbit liver homogenates which had been incubated with UDP-glucose, did not show any significant difference in their capacity for the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside when compared to microsomes prepared in the usual fashion using liver homogenates unfortified with uridine nucleotide sugars. These results are seen in (Table 9). The "UDP-glucose dependent" synthesis of 17α-estradiol-3-glucoside was slightly stimulated by this exposure of the homogenates to UDP-glucose. The normal procedure for the preparation of the microsomes would remove some but not all of this loosely attached UDP-glucose and the amount remaining would account for this increase in glucoside formation by the "UDP-glucose dependent" pathway.

Some tritium was incorporated from UDP-D-(6-^{3}H)glucose into the microsomal preparation but very little appeared in the 17α-estradiol-3-glucoside. The presence of magnesium chloride in the medium increased the incorporation of tritium into both the microsomes and the steroid glucoside. These results are illustrated in (Table 10). This incorporated radioactivity was washed out of the microsomes relatively easily and after three washes less than one per cent of the

THE CAPACITY OF MICROSOMES, PREPARED FROM HOMOGENATES WHICH HAD BEEN PRE-INCUBATED WITH UDP-GLUCOSE, TO EFFECT THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE

Microsomal Fraction Used	% Conversion of 17α-Estradiol to 17α-Estradiol-3-Glucoside	
	UDP-Glucose Independent	UDP-Glucose Dependent
Microsomes prepared from Homogenates pre-incubated with 1 micromole UDP-Glucose	11	9
Microsomes prepared from Homogenates pre-incubated without UDP-Glucose	13.4	6.1
Microsomes prepared as described in Chapter 2.	12.2	5.2

TABLE 9

TRITIUM INCORPORATION FROM [^{3}H] UDP-GLUCOSE INTO THE MICROSOMAL PREPARATION AND ALSO INTO THE 17α-ESTRADIOL-3-GLUCOSIDE FRACTION*

Method used to Separate the Microsomes from [^{3}H] UDP-Glucose	DPM Incorporated into Microsomes	DPM Found in the Ethyl Acetate Extract (Glucoside)
Sepharose 2B	15,537	
Filtration (teflon filters)	24,268	163
Centrifugation – Magnesium chloride	38,181	285
Centrifugation + Magnesium chloride	66,317	819

* Each preparation pre-incubated with 2.2×10^6 DPM [^{3}H] UDP-glucose

TABLE 10

added tritium counts were detected in either the microsomes or the ethyl acetate fraction.

The distribution of the small amount of tritium incorporated into the microsomes is shown in (Table 11). This gives the average results of seven different experiments. In these studies the presence of magnesium chloride inhibited the incorporation of tritiated glucose into the lipid fractions. When the different lipids were examined for their tritium content the neutral lipids were the chief acceptors of the radioactive sugar. These lipid extracts were chromatographed on 0.5 millimetre Silica Gel N plates in three different solvent systems, acidic, neutral and basic. Centimetre sections of the plate were counted to locate the tritium. The radioactive fractions were eluted and their effect on the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside was studied. The microsomal total, neutral and phospholipid extracts which had not been subjected to chromatography were also examined for their effect on the "UDP-glucose independent" synthesis of the steroid glucoside. The results obtained did not provide any definite evidence for the involvement of any of these lipids in the "UDP-glucose independent" reaction.

The inclusion of inhibitors of endogenous nucleotide pyrophosphatases did not provide conclusive evidence for or against the involvement of UDP-glucose as the sugar donor. In most of the studies involving cations, magnesium or manganous chloride caused a slight increase in the incorporation of

DISTRIBUTION OF THE TRITIUM INCORPORATED INTO THE MICROSOMES FROM[^{3}H]UDP-GLUCOSE*

Fraction Examined	% of Added Tritium Found	
	− Magnesium chloride	+ Magnesium chloride
Microsomes	4.7	4.9
Total Lipids from Microsomes	1.85	0.74
Neutral Lipids from Microsomes	1.75	0.40
Phospholipids from Microsomes	0.75	0.63

* The microsomes were pre-incubated for 30 minutes at room temperature with UDP-glucose (4.4×10^6 DPM)

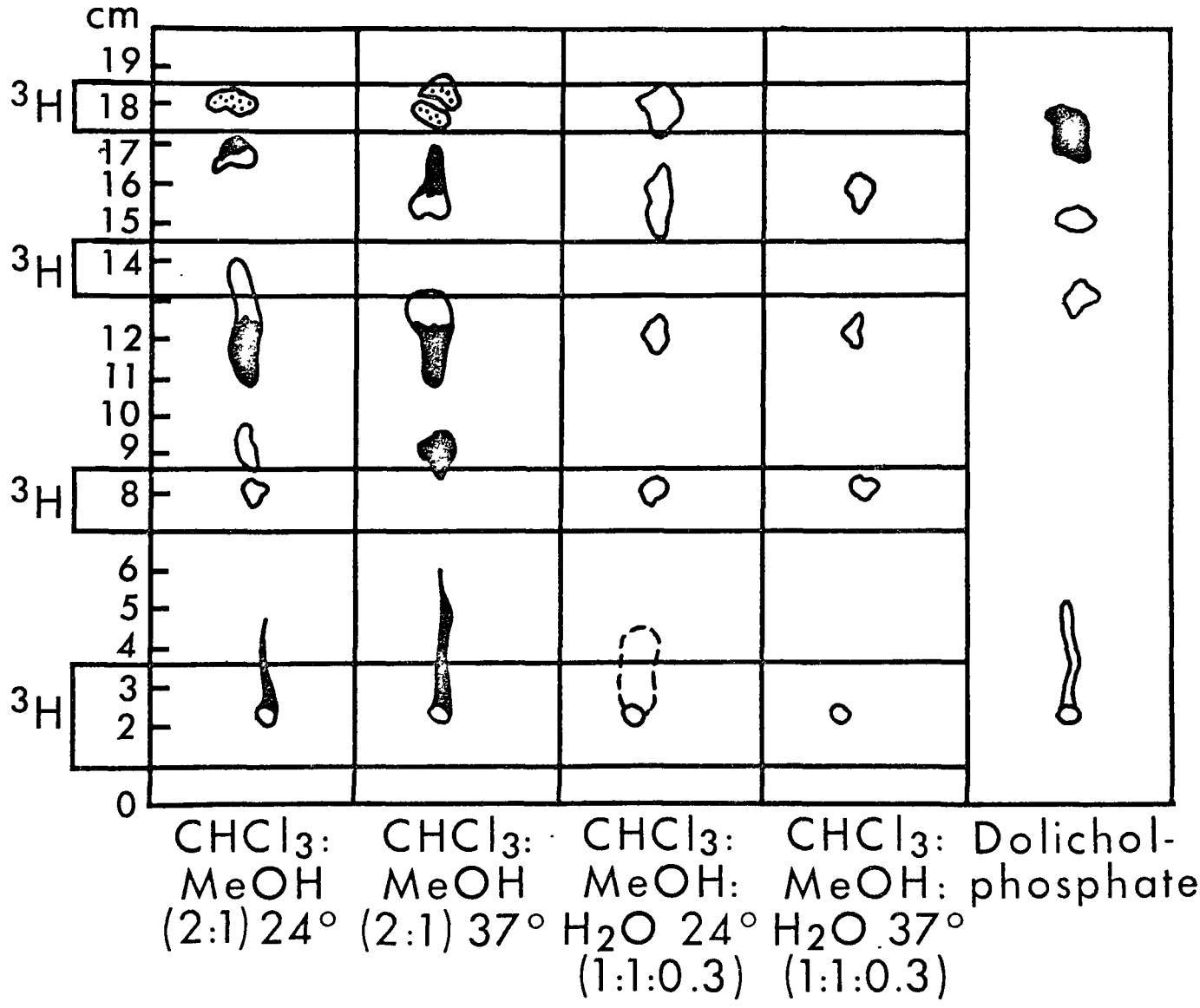
TABLE 11

tritium UDP-D-(6-^{3}H)glucose into both the chloroform : methanol : water (1:1:0.3) lipid fractions. This increase due to the presence of cations occurred in both the presence or absence of the nucleotide pyrophosphatase inhibitors ATP and AMP. These findings are in conflict with those depicted in (Table 11) where the presence of magnesium chloride reduced tritium incorporation into lipid fractions. However, the methods of lipid isolation were different in the two studies which might explain the discrepancy seen in the results. The presence of Triton X-100 at a concentration of 0.2 per cent and Cutscum at a concentration of 2 per cent did not have any significant effect on the amount of tritium incorporated into the microsomes.

Thin layer chromatographic examination of the lipid fractions as seen mapped in (Figure 10) showed that the incorporated radioactivity was separated into four different peaks. However, because of the lack of any pure standards at the time, it was impossible to determine which lipids were accepting the radioactive glucose. The lipid studies will be discussed in greater detail in Chapter 5.

If UDP-glucose was synthesized from non-radioactive UTP and glucose-1-phosphate it was not detected by UDP-glucose dehydrogenase (12) and it did not stimulate the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside either in the presence or absence of magnesium chloride either at 37^{o}C or 25^{o}C. (Table 12) illustrates the results obtained in the studies at 37^{o}C. A similar pattern was obtained at 25^{o}C.

THIN LAYER CHROMATOGRAPHY OF THE CHLOROFORM: METHANOL (2:1) AND CHLOROFORM: METHANOL: WATER (1:1:0.3) RADIOACTIVE LIPID FRACTIONS AGAINST A DOLICHOL-PHOSPHATE FROM CALF BRAIN



Solvent System: Chloroform: methanol: water (60:35:6)

Spray reagent : Anisaldehyde

FIGURE 10

ATTEMPTS TO SYNTHESIZE UDP-GLUCOSE AND ENSUING EFFECTS ON THE "UDP-GLUCOSE INDEPENDENT" SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE

Additions to the Standard Assay	% Conversion of 17α-Estradiol to 17α-Estradiol-3-Glucoside at 37°C	
	−Magnesium chloride	+250mM Magnesium chloride
None	39	63
4mM Mercaptoethanol	36	64
0.25mM Glucose-1-phosphate	37	67
0.25mM Glucose-1-phosphate +4mM Mercaptoethanol	40	65
0.25mM Glucose-1-phosphate +2.4mM UTP	30	55
0.25mM Glucose-1-phosphate +2.4mM UTP + 4mM Mercaptoethanol	24	50

TABLE 12

In the studies using (^{32}P)-UTP and non-radioactive glucose-1-phosphate a very small peak of ^{32}P co-chromatographed with UDP-D-(6-^{3}H)glucose in the solvent system butanol : acetone : acetic acid : ammonium hydroxide : water (4.5:1.5:1:1:2). The amount of UDP-glucose synthesized was not sufficient to have any significant effect on the synthesis of 17α-estradiol-3-glucoside.

The incubation of the microsomes with (^{32}P)-UTP did not produce any detectable UDP-glucose. This suggested that an endogenous sugar donor was unable to donate its sugar to UTP under the conditions used.

D) Discussion

The experiments discussed in this chapter provide strong evidence for the existence of two distinct mechanisms of steroid glycosylation in rabbit liver microsomes. One of these appeared independent of UDP-glucose and involved some water insoluble lipid intermediate, while the other was UDP-glucose dependent and involved the direct transfer of glucose from UDP-glucose to the 3α-hydroxyl group of the estrogen.

The high levels of conjugate synthesis seen in the unwashed microsomes, (Figure 8), can be attributed to the presence of endogenous nucleotide sugars. However, these nucleotide sugars were washed out of the microsomes relatively easily and were essentially absent after three or four washes, as shown in (Figure 9). The decline in the synthesis of the 17α-estradiol-3-glucuronide and the estradiol double conjugates on repeated washing of the microsomes can thus be attributed to the removal of endogenous nucleotide sugar donors. The ability of the microsomes to form considerable amounts of 17α-estradiol-3-glucoside and 3-galactoside even after removal of essentially all the endogenous UDP-glucose and UDP-galactose was unique and prompted the further investigation of this "nucleotide independent" glycoside synthesis.

The fact that the UDP-glucose specific enzymes, UDP-glucose dehydrogenase and UDP-glucose pyrophosphorylase did not cause any change in the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside but inhibited the "UDP-glu-

cose dependent" pathway also argued against UDP-glucose playing a role in the "UDP-glucose independent" synthesis of the glucoside. The studies shown in (Figure 10) and (Tables 9-12), indicated that UDP-glucose was not involved indirectly in the "UDP-glucose independent" glucosyl transferase reaction. The rabbit liver homogenates were unable to synthesize an intermediate sugar donor from UDP-glucose, since pre-incubation with UDP-glucose before preparation of the microsomes caused no change in the capacity of the microsomes for the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside. The studies examining the ability of the microsomes to incorporate glucose from UDP-D-(6-^{3}H)glucose, and to transfer any of the (^{3}H) glucose to the steroid also argued against the possible indirect involvement of UDP-glucose in the "UDP-glucose independent" pathway. Were exogenous UDP-glucose or an intermediate derived from it the only sugar donor in this pathway at least twenty to thirty per cent of the (^{3}H)glucose derived from exogenous UDP-glucose should be demonstrable in the 17α-estradiol-3-glucoside fraction to account for the normal rate of steroid glucoside formation by the "UDP-glucose independent" pathway.

The possible involvement of a lipid - sugar intermediate was indicated by both the washing studies and by the presence of much of the incorporated (^{3}H)glucose from UDP-glucose in the lipid fraction of the microsomes. The neutral lipids were the chief acceptors of the radioactive sugar. As discussed in

Chapter 1, dolichol is the polyprenol known to act as a lipid intermediate in many sugar transfer reactions in mammalian systems. The active sugar acceptors are usually the polyprenol - monophosphates but since to date there has been no evidence for a dolichol phosphokinase in mammalian systems, activation of the neutral lipid to a high energy acceptor form would appear unlikely. However, a neutral lipid sugar compound could participate in a transglucosylation reaction. The possible lipid involvement will be discussed in greater detail in Chapter 5.

Other evidence against a role for UDP-glucose in the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside was provided by the studies which demonstrated the inability of the microsomes to synthesize UDP-glucose from UTP and glucose-1-phosphate or from UTP and any endogenous sugar donor which might be present. This showed that the 3-glucoside conjugate synthesized by the washed microsomes when incubated in the absence of UDP-glucose was not due to the presence of UDP-glucose synthesized in the microsomes during the incubation.

These studies therefore provide definitive evidence for the existence of two pathways of steroid-3-glucoside formation. The properties of the 3-glucosyltransferase(s) will be discussed in detail in Chapter 4.

CHAPTER 4

AN INVESTIGATION OF THE PROPERTIES OF THE "UDP-GLUCOSE INDEPENDENT" AND "UDP-GLUCOSE DEPENDENT" STEROID-3-GLUCOSYL TRANSFERASE ACTIVITIES

A) Introduction

These studies were designed to investigate the specificity of the "UDP-glucose independent" and "UDP-glucose dependent" reactions, their characteristics and their requirement for co-factors. The involvement of sugar donors other than UDP-glucose was also examined in the case of the "UDP-glucose independent" estrogen-3-glucosyltransferase.

B) Methods

1) pH studies

Tris maleate and sodium carbonate buffers of 0.05 molarity and ranging in pH from 5.6 to 11.0 were used to determine the optimum pH for both the "UDP-glucose dependent" and "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside.

2) Effect of Time of Incubation

To determine the time period over which the velocity of both reactions was linear, a series of incubations were carried out at 37°C for time intervals ranging from 0 to 90 minutes.

3) Effect of Temperature

Both reactions were studied after a 30 minute incubation of the standard assay mixture at 4°, 25°, 37°, 46° and 60°C.

4) The Effect of Varying the Concentration of the Microsomal Preparation used

Both reactions were studied using microsomal preparations which contained from 1 to 10 milligrams of protein per 3 millilitre assay (212).

5) Substrate Specificity Studies (9, 67-69, 71)

(Table 13) shows the substrates examined, their specific activity and the concentrations used per 3 millilitre assay. For the estrogen substrates the assays were as described in the general methods in Chapter 2. For each substrate the validity of the assay was checked by chromatography of the ethyl acetate extracts against authentic standards of the free steroids and their glucosides in the solvent system chloroform : ethanol (4:1). One centimetre areas of the chromatograms were counted in the Mark III 6880 Liquid Scintillation Counter (Searle - Analytic Inc.) to locate the steroids and any glucosides formed. The chromatogram containing the ethyl acetate extract from the non-radioactive estriol incubation was sprayed with 20 per cent H_2SO_4/ethanol spray reagent (198) and heated for a few minutes at 100° to allow the development of the red and orange-yellow spots characteristic of the free estrogens and their glucosides.

The incubation mixtures containing the estrogen-glucuronide conjugates were treated with 3 volumes of ethanol to terminate the reaction after 30 minutes at 37°C. The mixtures were then centrifuged to precipitate the microsomal protein. The ethanol : water layer was concentrated to dryness, re-suspended in ethanol and centrifuged again to precipitate any of the remaining salts. The ethanol layer was evaporated to dryness under a stream of nitrogen and chromatographed in the solvents isopropanol : formic acid : water (5:3:1). The

SUBSTRATES USED TO STUDY THE SPECIFICITY OF THE 3-GLUCOSYLTRANSFERASE

Compounds Examined	Specific Activity	Concentration in Micromoles or Picomoles per 3 Millilitre Assay
1. Estrone -6, 7 [^{3}H]	47.9 Ci/millimole	1.85 picomoles
2. 17α-Estradiol-6, 7 [^{3}H]	46 Ci/millimole	1.85 picomoles
3. 17β-Estradiol-6, 7 [^{3}H]	46 Ci/millimole	1.85 picomoles
4. Estriol (nonradioactive)	-	30 picomoles
5. Estriol-2, 4, 6, 9 [^{3}H]	95 Ci/millimole	1.85 picomoles
6. 17α-Estradiol-6, 7[^{3}H]-3-Glucuronoside	48 Ci/millimole	1.85 picomoles
7. 17β-Estradiol-6, 7[^{3}H]-3-Glucuronoside	1 Ci/millimole	1.85 picomoles
8. p-Nitrophenol-2, 6 [^{14}C]	17.1 μCi/micromole	0.3 micromoles
9. Biochanin A [^{14}C]	0.08 μCi/milligram	0.25 micromoles
10. Genistein [^{14}C]	0.08 μCi/milligram	0.25 micromoles
11. Formononetin [^{14}C]	0.07 μCi/milligram	0.25 micromoles
12. Daidzein [^{14}C]	0.05 μCi/milligram	0.25 micromoles
13. Pregnan-3α-ol-20-one-7 [^{3}H]	13.9 mCi/micromole	1.8 picomoles
14. 17α-Hydroxypregnenolone-7 [^{3}H]	9.9 Ci/millimole	1.85 picomoles
15. Tetrahydro-cortisone [^{3}H]	24.4 Ci/millimole	1.85 picomoles
16. Testosterone [^{3}H]	1.7 Ci/millimole	1.85 picomoles
17. Epitestosterone [^{3}H]		1.85 picomoles
18. Diethylstilbestrol [^{14}C]	54 mCi/millimole	1.85 picomoles

TABLE 13

double conjugate 17α-estradiol-3-glucuronide-17β-glucoside was used as a standard. One centimetre zones, scraped from the chromatogram, were examined for their (^{3}H) content in order to locate the substrate and any product. The procedure followed by Labow and Layne (67) was used in the case of the isoflavone incubations. These microsomal preparations were also examined for their capacity to synthesize isoflavone glucosides in the absence of exogenous uridine nucleotide sugars. No UDP-glucose was added to these incubations. In the reactions containing the other substrates examined, (numbers 8 and 13-18 in Table 13), ethyl acetate rather than benzene was used to terminate the reaction. The ethyl acetate extracts were concentrated and chromatographed in the solvent system chloroform : ethanol (4:1) or chloroform : methanol (89:11).

Many of the studies undertaken during the course of this project utilized 17α-estradiol as the substrate for the 3-glucosyltransferase. Attempts were made to determine the Km of the "UDP-glucose dependent" and the "UDP-glucose independent" transferase activities towards this substrate. Concentrations of 17α-estradiol ranging from 0 to 6 x 10^{-6}M were used in the standard assay and the Km values were calculated from Lineweaver - Burke and Hanes plots (216).

6) Effect of Various Inhibitors

The concentrations of the inhibitors used are shown in (Tables 14, 15). The nucleotides, sulfhydryl reagents and

INHIBITORS USED IN THE STUDY OF
THE 3-GLUCOSYLTRANSFERASE REACTION

Compounds	Concentrations per 3 Millilitre Assay
Estrone	12 - 120 nanomoles
17β-Estradiol	12 - 120 nanomoles
Estriol	12 - 120 nanomoles
17α-Estradiol-3-Glucoside	1.5 - 3 nanomoles
17α-Estradiol-17-Glucoside	1.5 - 3 nanomoles
Diethylstilbestrol	0.03 - 1.2 micromoles
p-Nitrophenol	0.015 - 0.3 micromoles
Phenolphthalein	0.15 - 3.0 micromoles
p-Chloromercuribenzoate	0.02 - 0.05 micromoles
Dithiobisdinitrobenzoic acid	0.02 - 0.05 micromoles
Urea	6 - 12 millimoles
Guanidine - Hcl	3 - 15 millimoles

TABLE 14

INHIBITORS USED IN THE STUDY OF
THE 3-GLUCOSYLTRANSFERASE REACTION

Compounds	Concentrations per 3 Millilitre Assay
Methanol	450 micromoles - 1.35 millimoles
Ethanol	150 - 750 micromoles
Buthanol-1	150 - 450 micromoles
Ethylene Glycol	150 - 600 micromoles
Eugenol	3 - 12.5 micromoles
UTP	0.5 micromoles
UDP	0 - 5 micromoles
ATP	0.5 micromoles
ADP	0.5 micromoles
AMP	0.5 micromoles
GTP	0.5 micromoles
GDP	0.5 micromoles
GMP	0.5 micromoles
TTP	0.5 micromoles
TMP	0.5 micromoles

TABLE 15

protein denaturants were added to the standard incubation in 0.2M Tris pH 7.0 so as to give the final concentrations listed in (Tables 14-15). The steroids were added to the centrifuge tubes in methanol and evaporated to dryness before the addition of the other reagents. Increasing concentrations of 17β-estradiol, estrone and estriol were added to the standard incubations containing different fixed concentrations of the substrate, 17α-estradiol, in order to determine the extent of inhibition of 17α-estradiol-3-glucoside synthesis. These results were plotted according to the method of Dixon in an attempt to calculate the Ki of any of the added steroids and also to determine the type of inhibition involved. Since UDP is a product in the nucleotide dependent synthesis of 17α-estradiol-3-glucoside, similar studies were undertaken with UDP. All other assay and extraction procedures were exactly as described for the standard assay in the general methods, Chapter 2.

7) Effect of Metal Ions

The standard assay procedure was a slight modification of that described in the general methods, the only change being the addition of varying concentrations of magnesium chloride, manganous chloride, calcium chloride and sodium E.D.T.A. to the buffer. Ion concentrations rangingfrom 0 to 500mM were used.

8) Effect of Detergents

Six different detergents were examined for their effect on both the "UDP-glucose dependent" and the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside. In these studies the different detergents were added in the Tris buffer which represented a slight modification of the standard assay procedure described in the general methods. Cutscum, Tween 20 and Triton X-100 were examined using concentrations ranging from 0 to 0.2 per cent (w/v). The concentrations of sodium deoxycholate studied ranged from 0 to 0.15 per cent (w/v) while those of cetyltrimethylammonium bromide ranged from 0 to 0.5 per cent (w/v) and those of digitonin from 0 to 2 per cent (w/v).

9) Solubilization and Some Enzymatic Studies

a) Triton X-100 (9)

All the procedures for the solubilization and fractionation of the transferase activity were carried out in an ice water bath. A rabbit liver microsomal suspension (10 millilitres) in 0.15M KCl (10-12 milligrams of protein per millilitre) prepared as described in the general methods in Chapter 2 was diluted with 4 volumes of 0.2M Tris pH 7.0 and 0.1 millilitre of 100 per cent Triton X-100 to give a final Triton X-100 concentration of 0.2 per cent. This suspension was kept in ice for 30 minutes and then centrifuged at 105,000

X g (40,000 r.p.m., 40 K rotor) for 60 minutes. The supernatant was collected, concentrated in an Amicon cell to a volume of 10 millilitres and an aliquot of it examined for both "UDP-glucose dependent" and "UDP-glucose independent" transferase activities. The Triton X-100 precipitate was taken up in 10 millilitres of 0.15M KCl and an aliquot was again examined for each of the transferase activities. Blanks containing either boiled supernatant or precipitate were used where applicable.

b) Solubilization with Proteases (72)

The microsomal suspension was diluted with 4 volumes of 0.15M Tris - HCl pH 8.0 and incubated for 16 hours at 4°C in the presence of 0.03 milligram per millilitre of the protease, either alone or in the presence of 0.1 per cent Triton X-100 (w/v). The incubation mixture was then centrifuged at 105,000 X g (30,000 r.p.m., 30 K rotor) for 60 minutes. The supernatant was decanted and concentrated in an Amicon cell to the original volume of microsomal preparation used. The pellet was also re-suspended in a volume of 0.15M KCl so as to equal the volume of the microsomal preparation originally used. Both the precipitate and supernatant were assayed for "UDP-glucose dependent" and "UDP-glucose independent" glycosyltransferase activities.

c) Snake Venom, Phospholipase C and Phospholipase D Treatment (9, 72)

Both the 105,000 X g supernatant and precipitate, obtained after treatment of the diluted microsomal suspension with Triton X -100 in the absence of trypsin by the method of Collins et al, and the untreated microsomal suspension were treated with snake venom, phospholipase C and phospholipase D. The snake venom (Trimeresurus flavoviridis), which had previously been heated for 5 minutes at 100°C, was used at a concentration of 0.2 milligrams per millilitre in the Triton X-100 supernatant and precipitate incubations and at concentrations ranging from 0.05 to 0.2 milligram per 3 millilitres in the incubations containing untreated microsomal preparations. The phospholipases C and D were used at concentrations ranging from 0.02 to 0.1 milligram per millilitre. All the incubations were carried out in 0.15M Tris - HCl buffer pH 8.0 and 2.5×10^{-3}M calcium chloride. After 2 hours at 4°C the reactions were stopped by the addition of 0.5 millilitres of 0.15M EDTA, pH 8.0 and then assayed for the "UDP-glucose dependent and independent" transferase activities.

d) Treatment with phosphodiesterases

Since heating the crude snake venom, as described above in (c), inactivated the protease and phosphodiesterase activities of the preparation, some experiments were carried out

using both untreated and heated snake venom to assess the effect of the phosphodiesterase present in snake venom on both the "UDP-glucose dependent" and "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside.

The effect of pure phosphodiesterase on the transferase reaction was also examined using concentrations ranging from 3.3 to 32 micrograms per 3 millilitre assay. The conditions were similar to those employed for the snake venom phosphodiesterase studies. (9).

e) Treatment with Papain, Lysozyme and Neuraminidase

The microsomal suspensions were incubated with amounts of papain in 0.15M Tris - Hcl buffer pH 8.0 which gave a final concentration of enzyme equal to 50 or 250 micrograms per millilitre of incubation mixture and with amounts of lysozyme and neuraminidase in 0.066M potassium phosphate buffer pH 6.8 which gave a final enzyme concentration of 4 or 17 micrograms per millilitre of incubation mixture. All reaction mixtures were prepared in duplicate and each one incubated at either 37°C or 0°C for 2 hours. The incubation mixtures were then centrifuged for 60 minutes at 105,000 X g (40,000 r.p.m., 40 K rotor) in the Beckman L2-65B ultracentrifuge. The supernatant and the re-suspended pellet fractions were assayed for the "UDP-glucose dependent" and "UDP-glucose independent" transferase activities.

f) Treatment with Almond Emulsin and ∝-Glucosidase (217)

Microsomal preparations were pre-incubated with the glucosidases, and then assessed for their ability to form glycosides of 17∝-estradiol both in the presence and absence of UDP-glucose. The enzymes were added to the incubation mixture in 0.2M Tris pH 7.0. The glucosidases were added in quantities which gave a final concentration of either 1 or 2 milligrams per 3 millilitre standard assay.

