

ABSTRACT

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STUDIES ON THE STEROID ESTROGEN CONJUGATES IN HENS' URINE

Estrone-4- ^{14}C was injected intramuscularly into a laying hen. Urine collected thereafter was passed through a neutral ion exchange resin. The steroid conjugates eluted by methanol from the resin column were fractionated on a DEAE-Sephadex A-25 column by application of a linear gradient of sodium chloride (0.0 M-0.8 M). Monosulphate and monosulphate-monoglucosiduronate fractions were tentatively identified principally by elution patterns as compared with an elution pattern given by standard preparations and also those recorded in the literature.

In a second similar experiment estradiol-17 β -6,7- ^3H -17-glucosiduronate was injected. The fractionation of the radioactivity of the urine provided evidence for the tentative identification of estradiol-3-glucosiduronate, estradiol-3-sulphate and estradiol-3-sulphate-17 glucosiduronate. About 40 per cent of the urine radioactivity was present as free steroids. There were indications of the presence of estradiol-17-glucosiduronate and estrone-3-sulphate. A modified procedure for the exteriorization of the ureters of the fowl was devised and is described.

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CONJUGATES IN HENS' URINE

by

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Suggested Short Title

STEROID CONJUGATES IN HENS' URINE

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NOMENCLATURE OF ESTROGENS

<u>Trivial Name</u>	<u>Systematic Name</u>
Estrone	3-hydroxyestra-1,3,5(10)-trien-17-one
Estradiol-17 β	Estra-1,3,5(10)-triene-3,17 β -diol
Estradiol-17 α	Estra-1,3,5(10)-triene-3,17 α -diol
Estriol	Estra-1,3,5(10)-triene-3,16 α ,17 β -triol
16- <u>Epi</u> estriol	Estra-1,3,5(10)-triene-3,16 β ,17 β -triol
16,17- <u>Epi</u> estriol	Estra-1,3,5(10)-triene-3,16 β ,17 α -triol
17- <u>Epi</u> estriol	Estra-1,3,5(10)-triene-3,16 α ,17 α -triol
Estrone-3-sulphate	17-oxoestra-1,3,5(10)-trien-3-yl-sulphate
Estrone-3-glucosiduronate	17-oxoestra-1,3,5(10)-trien-3-yl- β -D-glucopyranosiduronate
Estradiol-17 β -3-sulphate	17 β -hydroxy-estra-1,3,5(10)-trien-3-yl-sulphate
Estradiol-17 β -3-glucosiduronate	17 β -hydroxy-estra-1,3,5(10)-trien-3-yl- β -D-glucopyranosiduronate
Estradiol-17 β -17-glucosiduronate	3-hydroxy-estra-1,3,5(10)-trien-17 β -yl- β -D-glucopyranosiduronate
Estradiol-17 β -3-sulphate-17-glucosiduronate	3-sulfo-estra-1,3,5(10)-trien-17 β -yl- β -D-glucopyranosiduronate

ABBREVIATIONS OF ESTROGENS

<u>Trivial Name</u>	<u>Abbreviations</u>
Estrone	E ₁
Estradiol-17 β	E ₂ 17 β
Estradiol-17 α	E ₂ 17 α
Estrone-3-sulphate	E ₁ 3S
Estrone-3-glucosiduronate	E ₁ 3G
Estradiol-17 β -3-sulphate	E ₂ 3S
Estradiol-17 β -3-glucosiduronate	E ₂ 3G
Estradiol-17 β -17-glucosiduronate	E ₂ 17G
Estradiol-17 β -3-sulphate-17-glucosiduronate	E ₂ 3S17G

GENERAL INTRODUCTION

Estrone and 16-epiestriol have been isolated from laying hens' urine and characterized in crystalline form. Estradiol-17 β has also been isolated from hens' urine and characterized in the form of its crystalline 3-methyl ether. Estriol and 16,17-epiestriol have been obtained from hens' urine and identified with reasonable certainty by thin-layer chromatography (TLC).

Other experiments based on the identification of radioactive urinary conversion products of injected phenolic steroids (usually estradiol-17 β -¹⁴C or estrone-¹⁴C) have permitted the identification of the following products: estradiol-17 α , 16-ketoestradiol-17 β and 17-epiestriol. The results of such experiments have also suggested that estrone, estradiol-17 α and estradiol-17 β are quantitatively the most important urinary steroid estrogens in the hen.

While it has been known for some years that at least most of the phenolic steroids of the hens' urine are present in conjugated form, no single steroid estrogen conjugate has hitherto been isolated in crystalline form from hens' urine. Evidence based on tracer methodology has indicated the presence of an appreciable proportion of doubly-

conjugated material. It has also been shown by similar methodology that singly- and doubly-conjugated sulphate fractions are present and it has even been shown that injected estradiol-17 β -3, 17-disulphate can lead to the appearance of a small proportion of the injected material in the urine in the form of the 17-monosulphate.

The elaboration by Hobkirk and his co-workers in Montreal of a new column gradient chromatographic technique for the separation of the steroid estrogen conjugates opened the possibility of separating and characterizing individual radioactive steroid estrogen conjugates in urine of hens that had been given injections of steroid estrogens labelled with ^{14}C or ^3H .

The present thesis describes an attempt to resolve the conjugate fraction of the phenolic steroids of the hens' urine into individual conjugates by this newer technique. It was hoped thereby to

- (a) secure confirmation or otherwise of the results of previous workers,
- (b) to arrive at more definite identifications of individual conjugates, and
- (c) to resolve the problem of whether glucuronides are among the phenolic steroid conjugates secreted by the hen.

PART I

HISTORICAL REVIEW

CHAPTER I

STEROID ESTROGENS FOUND IN HENS' URINE

A. Introduction

The presence of estrone (Ainsworth and Common, 1962) and of 16-epiestriol (Hertelendy and Common, 1964) in urine of the laying hen have been established by isolation and characterization of the steroid in crystalline form. The presence of estradiol-17 β has been established by separation of an impure estradiol fraction from laying hens' urine, methylation of this fraction and subsequent separation, purification and characterization of the crystalline 3-methyl ether of estradiol-17 β (Hertelendy et al., 1965).

Estriol and 16,17-epiestriol fractions have been separated from laying hens' urine and after purification by microsublimation, these two steroids were identified by TLC of the free steroids and of appropriate derivatives (Mathur and Common, 1967). Although the two steroids were not obtained in crystalline form, the evidence thus obtained for their presence was very strong indeed.

Radioactive estradiol-17 α appears in the urine of both laying and non-laying hens after the birds have been

given intramuscular injections of ^{14}C -labelled estrone or estradiol-17 β (Mulay and Common, 1967; Mulay and Common, 1968). Since the hen is known to produce estrone and estradiol-17 β endogenously, it may be presumed that this endogenous estrone and estradiol-17 β can also give rise to urinary estradiol-17 α and that estradiol-17 α is a normal urinary steroid estrogen in this species.

An earlier report (Ainsworth et al., 1962) of the presence of 16-ketoestrone as a urinary metabolite of injected estrone- ^{14}C has been shown to be erroneous (Mulay and Common, 1967; Mulay and Common, 1968). The fraction identified as 16-ketoestrone consists mainly (over 90%) of estradiol-17 α .

The urinary metabolites of injected ^{14}C -labelled estrone or estradiol-17 β which have been identified include 16-ketoestradiol-17 β and 17-epiestriol (Ainsworth et al., 1962; Hertelendy and Common, 1965), which may therefore be included among the phenolic steroids hitherto identified in hens' urine. The list may be summarized as follows (Table 1).

Table 1. Steroid estrogens hitherto identified in hens' urine

A. Identifications based on isolation as the crystalline phenolic steroid

estrone
16-epiestriol
estradiol-17 β

B. Identifications based on isolation and identification by TLC

estriol
16-epiestriol

C. Identifications as radioactive conversion products of injected radioactive estrone or estradiol-17 β

estradiol-17 α
16-ketoestradiol-17 β
17-epiestriol

B. Quantitative Aspects of Steroid Estrogens Excreted in the Hen

Information on the quantitative excretion of steroid estrogens in the urine of the fowl is still very limited. A series of studies conducted at Macdonald College (Common et al., 1965; Mathur et al., 1966; and Mathur and Common, 1966) has furnished average values for urinary estrone (corrected for methodological loss) for various non-laying hens that range from 0.9 micrograms (mg)/24 hr to 2.0 mg/24 hr

and from 1.2 mg/24 hr to 6.1 mg/24 hr for laying hens. In several of these studies, estrone excretion was followed for a single bird for some time, and it was found that estrone excretion rose from a relatively low level before laying to attain peak values sometime in the week preceding laying of the first egg (in one case on the day of laying of the first egg) and then declined slowly as laying continued. In birds which went out of lay, the value declined sharply to low non-laying levels. Only a few data were obtained for excretion of estradiol-17 β (Mathur and Common, 1969) but these suggest that the non-laying hen excretes more estradiol-17 β than estrone, whereas the laying bird excretes roughly similar amounts of estrone and estradiol-17 β .

More recently, Tang, Huston and Edwards (1970) have reported data for 24-hour urinary excretion of estrone and estriol, which they measured by a procedure based on gas liquid chromatography (GLC) instead of the fluorimetric Ittrich-Kober procedure used by the group at Macdonald College. Tang et al. (1970) obtained values for estrone which are roughly an order of magnitude greater than those reported by the Macdonald College group. Their values for estriol were about one fifth to one quarter of their values

for estrone. They reported that estradiol-17 β and 16-epiestriol were present in amounts too low for satisfactory assay. These workers have suggested that the wide discrepancies between their quantitative data and those obtained by the Macdonald College group may be due to the presence in hens' urine of inhibitors of the Ittrich-Köber procedure.

The foregoing observations have been supplemented by studies of the proportions in which identifiable metabolites of injected ^{14}C -labelled estrone or estradiol-17 β are found in the urine of the laying or non-laying hen. The first study of this kind (Ainsworth et al., 1962) showed that the major metabolites of injected ^{14}C -estrone recovered from the urine of the laying hen were estrone, estradiol-17 β and an acid-labile phenolic steroid erroneously identified as 16-ketoestrone but subsequently shown to be estradiol-17 α (Mulay et al., 1968).

Similar later studies (Common et al., 1969), in which either estrone-4- ^{14}C or estradiol-17 β -4- ^{14}C was injected into both laying and non-laying hens have in general confirmed that the major radioactive phenolic steroids obtained from the urine after such injections are estrone, estradiol-17 β and estradiol-17 α . The main results of these

studies are summarized in Table 2.

Table 2. Distribution patterns of the radioactive phenolic urinary conversion products of injected radioactive steroid estrogens in the non-laying and laying hen. Values expressed as percentages of the total radioactivity of the phenolic steroid fraction recovered from the urine (Common et al., 1969)

In urine	Non-laying	Laying
<u>Estradiol-17β-4-¹⁴C injected</u>		
Estrone	16.8	37.0
Estradiol-17 α	7.9	20.8
Estradiol-17 β	62.0	24.7
Other phenolic steroids	13.7	17.5
<u>Estrone-4-¹⁴C injected</u>		
Estrone	10.4	26.1
Estradiol-17 α	6.9	17.9
Estradiol-17 β	49.0	17.9
Other phenolic steroids	33.7	36.0

It will be seen that whether estrone-4-¹⁴C or estradiol-17 β -4-¹⁴C was injected, the most abundant radioactive urinary conversion product in the non-laying hens' urine was estradiol-17 β , followed by estrone and estradiol-17 α in that order; and in the laying hens' urine the most abundant urinary conversion product was estrone, with somewhat

smaller amounts of estradiol-17 β and of estradiol-17 α .

These results confirmed the finding of a higher ratio of estradiol-17 β to estrone in the urine of the non-laying bird than in the urine of the laying bird in the experiments based on the Ittrich-Köber determinations of these two steroids in hens' urine (Common et al., 1969).

C. State of Combination of Steroid
Estrogens in Hens' Urine

Investigations of this subject have been based entirely on tracer methodology. Early studies (Mulay, 1966) showed that the urine of hens which had received injections of estrone-¹⁴C or estradiol-17 β -¹⁴C contained most of its radioactivity in forms not extractable by chloroform or ethyl acetate. This naturally presented the problem of identification of the steroid conjugates present, and it seemed reasonable to suspect the presence of glucuronides or sulphates. It was known (Ainsworth et al., 1962) that 'Glusulase', a molluscan preparation containing both β -glucuronidase and sulphatase, liberates a high proportion of the urinary phenolic steroids into forms extractable by chloroform.

Mulay (1966) reviewed earlier observations on the

state of combination of the phenolic steroids in the urine of the hen. She also provided a brief review of the literature on the mechanisms of steroid conjugation in certain mammalian and other species. In the same thesis Mulay (1966) presented the results of her pioneer studies on the state of combination of steroid estrogens in hens' urine. Her experimental procedure was based on the intramuscular injection of estradiol- 17β -4- ^{14}C or estrone-4- ^{14}C into hens and the examination of the urine subsequently excreted by solvent partitions in combination with successive treatments by incubation with β -glucuronidase, solvolysis and acid hydrolysis. The results have also been published in summarized form (Mulay et al., 1968). In these experiments an average of 12.6% of the urinary radioactivity was present in forms extractable by chloroform, i.e. as 'free' steroid, and only a further 3.5% was removed by subsequent extractions with ethyl acetate. The residual urinary material was then incubated with β -glucuronidase and subsequently extracted successively with chloroform and with ethyl acetate as before. Three successive treatments of this kind were applied. In all, only 12.5% of the radioactivity was obtained in forms extractable by chloroform; whereas 35.5% had been altered to forms extractable by

ethyl acetate though not by chloroform. This observation which provided the first evidence for the existence of an appreciable proportion of doubly-conjugated material in the urinary conversion products of steroid estrogen in the fowl; for material extractable by ethyl acetate though not by chloroform could be presumed to represent singly-conjugated material. Subsequent solvolysis yielded a further 6.2% of radioactivity extracted by ethyl acetate, and another 4.8% was recovered by ether extraction after acid hydrolysis. The final aqueous residue accounted for 2.1% of the radioactivity. The recovery in the various fractions accounted for 77% of the total urinary radioactivity, a recovery that could be considered satisfactory for experiments of this type.

Mulay et al. (1968) also showed by appropriate TLC, that the 'free' steroid fraction contained radioactive estradiol-17 β , estradiol-17 α (erroneously equated mainly with 16-ketoestrone), estrone and a cis-estriol fraction, all in proportions which were in agreement with those previously reported by Ainsworth and Common (1962). An essentially similar distribution obtained for the 'free' estrogen fraction liberated by the incubation with β -glucuronidase save for an appreciably higher proportion of highly

polar material. By contrast, 95.4% of the material extractable by ethyl acetate, subsequent to the incubation and extraction with chloroform, consisted of highly polar material. This latter observation gave further support to the identification of this material as being singly-conjugated. Mulay et al. (1968), were even able, by appropriate TLC against reference singly-conjugated and doubly-conjugated materials, to obtain evidence that at least four singly-conjugated materials were present in this fraction. The principal criticism which may be levelled against the work of Mulay et al. (1968), is that it initially took insufficient account of the possibility that 'free' steroid and singly-conjugated material liberated by the incubation with β -glucuronidase might result from spontaneous hydrolysis of sulphates at the pH reaction (pH 4.5) used for incubation. In fact, the authors were able to show that comparable blank incubations with the enzyme omitted led to the conversion of an appreciable proportion of the originally conjugated material into forms extractable by ethyl acetate though not by chloroform.

