FACTORS AFFECTING THE IN VITRO FORMATION AND HYDROLYSIS OF STEROID GLYCOSIDES

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ABSTRACTS

PART I: On the Effects of Treatment with Estrogens and other Steroids on Steroid Glycosyl Transferases of Rabbit Tissues.

Administration of steroid estrogens and of diethyl stilbestrol to female rabbits at dose levels of 1.5 to 5.0 mgm/kg live weight increased the amount of steroid N-acetyl glucosaminyl transferase in kidney tissue. Significant increase of the enzyme in liver tissue was only noted in animals treated with 17β-estradiol. Small doses of estrogens not exceeding 75 μgm/kg body weight inhibit the N-acetyl glucosaminyl transferase in kidney tissue.

Neither the liver nor kidney levels of steroid glucuronyl transferase were affected. Testosterone, progesterone and cortisol in high doses had no effect on either transferase. Confirmation of previous observations about the increased size of livers of cortisol treated rabbits is also reported.

PART II: Studies on Steroid Glycosidases in the Serum of Pregnant and Non-pregnant Women.

Using a steroid substrate (Tritiated Estradiol 17α-N-Acetyl

glucosaminide) it was found that, consistent with findings of other investigators, serum N-Acetyl glucosaminidase is significantly raised during pregnancy. The substrate used in previous experiments was p-nitrophenol β-N-Acetyl glucosaminide. The peak of activity is reached during the second trimester and is maintained until the end of gestation.

It was found that a marked increase in serum glucosaminidase activity, exceeding the hydrolysis of 2.25 μgm of the substrate by one ml of serum when incubated for 22 hours at 37°C, would indicate placental dysfunction.

The present series is limited in number, but should these findings be substantiated this would provide an easy and rapid method of assessing placental function.

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PART I

ON THE EFFECTS OF TREATMENT WITH ESTROGENS AND OTHER STEROIDS ON STEROID GLYCOSYL TRANSFERASES OF RABBIT TISSUES

INTRODUCTION

Williams (1967) recently pointed out that our knowledge of the mechanism of glucuronic acid conjugation has been largely obtained from the study of the fate in the body of foreign organic compounds. He referred to the pioneer findings of Mering and Musculus (1875) who isolated trichloroethyl glucuronoside (Urochloralic acid) from human urine after the administration of chloral hydrate.

However, a number of natural metabolites can also undergo conjugation and detoxication in the body. In the steroid field, the glucuronoconjugation of estrogens was recognised in 1936 when Cohen and Marrian reported the isolation from human pregnancy urine of a non-crystalline substance containing 50% by weight of estriol. The substance gave a strong naphthoresorcinol reaction for glucuronic acid. Its elemental composition and the barium content of its barium salt were considered to be in fair agreement with those required for an estriol glucuronoside ($C_{24}H_{32}O_9$).

In 1938, Schacter and Marrian were the first to show that estrone sulfate occurred in pregnant mare's urine when they isolated it as the potassium salt.

Following these discoveries, and for almost two decades, glucuronosides and sulfates were believed to be the only hormonal steroid conjugates occurring naturally in mammals.

Rates of glucuronoconjugation and sulfoconjugation of

steroids are known to be different during the various phases of human life. Whilst the latter conjugation is predominant in the human fetus (Troen et al., 1961), steroids are largely glucuronoconjugated in normal human subjects and in pregnant women (Twombly and Levitz, 1960).

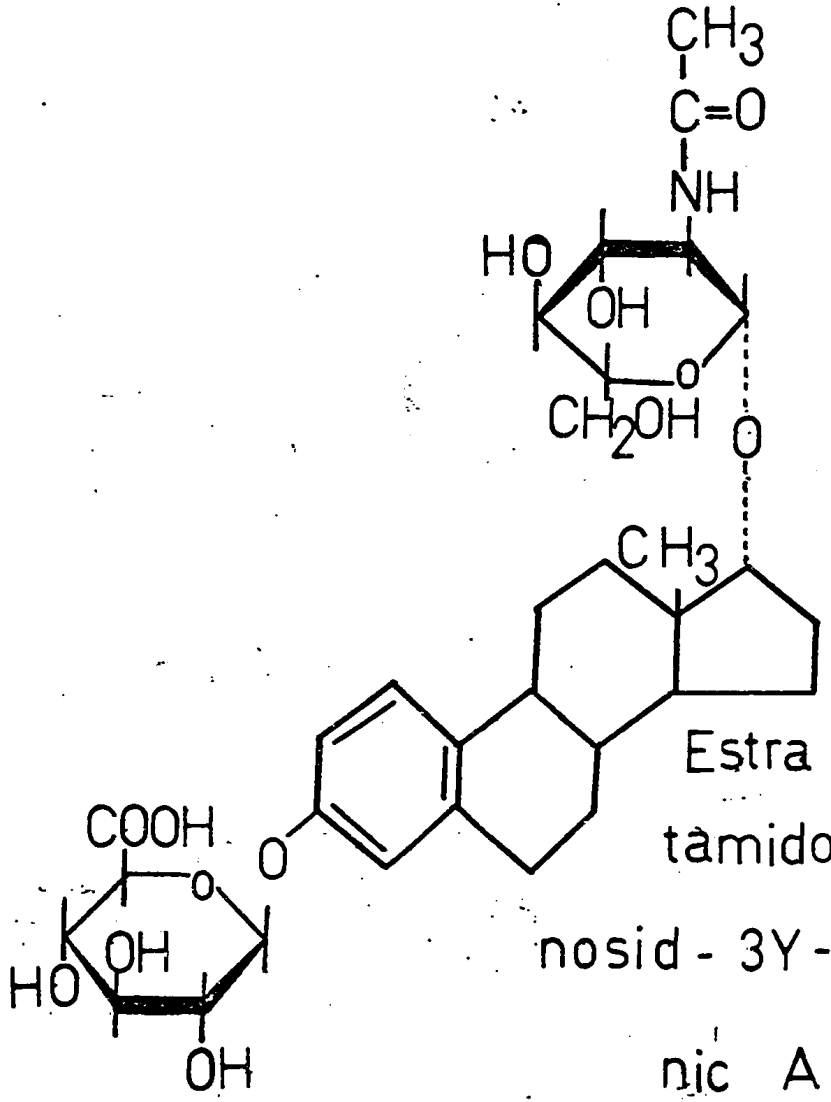
The past decade has witnessed the study of the biosynthesis of steroid glucuronosides following the administration of either non-radioactive or radioactive hormones to animals or to human subjects.

Various factors may affect the processes of conjugation and detoxication. Factors which may influence glucuronoconjugation have drawn the attention of research workers because of the development of jaundice in newborn human infants (Lathe and Walker, 1958).

A NEW STEROID CONJUGATE

The observation that steroids are excreted in the urine in combination with N-acetyl glucosamine represents a completely novel conjugation reaction. Work on this reaction has been reviewed by Layne (1969) and the salient points are outlined below.

Estradiol N-acetyl glucosaminide has been found in the urine of rabbits (Layne et al. 1964, Layne, 1964) and has been isolated as a double conjugate in which N-acetyl glucosamine is attached to the 17α position while glucuronic acid is attached to the hydroxyl on carbon 3 of the steroid (fig. 1). Using rabbit liver homogenate, Jirku and Layne



Estra 1,3,5(10) Trien-17α-YL-2'Acetamido-2''-Deoxy-ß-D-Glucopyranosid-3Y-YL-ß-D-Glucopyranosiduronic Acid.

Fig. 1. DOUBLE GLYCOSIDE OF 17α-ESTRADIOL.

(1965) were able to establish the presence of a mechanism for the transfer of N-acetyl glucosamine to the 17α hydroxyl group of estradiol. However, they found that the receptor is not the free steroid but the 3 glucuronoside.

Collins et al. (1968) confirmed the presence of a steroid N-acetyl glucosaminyl transferase enzyme in rabbit liver, kidney and intestine. Factors affecting this newly discovered transferase system have not yet been fully investigated.

SUBJECT OF PART I OF THE THESIS

It is the purpose of part 1 of this thesis to elucidate and compare the effects of the *in vivo* administration of estrogens and other steroids on the glucuronyl and N-acetyl glucosaminyl transferase enzymes of rabbit liver and kidney tissues as measured by *in vitro* techniques.

HISTORICAL DATA

I. On the use of Estrogen Substrates in Enzyme Assays

Foreword:

Non steroid substrates, such as O-amino phenol, p-nitrophenol and phenolphthalein have been used in classical experiments to study various conjugation reactions. On the other hand, the use of steroid substrates has allowed the study of conjugation at various sites on the steroid molecule.

Since the biological preparation of 6,7 tritiated 17α-estradiol (Layne et al, 1965), Layne and his co-workers

have used this radioactive steroid in their enzyme assays (Collins et al, 1968). A further report on the use of this steroid substrate is contained in the present work.

Historical Background:

The formation of estrone glucuronoside by the human intestinal tract was reported by Diczfalusy et al. (1962). 17β-estradiol was injected into an isolated intestinal loop with an intact blood supply. Large amounts of conjugated estrone were subsequently found in extracts of the intestinal wall and in the effluent venous blood. The conjugated estrone was identified as estrone glucuronoside.

In a previous paper, Diczfalusy et al. (1961) reported on the in vivo glucuronidation of estriol by the human intestinal mucosa.

In an attempt to find if the liver microsomal system is effective in the formation of glucuronosides of phenolic steroids, Smith and Breuer (1963) incubated estrone with a rabbit liver microsomal preparation and uridine diphosphoglucuronic acid. They reported the identification of estrone 3-glucuronoside as the reaction product. Estrone, which has only one hydroxyl group was chosen to avoid ambiguity about the reaction product. In a similar experiment, estradiol 17β-3-methyl ether was found to be conjugated to a less degree than estrone. These results suggest that in rabbit liver microsomes, the rate of glucuronidation may be

higher for the phenolic hydroxy group than for the 17 hydroxy group.

Slaunwhite et al. (1964) incubated homogenates of human liver with uridine diphosphoglucuronic acid and estriol. They were able to demonstrate the formation of estriol 16α-3-D-glucuronoside. They also reported that the inclusion of uridine diphospho N-acetyl glucosamine in the incubation mixture increases the yield of conjugate. It is believed that this increase is due to the inhibition of uridine diphosphoglucuronic acid pyrophosphatase.

II. BIOSYNTHESIS OF N-ACETYL GLUCOSAMINIDE

Foreword:

The preparation of 6,7 tritiated 17α-estradiol of high specific activity (Layne et al., 1965) and the characterisation of 17α-estradiol 3-glucuronoside (Collins et al., 1967) enabled Collins et al. (1968) to obtain information on the characteristics, tissue distribution, particulate localisation and substrate specificity of the N-acetyl glucosamine transferase in rabbit tissues.

The following account on the properties of the enzyme is extracted from the published data by Collins et al. (1968).

General Properties of UDP N-Acetyl Glucosaminyl Transferase

1. Location in the Cell

Microsomal suspensionshave been prepared by differential centrifugation of the tissue homogenate. The N-acetyl

glucosaminyl transferase activity was located in the washed microsomal fraction (105,000 x g precipitate). No transferase activity was detected in the 105,000 x g supernatant.

2. pH activity curve

The pH activity curve for the transfer of N-acetyl glucosamine to the 17α-hydroxyl group of 17α-estradiol-3-monoglucuronoside was different from that for the transfer of glucuronic acid to the 3 hydroxyl group of 17α-estradiol (fig. 2).

3. Distribution among tissues

Rabbit liver, kidney, small and large intestine showed UDP N-acetyl glucosaminyl transferase activity. No detectable amount of the enzyme was found in the rabbit ovary, adrenal, spleen, whole blood, erythrocytes and plasma.

4. Specificity of UDP N-Acetyl glucosaminyl transferase

Collins et al. (1968) tested a series of free steroids as substrates, none of them was conjugated by the liver microsomes in the presence of UDP N-acetyl glucosamine (table 1). When the monoglucuronosides of the steroids were used as substrates, or, when both UDP glucuronic acid and UDP N-acetyl glucosamine were present with the free steroid in the incubation medium, 17α-estradiol, 17 epiestriol, 16,17 epiestriol and 17β-methyl 17α-estradiol were converted to double glycosides. This evidence suggests that the

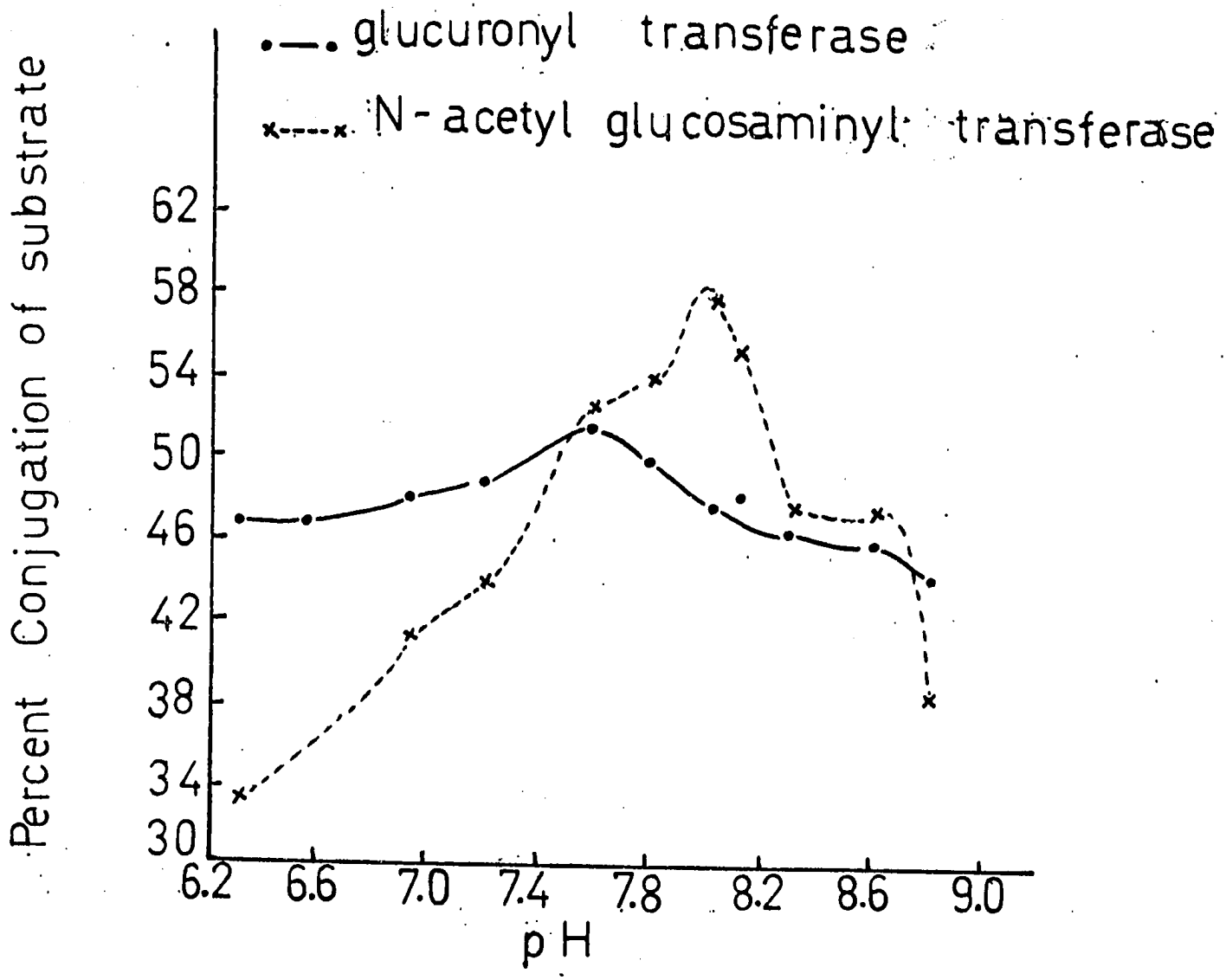


Fig. 2. pH activity curves for transferases

(from Collins, D.C. et al. J. Biol. Chem.,

243 : 2928 , 1968)

Table 1. Compounds tested as substrates for transferases of rabbit liver microsomes.

from Collins, D.C. et al. J. Biol. Chem. 243: 2928 (1968)

Compound	Trivial name	Formation of glucuronoside	Formation of N-acetyl glucosaminide
3-Hydroxyestra-1,3,5(10)-trien-17-one-6,7-^{3}H	Estrone	+	-
Estra-1,3,5(10)-trien-3,17β-diol-6,7-^{3}H	17β-Estradiol	+	-
Estra-1,3,5(10)-trien-3,17α-diol-6,7-^{3}H	17α-Estradiol	+	+*
17β-Methylestra-1,3,5(10)-trien-3,17α-diol	17β-Methyl-17α-estradiol	+	+*
3,15α-Dihydroxyestra-1,3,5(10)-trien-17-one-4-^{14}C	15α-Hydroxyestrone	+	-
Estra-1,3,5(10)-trien-3,16α,17β-triol-6,7-^{3}H	Estriol	+	-
Estra-1,3,5(10)-trien-3,16β,17β-triol-16-^{14}C	16-Epiestriol	+	-
Estra-1,3,5(10)-trien-3,16α,17α-triol-6,7-^{3}H	17-Epiestriol	+	+*
Estra-1,3,5(10)-trien-3,16β,17α-triol-6,7-^{3}H	16,17-Epiestriol	+	+*
3α,17α,21-Trihydroxy-5β-pregnan-11,20-dione 1,2-^{3}H	Tetrahydrocortisone	+	-
3α,11β,17α,21-Tetrahydroxy-5β-pregnan-20-one-1,2-^{3}H	Tetrahydrocortisol	+	-
3β,17α-Dihydroxy-pregn-5-ene-20-one-7α-^{3}H	17α-Hydroxypregnenolone	+	-
17α-Hydroxyandrost-4-en-3-one-1,2-^{3}H	Epitestosterone	+	-
Androst-4-en-3α,17α-diol-1,2-^{3}H	3α,17α-Androstenediol	+	-
Androst-4-en-3β,17α-diol-1,2-^{3}H	3α,17α-Androstenediol	+	-
4-Bis-(p-hydroxyphenyl)-3-hexene-(ethyl-1-^{14}C)	Diethylstilbestrol	+	-
17α-Hydroxyestra-1,3,5(10)-trien-6,7-^{3}H-3- L-β-D-glucopyranosiduronic acid	17α-Estradiol-3-glucuronoside	-	+
3-Hydroxyestra-1,3,5(10)-trien-6,7-^{3}H-17β- L-β-D-glucopyranosiduronic acid	Estradiol 17β-glucuronoside	-	-
3-Hydroxyestra-1,3,5(10)-trien-6,7-^{3}H-17α- L-2'-acetamido-2'-deoxy-β-D-glucopyranoside	Estradiol-17α-N-acetylglucosaminide	-	-

* These compounds formed N-acetylglucosaminides only after prior formation of a monoglucuronoside.

transfer of N-acetyl glucosamine requires, in addition to prior formation of the 3-glucuronoside of the phenolic steroid, the presence of an α-oriented hydroxyl group at position 17.