10) Sonication, Temperature Sensitivity and Stability Studies

a) Sonication

Microsomal preparations were sonicated at an intensity of 30 in a Bronwill Biosonik^{R} III sonicator (Bronwill Scientific, Division of Will Scientific, Inc., Rochester, N.Y.) for varying lengths of time (0 to 5 minutes) and then assessed for their capacity for the "UDP-glucose dependent" and "UDP-glucose independent" synthesis of 17∝-estradiol-3-glucoside.

b) Temperature Sensitivity Studies

Microsomal preparations were pre-heated at 50°, 52° and 60°C for varying lengths of time ranging from 0 to 20 minutes and then assayed for their ability to form 17∝-estradiol-3-glycosides in the presence and absence of UDP-glucose.

c) Stability to Storage at -4°C

The microsomal preparations which were stored in 0.15M KCl at -4°C for time periods ranging from 0 to 40 weeks were assayed for their ability to form glucosides of 17α-estradiol.

11) The Involvement of Sugar Donors Other than UDP-Glucose in the "UDP-Glucose Independent" Synthesis of 17α-Estradiol-3-Glucoside

The experiments described in Chapter 3 confirmed that UDP-glucose was not the sugar donor in the "UDP-glucose independent" synthesis of steroid glucoside. Glucose, glucose-1-phosphate, glucose-6-phosphate and glycogen were examined for their ability to act as sugar donors in this reaction. In each experiment the assay procedure was a slight modification of that described in the general methods in Chapter 2, the only change being the addition of varying concentrations of the sugars to the buffer. The glycogen concentrations studied ranged from 0 to 500 micrograms per 2 millilitre assay, whereas those of glucose, glucose-1phosphate and glucose-6-phosphate ranged from 0 to 5 micromoles per 2 millilitre assay.

C) Results

1) pH Studies

The pH activity curves for the "UDP-glucose dependent" and "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside in Tris - maleate and sodium carbonate buffers are shown in (Figure 11). Activity in both cases showed a pH optimum between 7.0 and 7.2. All further assays were carried out at pH 7.0 using Tris - HCl buffer. Initially it was found that at higher pH values the free estradiol was not extracted completely into the benzene layer but instead was extracted into the ethyl acetate fraction as was the 17α-estradiol-3-glucoside due to ionization of the phenolic OH above pH 8.0. This gave false high values for glucoside synthesis. To eliminate this extraction problem, sufficient 1N HCl was added to the reaction mixture before extraction to lower the pH to 2.0.

2) Effect of Time of Incubation

(Figure 12) shows the time relation of the activity for both the "UDP-glucose dependent" and the "UDP-glucose independent" glucoside synthesis in rabbit liver microsomal preparations. 30 minutes allowed for optimum synthesis of 17α-estradiol-3-glucoside in both the "UDP-glucose dependent and independent" assay systems.

FIGURE 11

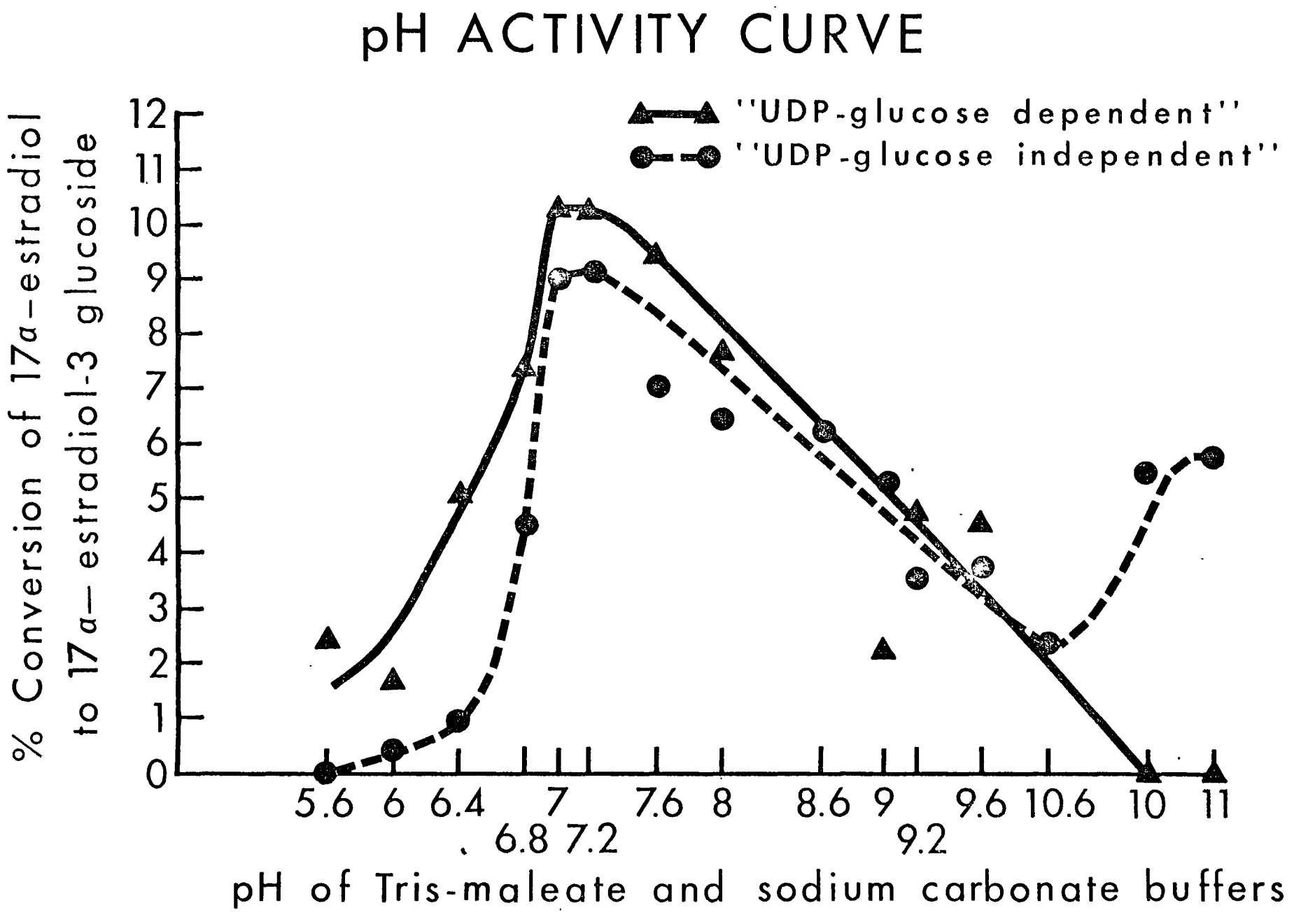
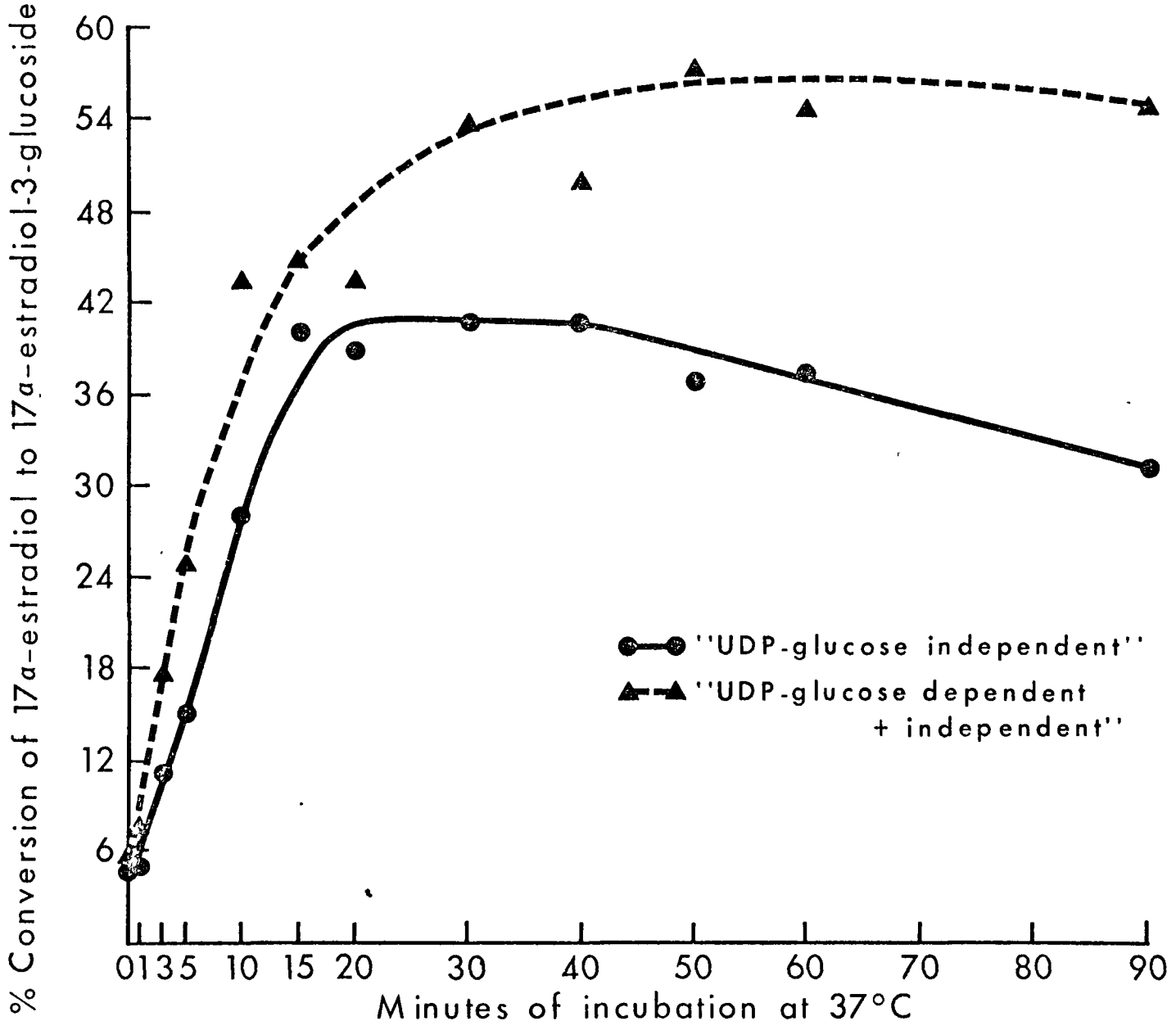

pH ACTIVITY CURVE
"UDP-glucose dependent"
"UDP-glucose independent"
% Conversion of 17a-estradiol to 17a-estradiol-3 glucoside
0
1
2
3
4
5
6
7
8
9
10
11
12
5.6
6
6.4
6.8
7
7.2
7.6
8
8.6
9
9.2
9.6
10.6
10
11
pH of Tris-maleate and sodium carbonate buffers

FIGURE 12

EFFECT OF TIME OF INCUBATION ON THE "UDP-GLUCOSE DEPENDENT" AND "UDP-GLUCOSE INDEPENDENT" SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE



3) Effect of Temperature

Both reactions had an optimum temperature of 37°C as illustrated in (Figure 13) and this temperature was used as the incubation temperature in all further studies.

4) Effect of Varying the Concentration of Microsomal Preparation

(Table 16) shows the effect of increasing the concentration of the microsomal preparation used on the amount of 17α-estradiol-3-glucoside synthesized by both the "UDP-glucose dependent and independent" synthetic pathways. Both pathways exhibit an increase in the amount of product formed on increasing the concentration of microsomal protein up to 0.1 millilitre per assay. The presence of 5 milligrams of microsomal protein allowed optimum synthesis of the glucoside and was used in all further studies.

5) Substrate Specificity Studies

The results of the substrate specificity studies are tabulated in a semi-quantitative fashion, ('+++' indicates the greatest production of conjugate and '–' no conjugate formation), in (Table 17). Estrone and 17α-estradiol were the best substrates in both the "UDP-glucose independent" and the UDP-glucose dependent" pathways of steroid glucoside synthesis. Estriol, which differs from the estradiol epimers and estrone

FIGURE 13

THE EFFECT OF THE INCUBATION TEMPERATURE ON THE "UDP-GLUCOSE DEPENDENT AND INDEPENDENT" SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE

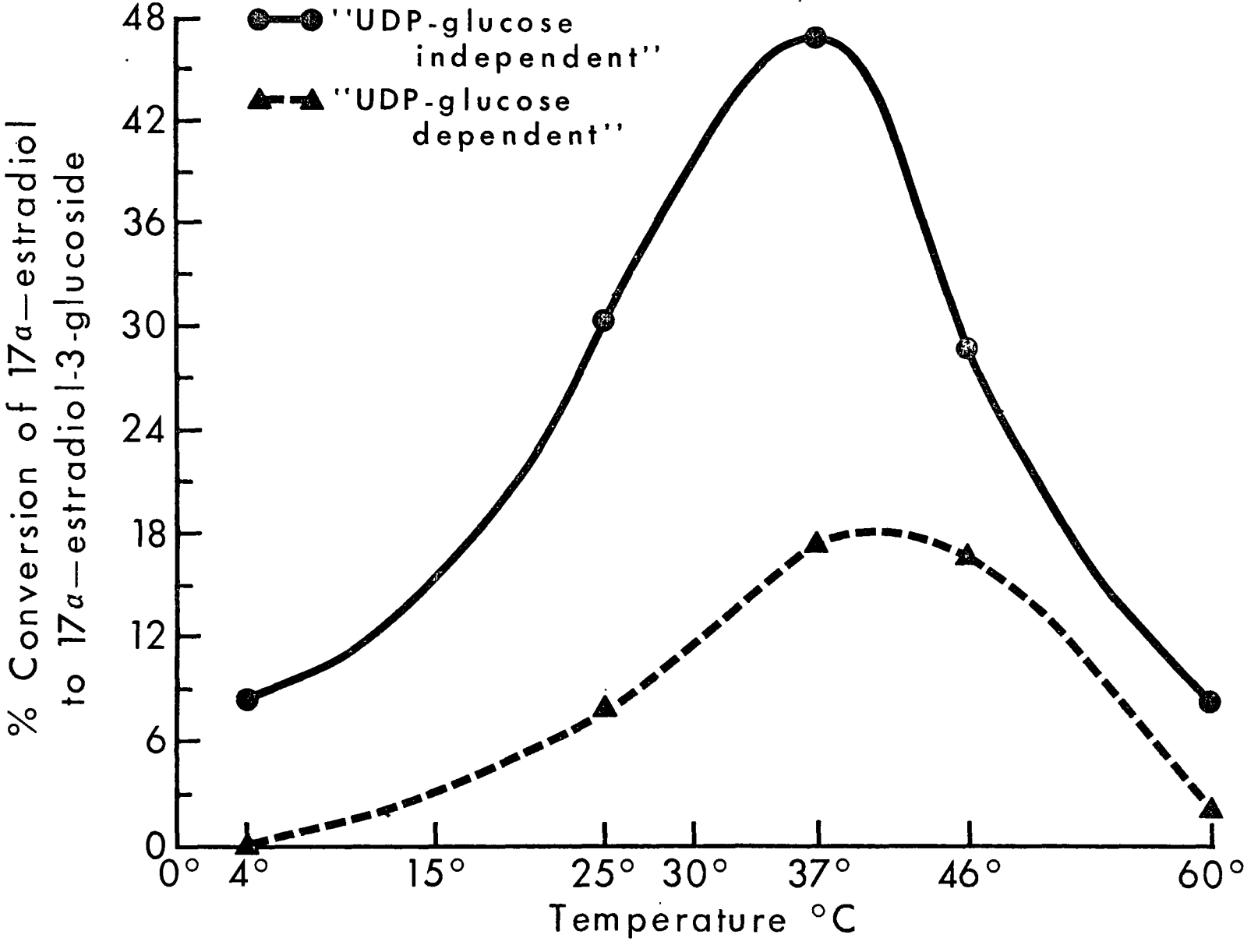
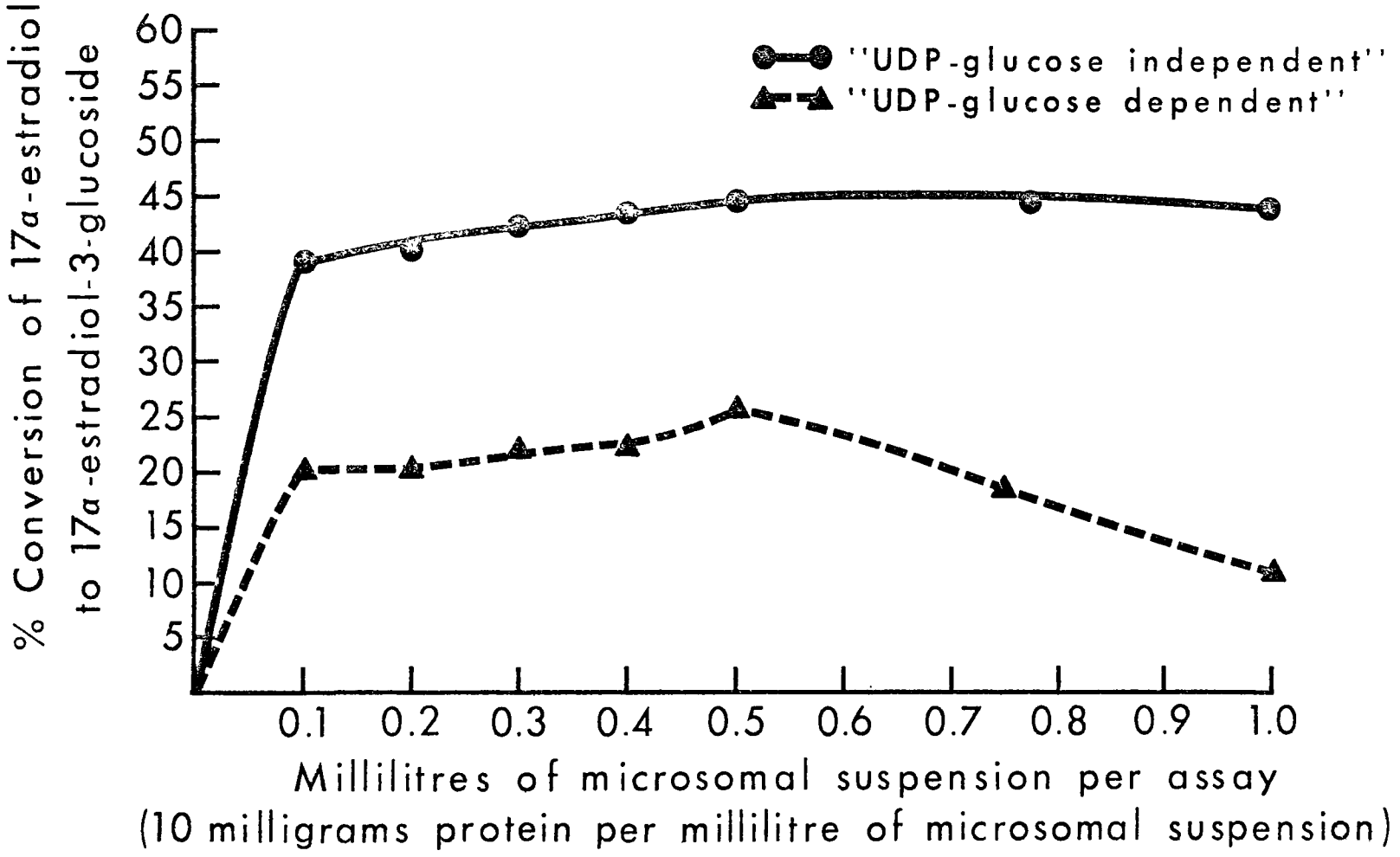


TABLE 16

THE EFFECT OF INCREASING THE CONCENTRATION OF THE MICROSOMAL PROTEIN ON THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE



A SEMI-QUANTITATIVE ESTIMATE OF GLUCOSIDE FORMATION WITH VARIOUS COMPOUNDS, +++ INDICATES MAXIMAL SYNTHESIS AND AND – INDICATES NO DEMONSTRABLE SYNTHESIS

Substrate	Uridine Nucleotide Added	
	UDP-Glucose	None
Estrone [^{3}H]	+++	++
17α-Estradiol [^{3}H]	+++	++
17β-Estradiol [^{3}H]	+++	++
Estriol [^{3}H]	–	–
17α-Estradiol-3-Glucuronide [^{3}H]	++	+
17β-Estradiol-3-Glucuronide [^{3}H]	–	–
p-Nitrophenol [^{14}C]	+	+
Biochanin A [^{14}C]	++	+
Genistein [^{14}C]	++	+
Formononetin [^{14}C]	++	+
Daidzein [^{14}C]	++	+
Pregnanolone [^{3}H]	–	–
17α-Hydroxypregnenolone [^{3}H]	–	–
Tetrahydrocortisone [^{3}H]	–	–
Testosterone [^{3}H]	–	–
Epitestosterone [^{3}H]	–	–
Diethylstilbestrol [^{14}C]	–	–

TABLE 17

only in the presence of a 16α-hydroxyl did not form glucosides. Diethylstilbestrol did not form a glucoside and the 17α but not the 17β-estradiol-3-glucuronide formed a double conjugate. The glucosides formed with these substrates and also with p-nitrophenol and the isoflavones were always produced in larger amounts when UDP-glucose was included in the incubation medium (7, 39, 40, 67). (Table 18) illustrates this in the case of the isoflavones. These glucosides, though formed in amounts insufficient to allow extensive characterization studies, had chromatographic properties similar to the conjugates produced in the presence of UDP-glucose, when examined by thin layer chromatography in two different solvent systems.

(Figure 14 and 15) show the Lineweaver - Burke and the Hanes plot for the determination of the Km towards the substrate 17α-estradiol. When the "UDP-glucose dependent" reaction was being examined UDP-glucose was present in excess. No difference in the Km towards 17α-estradiol could be demonstrated for the "UDP-glucose dependent" or the "UDP-glucose independent" transferases. The Km value as calculated from the Lineweaver -Burke plot was 1.67×10^{-7} M whereas that calculated from the Hanes plot was 1.99×10^{-5} M.

TABLE 18

FORMATION OF ISOFLAVONE GLUCOSIDES

Substrate	% Conversion of 17 α-Estradiol to 17 α-Estradiol-3-glucoside	
	UDP-glucose independent	UDP-glucose dependent + independent
Biochanin A	6.5	27.8
Genistein	7.3	31.4
Formononetin	8.4	27.7
Daidzein	4.7	14.4

FIGURE 14

LINEWEAVER-BURKE PLOT FOR THE DETERMINATION OF THE Km FOR THE GLUCOSYL TRANSFERASE ACTIVITY TOWARDS 17α-ESTRADIOL

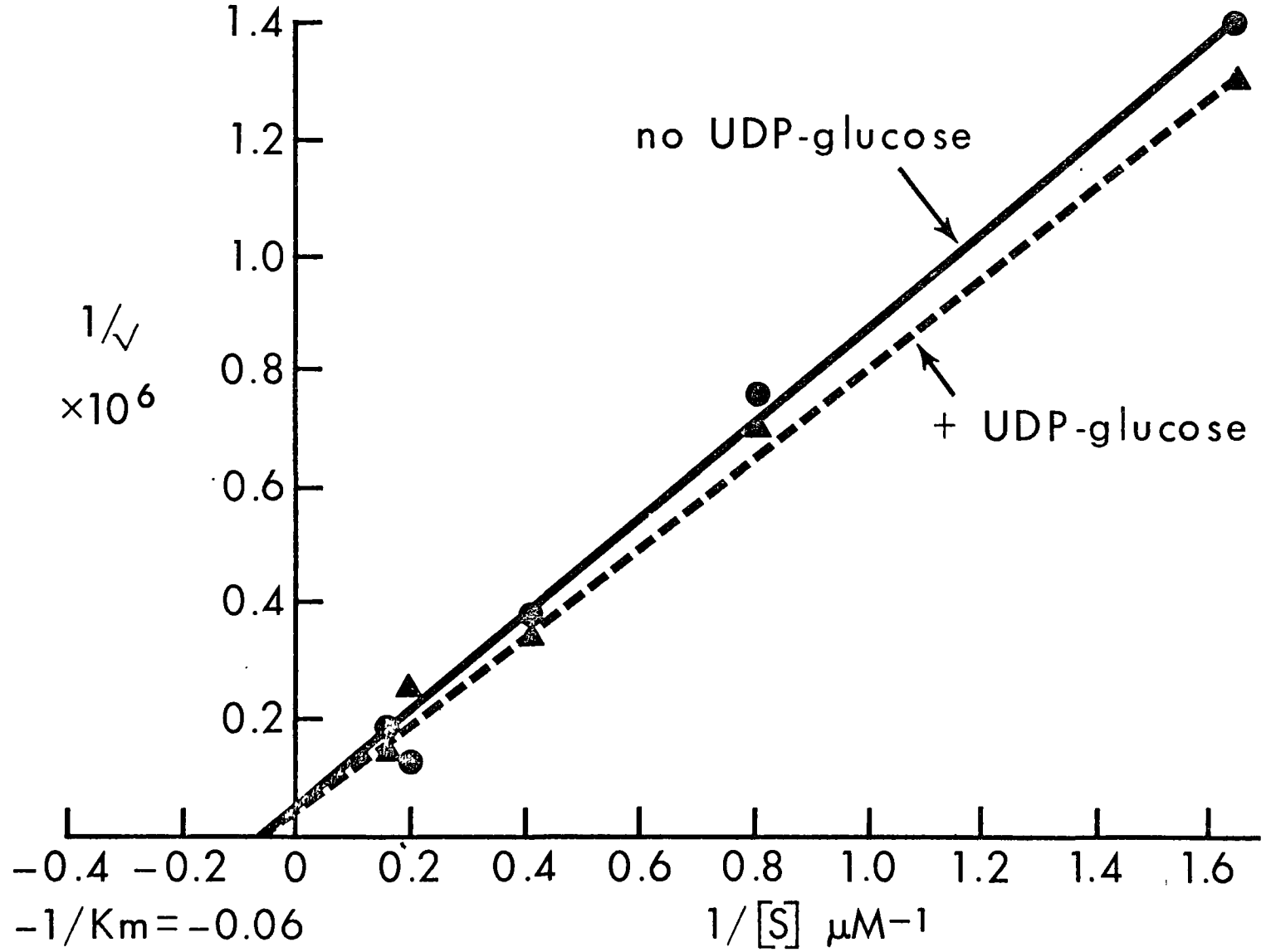
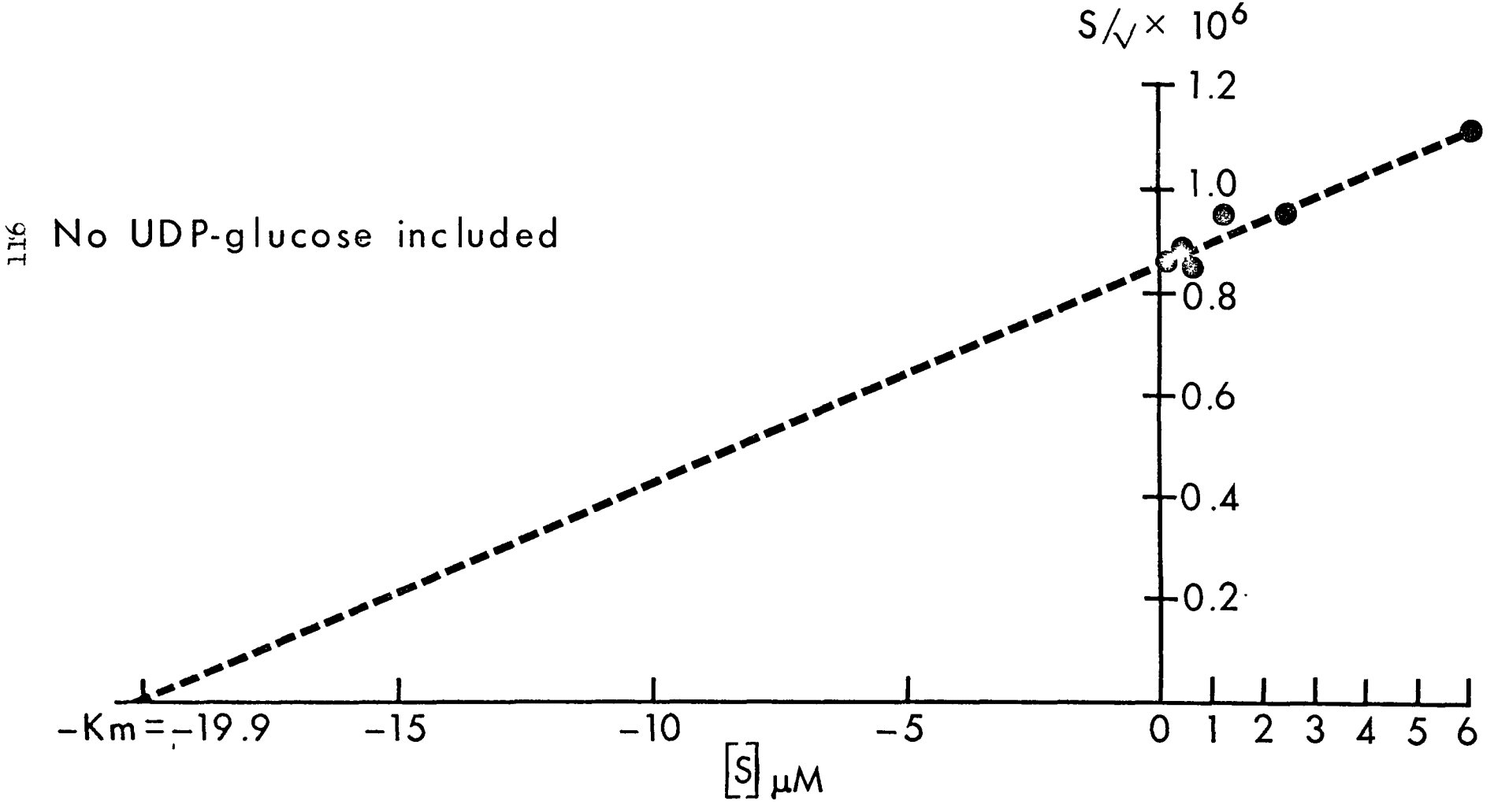


