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UPTAKE OF ESTROGEN BY RABBIT LIVER AND INTESTINE

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Thesis submitted to the Department of Biochemistry in partial fulfillment of the requirements for the degree of Master of Science

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Abstract

Studies with rabbit intestine and liver tissue and isolated cell preparations have shown that the in vitro uptake of estrone, 17β-estradiol and 17α-estradiol occurs by non saturable diffusion. There is a subsequent temperature dependent conjugation with glucuronic acid in position 3 of the steroid nucleus and export of the sequestered estrogen. Uptake of the non acidic 17β-estradiol 3-glucoside occurs only after prior hydrolysis to the steroid aglycone by a soluble steroid β-glucosidase that is released into the medium during incubation. The double conjugate 17α-estradiol-3-glucuronide 17-N-acetyl-glucosaminide does not enter liver or intestine and the entry of the monoglucuronide is restricted. However, uptake of intact 17β-estradiol 3-glucuronide by isolated hepatocytes may be a minor component of the overall transport process.

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LIST OF ABBREVIATIONS

BSA: bovine serum albumin

Ci: curie

DES: diethyl stilbestrol

DTT: dithiotheritol

E_1: estrone

E_2: estradiol

$E_2\beta 3G$: 17β-estradiol 3-glucoside

$E_2\beta 3GA$: 17β-estradiol 3-glucuronide

$E_2\alpha 3GA17NAG$: 17α-estradiol 3-glucuronide 17-N-acetyl glucosaminide

G: glucose

GA: glucuronic acid

HEPES: N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid

NAD: nicotinamide adenine dinucleotide

NADP: nicotinamide adenine dinucleotide phosphate

NAG: N-acetyl glucosamine

TES: N-Tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid

Tricine: N-Tris(hydroxymethyl)methyl glycine

UDPGA: uridine di-phospho glucuronic acid

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1. General Introduction

1.1 structure of steroid hormones

Steroid hormones are chemically simple molecules derived from cholesterol. The compounds are amphiphatic, having a hydrophobic steroid nucleus and hydrophilic oxygenated substituents. The unconjugated steroids do not carry an electrical charge at neutral pH and thus are liposoluble (King 1976, Giorgi 1980). The biological activities dictate the classification of these compounds into five main categories: estrogens, androgens, progestins, glucocorticoids and mineralocorticoids. The specificity of the response evoked by these low information molecules is believed to be a result of the interaction of the hormone with a receptor macromolecule in the cytosol of the target tissue. Characteristics that lead to the identification of a tissue as a target for a steroid hormone are prolonged hormone retention (Jensen and Jacobson 1962, Yamamoto and Alberts 1976), the presence of an extranuclear specific binding protein and a biological response to the hormone.

1.2 mechanism of action

Steroid hormones alter the pattern of gene expression of their target cells. For all classes of steroid hormones, the biologic effect ellicited was generally believed to be

the result of a 'Two Step Interaction Pattern'(Jensen, Suzuki, Kawashima, Stumpf, Jungblut and Desombre 1968). The sequence is initiated by a reversible and highly specific interaction of the steroid molecule with an extranuclear receptor (Dandliker, Braton, Hsi, Brawn, Levin, Meyers and Kolb 1978). This binding alters the receptor properties and promotes the translocation of the hormone-receptor complex to the target cell nucleus. Recently, a model explaining discrepancies in receptor localization and binding kinetics of estrogen to its receptor has been put forward. New enucleation techniques and immunocytochemistry using a monoclonal antibody against the estradiol receptor provide evidence for the nuclear distribution of both occupied and unoccupied receptor as expressed in a review by Gorski, Welshons and Sakai 1984. As well, the cooperativity of ligand receptor binding observed in vitro at high yet 'physiological' (1nM) concentrations of extracted receptor can be suppressed by immobilizing the receptor (ex. to a hydroxylapatite support), producing the binding behavior seen with intact cells or tissues. This new paradigm provides an interesting alternative to the two step model.

1.3 general aspects of steroid metabolism

Steroids can be metabolized quite extensively during their 'lifetime' in vivo. Reactions that are commonly encountered in steroid metabolism are: oxidation-reduction,

hydroxylation, conjugation and hydrolysis. The oxo group at position 17 of C_{18} and C_{19} steroids is susceptible to metabolic interconversion and dictates the potency of the steroid molecule. 17-ß-Estradiol ellicits the greatest estrogenic response in humans as well as other mammals, and the 17ß-hydroxysteroid dehydrogenase which reversibly converts estrone to 17ß-estradiol is present in all mammalian species studied in both steroid responsive and non responsive tissues (Williamson 1979). Oxido-reduction requires the participation of a cofactor that serves as electron donor and/or acceptor. 17ß-Hydroxysteroid dehydrogenases using NAD, NADP or both pyridine nucleotides as cofactor have been identified.