5. Some data on Kinetic studies

The Michaelis constant (K_m) value for UDP N-acetyl glucosaminyl transferase was 6.8×10^{-5} M for 17α-estradiol-3-glucuronoside at the optimum pH of 8.0 in phosphate buffer.

6. Inhibitors

Methanol and propylene glycol when present at low concentrations do not inhibit the transferase activity to any appreciable extent and are therefore suitable as solvents for the addition of steroids to the incubation medium. On the other hand, ethanol, butanol and eugenol are effective inhibitors. Estrone and diethylstilbestrol effectively inhibit the transfer of N-acetyl glucosamine to the 17α-estradiol-3-glucuronoside.

7. Storage

The washed microsomes resuspended in 0.15 M potassium chloride are stored at -10°C without any appreciable loss of activity.

8. Solubilization, Purification and Stability of the Enzyme

Treatment with deoxycholate and Triton x 100, butanol

extraction and sonic vibration have been investigated in an attempt to release the enzyme from the microsome.

It is hoped that studies on solubilisation, purification and stability of the enzyme will be soon available for publication.

III. SOME EFFECTS OF HORMONAL STEROIDS ON GLUCURONOCONJUGATION IN MAMMALS.

Lathe and Walker (1958) found that sera from pregnant women and newborn infants inhibited the conjugation of bilirubin with glucuronic acid by rat liver slices. The inhibitor was dialysable and heat stable. Conjugation in liver slices from rabbits and monkey was inhibited to a smaller extent. The conjugation of bilirubin with glucuronic acid by rat liver slices (but not rabbit or monkey) was also inhibited *in vitro* by several steroids including progesterone, etiocholanolone and deoxycorticosterone. On the other hand, the sera and steroids had no inhibitory effect on o-aminophenol conjugation by rat liver slices, or on bilirubin conjugation by rat liver suspensions.

Hsia et al. (1963) found that testosterone and progesterone inhibited guinea pig liver glucuronyl transferase whilst cortisone and estradiol did not. The aglycones used in their experiments were o-aminophenol, p-nitrophenol and 4-methyl umbelliferone. Halme et al. (1957) studied the effect of cortisone acetate on the glucuronoside synthesis of the liver and duodenum. 46 male albino rats were divided

into 2 groups (Group I 25 rats, Group II 21 rats). Fourteen rats in group I received daily doses of 12.5 mgm cortisone acetate intramuscularly over a period of 6 - 10 days. Twelve rats in group II received daily doses of 25 mgm cortisone acetate over a period of 10 days. The rest of the animals were used as controls. The detoxication synthesis was studied by using o-aminophenol as a substrate and measuring the glucuronoside formed with it. No marked changes were found in the glucuronoside synthesis of the liver after the administration of cortisone acetate. However, duodenal tissue slices showed a depression of the glucuronoside synthesis in cortisone treated animals (Table 2). Hartiala and Pulkkinen (1964) found that one month old Wistar rats possess a greater capacity than mature rodents for the synthesis of o-aminophenol glucuronoside in liver and duodenal tissue slices in vitro. However, they found no difference in either UDP GA transferase or β glucuronidase activity in the same groups of animals (Table 3). They postulate that the gradually increasing secretion of the sex hormone may be a causative factor.

Table 2

Effect of cortisone acetate on the glucuronoside detoxication synthesis of duodenum in rats.

from data by Halme et al. Acta Physiol. Scand. 42, Suppl. 145: 62 (1957)

	GROUP I (25 male albino rats)		GROUP II (21 male albino rats)	
	Control	Treated	Control	Treated
Number of Rats	11	14	9	12
Daily dose of cortisone	nil	125 mg	nil	25 mg
Number of days injected	-	6-10 days	-	10 days
o-aminophenol conjugated per 100 mg dry weight of duodenal tissue	217 ± 11.0	150 ± 9.2	237 ± 23.9	135 ± 5.4
Mean Difference	6.7 ± 14.3		102 ± 24.5	

Table 3

Formation of o-aminophenol glucuronoside in rat liver. µg o-aminophenol glucuronoside/100 mg dry weight/90 min.

from Hartiala et al. Ann. Acad. Scient. Fennicae A.V. 106/12 (1964)

	Rats aged 4 months	Rats aged 1 month
male	163 ± 30	445 ± 50
Female	174 ± 25	321 ± 26
UDP GA transferase in the same rats Δ O.D.		
male	363 ± 28	350 ± 51
Female	343 ± 27	296 ± 44

MATERIALS

A. RABBITS

Female immature white New Zealand rabbits weighing between 1.5 and 2.0 kilograms were used for all experiments.

B. HOUSING AND FEEDING THE RABBITS

The rabbits were housed in pairs. They received master rabbit pellets (Maple Leaf Mills Ltd.) and water ad libitum. The pellets contain crude Protein (min. 15.0%), crude fat (min. 3.0%), crude fibre (max. 15.0%), salt (0.5%), calcium (1.5%), phosphorus (0.68%) and Vit. A (min. 5,000 I.U./lb.) The cages were cleaned daily and their floors covered with fresh wood shavings.

C. ESTROGENS AND OTHER HORMONES

Estrogens and other hormones used in all experiments were pure as judged by thin layer chromatography.

Progesterone U.S.P. ("PROLUTON" Schering Corporation Ltd. Montreal) each cc. contains 50 mg crystalline progesterone U.S.P. in vegetable oil with 20% benzyl benzoate.

Testosterone U.S.P. ("ORETON" Schering Corporation Ltd. Montreal) each cc. contains 50 mg crystalline testosterone propionate in vegetable oil with 0.1% propyl ester of p-hydroxybenzoic acid as a preservative.

Estradiol benzoate ("PROGYNON B" Schering Corporation Ltd. Montreal) each cc. contains 3.333 mg of 17β-Estradiol benzoate in oil solution.

17β Estradiol benzoate was also obtained in the crystalline

form from Steroid Laboratories Ltd. (Montreal).

Hydrocortisone U.S.P. ("Solu-Cortef" from Upjohn Company of Canada) each cc. contains 50 mg hydrocortisone sodium succinate, 0.4 mg sodium biphosphate, 4.38 mg sodium phosphate, 1.24 mg methyl paraben, 0.14 mg propyl paraben in water suspension.

Diethyl Stilbestrol U.S.P. was provided in the crystalline form by Nutritional Biochemicals Corporation (Cleveland).

17α- and 17β-Estradiol were obtained in the crystalline form through Sigma Chemical Company (St. Louis, Missouri).

D. VEHICLES USED FOR INJECTIONS

During the early part of the experiment, the steroids were suspended in sesame oil. Later, a steroid suspending vehicle was available from the National Institutes of Health and was used. This vehicle contained per ml of water: Sodium chloride, 9 mg; Sodium carboxymethyl cellulose, 5 mg; Polysorbate 80, 0.004 ml; Benzyl alcohol, 0.009 ml.

E. SOLUTIONS FOR ENZYME ASSAY

Except for UDP-N-Acetyl glucosamine and UDP-Glucuronic acid which were purchased from Sigma Chemical Company, all other chemicals were ACS certified Fisher products.

1. 0.15 Molar Potassium Chloride

KCl F.W. 74.56

11.18 Gms of Potassium Chloride crystals were dissolved in a litre of distilled water.

2. Preparation of Phosphate Buffers

i. 0.15 M Dibasic Sodium Phosphate

Na_2HPo_4 F.W. 141.96

10.65 gms Na_2HPo_4 were dissolved in 500 mls of distilled water.

ii. 0.15 M Potassium Dihydrogen Phosphate

KH_2Po_4 F.W. 136.09

4.08 gms KH_2Po_4 were dissolved in 200 mls of distilled water.

iii. Phosphate Buffer pH 8.0

5.0 mls of 0.15 M KH_2Po_4 were added to 95.0 mls of 0.15 M Na_2HPo_4. The pH was brought up to 8.0 by careful addition of Na_2HPo_4 under the control of a pH meter.

iv. Phosphate Buffer pH 7.7

12.0 mls of 0.15 M KH_2Po_4 were added to 88.0 mls of 0.15 M Na_2HPo_4. The pH was brought up to 7.7 by careful addition of Na_2HPo_4 under the control of a pH meter.

3. Preparation of UDP Compounds

i. Uridine Diphosphate Glucuronic Acid Solution

8.33 mgm of UDPGA were dissolved in 50 mls of phosphate buffer of pH 7.7.

ii. Uridine Diphosphate N-Acetyl glucosamine Solution

8.33 mgm of UDPNAG were dissolved in 50 mls of phosphate buffer of pH 8.0.

iii. NOTES:

a) As 1 mgm of UDPNAG or UDPGA is equivalent to 1.5 μmole, 2.0 mls of the above prepared solutions would provide 0.5 μmole of UDPNAG or UDPGA.

b) Owing to the instability of the UDP compounds, they were prepared fresh just prior to the start of the experiment.

4. Preparation of 6,7 Tritiated 17α-Estradiol 3-Glucuronoside

A standard solution of the tritiated estradiol was used in all the experiments. The radioactivity had been incorporated two years previously and had a specific activity of 5.6 curies per millimole at that time. The 2 year decay as established from the chart (Table 4) is 0.89, this would reduce the specific activity to 4.98 curies per millimole.

5. Preparation of Tritiated 17α-Estradiol from a Standard Solution

i. Counting activity in standard solution

0.1 ml of the solution was counted in Toluene scintillation solvent. The result of the count showed 7,316,495 d.p.m. (disintegrations per minute) per 1.0 ml of the solution.

Table 4

Decay Chart for Tritium

YEARS	MONTHS 0	2	4	6	8	10
0	-	.99	.98	.97	.96	.95
1	.95	.94	.93	.92	.91	.90
2	.89	.88	.88	.87	.86	.85
3	.84	.84	.83	.82	.81	.80
4	.80	.79	.78	.78	.77	.76
5	.75	.75	.74	.73	.73	.72
6	.71	.71	.70	.69	.69	.68
7	.67	.67	.66	.65	.65	.64
8	.64	.63	.62	.62	.61	.61
9	.60	.60	.59	.58	.58	.57
10	.57	.56	.56	.55	.55	.54
11	.54	.53	.53	.52	.52	.51
12	.51	.50				

ii. Calculation of standard solution needed to provide 500,000 d.p.m./ml

a) Amount of d.p.m. in 100 ml for 500,000 d.p.m./ml

$500,000 \times 100 = 50 \times 10^6$ d.p.m./100 mls.

b) Number of millilitres needed from standard solution

50×10^6 d.p.m. $\div 7.316 \times 10^6 = 6.83$ mls in 100 mls

iii. Addition of 17α-Estradiol

a) µgms of tritiated 17α-estradiol in 50×10^6 d.p.m.

$$\frac{50 \times 10^6 \text{ d.p.m.}}{239.8 \times 10^6 \text{ d.p.m./µgm}} = 0.209 \text{ µgm}$$

b) Activity of standard solution

108 millicurie per microgram

c) Desired specific activity

5.6 curie per millimole

d) Activity of standard solution expressed per millimole

Since 1.0 µmole = 272 µgm

therefore $108 \times 272 = 29,276$ millicurie per micromole or 29.27 cu/m mole

e) Calculation of 17α-estradiol needed

$29.27 \div 5.6 = 5.22$

i.e. 5.22 Gms 17α-estradiol for each Gm of tritiated 17α-estradiol

0.209 µgm x 5.22 = 1.09 µgm 17α-estradiol

iv. Final count of prepared solution

0.1 ml counted in Toluene scintillation solvent. The result obtained was 516,820 d.p.m./ml.

6. Preparation of 17α-Estradiol

1 mgm in 100 mls methanol

i.e. 10 μgm in 1.0 ml

7. 1.0 N Hydrochloric Acid

Commercial concentrated hydrochloric acid was used in the preparation of 1.0 N HCl.

8. Ethyl Acetate

ACS certified Fisher product was used without further purification.

9. Benzene

ACS certified Fisher product used without further purification.

10. Preparation of Toluene Scintillation Solvent

4 Gms PPO (2,5 Diphenyloxazole) dissolved in one litre of Toluene.

11. Preparation of Dioxane Scintillation Solvent

Naphthalene		60 gms
PPO (2,5 diphenyloxazole)		4 gms
Methanol		100 mls
Ethylene glycol		20 mls
Dioxane	ad	one litre

F. SOLUTIONS FOR PROTEIN DETERMINATION

1. Reagent "A":

   2% w/v Sodium carbonate in 0.1 N Sodium hydroxide (NaOH M.W. 40)

2. Reagent "B":

   0.5% w/v Copper sulfate pentahydrate ($CuSo_4$ $5H_2O$) in 1% w/v Sodium tartrate.

3. Reagent "C": - "Alkaline Copper Solution"

   50 mls of reagent "A" were mixed with 1.0 ml of reagent "B". The solution was freshly prepared and discarded after one day.

4. Reagent "E":

   1.0 N Folin Ciocalteau phenol reagent

5. Standard Protein Solution:

   Solution of bovine albumen (500 µgm/ml)

G. SOLVENT SYSTEMS FOR THIN LAYER CHROMATOGRAPHY

1. Ethyl acetate and cyclohexane

   75 ml of each of ethyl acetate and cyclohexane. This system was satisfactory when separation of the three common estrogens i.e. Estrone (E_1), Estradiol (E_2) and Estriol (E_3) was required.

2. Chloroform, formic acid and isopropyl alcohol

   5 parts chloroform were added to 3 parts isopropyl alcohol and one part formic acid. In this solvent

system, diglycosides remain near the origin, they are well separated from both monoglucuronosides and mono-N-acetyl glucosaminides.

H. SILICA GEL FOR THIN LAYER CHROMATOGRAPHY

Silica Gel H (acc. to Stahl) is manufactured by E. Merck AG Darmstadt (Germany) and obtained through Canlab.

Type Analysis

Without $CaSo_4$. Iron 0.03%, chloride 0.02%, pH of a 10% aqueous slurry 7. Medium grain size of the adsorbent 10-40 μ.

APPARATUS

1. SYRINGES AND NEEDLES

Polypropylene disposable syringes were obtained from Burron Medical Products, Inc. Disposable injection needles gauge 20 and 1½ inches long were used.

These disposable syringes and needles were discarded at the end of each 9 day experiment.

2. DISSECTION TOOLS

Stainless steel Mayo Scissors 5½ inches long, straight Kelly scissors 6¼ inches long, 5 inch stainless steel thumb forceps and 5 inch stainless steel tissue forceps were required for the dissection of rabbits. These instruments were washed with running water and wiped dry after each dissection.

3. GRINDER

A modification of Potter Elvehjem tissue grinder with a teflon pestle (from A.H. Thomas Co., Philadelphia, Pa.) was utilised. A rotary motor was used to rotate the pestle.

4. SHAKER BATH

Incubations for enzyme assays were carried out in a shaker bath (Precision Scientific Co., Chicago). The temperature of the water bath was adjusted to 37°C and kept constant by a hydraulic thermostat with sensitivity of ±0.1°C.

5. CENTRIFUGE

International Equipment Co. (Boston, Mass.) centrifuge was used.

6. LIQUID SCINTILLATION VIALS

Spectravial IV (Nuclear Chicago Corporation, Des Plaines, Ill.) was chosen because of its low cost and its higher relative counting efficiency. This polyethylene vial exhibits less permeability to toluene than other non-glass vials. Spectravial is a standard 20 ml volume and has a plastic screw cap lined with cork and metal foil.

7. LIQUID SCINTILLATION SYSTEM

A Unilux T.M. II Liquid Scintillation System (Nuclear Chicago Co.) was used. This instrument is designed for high efficiency counting of soft beta emitting isotopes viz. Carbon 14 and tritium in a controlled temperature environment. Some of the features of this system are: 1) an automatic sample changer 2) a cooled sample environment 3) an automatic data lister.

8. SPECTROPHOTOMETER COLORIMETER

A spectronic 20 Colorimeter (Bausch & Lomb, Rochester, N.Y.) was chosen because of its easy operation and reliable results.

9. N-EVAP

Drying under nitrogen was facilitated by the use of N-EVAP (Organomation Assoc., Shrewsbury, Mass.)

OPERATIVE PROCEDURES

A. KILLING THE RABBITS

Stretching the cervical spine and acute angulation of the neck ensured a rapid way of killing the virgin female rabbits.

B. OBTAINING FRESH ORGANS

As soon as the animal was killed, the liver, kidneys and uterus were dissected out and placed in separate beakers containing 0.15 M Potassium Chloride. The beakers were completely surrounded by ice in an ice bucket.

The freshly obtained organs were weighed and an aliquot was taken for homogenisation.

C. CASTRATION OF FEMALE IMMATURE RABBITS

1. Introduction

Although the rabbits were chosen as sexually immature, it was thought that, to exclude any possible endogenous effect from the gonads, bilateral oophorectomy would limit the estrogenic effect, if such is observed, to a solely exogenous origin.

2. Anesthesia

Sodium pentobarbital 60 mgms in one ml was diluted in 4 mls of normal saline (0.9 Gms NaCl in 100 mls distilled water). The first rabbit was given an injection of 3.5 mls in the ear vein. This was followed by chloroform administered per funnel. The animal died early during the

operative procedure.

The second rabbit was injected with 2.0 mls of the diluted sodium pentobarbital solution (i.e. 24 mgm sodium pentobarbital) via the ear vein. This was followed by chloroform administered per funnel. Successful removal of both ovaries was achieved and the animal recovered well after the surgical procedure. The method employed in anesthetising the second rabbit was used for the remaining four rabbits, but despite due precautions, one further animal was lost during surgery.

Chloroform per funnel

A dropper bottle was filled with chloroform. An ordinary plastic funnel was utilised, the large end was placed over the animal snout and the narrow end was lightly packed with cotton wool.

Chloroform was poured dropwise to keep the cotton wool slightly wet during the period of operation.

Details of Anesthesia

As soon as the animal was given the injection of sodium pentobarbital into the ear vein, it was tied down to a dissecting board with its belly facing upwards. It was noted that at the time of tying the animal down it was limp but not completely anesthetised, the pupils were widely dilated and reflex blinking could be obtained by touching the eyelashes.

Just prior to a satisfactory degree of surgical anesthesia the animal gave a loud cry which was followed

by complete relaxation. At that time, the funnel was placed over the animal snout and the cotton wool kept constantly moist with chloroform.

Respiratory efforts of the rabbit under anesthesia were noted and any evidence of respiratory depression was followed by immediate removal of the funnel.

3. Shaving the Abdomen

Prior to surgery, the hind part of the abdomen of the rabbit was shaved using shearing scissors.