FIGURE 15

HANES PLOT FOR THE DETERMINATION OF THE Km FOR THE GLUCOSYL TRANSFERASE ACTIVITY TOWARDS 17α-ESTRADIOL



7) Effect of Various Inhibitors

Methanol did not inhibit glucoside synthesis to any significant extent even when present at a concentration of 1.35 millimoles per 3 millilitre assay. Ethanol and n-butanol were more effective inhibitors whilst ethylene glycol did not inhibit glucoside formation. Eugenol was the most potent inhibitor of the alcohols examined and almost completely abolished both the "UDP-glucose dependent and independent" synthesis of 17α-estradiol-3-glucoside at a concentration of 12.5 micromole per 3 millilitre assay. These results are shown in (Table 19) All the steroids examined except estriol inhibited glycoside formation. 17β-estradiol and estrone, which are substrates of the 17α-estradiol-3-glycosyltransferase, inhibited it competitively when the results were plotted according to Dixon. However, the lines representing activity at three different substrate concentrations did not intersect except in the case of the "UDP-glucose independent" studies with 17β-estradiol as the inhibitor. It was thus impossible to calculate the Ki with any degree of accuracy, (Figure 10). Diethylstilbestrol inhibited glucoside formation even though in the studies described earlier it appeared not to act as a substrate. The 17α-estradiol monoglucosides were weak inhibitors of the glucosyltransferase, but from the results obtained the type of inhibition could not be determined. TTP was the only nucleotide shown to cause any significant decline in 17α-estradiol-3-glucoside formation. The results seen in (Table 19) and

THE EFFECT OF VARIOUS COMPOUNDS ON THE GLUCOSYL-TRANSFERASE ACTIVITY OF RABBIT LIVER MICROSOMES

Compound Added to the Standard Incubation	% Inhibition of 17α-Estradiol-3-Glucoside Synthesis at the Highest Concentration of Inhibitor Examined	
	−UDP-Glucose (Ind.)	±UDP-Glucose (Ind. + Dep.)
Methanol (1.35 millimoles)	4.3	8.7
Ethanol (750 micromoles)	42.8	5.7
Butanol-1 (450 micromoles)	18.7	23.9
Ethylene glycol (600 micromoles)	0.5	0
Eugenol (12.5 micromoles)	93.2	75.7
UTP (0.5 micromoles)	8.4	9.9
UDP(0.5 micromoles)	9.3	5.3
(5 micromoles)	59.1	56.1
ATP (0.5 micromoles)	0	0
ADP	0	11.1
AMP	0	0
GTP	0	12.1
GDP	0	0
GMP	6.4	0
CTP	4.6	0
CMP	3.9	0
TTP	28.2	28.3
TMP	6.3	2.0

TABLE 19

DIXON PLOT FOR THE DETERMINATION OF THE Ki FOR 17β-ESTRADIOL FOR THE 3-GLUCOSYLTRANSFERASE ACTIVITY

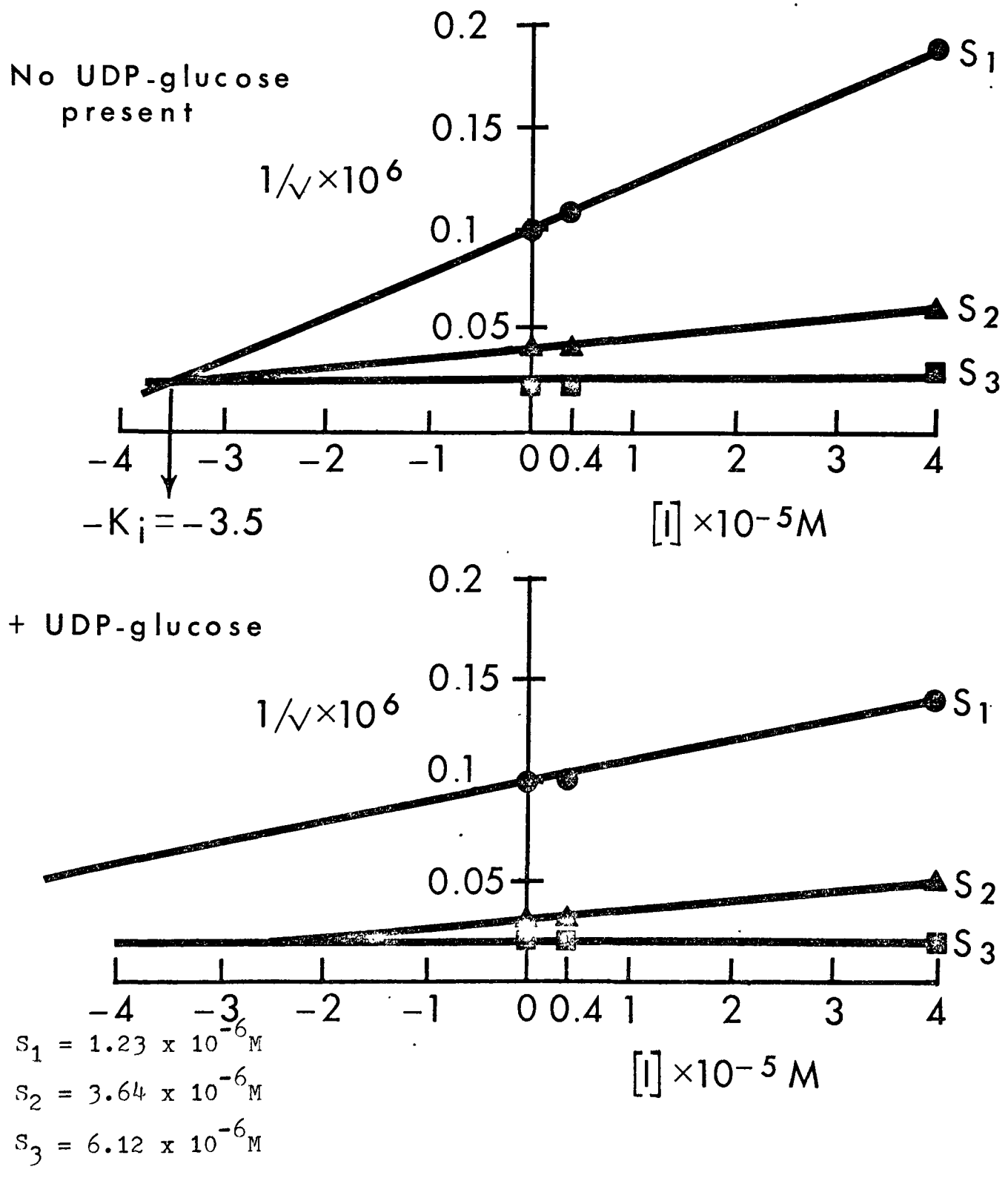


FIGURE 16

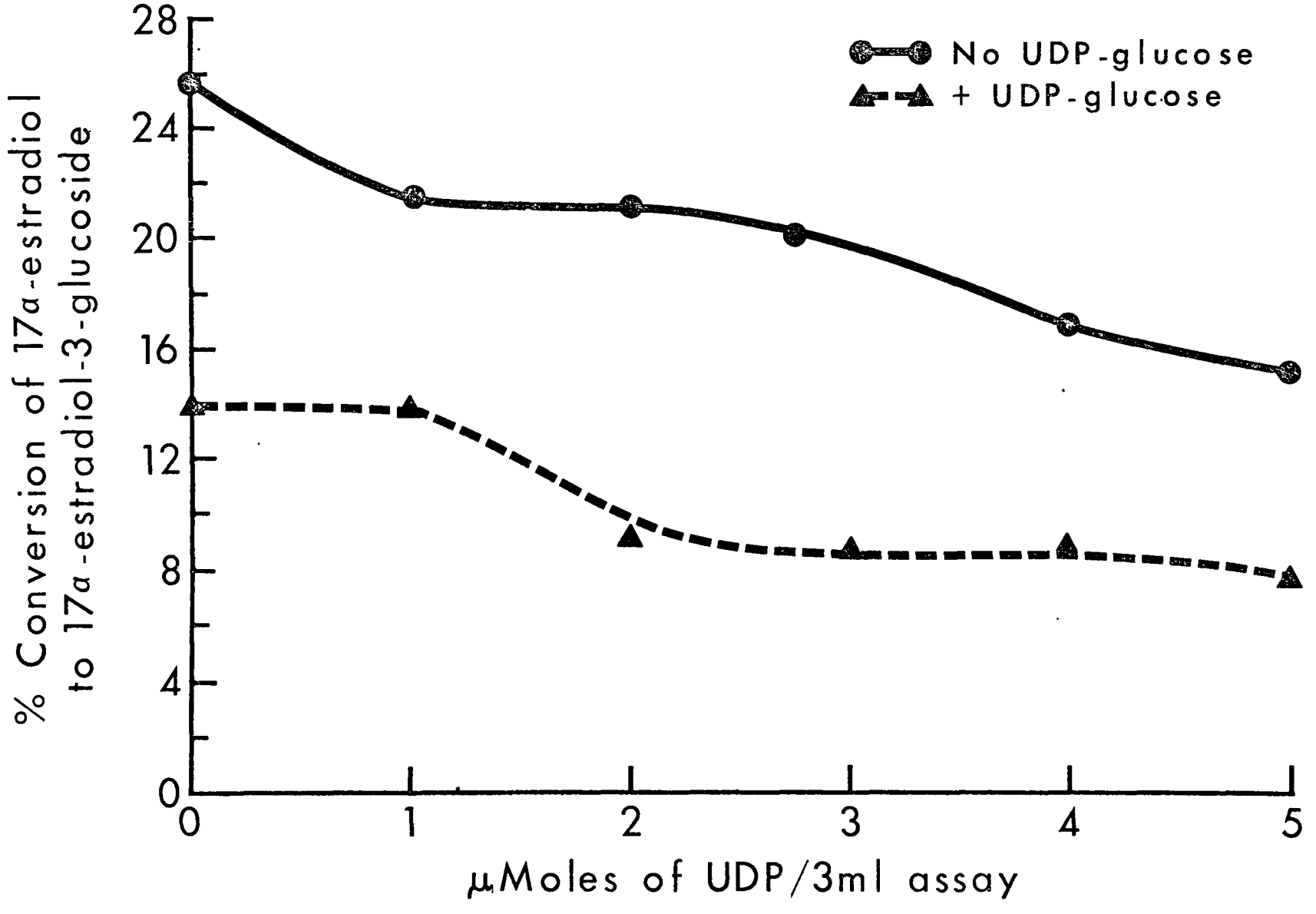
(Figure 17), showed that where inhibition did occur, the "UDP-glucose independent" pathway was usually more susceptible to inhibition than was the "UDP-glucose dependent" pathway. The pattern of inhibition did not give any definite indication as to why the nucleotides might exert such an effect. The results obtained when various concentrations of UDP were examined, (Figure 17), did not fit a Dixon plot so no Ki values were calculated. The sulphydryl reagents, p-chloromercuribenzoate and dithiobisdinitrobenzoic acid, inhibited the synthesis of 17α-estradiol-3-glucoside at a concentration of 16.7×10^{-6}M. Both the "UDP-glucose dependent and independent" pathways were susceptible to inhibition, the "UDP-glucose independent" being somewhat more sensitive to inhibition by dithiobisdinitrobenzoic acid. p-Nitrophenol and phenolphthalein were effective inhibitors of 17α-estradiol-3-glucoside synthesis, (Table 20), as were the protein denaturants, urea and guanidine - HCl when present at concentrations of 12 and 15 millimoles per 3 millilitre assay.

8) Effect of Metal Ions

Increasing concentrations of magnesium chloride, manganous chloride and calcium chloride caused a stimulation of the "UDP-glucose independent" pathway leading to 17α-estradiol-3-glucoside synthesis whereas the "UDP-glucose dependent" pathways was almost completely inhibited by these same ions.

FIGURE 17

THE EFFECT OF INCREASING CONCENTRATIONS OF UDP ON THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE



THE EFFECT OF VARIOUS COMPOUNDS ON THE
GLUCOSYLTRANSFERASE ACTIVITY OF RABBIT LIVER MICROSOMES

Compound Added to the Standard Incubation	% Inhibition of 17α-Estradiol-3-Glucoside Synthesis at the Highest Concentration of Inhibitor Examined	
	−UDP-Glucose (Ind.)	+UDP-Glucose (Ind. + Dep.)
Estrone (120 nanomoles)	35.7	30.0
17β-Estradiol (120 nanomoles)	21.4	26.7
Estriol (120 nanomoles)	0	0
17α-Estradiol-3-Glucoside (3 nanomoles)	9	13.1
17α-Estradiol-17-Glucoside (3 nanomoles)	6.4	9.7
Diethylstilbestrol (1.2 micromole)	26.9	9.3
p-Nitrophenol (0.3 micromoles)	93.3	95.3
Phenolphthalein (3 micromoles)	83.1	93.3
p-Chloromercuribenzoate (0.05 micromoles)	10.9	13.1
Dithiobisdinitrobenzoic acid (0.05 micromoles)	13.3	5.1
Urea (12 millimoles)	100	100
Guanidine-Hcl (15 millimoles)	60.5	79.5

TABLE 20

(Figure 18, 19, and 20) illustrate this effect. When concentrations of sodium EDTA ranging from 0 to 50mM were studied the effect on the two pathways leading to 17∝-estradiol-3-glucoside synthesis, was partially inhibitory, but the degree of inhibition varied. The combined effect of some of these ions was also examined. When manganous chloride and calcium chloride were studied, at concentrations ranging from 0 to 100mM, together with a fixed concentration of magnesium chloride, there was no significant change in the amount of 17∝-estradiol-3 -glucoside synthesis seen when two cations were present as compared to that seen when magnesium chloride was present alone. However, the presence of increasing concentrations of EDTA at a fixed concentration of magnesium chloride, caused almost complete loss of the ability to synthesize 17∝-estradiol-3-glucoside by either the "UDP-glucose dependent or independent" pathways. This inhibition of glucoside synthesis by EDTA in the presence of magnesium chloride was much greater than that seen when EDTA alone was added to the standard incubation mixture. These results are illustrated in (Figure 21).

9) Effect of Detergents

The "UDP-glucose independent" synthesis of 17∝-estradiol-3-glucoside was more susceptible to inhibition by all six detergents studied. The results are depicted in (Figure 22-26). At a concentration of 0.15 per cent deoxycholate, the "UDP-

FIGURE 18

THE EFFECT OF INCREASING CONCENTRATIONS OF MAGNESIUM CHLORIDE ON 17α-ESTRADIOL-3-GLUCOSIDE SYNTHESIS

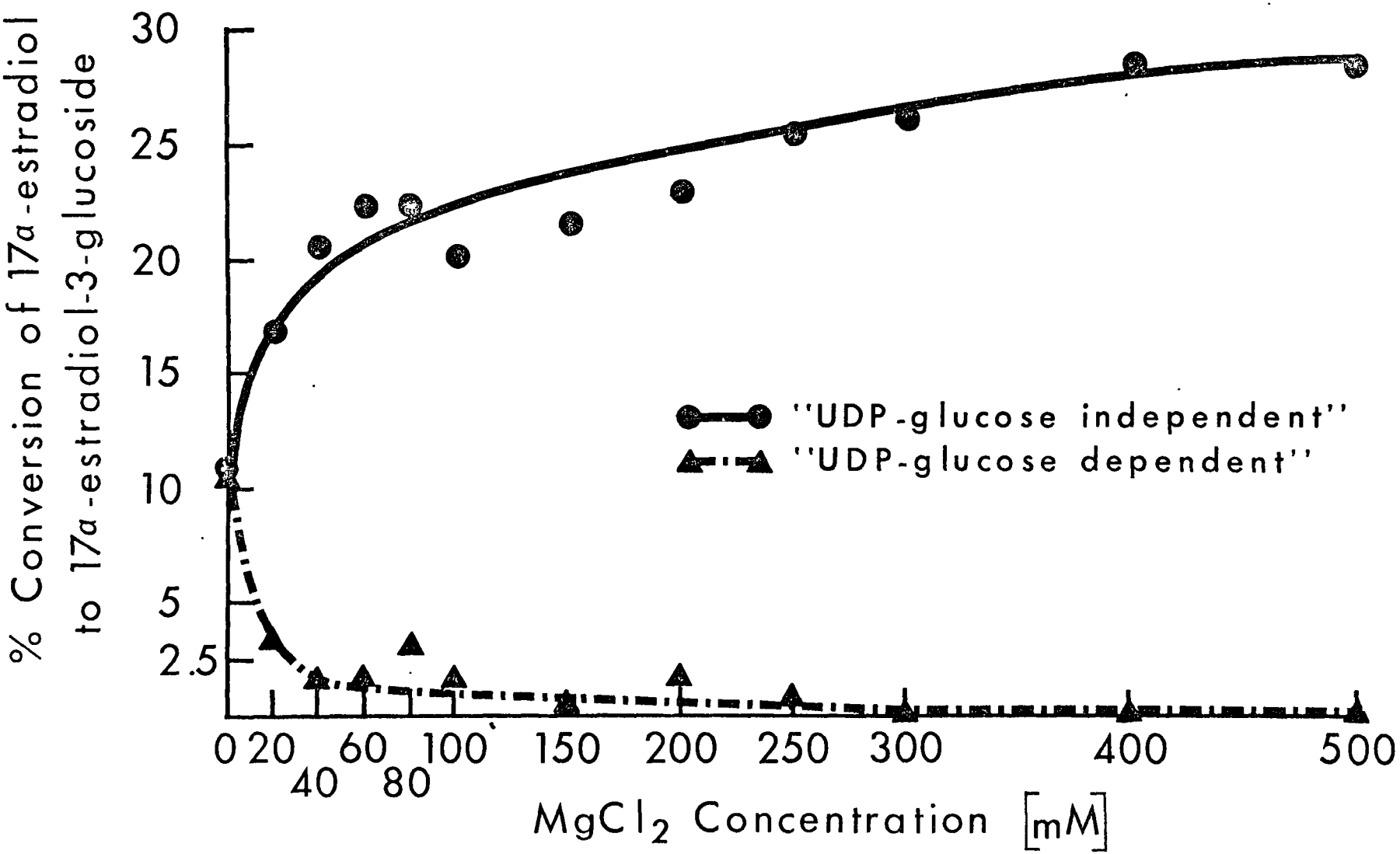


FIGURE 19

THE EFFECT OF INCREASING CONCENTRATIONS OF CALCIUM CHLORIDE ON 17α-ESTRADIOL-3-GLUCOSIDE SYNTHESIS

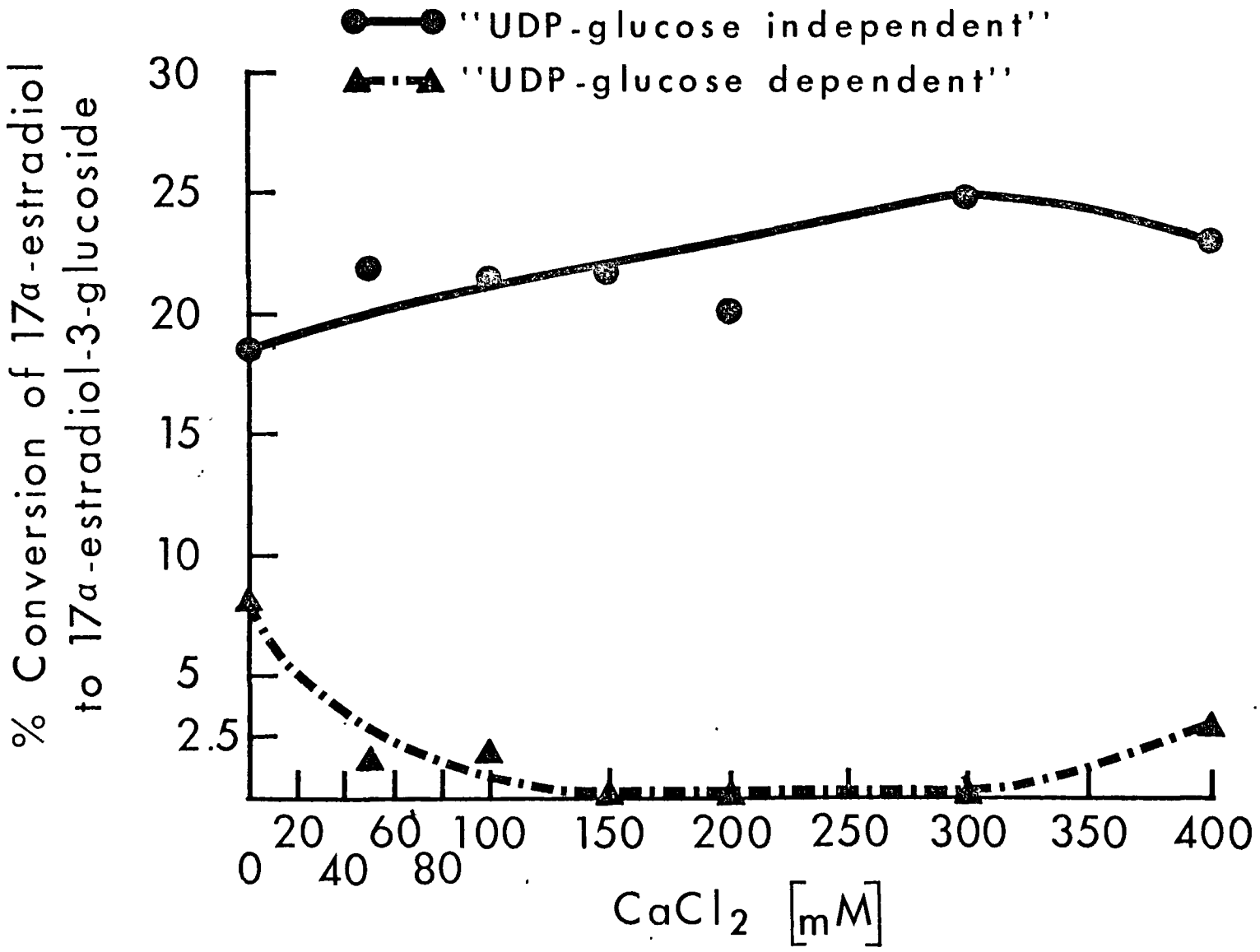


FIGURE 20

THE EFFECT OF INCREASING CONCENTRATIONS OF MANGANOUS CHLORIDE ON THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE

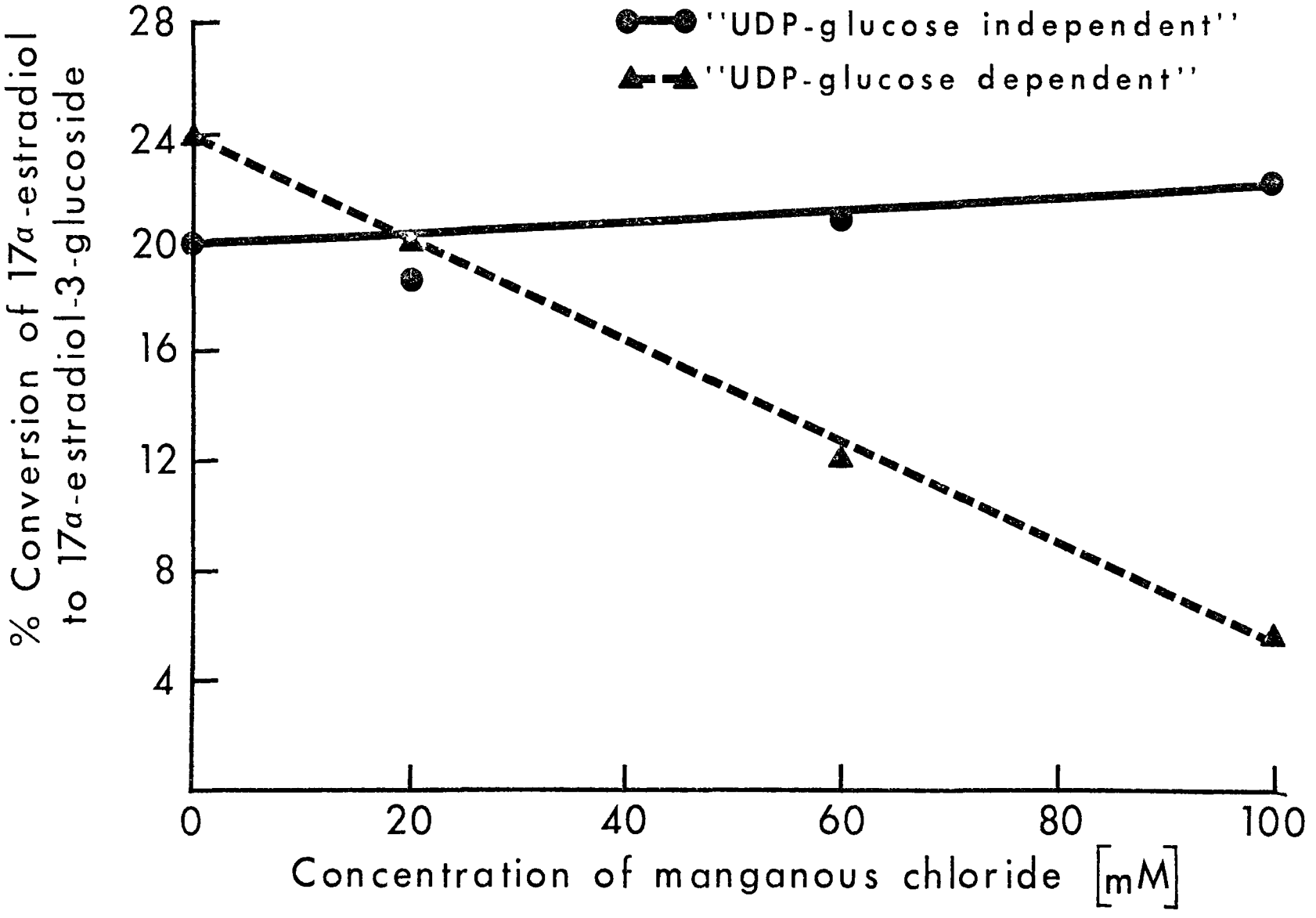


FIGURE 21

THE EFFECT OF INCREASING CONCENTRATIONS OF EDTA ON 17α-ESTRADIOL-3-GLUCOSIDE SYNTHESIS WHEN THE STANDARD ASSAY ALSO CONTAINED 250mM $MgCl_2$