The introduction of a polar substituent such as glucuronic acid (GA), glucose (G), N-acetylglucosamine (NAG), sulfate, amino acids, etc., is a means of rendering the parent molecule more hydrophilic. The transferases catalysing the conjugation of a sugar moiety to the steroid hydroxyl or phenolic group are usually particulate enzymes, and the sugar donors are the uridine nucleotides (ex. uridine diphosphate glucuronic acid (UDPGA). A simplified model would assume the water soluble steroid conjugate to be its final form in the body, however, in some cases, it may be an intermediate form. Glycosidases having a high affinity for estrogen glycosides are widespread in many species. For example, a ß-N-acetyl-glucosaminidase which

effectively removes N-acetyl glucosamine from 17α-estradiol-3glucoside-17N-acetyl glucosamine is present in rabbit liver, kidney, spleen, plasma, large intestine, small intestine, uterus, ovary, epididymis and testis (Whittemore and Layne 1965).

2. Steroid transport

The interconversions undergone by a steroid from synthesis to excretion are varied, but subcellular compartmentalization of the transforming enzymes reduces the accessibility of the latter and makes transport of the steroid to the site a prime consideration in the fate of the hormone. Because of the nonpolar nature of the steroid hormones, it was generally assumed that the plasma membranes of target cells allowed free passage of these solutes into the cytoplasmic compartment where they interacted with the soluble receptor and were consequently retained (Rao 1981; Gorski and Gannon 1976; Graff, Woklhueter and Plagemann 1977). This binding of hormone with receptor was thought to be the first 'specific' step in the chain of events resulting in the phenotypic effect. Of late, investigators have challenged the preceeding statements by presenting evidence for the occurrence of specific entry processes for steroids into their targets.

Uptake encompasses transport and metabolic trapping

(Heichal, Ish-Shalom, Koren and Stein 1979). The transport component may be by simple diffusion, where it can be monitored by the association of the hormone with the system and/or by metabolic conversion. Transport can also occur by facilitated diffusion, which has its own kinetic parameters. By whatever process the hormone makes its way into the tissue, in vitro studies have shown that the uptake is very rapid, with the linear phase of uptake versus time lasting only seconds at physiological temperature (Rao 1981; Peck, Burgner and Clark 1973; Giorgi and Stein 1981). Uptake is also often accompanied by adsorption onto the external surfaces of membranes.

The biological activity of the hormone has been attributed to the fraction that is not bound (Rao 1981), and the bulk of steroid permeating the plasma membrane is also in the free form (Giorgi 1980, Rao, Rao and Breuer 1977, Rao 1981). Yet 85-95% of all circulating steroids are bound to carrier proteins in the plasma. It would appear that the carrier proteins may regulate the concentration gradient across the cell membrane. Another factor, that must be considered is that the bound ligand crosses the capillary wall very slowly, with the exception of the liver sinusoids that are considerably more permeable to proteins (Bloom and Fawcett 1975).

Within the target cell, at saturation, approximately 20%

of the steroid molecules are specifically bound to receptors. The fraction nonspecifically bound may attain 80%. Thus, the amount of free steroid, or 'chemically active solute' within the tissue could ultimately depend on the relative affinity of the non-specific binding in the intracellular and extracellular spaces, and on the transit time of the ligand flow through the tissues (Pardridge and Mietus 1979, Giorgi 1980, Baird, Horton, Longcope and Tait 1968). The problem is complex, and this section will present some of the evidence for simple diffusion as well as for a carrier mediated process.

2.1 passive diffusion

The least complex mode of entry, given the lipophilic nature of the steroid molecule, is by diffusion, which involves the partionning of the hormone into the membrane from the aqueous environment, its movement within the membrane and its partionning out of the membrane (Giorgi 1980, Plagemann and Erbe 1976, Graff et al. 1977). The velocity of passage of a substance across a cell membrane, or its permeability coefficient, is a function of its partition coefficient, its diffusion coefficient and the

inverse of the thickness of the membrane (Graff et al. 1977). Giorgi and Stein (1981) determined that n-octanol was a good model solvent for membrane lipids. If uptake occurs by simple diffusion, then ease of entry of various steroids should follow the trend set by partition coefficients between n-octanol and water in response to the number of polar substituents. The diffusion coefficient describes the rate of movement of a substance within the membrane and is a property of the penetrating substance, related to its molecular size, shape and charge. The effect of temperature on permeation is mainly on the diffusion, possibly due to a decrease in fluidity of membrane lipids between 37°C and 4°C (Graff et al. 1977).

In vivo studies on steroid transport do not generally yield details about the mode of entry of steroid hormones into cells. However, Beckett and Pickup (1971) reported that steroid absorption in the oral mucosa of human subjects occurred by passive diffusion. The 'entry' was reversible, but open, as steroid made its way into the general circulation independent of initial concentration and unaffected by the presence of a mixture of steroids.