On the basis that the fowl is known to excrete benzoic acid in conjugation with ornithine as dibenzoylornithine (Jaffe, 1877), Mathur (1969) injected concurrently estrone-

6,7-³H and ornithine-5-¹⁴C into a hen. Investigation of the urine subsequently excreted for the possible presence of both ¹⁴C and ³H in the conjugated steroid fraction showed that the ¹⁴C was not accompanied by ³H and hence that none of the urinary ¹⁴C conjugated fraction contained ornithine. Mathur was careful, however, to point out that this negative result did not completely exclude the possibility that the fowl may conjugate phenolic steroids with ornithine. Such conjugates might be excreted by the biliary route. Again, the conjugation of estrone and ornithine may require an active form of ornithine. In order to ensure labelling of any such hypothetical active form it would be necessary to ensure that the entire body pool of ornithine in the hen's body was labelled.

Mathur et al. (1969), realizing the possible significance of sulphates, injected estradiol-17 β -4-¹⁴C into non-laying hens and then, by appropriate chromatography, separated from the urinary conversion products a mono-conjugate fraction and a diconjugate fraction. Both conjugate fractions proved to contain a high proportion of sulphuric acid ester. The proportion of glucuronide in either fraction was low, and evidence was obtained that an unexpectedly high proportion of phenolic steroids of the

urine was present as either monosulphate or disulphate. In fact, the results suggested that the fowl may excrete a higher proportion of its urinary phenolic steroids than does any mammalian species for which comparable information was available. Mathur et al. (1969) were also able to show that the ratio of monosulphate to disulphate in the urine was about 6:1. Solvolysis of monosulphate and di-conjugate fractions and subsequent chromatography of the liberated steroids, gave in two experiments the results shown in Table 3.

Table 3. Relative proportions of radioactivity in major phenolic steroids in monosulphate and disulphate fractions from hens' urine after injection of estradiol-17 β -4-¹⁴C (Mathur et al., 1969)

Steroid	Monosulphate fraction		Disulphate fraction	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Estriol*	0.8	0.9	1.2	1.0
16- <u>Epi</u> estriol**	4.3	5.2	6.1	5.5
Estradiol-17 β	62.6	41.4	72.8	33.9
Estradiol-17 α	7.9	39.8	9.0	51.7
Estrone	16.8	6.2	0.0	0.0

*May have contained some 16,17-epiestriol

**May have contained some 17-epiestriol

As was to be expected from the circumstance that

these experiments were restricted to non-laying hens, relatively large proportions of the radioactivity were present as estradiol-17 β .

The conclusions from the foregoing experiments may be summarized as follows:

- (a) the hen excretes most of its urinary steroid estrogen in conjugated forms,
- (b) the conjugated forms include both monoconjugate and diconjugate fractions with the former predominating, and
- (c) the monoconjugate and diconjugate fractions respectively contained mono- and disulphates of estradiols as their main constituents.

D. The Separation of Steroid Conjugates by Elution from Amberlite XAD-2 Resin Columns by Methanol, Followed by Elution from DEAE-Sephadex A-25 Columns by a Sodium Chloride Gradient

The quantitative and efficient separation of free and conjugated steroid fractions by resin chromatography was elaborated by Bradlow (1968). Bradlow used a neutral cross linked polystyrene polymer, Amberlite XAD-2, to effect separation of free and conjugated steroids from urine at a level of 90% recovery, using synthetic conjugates at levels

up to 250 mg of steroid conjugates per litre of urine. Amberlite XAD-2 adsorbs the conjugates of steroid metabolites from urine which may subsequently be easily eluted from the resin with methanol or ethanol. It was demonstrated that the greater part of the steroid conjugates was eluted in the first volume of solvent. The Amberlite XAD-2 column also effects an appreciable level of purification, as it also removes from urine percolated through it, pigments and other undesirable extraneous materials which are held back on the resin.

Evaporation of the methanolic eluate from the Amberlite XAD-2 column to dryness yielded a residue which contained the conjugates of steroid metabolites from the urine. This residue was taken up in 5 ml water and transferred to DEAE-Sephadex A-25 columns along with further washings of 2 x 2.5 ml water. DEAE-Sephadex A-25 is a weakly basic anion exchanger prepared by incorporating diethylaminoethyl groups into a dextran obtained from a bacterial source. This ion exchanger combines the advantages of resin-based exchangers with those of cellulose based exchangers. It can be prepared to a high standard of purity. DEAE-Sephadex is strongly hydrophilic and is available in the form of small spherical beads, 40-120 μ in diameter, thus ensuring

good flow rate through column beds. The notation 'A-25' denotes the porosity type, the porosity being a function of the degree of cross-linkage in the gel. The 'A-25' type, which is highly cross-linked, has a lower porosity than the moderately cross-linked 50 types. The porosity of a gel determines the extent to which solutes of a certain molecular size are able to diffuse into the granules and consequently the available capacity for these molecules.

The application of Sephadex ion exchangers to separate conjugated urinary estrogens was pioneered by Hähnel (1965); Hähnel and Ghazali (1967). Hähnel had originally used columns packed with an anion-exchange resin "D acidite FF", and eluted with a buffer-ethanol mixture (Hähnel, 1962, 1963). He obtained effluents containing significantly less nonestrogenic chromogens than the original urine and the main portion of the estrogens in a conjugated form. Hähnel (1965) then explored the possible applicability of DEAE-Sephadex A-25 to the separation of steroid estrogen conjugates. Hähnel's procedure was elaborate and time-consuming. Hobkirk et al. (1969a) later developed an elegant and efficient application of DEAE-Sephadex A-25 to the separation of conjugated urinary estrogens. In Hobkirk's method the individual conjugates are eluted from the exchanger column

by applying a simple gradient elution with sodium chloride.
The methodology of Hobkirk's gradient system is given in
the section on materials and methods of this thesis.

CHAPTER II

OCCURRENCE OF STEROID ESTROGEN CONJUGATES IN VERTEBRATE SPECIES OTHER THAN AVIAN SPECIES

A. Human

The presence of estrogen conjugates in human blood, particularly in the pregnant state, was first established by Mühlbock (1939). Cohen and Marrian (1936) reported the isolation of an estriol glucosiduronate, which they suggested was coupled in the 16 or 17 position, from human pregnancy urine. It was not until many years later that Carpenter and Kellie (1961, 1962) showed that the estriol glucosiduronate fraction from human pregnancy urine contained estriol-16 α -glucosiduronate and estriol-17 β -glucosiduronate. Beling (1961, 1962) identified estriol-3-glucosiduronate in human pregnancy urine. Following intraamniotic administration or perfusion of estradiol-16-¹⁴C and estriol-16-¹⁴C in foetal in vivo studies, Diczfalusy et al. (1961a, 1961b) identified radioactive estradiol-3-sulphate and radioactive estrone sulphate in the lung, liver and kidney. Diczfalusy et al. (1962) investigated the formation of estrone glucosiduronate in an isolated jejunum loop but found very little estrone or estradiol sulphate. Levitz et al. (1960) injected estrone-¹⁴C

into a mother and thereafter found both estrone sulphate and estradiol sulphate in the umbilical circulation of the foetus. Purdy et al. (1961) administered estradiol-¹⁴C to women and then identified estrone sulphate in extracts of their plasmas, including late pregnancy plasma. These workers found that the molar concentration of estrone sulphate in pooled pregnancy plasma was about four times that of free estrone. Consequently, Purdy et al. (1961) concluded that estrone sulphate is a notable circulating form of estrogens in human. Purdy's (1961) conclusions were supported by studies of pregnancy urine by other workers (McKenna et al., 1961). Evidence for the occurrence of estrone sulphate in non-pregnant urine remained inconclusive until positive proof was given by Hobkirk et al. (1969b).

The literature on steroid estrogen conjugates is the subject of a monograph by Bernstein, Cantrall, Dusza and Joseph (1968).

B. Primates Other than the Human Species

Research on steroid estrogen conjugation in primates other than the human has been restricted to a few studies on the monkey, the testes of which have been shown to form

estrone-3-sulphate from estrone (Holcenberg and Rosen, 1964).

C. Dog

In vivo studies on dog liver microsomes incubated with estradiol produced estradiol-17-glucosiduronate (Breuer and Wessendorf, 1966).

Collins et al. (1970) recently studied in vivo the metabolism of arterial plasma estrogens by the splanchnic organs of the dog. Estrone-6,7-³H was infused constantly into dogs and arteriovenous differences at equilibrium were determined for estrone-6,7-³H, estradiol-17 β , estrone sulphate, estrone glucosiduronate and estradiol-17 β glucosiduronate across the splanchnic bed (artery-hepatic vein), the small intestine (artery-superior mesenteric vein) and the spleen (artery-splenic vein). The mean plasma metabolic clearance rate for estrone was found to be 731 liters/day per m² (standard error of mean-SEM=50). These values, in comparison, are much lower than those reported respectively by Longcope et al. (1968) and Hembree et al. (1969) which were 1310 liters/day per m² (SEM 80) and 1168 liters/day per m² (SEM 95).

The mean per cent extractions were found to be as follows:

(a) splanchnic bed:

estrone	85.9 (SEM 1.92)
estradiol-17 β	88.11 (SEM 3.36)
estrone sulphate	27.9 (SEM 5.22)
estrone glucosiduronate	-48.5 (SEM 9.33)
estradiol-17 β	
glucosiduronate	-33.3 (SEM 8.03)

(b) small intestine:

estrone	45.3 (SEM 2.60)
estradiol-17 β	46.1 (SEM 12.9)
estrone glucosiduronate	-30.8 (SEM 7.9)

(c) spleen:

estrone	35 (SEM 3.8)
estrone glucosiduronate	12 (SEM 3.7)

Collins et al. (1970) from their results, concluded that the estrone and estradiol-17 β were nearly completely extracted in a single passage through the splanchnic bed. The splanchnic bed showed a net uptake of estrone sulphate and a net production of estrone glucosiduronate and of estradiol-17 β glucosiduronate. The intestine exhibited a net uptake of estrone and estradiol-17 β which Collins et al. (1970) associated with the significant net production of estrone glucosiduronate. In the case of the spleen, although

there was a small but significant uptake of estrone glucosiduronate, there was a definite net uptake of estrone.

Studies involving the technique of constant infusion of radioactive estrone and estradiol-17 β presented difficulties in obtaining a steady state of both precursor and of products (Hembree et al., 1969).

Consequently, studies based on constant infusion applications produce meaningful yet relative results within the limits of existing present experimental parameters.

D. Rabbit

Microsome-free rabbit liver homogenates, with ATP added can convert estrogens into sulphates (Schneider and Lewbart, 1956; and De Meio et al., 1958). Layne et al. (1964) and Layne (1965), in radioactive tracer studies found that the rabbit is capable of conjugating estradiol-17 α with N-acetyl glucosamine. Layne et al. (1964) and Layne (1965) demonstrated the presence of estradiol-3-glucuronide-17 α -N-acetyl glucosaminide in rabbit's urine. Conjugation of the N-acetyl glucosamine occurs only at the 17 α and never at the 17 β position, and this conjugation follows the prior conjugation with glucuronic acid at the C-3 position. More recently, Williamson et al. (1969) have reported the presence

of estradiol-17 α - β -glucoside in rabbit's urine.

E. Ruminants

A check of the literature failed to elicit information on the occurrence of steroid estrogen conjugates in the sheep and goat. In the case of the cow, a species in which a considerable proportion of steroid estrogen is excreted via the feces, the presence of combined forms of steroid estrogen in the feces has been reported by Pearson and Martin (1966). Pearson and Martin (1966), following the injection of radioactive estradiol-17 β into bulls, found estrone glucuronide and estradiol-17 α glucuronide to be the major biliary metabolites. In 1963 Sneddon and Marrian demonstrated in vivo that bovine adrenal tissue can convert estrone to estrone sulphate.

F. Rat

Estrogen steroid conjugates of sulphates and glucuronides have been shown, either by in vivo or in vitro studies, to be present in the rat (Felger, 1961; and Sjoval1 et al., 1968).

G. Other Species

Schachter and Marrian (1938) isolated and identified

estrone sulphate from pregnant mare's urine. Estrogen conjugates have been shown to be present in equine blood (Mühlbock, 1937). Recently, Raeside (1969) isolated estrone sulphate and estradiol-17 β sulphate from stallion testes.

The greater part of contemporary research on estrogen conjugates is concerned with enzymatic in vitro systems. Valuable tabulations of the main results of such work up until about 1967 have been furnished in the text (Chapters II and III) by Hadd and Blickenstaff (1969). It may be well to mention, however, that the results of in vitro experiments do not provide a reliable guide to the types of conjugates excreted in the urine, a point emphasized in a recent paper by Quamme et al. (1971).

CHAPTER III

MECHANISMS OF CONJUGATION

A. Introduction

Sterols, bile acids and steroid hormones and their metabolites constitute one group of naturally-occurring endogenous compounds which has been investigated to provide basic information on metabolic conjugation. The processes of conjugation and detoxication have also been assessed by studying the fate of foreign organic compounds in the body. The main conjugation reactions which occur in laboratory animals are those involving glucosiduronic acid, sulphate, glycine, cysteine (i.e. mercapturic acid synthesis), methylation and acetylation. A few other conjugations occur in other species (Table 4). Glucosiduronic acid conjugation is considered to be of prime importance because it occurs extensively in man and all laboratory animals except the cat. Some glucosiduronic acid conjugation does occur in the cat, but at a relatively low level as compared with other mammalian species.

Conjugations have, in general, been regarded as

detoxication mechanisms, on the ground that compounds which undergo these reactions are converted into products which are usually less toxic and more rapidly excreted than their precursors. Conjugation relative to detoxication or an increase in excreatability, usually follows oxidation, reduction or hydrolysis of foreign molecules, although some compounds are conjugated without previous alteration. Glucosiduronic acid conjugates involve an ester or glycosidal linkage with glucosiduronic acid, e.g. with a carboxyl or hydroxyl group of the foreign compound. The body readily produces glucosiduronic acid from glucose. Various compounds with phenolic hydroxyl groups are conjugated with sulphate in the animal body to yield sulphuric acid esters. The ethereal sulphate conjugates thus formed are excreted in the urine as salts. Peptide type linkages are involved in conjugates of cholic acid with glycine and taurine. Acetylations and methylations occur in lesser degree than conjugations with glucosiduronic acid or with sulphuric acid.

B. Conjugation with:

1. Glucosiduronic Acid

The common occurrence of glucosiduronic acid conjugation

may be associated in part with the ease with which the body produces glucosiduronic acid from carbohydrate sources and the variety of chemical groups to which glucosiduronic acid can be transferred enzymatically. The limited availability of conjugating agents, such as glycine and cysteine, (via glutathione) and the small number of chemical groupings to which conjugating agents such as sulphates, glycine, cysteine, methyl and acetyl can be transferred, may explain the restricted occurrence of other conjugation mechanisms. In addition, glucosiduronic acid may be attached to any position on various steroids, as reviewed by Jayle and Pasqualini (1966).

Specific cellular sites of conjugation have been observed. Thus, UDP-glucuronyl transferase, which effects the catalytic formation of testosterone 17 β -glucosiduronate, has been located in the microsomal fraction of human intestine (Dahm et al., 1966).

Enzyme specificity to effect conjugation at specific sites on the steroid molecule has also been demonstrated in the glucuronidation of estriol at 16 α and also at the 17 β positions (Dahm and Breuer, 1966a, 1966b). Indications from double conjugates of glucosiduronic acid support the idea that not only may specific enzymes be involved with

specific positions on the steroid nucleus, but also that another enzyme may effect glucuronidation of the 3C phenolic hydroxyl (Slaunwhite et al., 1964; Dahm and Breuer, 1966b).