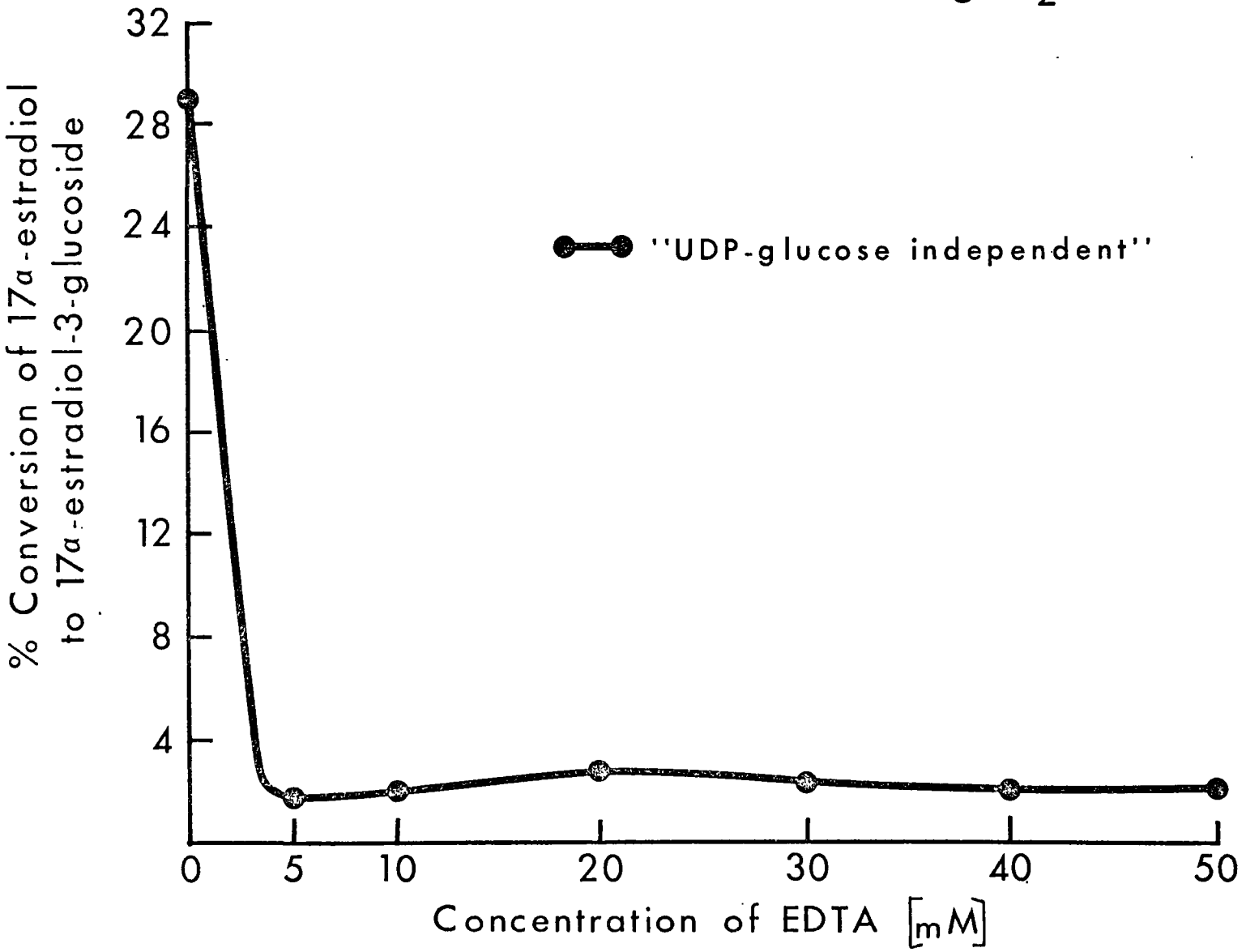


FIGURE 22

THE EFFECT OF INCREASING CONCENTRATIONS OF DEOXYCHOLATE ON THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE

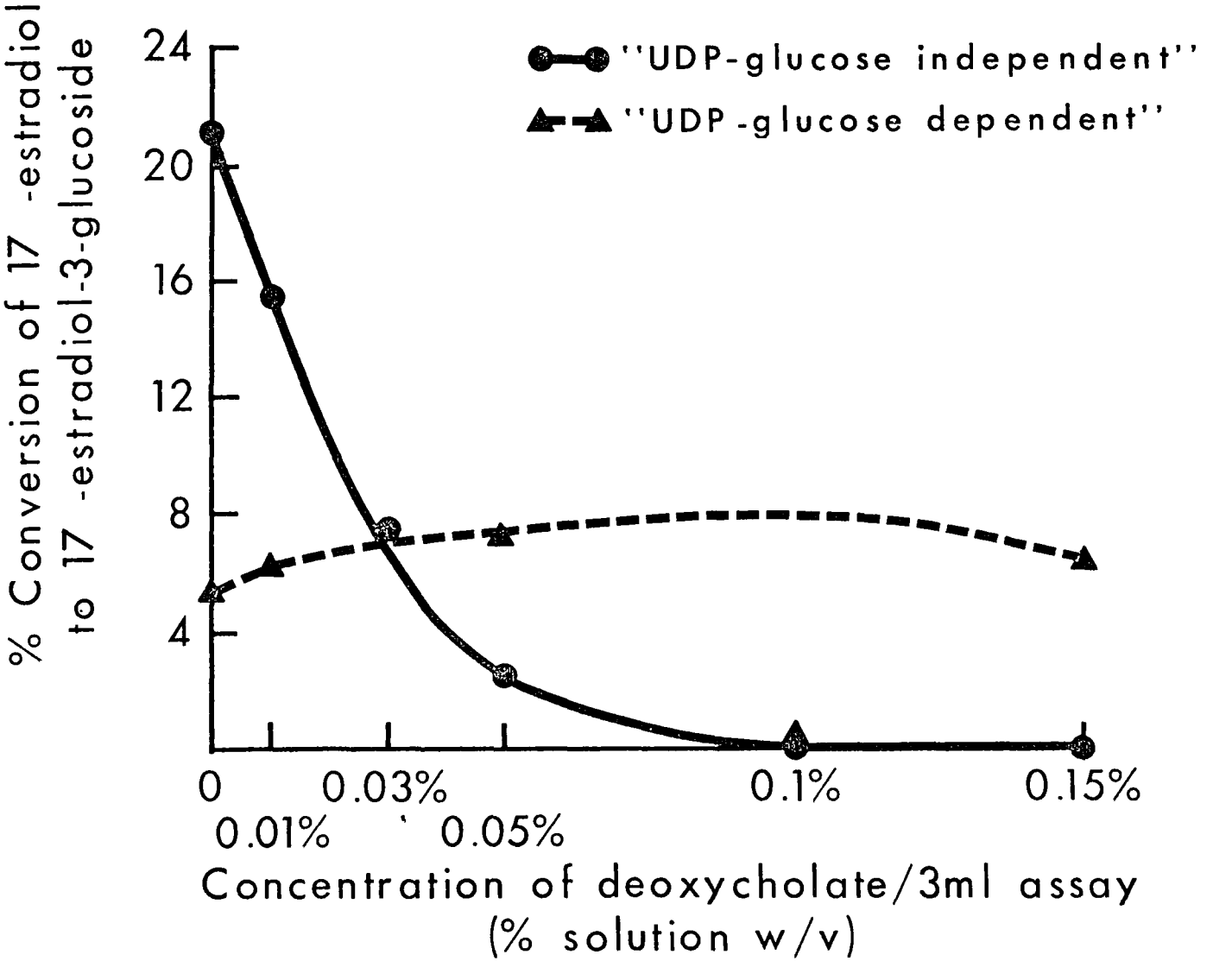


FIGURE 23

THE EFFECT OF INCREASING CONCENTRATIONS OF TWEEN 20 ON THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE

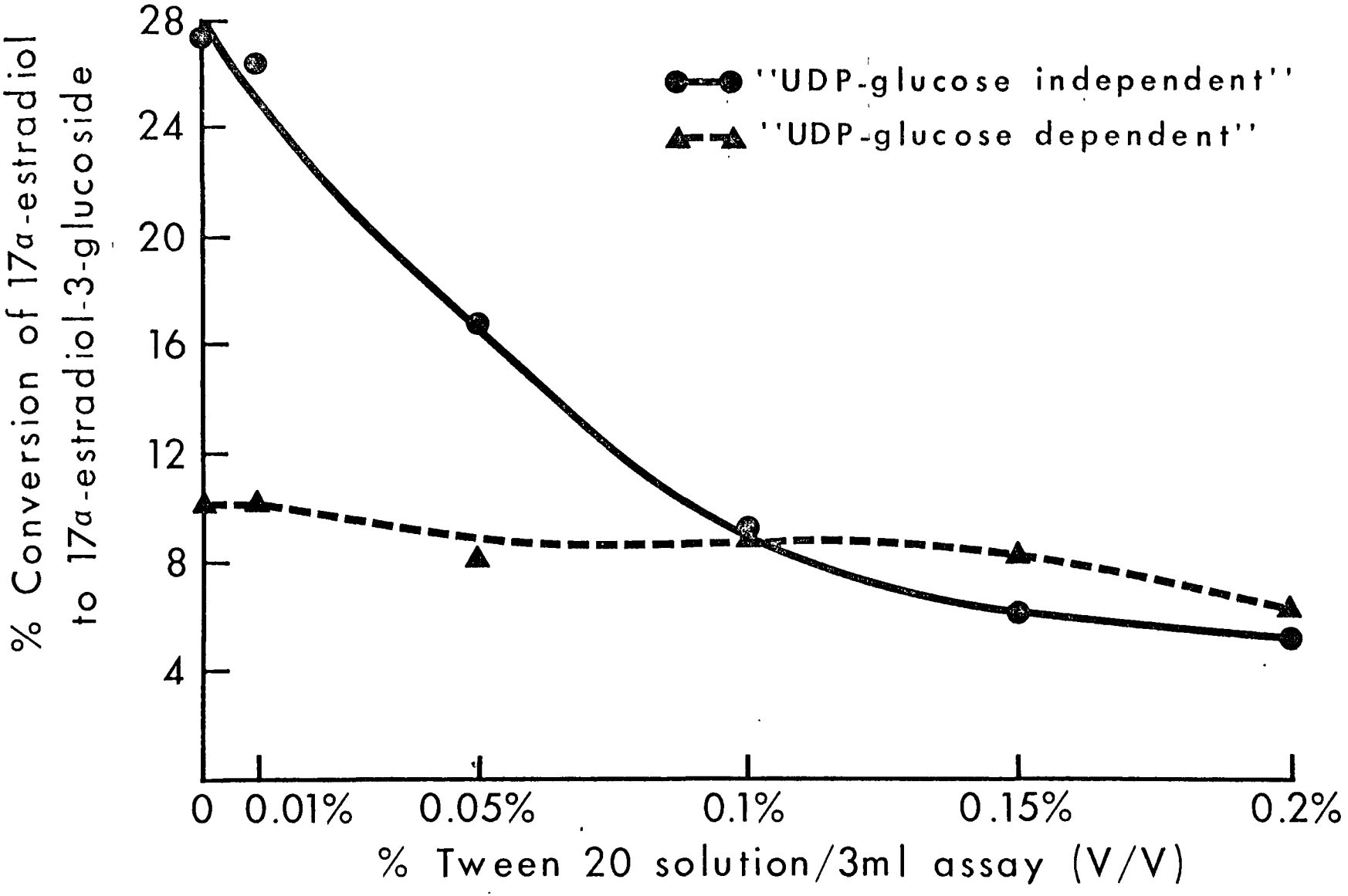


FIGURE 24

THE EFFECT OF INCREASING CONCENTRATIONS OF TRITON X-100 ON THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE

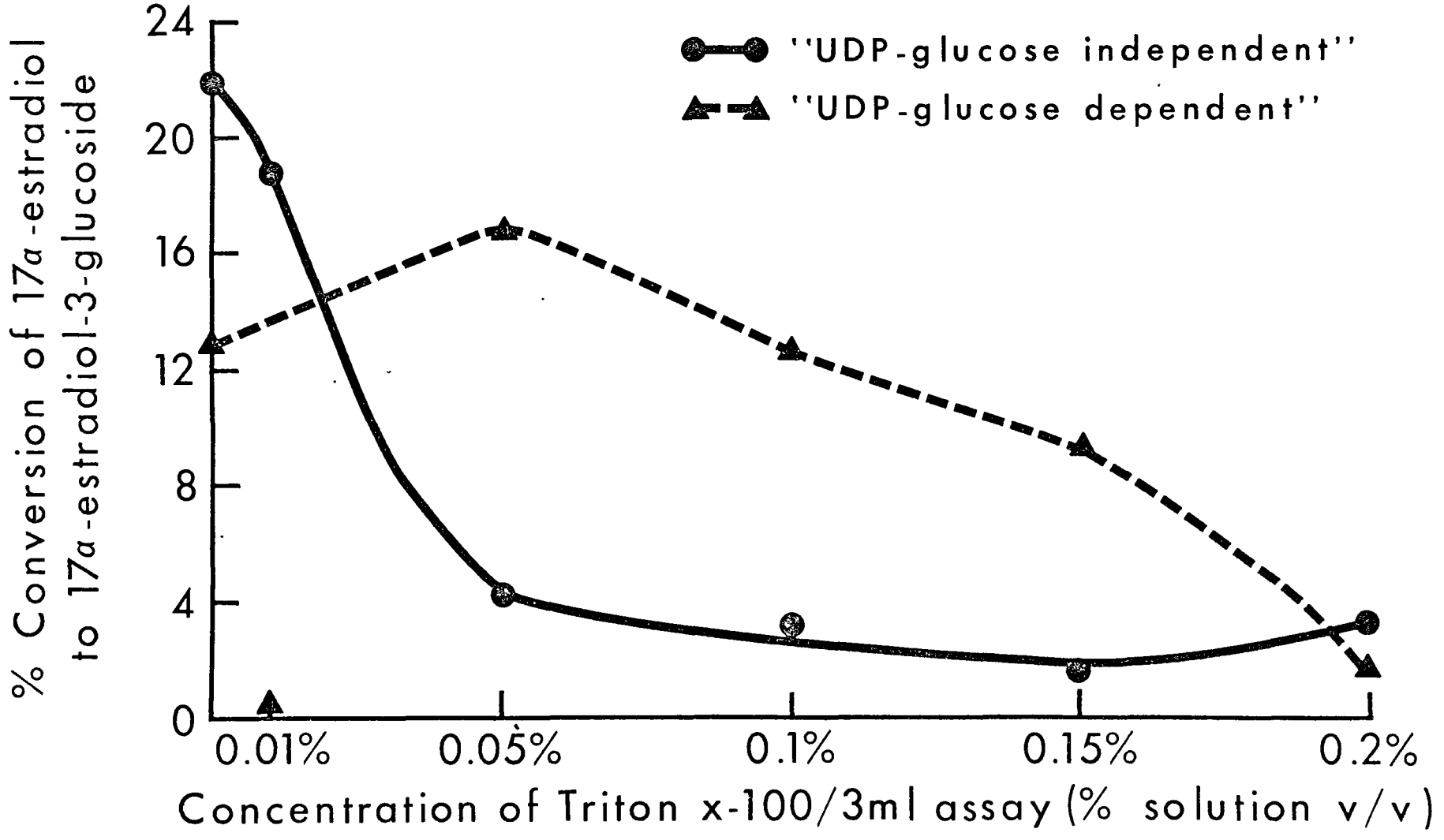


FIGURE 25

THE EFFECT OF INCREASING CONCENTRATIONS OF CETYLTRIMETHYL AMMONIUM BROMIDE ON THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE

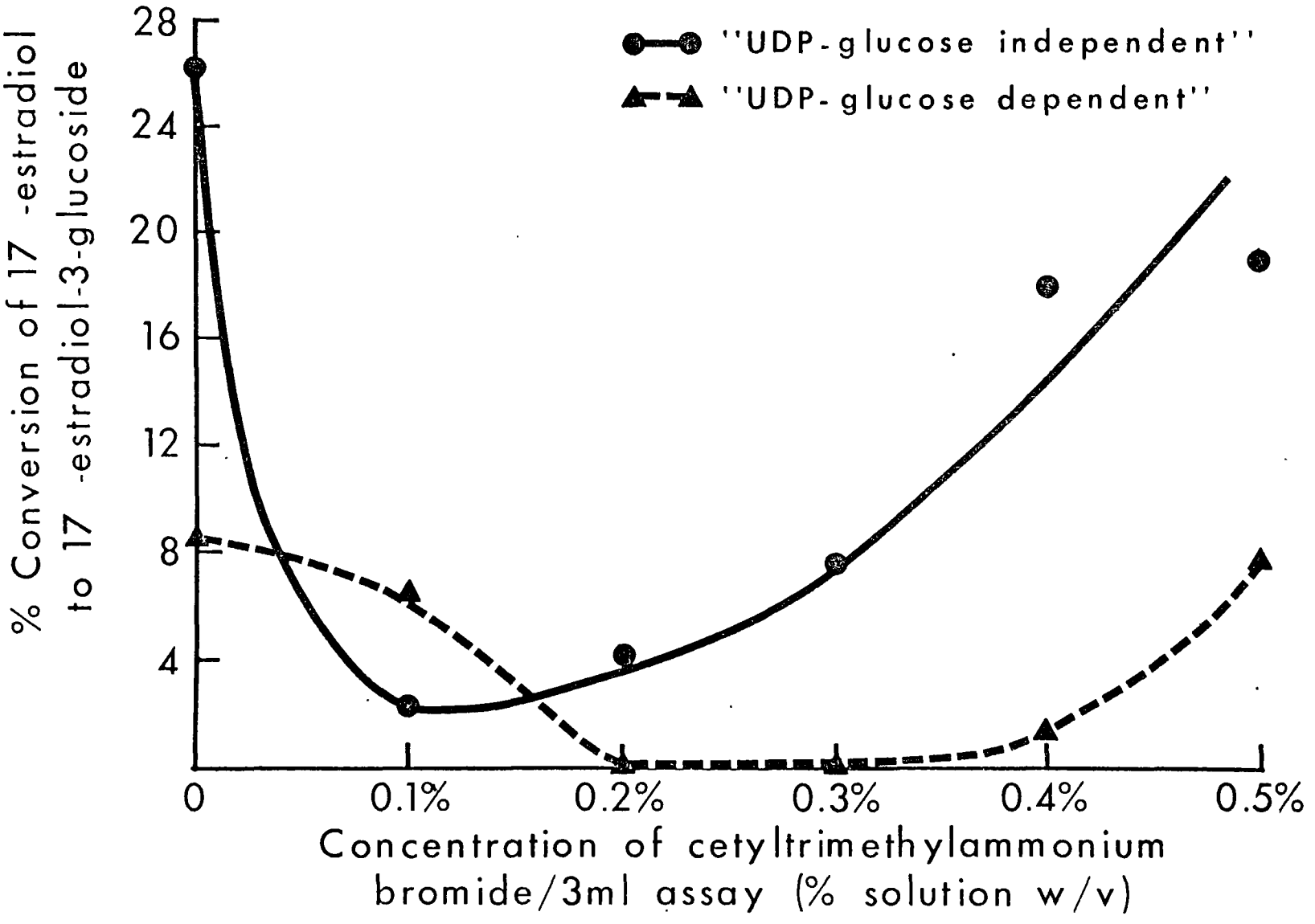
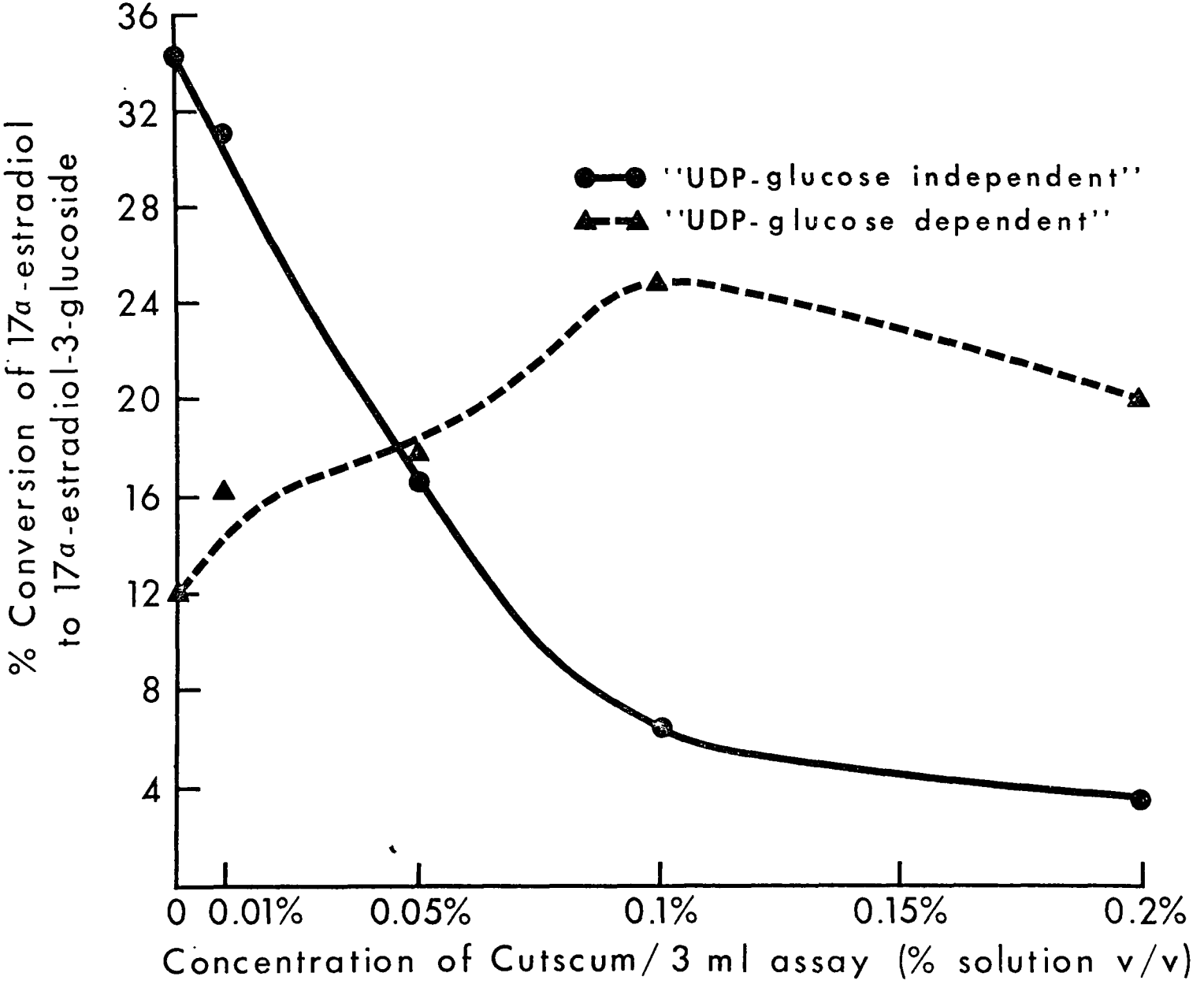


FIGURE 26

THE EFFECT OF INCREASING CONCENTRATIONS OF CUTSCUM ON THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE



glucose independent" synthesis of 17α-estradiol-3-glucoside was completely inhibited, whereas the "UDP-glucose dependent" pathway was slightly stimulated. At a concentration of 0.2 per cent Tween 20 the "UDP-glucose independent" pathway was 82 per cent inhibited whereas the "UDP-glucose dependent" pathway was only 39 per cent inhibited. The presence of increasing concentrations of digitonin caused a slight inhibition of both pathways leading to 17α-estradiol-3-glucoside synthesis. At a concentration of 0.15 per cent Triton X-100 the "UDP-glucose independent" pathway was inhibited 92. per cent whereas the "UDP-glucose dependent" pathway was only 28. per cent inhibited. The pattern of inhibition seen with cetyltrimethylammonium bromide differed from that seen in the other detergent studies, but was quite reproducible. At low cetyltrimethylammonium bromide concentrations the "UDP-glucose independent" and the "UDP-glucose dependent" pathways were inhibited to a much greater extent than at concentrations greater than 0.3 per cent. As in the other detergent studies discussed above the "UDP-glucose independent " pathway leading to 17α-estradiol-3-glucoside synthesis was more susceptible to inhibition by cetyltrimethylammonium bromide than was the "UDP-glucose dependent"pathway. Some interesting results were obtained when the effect of Cutscum was examined. These are shown in (Figure 26). At a concentration of 0.2 per cent Cutscum the "UDP-glucose independent" pathway was 90 per cent inhibited whereas the "UDP-glucose dependent" pathway was 67

per cent stimulated.

Much of the later work with lipids, which will be discussed in Chapter 5, involved the detergent Triton X-100. (Table 21) depicts some of the results obtained when the effect of Triton X-100 on the "UDP-glucose independent" reaction was studied at different times during 1975 and 1976. The inconsistency and the variation in the extent of inhibition seen, as well as the stimulation seen in some preparations is interesting and reminiscent of the variation in the levels of the "UDP-glucose independent" and "UDP-glucose dependent" 17α-estradiol-3-glucoside synthesis depicted in (Table 7) in Chapter 3. This effect seems to depend on the rabbit preparation used, which suggests that the concentration of lipid in the microsomal preparation varies from rabbit to rabbit, and maybe even from season to season. A study of the lipid content of each preparation was not undertaken since this would have been a very complex problem both quantitatively and qualitatively. However, Inque and Kitagawa have reported that the sensitivity of the membranes to Triton X-100 does indeed depend on the composition of the lipids present in the membrane (218).

10) Solubilization and Some Enzymatic Studies

a) Triton X-100

The Triton X-100 solubilization studies provided some interesting results which are depicted in (Table 22). The

THE EFFECT OF TRITON X-100 ON THE "UDP-GLUCOSE INDEPENDENT" SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE

	% Conversion of 17 α -Estradiol to 17 α -Estradiol-3-Glucoside Concentration of Triton-X-100/3 Millilitre Assay (% Solution V/V)											
	0	0.01	0.04	0.05	0.08	0.1	0.12	0.15	0.16	0.2	0.3	0.5
17 Jul 75	22.1	18.8		4.2		3.2		1.6		3.4		
19 Jan 76	36.1									3.2		
22 Mar 76	7.8									0.74		
4 Oct 76	18.9									22.8		
14 Oct 76	24.2									26.1		
19 Oct 76	17.8		18.6		14.1		14.9		13.5	12.4	10.7	9.3
25 Oct 76	13.7		13.3		13.2		10.9		9.8	9.0	7.1	7.5
26 Oct 76	20.75		18.0		16.5		14.8		14.9	12.7	11.4	9.2

TABLE 21

SOLUBILIZATION WITH TRITON X-100

Concentration of Triton X-100	% Conversion of 17α-Estradiol to 17α-Estradiol-3-Glucoside			
	UDP-Glucose Independent		UDP-Glucose Dependent	
Microsomal Suspension	15.6		12.4	
	Supernatant	Pellet	Supernatant	Pellet
0.05%	1.2	16.8	1.8	4.8
0.1%	0.8	15.0	7.1	2.0
0.15%	0.7	12.6	6.3	1.4
0.2%	1.1	9.6	2.7	1.4

TABLE 22

100,000 X g soluble fractions could only form steroid glucosides when fortified with UDP-glucose, while the ability to form the glucoside without the addition of UDP-glucose was retained in the Triton X-100 insoluble pellet. At a concentration of 0.15 per cent Triton X-100, the microsomal pellet, retained 80.8 per cent of the untreated microsomal suspension's capacity for the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside, but only 11.1 per cent of the microsomes' capacity for the"UDP-glucose dependent" synthesis of the steroid glucoside. However, at the same detergent concentration, the supernatant fraction retained only 4.4 per cent of the microsomes' capacity for the "UDP-glucose independent" glucoside synthesis, but on the addition of UDP-glucose exhibited 56.6 per cent of the microsomes' capacity for the UDP-glucose dependent synthesis of 17α-estradiol-3-glucoside.

b) Solubilization with proteases

(Table 23) shows the results obtained in the solubilization studies with the proteases, trypsin and chymotrypsin. Both the "UDP-glucose independent" and the "UDP-glucose dependent" synthesis of 17α-estradiol-3-glucoside were inhibited by treatment with these enzymes alone or in the presence of Triton X-100. The "UDP-glucose independent" pathway was more susceptible than the "UDP-glucose dependent" pathway to inhibition, being completely inhibited by chymotrypsin, Triton X-100-trypsin

and Triton X-100-chymotrypsin treatment. Despite the ready solubilization by treatment with Triton X-100, as illustrated in (Tables 22 and 23), the "UDP-glucose dependent" glucosyltransferase activity was not solubilized to any significant extent by this protease treatment, except when Triton X-100 was also present.

c) Snake venom, phospholipase C and D treatment

Both the "UDP-glucose dependent" and the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside was inhibited by increasing concentration of snake venom which had been pre-heated in order to destroy the protease and phosphodiesterase activities present. The pattern of inhibition was similar for both pathways. In this particular study the "UDP-glucose independent" activity was lower than the "UDP-glucose dependent" activity when no snake venom was present. This was another example of the variation seen in the level of these synthetic activities when different microsomal preparations were examined and this has already been mentioned with respect to (Table 7) in Chapter 3.