4. Incision

A midline incision over the hind part of the abdomen was effected using a sharp knife.

5. Castration and Closure of Incision

Both ovaries were removed with no attempt at ligation of bleeding vessels. Bleeding was minimal and did not give rise to any concern. The peritoneum was closed with oo plain catgut using a continuous stitch. The abdominal wall including the skin was closed with ooo silk using a continuous stitch.

6. Post-operative Recovery

After surgery the animals were placed on their side and respiratory obstruction was avoided by proper positioning of the head.

7. Convalescence

The four surviving rabbits were left to convalesce for a period of two weeks before injections were started.

METHODOLOGY

A. THIN LAYER CHROMATOGRAPHY

1. Testing purity of radioactive steroids

An activated 0.25 mm thick silica plate was used. The plate was impregnated with standard steroids and the radioactive steroid to be tested.

A chloroform, isopropanol and formic acid solvent system was used. The plate was then sprayed with a saturated solution of phosphomolybdic acid in methanol. The plate was gently heated in an oven and the coloured spots with the description of their corresponding standard solutions were noted, scraping off small sections of the silica gel and counting the radioactivity in a scintillation counter determined the site of maximum steroid concentration and its relative purity as compared to the standard solutions.

2. Purifying radioactive steroid compounds

Using a 1 mm thick silica plate, the same procedure as for testing the purity of the steroid was adopted. The substance to be purified whilst impregnated in silica gel was protected from the phosphomolybdic acid spray.

The silica gel was scraped off opposite the site where the pure steroid was judged to be concentrated. Dissolving in methanol and filtering off the silica gel under vacuum resulted in the acquisition of a solution of

the relatively pure steroid. Testing the purity of the steroid thus obtained was again required.

B. RANDOMISATION

Randomisation by number was adopted. Each animal was given a serial number before being weighed and housed. The type of treatment was assigned according to the number.

C. ADMINISTRATION OF STEROIDS

Steroids suspended in 1 ml of vehicle (p. 17) were injected subcutaneously. All animals received a daily dose for eight days and were killed on the ninth day. Control animals received 1.0 ml of the suspending vehicle only.

D. METHODOLOGY OF ENZYME ASSAY

1. Homogenisation of tissues obtained

Aliquots of the fresh organs were homogenised with 4 volumes of 0.15 M potassium chloride solution in a Potter Elvehjem tissue grinder (p. 25).

2. Preparation of samples

15 mls centrifuge tubes were used for this purpose and adequately labelled. Measured quantities of the steroid substrates were pipetted out and dried under nitrogen.

3. Mixing of samples

N.B. In all samples, the incubation medium was brought to a final volume of 3.0 ml with 0.15 M potassium chloride.

a) For liver N-acetyl glucosaminyl transferase

i. 0.05 ml liver homogenate

ii. 2.4×10^6 d.p.m. tritiated 17α-estradiol 3 glucuronoside (p. 19)

iii. 0.5 μmole uridine diphosphate N-acetyl glucosaminide in 2.0 mls of phosphate buffer (p. 19)

b) For liver glucuronyl transferase

i. 0.1 ml liver homogenate *

ii. 10 μgm 17α-estradiol (p. 22)

iii. 517,000 d.p.m. tritiated 17α-estradiol (p. 22)

iv. 0.5 μmole uridine diphosphate glucuronic acid in 2.0 mls phosphate buffer (p. 19)

* 0.05 ml was used in some experiments

c) For kidney N-acetyl glucosaminyl transferase

i. 0.05 ml kidney homogenate

ii. 1.9×10^6 d.p.m. tritiated 17α-estradiol 3 glucuronoside (p. 19)

iii. 0.5 μmole UDP NAG (p. 19)

d) For kidney glucuronyl transferase

i. 0.2 ml kidney homogenate *

ii. 517,000 d.p.m. tritiated 17α-estradiol (p. 22)

iii. 0.5 μmole UDP GA (p. 19)

* 0.5 ml was used in some experiments

4. Incubation

The samples were shaken well using a Parafilm "M" (American Can Company, Neenah, Wisconsin) to seal the tube. The centrifuge tubes containing the samples were then placed in a shaker water bath at 37°C.

Incubations were allowed to proceed for 30 minutes with air as the gas phase.

5. Extraction of non-conjugated substrates

a) 17α-estradiol 3 monoglucuronoside

i. Addition of 1.0 N Hydrochloric acid

0.5 ml was added to the incubation medium to reduce the pH to 2.0.

ii. Extraction with ethyl acetate

5.0 mls ethyl acetate were added to the incubation medium and the tubes well shaken. The tubes were then centrifuged at low speed for 5 minutes.

b) 17α-Estradiol

5.0 mls of benzene were added to the incubation medium and the tubes well shaken. The tubes were then centrifuged at low speed for 5 minutes.

6. Counting Radioactivity

a) Ethyl acetate extract

0.5 ml of ethyl acetate was pipetted out and transferred to a polyethylene vial;10.0 mls of dioxane scintillation solvent were added using an automatic dispenser. Counting was effected in a liquid scintillation system.

b) Benzene extract

The procedure is similar to that for the ethyl acetate extract except that toluene scintillation solvent was used.

E. ESTIMATION OF PROTEIN CONTENT OF HOMOGENATES

(for solutions required see p. 23)

Introduction

The rabbit liver is known to develop fatty changes when the animal is subjected to estrogen therapy. This phenomenon was observed during the course of this experiment. In order to obviate possible changes in the results due to this effect, the protein content of all homogenates was estimated.

The method is that of Lowry et al. (1951).

1. Preparation of standard solutions

Using colorimeter tubes, 50, 100, 150, 200, 250 and 300 μgm of a standard protein solution were brought to a volume of 1.0 ml with water. 5.0 mls of reagent "C" were added and the mixture was well shaken. Ten minutes later 0.5 ml reagent "E" was added and well mixed.

Using a spectrophotometer colorimeter (p. 26) reading was effected after 30 minutes at 750 μ (Additional readings may be also obtained at 650 and 500 μ).

2. Dilution of Homogenates

0.05 ml of the homogenate was added to 9.95 mls of distilled water and the mixture well shaken.

3. Estimation of Protein Content

1.0 ml of the diluted homogenate (0.005 ml original homogenate) was transferred to a colorimeter tube ... 5.0 mls of reagent "C" added and the mixture well shaken. After 10 minutes, 0.5 ml of reagent "E" was added and well mixed.

Estimating the percent transmittance after 30 minutes was effected in a spectrophotometer colorimeter at a wave length of 750 μ. The amount of protein was calculated from the standard curve (fig. 3).

F. CALCULATIONS OF DPM IN A QUENCHED SAMPLE

1. Background Measurement

The background CPM was simply measured by using a blank (unlabelled) sample similar in volume and composition to the unknown sample.

To obtain the net channel CPM for a sample the background CPM was subtracted from gross sample CPM in each channel.

2. Quenching Correction Curve and Efficiency

The ratio of the count rates in the two counting channels A & B is directly related to the counting efficiency of the sample. A quench correction curve is constructed

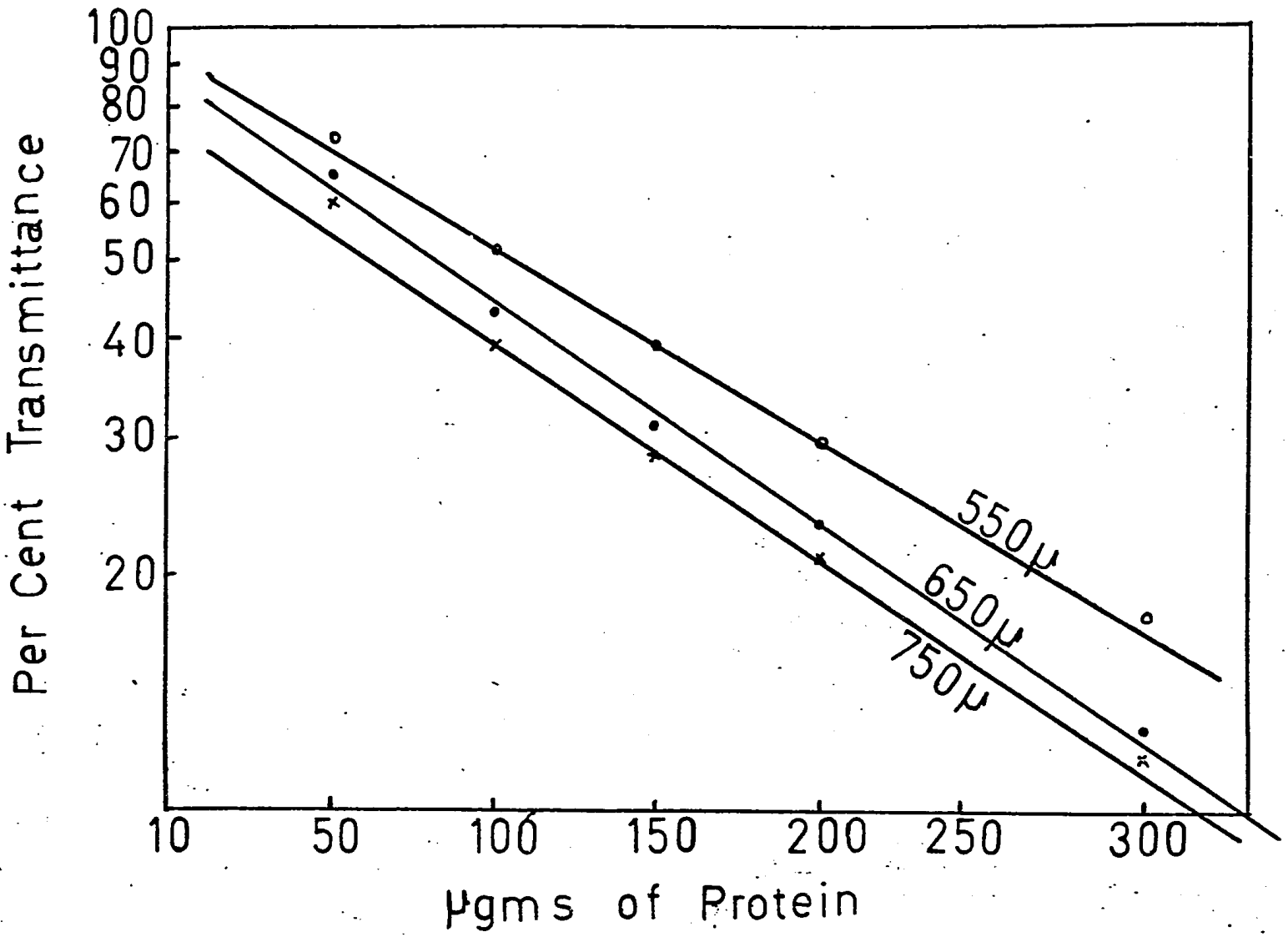


Fig. 3. Typical Results in an Experiment for Protein Determination (Curve for Standard Protein Solutions.)

by using the Tritium Quench Standards Set (Nuclear Chicago). This set is a series of standard samples of known activity and varying degrees of quenching.

The counting efficiency for any unknown sample is read directly from the graph using the ratio:

$$\frac{\text{Net Channel B CPM}}{\text{Net Channel A CPM}}$$ (see fig. 4)

3. DPM Calculations

Knowing the counting efficiency and the net Channel CPM for a sample, it is possible to calculate the isotope disintegration rate in the unknown sample.

G. CALCULATION OF MICROMOLES OF SUBSTRATES IN LIVER AND KIDNEY SAMPLES

1. DPM of 17α-estradiol 3 monoglucuronoside per micromole

i. specific activity of solution used:
5.6 curie/millimole.

ii. 2 year decay from chart (p. 20) 0.89

iii. Present specific activity = 4.984 cu/millimole or 4.98 millicurie per micromole (4.98 mcu/μmole)

iv. As one mcu = 2.22×10^9 d.p.m.

v. Therefore,

$4.98 \times 2.22 \times 10^9 = 11.0556 \times 10^9$ d.p.m./μmole

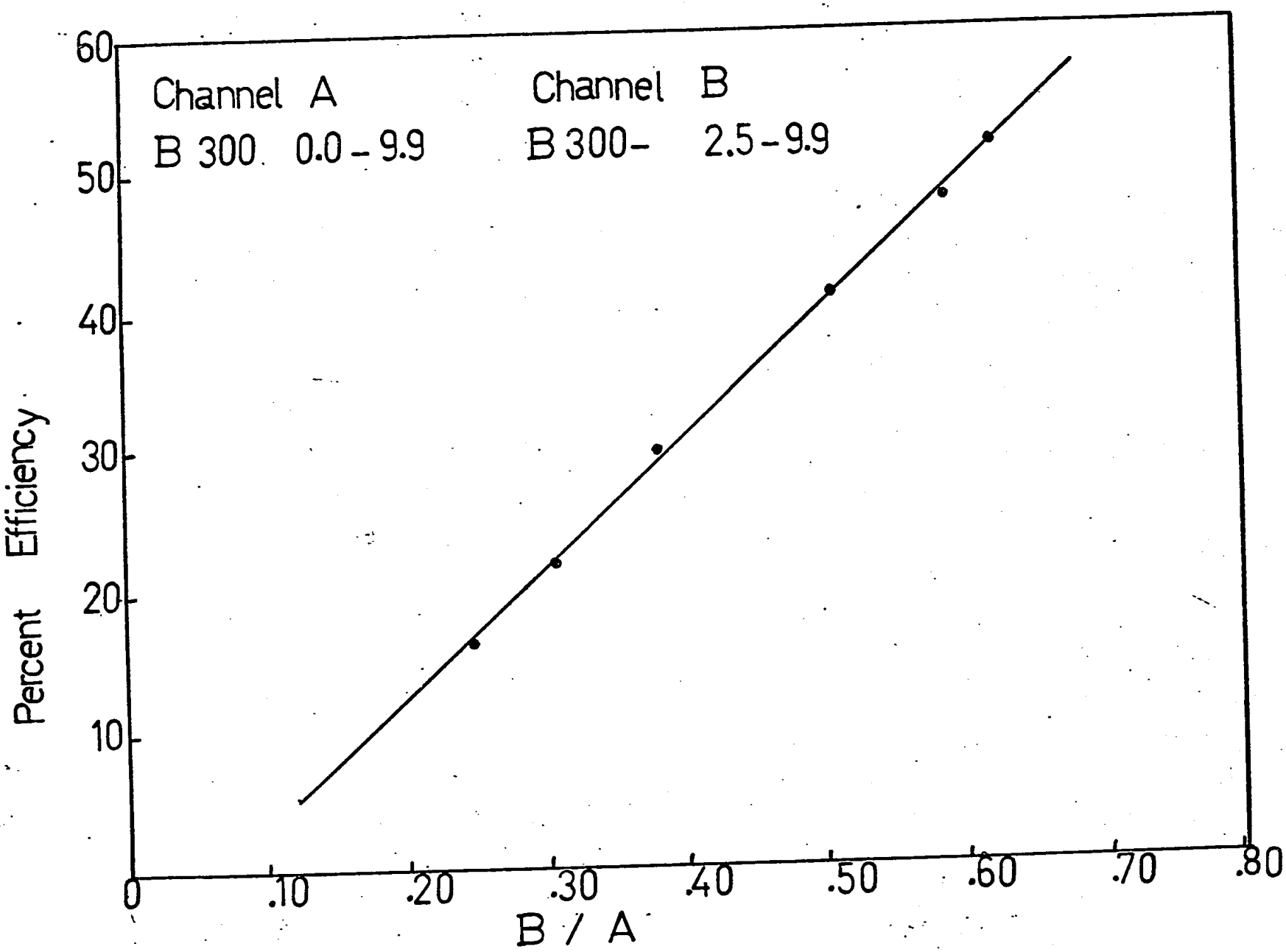


Fig. 4. H^3 Channel Ratio Quench Correction Curve

2. Micromoles of 17α-estradiol 3 monoglucuronoside in liver and kidney samples

i. DPM in liver sample 2.4×10^6 dpm (p. 33)

ii. Therefore

$$\frac{2.4 \times 10^6 \text{ dpm/sample}}{11.0556 \times 10^9 \text{ dpm/}\mu\text{mole}} = \frac{2.17 \times 10^{-4} \ \mu\text{mole/}}{\text{liver sample}}$$

iii. DPM in kidney sample 1.9×10^6 dpm (p. 33)

iv. Therefore

$$\frac{1.9 \times 10^6 \text{ dpm/sample}}{11.0556 \times 10^9 \text{ dpm/}\mu\text{mole}} = \frac{1.72 \times 10^{-4} \ \mu\text{mole/}}{\text{kidney sample}}$$

3. DPM of 17α-estradiol per Micromole

i. specific activity of solution used (Aug. 1966)

5.6 curie/millimole

ii. 2 year decay from chart (p. 20) 0.89

iii. Present specific activity = 4.984 cu/millimole or 4.98 millicurie per micromole

(4.98 mcu/μmole)

iv. As one mcu = 2.22×10^9 dpm

v. Therefore

$4.98 \times 2.22 \times 10^9 = 11.0556 \times 10^9$ dpm/μmole

4. Micromoles of 17α-estradiol in liver and kidney samples

i. Liver sample

0.517×10^6 dpm + 10 μgms (p. 33)

ii. Therefore

$$\frac{10 \ \mu\text{gms}}{272 \ \mu\text{gms/}\mu\text{mole}} + \frac{0.517 \times 10^6 \text{ dpm/sample}}{11.0556 \times 10^9 \text{ dpm/}\mu\text{mole}}$$

$0.036764\ \mu mole + 0.0000467\ \mu mole =$

$0.036810\ \mu mole =$

$\underline{3.68 \times 10^{-2}\ \mu mole/liver\ sample}$

iii. Kidney sample

0.517×10^{6} dpm (p. 33)

iv. Therefore

$$\frac{0.517 \times 10^{6}\ dpm/sample}{11.0556 \times 10^{9}\ dpm/\mu mole} = 4.67 \times 10^{-5}\ \mu mole/kidney\ sample$$

H. STATISTICAL ANALYSIS

For rapid statistical analysis of our preliminary data, a rapid approximate statistical procedure was adopted (Wilcoxon & Wilcox 1964).

In all other experiments Z was calculated

where $Z = \dfrac{\bar{x}_1 - \bar{x}_2}{b\,\bar{x}_1 - \bar{x}_2}$

when $b\,\bar{x}_1 - \bar{x}_2 = \sqrt{\dfrac{(s_1)^2}{n_1} - \dfrac{(s_2)^2}{n_2}}$

s = standard Deviation

$\bar{x}$ = mean of samples

n = number of samples

PILOT EXPERIMENT

OBJECTIVE

It is an attempt to determine the possible in-vivo effects of a wide range of steroids on the activity of N-acetyl glucosaminyl and glucuronyl transferases of rabbit liver and kidney.