Diglucosiduronates of 17 β -estradiol (Breuer and Wessendorf, 1966) and of estriol and 17-epiestriol (Collins et al., 1968) are reported to be formed in small amounts by rabbit liver in vitro.

Double conjugates with glucosiduronic acid in one position and a different conjugating group at another position are known (e.g. estriol-3-sulphate 16(17)-glucosiduronate (Straw et al., 1955; Levitz et al., 1965).

Conjugation of a substance with glucosiduronic acid produces a strongly acidic compound which is more water-soluble at physiological pH values than the precursor. The pK_a values (apparent dissociation constants) for about fifteen glucuronides have been determined and are in the range of pK_a 3.0 - 4.0. Although the solubilities of the alkali salts of glucuronides have not been measured, practical experience shows that they are highly water-soluble with the exception of the sodium salt of stilbestrol monoglucuronide, which is sparingly soluble in water. These properties of relatively high acidity and high water

solubility in the physiological environment, support the concept that glucuronide formation is a detoxication mechanism.

2. Sulphuric Acid

As is the case for most other conjugations, recent investigations have raised some doubt as to the liver being the major site of steroid sulphate formation.

Estrone sulphate has been synthesized in vitro by bovine adrenal tissue by Sneddon and Marrian (1963), while Wallace and Silberman (1964) have demonstrated the ability of the ovary to synthesize steroid-3-monosulphates. The sulphation of dehydroepiandrosterone and its secretion by the adrenal has been reviewed by Van de Wiele et al. (1963). Other endocrine tissues effect sulphation, e.g. testis, placenta and corpus luteum (Payne and Mason, 1965). Wang and Bulbrook (1968) tabulated steroids sulphated by various animal tissues. As far as is presently known, steroid sulphates appear to be synthesized in active endocrine tissue to a greater relative degree than are glucuronides.

Several steroid disulphates are known and have been catalogued by Bernstein et al. (1968), but only a few have been isolated from natural sources.

Of comparative significance is the work by Mathur

et al. (1969). These workers injected estradiol-17 β -4-¹⁴C intramuscularly into hens and showed that the radioactive monoconjugate and diconjugate fractions subsequently excreted in the urine both contained a high proportion of sulphate and that the main constituents of the diconjugate fraction were disulphates of estradiols.

The chief bile salt of the hagfish, a primitive chordate, is the disulphate of myxinol (Haslewood, 1965). This observation lends support to the idea that sulphation of steroids may be the most primitive mechanism of detoxication of steroids.

3. N-acetyl Glucosamine

The discovery that steroid N-acetylglucosamides occur in rabbits' urine (Layne et al., 1964; Layne, 1965) represented the discovery of a novel type of metabolic conjugate, for although N-acetylglucosamine transfer was known to occur in animal tissues in the biosynthesis of polysaccharidic and nucleotidic material, it had not previously been observed in biosynthesis of small molecules. Steroid N-acetylglucosamides have now been found in the human (Arcos and Lieberman, 1967) and in human bile (Jirku and Levitz, 1969). Structurally, N-acetylglucosamine is attached to a ring D hydroxyl or on the 17 side chain, with glucuronic or sulphuric acid

attached to the 3C hydroxyl of the steroid nucleus. All steroid N-acetylglucosaminides isolated to date from animal sources have proved to be double conjugates.

4. D-glucose

Uridine diphosphate (UDP) glucose is an important precursor for UDP glucosiduronic acid and UDP-iduronic acid and as such is involved in conjugation of glucosiduronic acid. However, recent work by Collins et al. (1968) has demonstrated the direct conjugation of UDP-glucose to the steroid nucleus in rabbit liver tissue. UDP-glucose, like UDP-N-acetyl glucosamine was found to form a double conjugate of 17 α -estradiol. β -Glucuronidase ('Ketodase'), which is specific for cleavage of the β -linkage of glucosiduronic acid, was used to remove glucosiduronic acid from the 3 position, yielding the 17-monoconjugate.

C. Other Conjugations

1. Acetylations

Acetylation in general is confined to some amino hydrazino groups. Acetylation may be regarded as a detoxication mechanism utilized by the body to remove amines and drugs containing amino groups, such as sulphanilamide,

and is also used in the course of metabolic formation of physiological compounds such as acetylcholine and acetylhexosamines. Only recently has evidence been presented in support of metabolic acetylation of steroids in vivo (Weichselbaum and Margraf, 1960; Margraf et al., 1963).

These are the corticosteroid acetates present in human blood. King et al. (1964) reported a small production of acetylated testosterone by animal tissue in vivo. Grosser and Axelrod (1967, 1968) have demonstrated the formation of the C21-acetates of cortisol and of cortisone in rats and in fetal and newborn baboons.

2. Phosphorylation

Synthetic steroid phosphates have been used therapeutically. They are converted in vivo to the parent steroids. The high inhibitory effect of estrogen phosphates compared to that of sulphates as inhibitors of the reconstitution of kynurenine-amino transferase and aspartate-amino transferase has been demonstrated. Concrete evidence as to the presence of steroid phosphokinases is lacking, as is also evidence for their natural occurrence.

3. Methylation

Methylation of steroids in animals was proven with

the isolation and characterization of 2-methoxyestrone (Kraychy and Gallagher, 1957). Other 2-methoxy derivatives, such as those of 17 β -estradiol and estriol have also been found in man (Frandsen, 1959; Fishman and Gallagher, 1958). Methylation of steroids proceeds by way of the corresponding catechols or 2-hydroxy-estrogens. The methylation reaction requires oxygen-methyl transferase and S-adenosylmethionine as the methyl group donor (Breuer et al., 1961; King, 1961b).

4. Glutathione Conjugation

Radioactive tracer work with rat liver homogenates has demonstrated formation of water-soluble conjugates of 2-hydroxyestrone and of 2-hydroxyestradiol, each joined by a thio-ether linkage at either the C-4 or C-1 of the steroid to the cysteine moiety of glutathione (Kuss, 1968, 1969).

5. Amino Acids

Glycocholic and taurocholic acids are known conjugates of cholic acid with glycine and taurine, respectively. Because these conjugations require taurocholic acid and a carboxylic acid group in the steroid, occurrence among steroids would be limited, and to date no definitive proof

of such steroid conjugates has been forthcoming.

6. Other Steroid Conjugates

An androgen conjugate with urea has been reported by Gallagher et al. (1966); an estrogen glucoside has been found in the rabbit by Williamson et al. (1969).

Table 4. The commoner conjugation reactions*

Mechanism	Conjugating agent
<u>Occurring widely in species</u>	
Glucuronide synthesis	Glucuronic acid
Ethereal sulfate synthesis	Sulfate
Hippuric acid synthesis	Glycine
Mercapturic acid synthesis	N-Acetylcysteine
Methylation	Methyl group
Acetylation	Acetyl group
Thiocyanate synthesis	Thio group
<u>Of limited occurrence</u>	
Ornithuric acid synthesis (certain birds and reptiles)	Ornithine
Glutamine conjugation (man and higher apes)	Glutamine
Glucoside synthesis (insects)	Glucose

*From the monograph (Chapter 7) edited by Dutton (1966).

PART II
EXPERIMENTAL

CHAPTER I

MATERIALS AND METHODS

A. Reference Standards

Crystalline estrone, estradiol-17 α and estradiol-17 β were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A..

Crystalline estradiol-17 β -D-glucuronide was obtained from Calbiochem, Los Angeles, California, U.S.A..

Estriol was obtained from Steraloids, New Jersey, U.S.A..

B. Solvents

Methanol (spectraanalyzed grade), chloroform (A.R. grade), isopropanol (Fisher's certified grade, boiling range 82.2 - 82.5°C) and benzene (thiophene-free) were obtained from Fisher Scientific Company, Montreal, Quebec, Canada.

Ethyl acetate, cyclohexane, formic acid and propylene glycol (laboratory grade, boiling range 186 - 188°C) were obtained from British Drug Houses Ltd., Toronto, Ontario, Canada.

With the exception of methanol, all above mentioned solvents were redistilled just before use.

Absolute ethanol was obtained from Consolidated Alcohols Ltd., Montreal, Quebec, Canada. This alcohol was purified by refluxing with 5 g NaOH pellets and 5 g Zn metal for each 100 ml for 12 hours and then distilling and re-distilling.

Diethyl ether U.S.P. was obtained from Fisher Scientific Company, Montreal, Quebec, Canada. Immediately before use, diethyl ether was purified according to the procedure of Bauld (1956).

C. Chemicals

Saccharo-1,4-lactone was obtained from Calbiochem, Los Angeles, California, U.S.A.

All chemicals used were of analytical reagent grade unless stated otherwise.

Medical X-ray film, Kodak liquid X-ray developer and Kodak X-ray fixer were obtained from Picker X-ray Engineering Ltd., Montreal, Quebec, Canada.

D. Radiochemicals and Materials For Counting

Estrone-4-¹⁴C-3-glucosiduronate, 17 β -estradiol-4-

^{14}C -3-glucosiduronate, estrone-6,7- ^3H -3-sulphate, 17β -estradiol-6,7- ^3H -17-glucosiduronate and 17β -estradiol-6,7- ^3H -3-sulphate-17-glucosiduronate were kindly donated by Dr. R. Hobkirk, Department of Experimental Medicine, McGill University, Montreal, Quebec, Canada.

Estrone-4- ^{14}C (53.4 mc/mM), 0.01 mc in 10% ethanol in benzene solution and estradiol- 17β -4- ^{14}C (51.4 mc/mM), 0.01 mc in 90:10 benzene:ethanol solution were obtained from Radiochemical Centre, Amersham, England.

Estrone-4- ^{14}C (45.2 mc/mM), 0.01 mc in 10% ethanol in benzene solution and estradiol-6,7- ^3H (N)- 17β -D-glucuronide (1.0 c/mM), 0.25 mc in ethanol were obtained from New England Nuclear Corp., Boston, Mass., U.S.A.

^{14}C -Toluene and ^3H -toluene for internal standards, PPO (2,5-diphenyloxazole) and POPOP (1,4-bis-2,5-phenyloxazolyl benzene) were obtained from Packard Instrument Company, Downer's Grove, Illinois, U.S.A.

Toluene, scintillation grade, was obtained from Nuclear Enterprises, Winnipeg, Manitoba, Canada.

Scintillation mixture for counting radioactive samples in the Packard Tri-Carb Liquid Scintillation

Spectrometer Model No. 3365, consisted of 3.0 g PPO and 0.1 g POPOP per litre of scintillation grade toluene.

Cab-O-sil M-5 silica gel was obtained from Cabot Corporation, Boston, Mass., U.S.A..

E. Enzyme Preparations

'Ketodase', a commercial enzyme preparation, was obtained from Warner-Chillcott Company, Morris Plains, New Jersey, U.S.A.. Each ml of 'Ketodase' contains 5,000 Fishman Units (F.U.) of β -glucuronidase buffered in acetate to pH 5.

'Phenol sulfate; Aryl sulfatase, Type III' was obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.. This preparation is described by the suppliers as 'from limpets, contains low β -glucuronidase activity and 0.837 sulfatase units/mg phenol sulfatase'.

F. Colour Reagent

Folin and Ciocalteu phenol reagent, laboratory reagent grade, was obtained from British Drug Houses Ltd., Toronto, Ontario, Canada.

G. Column Chromatographic Equipment

Amberlite XAD-2 resin was purchased from Rohm and

Haas, Philadelphia, Pa., U.S.A.. It was soaked in H₂O and washed several times, suspended fines being removed, prior to use. XAD-2 resin was used to remove pigments and other extraneous materials from urine samples and to effect separation of free from conjugated steroids in urine samples. XAD-2 resin columns were also used to effect the recovery of conjugates from NaCl solutions following DEAE-Sephadex A-25 chromatography. Salt solutions after application to XAD-2 resin columns were washed with water and eluted with methanol as described by Bradlow (1968).

DEAE-Sephadex A-25, particle size 40-120 μ , and no. K 9/60 Sephadex columns were obtained from Pharmacia (Canada) Ltd., Montreal, Quebec, Canada. Before use, DEAE-Sephadex A-25 was steeped in water for at least 24 hours, fine particles being removed by suction. DEAE-Sephadex A-25 was added to Sephadex columns as a slurry in water and was allowed to settle under gravity to yield a gel column of dimensions 0.9 x 50 cm.

Pure 'free' steroids and steroid conjugates as well as steroid conjugates recovered through XAD-2 resin from urine samples, were applied in 5 ml water and washed into the column with a further 2 x 2.5 ml water. Linear NaCl gradients were applied from two identical 1,000 ml polyethylene bottles (obtained from Fisher Scientific Co.,

Montreal, Quebec, Canada) connected by polyethylene tubing as shown in Figure 1, pp 43a and 43b. The mixer vessel was provided with a magnetic stirrer.

When linear gradient A was to be applied, the mixer vessel was charged with 500 ml water and the reservoir vessel with 500 ml 0.8 M NaCl.

When linear gradient B was to be applied, the mixer vessel was charged with 500 ml 0.8 M NaCl and the reservoir vessel with 500 ml 2.0 M NaCl.

When linear gradient B was applied, it was always applied subsequent to application of linear gradient A.

H. Thin-layer Chromatographic Equipment

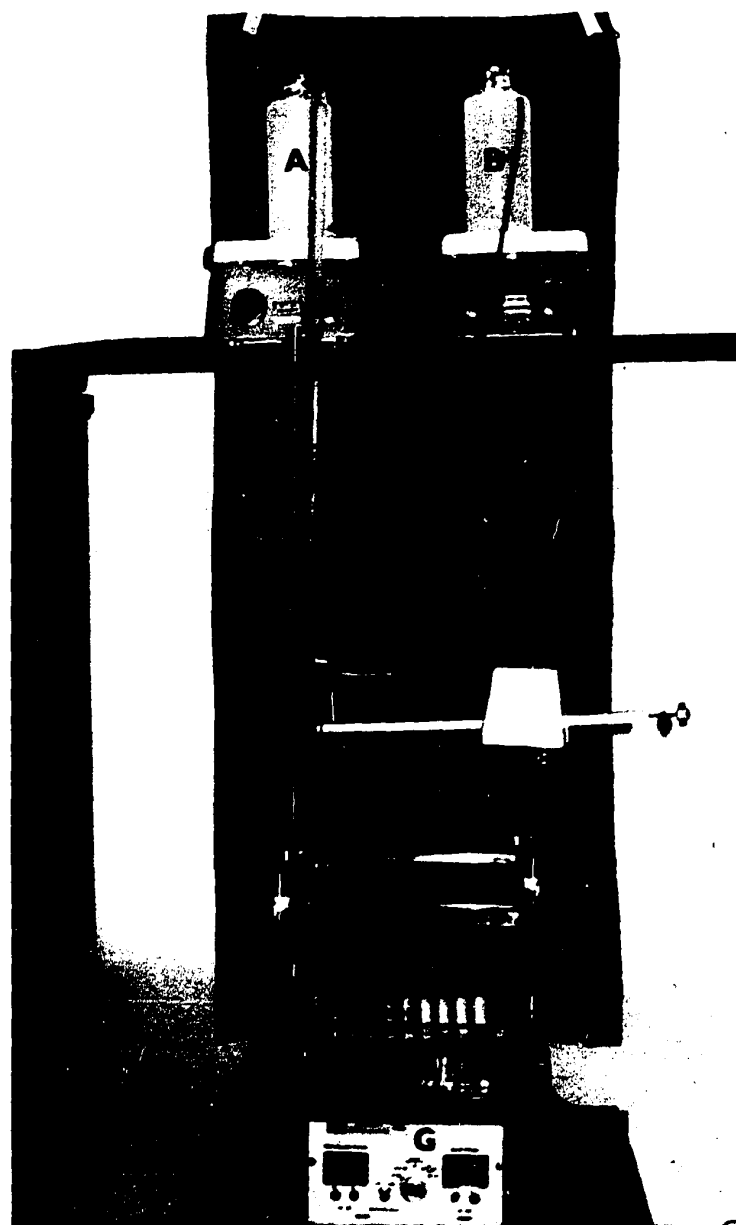
Silica gel G - Stahl (Merck, Darmstadt, West Germany) was obtained from Canlab, Montreal, Quebec, Canada. The Shandon thin-layer chromatography outfit was purchased from Consolidated Laboratories Ltd., Toronto, Ontario, Canada.