The results obtained when the 0.1 per cent Triton X-100 pellet and supernatant were treated with snake venom and the phospholipases C and D are shown in (Table 24). Increasing concentrations of snake venom caused an increase in the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside in

SOLUBILIZATION WITH TRITON X-100,
TRYPSIN AND CHYMOTRYPSIN

Additions	% Conversion of 17α-Estradiol to 17α-Estradiol-3-Glucoside*			
	UDP-Glucose Independent		UDP-Glucose Dependent	
None (untreated microsomal preparation)	28.6		5.51	
	Pellet	Supernatant	Pellet	Supernatant
0.1% Triton X-100	21.1	1.3	0.6	2.8
Trypsin	1.4	2.1	1.9	0.3
Chymotrypsin	0	0	4.6	0.1
Trypsin-Triton X-100	0	0	1.0	1.4
Chymotrypsin-Triton X-100	0	0	0.7	1.8

* Average of 3 experiments

TABLE 23

TREATMENT WITH TRITON X-100, SNAKE VENOM
AND PHOSPHOLIPASE C AND D

Additions	% Conversion of 17α-Estradiol to 17α-Estradiol-3-Glucoside*			
	UDP-Glucose Independent		UDP-Glucose Dependent	
None	17.0		4.1	
	Pellet	Supernatant	Pellet	Supernatant
0.1% Triton X-100	12.0	0.75	0.4	2.05
0.1% Triton X-100–Snake Venom				
0.02 milligrams enzyme	29.8	10.4	0	0
0.1 milligrams enzyme	14.4	14.7	12.8	0
0.1% Triton X-100-Heated Snake Venom				
0.02 milligrams enzyme	14.2	10.1	0	0
0.1 milligrams enzyme	16.0	13.6	0	0
0.1% Triton X-100–Phospholipase C				
12.5 micrograms enzyme	6.4	7.9	8.4	0
25 micrograms enzyme	6.2	10.2	7.4	0
0.1% Triton X-100–Phospholipase D				
12.5 micrograms enzyme	12.9	6.8	2.5	0
25 micrograms enzyme	7.2	11.4	8.8	0

* Average results of two experiments

TABLE 24

both the Triton X-100 pellet and supernatant fractions. This indicated that partial solubilization of the "UDP-glucose independent" activity occurs, since prior to snake venom treatment it was confined to the Triton X-100 pellet. The "UDP-glucose dependent" activity was completely inhibited by this Triton X-100-Snake venom treatment. Phospholipase C and D caused some solubilization of the "UDP-glucose independent" glucosyltransferase, since 17α-estradiol-3-glucoside synthesis occurred in both the Triton X-100 pellet and supernatant when these fractions had been treated with the phospholipases. As described previously in (Table 22), Triton X-100 caused solubilization of the "UDP-glucose dependent" glucosyltransferase. However, when these fractions were subjected to both Triton X-100 and phospholipase treatment, the supernatant lost all its capacity for the "UDP-glucose dependent" synthesis of 17α-estradiol-3-glucoside while glucoside synthesis in the pellet was stimulated

d) Treatment with phosphodiesterases

Pure phosphodiesterase, at the concentrations studied, did not have any significant effect on either the "UDP-glucose independent" or the "UDP-glucose dependent" synthesis of 17α-estradiol-3-glucoside. These results are shown in (Table 25). Some solubilization of the "UDP-glucose independent" activity did occur with increasing concentration of phosphodiesterase

THE EFFECT OF INCREASING CONCENTRATIONS OF PURE PHOSPHODIESTERASE ON THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE

Concentration of Phosphodiesterase Added/3 Millilitre Assay	% Conversion of 17α-Estradiol to 17α-Estradiol-3-Glucoside			
	UDP-Glucose Independent		UDP-Glucose Dependent	
0 (Untreated microsomal suspension)	26.4		9.8	
	Pellet	Supernatant	Pellet	Supernatant
0	18.5	6.9	13.7	0.4
3.3 μg or 0.05 units	18.3	9.7	12.9	0
16 μg or 0.125 units	20.4	8.1	12.9	2.6
32 μg or 0.25 units	22.5	11.2	9.5	0

TABLE 25

but very little "UDP-glucose dependent" activity was detected in the supernatant fractions.

e) Treatment with papain, lysozyme or neuraminidase

Papain, lysozyme or neuraminidase did not solubilize either the "UDP-glucose independent" or the "UDP-glucose dependent" steroid glucosyl transferase activities, since neither activity could be detected in the 105,000 X g supernatants. All three enzymes inhibited the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside to a greater degree than the "UDP-glucose dependent" synthesis and inhibition was always greater at 37°C than at 0°C.

f) Treatment with almond emulsin and α-glucosidase

The results obtained when studies with the glucosidases were undertaken are depicted in (Table 26). α-glucosidase had no significant effect on 17α-estradiol-3-glucoside synthesis. The presence of β-glucosidase inhibited both the "UDP-glucose independent" and the "UDP-glucose dependent" glucosyltransferases. At a concentration of 2 milligrams of β-glucosidase per 3 millilitre assay the "UDP-glucose dependent" pathway was completely inhibited, whilst the "UDP-glucose independent" synthesis was about 50 per cent inhibited.

THE EFFECT OF ALMOND EMULSION AND α-GLUCOSIDASE ON THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE

Additions	% Conversion of 17α-Estradiol to 17α-Estradiol-3-Glucoside*	
	UDP-Glucose Independent	UDP-Glucose Dependent
None	37	9
1 mg α-Glucosidase	37	13
2 mg α-Glucosidase	38	8
1 mg β-Glucosidase (almond emulsion)	20	8
2 mg β-Glucosidase	19	0

* Average of 3 experiments

TABLE 26

11) Sonication, Temperature Sensitivity and Stability Studies

a) Sonication

The effect of sonication of the microsomal preparations for varying lengths of time on their capacity for steroid gluside synthesis is shown in (Table 27). Sonication for 30 seconds at an intensity of 30 caused a slight increase in the level of "UDP-glucose independent" 17α-estradiol-3-glucoside synthesis but caused a decline in glucoside synthesis by the "UDP-glucose dependent" pathway. When the length of sonication was increased even up to 5 minutes, there was no significant change in the level of stimulation or inhibition as compared to that seen after a 30 second sonication of the microsomal preparation.

b) Temperature sensitivity studies

At the temperature studied the "UDP-glucose independent" glucosyltransferase was more heat sensitive than the "UDP-glucose dependent" transferase. Some of the results obtained are seen in (Figure 27). After pre-heating the microsomal preparation for 90 seconds at 60°C only 20.6 per cent of the "UDP-glucose independent" transferase activity remained whereas the "UDP-glucose dependent" activity has increased by 100 per cent. Similar trends were obtained in the studies at 50° and 52°C.

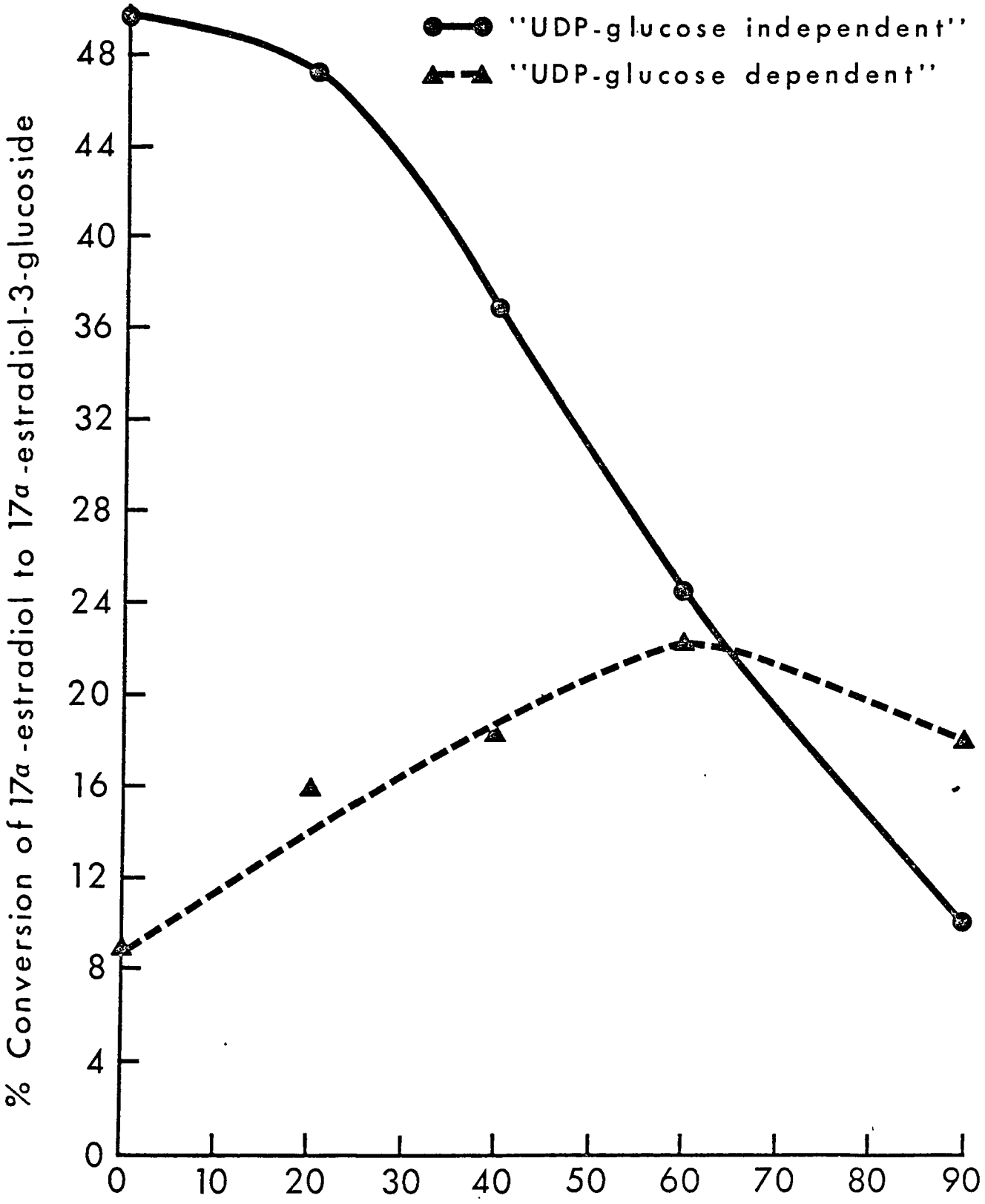
THE EFFECT OF SONICATION ON THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE

Length of Time Sonicated at an Intensity of 30 (Secs or mins)	% Conversion of 17α-Estradiol to* 17α-Estradiol-3-Glucoside	
	UDP-Glucose Independent	UDP-Glucose Dependent
0	25.2	10.6
30 sec	28.7	4.3
60 sec	26.5	6.1
90 sec	27.1	6.3
2 min	27.2	6.8
3 min	27.1	8.4
4 min	26.7	7.9
5 min	27.2	6.1

* Average of two experiments

TABLE 27

TEMPERATURE SENSITIVITY OF THE 3-GLUCOSYLTRANSFERASE



(Activity measured at 37°C)

c) Stability to Storage at -4°C

Different microsomal preparations lost glucosyltransferase activity at different rates when stored at -4°C. Some retained detectable glucosyltransferase activity even after 32 weeks of storage whereas other preparations had lost all activity after 8 weeks. However, there was no significant difference in the rate at which the "UDP-glucose independent" and the "UDP-glucose dependent" transferase(s) lost activity on storage.

12) The Involvement of Sugar Donors other than UDP-Glucose in the "UDP-Glucose Independent" Reaction

The presence of glucose, glucose-1-phosphate, glucose-6-phosphate and glycogen in the standard incubation mixture did not have any significant effect on the capacity of the microsomes to effect the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside.

D) Discussion

The results reported in this chapter showed that the rabbit liver 3-glucosyltransferase activities had a number of common properties but several others which differed when the transferase was studied in the presence and absence of UDP-glucose. These similarities and differences are summarized in (Tables 28 and 29).

(Figure 11) showed the pH activity curves for the transfer of glucose to the 3α-hydroxyl group of 17α-estradiol from either UDP-glucose or some endogenous sugar donor. The pH optimum for both activities was 7.0. Earlier studies in this laboratory had demonstrated that the pH activity curve of the "UDP-glucose dependent" 3-glucosyltransferase differed from those obtained for the nucleotide dependent 3-glucuronyltransferase, 17-glucosyl and 17-N-acetylglucosaminyltransferases (9, 69, 219). The present studies confirmed these findings and also showed that the pH activity curve of the "UDP-glucose independent" transferase, having been similar to that of the "UDP-glucose dependent" 3-glucosyltransferase, differed from those of the other steroid glycosyltransferases.

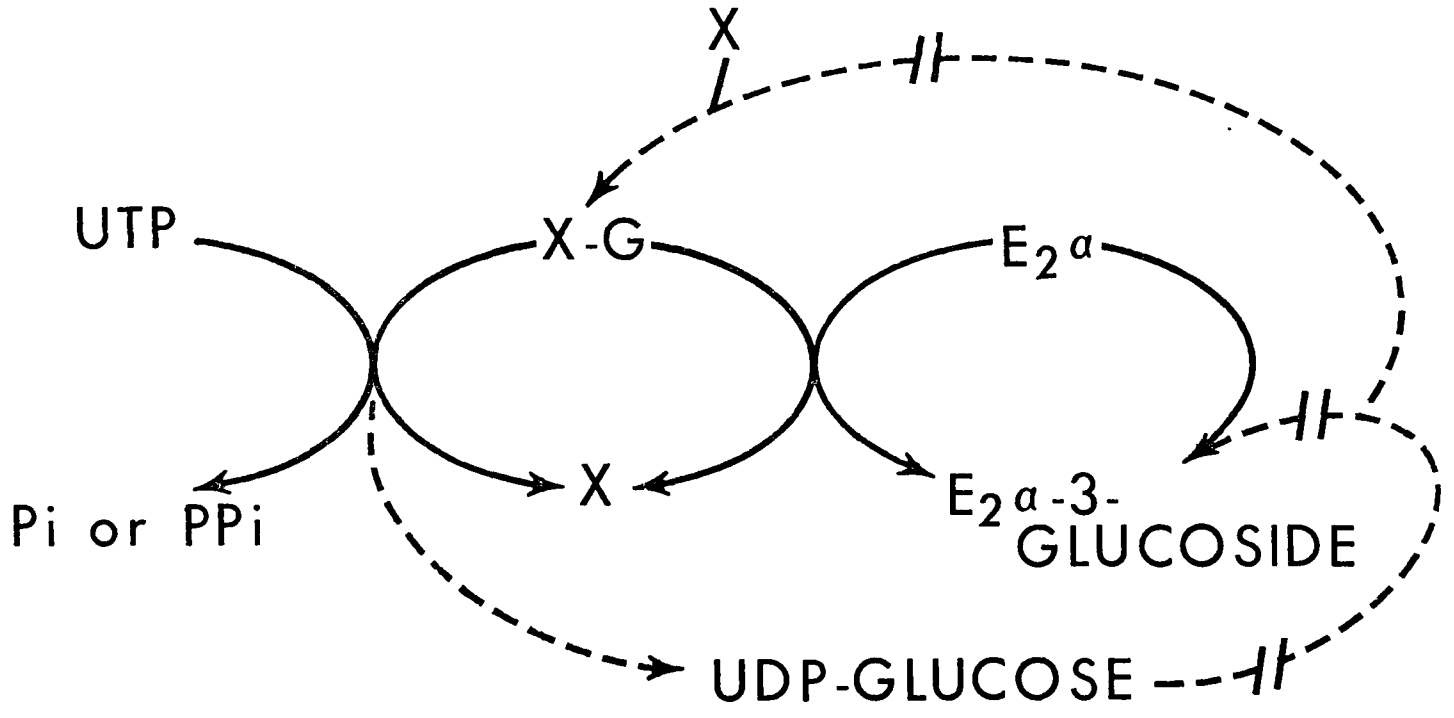
Many of the compounds examined for their ability to act as substrates for the "UDP-glucose dependent" and the "UDP-glucose independent" 3-glucosyltransferases were previously tested in studies with the other steroid glucosyltransferases (9, 12, 19, 67, 69). These studies, which showed that the

PROPERTIES COMMON TO THE "UDP-GLUCOSE INDEPENDENT" AND "UDP-GLUCOSE DEPENDENT" PATHWAYS OF 17α-ESTRADIOL-3-GLUCOSIDE SYNTHESIS

Property Studied	Result Obtained in Both Pathways
pH Optimum	7.0
Optimum Temperature of Incubation	37°C
Optimum Length of Incubation at 37°C	30 minutes
Optimum Concentration of Microsomal Protein	5 milligrams
Substrate Specificity	same
Sensitivity to Inhibitors	same
Stability to Storage at −4°C	same
Treatment with Snake Venom, Phospholipases, Papain Lyzozyme and Neuraminidase	same

TABLE 28

A POSSIBLE EXPLANATION FOR THE INHIBITION OF THE "UDP-GLUCOSE-INDEPENDENT" SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE BY UTP



X-G = ENDOGENOUS GLUCOSE DONOR
$E_2\alpha$ = 17α-ESTRADIOL
$E_2\alpha$-3-GLUCOSIDE = 17α-ESTRADIOL-3-GLUCOSIDE

PROPERTIES WHICH VARIED IN THE PRESENCE AND ABSENCE OF UDP-GLUCOSE

Property Examined	– UDP-Glucose	+ UDP-Glucose
Effect of Cations	Stimulation	Inhibition
Sensitivity to detergents	Yes	No
Solubilization with Triton X-100	No	Yes
Sensitivity to Preheating	Heat labile	Heat stable
Effect of Sonication	Stimulation	Inhibition
Susceptibility to Inhibition by Protease-Triton X-100 Treatment	Sensitive	Insensitive

TABLE 29

A SCHEME FOR THE INVOLVEMENT OF A POLYPRENOL PHOSPHATE IN SUGAR TRANSFER REACTIONS

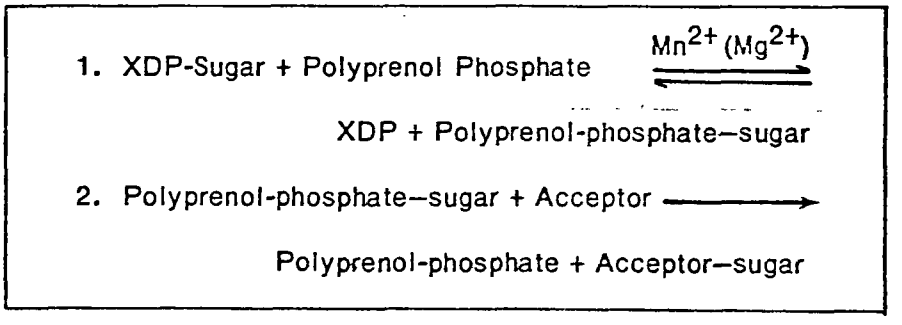


FIGURE 29

"UDP-glucose dependent" and "UDP-glucose independent" 3-glucosyltransferase had the same specificity towards seventeen different substrates, gave further evidence that the steroid glycosyltransferases show a high specificity with regard to their steroid substrates. Diethylstilbestrol glucoside formation by rabbit liver microsomes has been demonstrated on other occasions when diethylstilbestrol was incubated in the presence of high specific activity UDP-D-(6-^{3}H)glucose (9, 67, 69). The failure to show glucoside formation in the present studies, where low specific activity (^{14}C)diethylstilbestrol was incubated either in the presence or absence of non-radioactive UDP-glucose, was not proof that glucosylation of this compound did not occur, but it was undetectable under the conditions used.

The Km values calculated for the"UDP-glucose dependent" and the "UDP-glucose independent" 3-glucosyltransferase activities towards 17α-estradiol, 1.67 x 10^{-7}M and 1.99 x 10^{-7}M, were a little higher than the Km value of 7.14 x 10^{-8}M, calculated by Collins et al for the transfer of glucose from UDP-glucose to 17α-estradiol-3-glucuronide by the steroid 17-glucosyltransferase (9). However, this difference in the affinity of the glucosyltransferases towards their substrates was not great enough to provide an argument for two possibly different roles for the 3- and the 17-transferases, namely directing the substrate either towards excretion or towards a role as a metabolic intermediate.

The inhibition of glucose transfer to the steroid caused by the presence of the aliphatic alcohols was similar for both the "UDP-glucose dependent" and "UDP-glucose independent" transferase activities except in the case of ethanol, which inhibited the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside much more than the "UDP-glucose dependent" synthesis. These studies demonstrated a pattern of inhibition similar to that seen when these alcohols were examined for their inhibitory effects on the steroid N-acetylglucosaminyltransferase of rabbit liver (19). The levels of the alcohols required to cause significant inhibition in both of these studies were greater than the concentrations of the substrates present, suggesting that if the inhibition was of the competitive nature the affinity of the sugar transferases for the alcohols examined must be quite small.

The inhibition of the 3-glucosyltransferases by estrone, 17β-estradiol, p-nitrophenol and phenolphthalein was not surprising since these compounds were known to form glucosides in rabbit liver (67). The range of solubility of the steroids prevented their use in concentrations high enough to allow accurate Ki determinations from Dixon plots.

The inhibition of the transferases by 17α-estradiol-3-glucoside was not unexpected since this was a product in both the "UDP-glucose dependent" and "UDP-glucose independent" pathways. Diethylstilbestrol did act as an inhibitor of 17α-estradiol-3-glucoside synthesis which would agree with the

earlier reports of its ability to act as a substrate for the 3-glucosyltransferase (9, 67, 69).

The inhibition of steroid glucoside formation by steroids and other compounds which themselves do not form the corresponding glucoside has been reported (19). The inhibition of the 3-glucosyl transferase by 17α-estradiol-17-glucoside which is neither a substrate nor a product of this reaction, may be due to the attachment of this compound to the enzyme in the form of a 'dead end' complex.

Some of the nucleotides examined inhibited both the "UDP-glucose dependent" and the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside. The inhibition of the "UDP-glucose independent" pathway could be explained by the hypothesis shown in (Figure 28). UTP would compete with the steroid for the endogenous glucose donor. However, as discussed in Chapter 3, the microsomes were unable to form significant amounts of UDP-glucose when incubated with UTP alone, suggesting that any UDP-glucose formed could not participate either in the glucosylation of the steroid by the "UDP-glucose dependent" pathway or in the regeneration of the endogenous sugar donor. Labow et al have reported that UTP inhibited the rabbit liver N-acetylglucosaminyl and glucuronyltransferases in a non-competitive fashion (71). The inhibition of the 3-glucosyltransferase seen in the above studies suggested that UTP might act as a general inhibitor of the steroid glycosyltransferases.

UTP has also been reported to inhibit other glycosyltransferases which employ UDP-N-acetylglucosamine as the donor nucleotide (220).

The sulfhydryl reagents caused some inhibition of the 3-glucosyltransferase activities. These compounds have previously been shown to inhibit the steroid N-acetylglucosaminyltranferase but did not inhibit the glucuronyltransferase (71). As discussed earlier p-nitrophenol acted as a substrate for the 3-glucosyltransferase and this would explain its ability to act also as an inhibitor. Phenolphthalein has on some occasions been shown to act as a substrate for the steroid glucosyltransferase which would explain its inhibitory effect seen in (Table 19) (71).

The different response of the "UDP-glucose dependent" and the "UDP-glucose independent" glucosyltransferases to magnesium chloride, manganous chloride and calcium chloride was especially interesting. The requirement for manganese or magnesium ions in many of the sugar transfer reactions involving lipid intermediates has been well documented (83-88, 119, 136-140, 146-149, 152, 158, 176, 179, 187, 189). (Figure 29), page 152, illustrates the sugar transfer reactions with an intermediate lipid and also the step which usually exhibits an ion requirement. Thus the stimulation of the "UDP-glucose independent" synthesis of 17αestradiol-3-glucoside by these ions, which inhibited the "UDP-glucose dependent" pathway, substantiated earlier evidence for the involvement of a lipid

intermediate in the "UDP-glucose independent" pathway. This possible lipid involvement will be investigated in greater detail in Chapter 5.

EDTA is a well known cation chelator which partly explains the inhibition seen when it was added to the standard incubation mixture either alone or in the presence of metal ions. As mentioned above the "UDP-glucose independent" pathway of 17α-estradiol-3-glucoside synthesis was stimulated by the presence of magnesium chloride. This stimulation was abolished with the addition of increasing concentrations of EDTA, (Figure 21). This result could be explained by the reaction of the added EDTA with both exogenous and endogenous cations which would make them unavailable to the "UDP-glucose independent" glucosyltransferase. This reaction with the endogenous cations would also explain why the level of synthesis of glucoside in the presence of magnesium chloride and EDTA was below that seen when no exogenous ions were included in the standard incubation mixture. However, EDTA must also have exerted some other inhibitory effect on these transferases since its presence, which would be expected to chelate the exogenous ions which are inhibitory to the "UDP-glucose dependent" transferase did not restore the level of 17α-estradiol-3-glucoside synthesis by the "UDP-glucose dependent" pathway to that seen prior to the addition of any exogenous ions.

As mentioned earlier the effect of magnesium chloride in combination with either calcium chloride or manganese chloride

on 17α-estradiol-3-glucoside synthesis was similar to that obtained when magnesium chloride was added alone. This suggested that these ions compete with each other for the enzyme in the reaction.

The different sensitivity of the "UDP-glucose dependent" and "UDP-glucose independent" 3-glucosyltransferase activities to inhibition by detergents, especially the detergents, deoxycholate, Triton X-100 and Cutscum, was further evidence for two distinct mechanisms of steroid glucosylation. Further evidence was provided by the Triton X-100 solubilization studies. The "UDP-glucose dependent" activity was readily solubilized, being detected almost free of the "UDP-glucose independent" 3-glucosyltransferase in the Triton X-100 supernatant. However the Triton X-100 pellet while rich in the "UDP-glucose independent" activity also contained some of the unsolubilized "UDP-glucose dependent" 3-glucosyltransferase which made it impossible to study the "UDP-glucose independent" 3-glucosyltransferase in its absence.

Treatment of the microsomes with the proteases, trypsin and chymotrypsin, either alone or in the presence of Triton X-100 inhibited both the "UDP-glucose dependent" and "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside. These results resembled those obtained by Labow et al when similar studies were carried out with the other nucleotide dependent glycosyltransferases from rabbit liver (71). The "UDP-glucose dependent" glucosyltransferase resembled the 3-glucu-

ronyltransferase in its pattern of inhibition and solubilization but the "UDP-glucose independent" transferase differed from both of these.

Pure phosphodiesterase did not cause any decline in the "UDP-glucose independent" pathway of 17α-estradiol-3-glucoside synthesis as seen in (Table 25). This suggests that if, as has been proposed earlier, an endogenous lipid-phosphoryl-sugar intermediate participated in the "UDP-glucose independent" pathway, it did not appear susceptible to degradation by the added phosphodiesterase under the condition employed in this study.

The significance of the inhibition of both the transferase activities by papain, lysozyme and neuraminidase is not obvious but perhaps indicates a structural role for the membrane proteins and glycoproteins susceptible to hydrolysis by these enzymes. The degradation of these membrane components may disrupt the environment which is essential for the 3-glucosyl transferase activities.

The inhibition of the synthesis of 17α-estradiol-3-glucoside by β-glucosidase suggested the involvement of a β-glucolipid or glycoprotein in glucoside formation. In the "UDP-glucose independent" pathway the inhibition might be explained by the involvement of a β-glucolipid in glucoside synthesis, however,no such intermediate has been implicated in the "UDP-glucose dependent" pathway.