PRELIMINARY EXPERIMENT

The results of this experiment are presented in a graphic form (fig. 5). 17β-estradiol benzoate was noted to have an effect on all the transferases except the liver glucuronyl transferase (fig. 5).

CONFIRMATORY EXPERIMENT

17α-Estradiol was injected into rabbits instead of 17β-estradiol. Preparations of testosterone, progesterone and cortisol were similar to those used in the preliminary experiment. The results are presented in table forms for ease of reference (Tables 5, 6, 7, 8, 9 & 10)

RESULTS

On perusal of table 5, it is noted that one animal weighed less than 1.5 kilos which was the accepted minimal weight for one experimental animal. The daily dose of testosterone given to this relatively small animal was slightly more than two thirds of the dose given to the other rabbit treated with male hormone. It is rather surprising to note (table 6) that the liver and

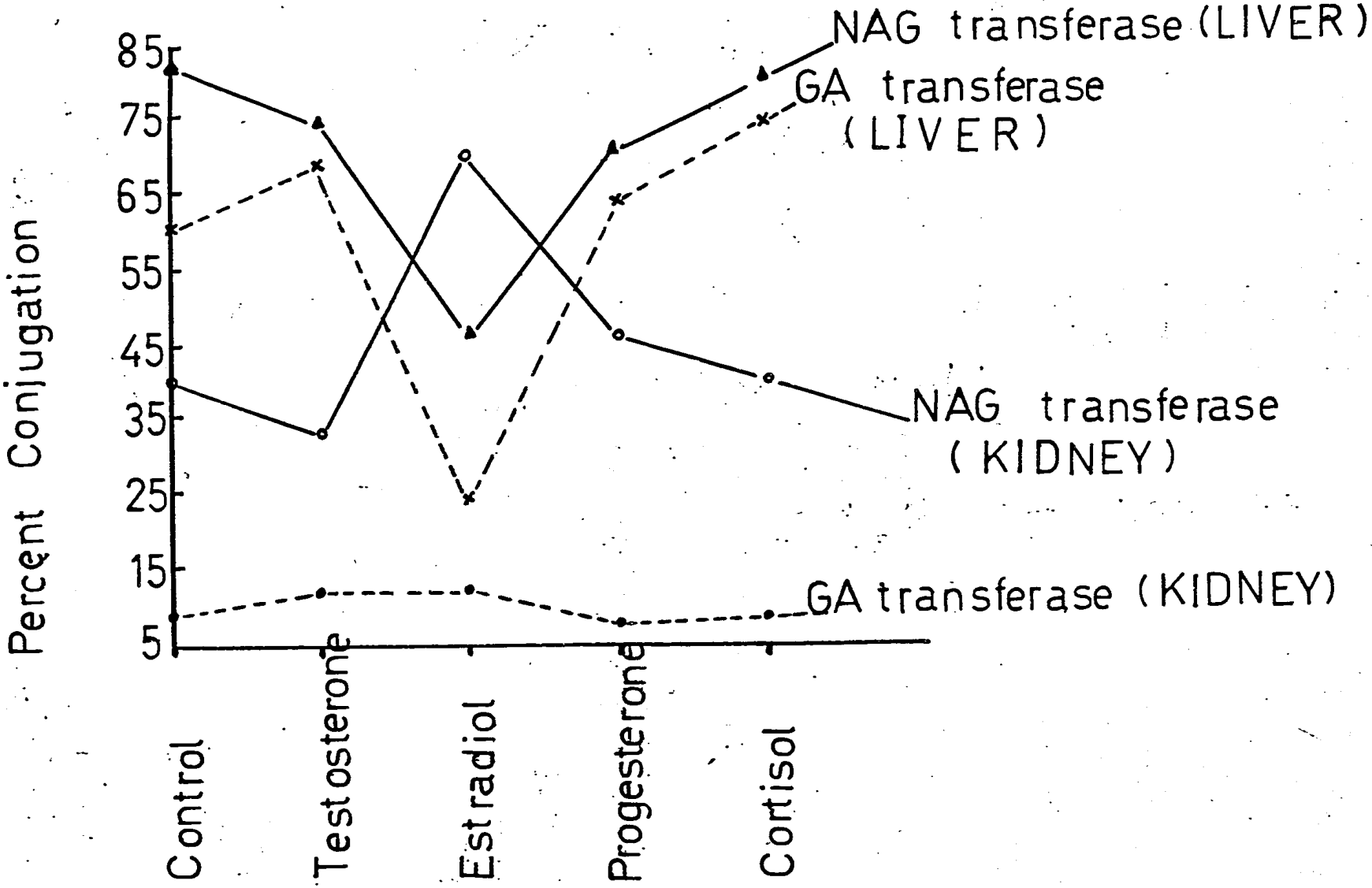


Fig. 5. Effect of Steroids on Transferase Enzymes of Rabbit Liver & Kidney

Table 5

Rabbits used in pilot experiment with doses of steroid injected

RABBIT SYMBOL AND NUMBER	WEIGHT IN GRAMS	DAILY INJECTIONS DOSE 2 mgm/kilo	DOSE INJECTED (in μgms)	ML INJECTED	REMARKS
CON # 52	1635	ster. susp. veh.	none	1.0 ml	
TEST # 53	1293	Testosterone	2586	1.0 ml	
$E_{2\sigma}$ # 54	1623	17$_\sigma$-estradiol	3246	1.05 ml	
PROG # 55	1808	Progesterone	3616	1.0 ml	
CORT # 56	1877	Cortisol	3754	1.16 ml	
CON # 57	1784	ster. susp. veh.	none	1.0 ml	
TEST # 58	1774	Testosterone	3548	1.37 ml	
$E_{2\sigma}$ # 59	1543	17$_\sigma$-estradiol	3086	1.0 ml	
PROG # 60	1829	Progesterone	3658	1.01 ml	
CORT # 61	1617	Cortisol	3234	1.0 ml	

Testosterone 1 ml = 2586 μgms

17$_\sigma$-estradiol 1 ml = 3086 μgms

Progesterone 1 ml = 3616 μgms

Cortisol 1 ml = 3234 μgms

Table 6

Weights of dissected out organs and a comparison between the liver-body weight ratios of various treatments

RABBIT	WEIGHT LIVER (Gms)	ALIQUOT (Gms)	WEIGHT KIDNEYS (Gms)	ALIQUOT	UTERUS (Gms)	LIVER-BODY WEIGHT RATIO	TOTAL PROTEIN (LIVER) (Gms)
CON # 52	68.43	3.0	11.54	3.22	0.21	4.18%	19.50
TEST # 53	66.98	3.22	9.71	2.23	2.60	5.18%	19.75
$E_2\alpha$ # 54	59.09	3.15	11.07	3.17	7.16	3.64%	17.72
PROG # 55	88.7	4.28	11.81	2.65	1.87	4.90%	21.95
CORT # 56	158.81	2.69	12.40	3.64	1.82	8.46%*	31.76*
CON # 57	77.22	3.36	11.54	2.51	0.34	4.32%	16.02
TEST # 58	56.77	2.52	9.55	2.70	2.62	3.20%	15.04
$E_2\alpha$ # 59	57.51	2.44	11.53	3.54	2.84	3.72%	16.39
PROG # 60	51.87	2.99	10.86	3.13	2.28	2.83%	15.82
CORT # 61	116.21	3.51	12.96	3.67	1.24	7.18%*	24.98*

* Significantly higher than control ($p < 0.01$)

TABLE 7

Specific Activity of Kidney N-Acetyl Glucosaminyl Transferase of Rabbits Treated with Various Steroids.

RABBIT	% Conjugation	10^6 x μmoles conjugated/ sample	10^4 x μmoles conj/ml homog. (μmoles/0.2 gm tissue)	10^3 x μmoles conj/gm wet tissue	μgms ptn/ homog. used	10^2 x μmoles conj/gm prot.
CON # 52	36.95	63.55	12.71	6.35	2600	2.44
CON # 57	32.06	55.14	11.02	5.51	2850	1.93
TEST # 53	36.15	62.18	12.43	6.21	2850	2.18
TEST # 58	45.96	79.05	15.81	7.90	2750	2.87
$E_2\sigma$ # 54	65.59	112.81	22.56	11.28	2400	4.70*
$E_2\sigma$ # 59	43.68	75.13	15.02	7.51	2750	2.73*
PROG # 55	51.94	89.34	17.86	8.93	2850	3.13
PROG # 60	31.19	53.65	10.73	5.36	2475	2.16
CORT # 56	31.99	55.02	11.00	5.50	2400	2.29
CORT # 61	38.64	66.46	13.29	6.64	2475	2.68

* Statistically not significant (p = 0.05)

TABLE 8

Specific Activity of Liver N-Acetyl Glucosaminyl Transferase of Rabbits Treated with Various Steroids.

RABBIT	% Conjugation	10^6 x μmoles conjugated per sample	10^4 x μmoles conj. per ml homog. (μmoles/0.2 gm tissue)	10^2 x μmoles conj./ gm wet tissue	μgm protein/ homogenised used	10^2 x μmoles conj./gm protein
CON # 52	74.55	161.77	32.35	1.61	2850	5.68
CON # 57	51.76	112.32	22.46	1.12	2075	5.41
TEST # 53	72.29	156.87	31.37	1.56	2950	5.32
TEST # 58	72.50	157.32	31.46	1.57	2650	5.94
$E_2\alpha$ # 54	68.09	147.75	29.55	1.47	3000	4.93
$E_2\alpha$ # 59	68.27	148.15	29.63	1.48	2850	5.20
PROG # 55	80.32	174.29	34.85	1.74	2475	7.04
PROG # 60	73.60	159.71	31.94	1.59	3050	5.24
CORT # 56	52.16	113.19	22.63	1.13	2000	5.66
CORT # 61	49.89	108.26	21.65	1.08	2150	5.04

TABLE 9

Specific Activity of Kidney Glucuronyl Transferase of Rabbits Treated with Various Steroids

RABBIT	% Conjugation	10^6 x μmoles conjugated per sample	10^6 x μmoles conj/ml homogen. (μmoles/0.2 gm tissue)	10^6 x μmoles conj/gm wet tissue	μgms protein/homogen. used	10^4 x μmoles conj/gm protein
CON # 52	21.96	10.25	20.51	102.55	26,000	7.88
CON # 57	33.83	15.79	31.59	157.98	28,500	11.08
TEST # 53	34.30	16.01	32.03	160.18	28,500	11.24
TEST # 58	36.45	17.02	34.04	170.22	27,500	12.37
E_2 # 54	37.12	17.33	34.67	173.35	24,000	14.44
E_2 # 59	39.19	18.30	36.60	183.01	27,500	13.30
PROG # 55	27.63	12.90	25.80	129.03	28,500	9.05
PROG # 60	26.91	12.56	25.13	125.66	24,750	10.15
CORT # 56	28.55	13.33	26.66	133.32	24,000	11.11
CORT # 61	19.45	9.08	18.16	90.83	24,750	7.33

TABLE 10

Specific Activity of Liver Glucuronyl Transferase of Rabbits Treated with Various Steroids

RABBIT	% Conjugation	10^3 x μmoles conj./sample	μmoles conj. al homogen. (μmoles/0.2 gm tissue)	μmoles conj./ gm wet tissue	μgm protein/ homogen. used	μmoles conj./ gm protein
CON # 52	53.12	19.54	0.39	1.95	2850	137.17
CON # 57	42.03	15.46	0.30	1.55	2075	149.07
TEST # 53	56.50	20.79	0.41	2.08	2950	140.69
TEST # 58	55.28	20.34	0.40	2.03	2650	153.53
$E_{2\alpha}$ # 54	48.53	17.85	0.35	1.79	3000	119.06
$E_{2\alpha}$ # 59	60.24	22.16	0.44	2.22	2850	155.56
PROG # 55	62.10	22.85	0.45	2.29	2475	184.66
PROG # 60	44.92	16.53	0.33	1.65	3050	108.39
CORT # 56	51.17	18.83	0.37	1.88	2000	188.30
CORT # 61	33.94	12.49	0.24	1.25	2150	116.18

kidneys of this relatively small animal weighed more that those of its counterpart.

The livers of cortisol treated rabbits were significantly larger than those of the control group ($p < 0.01$; see table 6).

Table 6 also shows that the total protein content of rabbit livers treated with cortisol is significantly higher than those of control rabbits (see conclusions and discussion, p. 59).

The rabbit which responded to 17α-estradiol therapy by a marked increase in uterine weight (table 6) showed a rise in the activity of the kidney N-acetyl glucosaminyl transferase enzyme (table 7). However, a correlation between uterine size and enzyme activity could not be obtained in other experiments (fig. 6).

To calculate the statistical significance of our results, data from both pilot experiments (preliminary and confirmatory) were compiled. Our attention was drawn to the N-acetyl glucosaminyl transferase levels of rabbit kidneys of estradiol treated and control animals when a rapid test of statistical significance was applied to our results (Table 11). Percent conjugation of substrates was chosen for analysis. The rank sum test as suggested by Wilcoxon and Wilcox (1964) showed a difference in the rank sum between the control and the treated animals of 41.5 which is higher than the number 41.2 required to obtain statistical significance. In

TABLE 11

Per Cent Conjugation of 17α-Estradiol Monoglucuronoside in the Estimation of Kidney N-Acetyl Glucosaminyl Transferase Activity. The Application of the "RANK SUM TEST" on Rabbits Treated with Various Steroids.

Rank	CONTROL	Rank	TESTOS-TERONE	Rank	ESTRA-DIOL	Rank	PROGES-TERONE	Rank	CORTISOL
8	36.9	7	36.1	18	65.6	17	51.9	2.5	32.0
2.5	32.0	15	45.9	12	43.7	1	31.2	9	38.6
6	36.0	5	33.5	20	73.3	13	44.9	10	39.8
11	41.9	4	32.5	19	67.2	14	45.4	16	46.1
27.5		31		69		45		37.5	

Rank sum difference (Estradiol Control)
41.5 (for P = 0.1 41.2)

this test K = 5 and N = 4 (where "K" is the number of treatments and "N" the number of observations per treatment). Results were further tabulated as to the activity of the enzyme per gram of wet kidney tissue (Table 12). Statistical significance ($P < 0.01$) was noted when comparing the N-acetyl glucosaminyl transferase levels of rabbit kidneys of estradiol treated and control animals.

TABLE 12

Kidney N-Acetyl Glucosaminyl Transferase of Rabbits Treated with Various Steroids. Comparative Study of Activities.

10^5 x μmoles conjugated per Gm wet kidney tissue

	CONTROL	TESTOS-TERONE	ESTRA-DIOL	PROGES-TERONE	CORTISOL
RABBIT # 1	635	621	1128	893	550
RABBIT # 2	551	790	751	536	664
RABBIT # 3	619	576	1260	772	684
RABBIT # 4	720	559	1155	780	792
MEAN	631	636	*1073	745	672
STANDARD ERROR	34.64	51.96	*110.90	74.83	48.98
STAND. DEVIATION	69.28	103.92	*221.80	149.66	97.96

*Statistically significant from control ($P \leq 0.01$)

MAJOR EXPERIMENTS

INTRODUCTION

The points raised by the pilot experiment were considered:

1. It was necessary to determine whether the increased kidney N-acetyl glucosaminyl transferase level was an effect of estrogen. If so, stilbestrol and other non-steroid estrogens should give it.

2. The effect of dosage in relation to enzyme activity had to be further elucidated.

3. The action of estradiol on kidney N-acetyl glucosaminyl transferase may be a replica of the action of benzpyrene on glucuronyl transferase (see Inscoe and Axelrod, 1960). Such induction of an enzyme by a specific compound had to be considered.

4. Achieving sexual maturity by exogenous estrogenic compounds may bear a causal relationship to enzyme activity. Study of this point was envisaged.

EXPERIMENTS AND RESULTS

In an early experiment, castrated rabbits were used (p. 28). Due to the great wastage of animals during and after surgery it was decided to limit the number of castrated rabbits to four. However, one rabbit died of diarrhea during injections with estradiol. It was noted (Table 13) that the N-acetyl glucosaminyl transferase activity of kidney tissues in the estradiol treated animal was markedly higher than that in the controls.

TABLE 13

Effect of Estradiol Benzoate Treatment on Glycosyl Transferase Enzymes of Liver and Kidney in Castrated Rabbits.

	CONTROL # 1	CONTROL # 2	ESTRADIOL
Daily dose of Estradiol benzoate	nil	nil	3.325 mgm
Uterine weight (Gms)	0.25	1.30	2.93
Kidney glucuronosyl transferase (10^5 x μmoles conjugated/Gm protein)	3.29	6.02	9.45
Kidney N-Acetyl glucosaminyl transferase (10^2 x μmoles conjugated/gm protein)	3.23	4.18	6.75*
Liver glucuronosyl transferase (μmoles conjugated/gm protein)	6.56	7.63	9.73
Liver N-Acetyl glucosaminyl transferase (10^2 x μmoles conjugated/gm protein)	4.65	6.59	6.11

* Statistical analysis of results not possible

Stastistical analysis of the scant results was, however, not possible.

In another series of experiments, female immature New Zealand rabbits were injected with 17α and 17β estradiol and diethyl stilbestrol. The dosages used ranged between 1.5 and 5 mgm per kilogram of live body weight. The results are presented in table form (Table 14). Statistical significance ($P < 0.01$) was observed when the N-acetyl glucosaminyl transferase activity of kidney tissues of rabbits treated with estrogens was compared with that of control animals. It is also noted that N-acetyl glucosaminyl transferase activity of liver tissues is significantly higher than the control ($P < 0.01$) in rabbits treated with 17β-estradiol. No correlation was observed between uterine weight and kidney N-acetyl glucosaminyl transferase in individual animals (fig. 6).