Figure 1. Photograph of LKB 7000 UltroRac fraction collector with NaCl gradient system and DEAE-Sephadex A-25 column.

Legend:

- A. Reservoir vessel
- B. Mixer vessel containing a magnetic stirrer
- C. Corning Model P.C. 351 Heater and Stirrer Unit
- D. Bent glass tubing with rubber tubing delivering into DEAE-Sephadex A-25 Column
- E. DEAE-Sephadex A-25 Column
- F. Siphon
- G. LKB 7000 UltroRac Fraction Collector

Figure 1



CHAPTER II

MEASUREMENT OF RADIOACTIVITIES USING A PACKARD TRI-CARB LIQUID SCINTILLATION SPECTROMETER MODEL NO. 3365

A. Calibration Curves

Three calibration curves were needed for the Packard Tri-Carb Liquid Scintillation Spectrometer, because the experimental procedures required the use of compounds which were labelled with ^{14}C or ^3H or both isotopes. Two standard solutions were prepared therefore, one using toluene- ^{14}C , one with tritiated toluene and one for samples containing both ^{14}C and ^3H .

1. ^{14}C Calibration Curve, Single-label

An aliquot (0.6 ml) of toluene- ^{14}C , which had a specific activity at 25°C of $4.25 \times 10^5 \pm 1\%$ disintegrations per minute (dpm) per ml, was transferred to a 250 ml volumetric flask. Scintillation mixture was added to yield 250 ml of standard solution. The mixture was thoroughly mixed. The total radioactivity in this 250 ml volume was 2.55×10^5 dpm.

Twelve 15 ml aliquots of this standard solution,

each equivalent to 1.53×10^4 dpm as ^{14}C , were transferred to counting vials. The samples were each counted six times in the liquid scintillation spectrometer, and the average for each vial was recorded. The counting data showed that the average activity per vial was 12,487 (S.D. = ± 38) counts per minute (cpm), except for one in which a count of 10,934 cpm was observed. This was probably due to loss incurred in the transfer of the aliquot to the vial. The vials were numbered from 1 to 12. Vials 1 and 2 were left unquenched, but to each of the remaining vials, in order, was added increasing amounts of pyridine. Vial 3 received 0.02 ml of dry pyridine, vial 12 received 0.20 ml. Vials 1 and 2 were used to determine the proper control settings that were needed to maximize the cpm for ^{14}C registered in the Green Channel, with the minimum amount of background noise. These settings (C = 50, D = 1,000, Gain 9.0% and a window width of C - D) gave a counting efficiency of 81.6%.

The vials to which pyridine had been added were shaken to mix the contents thoroughly. The complete set of twelve was again counted six times and the average value for each vial was recorded along with the average corresponding external standard count. The counting efficiency

for each vial was determined as follows. The average of the counts per minute after quenching was divided by the average counts per minute when unquenched and the result multiplied by 81.6%. A graph was prepared on two-cycle, semi-logarithmic paper on which the external standard counts were plotted on the logarithmic axis versus the counter efficiency.

2. ^3H Calibration Curve, Single-label

The calibration curve for single-label ^3H counting was obtained in the same way as that for ^{14}C . The 250 ml standard sample contained 4.80×10^5 dpm (0.25 ml aliquot of toluene - ^3H specific activity $1.92 \times 10^6 \pm 1\%$ dpm per ml). Each vial received a 15 ml aliquot of this mixture and thus contained 2.88×10^4 dpm of radioactivity. The spectrometer settings for ^3H (A = 50, B = 1,000, Gain 50%, window width A - B) gave a counting efficiency of 35.6%.

3. Double-label Counting

Two isotopes may be counted simultaneously, provided that the scintillations from one may be completely excluded from the other channel. The ^{14}C and ^3H spectra overlap slightly, the degree of overlap being somewhat enhanced by quenching. In general, quenching tends to

shift the distribution of the pulse energies toward the lower energy ranges. Thus, the quenching of samples containing ^{14}C and ^3H causes more counts from ^{14}C to spill over into the ^3H channel.

The control settings of the Red and Green Channels for dual label counting were determined with the aid of the standard unquenched samples of ^3H and ^{14}C . A standard ^3H sample was placed in the counter and the sample changer switch was set to 'repeat'. The settings of the Green Channel were adjusted until the efficiency for ^3H was reduced to an acceptably low level. The Green Channel settings for a ^3H efficiency of 0.014% were $C = 100$, $D = 1,000$, Gain 4.0%, window width $C - D$. These settings gave a ^{14}C efficiency of 50.0%.

The control settings of the Red Channel were determined with a ^{14}C standard sample in the counter. The settings were adjusted until a spill over of approximately 5% from the ^{14}C was obtained. With the following settings, $A = 50$, $B = 500$, Gain 80.0%, window width $A - B$; the Red Channel showed an efficiency of 5.05% for an unquenched sample containing ^{14}C . The ^{14}C efficiency increased to approximately 15% when strongly quenched samples were used. The above mentioned settings for the Red Channel gave a

^3H counting efficiency of 20.7%.

The complete series of quenched ^{14}C and ^3H standards was placed in the counter and counted in triplicate. The averages for the external standard registered in the Blue Channel were plotted against the average of the Red Channel counts and the average of the Green Channel counts in the same manner as for the single-label data. In addition, the efficiency with which ^{14}C was counted in the Red Channel was also plotted against the Blue Channel data. The Blue Channel settings for the external standard remained the same for both single- and double-label measurements, viz. $E = 350$, $F = 100$, $\text{Gain} = 2.0\%$ and window width $E - 00$.

B. Calculation of Sample Activity (dpm)
In Double-labelled Samples

1. Carbon-14

Each vial which contained ^{14}C was counted for the required time and then was counted for one minute with the external standard in the counting well. The cpm due to the external standard had previously been determined for vials containing only unquenched scintillation mixture. The ratio of the counts in the Blue Channel for the sample and the external standard to the counts for an unquenched

sample indicated the degree to which the sample was quenched. A calibration curve was prepared on which the efficiency was plotted against the Blue Channel counts at different degrees of quenching. Highly quenched samples absorb much of the light energy and thus the instrument counts at an apparent low efficiency for quenched samples. Thus, to determine the efficiency of the instrument when counting a sample of material which may be quenched, it is only necessary to record the count in the Blue Channel with the external standard in place and from those figures and the calibration curve the efficiency may be ascertained. The counts per minute datum for ^{14}C (Green Channel), corrected for background, was divided by the efficiency to obtain the dpm due to ^{14}C .

2. Tritium

The efficiency with which the ^{14}C in the vial was counted in the Red Channel was determined similarly from the standard curve for efficiency for the Red Channel. The dpm due to ^{14}C multiplied by this efficiency gave the number of counts per minute that had spilled over into the Red Channel.

The value for counts per minute in the Red Channel less background, less spill over from ^{14}C was divided by

the efficiency with which ^3H was counted in the Red Channel. This efficiency like that for ^{14}C , was found with the aid of the external standard count and the standard curve for ^3H .

C. Data Processing For Double-label Counting

$$1. \text{ Carbon-14 dpm} = (A - a) \div \alpha$$

Where A = counts per minute in Green Channel

a = background counts per minute in Green Channel

α = Green Channel efficiency for ^{14}C

$$2. \text{ Spill over of } ^{14}\text{C} \text{ into Red Channel}$$

$$= \{(A - a) \div \alpha\} \times \beta$$

Where β = Red Channel efficiency for ^{14}C

$$3. \text{ Tritium dpm} = [(B - b) - \{(A - a) \div \alpha\} \times \beta] \div \gamma$$

Where B = counts per minute in Red Channel

b = background counts per minute in Red Channel

γ = Red Channel efficiency for ^3H

To expedite the processing of counting data a table was prepared from the standard curves in which the values for α , β and γ were given for different external standard counts per minute (Appendix). The values for α , β and γ

were taken from the curves at intervals of 5×10^3 cpm in the Blue Channel. Intermediate values were determined easily by interpolation.

Following is the programme which was used for processing the counting data on a Monroe Calculator Model 'Epic 3000'.

<u>Number Entered</u>	<u>Instruction Entered Via Key Marked</u>
A	Enter tab
a	Minus
α	Divide, store
B	Enter tab
b	Minus, invert
β	Multiply, minus
γ	Divide, record
	Print I, clear
	Print II

Print I gives the ^{14}C dpm
 Print II gives the ^3H dpm

Once the calculation had been programmed, it was only necessary to punch in the numerical data in the order given. When each complete set of seven numbers had been entered for each sample, the calculator automatically printed out the dpm for each isotope.

CHAPTER III

EXPERIMENTAL PROCEDURES

A. Collection, Preparation and Storage of Urine Samples

1. In Vivo Experiments

In the earlier experiments, mature Rhode Island Red hens were surgically modified so as to provide exteriorized ureteral openings by the procedure described by Ainsworth (1965). In the later experiments a modification of this procedure was introduced by the author. This modification is described in an appendix to the present thesis.

Radioactive material for in vivo experiments was dissolved in 2.0 ml propylene glycol and was injected intramuscularly into the breast muscles of the hen. The particular radioactive material injected in each experiment is indicated in the appropriate sections of this thesis.

The urine voided subsequent to the injection was collected for three 24-hour periods in each in vivo experiment. Urine samples were immediately filtered through a Büchner funnel. The urates were further extracted by grinding in a mortar with successive small volumes of water. The bulked filtered urine plus washings was made up to

500 ml or other convenient volume. Following the removal of aliquots for measurement of recovery of radioactivity injected, the urine samples were passed through a column containing Amberlite XAD-2 resin. Free and conjugated steroids held on the column were then eluted with methanol. The methanolic extracts were evaporated to dryness and then redissolved in methanol and made up to 100 ml. At this stage, samples were stored in a cold room at 4°C, if necessary, before subjection to DEAE-Sephadex A-25 chromatography.

B. Enzymatic Hydrolysis

1. With β -Glucuronidase ('Ketodase')

Following desalting procedure, peaks of presumptive estrogen glucuronides in methanol were evaporated to dryness and then redissolved in 50 ml of 0.5 M sodium acetate buffer adjusted to pH 4.5 with glacial acetic acid. The glucuronidase preparation ('Ketodase') was added at the rate of 500 Fishman Units per ml of buffer. In a parallel experiment, saccharo-1,4-lactone (3 mg per ml buffer solution) was added as well as 'Ketodase' in order to establish the extent to which liberation of free steroids was in fact due to β -glucuronidase action. The liberated free steroids were extracted with 3 x 1 volume of diethyl ether.

The ethereal solutions were washed with water ($1 \times \frac{1}{10}$ vol), dried over anhydrous Na_2SO_4 and evaporated to dryness prior to thin-layer chromatography.

2. With Aryl Sulfatase

Following evaporation of methanol, presumptive estrogen sulphate peaks were redissolved in 50 ml of 0.15 M sodium acetate buffer adjusted to pH 5 with glacial acetic acid. The enzyme aryl sulfatase was added at a rate of 2 sulfatase units per ml of buffer solution, then incubated for 24 hours at 37°C . The liberated free steroids were extracted with diethyl ether (3×1 vol). Evaporation of the diethyl ether preceded thin-layer chromatography.

C. Thin-layer Chromatography

1. Preparation of Thin-layer Plates

Glass plates (20 x 20 cm) were coated with a 0.25 mm thick layer of silica gel G. The slurry was prepared by vigorously shaking 30 g of silica gel G with 60 ml of distilled water for two to three minutes in a glass-stoppered 250 ml Erlenmeyer flask. The slurry was then applied to the glass plates with a suitable applicator. The plates were left on the bench for 30 minutes, activated at $100 - 110^\circ\text{C}$ for 30 minutes, then cooled in a desiccator. The

plates were then washed with methanol in chromatographic developing tanks to remove extraneous materials from the silica gel. After air drying the plates were reactivated for one hour at 100 - 110°C and then stored in a desiccator over anhydrous silica gel.

2. Application of Sample to Thin-layer Plates

The sample being subjected to separation and/or purification was taken up in a minimum volume of methanol and volumes of 10 μ l were withdrawn into a Hamilton syringe (of volumetric capacity 100 μ l) attached to a sample applicator and applied to a plate along a line (line of application). Sample washings were also applied to the plate.

For the identification of metabolites, radioactive or 'cold' reference compounds were applied by means of a micro-pipette on lateral channels.

3. Solvent Systems

The composition of all the solvent systems used in this thesis are listed in Table 5. All solvents, except spectraanalysed methanol were redistilled just before use.

4. Visualization of Steroid Estrogens

Phenolic estrogens were detected by use of the

Table 5. Composition of the solvent systems used for thin-layer chromatography

System	Composition by Volume	References
A	Cyclohexane:ethyl acetate:ethanol (45:45:10)	Lisboa and Diczfalusy (1962)
X	Chloroform:diethyl ether (60:40)	Sobrevilla, Hagerman and Villee (1964)
Y	Benzene:methanol (95:5)	Ladany and Finkelstein (1963)
Fishman	Chloroform:isopropanol:formic acid (15:5:3)	Fishman, Harris and Green (1965)
CE	Chloroform:absolute ethanol (7:3)	

reagent of Folin and Ciocalteu (1927) according to the method of Mitchell and Davies (1954). The thin-layer plate was sprayed with a solution made by diluting one volume of the reagent with two volumes of distilled water and then exposing the plate to ammonia fumes. A dark blue colour was developed by all the phenolic estrogens. Phenolic estrogens were also detected by spraying plates with 'H₂SO₄-ethanol' reagent (2% conc. H₂SO₄ in 50% aqueous ethanol v/v) and heated in an oven at 105°C for 20 - 25 minutes. Characteristic colours were developed by different phenolic estrogens.

D. Measurement of Radioactivity

1. Measurement of Radioactivity in Aqueous Fractions and Methanolic Extracts

Aqueous fractions, such as filtered urine or aqueous residue after enzymatic hydrolysis, were assayed for radioactivity in the following manner. Samples of 0.2 or 0.5 ml volume were taken, evaporated to dryness, then 0.5 ml spectraanalysed methanol was added, evaporated to dryness, then 15 ml scintillation mixture was added and the counting was performed in triplicate. The quenching was determined by use of an automatic external standard as described above. The total radioactivity, after correction for quenching, was expressed in disintegrations per minute.

A suitable aliquot of methanolic extracts was taken (usually 0.2 ml) and counting was done as described above.

2. Measurement of Radioactivity in NaCl Solution Fractions from DEAE-Sephadex A-25 Columns

Aliquots of 0.2 ml of each 10 ml fraction were taken and counting was done as described above for aqueous fractions.