The stimulation of the "UDP-glucose independent" pathway of 17α-estradiol-3-glucoside synthesis by sonication of the

microsomal preparations suggests that sonication may expose some membrane components, perhaps lipids, which are required in this pathway but not in the "UDP-glucose dependent" reaction. The temperature sensitivity studies also indicate the involvement of a heat labile component in the "UDP-glucose independent" pathway which is absent from or non-essential to the "UDP-glucose dependent" glucosyltransferase reaction.

As discussed in Chapter 3, UDP-glucose was not involved either directly or indirectly in the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside. The studies which examined the effect of glucose, glucose-1-phosphate, glucose-6-phosphate and glycogen on the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside indicated that none of these four sugars were the principal sugar donors in this pathway of glucoside synthesis.

Many of the properties of the "UDP-glucose dependent" and "UDP-glucose independent" 3-glucosyltransferases discussed above and also in Chapter 3 fit the hypothesis of the possible involvement of a lipid intermediate in the "UDP-glucose independent" pathway of glucoside synthesis. This prompted the further investigation of this possibility in the experiments described in Chapter 5.

CHAPTER 5

EVIDENCE FOR A POSSIBLE LIPID INTERMEDIATE IN THE UDP-GLUCOSE INDEPENDENT SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE

A) Introduction

The involvement of a lipid intermediate in the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside and the nature of such a compound are examined in this chapter. Rabbit and pig liver lipids as well as some pure isoprenoid compounds were assessed for their ability to participate in the "UDP-glucose independent" pathway.

B) Methods

1) The Effect of Rabbit Liver Lipid Fractions on the "UDP-Glucose Independent" Synthesis of 17α-Estradiol-3-Glucoside

The lipid fractions were prepared as described in the general methods, Chapter 2, Section 4. (Table 30), gives the concentrations of the lipids used in these studies. The lipid extracts were concentrated to dryness under nitrogen and then sonicated at an intensity of 30 for one minute in a mixture of Triton X-100 and 0.2M Tris buffer, pH 7.0. The detergent was used to help to solubilize the lipids and the above sonication procedure would be expected to ensure even suspension of the lipid in the aqueous assay mixture. Triton X-100 was used since in many of the other sugar transfer reactions involving a lipid intermediate there has been a requirement demonstrated for this detergent (116, 134, 135, 154, 156, 171, 189, 192).

Aliquots of the stock solutions were added to the standard incubation mixture both in the presence and absence of 80mM magnesium chloride, so as to give the required lipid concentrations and a final Triton X-100 concentration of 0.2 per cent. As mentioned earlier, (Figure 24 and Tables 21 and 22, Chapter 4) Triton X-100, when present at this concentration, partially inhibited the "UDP-glucose independent" synthesis

THE CONCENTRATIONS OF RABBIT AND PIG LIVER LIPIDS USED PER 3 MILLILITRE ASSAY

Lipid Fraction	Microgram Added	Weight per Gram of Liver Wet Weight
Rabbit Liver Total Lipids	105 - 421	63.2 Milligrams
Rabbit Liver Neutral Lipids	36.3 - 726	43.2 Milligrams
Rabbit Liver Phospholipids	0.05 - 0.8 Micrograms Pi	41.2 Micrograms Pi
Rabbit Liver Microsomal Phospholipids	0.3 - 2.0 Micrograms Pi	
Rabbit Liver Microsomal Chloroform: Methanol (2:1) Extract	1.1 - 44 Micrograms Pi	75.31 Micrograms Pi
Rabbit Liver Microsomal Chloroform: Methanol:Water (1:1:0.3) Extract	1.1 - 44 Micrograms Pi	94.1 Micrograms Pi
Pig Liver Chloroform: Methanol (2:1) Extract	17.12 - 284.45 Micrograms Pi	57.29 Micrograms Pi

TABLE 30

of 17α-estradiol-3-glucoside. Any increase in the level of glucoside synthesis, in these Triton X-100 treated microsomal preparations, with the addition of the lipid extracts would suggest some role for a lipid in the "UDP-glucose independent" pathway. The ability of the microsomal lipid extracts to restore this "UDP-glucose independent" synthesis of glucoside in microsomal preparations where this capacity had been lost due to treatment with deoxycholate was also examined. The final concentration of deoxycholate was 0.01 per cent per 3 millilitre assay.

2) The Effect of Pig Liver Lipid Fractions on the "UDP-Glucose Independent" Synthesis of 17α-Estradiol-3-Glucoside

As discussed in Chapter 1 polyprenols have been shown to be involved in a number of sugar transfer reactions (83-180, 183-194). In mammalian systems, the polyprenol dolichol, which is found in high concentrations in pig liver, (221) plays an analogous role to that played by bactoprenol in bacterial cell wall biosynthesis (83-91). Thus the ready availability of this source rich in dolichol prompted the study of the effect of the pig liver lipid extracts on the capacity of the rabbit liver microsomes for the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside. The procedures used for lipid isolation were described in the general methods in

Chapter 2 and in all cases the fresh liver was the starting material. The concentrations of lipid extract used are listed in (Table 30). Due to the presence of such high lipid concentrations in the standard incubation mixture some extraction problems arose. The manipulations of the standard assay and extraction procedures which were undertaken in an effort to overcome the interference by the presence of high concentrations of pig liver extract are illustrated in (Table 31).

3) Purification of the Lipid Extracts by DEAE - Cellulose Chromatography and Thin Layer Chromatography

The acetate form of the DEAE - cellulose column was prepared as described in Chapter 2 (206). Approximately 350 milligrams of the crude pig liver chloroform : methanol (2:1) extract or 175 milligrams of the rabbit liver microsomal chloroform : methanol (2:1) extract were applied to the column in 10 millilitres of chloroform : methanol (2:1). The column was washed with chloroform : methanol (2:1) and then eluted with 0.1M ammonium formate, pH 4.0, in chloroform : methanol (2:1). Ten millilitre fractions were collected, evaporated to dryness, re-suspended in 10 millilitres of chloroform : methanol (2:1) and then extracted with 4 millilitres of water in order to remove the ammonium acetate. This was repeated and the final volume was adjusted to 10 millilitres. Five millilitre aliquots of each fraction were added to the standard assay and

MANIPULATIONS OF THE STANDARD ASSAY AND EXTRACTION PROCEDURE FOR THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE IN AN EFFORT TO OVERCOME THE INTERFERENCE BY THE PRESENCE OF PIG LIVER ORGANIC EXTRACTS

	Modifications of the 3 Millilitre Incubation	First Extracting Solvent	Second Extracting Solvent	Other Modifications
1.	Concentrated Triton X-100 added instead of a Triton X-100-lipid-Tris or Triton X-100-Tris mixture	2 x 5 Millilitres benzene	2 x 5 Millilitres ethyl acetate	-
2.	0.05 Millilitre of organic extract (chloroform:methanol 2:1	As above	As above	-
3.	0.2 Milligrams bovine serum albumin just before stopping the reaction	As above	As above	-
4.	None	2 x 5 Millilitres Chloroform	As above	-
5.	None	2 x 5 Millilitres Chloroform: Benzene (1:1)	As above	-
6.	None	2 x 5 Millilitres ethyl acetate	-	-
7.	None	7 Millilitres methanol	7 Millilitres hexane	5 Millilitres benzene used to extract the methanol layer of the methanol: hexane mixture
8.	None	7 Millilitres ice cold acetone	2 x 5 Millilitres benzene	-
9.	None	2 x 5 Millilitres benzene (to extract steroid from methanol)	2 x 5 Millilitres ethyl acetate	Incubation put through an Amberlite -XAD column methanol used to elute the steroids
10.	100 Micrograms non-radioactive 17α-estradiol 5 minutes before stopping the reaction	2 x 5 Millilitres benzene	2 x 5 Millilitres ethyl acetate	-

TABLE 31

examined for their ability to restore the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside in microsomal preparations where this capacity had been lost due to treatment with Triton X-100. A five millilitre aliquot of each fraction was also assayed for lipid glycosylation in the presence of UDP-D-(U-^{14}C)glucose. The procedure of Behrens and Leloir was followed in the assay for lipid glycosylation (148).

The silica gel plates for thin layer chromatographic studies were prepared as described in Chapter 2. The thin plates were 0.25 millimetres and the thick plates were 0.75 millimetres thick. The rabbit or pig liver lipid fractions chromatographed had not been purified by DEAE - cellulose chromatography or by any other column chromatography. (Table 32) illustrates the solvent systems and (Table 33) the spray reagents used in these studies. The various rabbit liver lipid extracts were chromatographed against dolichol phosphate from calf brain and against commercial preparations of dolichol and dolichol phosphate in the solvent systems, chloroform : methanol : ammonia : water (80:20:0.5:3 v/v), chloroform : methanol: water (65:25:4 v/v) and chloroform : methanol : water (10:10: 3 v/v). Phosphomolybdic acid, Hanes reagent, iodine and a reagent containing vanadyl chloride and a zinc reducing solution were used to detect the different lipids present. When good fractionation of the lipids present in the organic extract was obtained, the chromatogram was sub-divided and the different lipids were eluted with chloroform : methanol : water

SOLVENT SYSTEMS USED IN THIN LAYER
CHROMATOGRAPHIC STUDIES OF LIPID EXTRACTS

	Solvent System (v/v)	Reference
1.	Chloroform:Methanol:Water (65:25:4)	(105,148)
2.	Chloroform:Methanol:Water (60:35:6)	(223),(215)
3.	Chloroform:Methanol:Water (10:10:3)	(223)
4.	n-Propanol:Water (70:30)	(222)
5.	Chloroform:Methanol:Ammonia:Water (80:30:0.5:3)	(105,148)
6.	Chloroform:Methanol:Ammonium Hydroxide (75:25:4)	(200)
7.	Chloroform:Methanol:Ammonium Hydroxide (65:35:5)	
8.	Chloroform:Methanol:Formic acid:Water (70:18.5:8:0.5)	(105,148)
9.	Chloroform:Methanol:Acetic acid:Water (25:15:4:2)	(200)
10.	Chloroform: 90% Acetic acid:Methanol (30:20:4)	
11.	Acetone	(201)

TABLE 32

SPRAY REAGENTS USED TO DETECT
LIPIDS ON THIN LAYER CHROMATOGRAMS

Spray Reagent Used	Compound Detected by Reagent	Reference
1. Phosphomolybdic acid (5 gm/50 ml methanol)	Reducing compounds, lipids, sterols and steroids	(202)
2. Hanes reagent	Phosphate ester	(202)
3. Anisaldehyde-sulphuric acid	Sugars, steroids, terpenes and phenols	(201,202,207)
4. Iodine	Non specific	(202)
5. Vanadyl chloride and zinc reducing solution	Phosphate containing compounds	(204)
6. Phosphate stain	Phospholipids	(224, 225)

TABLE 33

(1:2:0.8 v/v). Each eluted fraction was then diluted with an equal volume of benzene and evaporated almost to dryness on a rotary evaporator under reduced pressure at 30°C. More benzene was added gradually to aid in the removal of the water and the extract was finally brought to dryness. The residue was dissolved in chloroform : methanol (1:1 v/v) and centrifuged so as to remove any residual silica gel. Aliquots of these fractions were then examined either in the absence or presence of 250mM magnesium chloride for their ability to restore the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside in Triton X-100 treated microsomal preparations.

Similar studies were undertaken with the pig liver chloroform : methanol (2:1) extracts except that in this case the lipid extracts were chromatographed in all the solvent systems listed in (Table 32). Anisaldehyde and Vaskovsky's phosphate stain were the spray reagents used to detect the lipids. The crude pig liver extracts were also chromatographed using a number of different phospholipids as reference standards in chloroform : methanol : ammonium hydroxide (65:25:5 v/v) and chloroform : 90 per cent acetic acid : methanol (30:20:4 v/v). The fractionated pig liver lipids from these chromatograms were eluted as described above, and then examined either alone or in various combinations with the other fractions eluted from the chromatogram for their ability to influence the synthesis of 17α-estradiol-3-glucoside in the

microsomes which had lost this capacity due to treatment with Triton X-100.

4) Studies to Examine the Nature of the Lipid Intermediate

Some isoprenoid and other lipid compounds were examined for their effect on the "UDP-glucose independent" synthesis of 17α-estradiol-3 -glucoside and also for their ability to act as precursors for the synthesis of an "active" lipid-sugar intermediate.

a) The Ability of Rabbit or Rat Liver Microsomes to Synthesize Dolichol Monophosphate Glucose

The microsomes were prepared as described in the general methods, Chapter 2. The incubation mixture had a final volume of 50 microlitres and contained (25 microlitres) 1M glycylglycine buffer pH 7.5, (5 microlitres) 1M 2-mercaptoethanol, (5 microlitres) 0.1M magnesium EDTA, (5 microlitres) 5 per cent Triton X-100, (10 microlitres) microsomal preparation which contained approximately 0.52 milligrams protein, (10 microlitres) 5 nanomoles dolichol phosphate and (16microlitres) 1.4 nanomoles UDP-D-(U-^{14}C)glucose (643,000 d.p.m. per nanomole) (226).

The UDP-D-(U-^{14}C)glucose and dolichol phosphate were added to a 1.5 millilitre glass culture tube and evaporated to dryness under a stream of nitrogen before the addition of any

of the other reactants. The mixture was shaken vigourously on a vortex mixer and then incubated for 30 minutes at 37°C. The reaction was stopped by adding 0.4 millilitres of methanol and 0.6 millilitres of chloroform. The mixture was centrifuged and the chloroform phase was collected. 4M magnesium chloride (0.2 millilitres) was added to the chloroform phase which was then washed according to the procedure of Folch, Lees and Sloane - Stanley (199). The chloroform fraction would be expected to contain dolichol monophosphate glucose if any were synthesized.

b) <u>The Ability of the Chloroform Extract to Influence the Synthesis of 17α-Estradiol-3-Glucoside</u>

The procedure was a modification of that described in the general methods, <u>Chapter 2</u> for the standard incubation. In this case the final volume was 0.3 millilitres instead of 3 millilitres. Triton X-100 was added so as to give a final concentration of 0.2 per cent per 3 millilitre assay. The chloroform fraction labelled with (^{14}C)glucose was added. The reaction mixture was incubated for 30 minutes at 37°C and then stopped with benzene. The volume of the assay was adjusted to 3 millilitres with water before the addition of 5 millilitres of benzene. The rest of the extraction procedure was as described previously. The ethyl acetate extract, which would be expected to contain the 17α-estradiol-3-glucoside, was coun-

ted for ^{3}H and ^{14}C and also chromatographed in propanol : water (7:1) and chloroform : methanol (4:1) against authentic standards of dolichol, dolichol phosphate, 17α-estradiol, 17α-estradiol-3-glucoside, UDP-glucose, UDP, glucose and glucose-1-phosphate. One centimetre areas from that part of the chromatogram which contained the ethyl acetate were scraped and counted in order to determine if ^{14}C chromatographed with the 17α-estradiol-3-glucoside. This procedure would also be expected to indicate the presence of any residual UDP-D-(U-^{14}C)-glucose or its degradation products.

c) The Specificity of the "UDP-Glucose Independent" 3-Glucosyltransferase towards the Lipid Required for the Synthesis of 17α-Estradiol-3-Glucoside by this Pathway

The stimulation of 17α-estradiol-3-glucoside synthesis by the rabbit and pig liver crude organic extracts suggested that the lipid requirement in this "UDP-glucose independent" pathway might be non-specific. To examine this hypothesis some lipids unrelated to the polyprenols were added to examine their ability to restore the "UDP-glucose independent" synthesis of glucoside in Triton X-100 treated microsomal preparations. Synthetic and soybean lecithin were added to the standard 3 millilitre assay at concentrations ranging from 100 to 500 micrograms.

A significant stimulation of the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside in microsomal preparations which had been treated with Triton X-100, by any added pure isoprenoid type compound would provide a strong indication of the involvement of that compound in the reaction and would provide a standard for comparison with the different pig and rabbit liver lipid extracts of the earlier studies.

(Table 34) lists the compounds examined in these studies. These lipids were examined for their effect on the "UDP-glucose independent" and in some cases the "UDP-glucose dependent" synthesis of 17α-estradiol-3-glucoside in untreated microsomes and also in microsomes which had been treated with 0.2 per cent Triton X-100. As in the other lipid studies described previously, a stock solution of lipid was prepared by sonicating the lipid in a mixture of Triton X-100 and Tris buffer pH 7.0. Aliquots of the stock solution were then added to the incubation mixture so as to give the required lipid concentrations. This addition of the sonicated lipid in Tris was the only modification to the procedure for the standard assay which was described in the general methods Chapter 2. All the studies involving retinol were carried out in dim light and in centrifuge tubes which were completely covered with aluminium foil. These precautions would be expected to minimize the degradation of this light sensitive vitamin. The preliminary studies examined the suitability of the conditions, used in the study of retinol glycolipids by other groups of workers, for use in

ISOPRENOID AND RELATED COMPOUNDS TESTED FOR THEIR EFFECT ON 17α-ESTRADIOL-3-GLUCOSIDE SYNTHESIS

Compound Examined	Concentration per 3 3 Millilitre Assay	Reference
Dolichol	~300 micrograms	(218)
Dolichol phosphate	~280 micrograms	(218)
^{14}C-Retinol	53 micrograms (~100,000 dpm/ microgram, 13.7 mCi/ millimole)	-
Retinol	400 micrograms-2 milligrams	(42,118,222-226)
Farnesol	320 micrograms - 1.49 milligrams	-
Geranylgeraniol	320 micrograms - 1.5 milligrams	-
D-α-Tocopherol	200 - 400 micrograms	-

TABLE 34

the study of the involvement of retinol in the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside (48, 123, 227-230). The standard 3 millilitre assay for the "UDP-glucose independent" synthesis of glucoside was modified so as to contain (10mM) manganous chloride, (2.5mM) EDTA, 3μmole ATP, 0.2 per cent Triton X-100 and (1.125 milligram) retinol, alone or in various combinations with each other.

(^{14}C) Retinol was also used to assess the ability of the rabbit liver microsomes to effect the synthesis of retinol-glucoside. The method used in this study was a modification of that used by Rodriguez, Bello and Gaede when examining the capacity of rat thyroid homogenates to synthesize retinol glycosides (48). The total assay volume was 1.5 millilitres and contained (5milligrams) microsomal protein, (0.25 micromole) UDP-glucose, (1.5 micromole) ATP, (0.2 per cent) Triton X-100, (700 micrograms) retinol, (53 micrograms) (^{14}C) retinol, 100,000 d.p.m. per microgram, and 0.2M Tris pH 7.0. The mixture was incubated for 60 minutes at 37°C. The reaction was stopped by the addition of 30 millilitres of chloroform : methanol (6:4 v/v). This mixture was shaken for 10 minutes, then centrifuged and the chloroform phase concentrated to near dryness. This extract was chromatographed overnight on Whatman #3M paper in a descending solvent system containing N-butanol : acetic acid : water (78:5:17). The paper chromatogram was scanned for ^{14}C in a Nuclear Chicago Scanner (Model Actigraph III) (48) and later cut into one centimetre pieces

and counted for ^{14}C in aquasol : xylene (1:1) in a Liquid Scintillation Counter.

C) Results

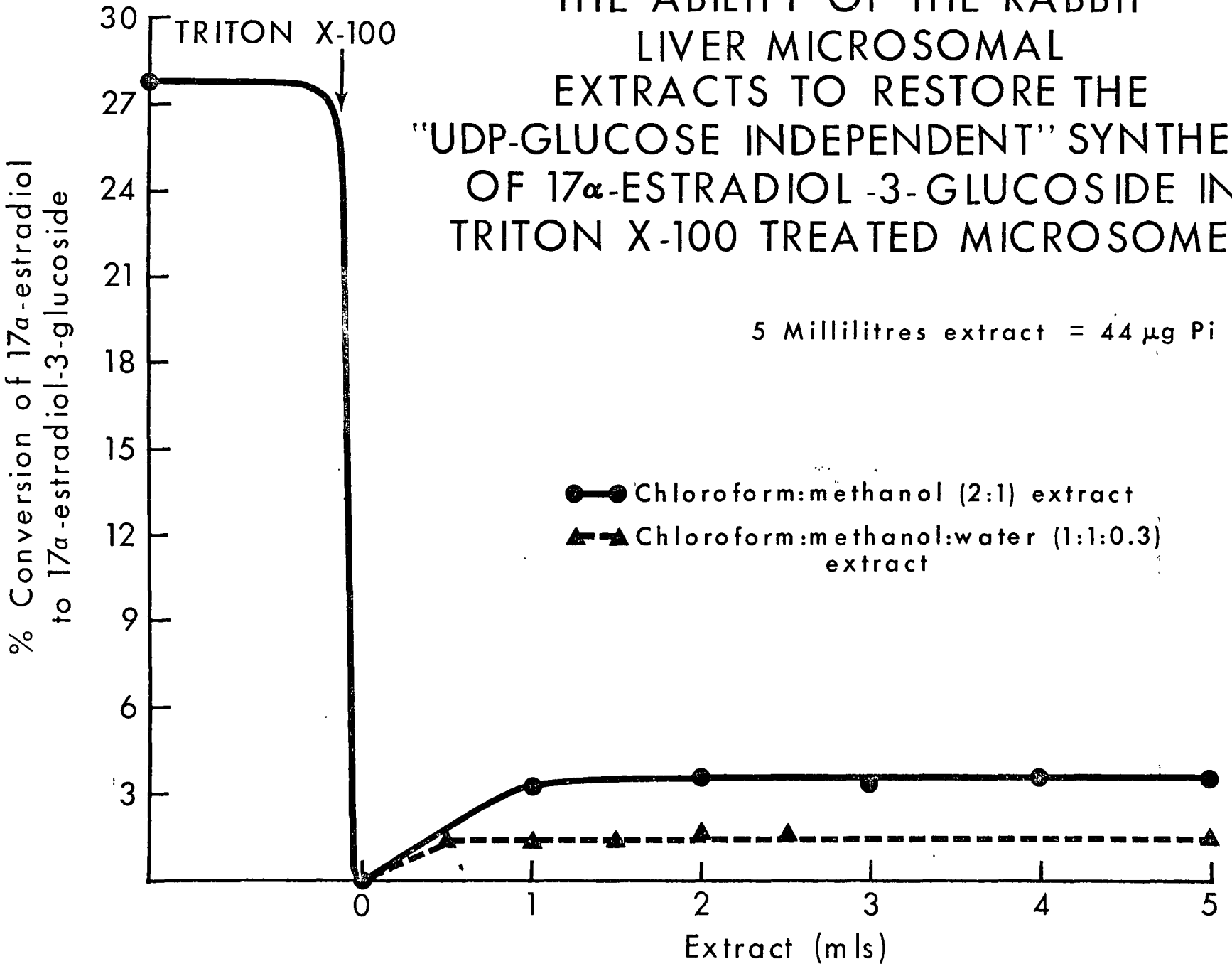
1) Rabbit Liver Lipid Studies

The presence of rabbit liver total, neutral or phospholipids incubated in the presence and absence of 50mM magnesium chloride did not cause any significant change in the amount of 17α-estradiol-3-glucoside synthesized by the "UDP-glucose independent" pathway. Similar results were obtained with the rabbit liver microsomal phospholipid extracts. However, the chloroform : methanol (2:1) and chloroform : methanol : water (1:1:0.3) extracts, prepared from lyophilized rabbit liver microsomes, were able to partially restore the synthesis of 17α-estradiol-3-glucoside in Triton X-100 treated microsomal preparations. These results are seen in (Figure 30). The addition of 0.2 per cent Triton X-100 completely inhibited the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside in this particular microsomal preparation. The addition of even 1 millilitre of organic extract, (equivalent to 8.8 micrograms inorganic phosphorus) caused some restoration of steroid glucoside synthesis. The chloroform : methanol (2:1) extract was better able to effect restoration of synthesis than was the chloroform : methanol : water (1:1:0.3) extract.

As mentioned in Chapter 4 the presence of magnesium chloride stimulated the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside in untreated microsomal preparations.

FIGURE 30

THE ABILITY OF THE RABBIT LIVER MICROSOMAL EXTRACTS TO RESTORE THE "UDP-GLUCOSE INDEPENDENT" SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE IN TRITON X-100 TREATED MICROSOMES



However, it did not significantly affect the ability of added organic extract to restore steroid glucoside synthesis in Triton X-100 treated microsomal preparations. These results are illustrated in (Table 35).

Even though the detergents Triton X-100 and deoxycholate both inhibit the "UDP-glucose independent" synthesis of steroid glucoside, the added organic extracts were better able to restore this synthesis in microsomal preparations which had been treated with Triton X-100 than in those treated with 0.01 per cent deoxycholate. Triton X-100 was used in all further lipid studies.

2) Pig Liver Lipid Studies

The initial studies with the crude pig liver chloroform : methanol (2:1) extract suggested a much higher restoration of 17α-estradiol-3-glucoside synthesis in Triton X-100 treated microsomal preparations on addition of the pig liver organic extract, than on addition of rabbit liver organic extracts. The results obtained in the pig liver studies are depicted in (Table 36). Since high lipid concentrations were used in these studies, their possible interference with the normal extraction procedure for 17α-estradiol-3-glucoside was examined . Microsome blanks were set up containing (^{3}H)17α-estradiol in the presence and absence of the lipid Triton X-100 extract. As shown in (Table 37) the presence of the lipid extract caused the 17α-estradiol to extract into the ethyl acetate rather than

THE EFFECT OF MAGNESIUM CHLORIDE ON THE ABILITY OF RABBIT LIVER MICROSOMAL ORGANIC EXTRACT TO RESTORE 17α-ESTRADIOL-3-GLUCOSIDE SYNTHESIS IN TRITON X-100 TREATED MICROSOMES

Additions	% Conversion of 17α-Estradiol to 17α-Estradiol-3-Glucoside "UDP-Glucose Independent"	
	–Magnesium Chloride	+ 80 mM Magnesium Chloride
None	22.7	29.0
+ 0.2% Triton X-100	0	0
+ Triton + 0.5 Millilitres Extract	0	0.3
+ Triton + 1 Millilitre Extract	0.4	0.4
+ Triton + 2.5 Millilitres Extract	0.5	0.9
+ Triton + 4 Millilitres Extract	1.8	2.1
+ Triton + 5 Millilitres Extract	5.5	3.5

Extract = Rabbit Liver Microsomal Chloroform:Methanol:Water (1:1:0.3)

5 Millilitre Extract Contains 44.3 Micrograms P_i

TABLE 35

THE EFFECT OF PIG LIVER CHLOROFORM METHANOL (2:1) EXTRACT ON THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE IN TRITON X-100 TREATED MICROSOMES

Additions	"UDP-Glucose Independent" Synthesis of 17α-Estradiol-3-Glucoside	
	DPM in Ethyl Acetate	% Conversion of 17α-Estradiol to 17α-Estradiol-3-Glucoside
None	11,733	21.3
0.2% Triton X-100	1,110	2.0
0.2% Triton X-100 + 1 Millilitre Organic Extract	2,696	4.9
0.2% Triton X-100 + 2 Millilitres Organic Extract	4,601	8.4
0.2% Triton X-100 + 3 Millilitres Organic Extract	5,671	10.3
0.2% Triton X-100 + 4 Millilitres Organic Extract	7,045	12.8
0.2% Triton X-100 + 5 Millilitres Organic Extract	10,952	19.9
0.2% Triton X-100 + 7.5 Millilitres Organic Extract	14,525	26.4
0.2% Triton X-100 + 10 Millilitres Organic Extract	16,554	30.1
0.2% Triton X-100 + 15 Millilitres Organic Extract	24,990	45.4
0.2% Triton X-100 + 20 Millilitres	29,771	54.1

1 Millilitre Chloroform:Methanol (2:1) Extract is Equivalent to 14.2 Micrograms Pi

TABLE 36

THE INTERFERENCE BY PIG LIVER ORGANIC EXTRACT
WITH THE EXTRACTION OF 17α-ESTRADIOL
AND 17α-ESTRADIOL-3-GLUCOSIDE

Contents of the Blank	D.P.M. in the Ethyl Acetate 17α-Estradiol-3-Glucoside	% of the Added Tritium in the Ethyl Acetate Fraction
[^{3}H] 17α-Estradiol, KCl, Tris (550,000 d.p.m.)	5,786	1.1
[^{3}H] 17α-Estradiol, KCl, Tris Organic Extract in 0.1% Triton X-100	55,331	10
[^{3}H] 17α-Estradiol, KCl, Tris Organic Extract in 0.2% Triton X-100	106,043	19.3
[^{3}H] 17α-Estradiol, KCl, Tris Organic Extract in 0.1% Triton X-100 + Boiled Microsomes	35,599	6.5

TABLE 37

into the benzene layer as would be expected. The counts depicted in (Table 36) thus represent both unreacted 17α-estradiol and also 17α-estradiol-3-glucoside and therefore are not an accurate measure of the per cent conversion of 17α-estradiol to its glucoside.