THE EFFECT OF DOSAGE

Rabbits were injected with varying doses of 17β-estradiol benzoate. The lowest dose was 20 μgm of 17β-estradiol benzoate per kilogram of live body weight (equivalent to 15 μgm of 17β-estradiol). The highest dose was 1500 μgm (1127 μgm 17β-estradiol). As our main interest was to establish the effective dosage of the steroid estrogen on kidney N-acetyl glucosaminyl transferase, it was decided to limit our observations to

TABLE 14

Glycosyl Transferases in Rabbits Treated with Various Estrogens

Weight Uterus	10^2 x μmoles conj/gm ptn KIDNAG	10^4 x μmoles conj/gm ptn KIDGA	10^2 x μmoles conj/gm ptn LIVNAG	μmoles conj/gm ptn LIVNAG	
(A) CONTROLS					
3.63	1.87	5.17	3.92	11.81	Mean KIDNAG 2.17 S.E. ± 0.13
1.69	2.18	3.66	5.28	27.29	Mean KIDGA 6.25 S.E. ± 1.19
2.98	1.85	3.25	4.08	14.17	Mean LIVNAG 5.16 S.E. ± 0.40
0.21	2.50	7.88	5.75	13.71	Mean LIVGA 15.18 S.E. ± 8.10
0.34	1.97	11.08	5.48	14.90	Mean wt. ut. 1.81 S.E. ± 0.46
2.05	2.64	6.49	6.45	9.17	
(B) 17α-ESTRADIOL					
3.96	4.00	5.91	4.23	11.36	Mean KIDNAG 3.53* S.E. ± 0.32
4.30	2.95	5.71	5.07	13.33	Mean KIDGA 8.55 S.E. ± 2.22
3.39	3.13	3.41	4.66	10.33	Mean LIVNAG 4.84 S.E. ± 0.17
7.16	4.80	14.44	4.99	11.90	Mean LIVGA 12.49 S.E. ± 0.90
2.84	2.79	13.30	5.26	15.55	Mean wt. ut. 4.33 S.E. ± 0.74
(C) 17β-ESTRADIOL					
2.17	2.27	5.54	5.31	24.57	Mean KIDNAG 4.54* S.E. ± 0.74
7.31	4.04	5.12	6.11	14.70	Mean KIDGA 6.85+ S.E. ± 0.97
2.64	2.73	4.11	5.20	15.46	Mean LIVNAG 6.27* S.E. ± 0.40
5.04	5.70	6.92	7.79	9.28	Mean LIVGA 14.71 S.E. ± 2.25
5.53	5.62	10.21	7.07	14.54	Mean wt. ut. 4.27 S.E. ± 0.82
2.93	6.90	9.08	6.18	9.73	
(D) STILBESTROL					
4.79	2.64	4.54	5.95	19.20	Mean KIDNAG 4.83* S.E. ± 0.85
2.89	3.48	5.98	4.64	9.36	Mean KIDGA 8.59 S.E. ± 1.75
3.35	2.74	5.78	4.11	12.87	Mean LIVNAG 6.15 S.E. ± 0.66
2.08	7.07	16.32	7.49	8.66	Mean LIVGA 11.27 S.E. ± 1.73
2.92	6.41	10.03	8.41	7.72	Mean wt. ut. 2.99 S.E. ± 0.41
1.96	6.66	8.90	6.30	9.78	

μmoles conj./Gm ptn = Micromoles conjugated per gram of tissue protein;
KIDNAG = Kidney N-acetyl glucosaminyl transferase activity
KIDGA = Kidney glucuronosyl transferase activity
LIV = Liver; S.E. = Standard error; wt. ut. = weight of uterus.

* significantly greater than control ($P < 0.01$)
\+ significantly greater than control ($P < 0.01$)

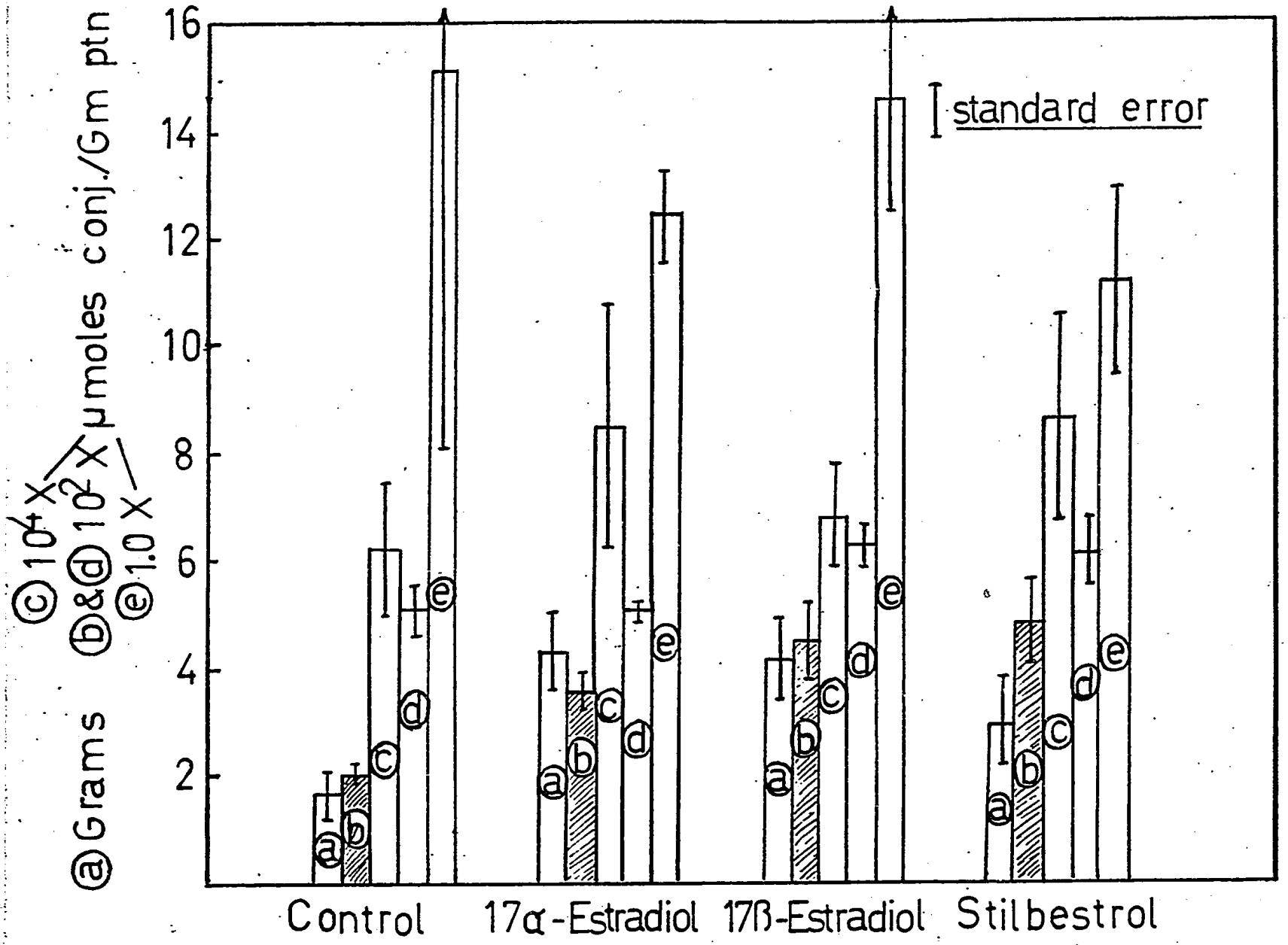


Fig.6. Effect of Estrogens on Uterus & Transferase Enzymes

ⓐ Ut.Wt ⓑ KID NAG ⓒ KID GA ⓓ LIV NAG ⓔ LIV GA

(For Details of Values and abbreviations, see Table 14)

this enzyme. The results are presented in table 15. It is noted that low doses of estrogens viz 15.0 µgm and 75.1 µgm per kilogram body weight would significantly inhibit the enzyme activity (fig. 7)

TRANSFERASE ACTIVITY AS A FUNCTION OF THE LENGTH OF TREATMENT

Rabbits were injected daily with estradiol benzoate (dose 2.5 mgm per kilo body weight). The first group of control and treated rabbits were killed on the third day after two days of treatment. The second group was allowed 4 days of injections and killed on the 5th day. The third group was killed on the 7th day after 6 days of treatment.

The results pertaining to the first two groups are presented in tables 16 and 17. Increased kidney N-acetyl glucosaminyl transferase activity was noted after two days of treatment and was again demonstrated after four days.

CONCLUSIONS AND DISCUSSION

The increase in liver weight noted in cortisol treated rabbits (see table 6) confirms previous reports on this phenomenon. Silber and Porter (1953) postulated that the increased size of livers caused by cortisone may be due primarily to a laying down of cytoplasm

TABLE 15

Effect of Various doses of 17β-Estradiol on Rabbit Kidney N-Acetyl Glucosaminyl Transferase *in vivo*

RABBIT	μgms 17β-estradiol per kilo body weight	Uterine weight (gms)	10^2 x μmoles conj./gm ptn	
CONTROL # 76	none	0.94	3.23	Mean KIDNAG 3.35 S.E. ± 0.158
CONTROL # 81	none	2.08	3.67	Mean wt ut. 1.40 S.E. ± 0.344
CONTROL # 86	none	1.19	3.15	
E_1 # 77	1127.8	3.64	2.32	Mean KIDNAG 3.64* S.E. ± 0.860
E_1 # 82	1127.8	3.28	5.26	Mean wt ut. 3.45 S.E. ± 0.10
E_1 # 87	1127.8	3.44	3.34	
E_2 # 78	375.9	4.24	3.28	Mean KIDNAG 2.69* S.E. ± 0.448
E_2 # 83	375.9	4.29	2.98	Mean wt ut. 4.00 S.E. ± 0.264
E_2 # 88	375.9	3.47	1.81	
E_3 # 79	75.1	6.23	3.26	Mean KIDNAG 3.07+ S.E. ± 0.122
E_3 # 84	75.1	3.51	3.13	Mean wt ut. 3.96 S.E. ± 1.195
E_3 # 89	75.1	2.16	2.84	
E_4 # 80	15.0	4.88	3.24	Mean KIDNAG 2.61⊗ S.E. ± 0.392
E_4 # 85	15.0	4.52	1.89	Mean wt ut. 5.46 S.E. ± 0.770
E_4 # 90	15.0	6.99	2.71	

* Statistically not significant at 0.05 level of confidence
\+ Significantly lower than control ($p < 0.01$)
⊗ Significantly lower than control ($p < 0.05$)

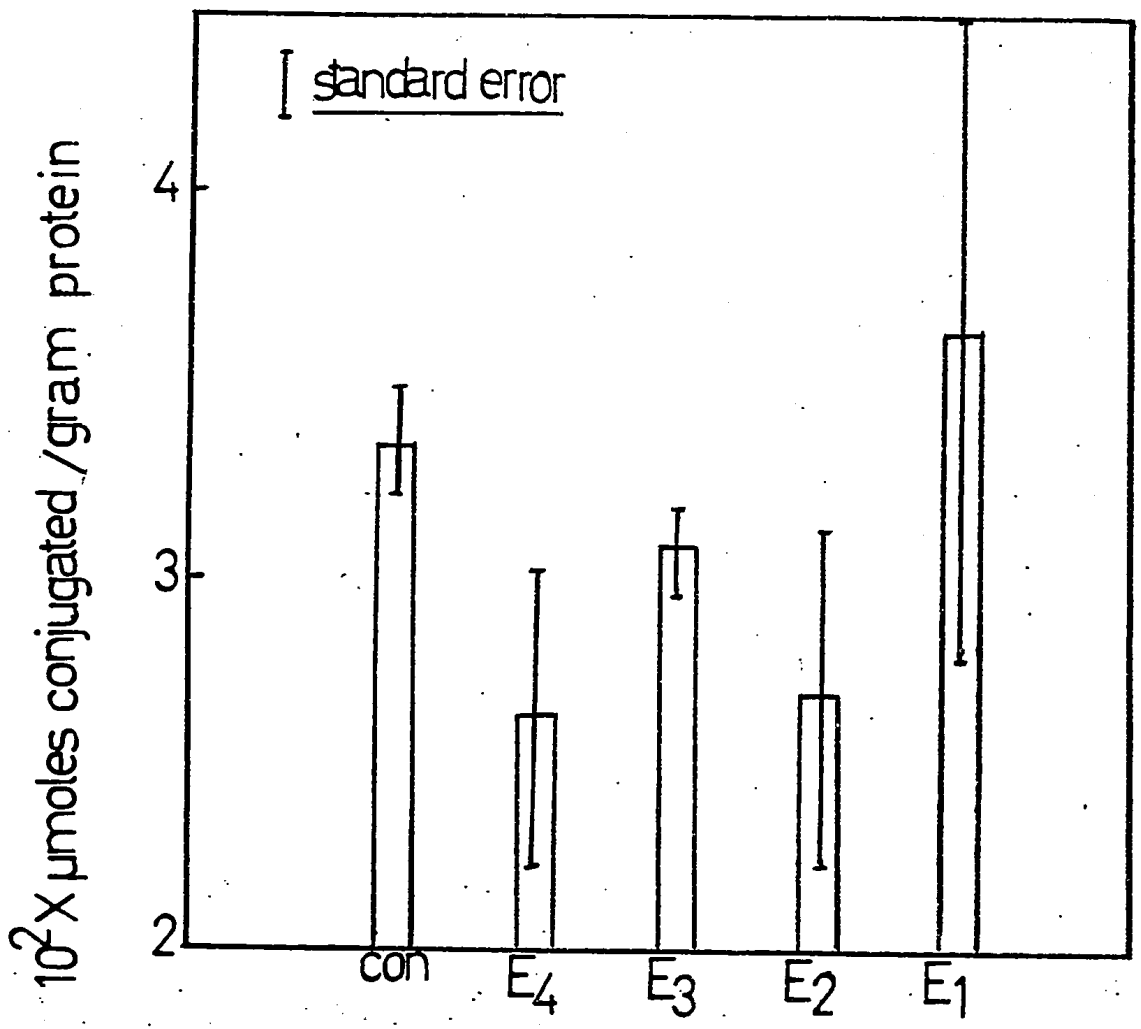


Fig. 7. N-acetyl glucosaminyl transferase activity in kidneys of rabbits treated with different doses of 17β-estradiol.

con = control; E_4 = 15.0 µgms/kilo; E_3 = 75.1 µgms/kilo; E_2 = 375.9 µgms/kilo; E_1 = 1127.8 µgms/kilo.

TABLE 16

Effect of two-day treatment with 17β-estradiol
on rabbit kidney N-acetyl glucosaminyl transferase

	Total dose (mgm) estrogen given in 2 days	Uterine weight (gms)	10^2 x μmoles conj./gm ptn	
CONTROL # 17	none	2.47	1.901	Mean KIDNAG 1.949 S.E. ± 0.077
CONTROL # 15	none	2.69	1.835	Mean wt ut. 1.786 S.E. ± 0.759
CONTROL # 3	none	0.20	2.112	
ESTRADIOL # 18	9.064	2.73	2.453	Mean KIDNAG 2.968* S.E. ± 0.258
ESTRADIOL # 16	9.220	1.02	3.188	Mean wt ut. 1.53 S.E. ± 0.601
ESTRADIOL # 4	8.840	0.84	3.265	

* Significantly higher than control ($p < 0.01$)

TABLE 17

Effect of four-day treatment with 17β-estradiol on rabbit kidney N-acetyl glucosaminyl transferase

	Total dose (mgm) estrogen given in 4 days	Uterine weight (gms)	10^2 x μmoles conj./gm ptn	
CONTROL # 11	none	1.56	2.126	Mean KIDNAG 1.566 S.E. ± 0.303
CONTROL # 9	none	1.48	1.080	Mean wt ut. 2.056 S.E. ± 0.536
CONTROL # 1	none	3.13	1.492	
ESTRADIOL # 12	17.868	2.45	3.785	Mean KIDNAG 3.307^{+} S.E. ± 0.824
ESTRADIOL # 10	15.448	1.28	1.701	Mean wt ut. 3.05 S.E. ± 1.228
ESTRADIOL # 2	17.620	5.42	4.436	

+ Significantly higher than control ($p < 0.05$)

(ribonucleic acid increased) without the formation of new cells (deoxyribonucleic acid not increased).

In our series of experiments livers of cortisol treated rabbits showed a significant increase in total protein content when compared with those of control animals (see table 6). Feigelson et al. (1962) studied the effects of cortisone on nucleic acid and protein metabolism of rat liver. They found that parenteral administration of a single low dose of cortisone acetate exerts an anabolic influence on nucleic acids and proteins of both normal and regenerating rat livers. The stimulatory influence of cortisone on RNA metabolism was more marked when [2 ^{14}C] glycine served as precursor than when radioactive orthophosphate was employed, suggesting a hormonally induced enhancement of purine nucleotide biosynthesis as well as stimulated nucleotide polymerization into RNA.

As regards glucuronoside synthesis one may consider the work of Lathe and Walker (1958). These workers have used the same experimental animal as that used in the present experiments, namely the rabbit. They were able to compare the results obtained when using other animals viz. the rat and the monkey under similar experimental conditions (see p. 12). The observation that various steroids do not affect the <u>in vitro</u> conjugation of bilirubin with glucuronic acid in rabbit liver slices

correlates rather well with our *in vivo* findings. It is interesting to note that not only species difference was important but the use of a different substrate viz. o-aminophenol or the replacement of liver slices by a liver suspension in the same experimental animal led to a different result under similar experimental conditions.

The increased activity of kidney N-acetyl glucosaminyl transferase of rabbits treated with adequate doses of estrogens for 2 days or more is the main subject of this discussion.

Whether estrogens enhance the activity of the enzyme or increase its production *in vivo* is not known. The finding that stilbestrol as well as the steroid estrogens produce this effect may suggest that the enhanced activity is due to the estrogenic effect of the injection rather than to a specific action of the estradiol steroid.

Wayne et al. (1964) found that a non specific serum hyaluronidase inhibitor increases following intravenous administration of estrogens in the human. The increased activity of kidney N-acetyl glucosaminyl transferase under estrogen therapy may be in fact due to a decreased activity of hyaluronidase under the effect of an inhibitor similar to that found in human serum.

The liver is not affected similarly because of its capacity to metabolise the estrogen it fixes (Segaloff, 1963). On the other hand, table 14 shows a significant

increase in the activity of liver N-acetyl glucosaminyl transferase of rabbits treated with 17β-estradiol. One may speculate that as the rabbit metabolises 17β-estradiol into its 17α isomer before conjugation and excretion (Heard et al., 1941) the estrogen may be fixed to the liver parenchyma for a sufficiently long period to produce a measurable effect.

An increased synthesis of the transferase enzyme by the kidneys is another possibility. Jensen (1962) envisions that estrogens have an indirect effect on the synthetic machinery which is already present in the cells. He postulates that high energy phosphate fuel is made available by blocking the enzyme adenosine triphosphatase. The latter enzyme is considered to be an efficient shunt mechanism which drains off high energy phosphate in the absence of estrogens in estrogen sensitive tissues.

The increased activity of the kidney enzyme may be ascribed to a specific action of the estradiol molecule (Layne, 1969). The theory is based on the finding that the rabbit excretes 17α-estradiol as the double conjugate. It seems probable that the kidney is unable to excrete the estradiol as a monoglucuronoside. It is suggested that following injections with estradiol, the kidney N-acetyl glucosaminyl transferase is actively involved in transferring N-acetyl glucosamine to the steroid. This, however, does not explain the similar

effect obtained on the transferase system by treatment with stilbestrol.