3. Assay of Radioactivity on Thin-layer Plates

Zones separated by TLC were assayed for radioactivity, as described below, to eliminate elution from the

silica gel. The different zones were scraped into separate scintillation vials. A modification of the method described by Snyder and Stevens (1962) was used to prepare the vials for assay. Each vial was filled with Cab-O-sil M-5 silica gel up to the neck of the vial, 15 ml scintillation fluid (5.0 g PPO + 0.1 g POPOP per litre of toluene) were added and then the vial was capped and shaken. The samples were counted in the way described above for other radioactive fractions.

Measurement of radioactivity of metabolites labeled with tritium and separated by TLC was done by scraping serial strips 5 mm wide into vials and counting as described above. A histogram was plotted and the radioactivity peaks were compared with the reference standards on pilot strips. The R_f value of radioactive metabolites were determined from the histograms.

4. Radioautography

The developed chromatoplate was placed in a cassette and a medical X-ray film was cut to the appropriate size and placed in contact with the plate. The cassette was left in the dark for an appropriate period of time. The time of exposure was determined empirically. The cassettes were constructed as described by Richardson et al. (1963).

PART III

A STANDARD REFERENCE CURVE OF SOME LABELLED ESTROGEN CON-
JUGATES AND IN VIVO STUDIES OF LABELLED ESTRONE AND ESTRADIOL-
17 β -6,7-³H-17-GLUCOSIDURONATE

CHAPTER I

STANDARD CURVE OF SOME LABELLED ESTROGEN CONJUGATES

Three tritiated estrogen conjugates, viz., estrone-3-sulphate ($^3\text{H-E}_1\text{3S}$), estradiol-17 β -17-glucosiduronate ($^3\text{H-E}_2\text{17G}$) and estradiol-17 β -3-sulphate-17-glucosiduronate ($^3\text{H-E}_2\text{3S17G}$) were subjected in combination to DEAE-Sephadex A-25 chromatography with an applied linear gradient of 0-0.80 M NaCl. Table 6 gives the amount of radioactivity for each of the above mentioned conjugates used.

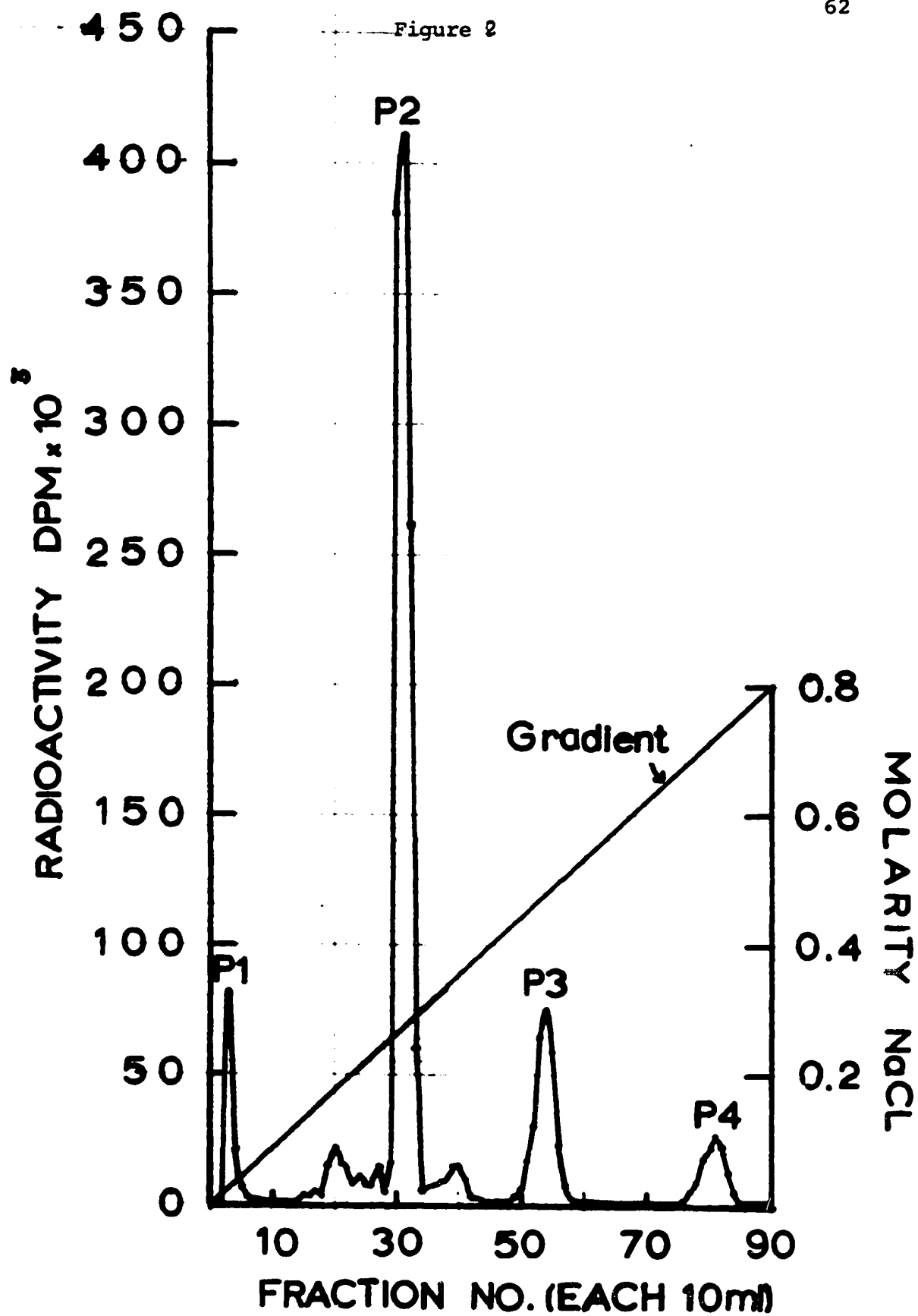
Table 6. Amounts of radioactivity of certain estrogen conjugates subjected to DEAE-Sephadex A-25 chromatography using a 0-0.8 M NaCl linear gradient.

Material	Amount of radioactivity (dpm)
$^3\text{H-E}_2\text{17G}$	1358×10^3
$^3\text{H-E}_1\text{3S}$	231×10^3
$^3\text{H-E}_2\text{-3S-17G}$	168×10^3

The positions of the estrogen conjugates recovered from the DEAE-Sephadex A-25 column are shown in Figure 2 (page 62) and were in reasonable agreement with those given by Hobkirk et al. (1969a). Table 7 illustrates

Figure 2. Standard reference curve of certain
labelled estrogen conjugates.

Legend -	P1	Peak 1
	P2	Peak 2
	P3	Peak 3
	P4	Peak 4



the author's peak positions compared to those Hobkirk et al. (1969a).

Table 7. Comparison of peak positions for some pure tritiated estrogen conjugates.

Material	Tube Nos. (10 ml fractions)*	Tube Nos. (10 ml fractions) [†]
³ H-E ₂ 17G	28-33	29-34
³ H-E ₁ 3S	46-50	50-58
³ H-E ₂ 3S17G	70-75	76-85

*Data from Hobkirk et al. (1969a).

[†]Author's data from Figure 2.

From the nature of the materials loaded on to the column and the positions of the peaks in Figure 2, the following presumptive identifications were possible:

Peak 1	Free steroid
Peak 2	E ₂ -17G
Peak 3	E ₁ ² -3S
Peak 4	E ₂ ¹ -3S-17G

The free steroid represented only 6.1% of the total radioactivity applied to the column and 42.5% of the total recovered from the column. It may have represented free steroid present in the materials applied or formed therein by partial cleavage. The total radioactivities applied to the column in

the three standard preparations and the radioactivities recovered in the eluted fractions are shown in Table 8.

Table 8. Recovery of radioactivity from column, all values as dpm.

	Applied	Recovered	%
Free steroid	0	108×10^3	(00)
E ₂ -17G	1358×10^3	1142×10^3	84
E ₁ -3S	231×10^3	291×10^3	126
E ₂ -3S-17G	168×10^3	126×10^3	75
Other	0	254×10^3	
Total	1757×10^3	1921×10^3	

The total recovery of radioactivity was satisfactory. Recoveries from the three preparations were less satisfactory. No explanation was evident for the high recovery from the E₁-3S preparation. If E₁-3S had broken down to give free steroid, one would have expected a relatively low recovery of radioactivity from this fraction. So far as they go, the results support the identifications of Peaks 2, 3 and 4 in the eluate, the relative magnitudes of the radioactivities in the preparations applied and in the corresponding fractions recovered being reasonable agreement.

	E ₂ -17G	E ₁ -3S	E ₂ -3S-17G
On to column	100	17	12
Off column	100	25	11

CHAPTER II

IN VIVO STUDIES OF LABELLED ESTRONE (EXPERIMENT I) AND LABELLED ESTRADIOL- -17 β -6,7-³H-17-GLUCOSIDURONATE (EXPERIMENT II)

A. Object: To determine the presence of singly and/or doubly conjugated estrogens in hens' urine.

B. Experimental Procedure

In Experiments I and II respectively, estrone-4-¹⁴C and estradiol-17 β -6,7-³H-17-glucosiduronate was injected into a laying hen intramuscularly and urine subsequently excreted was collected in three 24-hour lots and processed as given below.

Procedure for processing urine:

Filtered urine - - - - -count

Urine passed through a pre-prepared Amberlite XAD-2 column, urine on column washed 2x with 1000 ml water, column allowed to drain dry.

Conjugates eluted with 3 litres methanol

Methanol was evaporated completely and residue taken up in 100 ml methanol - - - - -count

Methanol evaporated

Residue dissolved in 5 ml water and transferred to DEAE-Sephadex A-25 column with a further 2 x 2.5 ml water. An 0-0.8 M NaCl gradient was applied.

Ninety tubes each of 10 ml volume eluate were collected. From each tube an aliquot (0.2 ml) was removed for counting - - - - - count

The elution pattern of the radioactivity was compared with the elution patterns of known radioactive conjugates. On the basis of correspondence of peaks, appropriate tubes from the experimental run were pooled for further examination. These "pooled peak" samples were incubated with either aryl sulfatase and/or 'Ketodase'.

Cleavage of β -glucosiduronate linkages was effected by 'Ketodase'. Control incubations including saccharolactone as β -glucuronidase inhibitor were included.

Sulphate groups were cleaned by aryl sulfatase incubation and any free steroid liberated was extracted with diethyl ether (3 x 1 vol).

These ether extracts were examined by TLC against appropriate standards, either 'hot' or 'cold' and the position of the radioactivity on the plate was ascertained either by radioautography or by scraping serial strips from the gel.

C. Results and Discussion

In Experiment I peak 1 (Figure 3) was examined by TLC in System X (Sobrevilla et al., 1964) against 'cold' estradiol-17 β and 'cold' estradiol-17 α with subsequent

Figure 3. Elution pattern of fractions from DEAE-Sephadex A-25 for Experiment I, day 1 urine sample.

Legend -	P1	Peak 1
	P2	Peak 2
	P3	Peak 3
	P4	Peak 4
	P5	Peak 5
	P6	Peak 6
	P7	Peak 7

Figure 3

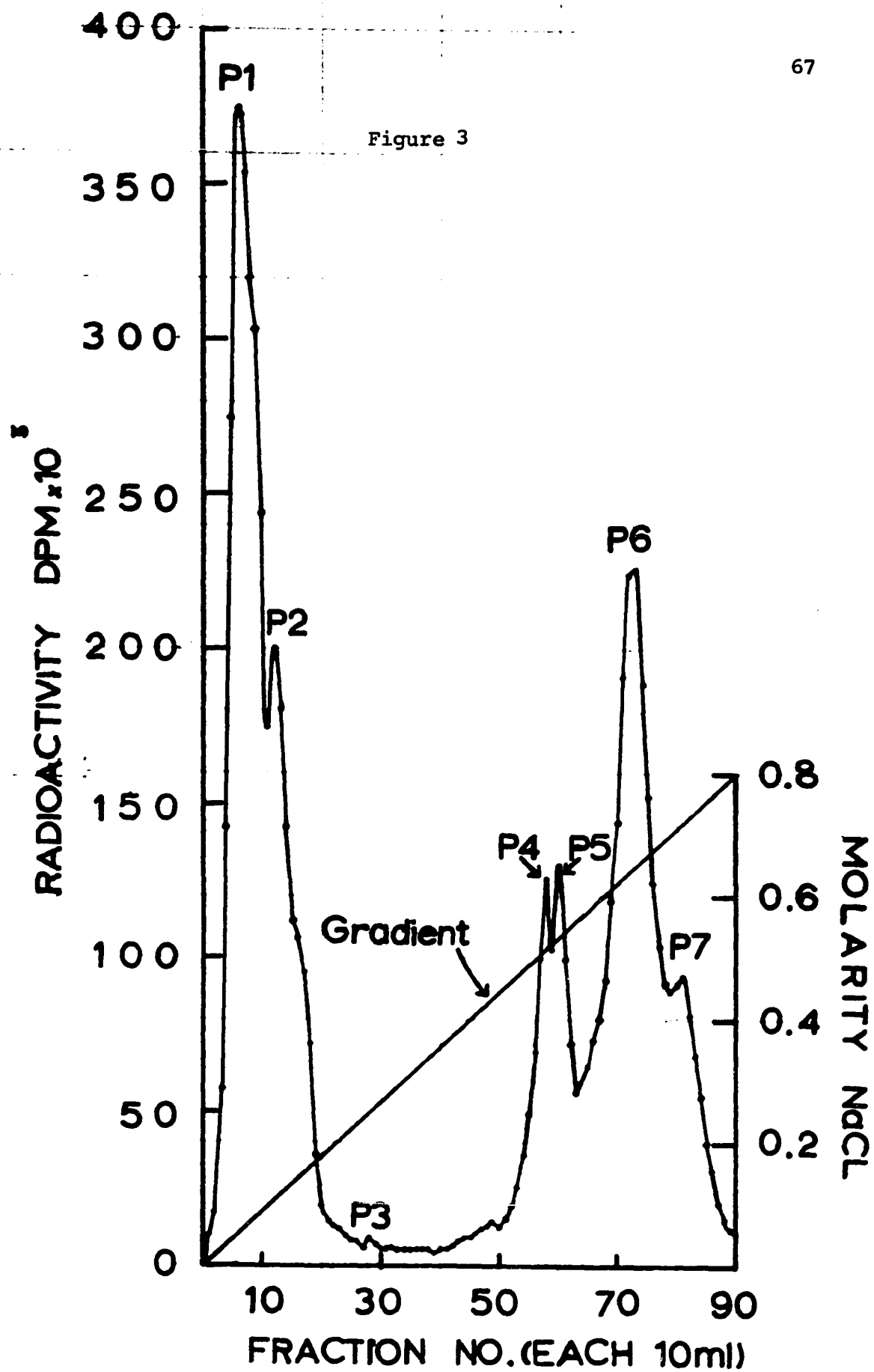
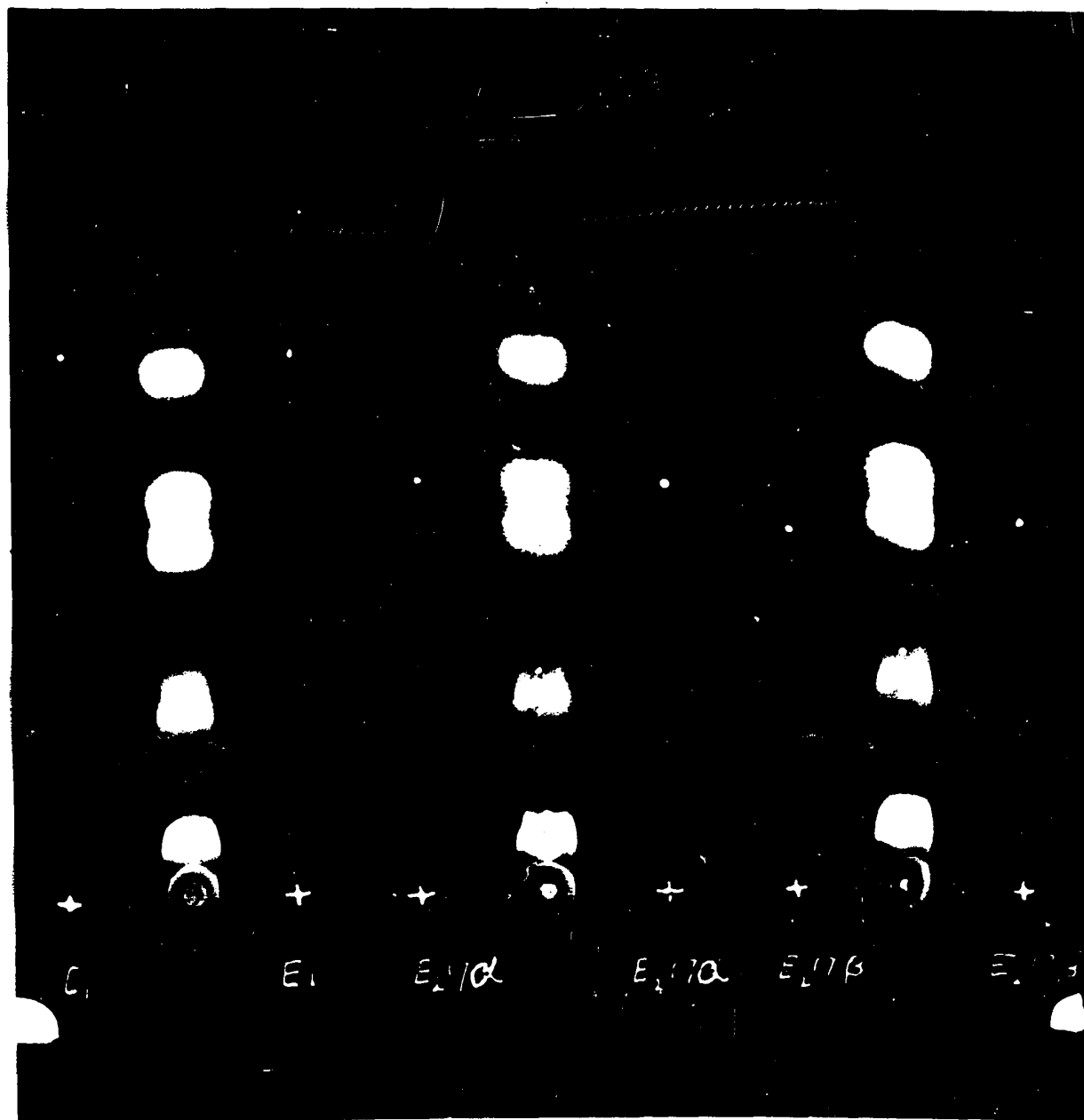