In some of the manipulations listed in (Table 21) it was impossible to eliminate the interference by the lipid, i.e. numbers 1, 2, 3, 4, 5 and 9. Others, i.e. 6, 7 and 8, permitted the separation of 17α-estradiol from 17α-estradiol-3-glucoside, but were tedious and time consuming. Each manipulation of the assay involved a number of extra incubations, blanks and various controls, and often meant numerous calculations for the simplest experiment. The addition of non-radioactive 17α-estradiol to the standard incubation 5 minutes before stopping the reaction minimized any interference by the lipid with the extraction procedure. For all further pig liver lipid studies, a lipid blank was included for each pig liver lipid concentration assayed and 100 micrograms of non-radioactive 17α-estradiol was added to the incubation as described above. These precautions ensured that the counts obtained in the ethyl acetate extract were a true representation of the 17α-estradiol-3-glucoside synthesized. (Table 38) illustrates the results obtained in these studies and show that when the ethyl acetate extracts were subjected to chromatographic examination in chloroform : ethanol (4:1), there was a good correlation between the increasing counts obtained in the area of the chroma-

THE EFFECT OF CRUDE PIG LIVER ORGANIC EXTRACTS
ON THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE

Additions to Standard 3 Millilitre Assay (Contains 100 micrograms nonradioactive estradiol)	% Conversion of 17α-Estradiol to 17α-Estradiol -3-Glucoside	C.P.M. in 17α-Estradiol-3-Glucoside Area of Chromatogram	C.P.M. in 17α-Estradiol Area of the Chromatogram
None	46	1928	423
0.2% Triton X-100	3.3	598	420
0.2% Triton X-100 + 0.5 Millilitre Lipid Extract	2.8	846	533
Blank	-	-	147
0.2% Triton X-100 + 1 Millilitre Lipid Extract	4.0	612	541
Blank	-	-	439
0.2% Triton X-100 + 1.5 Millilitre Lipid Extract	6.0	881	594
Blank	-	-	117
0.2% Triton X-100 + 2.0 Millilitre Lipid Extract	5.4	758	414
Blank	-	-	215
0.2% Triton X-100 + 2.5 Millilitre Lipid Extract	7.0	751	254
Blank	-	-	318
0.2% Triton X-100 + 3 Millilitre Lipid Extract	7.5	1,621.3	517
Blank	-	-	528
0.2% Triton X-100 + 5 Millilitres Lipid Extract	10.8	424	880
Blank	-	-	383

5 Millilitre Lipid Extract Contains 71.11 Micrograms Pi

TABLE 38

togram where 17α-estradiol-3-glucoside would be expected to run and the presence of increasing concentrations of pig liver lipid extracts.

3) Purification Studies

DEAE cellulose chromatography

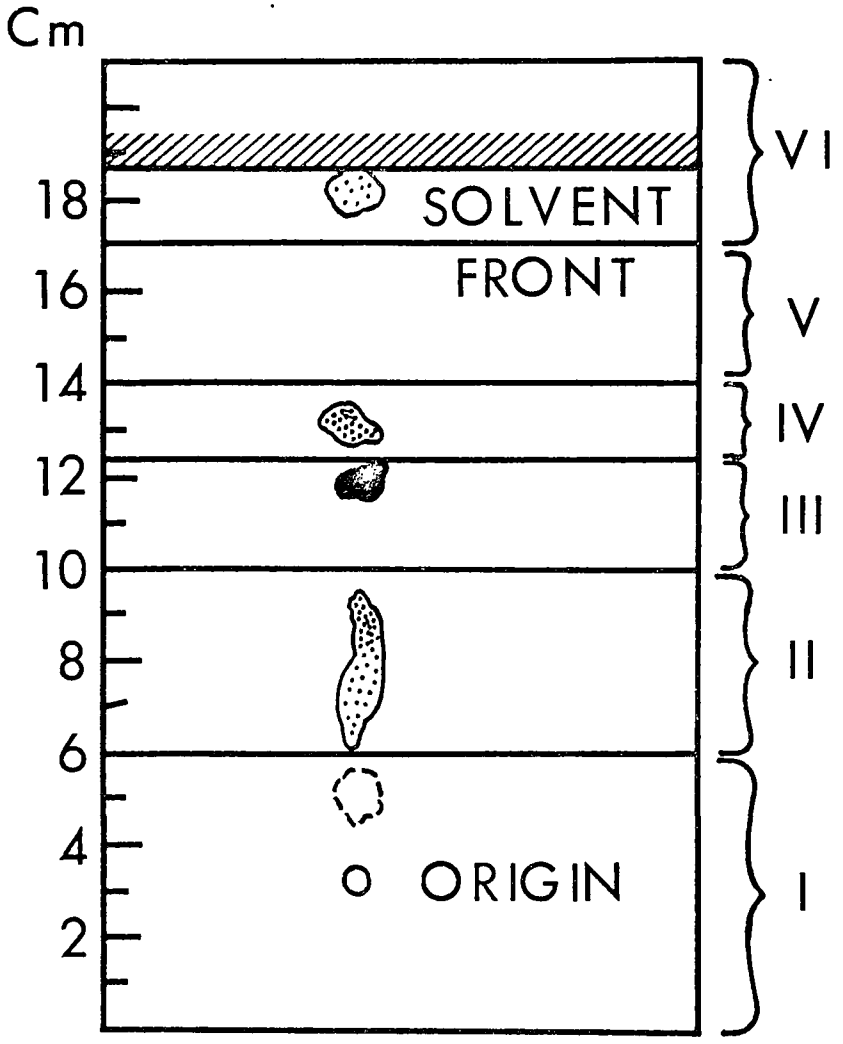
Attempts at purification of the lipid extracts by DEAE cellulose chromatography abolished their ability to restore "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside in microsomal preparations which had lost this capacity due to treatment with Triton X-100. None of the fractions collected from the column, to which the rabbit liver microsomal chloroform : methanol (2:1) extract had been applied, were able to stimulate steroid glucoside synthesis when incubated with Triton X-100 treated microsomal preparations either in the presence or absence of 250mM magnesium chloride. The initial studies with the pig liver extracts suggested three different peaks of restorative activity among the fractions collected from the DEAE - cellulose column. However, these results were not reproducible and when these and all the other fractions collected were examined for lipid glucosylation no glycosylation activity could be demonstrated.

Thin layer chromatographic examination of the lipid extracts

(Figure 31) illustrates the separation achieved when a rabbit liver acceptor lipid extract was chromatographed in chloroform : methanol : ammonia : water (80:30:0.5:3 v/v). When the different fractions eluted from this chromatogram were examined for their effect on the synthesis of 17∝-estradiol-3-glucoside in Triton X-100 treated microsomal preparations no increase in steroid-3-glucoside synthesis was observed. When other rabbit liver lipid extracts were subjected to chromatographic examination in the above solvent system and also in chlorofrom : methanol : water (10:10:3 v/v) and then assessed for their ability to restore 17∝-estradiol-3-glucoside synthesis in microsomal preparations where this capacity had been lost due to treatment with Triton X-100 the same results were obtained.

Similar results were also obtained when the above studies were carried out in the presence of 250mM magnesium chloride. The pig liver lipid fractions, (Figure 32), eluted from the chromatograms also proved unable to restore 17∝-estradiol-3-glucoside synthesis in Triton X-100 treated microsomal preparations either when added alone, or in various combinations with the other fractions eluted from the chromatogram.

CHROMATOGRAPHIC EXAMINATION OF THE RABBIT LIVER ACCEPTOR LIPID EXTRACT



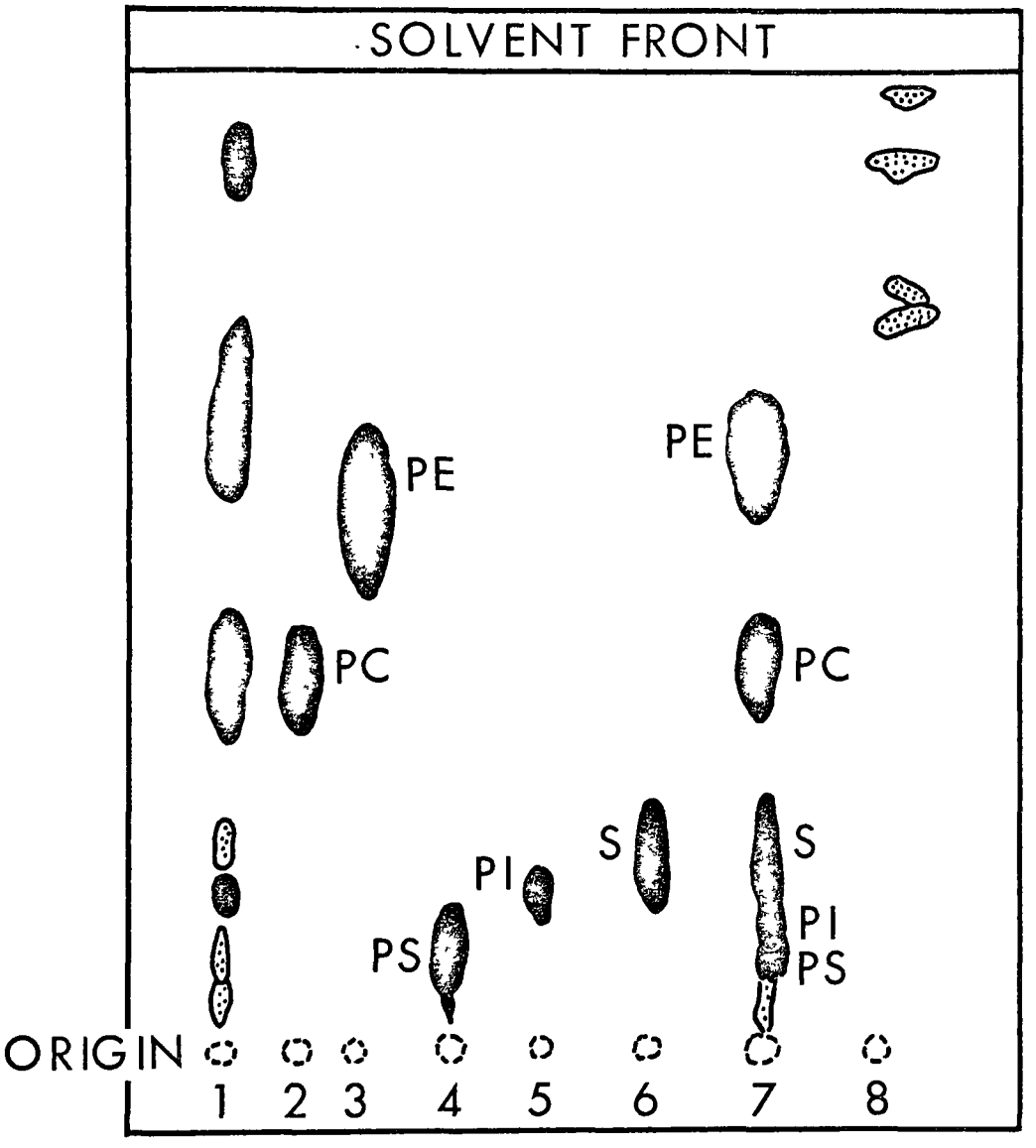
SOLVENT SYSTEM = CHLOROFORM:METHANOL:AMMONIA: WATER (80:30:0.5:3 V/V)

SPRAY REAGENT = PHOSPHOMOLYBDIC ACID

FIGURE 31

CHROMATOGRAPHIC EXAMINATION OF THE PIG LIVER CHLOROFORM: METHANOL (2:1) EXTRACT

CHLOROFORM:METHANOL:AMMONIUM HYDROXIDE (65:35:5 V/V)



(1) CRUDE PIG LIVER CHLOROFORM:METHANOL (2:1) EXTRACT
(2) PHOSPHATIDYL CHLORINE,
(3) = PHOSPHATIDYL ETHANOLAMINE
(4) = PHOSPHATIDYL SERINE, (5) = PHOSPHATIDYL INOSITOL,
(6) = SPHINGOMYELIN,
(7) = COMBINATION OF 2-6 STANDARDS,
(8) = DOLICHOL PHOSPHATE FROM CALF BRAIN

FIGURE 32

4) Studies to Examine the Nature of the Lipid Intermediate

The chloroform layer, which would be expected to contain any of the dolichol monophosphate glucose synthesized by the rabbit or rat microsomes, was found to contain 2.2 per cent of the radioactivity added to the assay as UDP-D-(U-^{14}C)glucose.

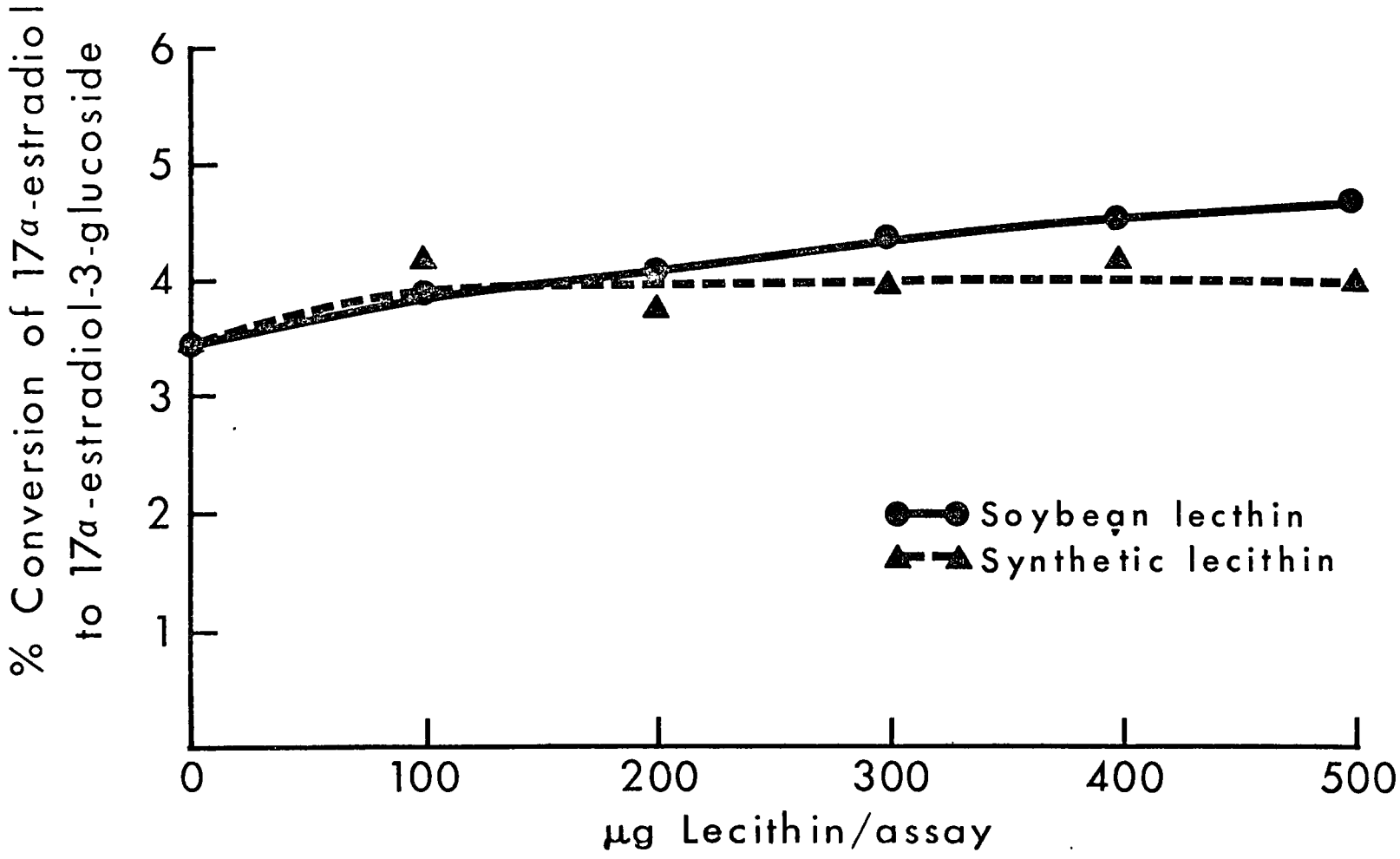
However, when this chloroform fraction was examined for its ability to increase the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside in Triton X-100 treated microsomal preparations, no increase in glucoside synthesis was observed, and no ^{14}C was detected in the area of the chromatogram where 17α-estradiol-3-glucoside would be expected to run when chromatographed in the solvent systems chloroform : ethanol (4:1 v/v) or propanol : water (7:1 v/v).

As seen in (Figure 33), the addition of synthetic or soybean lecithin at concentrations ranging from 100 to 500 micrograms per 3 millilitre assay did not cause a significant change in 17α-estradiol-3-glucoside in the Triton X-100 treated microsomal preparations.

(Tables 39-41) depict the results obtained when dolichol, dolichol phosphate, farnesol, geranylgeraniol and vitamin E were examined for their effect on 17α-estradiol-3-glucoside synthesis. Dolichol rather than dolichol phosphate caused an increase in the "UDP-glucose independent" glucoside synthesis in both the untreated microsomal preparations and those treated with Triton X-100. The "UDP-glucose dependent" pathway was not influenced by the presence of these isoprenoid com-

FIGURE 33

THE EFFECT OF ADDING VARYING CONCENTRATIONS OF SOYBEAN LECITHIN AND SYNTHETIC LECITHIN ON THE "UDP-GLUCOSE INDEPENDENT" SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE IN TRITON X-100 TREATED MICROSOMAL PREPARATIONS



THE EFFECT OF DOLICHOL AND DOLICHOL PHOSPHATE
ON THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE

Additions	% Conversion of 17α-Estradiol to 17α-Estradiol-3-Glucoside			
	"UDP-Glucose Independent"		"UDP-Glucose Dependent"	
	−Triton X-100	+ 0.2% Triton X-100	− Triton X-100	+ 0.2% Triton X-100
None	32.9	11.5	20.8	15.1
+ 30 Micrograms Dolichol	42.3	15.7	19.9	14.7
+ 28 Micrograms Dolichol Phosphate	25.8	11.2	20.3	15.8

TABLE 39

THE EFFECT OF FARNESOL AND GERANYLGERANIOL
ON THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE

Concentration of the Isoprenoid per 3 Millilitre Assay	% Conversion of 17α-Estradiol to 17α-Estradiol-3-Glucoside*			
	"UDP-Glucose Independent"		"UDP-Glucose Dependent"	
	−Triton X-100	+Triton X-100	−Triton X-100	+Triton X-100
0	25.95	3.2	6.3	6.65
1.49 Milligrams Farnesol	5.65	2.3	0.25	1.6
1.5 Milligrams Geranylgeraniol	12.3	2.4	0.65	2.88

* Average result of two experiments

TABLE 40

THE EFFECT OF VITAMIN E (D-α-TOCOPHEROL)
ON THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE

Concentration of Vitamin E per 3 Millilitre Assay (Micrograms)	% Conversion of 17α-Estradiol to 17α-Estradiol-3-Glucoside			
	"UDP-Glucose Independent"		"UDP-Glucose Dependent"	
	−Triton X-100	+Triton X-100	−Triton X-100	+Triton X-100
0	22.1	4.5	10.3	4.9
200	22.6	4.4	10.0	5.5
300	23.7	2.3	10.1	5.5
400	20.2	3.3	9.9	3.6

TABLE 41

pounds. The ethyl acetate extracts from these studies were chromatographed in chloroform : ethanol (4:1 v/v) using authentic standards of 17α-estradiol and 17α-estradiol-3-glucoside as reference compounds in order to ensure that the assay was operating satisfactorily.

The presence of farnesol and geranylgeraniol inhibited the synthesis of steroid 3-glucoside by the "UDP-glucose independent" and the "UDP-glucose dependent" pathways in the untreated and Triton X-100 treated microsomal preparations, (Table 40). Vitamin E had no significant effect on 17α-estradiol-3-glucoside synthesis as seen in (Table 41).

The results obtained in studies with retinol are shown in (Tables 42-43). Retinol, when added alone or in the presence of manganous chloride, caused an increase in the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside in Triton X-100 treated microsomal preparations. This partial restoration of the glucoside synthesis was abolished when EDTA or ATP was included in the incubation mixture. Increasing concentrations of retinol, up to 1.2 milligram per 3 millilitre assay, caused a gradual increase in the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside. At concentrations of retinol above 1.2 milligrams per 3 millilitre assay, glucoside synthesis declined. The presence of retinol inhibited the "UDP-glucose dependent" synthesis of steroid 3-glucoside at all concentrations examined.

When the rabbit liver microsomes were examined for their

THE EFFECT OF RETINOL ON THE SYNTHESIS OF
17α-ESTRADIOL-3-GLUCOSIDE

Additions	% Conversion of 17α-Estradiol to 17α-Estradiol-3-Glucoside	
	"UDP-Glucose Independent"	"UDP-Glucose Dependent"
None	22.6	12.3
+ 0.2% Triton X-100	11.4	2.3
+ 0.2% Triton X-100 + 400 Micrograms Retinol	3.9	1.4
+ 0.2% Triton X-100 + 1.125 Milligrams Retinol	18.0	
+ 0.2% Triton X-100 + 1.52 Milligrams Retinol	25.5	
+ 0.01 M $McCl_2$	20.1	
+ 0.01 M $MnCl_2$ + 0.2% Triton X-100	7.75	
+ 0.01 M $MnCl_2$ + 0.2% Triton X-100 + 1.52 Milligrams Retinol +	16.3	

TABLE 42

THE EFFECT OF INCREASING CONCENTRATIONS OF RETINOL ON THE SYNTHESIS OF 17α-ESTRADIOL-3-GLUCOSIDE

Additions to Standard 3 Millilitre Assay	% Conversion of 17α-Estradiol to 17α-Estradiol-3-Glucoside	
	"UDP-Glucose Independent"	"UDP-Glucose Dependent"
None	22.3	4.7
0.2% Triton X-100	8.4	7.8
0.2% Triton X-100 + 400 Micrograms Retinol	15.3	0.8
0.2% Triton X-100 + 800 Micrograms Retinol	14.0	3.9
0.2% Triton X-100 + 1.2 Milligrams Retinol	19.9	-
0.2% Triton X-100 + 1.6 Milligrams Retinol	8.6	2.6
0.2% Triton X-100 + 2 Milligrams Retinol	8.7	-

TABLE 43

ability to synthesis retinol-glucoside from UDP-glucose and (^{14}C)retinol no ^{14}C was detected in the area of the chromatogram where retinol glucoside would be expected.

D) Discussion

The stimulation of the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside in Triton X-100 treated microsomes by the organic extracts from pig liver and lyophilized rabbit liver microsomes indicates that there is a lipid involved in this particular reaction as in many other sugar transfer reactions (83-180, 183-194). The chloroform : methanol (2:1) extracts would be expected to contain a mixture of phospholipids, including dolichol monophosphate glucose or a similar type lipid intermediate, if these had been present in the pig liver or rabbit liver microsomes. The chloroform : methanol : water (1:1:0.3) extract, on the other hand, might contain, in addition to dolichol monophosphate glucose, other isoprenoid monosaccharide derivatives and various other phospholipids, such as a dolichol pyrophosphate oligosaccaride type of compound (118, 152, 168, 175, 176, 187, 188)

As seen in (Table 35), the presence of 80mM magnesium chloride did not enhance the stimulation of steroid glucoside synthesis caused by the addition of the rabbit liver microsomal organic extract to the Triton X-100 treated microsomes. This insensitivity to the presence of magnesium chloride could be expected if the exogenous organic extract contained an intact gluco-lipid intermediate, since a requirement for manganous or magnesium chloride has been demonstrated in the synthesis of a glycolipid from isoprenol phosphate and the relevant

nucleotide sugar donor. This requirement does not always pertain to the step which involves transfer of the sugar from this monosaccharide derivative of polyprenol phosphate to an acceptor, (Figure 29) (88, 101, 133, 190, 193).

The extraction problems which arose due to the presence of high pig lipid concentrations in the standard incubation were overcome by the addition of non-radioactive 17α-estradiol to the standard assay mixture and by the inclusion of adequate lipid blanks. The high concentrations of non-radioactive 17α-estradiol would be expected to compete with the unreacted radioactive substrate 17α-estradiol and the radioactive product 17α-estradiol-3-glucoside for the lipid since the lipid preferentially binds to the free steroid. This non-radioactive estrogen - lipid complex, even if extracted into the ethyl acetate fraction would not be detected when the fractions were counted for their tritiated conjugate content and thus the counts obtained would only be representative of the amount of glucoside produced, (Table 38). These studies using pig and rabbit liver organic extracts, provided further proof for the involvement of a lipid in the "UDP-glucose independent" synthesis of 17α-estradiol and prompted the studies designed to determine the nature of such a lipid.

Much of the work dealing with the lipid extracts was complicated by the lack of pure isoprenoid compounds for use as standards. Dolichol phosphate from calf brain was used as a standard in some of the earlier chromatographic studies, but

as seen in (Figure 32) the sample available was unstable or impure, since more than one spot was obtained upon chromatographic examination. Non-radioactive dolichol and dolichol phosphate recently became available commercially and these were used in some of the other studies described. However, neither radioactive nor non-radioactive pure dolichol monophosphate glucose, dolichol pyrophosphate - oligosaccharide or similar isoprenoid sugar derivatives were available commercially so that the identification of the "active" lipid component remains a difficult task.

The inability of any of the fractions collected from the DEAE cellulose column or eluted from the various thin layer chromatograms to restore 17α-estradiol-3-glucoside synthesis in Triton X-100 treated microsomal preparations cannot be taken as definite proof for the non-involvement of a lipid in the "UDP-glucose independent" reaction. Some of these fractions might contain the lipid intermediate but at concentrations too low to effect lipid glycosylation or to cause an increase of the synthesis of 17α-estradiol-3-glucoside. This negative result might also be explained by the breakdown of an unstable intermediate in the course of the purification procedures. The availability of radioactive polyprenol phosphate sugar derivatives would make the detection and identification of any similar lipid intermediates present in the fractions isolated from the DEAE cellulose column or from the thin layer chromatograms more practicable. The fraction found

to contain an "active" lipid might then be subjected to further purification and characterization.

This lack of a lipid - sugar standard also caused a certain degree of uncertainty in the studies undertaken to examine the ability of the rabbit and rat liver microsomes to synthesize dolichol monophosphate glucose from pure dolichol phosphate and UDP-D-(U-^{14}C)glucose. Even though some radioactivity was found in the chloroform fraction thought to contain the dolichol monophosphate glucose, thin layer chromatographic examination of the extract suggested at least partial breakdown of the compound present. This instability of the lipid would explain the absence of any detectable transfer of (^{14}C)-glucose from this chloroform fraction into the steroid.

The stimulation of 17α-estradiol-3-glucoside synthesis by dolichol rather than dolichol phosphate, as seen in (Table 39), was unexpected, since dolichol monophosphate glucose would be the most likely "active" lipid intermediate, and no dolichol phosphorylating enzyme has been reported in mammalian systems. The transfer of glucose from a low energy dolichol glucoside would appear unlikely unless a transglucosylation type reaction could occur. The studies. depicted in (Tables 42 and 43) indicated an effect of retinol in the "UDP-glucose independent" reaction. However, the fact that the rabbit liver microsomes were unable to synthesize a retinol glucoside suggested that tne stimulatory effect on 17α-estradiol-3-glucoside synthesis might be due to retinol-phosphate-glucose.

These studies are consistent with the involvement of a lipid in the "UDP-glucose independent" synthesis of 17α-estradiol-3-glucoside, but not in the "UDP-glucose dependent" reaction since the addition of the pure isoprenoid compounds either inhibited or had no effect on the "UDP-glucose dependent" reaction. Even though the exact nature of the lipid has not been defined it does not appear to be farnesol, geranylgeraniol or Vitamin E. The possibility exists that it may be a highly unstable isoprenoid compound of the dolichol type.