Mazur and Shorr (1942) have found that stilbestrol is mainly excreted by the rabbit as the monoglucuronoside. Collins et al. (1968) found that diethylstilbestrol inhibits the transfer of N-acetyl glucosamine to the 17α-hydroxy of 17α-estradiol-3-glucuronoside. In view of these findings, it is suggested that a reexamination of the mode of conjugation of stilbestrol in the rabbit would be of interest in order to definitely establish whether this compound is an in-vivo substrate for the N-acetyl glucosaminyl transferase (Himaya et al., 1969). Finally the possibility that the increased transferase activity is a function of neoprotein synthesis may be entertained. In such a case, the administration of puromycin may stop the entire process (see Mueller et al., 1961).

A plausible explanation for the inhibition of the kidney N-acetyl glucosaminyl transferase by small doses of 17β-estradiol (see table 15 and fig. 7) is yet to be found.

As a final thought for this discussion, the use of microsomal preparations may elucidate some of the actions of this transferase system. The addition of the supernatant fluid obtained at the various phases of differential centrifugation may reveal the presence of

an inhibitor for the hydrolase enzyme or an activator of the transferase system or both.

PART 2

STUDIES ON STEROID GLYCOSIDASES IN THE SERUM OF PREGNANT AND NON PREGNANT WOMEN

INTRODUCTION

A number of enzymes and hormones produced by the human placenta are believed to be necessary for the maintenance of gestation. The recognition of the functional capacity of the placenta may therefore help in predicting the outcome of pregnancy. Clinical methods, viz. auscultation of the fetal heart, naked eye observation of the liquor amnii etc., when taken as the only criteria for assessment are inadequate and have resulted in sudden unexpected fetal death. Placental insufficiency is also known to be responsible for cases of intrauterine fetal growth retardation. This so-called "Small for Dates" or dysmature infant is a child at risk to various hazards (Brit. Med. J., 1967).

These unfortunate outcomes of pregnancy have led to extensive investigation of the placenta. Tremblay et al. (1965) found that placentae from cases of fetal malnutrition utilize oxygen at a lower rate than do those from normal cases. According to these workers the fault seems to lie in the enzyme systems of the placenta. However, the reduced oxygen uptake, being a blanket test for metabolic activity, cannot detect which function is depressed.

On the other hand, placental insufficiency was found to be associated with a reduction in urinary pregnanediol excretion (Russell et al., 1960) thus indicating

a depression of the endocrine function of the placenta (Russell, 1961).

During recent years, Clinicians have largely relied on urinary estrogen estimations to assess the condition of the fetus in utero. However, the wide fluctuations in serial estriol excretion values has led to a search for a substance which is mainly produced by the placenta (Morris, 1968). As a logical sequence to the above discussion, research should be concerned with an enzyme (or a number of enzymes) which is almost entirely produced by the placenta and closely connected to its endocrine function.

This part of the thesis deals with some preliminary findings on steroid glycosidases in the sera of pregnant and non-pregnant women.

HISTORICAL REVIEW

A. STUDIES ON N-ACETYL β GLUCOSAMINIDASE

Foreword:

The hydrolysis of glucosaminides by an enzyme in Helix Pomatia has been investigated by Neuberger et al. (1939). Pugh et al. (1957) reported on N-acetyl β glucosaminidase in rat kidney. However, work on the kinetics and specificity of N-acetyl β glucosaminidase in human serum is largely attributable to Walker et al. (1960). The following account on the properties of this glycosidase is mainly based on the findings of Walker and his co-workers.

Activity in acetate and citrate buffers:

Pugh et al. (1957) found that the activity of the enzyme in acetate buffer was considerably lower than that in citrate buffer of equimolar concentration (fig. 8.). It was also noted that the enzyme activity fell progressively with increasing acetate concentration. Pugh et al. (1957) concluded from experimental evidence that the inhibition of the enzyme produced by acetate might be competitive.

Effect of pH:

The pH optimum of 4.3 was obtained when the enzyme activity was determined over the range pH 3 - 6.5 in 0.05 M citrate buffer at a substrate concentration of 0.01 M (fig. 8).

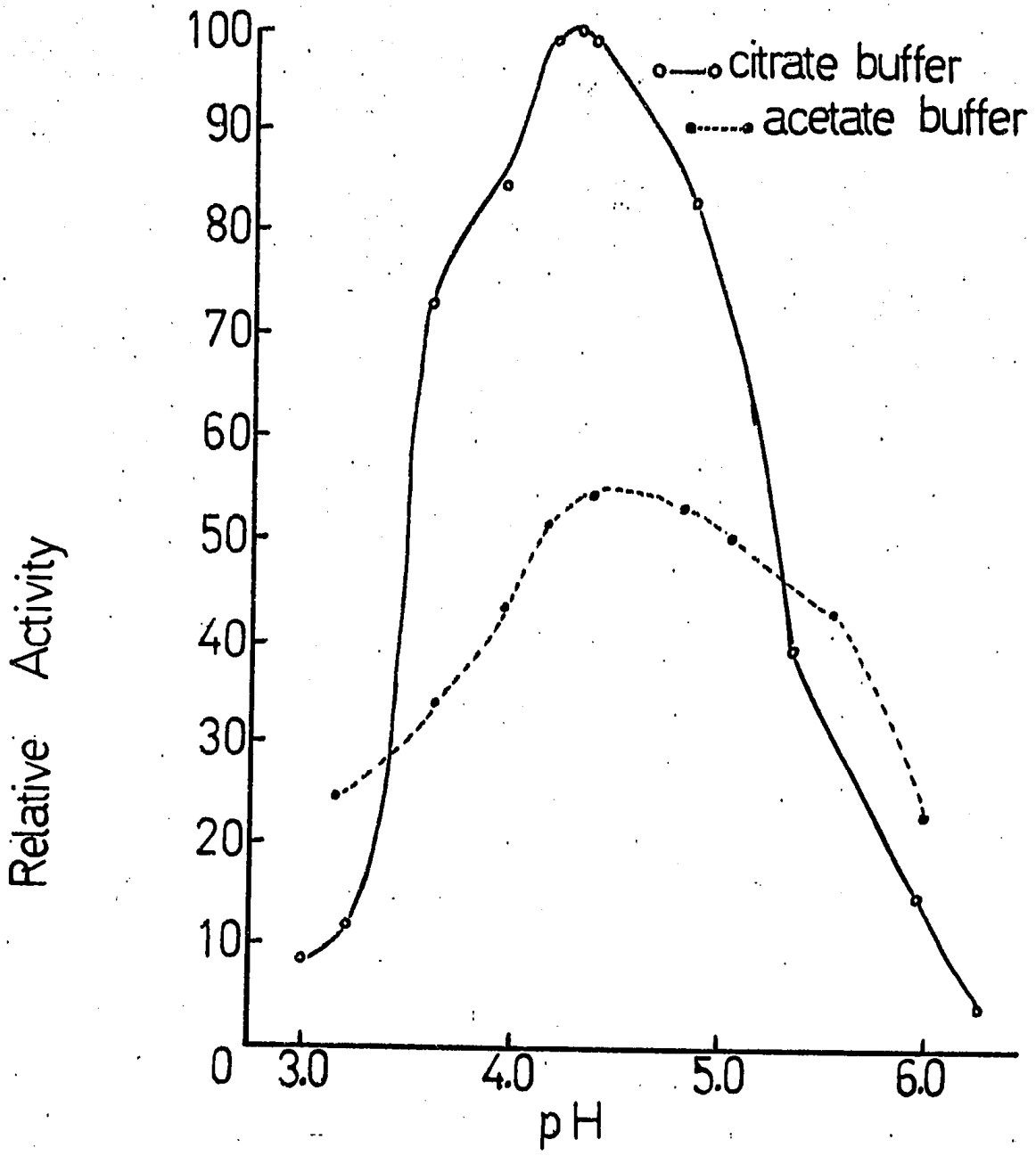


Fig.8. Effect of pH on Reaction Velocity of N-Acetyl ß-Glucosaminidase (Pugh et al., Biochem. J. 65:465(1957)

Effect of time of incubation:

Pugh et al. (1957) reported that the rate of hydrolysis of phenyl-N-acetyl β glucosaminide was proportional to the time of incubation up to one hour but after this it declined progressively (fig. 9). This fall has been attributed to a probable instability of the enzyme on prolonged incubation.

Serum N-acetyl-β-glucosaminidase in women:

Walker et al. (1960) studied the activity of serum N-acetyl-β-glucosaminidase during the course of a normal menstrual cycle, they found slight variations in the enzyme activity. They report, however, that the mean N-acetyl-β-glucosaminidase activity of serum rose throughout pregnancy. The increase was logarithmic, the activity being approximately double in each trimester.

Localisation of N-acetyl-β-glucosaminidase in placenta and membranes

The histochemical localisation of N-acetyl-β-glucosaminidase in formalin fixed placenta embedded in gelatin, as well as in the membranes was studied by Walker et al. (1960).

The histochemical reaction depends on the liberation of α-naphthol by the enzyme from α-naphthyl N-acetyl-β-glucosaminide and its precipitation in the section as insoluble azo dye by simultaneous coupling with diazotised o-aminoazotoluene (see Pugh & Walker, 1958).

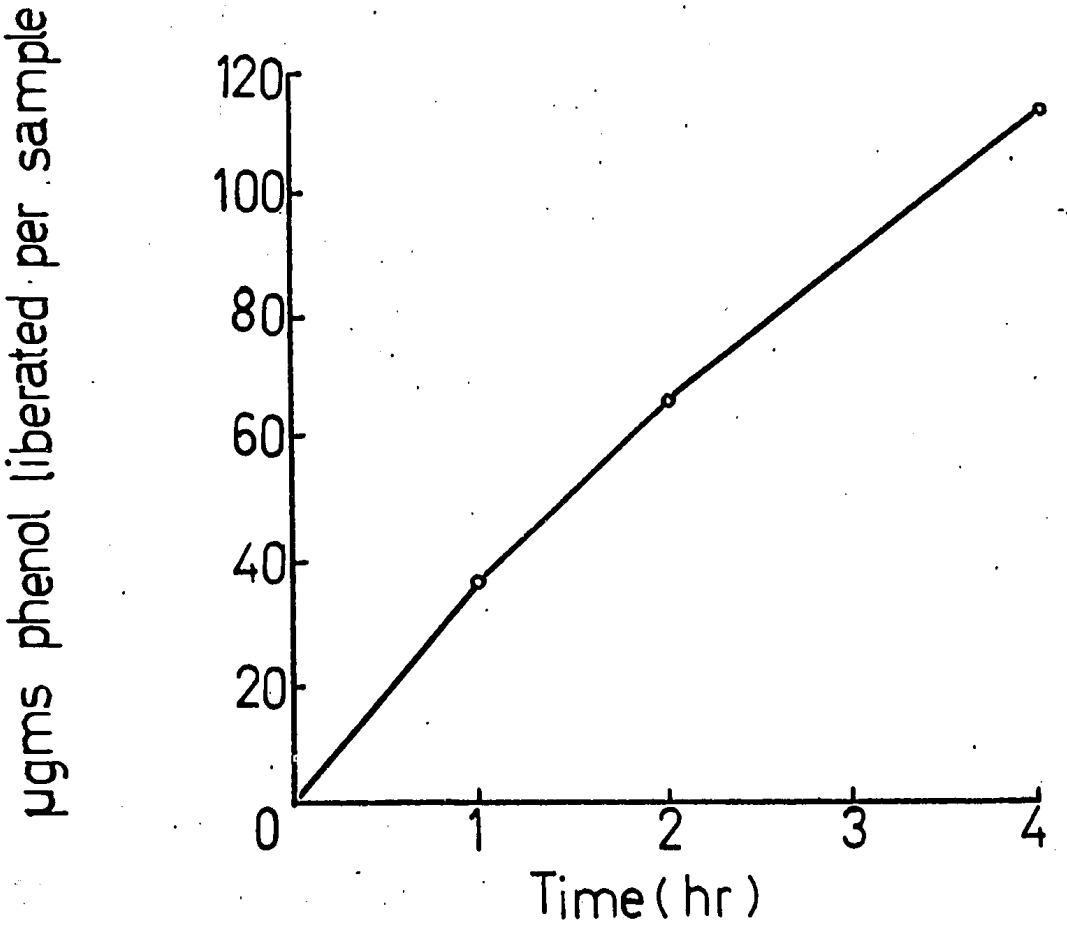


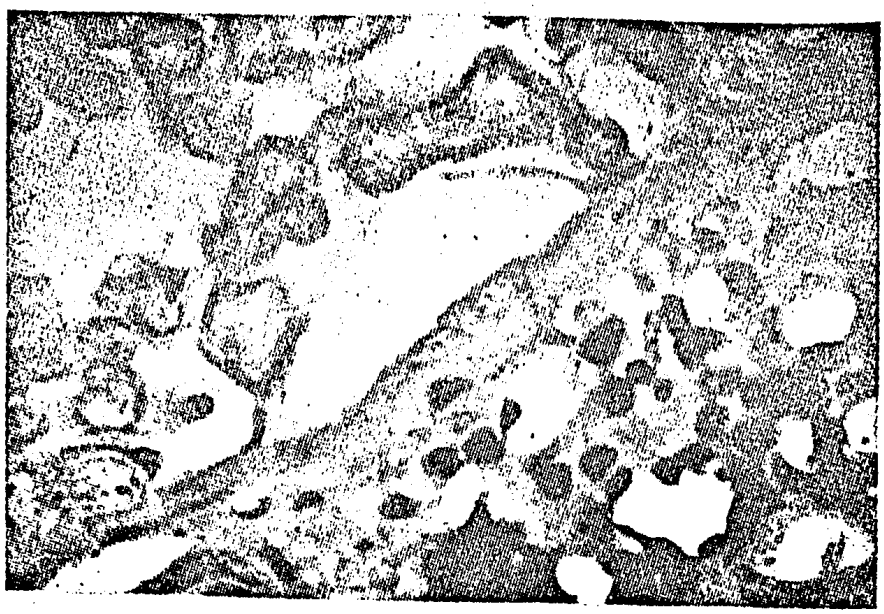
Fig. 9. Effect of Time of Incubation on Rate of Hydrolysis of Phenyl-N-Acetyl-ß-Glucosaminide (from Pugh et al. Biochem. J. 65:466(1957)

Decidual cells in the basal and chorionic plates of the placenta and on the maternal surface of the chorion show an intense reaction (fig. 10). Amnion chorionic connective tissue and placental villi are much less active. These studies suggest that the fetal contribution to the enzyme is negligible.

Studies on serum N-acetyl-β-glucosaminidase in abnormal pregnancy

In 10 out of 15 pregnant women suffering from moderate or marked edema Furuya (1966) found that the serum NAG activity was higher than that expected for normal pregnancy. On the other hand, he observed that there were minimal or no changes in enzyme activity in 7 cases with hypertension in the late stage of pregnancy.

However, herreported that 2 cases with persistent hypertension in pregnancy had lower serum NAG activity than normal. The abnormal higher and lower levels were found to revert to normal on subsidence of the symptoms.



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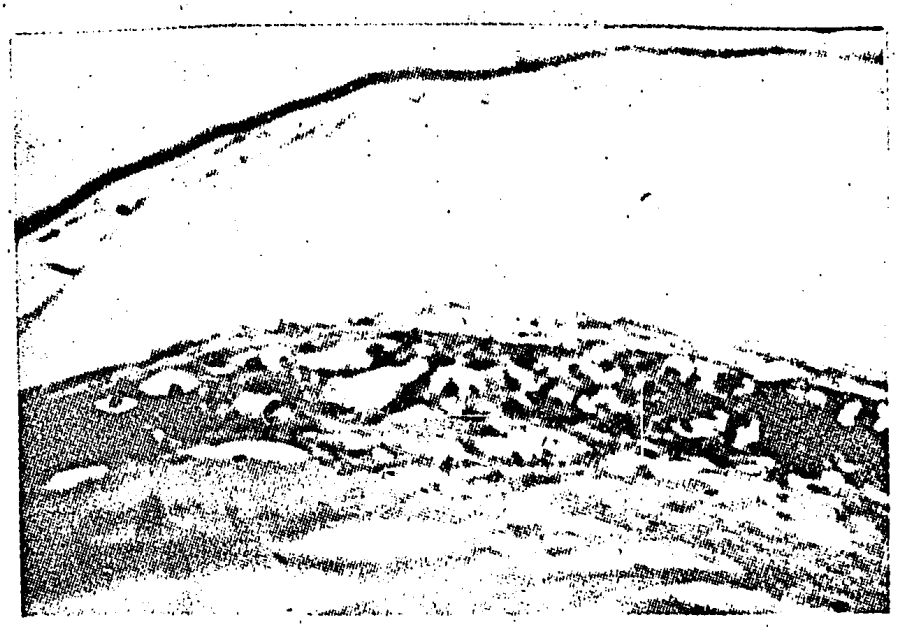

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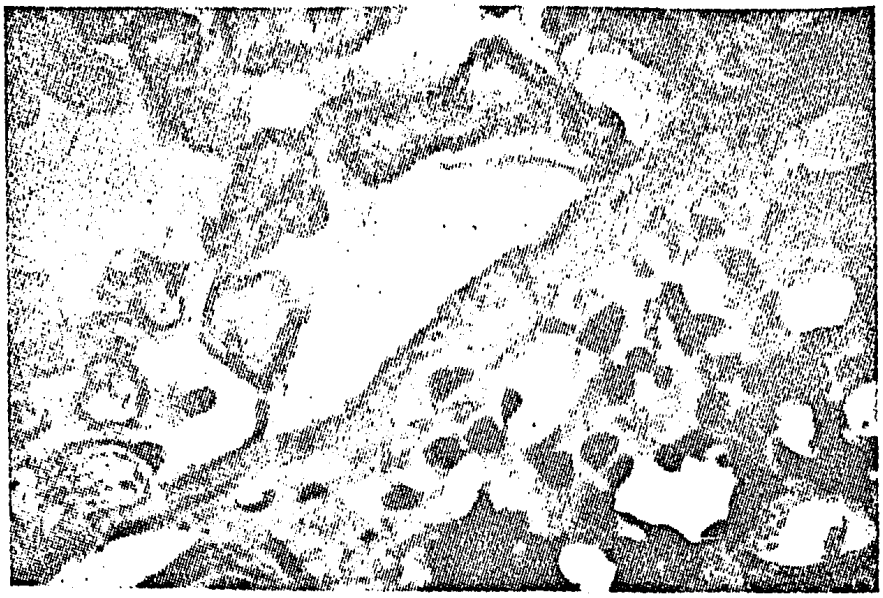
Fig. 10. Localisation of N-acetyl ß glucosaminidase in (i) basal plate of placenta (ii) fetal membranes from Walker et al. J. Clin. Path. 13 : 353 (1960)



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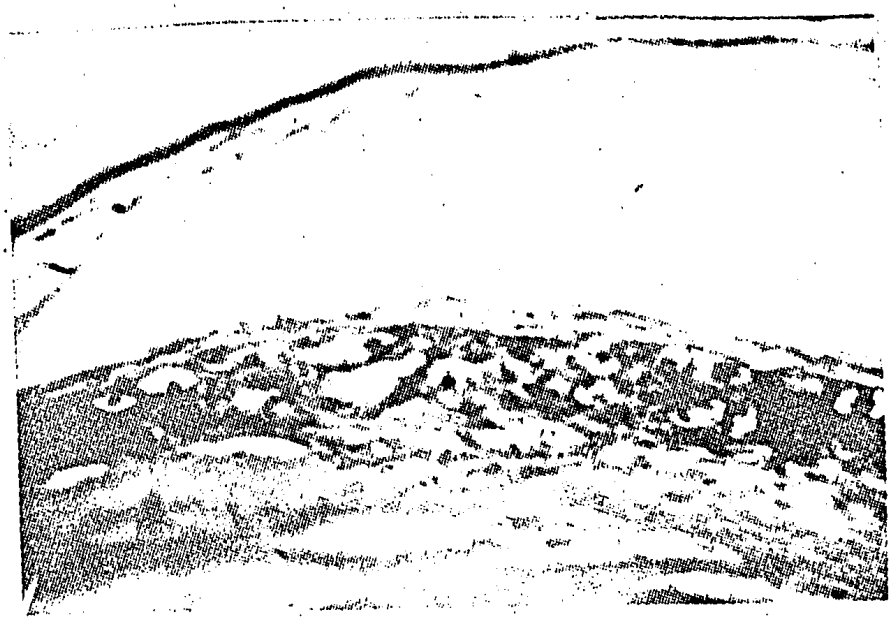

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Fig. 10. Localisation of N-acetyl ßglucosaminidase in (i) basal plate of placenta (ii) fetal membranes from Walker et al. J. Clin. Path. 13 : 353 (1960)

B. STUDIES ON β GLUCURONIDASE

Foreword:

The observation that urinary excretion of estriol and pregnanediol glucosiduronate increases during pregnancy together with the introduction of a simple colorimetric method for enzyme assay have stimulated the interest of McDonald and Odell (1947) to study the serum glucuronidase activity during the course of gestation.