Figure 4

Radioautogram of peak 1: presumptive estrone and estradiol
from Experiment I, System X.



radioautography. The radioautogram (Figure 4) confirmed the presence of 'free steroids'. Rf values are given in Table 12 below.

Table 9. Recoveries of injected radioactivity in hens' urine.

Fraction	Experiment I	Experiment II
Dose injected dpm	3.98×10^7 dpm	2.5×10^6 dpm
Radioactivity in urine as % of that injected		
Day 1	23.90	25.20
Day 2	0.82	5.18
Day 3	0.13	0.45
Total	24.85	30.83

Table 10. Recoveries of radioactivity in the urinary fractions following injection of radioactive material.

Fraction	Experiment I	Experiment II
Dose injected dpm	3.98×10^7 dpm	2.5×10^6 dpm
Total radioactivity (dpm) in day 1 urine	9.94×10^6	6.2×10^5 dpm
% Recovery in methanolic eluate from XAD-2 column for day 1 urine sample	68.6	42.0

Table 11. Percentage radioactivity recovered in fractions from DEAE-Sephadex A-25 column for day 1 urine sample.

Fraction	Peak No.	<u>Experiment I</u>	
		Presumptive identity	% Recovery
1-10	1	$E_1/E_2^{17\beta}/E_2^{17\alpha}$	30.4
11-20	2	$E_2^{17\beta}/E_2^{17\alpha}$	16.4
27-32	3	E_2^3G	0.6
52-59	4	E_2^3S	7.6
60-63	5	E_2^3S	4.9
64-79	6	$E_2^3S^{17}G$	29.4
80-90	7	-	7.5
Total			96.8
<u>Experiment II</u>			
1-18	1	$E_1/E_2^{17\beta}/E_2^{17\alpha}$	42.6
19-21	2	E_1^3G	3.5
22-26	3	E_2^3G	30.7
28-35	4	$E_2^{17}G$	3.4
40-50	5	E_1^3S	3.0
51-64	6	E_2^3S	6.9
67-76	7	$E_2^3S^{17}G$	6.5
77-90	8	-	2.2
Total			98.8

Table 12. Chromatographic mobilities of radioactive phenolic steroids in peak 1.

Steroid	Presumptive		Reference	
	R _f	R _{E1}	R _f	R _{E1}
Estrone	0.59	-	0.61	-
Estradiol-17 α	0.45	0.76	0.46	0.76
Estradiol-17 β	0.39	0.66	0.41	0.67
17- <u>Epi</u> estriol	0.25	0.42	-	-
16- <u>Epi</u> estriol	0.20	0.34	-	-
'Estriol'	0.05	0.08	-	-

The chromatographic mobilities of the radioactive spots indicate that all the usual major metabolic products of injected estrone-¹⁴C were present.

The material of peak 2 was run similarly in system X with reference estrone, estradiol-17 α and estradiol-17 β in lateral columns. The position of radioactive spots was monitored by scraping serial strips of gel from the plate and then measuring their radioactivity. There were two major peaks of radioactivity, the greater corresponding in position with reference estradiol-17 α plus estradiol-17 β , the lesser with estrone. The material of peak 4 was incubated with aryl sulfatase as described in Part III of this thesis. The

steroids extracted from the hydrolysis mixture were subjected to TLC (system X) and the distribution of radioactivity was examined by serial strip counting. A high proportion of the radioactivity was in a position corresponding to that of estrone and relatively little in the positions of the estradiols. This result was unexpected and no explanation can be offered at this point.

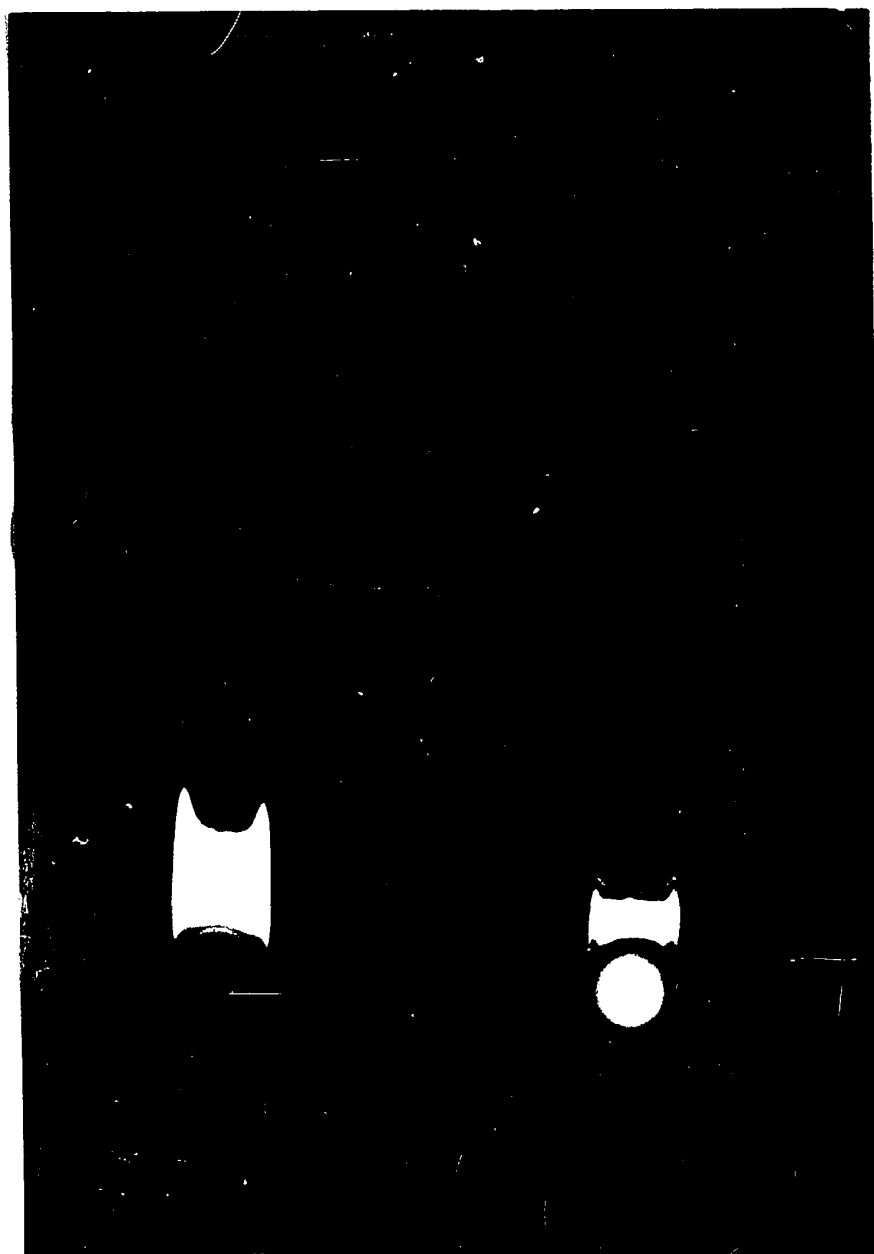
Peak 5 was examined in the same manner as was peak 4. The outcome was qualitatively the same, except for more indication of the presence of cis-estriols.

In Experiment I, peaks 6 and 7 were provisionally equated with mono sulphate-monoglucosiduronate. Following purification the materials of these peaks were examined by TLC in the Tubman system (Fishman et al., 1965) and in system CE (chloroform:ethanol, 7:3). Radioautograms of the TLC plates are presented in Figure 5. By far the greater part of the radioactivity remained near the point of application in either system with relatively minor amounts of free steroid. This observation suggests that most of the radioactive material of peaks 6 and 7 was present as doubly conjugated phenolic steroid.

Other portions of the material of peaks 6 and 7 were incubated with aryl sulfatase followed by incubation with

Figure 5

Radioautogram of peaks 6 and 7: presumptive mono-sulphate-mono-glucosiduronate from Experiment I, System CE.



'Ketodase' (pH 4.5) but recoveries of radioactivity were inadequate to permit of chromatographic examination. This situation was probably a consequence of

In Experiment II (Figure 6), the losses incurred during desalting and purification of the materials of peaks 4, 5, 6 and 7 were so heavy that the further examination of these materials by enzymic hydrolysis and TLC did not yield meaningful information.

Peak 3 had been tentatively identified as estradiol-3-glucosiduronate. Enzymic treatment with Ketodase, however, liberated estrone as well as estradiol-17 α and estradiol-17 β as evidenced by TLC in system X followed by strip counting.

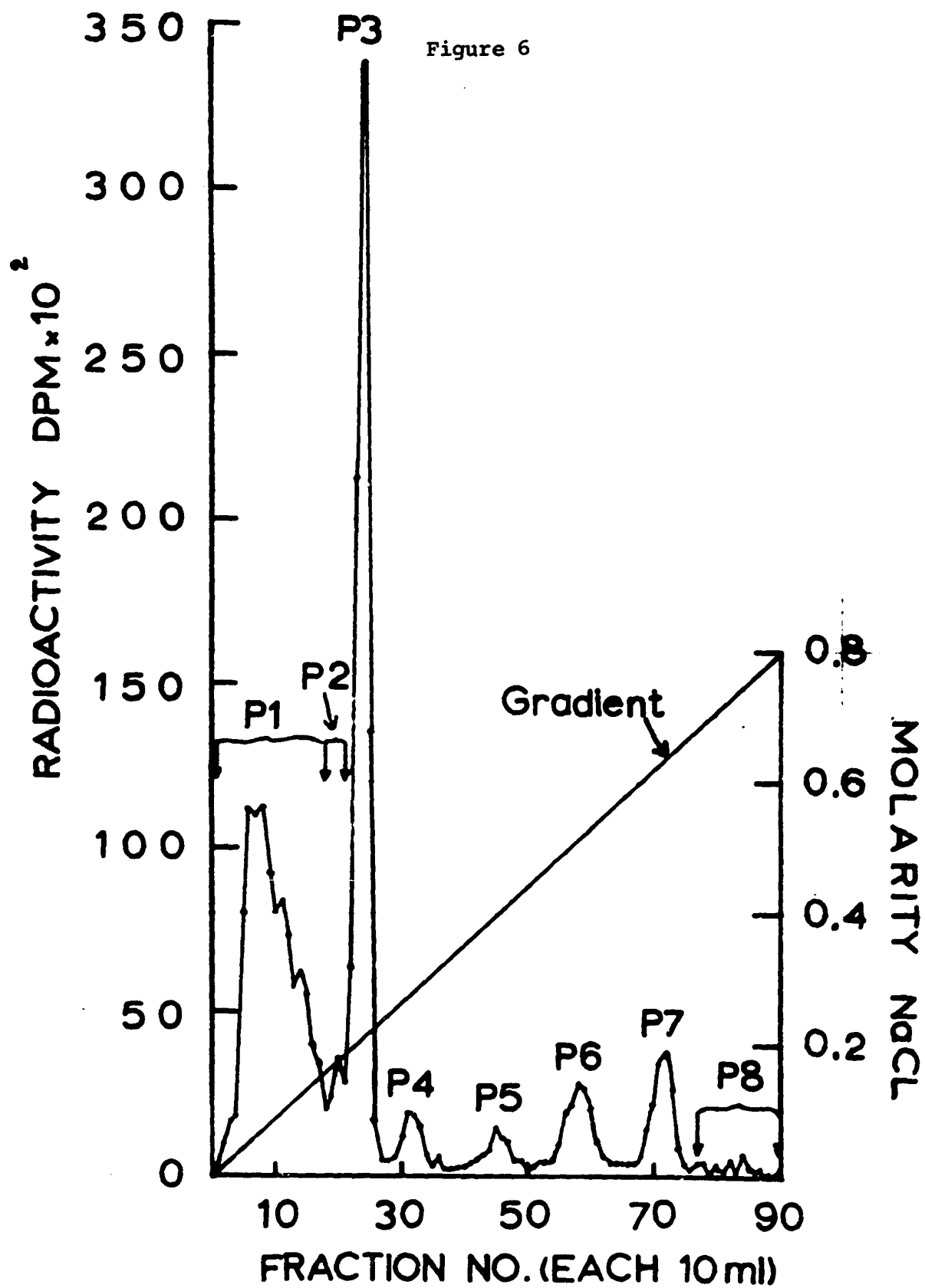
A portion of the material of peak 7 was subjected to TLC in the Fishman and CE systems and the distribution of radioactivity was monitored by serial strip counting. The bulk of the radioactivity remained close to the point of application in both systems and there were no indications of the presence of minor amounts of 'free steroid'. These foregoing observations were consistent with the identification of the material of peak 7 as deconjugate.