This area is worthy of further investigation, since it is the first report of a lipid involvement in steroid glycoside formation or in a sugar transfer reaction to a small molecule. If the lipid in question does prove to be of the isoprenoid family there arises the interesting possibility of an inter-relationship between steroid conjugate formation and glycoprotein synthesis. Since glucose is not one of the sugars commonly found in glycoproteins, the involvement of a dolichol sugar derivative or a related compound in steroid glucosylation would explain the detection of these compounds in a number of animal systems (143, 162-165). Hopefully, in the future, with the increased availability of pure radioactive and non-radioactive isoprenoid sugar and isoprenoid phosphate sugar compounds, the exact nature of the lipid will be more easily defined and its significance in estrogen metabolism and glycoprotein synthesis established.

BIBLIOGRAPHY

1. Williams, R. T. in "Biogenesis of Natural Compounds" (P. Bernfield, ed.) 2nd ed. Macmillan (pergamon) New York. pp 589-639 1967
2. Venning, E. H. and Browne, J.S.L. Proc. Soc. Exp. Biol. Med, 34, 792 1936
3. Cohen, S.L. and Marrian, G.F. Biochem. J., 30, 57 1936
4. Schacter, B. and Marrian, G.F. J. Biol. Chem., 126, 663 1938
5. Layne, D.S. Endocrinology, 76, 600 1965
6. Layne, D.S., Sheth, N.A. and Kirdani R.Y. J. Biol. Chem., 239, 322, 1964
7. Williamson, D.G., Collins, D.C., Layne, D.S., Brown Conrow, R. and Bernstein, S. Biochemistry, 8, 4299 1969
8. Mellor, J.D. and Layne, D.S. J. Biol. Chem., 246, 4377 1971
9. Collins, D.C., Williamson, D.G. and Layne, D.S. J. Biol. Chem., 245, 873 1970
10. Quamme, G.A. and Layne, D.S. unpublished
11. Strominger, J.L., Maxwell, E.S. and Kalckar, H.L. in Methods in Enzymology, Editors: S.P. Colowick and N.O. Kaplan. Academic Press, New York. N.Y. Vol. III pp 974 1957

12. Williamson, D.G., Polakova, A. and Layne, D.S.
Biochem. Biophys. Res. Commun., 42, 1057 1971

13. Dutton, G.J.
in "Glucuronic Acid, Free and Combined"
Academic Press, London. pp. 185 1966

14. Jayle, M.F. and Pasqualini, J.R.
in "Glucuronic Acid, Free and Combined. Chemistry, Biochemistry, Pharmocology and Medicine". (G.J.Dutton, ed)
Academic Press, New York. pt III pp. 507 1966

15. Layne, D.S.
in "Metabolic Conjugation and Metabolic Hydrolysis", (N.H. Fishmen, ed.) Academic Press. Vol. 1, pp 21-52 1970

16 Layne, D.S., Labow, R.S. and Williamson, D.G.
in Molecular Mechanisms of Gonadal Hormone Action in Sex Hormone Research Volume 1 University Park Press, Baltimore. London. Tokyo. Edited by John A. Thomas, Radhey L. Singhal.

17. Felger, C.B. and Katzman, P.A.
Fed. Proc., 20, 199 1961

18. Levitz, M., Katz, I. and Twombly, G.H.
Steroids 6, 553 1965

19. Collins, D.C., Jirku, H. and Layne, D.S.
J. Biol. Chem., 243, 2928 1968

20. Dahm, K. and Breuer, H.
Acta Endocrinol., 52, 43 1966_a

21. Dahm, K. and Breuer, H.
Z. Clin, Chem., 4, 153 1966_b

22. Breuer, H. and Wessendorf, D.
Z. Physiol. Chem., 345, 1 1966

23. Williamson, D.G., Layne, D.S., Nilsen, M. and Hobkirk, R.
Can. J. Biochem., 50, 958 1972

24. Kirdani, R.Y., Slaunwhite, W.R. Jr. and Sandberg, A.A.
Steroids, 12, 171 1968

25. Hobkirk R. Nilsen, M., Williamson, D.G. and Layne, D.S.
J. Clin. Endocrinol., 34, 690 1972

26. Kirdani, R.Y., Sampson, D., Murphy, G.P. and Sandberg, A.A.
J. Clin. Ecdocrinol., 24 546 1972

27. Sa'at, Y.A. and Slaunwhite, W.R. Jr.
Steroids, 13, 545 1969

28. Hobkirk, R., Green, R.N., Nilsen, M. and Jennings, B.A.
Can. J. Biochem., 52, 9 1974

29. Mellor, J.D. and Hobkirk, R.
Can. J. Biochem., 53, 779 1975

30. Williams, K.I.H., Henry, D.H., Collins, D.C. and Layne, D.S.
Endocrinology, 83, 113 1968

31. Jirku, H. and Layne, D.S.
Biochemistry, 4, 2126 1965

32. Collins, D.C., Williams, K.I.H. and Layne, D.S.
Archiv. Biochem. Biophys., 121, 609 1967

33. Cable, R.G., Jirku, H. and Levitz, M.
Biochemistry, 9, 4587 1970

34. Eichenberger, W. and Newman, D.W.
Biochem. Biophys. Res. Commun., 32, 366 1968

35. Prochazka, Z. Collection Czech. Chem. Commun., 33, 4039 1968

36. Tabone, D. and Tabone, J. Compt. Rend., 242, 302 1956

37. Smith, J.N. and Turbert, H. Biochem. J., 92, 127 1964

38. Dutton, G.J. Archiv. Biochem. Biophys., 116, 399 1966

39. Gessner, T. and Vollmer, C.A. Fed. Proc. Fed. Amer. Soc. Exp. Biol., 28, 545 1969

40. Gessner. T., Jacknowitz, A. and Vollmer, C.A. Biochem. J., 132, 249 1973

41. Kuenzle, C.C. Biochem. J., 119, 387 1970
395 1970
411 1970

42. Compernolle, F., Van Hees, G.P., Jevery, J. and Heirwegh, K.P.M. Biochem. J., 125, 811 1971

43. Fevery, J., Van Hees, G.P., Leroy, P., Compernolle, F. and Heirwegh, K.P.M. Biochem. J., 125, 803 1971

44. Wong, K.P. Biochem. J., 125, 929 1971

45. Heirwegh, K.P.K., Mellwissen, J.A.T.P. and Fevery J. Biochem. J., 125, 28p 1971

46. Fevery, J., Leroy, P. and Heirwegh, K.P.M. Biochem. J., 129, 619 1972

47. Van Heyningen, R.

Nature, Lond. 230, 393 1971

48. Rodriguez, P., Bella, O. and Gaede, K.

FEBS Letters, 28, 133 1972

49. Williamson, D.G. and Layne, D.S.

J. Clin. Endocrinology, 33, 157 1971

50. Labow, R.S., Williamson, D.G., Layne, D.S. and Collins, D.C.

Can. J. Biochem., 53, 1028 1975

51. Layne, D.S., Labow, R.S., Paquet, A. and Williamson, D.G.

Biochemistry, 15, 1268 1976

52. Sneddon, A. and Marrian, G.F.

Biochem, J., 86, 385 1963

53. Wallace, E. and Silberman, N.

J. Biol. Chem., 239, 2809 1964

54. Wang, D.Y. and Bulbrook, R.D.

in "Advances in Reproductive Physiology" (A. McLaren, ED.)

Logos Press London Vol. 3, pp. 113 1968

55. Jirku, H. and Levitz, M.

J. Clin. Endocrinol. Metab., 29, 615 1969

56. Touchstone, J.C.

in "Estrogen Assays in Clinical Medicine"

(C.A. Paulsen ed.) Univ. of Washington Press,

Seattle. Washington. pp. 164-172 1965

57. Rao. G.S. and Breuer, H.

J. Biol. Chem., 244, 5521 1969

58. Rao, G.S., Rao, M.L. and Breuer, H.

Biochem. J., 118, 625 1970_a

59. Rao, G.S., Rao, M.L. and Breuer, H.
Biochem. J., 119, 635 1970b

60. Rao, G.S., Rao, M.L. and Breuer, H.
J. Steroid Biochem., 3, 1 1972

61. Gotze, W., Grube, E., Rao, G.S., Rao M.L. and Breuer, H.
Hoppe - Seyler's Zirt. Physiol. Chem., 352,
1223 1971

62. Jirku, H., Kadner, S. and Levitz, M.
Steroids, 19, 525 1972

63. Arcos, M. and Lieberman, S.
Biochemistry, 6, 2032 1967

64. Abdel-Aziz, M.T. and Williams, K.I.H.,
Steroids, 13, 809 1969

65. Collins, D.C. and Layne, D.S.
Steroids, 13, 783 1969

66. Layne, D.S., Quamme, G.A., Labow, R.S., Mellor, J.D.,
Polakova, A. and Williamson, D.G.
Proceedings of the 3rd International Congress
on Hormonal Steroids, Hamburg, Editors V.H.T.
James and L. Martini pp 217 1970

67. Labow, R.S. and Layne, D.S. Biochem. J., 128, 491 1972

68. Labow, R.S., Williamson, D.G. and Layne, D.S.,
Can. J. Biochem., 52, 203 1974

69. Labow, R.S. and Layne, D.S.
Biochem. J., 142, 75 1974

70. Mulder, G.J., Biochem. Biophys, Acta, 289, 284 1972

71. Labow, R.S., Williamson, D.G. and Layne, D.S.
Biochemistry, 10, 2553 1971

72. Labow, R.S., Williamson, D.G. and Layne, D.S.
Biochemistry, 12, 1548 1973

73. Hasnain, S. and Williamson, D.G.
Can. J. Biochem., 52, 120 1974

74. Hasnain, S. and Williamson, D.G.
Biochem. J., 147, 457 1975

75. Hasnain, S. and Williamson, D.G.
Biochem. J., 161, 279 1977

76. Williamson, D.G., Layne, D.S. and Collins, D.C.
J. Biol. Chem., 247, 3286 1972

77. Mellor, J.D., Layne, D.S., Irwin, J.E. and Mellor, A.
Can. J. Biochem., 51, 1292 1973

78. Williamson, D.G. and Layne, D.S.
Can. J. Biochem., 48, 523 1970

79. Hobkirk, R. and Nilsen, M.
Steroids (Shrewsbury, Mass), 15, 649 1970

80. Conn E.E.
Enzymology of phenolic biosynthesis. In: "Biochemistry of Phenolic Compounds" Editor: J.B. Harborne.
Academic Press, London pp 423 1964

81. Williamson, D.G. and Layne, D.S.
Biochem. Biophys. Res. Commun., 64, 256 1975

82. Burns, J.J. and Conney, A.H.
Metabolism of glucoronic acid and its lactone. In "Glucuronic acid, Free and Combined".
Editor, G.J. Dutton, Academic Press, New York, N.Y.
pp 366 1966

83. Leloir, L.F.

Science, 172, 1299 1971

84. Osborn, M.J.

Ann. Rev. Biochem., 38, 501 1969

85. Lennarz, W.J.

Ann. Rev. Biochem., 39, 359 1970

86. Heath, E.C.

Ann. Rev. Biochem., 40, 29 1971

87. Wright, A. and Kanegasaki, S.

Physiol. Rev., 51, 748 1971

88. Lennarz, W.J. and Scher, M.G.,

Biochem. Biophys. Acta, 265, 417 1972

89. Lennarz, W.J. and Wakil, S.J.

in Lipid Metabolism. Academic Press, N.York pp 155 1970)

90. Baddiley, J.

in Essays in Biochemistry 8, 35 1972

91. Hemming, F.W.

in Biochemistry of Lipids. (Goodwin, T.W. ed. Butterworths, London and University Park Press, Baltimore Vol. 4 pp 39-97 1974

92. Higashi, Y., Strominger, J.L. and Sweeley, C.C.

Proc, Natl. Acad. Sci. U.S.A., 57, 1878 1967

93. Wright, A. Dankert, M., Fennessey, P. and Robbins, P.W.

Proc. Natl. Acad. Sci. U.S.A. 57, 1798 1967

94. Weiner, I.M., Higuchi, T., Rothfield, L., Saltmarch Andrew, M., Asborn, M.J. and Horecker, B.L.

Proc. Natl. Acad, Sci. U.S.A., 54, 228 1965

95. Wright, A., Darkert, M. and Robbins, P.W.

Proc. Natl. Acad. Sci. U.S.A., 54, 235 1965

96. Badiley, J.

in Biochem. Soc. Trans., 1, 1026 1973

97. Troy, F.A., Frerman, F.E. and Heath, E.C.

J. Biol. Chem., 246, 118 1971

98. Scher, M., Lennarz, W.J. and Sweeley, C.C.

Proc. Natl. Acad. Sci. U.S.A., 59, 1313 1968

99. Behrens, N.H. and Cabil, E.J.

Biol. Chem., 243, 502 1968

100. Lahav, M., Chill, T.H. and Lennarz, W.J.

J. Biol. Chem., 244. 5890 1969

101. Tanner, W.

Biochem. Biophys. Res. Commun., 35, 144 1969

102. Sentandreu, R. and Lampen, J.O.

FEBS Letters, 14, 109 1971

103. Tanner, W., Jung, P. and Behrens, N.H.

FEBS Letters, 16, 245 1971

104. Bretthauer, R.K., Wu, S. and Irwin, W.E.

Biochim. Biophys. Acta, 304, 736 1973

105. Jung, P. and Tanner, W.

Eur. J. Biochem., 37,1 1973

106. Parodi, A.J.

Eur. J. Biochem., 75, 171 1977

107. Sentandreu, R. and Northcote, D.H.

Biochem. J., 109, 419 1968

108. Nakajima, T. and Ballow, C.E.

J. Biol. Chem., 249, 7685 1974

109. Babczinski, P. and Tanner, W.

Biochim. Biophys. Res.Commun., 54, 1119 1973

110. Sharma, C.B., Babczindki, P., Lehle, L. and Tanner, W.
Eur. J. Biochem., 46, 35 1974

111. Lehle, L. and Tanner, W.
Biochim. Biophys. Acta, 350, 225 1974

112. Bretthauer, R.K. and Wu, S.
Archiv. Bioche,. Biophys., 167, 151 1975

113. Lehle, L. and Tanner, W.
Biochim. Biophys. Acta, 399, 364 1975

114. Parodi, A.J.
FEBS Letters, 71, 283 1976

115. Behrens, N.H.
Proc. Natl. Acad. Sci. U.S.A., 66, 153 1970

116. Bretthauer, R.K. and Wu. S.
in Miami Winter Symposia Biology and Chemistry of Eucaryotic Cell Surfaces. Edited by Lee and Smith.
Vol. 7 pp 335 1974

117. Barr. R.M. and Hemming, F.W.
Biochem. J., 126 1203 1972

118. Letoublon, R.C.P., Comte, J. and Got, R.
Eur. J. Biochem., 40, 95 1973

119. Waechter, C.J. and Lennarz, W.J.
Ann. Rev. Biochem., 45, 95 1976

120. Kauss, H.
FEBS Letters, 5, 81 1969

121. Villemez, C.C. and Clark, A.F.
Biochem. Biophys. Res. Commun,. 36, 57 1969

122. Villemez, C.C.
Biochem. Biophys. Res. Commun., 40, 636 1970

123. Alam, S.S., Barr, R.M., Richards, J.B. and Hemming F.W.
Biochem. J., 121, 19p 1970

124. Alam, S.S. and Hemming, F.W.
Phytochemistry, 12, 1641 1973

125. Clark, A.F. and Villemez, C.C.
FEBS Letters, 32, 84 1973

126. Forsee, W.T. and Elbein, A.D.
J. Biol. Chem., 248, 2858 1973

127. Forsee, W.T. and Elbein, A.D.
J. Biol. Chem., 250, 9283 1975

128. Pont Lezica, R., Brett, C.T., Romero Martinez, P. and Darkert,M.A
Biochem. Biophys. Res. Commun., 66, 980 1975

129. Roberts, R.M. and Pollard W.E.
Plant Physiology, 55, 431 1975

130. Brett, C.T. and Leloir, L.F.
Biochem. J., 161, 93 1977

131. Sharon, N.
in Plant Carbohydrate Chemistry (Pridhan, J.B., ed.)
Academic Press London - N.York. pp 244-252 1974

132. Keenan, R.W., Matula, J.M. and Holloman, L.
Biochim. Biophys. Acta, 326, 84 1973

133. Keenan, R.W., Kruczck, M. and Fusinato, L.
Archiv. Bioche,. Biophys., 167, 697 1975

134. Quesada Allué, L.A.,Belocopiton, E. and Marechal, L.R.
Biochem. Biophys. Res.Commun., 66, 1201 1975

135. Quesada Allué, L.A., Marechal, L.R. and Belocopitow, E.
FEBS Letters, 67, 243 1976

136. Behrens, N.H.

In "Biology and Chemistry of Eucaryotic Cell Surfaces. Miami Winter Sympl ed. E.Y.C. Lee and E.E. Smith, N. York. London Academic 7, pp 159-180 1974

137. Heath, E.C., Baynes, J.W. and Hsu, A.J.,

Ref. 131 7, pp 181-211 1974

138. Leloir, L.F.

See Ref. 131 7, pp 1 1974

139. Lucas, J.J. and Waechter C.J.

Mol. and Cell Biochem., 11, 67 1976

140. Lennarz, W.J.

Science, 188, 986 1975

141. Burgos, J., Hemming, F.W., Pennock, J.F. and Morton R.A.

Biochem. J., 88, 470 1963

142. Butterworth, P.H.W. and Hemming, F.W.

Archiv. Biochem. Biophys., 128, 503 1968

143. Hemming, F.W.

Symp. Biochem. Soc., 29, 105 1970

144. Richards, J.B. and Hemming, F.W.

Biochem. J., 128, 1345 1972

145. Hemming,, F.W.

Biochem. Soc. Trans., pp 1029 1973

146. Caccam, J.F., Jackson, J.J. and Eylar, E.H.

Biochem. Biophys. Res.Commun., 35, 505 1969

147. Zatz, M. and Barondes, S.H.

Biochem. Biophys. Res.Commun., 36, 511 1969

148. Behrens, N.H. and Leloir, L.F.

Proc. Natl. Acad. Sci. U.S.A., 66, 153 1970

149. Behrens, N.H. , Parodi, A.J., Leloir, L.F. and Krisman, C.R.,
Archiv. Biochem. Biophys., 143, 375 1971

150. Herscovics, A., Bugge, B. and Jeanloz, R.W.
J. Biol. Chem., 252, 2271 1977

151. Palamarczyk, G. and Hemming, F.W.
Biochem. J., 148, 245 1975

152. Waechter, C.J., Lucas, J.J. and Lennarz, W.J.
J. Biol. Chem., 248, 7570 1973

153. Chambers, J. and Elbein, A.D.
J. Biol. Chem., 250, 6904 1975

154. Thacz, J.S., Herscovics, A., Warren, C.D. and Jeanloz, R.W.
J. Biol. Chem., 249, 6372 1974

155. Waechter, C.J., Kennedy, J. and Harford, J.
Archiv. Biochem. Biophys., 174, 726 1976

156. Hedgewood, J.F., Strominger, J.L. and Warren C.D.
J. Biol. Chem., 249, 6316 1974

157. Richards, J.B., Evans, P.J. and Hemming, F.W.
Biochem. J., 124, 957 1971

158. Baynes, J.I., Hsu, A.F. and Heath, E.C.
J. Biol. Chem., 248, 5693 1973

159. Evans, P.J. and Hemming, F.W.
FEBS Letters, 31, 335 1973

160. Warren, C.D. and Jeanloz, R.W.
Biochemistry, 12, 5038 1973

161. Warren, C.D. and Jeanloz. R.W.
Carb. Res., 37, 252 1974

162. Wedgewood, J.F., Warren, C.D., Jeanloz, R.W. and
Strominger, J.L.
Proc, Natl. Acad. Sci. U.S.A., 71, 5022 1974

163. Warren, C.D. and Jeanloz. R.W.
Biochemistry, 14, 412 1975

164. Warren, C.D., Liu, I.Y., Herscovics, A. and Jeanloz, R.W.
J. Biol. Chem., 250, 8069 1975

165. Tkacz, J.S. and Herscovics, A.
Biochem. Biophys. Res. Commun., 64, 1009 1975

166. Herscovics, A., Warren, C.D. and Jeanloz, R.W.
J. Biol. Chem., 250, 8079 1975

167. Herscovics, A., Warren, C.D., Jeanloz, R.W.,
Wedgewood, J.F., Liu, I.Y. and Strominger, J.L.
FEBS Letters, 45, 212 1974

168. Behrens, N.H., Parodi, A.J. and Leloir, L.F.
Proc. Natl. Acad. Sci. U.S.A., 68, 2857 1975

169. Richards, J.B. and Hemming, F.W.
Biochem. J., 130, 77 1972

170. Hsu, A.F., Baynes, J.W. and Heath, E.C.
Proc, Natl. Acad, Sci. U.S.A., 71, 2391 1974

171. Wolfe, L.S., Breckenridge, W.C. and Shelton, P.P.C.
J. Neurochem., 23, 175 1974

172. Levy, J.A., Carminatti, H., Cantarella, A.I., Behrens, N.H., Leloir, L.F. and Tabora, E. Biochem. Biophys, Res. Commun., 60, 118 1974

173. Parodi, A.J., Behrens, N.H., Leloir, L.F. and Dankert, M. Biochim. Biophys. Acta., 270, 529 1972

174. Lucas, J.J., Maechter, C.J. and Lennarz, W.J. J.Biol. Chem., 250, 1992 1975

175. Forsee, W.T. and Elbein, A.D. Fed. Proc., 34, 2607 1975

176. Behrens, N.H., Carminatti, H., Staneione, R.J., Leloir, L.F. and Cantarella, A.I. Proc. Natl. Acad. Sci. U.S.A., 70, 3390, 1973

177. Chen, L.W., Lennarz, W.J., Tarentino. A.L. and Maley, F. J. Biol. Chem., 250, 7006 1975

178. Eagon, P.K., Hsu, A.F. and Heath, E.C. Fed. Proc., 34, 2609 1975

179. Vessey, D.A. and Zakim, D. Eur. J. Biochem., 53, 499 1975

180. Leloir, L.F., Staneloni, R.J., Carminatti, H. and Behrens, N.H. Biochem. Biophys. Res. Commun., 52, 1285 1973

181. Collins. D.C. and Layne, D.S. unpublished results

182. Collins, D.C. and Layne, D.S. Can. J. Biochem., 46, 1089 1968

183. Waechter, C.J., Lucas, J.J. and Lennarz, W.J.
Biochem. Biophys. Res. Commun., 56, 343 1974

184. Ghalambor, M.A., Warren, C.D. and Jeanloz. R.W.
Biochem. Biophys. Res. Commun., 56, 407 1974

185. Breckenridge, W.C. and Wolfe, L.S.
FEBS Letters, 29, 66 1973

186. Jankowski, W. and Chojnacki, T.
Biochem. Biophys. Acta, 260, 93 1972

187. White, D.A. and Waechter, C.J.
Biochem. J., 146, 655 1975

188. Parodi, A.J., Staneloni, R., Cantarella, A.I., Leloir, L.F., Behrens, N.H., Carminatti, H. and Levy, J.A.
Carb. Res., 26, 393, 1973

189. Martin, H.G. and Thorne, K.J.I.
Biochem. J., 138, 281 1974

190. Herscovics, A., Golovtchenko, A.M., Warren, C.D., Bugge, B. and Jeanloz, R.W.
J. Biol. Chem., 252, 224 1977

191. Herscovics, A., Tkacz, J.S. and Warren, C.D.
Fed. Proc. 1708 1973

192. Oliver, G.J.A. and Hemming, F.W.
Biochem. J., 152, 191 1975

193. Schutzbach, J.S. and Verma, A.K.
Biochem. Biophys. Res. Commun., 71, 203 1976

194. Schutzbach, J.S. and Verma, A.K.
Biochem. Biophys. Res. Commun., 75, 799 1977

195. Bradlow, H.L. Steroids, 11, 265 1968

196. Layne, D.S., Roberts, J.B., Gibree, N. and Williams, K.I.H., Steroids, 6, 855 1965

197. Moulé, Y., Rouiller, C. and Chauveau, J. J. Biophys. and Biochem. Cytol., 7, 547 1960

198. Artom, C. Methods Enzymol., 4, 809 1957

199. Folch, J., Lees, M. and Sloane - Stanley, G.H. J. Biol. Chem., 226, 497 1957

200. Chambers, J. and Elbein, A.D. J. Biol. Chem., 250, 6904 1975

201. Dunphy, P.J., Kerr, J.D., Pennock, J.F., Whittle, K.J. and Feeney, J. Biochim. Biophys. Acta., 136, 136 1967

202. in Thin Layer Chromatography, edited by Egon Stahl, 2nd. Edition, Springer - Vertag, New York. pp 854 1969 pp 900 1969

203. Gregonis, D.E. and Rilling, H.C. Biochem. Biophys. Res. Commun., 54, 449 1973

204. Rosenberg, A. J. Chromat., 2, 487 1959

205. Bartlett, G.R. J. Biol. Chem., 234, 466 1959

206. Houser, G., Kritchevsky, G. and Yamamoto A. in Lipid Chromatographic Analysis edited by Guido V. Marinetti, New York, M, Dekker 1, 123 1967

207. Thin Layer Chromatography, A Laboratory Handbook
edited by Krebs, Heisser and Wimmer
pp 857,

208. Kates M. in Techniques of Lipidology, Isolation,
Analysis and Identification of Lipids
Edited by T.S. Work and E. Work, American
Elsevier Publishing Co., Inc. - New York
pp 428 1972

209. Dowex: Ion Exchange, The Dow Chemical Company Lakeside
Press
pp 39 1958

210. Das, I., Sie, H.G. and Fishmen, W.H.
Archiv. Biochem. Biophys., 144, 715 1971

211. Gornall, A.G., Bardawill, C.J. and David, M.M.
J. Biol. Chem., 177, 751 1959

212. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall,
R.J.
J. Biol. Chem., 193, 265 1951

213. Zalta, P., Zakim. D. and Vessey, D.A.
Biochim. Biophys. Acta, 392, 361 1975

214. Parodi, A.J., Behrens, M.H., Leloir, L.F. and
Carminetti, H.
Proc. Natl. Acad. Sci. U.S.A., 69, 3268 1972

215. Berthillier, G. and Got, R.
Biochim. Biophys. Acta, 362, 390 1974

216. Dixon, M. and Webb, E.C.
Enzymes, 2nd. ed. New York, N.Y., Academic Press pp 329 1967
pp 69 1967

217. Munch - Peterson, A., and Kalckar, H.M.,
Methods in Enzymology, Editors S.P. Colowick and N.O. Kaplan, Academic Press, New York, N.Y. Vol. II, pp 675 1955

218. Inque, K. and Kitagawa, T.
Biochim. Biophys. Acta, 426, 1 1976

219. Labow, R.S. and Layne, D.S.
unpublished results

220. Tuppy, H. and Schenkel - Brunner, G.
Eur. J. Biochem., 10, 152 1969

221. Ghei, O.K., Rupar, C.A. and Carroll, K.K.
Can, Fed. Biol. Soc, Abstract 516 1977

222. Evans, P.J. and Hemming, F.W.
J. Chromatography, 97, 293 1974

223. Warren, C.D. and Jeanloz, R.W.
FEBS Letters, 31, 332 1973

224. Dittmer, J.D. and Lester, R.L.
J. Lipid Res., 5, 126 1964

225. Vaskovsky, V.E. and Kostetsky, E.Y.
J. Lipid Res., 9, 396 1968

226. Mańkowski T., Sasak, W. and Chojnacki, T.
Biochem. Biophys. Res. Commun., 65, 1292 1975

227. Helting, T. and Peterson, P.A.
Biochem. Biophys. Res. Commun., 46, 429 1972

228. Luca, L., Maestri, N., Rosso, G.C. and Wolf, G.
J. Biol. Chem., 248, 641 1973

229. Rosso, G.C., de Luca, L. Warren, C.D. and Wolf, G.
J. Lipid Res. , 16, 235 1975

230. Barr, R. M. and de Luca, L.
Biochem. Biophys. Res. Commun., 60, 355 1974

231. de Luca, L., Rosso, G.C. and Wolf, G.
Biochem. Biophys. Res. Commun., 41, 615 1970

232. Oliver, G.J.A., Harrison, J. and Hemming, F.J.
Eur. J. Biochem., 58, 223 1975

233. Arnold, D, Hommel, E. and Risse, H.J.
Mol. and Cell. Biol., 11, 137 1976