Serum β glucuronidase during normal pregnancy:

McDonald and Odell (1947) found that during normal pregnancy, the mean curve for serum glucuronidase activity showed a downward inclination at its inception between 6 to 12 weeks (see fig. 11). This fall in enzyme activity was coincident with that of serum gonadotropins. The subsequent rise in serum glucuronidase parallels that of serum estrogens (Smith and Smith, 1937; McDonald and Odell, 1947).

Toxemia of pregnancy:

In 12 cases of pregnancy associated with hypertension, McDonald and Odell (1947) found that the serum glucuronidase activity was not significantly different from values for normal pregnancy. On the other hand, much higher values were obtained when the pregnancy was complicated by pre-eclampsia (fig. 11).

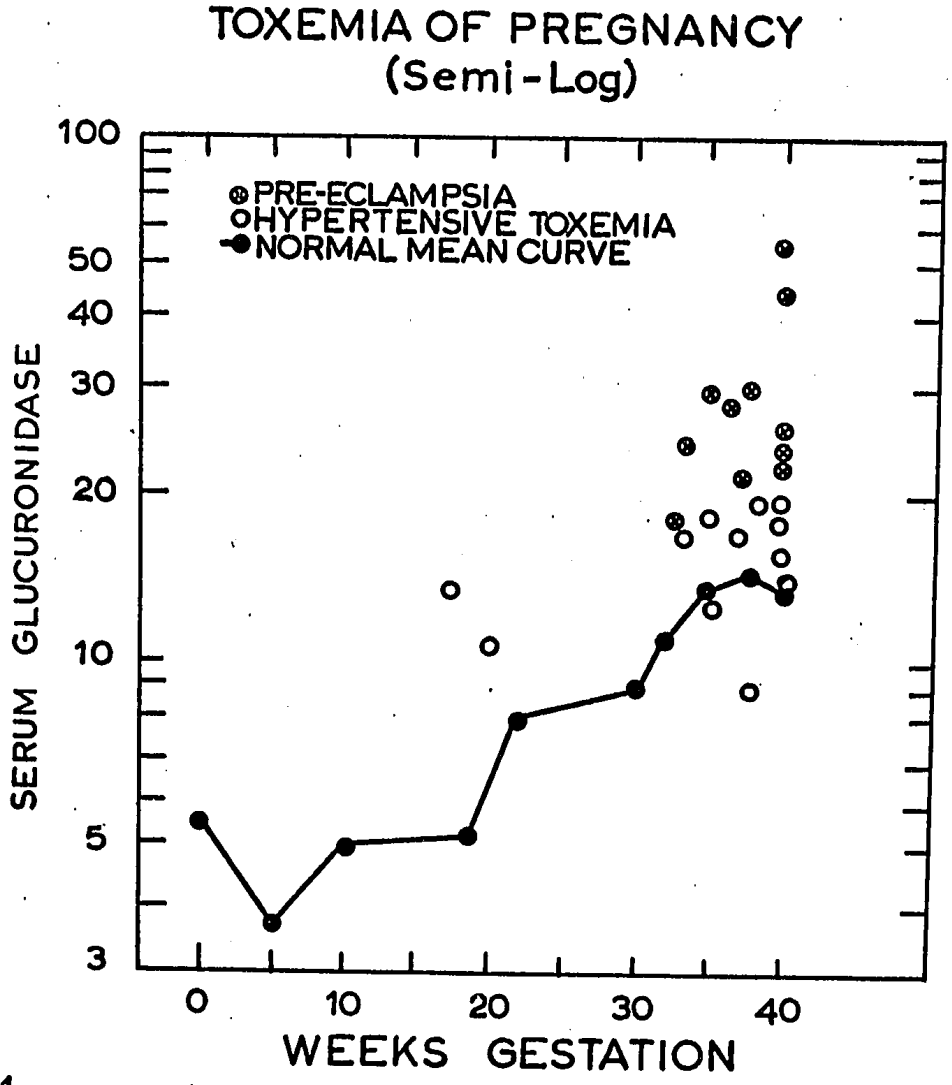


Fig.11. Serum glucuronidase activity in micrograms per ml. in twenty-four cases of pregnancy toxemia. The mean curve of normal pregnancy is plotted for comparison. From McDonald and Oddell J.Clin.Endocr.(1947) 7:535

Source of serum glucuronidase during pregnancy:

The findings of McDonald and Odell (1947) seem to indicate that the source of serum β glucuronidase activity during pregnancy is not the gravid endometrium or the placenta. This conclusion was based on glucuronidase assays of various organs during pregnant and non-pregnant states (see fig. 12).

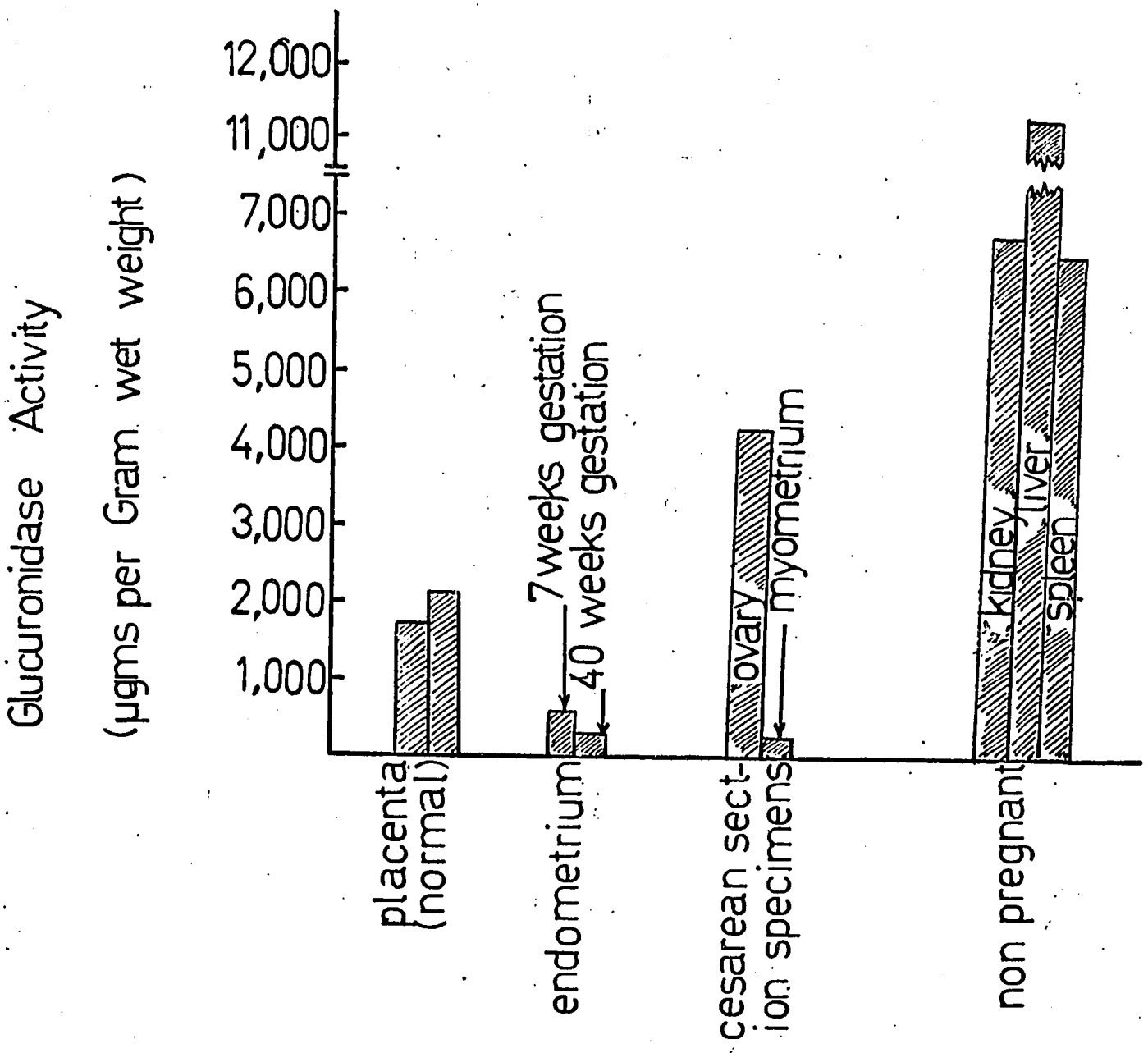


Fig. 12. Glucuronidase Activity in Various Tissues
from data by Mc Donald & Odell J. Clin. Endocr. 7:535 (1947)

MATERIALS AND APPARATUS

A. PATIENTS INCLUDED IN THE STUDY

Female patients attending the out patient clinic and those admitted to the gynecological and obstetrical wards of the Ottawa General Hospital were randomly ascribed to the study. No attempt was made for the selection of patients although most of them were from the clinic service.

B. THE SERUM

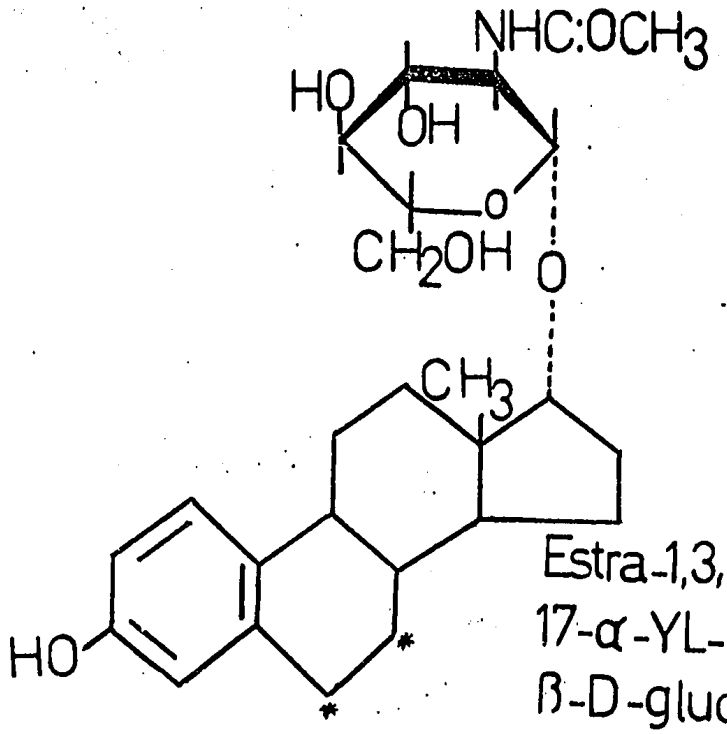
Fasting blood was collected from the antecubital vein by venipuncture using a disposable vacutainer needle and a sealed vacutainer test tube. About 5 mls were collected from each patient. The time elapsed between the collection of the blood sample and the incubation of the specimen for enzyme assay did not exceed one hour.

Blood was centrifuged at low speed and the supernatant serum pipetted out and transferred to another test tube adequately labelled.

C. THE SUBSTRATES

1) Tritiated Estradiol 17α N-Acetyl β D Glucosaminide

was prepared by Dr. D.S. Layne (fig. 13a) 2.65 mgm of the crystals were dissolved in 10 mls methanol and stored at -10°C. 50 μl of the prepared



Estra-1,3,5(10)-trien-3-ol-
17-α-YL-2'acetamido-2'deoxy
β-D-glucopyranoside

Fig.13(a') Estradiol 17α-N-acetyl β-D-glucosaminide

Estra-1,3,5(10)-trien-17α-ol-3-YL-β-D-
glucosapyranosiduronic acid

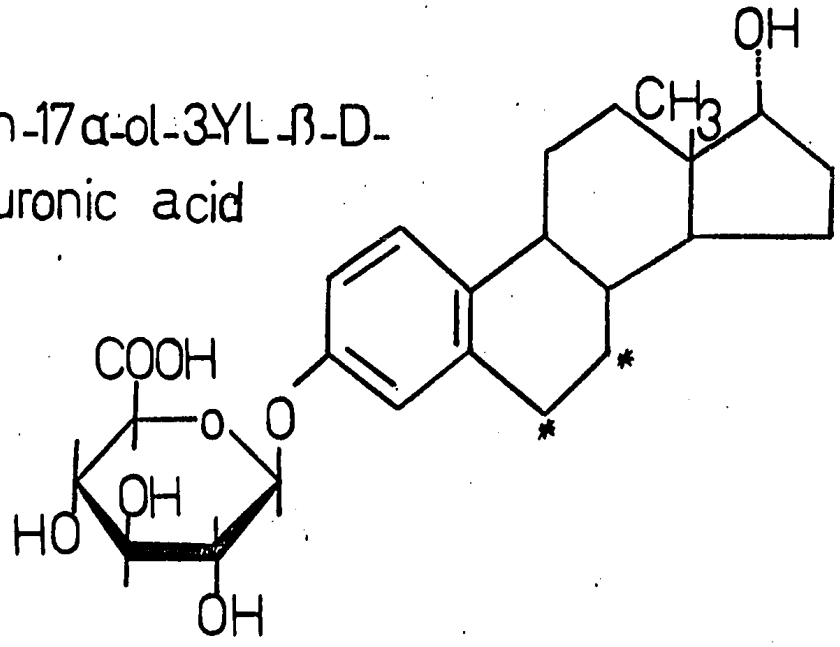


Fig.13(b)Estradiol 17α-hydroxy-3-monoglucuronoside

solution showed an activity of 4,250 d.p.m. It was previously known that one μgm of the substrate had 318 d.p.m.

2) Tritiated Estradiol 17α-hydroxy 3 monoglucuronoside

was also prepared by Dr. D.S. Layne (fig. 13b). 3.39 mgm of the powder were dissolved in 10 mls methanol and stored at -10°C. 50μl of the prepared solution showed an activity of 6,000 d.p.m.

D. BUFFER SOLUTIONS

1) 0.1 M Acetate Buffer pH 5.0

a) Preparation of 1.0 M sodium acetate

($CH_3COONa.\ 3H_2O$ Mol. wt. 136)

68 gms of sodium acetate are dissolved in 500 mls of distilled water.

b) Preparation of 0.1 M sodium acetate

180 mls of distilled water are added to 20 mls of 1.0 M sodium acetate solution.

c) Adjusting pH

Under the control of a pH meter, glacial acetic acid is added dropwise to lower the pH to 5.0.

2) 0.1 M Citrate Buffer pH 4.2

a) Preparation of 1.0 M sodium citrate

($Na_3C_6H_5O_7.\ 2H_2O$ F.W. 294.10)

147 gms of sodium citrate are dissolved in 500 mls of distilled water.

solution showed an activity of 4,250 d.p.m. It was previously known that one μgm of the substrate had 318 d.p.m.

2) Tritiated Estradiol 17α-hydroxy 3 monoglucuronoside

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2) 0.1 M Citrate Buffer pH 4.2

a) Preparation of 1.0 M sodium citrate

($Na_3C_6H_5O_7.\ 2H_2O$ F.W. 294.10)

147 gms of sodium citrate are dissolved in 500 mls of distilled water.

b) Preparation of 0.1 M sodium citrate

180 mls of distilled water are added to 20 mls of 1.0 M sodium citrate solution.

c) Preparation of 1.0 M citric acid

($H_3C_6H_5O_7$ F.W. 192.13)

96 gms of citric acid are dissolved in 500 mls of distilled water.

d) Preparation of 0.1 M citric acid

180 mls of distilled water are added to 20 mls of 1.0 M citric acid solution.

e) Adjusting pH

Under the control of a pH meter 0.1 M citric acid solution is added to 0.1 M sodium citrate solution to lower the pH to 4.2.

E. OTHER LIQUIDS REQUIRED

1) Benzene:

ACS certified Fisher product used without further purification.

2) Toluene Scintillation Liquid:

4 gms PPO (2.5 Diphenyloxazole) are dissolved in one litre of toluene.

F. OTHER APPARATUS USED

1) Centrifuge:

This is obtained from International Equipment

Co. (Boston, Mass.).

2) Incubator:

Fisher Isotemp incubator (Senior Model) is used because of its accurate temperature regulation.

3) Lang Levy Pipette:

A 50 µl capacity Lang Levy pipette is used for measuring the substrates.

4) Centrifuge tubes:

15 ml pyrex centrifuge tubes fitted with stoppers are used for the incubation media.

5) Scintillation vials & liquid scintillation system

As described in Part 1, page 25.

METHODOLOGY

A. MIXING THE INCUBATION MEDIA

1) For a glucuronidase assay

Using a 15 ml centrifuge tube, 50 µls of estradiol 17α 3 monoglucuronoside solution are added to 4.0 mls of acetate buffer pH 5.0. One ml of the serum to be tested is then added to the above mixture. The control tube contains 5.0 mls of the acetate buffer in order to equalize the volumes of the incubation media.

2) For N-acetyl a glucusaminidase assay

Using a 15 ml centrifuge tube 50 µls of estradiol 17α-N-acetyl glucosaminide solution are added to 4.0 mls of citrate buffer pH 4.2. One ml of the serum to be tested is added to the above mixture. The control tube contains 5.0 mls of the citrate buffer in order to equalize the volumes of the incubation media.