Summary

Following the injection of estrone-4-¹⁴C or of

Figure 6. Elution pattern of fractions from DEAE-Sephadex A-25 for Experiment II, day 1 urine sample.

Legend -	P1	Peak 1
	P2	Peak 2
	P3	Peak 3
	P4	Peak 4
	P5	Peak 5
	P6	Peak 6
	P7	Peak 7
	P8	Peak 8



estradiol-17 β -6,7-³H-17-glucosiduronate into laying hens, the conjugated materials were separated from the urine by use of neutral ion exchange resin. The conjugated material was then subjected to fractionation on a DEAE-Sephadex A-25 column in a sodium chloride gradient. The position of the peaks of radioactivity provided evidence for the presence of both singly and doubly conjugated phenolic estrogens in hens' urine.

Attempts to identify further the materials in these peaks were largely unsuccessful because of heavy losses of radioactivity during the necessary preliminary desalting and purification processes.

Some additional evidence in support of the identification of doubly conjugated material was obtained in one experiment by use of appropriate thin-layer chromatographic systems. Attempts to identify monoglucosiduronate by liberation of radioactivity on incubation with β -glucuronidase ('Ketodase') as compared with control incubations in the presence of excess saccharo-1,4-lactone (β -glucuronidase inhibition) yielded negative results, again on account of the low amounts of radioactivity recovered from the appropriate peaks.

PART IV

**A MODIFIED SURGICAL PROCEDURE FOR THE EXTERIOR-
IZATION OF THE URETERAL OPENINGS OF THE HEN**

CHAPTER I

B. Literature Review of the Surgical Methods for the Separation of Urine and Feces in the Hen

Avian metabolic studies have been restricted to a considerable degree by the difficulties of collecting the urine and the feces separately over periods of time sufficient for purposes of metabolic studies.

The techniques which have been used to effect separate collection of urine and/or feces include catheterization, ligation of the ureters, colostomy, artificial anus and exteriorization of the ureteral openings

1. Artificial Anus and Colostomy

Earlier reports on the artificial anus procedure with the domestic fowl include those of Milroy (1901), Paraschtschuk (1902), Lehmann (1904), Völtz (1909, 1910), Szalagyi and Kriwuscha (1914, 1918) and Katayama (1924). The same technique was used on turkeys by Milroy (1901), on geese by Szalagyi and Kriwuscha (1918) and on ducks by Szalagyi and Kriwuscha (1914, 1918) and also by Hari and Kriwuscha (1918).

Hart and Essex (1942), Rothchild (1947), Imabayashi

et al. (1956), Fussel (1960) and Calvin et al. (1966) reported successful operations to effect an artificial anus in birds. More recently Paulson (1969) reported an improved method to collect urine, feces and expiratory gases from mature chickens. Paulson was able to maintain healthy birds following the operation for as long as a month, after which time the skin surrounding the artificial anus gradually lost tonicity and the lumen eventually closed due to constriction of the abdominal muscle.

In 1965 Squance and Brown used colostomised laying pullets in a study of digestibility and biological value of protein diets. Recently, Robert Tao et al. (1969) and Tang et al. (1970) reported colostomies in mature chickens. Tao et al. (1969) found that their colostomised birds required very little post-operative care and had normal fecal excretion for several months. Tao et al. (1969) further reported that there was no intestinal blockage due to many types of rations. Tang et al. (1970) in order to prevent occlusion of the exteriorized rectum inserted a self-retaining tygon cannulae.

2. Exteriorization of Ureters

Successful exteriorization of the ureters permit direct collection of the urine over long periods of time

and is especially useful for studies of the in vivo metabolism of individual substances, e.g., in studies of the in vivo metabolism of labelled steroids. Unfortunately, the surgical procedure is somewhat tricky and the proportion of really successful operations is low.

In 1957 Dixon and Wilkinson reported a surgical procedure for exteriorization of the ureters based upon an earlier procedure described by Hart and Essex (1942). Dixon and Wilkinson were able to obtain birds which resumed normal laying after the operation. Newberne et al. (1957) were successful in exteriorizing the ureteral orifices of young chickens but found that the chickens were only useful for two weeks after the operation. Two hens which MacRae (1960) operated by the procedure of Dixon and Wilkinson came back into full lay and remained useful for at least 80 days. Ainsworth (1965) reported a modification of the technique of Dixon and Wilkinson (1957). This modification consisted of isolating the ureteral openings from the cloacal mucosa immediately after elevation of the ureteral openings below the pygostyle. Suturing of the wound following isolation of the ureteral openings from the mucosa of the cloaca was another modification made by Ainsworth on Dixon and Wilkinson's technique. Birds operated by Ainsworth's technique permitted the

separate collection of urine and feces over an indefinite period of time and the birds also resumed their normal laying cycle.

A subsequent modification by the author of this thesis is described separately below.

3. Catheterization and Cannulation

Some non-surgical methods for the collection of urine only have been done by Davis (1927), Coulson and Hughes (1930) and Pitts (1938). Hester et al. (1940) cannulated hens' ureters in order to measure urine flow.

In 1960 Richardson et al. surgically modified hens by cannulation of exteriorized recta. Surgical exteriorization of the rectum has been reviewed by Hartfiel (1962).

C. A Modification of the Surgical Procedure Previously Used for the Exteriorization of the Ureters of the Hen

The surgical procedure previously used with some success in the earlier stages of experimentation for this thesis was a modification of the surgical procedure of Dixon and Wilkinson (1957) as described by Ainsworth (1965). Despite daily postoperative care to wash away at least three times daily solid waste material from the operated area, the author lost several operated birds as a result

of blocking of the ureteral openings as well as by fistulation of the cloaca. Due note was taken, as indicated by Ainsworth, to avoid fistulation by making the necessary sutures to effect union of the mucosa of the cloaca with the tissue of the dorsal lip of the vent, but without much success. In addition, the author lost several birds as a result of repeated dehiscence of the cloacal incision. Eventually the following modified surgical procedure was tried, found successful and used for further work described in this thesis.

Mature laying and non-laying Rhode Island Red hens were used for all operations. The weight of the birds used lay within the range of 2.0 to 2.5 kg.

Procedure

The bird is first anaesthetized by injecting Nembutal (sodium pentobarbital, obtained from Abbott Laboratories Ltd., Montreal, Quebec containing 60 mg per ml) very slowly into the brachial wing vein until the bird was non-responsive to pinching of the comb. The amount of Nembutal required ranges from 0.5 ml to 1.0 ml, depending on the weight of the bird. While the anaesthetic is being administered, care should be taken to keep the head of the bird above the level of the crop in order to avoid

strangulation (Newberne, Laerdal and O'Dell, 1957) as shown in Figure 4A. Following the induction with Nembutal, 1.0 ml of 'Luminal Sodium' (Winthrop, containing 120 mg phenobarbitone sodium per ml) was administered intramuscularly.

The anaesthetized bird is then placed on a wooden stand (Figure 7) made to fit its body snugly. Figures 8A and 8B illustrate the bird breast down on the stand, legs extended over the rear end of the stand and tail hoisted out of the operation area. In order to maintain the anaesthesia of larger birds, it was necessary now and then to administer a little diethyl ether by face mask and as required. The feathers are then removed from the area below the tail. Aseptic conditions should be observed throughout the entire operation. A 1% solution of Bradosol (Ciba Co. Ltd., Dorval, Quebec) was used during the operation, as well as for washing of the operated area after the operation.

An initial transverse incision is made through the skin at the base of the pygostyle (Figure 9, Step 1). This incision should not extend more than 0.75 cm on either side of the midline but should be long enough to permit easy passage of forceps inside to lift the urodeum through the opening. This lifting of the urodeum through the incision and its subsequent correct placement is a critical stage in the

operation and care has to be taken to avoid injury to the urodeumal tissue. Immediately dorsal to the incision, from an area of about 2 cm in diameter, the cutaneous and subcutaneous tissues and fatty tissue were excised such that the reflected urodeum would fill the defect (Figure 10, Step 2). The exposed urodeum was then retracted caudally through the transverse incision and the lateral folds of tissue so formed were incised to permit unrestricted dorsal reflection of the urodeum (Figure 11, Step 3A and Figure 12, Step 3B), as well as to ensure minimal displacement of the oviduct and rectum. Great care must be taken not to stretch the urodeum beyond its normal elasticity. A series of suburodeumal single interrupted sutures (3/0 polypropylene) were used to fix the urodeum to the exposed area below the tail (Figure 13, Step 4). The edge of the urodeum was then fixed to the cut skin edge of the exposed area by mattress sutures (3/0 polypropylene; Figure 13, Step 4). Finally, the base of the exposed area of the urodeum was sutured by mattress sutures (3/0 polypropylene) to the cut edge, including the skin of the transverse incision (Figure 14, Step 5). In closing the incision great care must be taken to avoid trauma to the two ureters.

The entire operation takes about an half hour. Post-operative care consists of treating the operated area with

"Eye and Wound Powder" (chlorhexidine dihydrochloride powder, obtained from Ayerst Laboratories division of Ayerst, McKenna and Harrison Ltd., Montreal, Quebec) three times daily for four days, as well as washing of the operated area three times daily with Bradosol solution to remove solid waste material. Birds were fully recovered from the operation within 12 hours and usually came back into lay within two weeks after the operation.

The author found Crossbred Leghorn strain birds unsuitable for the operation as described by Ainsworth (1965) due to the close proximity of the ureteral openings to the oviduct, as well as the fact that the ureteral openings were often asymmetrically situated. In addition, Crossbred Leghorn strain birds were found to be less tolerant to the operation than Rhode Island Red birds. Mature Rhode Island Red birds were found to be distinctly more suitable for the operation and they also tended to come back into lay after operation more readily than did younger birds of the same breed.

Photographs of the Modified Surgical Procedure

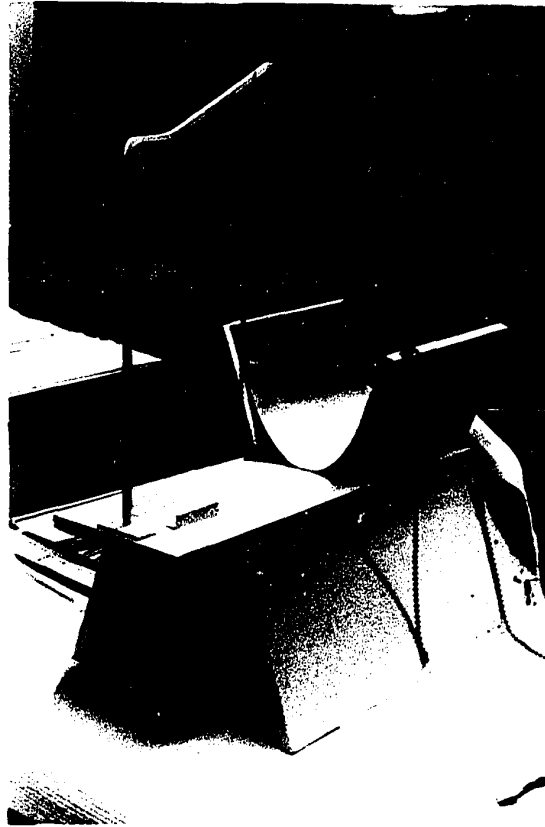


Figure 7. Operation stand.

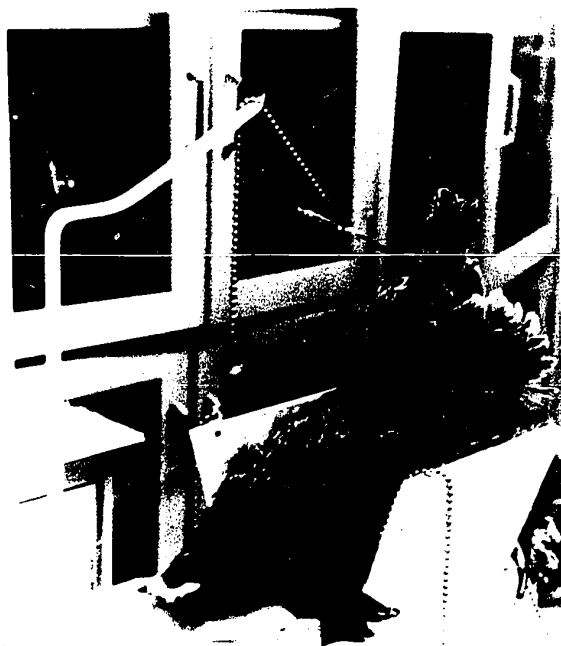


Figure 8A



Figure 8B



Figure 9
Step 1



Figure 10
Step 2



Figure 9
Step 1



Figure 10
Step 2



Figure 11
Step 3A



Figure 12
Step 3B



Figure 13
Step 4



Figure 14
Step 5

APPENDIX

- A. Quenching Curves for the Packard Tri-Carb Liquid
Scintillation Spectrometer Model No. 3365

Figure15. Quenching curve for carbon-14, single-label.

Figure 15

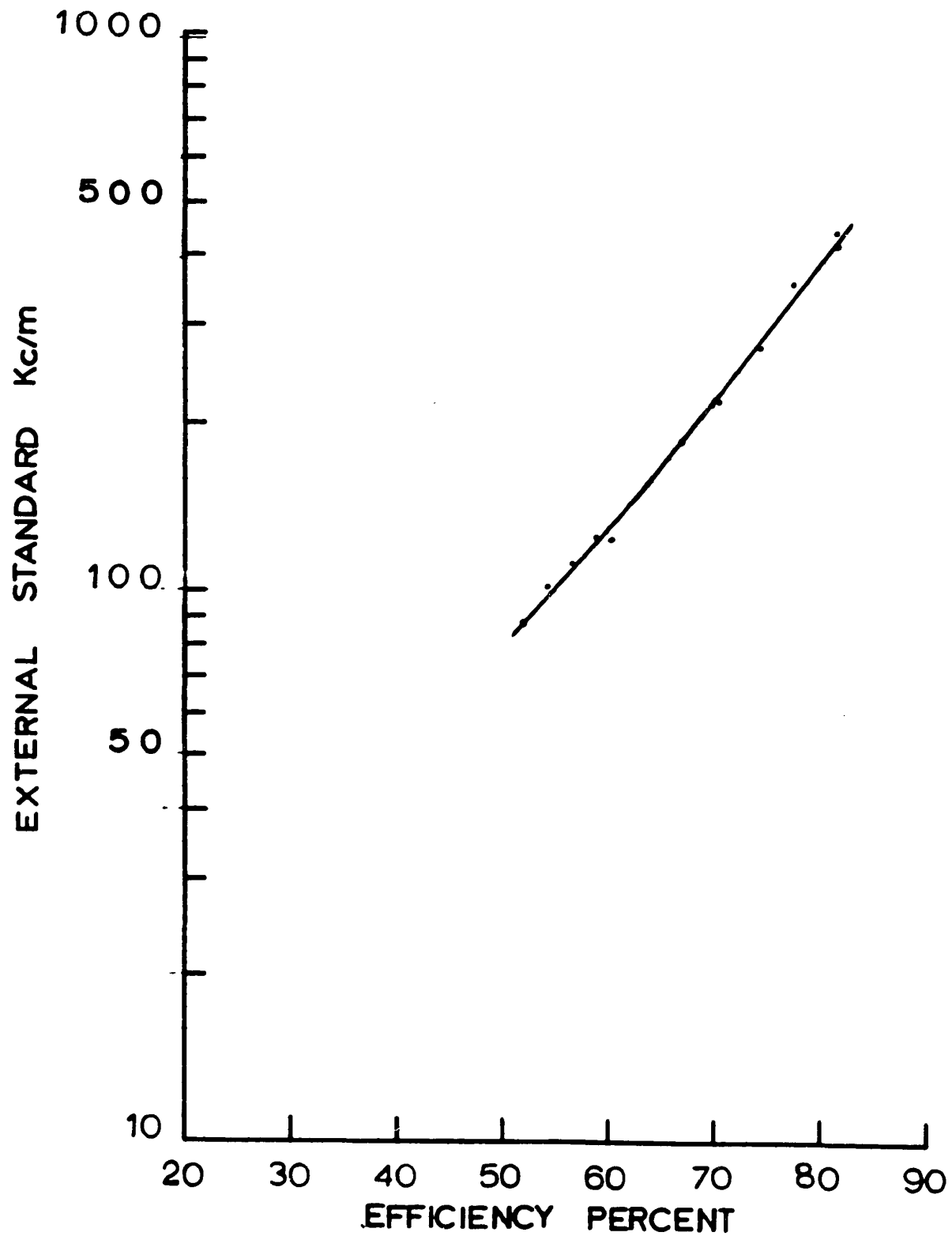


Figure 16. Quenching curve for tritium, single-label.