B. INCUBATION

The centrifuge tubes are fitted with stoppers and kept in an incubator at 37°C for 22 hours.

C. EXTRACTION

At the end of the incubation period, 5 mls of benzene are added to each incubation medium and the mixture is gently but thoroughly shaken. Vigorous

shaking results in a milky suspension especially when dealing with the N-acetyl-β-glucosaminidase assay.

D. CENTRIFUGATION

After adequate shaking the centrifuge tubes are centrifuged at low speed for about 5 minutes. If the formation of a clear upper benzene phase is prevented by a milky emulsion, the thick curd is gently broken up using a glass rod or a disposable Pasteur pipette and centrifugation is allowed to be repeated.

E. COUNTING RADIOACTIVITY

One ml of the clear upper benzene phase is obtained and transferred to a scintillation vial. Ten mls of toluene scintillation liquid are added to each sample and the vial closed and well shaken. Counting is effected in a liquid scintillation system.

F. CALCULATING PERCENT HYDROLYSIS

The DPM count is calculated in each sample after accounting for quenching, efficiency and the blank specimen.

As the number of d.p.m. contained in each substrate sample is previously known, the calculation of the percentage hydrolysis is facilitated by applying the following equation:

$$\frac{\text{dpm counted} \times 100}{\text{Total dpm added}}$$

G. CALCULATING AMOUNT OF SUBSTRATE HYDROLYSED

As each μgm of the prepared radioactive substrate gives a fixed number of d.p.m. it is possible to calculate the amount of substrate hydrolysed after obtaining the d.p.m. count on any sample.

RESULTS

It was noted that serum N-acetyl glucosaminidase activity rises during pregnancy. The peak of activity is reached towards the end of the second trimester and is maintained until the end of gestation. Non-pregnant women receiving progestogen therapy do not show any change from the non-pregnant level (fig.14). However, a post menopausal woman showed a high level of the enzyme, she was receiving cortisone therapy for arthritis.

Early in the experiment a level markedly higher than that obtained in normal pregnancy prompted us to study the problem of placental dysfunction; this was the case of a pregnancy complicated by excessive weight gain, peripheral edema, hypertension and minimal albuminuria. Treatment with hydrochlorothiazide was successful in abating the clinical signs but did not affect the serum N-acetyl glucosaminidase activity. Up to the present time, the sera of 48 pregnant and non-pregnant women have been examined for glycosidase activity. The results are represented in a graph form (fig. 14). In the two cases of toxemic pregnancy, there is marked increase in N-acetyl glucosaminidase activity. A case of Wilson's disease (Hepatolenticular degeneration) and two cases of Diabetes have been studied, the results do not show any significant change from normal pregnancy.

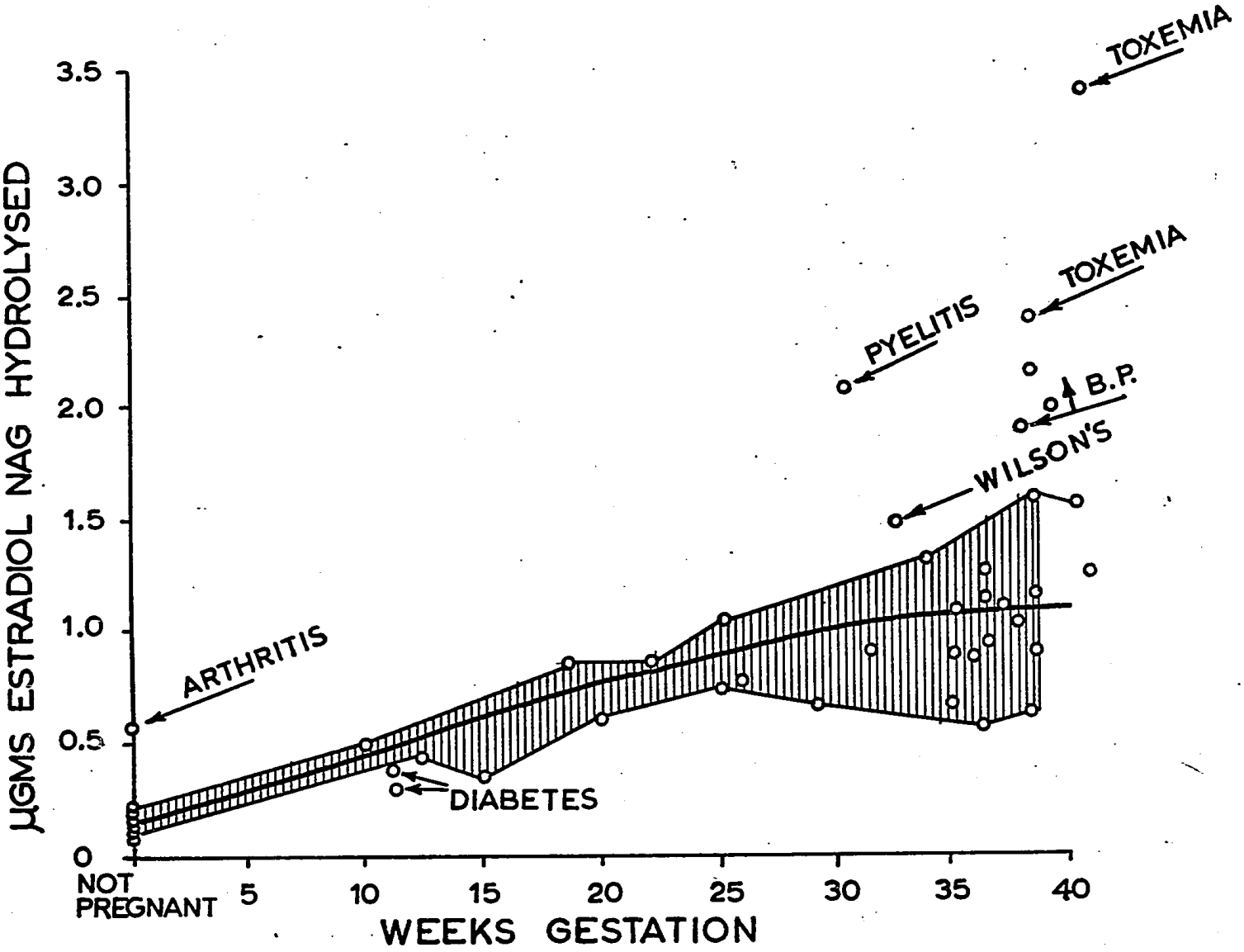

Fig.14. Serum N.acetyl Glucosaminidase activity per ml.

The paucity of our abnormal obstetric cases is apparent. However, the few cases we have confirm the hypothesis that a high level of N-acetyl-β-glucosaminidase activity may be indicative of placental dysfunction (Himaya & Layne, 1969). Table 18 shows our results in table form excluding the cases of toxemic pregnancy which are not sufficient for statistical analysis. The various clinical conditions encountered are outlined but these do not seem to affect the serum glycosidase activity.

Comparison between serum glycosidases

The parallel changes in activity of N-acetyl-β-glucuronidase in serum during pregnancy as demonstrated by Walker et al. (1960) suggest that these enzymes may be related (see fig. 15). McDonald and Odell report a higher level of β-glucuronidase in toxemia of pregnancy (see fig. 11). Using a steroid substrate, we were able to confirm the latter finding in only one of our two cases (fig. 16). In our experiment the results of serum β-glucuronidase activity are unpredictable and do not seem to be of practical value. It is envisaged in the future to investigate possible avenues to improve our results.

TABLE 18

Values for serum N-acetyl-β-glucosaminidase activity in pregnant and non-pregnant women

Condition	$E_2\alpha$NAG*	Standard Error	Standard Deviation	Clinical Conditions
Non-Pregnant	0.21	±0.017	±0.044	Oligomenorrhea for investigation; Stein-Leventhal; Menorrhagia; Progestogen Depôt; Endometrial hyperplasia; stress incontinence.
First Trimester Pregnancy	0.38	±0.031	±0.087	Inevitable abortion; Ectopic pregnancy; threatened abortion; Diabetes mellitus.
Second Trimester Pregnancy	1.01	±0.063	±0.166	Urinary infection; Pyelonephritis; Shirodkar's procedure (cerclage).
Third Trimester Pregnancy	1.01	±0.1	±0.316	Wilson's disease; Urinary infection.

* $E_2\alpha$NAG = mean value for μgms estradiol 17α-N-acetyl β-glucosaminide hydrolysed by one ml. of serum when incubated at 37°C for 22 hours.

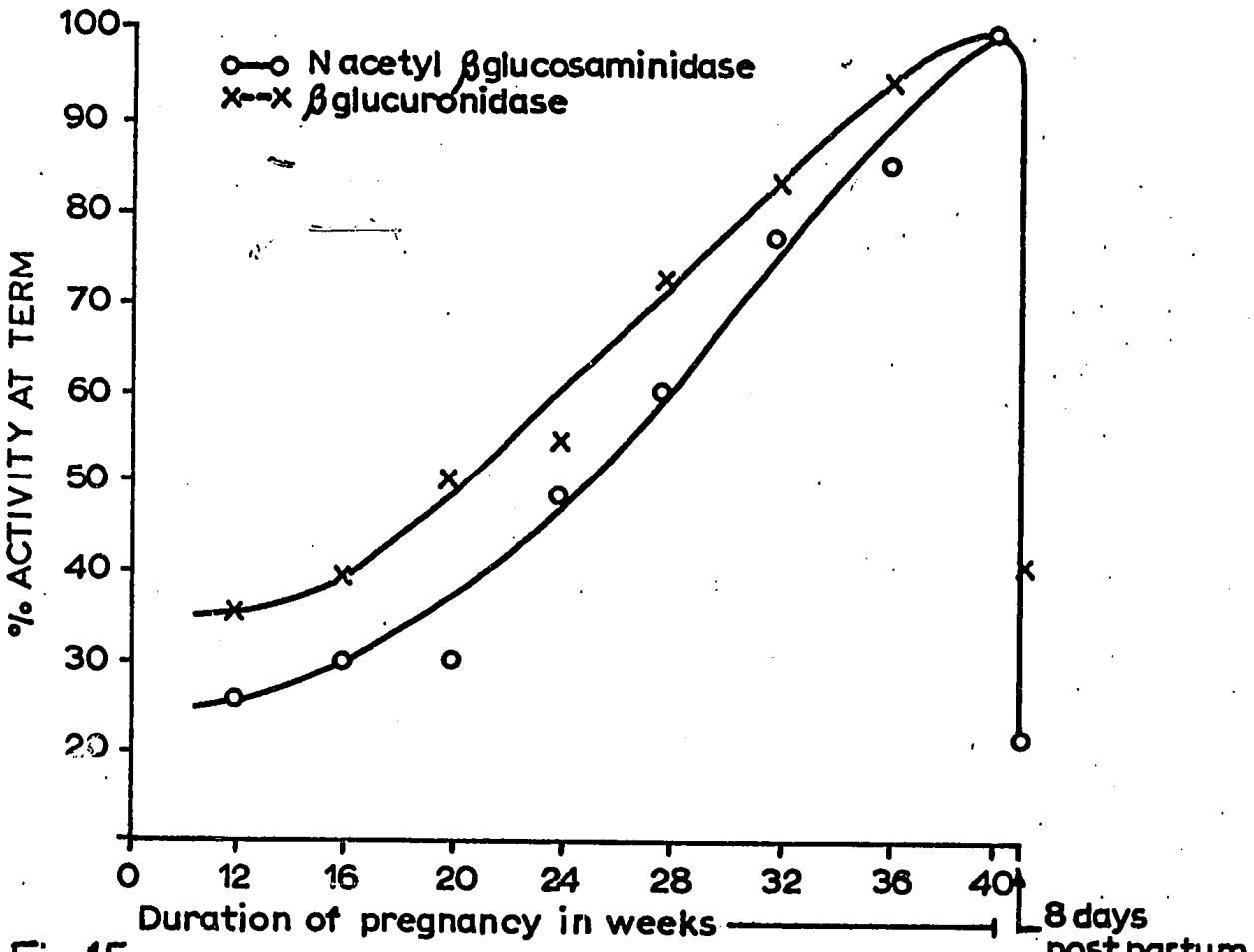

Fig.15. Variation in mean enzyme activity of serum during normal pregnancy.

From Walker et al. J. Clin. Path. (1960) 13:353

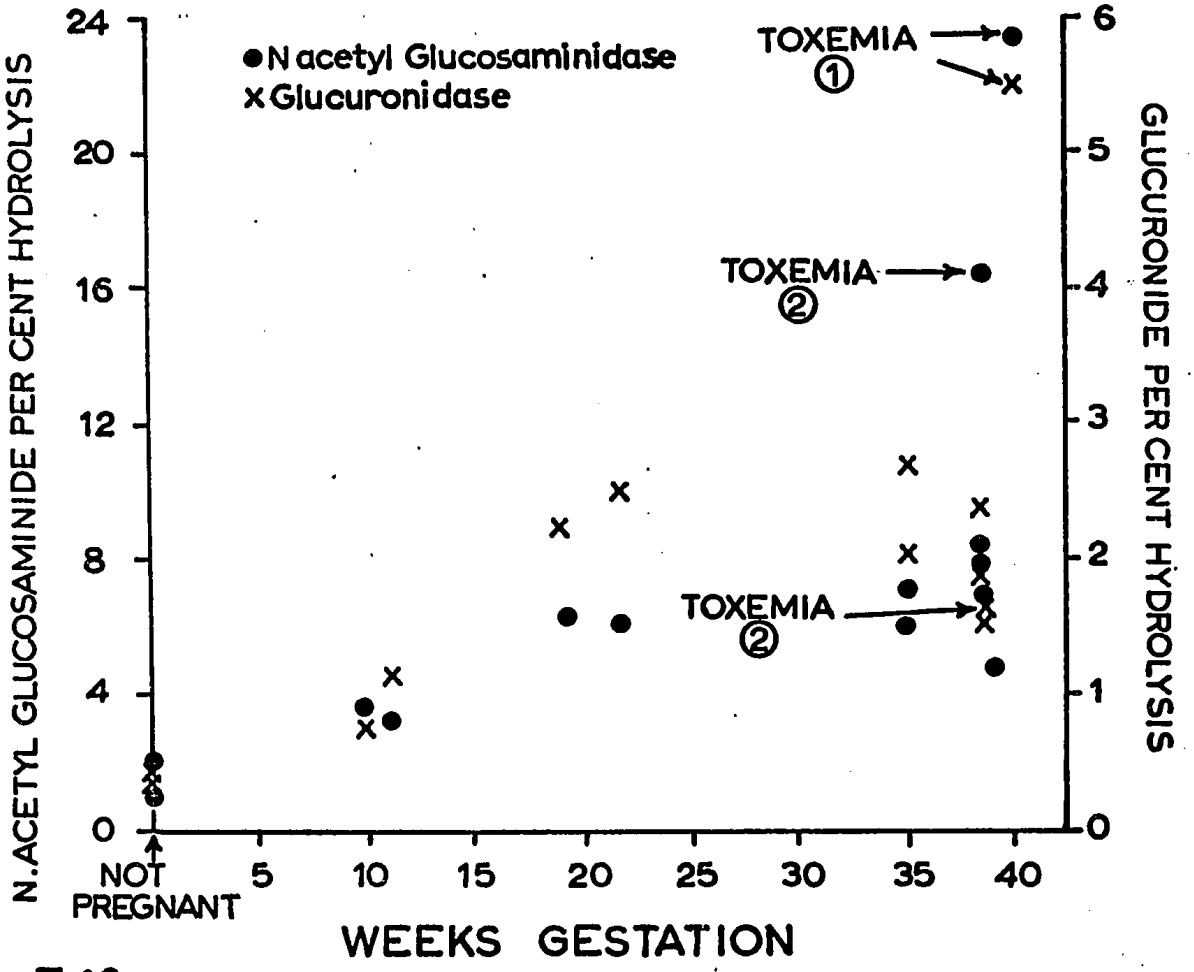

Fig16. Serum N-acetyl Glucosaminidase and Glucuronidase in normal and toxemic pregnancy.

PRESENT VIEWS ON PLACENTAL FUNCTION

PLANS FOR FUTURE STUDIES

Serial urinary estriol estimations have gained rapid popularity as the accepted parameters for fetal health.

Cassmer (1959) found that following an artificially induced fetal death in utero, there was a marked reduction in urinary excretion of estrogens. The same author further noted that, of the three urinary estrogens, estriol disappears more rapidly than does estrone and estradiol.

The observations of previous investigators (Zondek, 1957; Taylor, 1958) were thus irrefutably confirmed by experimental evidence.

Despite the declining interest in estriol values (Booth et al., 1965) urinary estrogen excretion during pregnancy is the universally accepted laboratory investigation at present available for assessing fetal health. A parallel study using both urinary estriol and serum glycosidase is planned for the future.

It is hoped that the time of incubation can be reduced so that the results of the enzyme assay, would be available in a few hours. Enzymatic and histologic studies of the placentae of the pregnan-

cies involved in the experiment must be well planned. An accurate record of the infant's health for the first 2 years of his life will add to the value of the data collected.

CONCLUSIONS AND DISCUSSION

The search for a substance which is largely or solely manufactured by the placenta and whose production could be used to measure placental activity is still in its early stages. The increase in serum N-acetyl glucosaminidase during pregnancy is believed to be due to the production of this enzyme by the placenta. Histochemical localisation of this glycosidase in the placenta and the limited N-acetyl a glucosaminidase activity in umbilical vein serum (Walker et al., 1960) suggest that the fetal contribution is negligible.

Furuya (1966) found high levels of N-acetyl glucosaminidase in pregnancies complicated by edema and low levels in 2 out of 9 cases with hypertension. He relates these findings to the possible action of this glycosidase on the mucopolysaccharide and hyaluronic acid of the wall of the blood vessels and connective tissues.

We have been able to confirm the presence of higher N-acetyl glucosaminidase activity in cases of edema which was part of the pre-eclampsia syndrome.

On the other hand, Browne and Veall (1953) studied the role of placental ischemia in the etiology of pre-eclampsia, they found that the maternal placental blood flow in pre-eclampsia and chronic hypertension is reduced to about one third of the normal.

As it is usually assumed that an increase in the activity of an enzyme in serum reflects either an increased production by the tissues or breakdown of the cells producing it causing a release of their enzymatic content, it is reasonable to presume that the increased serum N-acetyl glucosaminidase activity is a function of cellular breakdown due to ischaemia. Extensive research is required before the validity of this test can be established. A protocol which can be used for further studies is proposed (see Appendices 1 and 2).

As a closing comment one must admit that at present our results for β-glucuronidase assay are inconclusive and comparison with other studies is not envisaged (see McDonald and Odell, 1947).

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