Figure 16

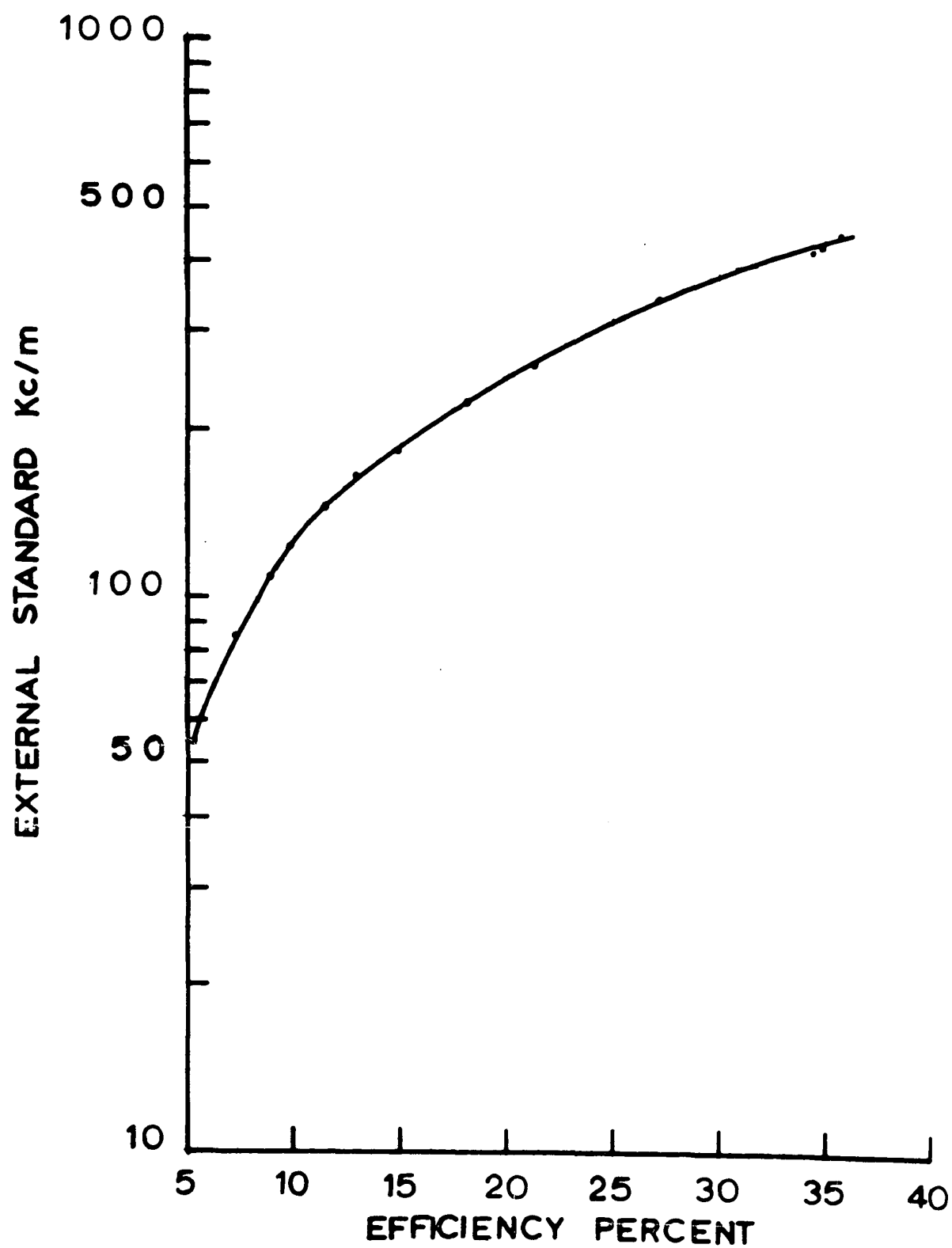


Figure 17. Quenching curve for carbon-14, double-label.

Figure 17

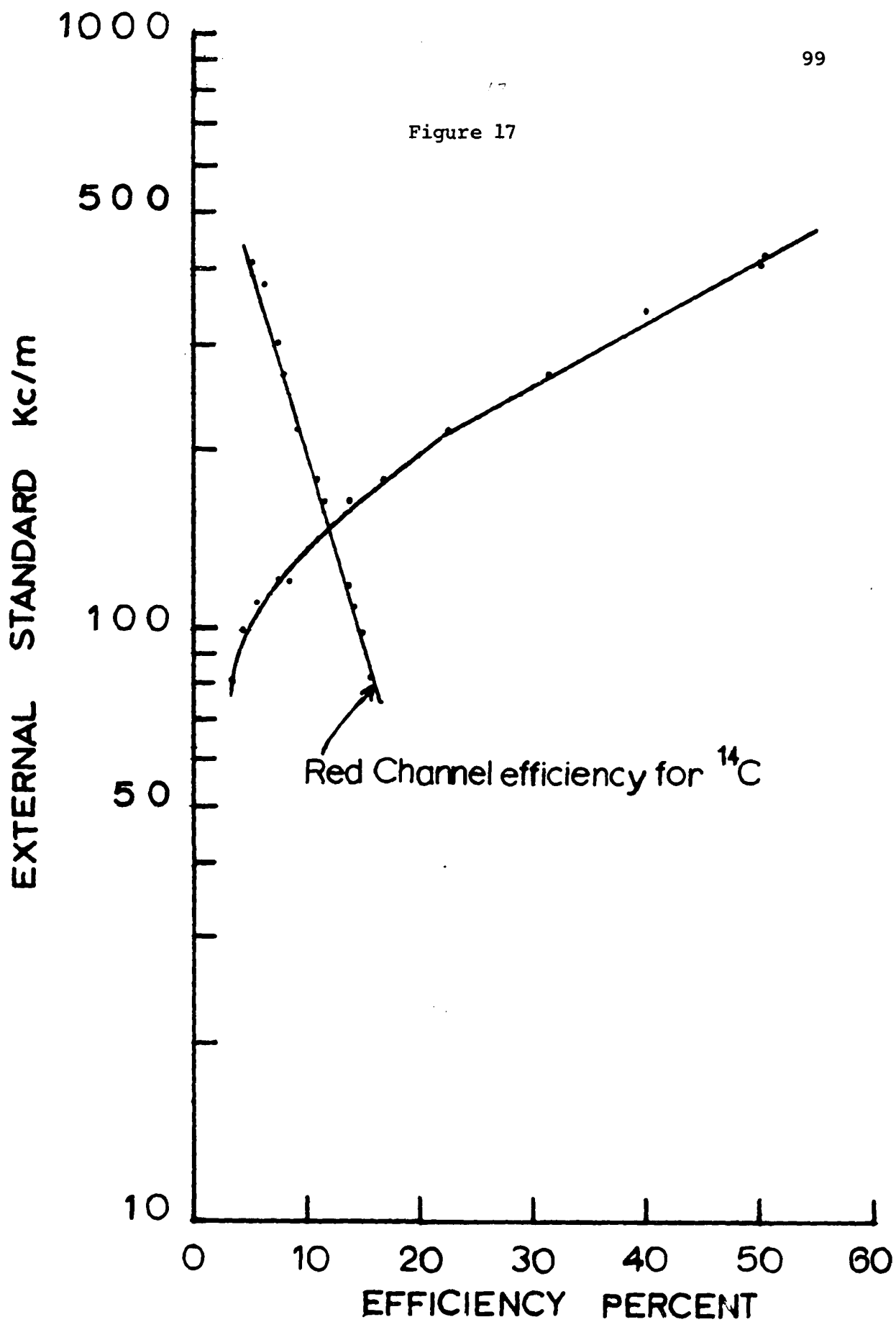
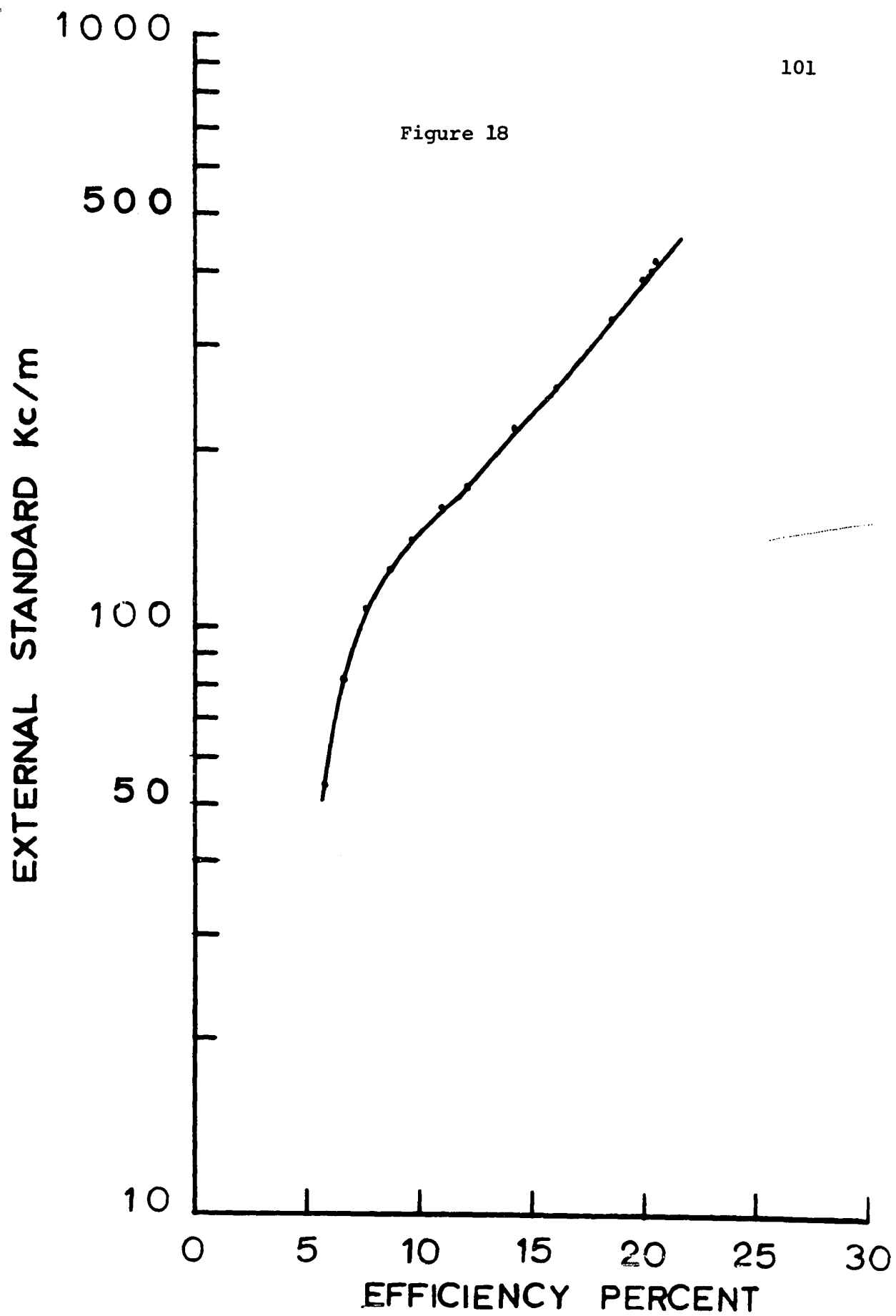


Figure 18. Quenching curve for tritium, double-label.

Figure 18



- B. Quenching Factor Tables for the Packard Tri-Carb
Liquid Scintillation Spectrometer Model No. 3365

Table 13. Quenching factors for carbon-14, single-label.

Table 13

External Standard K c/m	Factor α	External Standard K c/m	Factor α
440	.816	240	.712
430	.813	230	.705
420	.809	220	.698
410	.805	210	.690
400	.802	200	.681
390	.797	190	.672
380	.792	180	.663
370	.788	170	.653
360	.783	160	.643
350	.778	150	.631
340	.773	140	.619
330	.768	130	.606
320	.763	120	.591
310	.757	110	.574
300	.751	100	.555
290	.745		
280	.739		
270	.733		
260	.727		
250	.720		

Table 14. Quenching factors for tritium, single-label.

Table 14

External Standard	Factor	External Standard	Factor
K c/m	β	K c/m	β
440	.356	240	.194
430	.345	230	.187
420	.332	220	.177
410	.323	210	.168
400	.313	200	.161
390	.305	190	.153
380	.297	180	.143
370	.288	170	.135
360	.282	160	.127
350	.275	150	.118
340	.267	140	.111
330	.260	130	.103
320	.253	120	.096
310	.246	110	.088
300	.238	100	.083
290	.232		
280	.224		
270	.217		
260	.209		
250	.202		

Table 15. Quenching factors for carbon-14 and tritium,
double-label.

Table 15

Standard Blue Channel K c/m	^{14}C effy* (2) α	^{14}C effy* (4) β	^3H effy* (5) γ
425	.500	.050	.206
420	.495	.051	.204
415	.490	.053	.203
410	.485	.054	.202
405	.480	.054	.201
400	.474	.055	.200
395	.468	.056	.199
390	.463	.056	.198
385	.457	.057	.197
380	.453	.057	.196
375	.446	.058	.195
370	.440	.058	.194
365	.435	.059	.193
360	.429	.060	.192
355	.423	.061	.191
350	.417	.062	.190
345	.412	.063	.188
340	.406	.063	.187
335	.399	.064	.186
330	.393	.064	.185
325	.385	.065	.183
320	.380	.065	.182
315	.373	.067	.180
310	.367	.068	.179
305	.360	.069	.177
300	.353	.070	.176
295	.346	.071	.175
290	.340	.072	.173
285	.333	.073	.171
280	.327	.074	.170
275	.319	.076	.168
270	.313	.076	.167
265	.305	.077	.165
260	.298	.078	.163
255	.292	.080	.162
250	.284	.081	.158

Table 15 (cont'd)

Standard Blue Channel K c/m	^{14}C effy* (2) α	^{14}C effy* (4) β	^3H effy* (5) δ
245	.276	.083	.157
240	.268	.083	.154
235	.262	.085	.152
230	.255	.086	.150
225	.248	.088	.148
220	.240	.090	.145

*Efficiency

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