Isolated tissue or slices offer the advantage of relatively intact cells still in contact with cell borders and natural barriers but devoid of endocrine control. Peck et al. (1973) showed that the initial rate of movement of

^{3}H-17-β-estradiol into rat uterus (a target tissue) and diaphragm (a non target tissue) was independent of the initial 17β-estradiol concentration in the medium, between 1 and 40 nM. Exposure of these tissues to the metabolic inhibitor 2-4 dinitrophenol and/or the sulfhydryl reagent N-ethylmaleimide had no effect on the initial uptake velocity. The inclusion of serum albumin in the incubations decreased the estrogen uptake at physiological temperature, a result consistent with a partition phenomenon. Another criterion for passive diffusion fulfilled by this experimental system was the noncompetition of a 100 fold excess of an unlabelled receptor ligand analog diethylstilbestrol (DES) for the uptake.

In studies on the binding of ^{3}H-17β-estradiol by isolated uterine cells Williams and Gorski (1971,1973) demonstrated that the initial binding process occurred much more slowly at 0°C than at 37°C. The total ^{3}H-estradiol binding by the cell suspension could be divided into two components. The first was non specific, non saturable and had an apparent activation energy of 2.5 Kcal/mole under initial uptake conditions (4min, 0-37°C), consistent with free diffusion. The second component was specific, saturable, and had an apparent activation energy of 20.7 Kcal/mole. The authors state that this value is consistent with either significant conformational contributions to binding, or a temperature dependent penetration of estradiol

into uterine cells. If in fact the entry process involves an additional interaction with membrane constituents, these constituents are not susceptible to the proteases used in cell isolation (pronase, trypsin).

Tseng, Stolee and Gurpide (1972) also failed to observe saturation of uptake during in vitro perfusion of slices of human endometrium and placenta with labelled estrone and 17β-estradiol, and concluded that the behavior indicated passive diffusion, or at best, a carrier system with a high dissociation constant that could not be saturated at physiological concentrations of the hormones.

Giorgi and Stein (1981) incubated cultured hepatoma cells (HTC-NIL) with 17β-estradiol, progesterone, testosterone, corticosterone and dexamethasone and found that the entry of steroid was linearly proportional to the substrate concentration in the medium (up to 28 nM). The uptake was not reduced by the presence of competing steroids, and the rate of passage of steroids through the membrane could be correlated to the partition coefficient of the steroid between water and n-octanol.

Plagemann and Erbe (1976) compared the entry of the synthetic glucocorticoid, prednisolone into Reuber hepatoma cells, a glucocorticoid responsive line and into Novikoff

cells,a cell line devoid of the cytosolic receptor. Prednisolone entered both cell lines to the same extent, the initial rates being similar. No saturation of steroid uptake was attained with a steroid concentration ranging 0.01 μM to 5 mM and there was no competition for entry by a two hundred fold excess of unlabelled corticosterone. Metabolic inhibitors such as KCN did not alter uptake, nor did preincubation of the cells with 5mM iodoacetate. Membrane perturbing agents such as phospholipase C and neuraminidase did not inhibit entry. From these results, it was concluded that prednisolone is taken up by both types of cells equally well and that simple diffusion is the mechanism of entry into both responsive and unresponsive cells.

2.2 facilitated transport

It is possible that there are specialized steroid transport systems in classical and nonclassical target cells. Antecedents for carrier systems for lipophilic molecules exist, for example, the low density lipoprotein receptor system by which free cholesterol is made available for use by cells (Goldstein and Brown 1977). Steroid transport systems could function to ensure an adequate supply of the hormone, to allow the direct transfer of steroid to its soluble receptor, to act itself as a receptor precursor (Jackson and Chalkey 1974), or to regulate the

metabolic fate of the hormone in tissues processing large quantities of steroids such as the liver and kidney.

Milgrom, Atger and Baulieu (1973) measured the uptake of tritiated 17β-estradiol by whole uterine horns from immature rats. They measured 17β-estradiol uptake indirectly by monitoring the amount of steroid specifically bound to the high affinity sites in cytosol and nuclear extracts after incubation, making the basic assumption that the rate of association of 17β-estradiol with the soluble receptor was not a limiting step. Also, initial velocities were measured during the first 5 to 15 minutes, a time during which uptake was found to be linear at 37°C. A scatchard type plot revealed a saturable component for entry at steroid concentrations up to 3ηM in the medium with an apparent K_D of 3.21ηM and a nonsaturable component at high concentrations, i.e. up to 40ηM. The tissue uptake also exhibited a temperature dependence, unlike receptor-estradiol binding that has a high association constant even at 0°C. The effect of sulfhydryl reagents such as iodoacetamide, 5,5'dithiobis(2-nitrobenzoic acid) and p-chloromercurophenylsufonate was evident on both the entry process and on the cell-free receptor binding, but the inhibition was greater when whole tissue was incubated. Metabolic inhibitors, or a one hundred fold molar excess of cortisol, corticosterone, testosterone or progesterone did not alter the entry process, whereas unlabelled

17β-estradiol and to a lesser extent, DES, competed for the apparent uptake. The results differ markedly from those of Peck et al. (1973) and Williams and Gorski (1973) and cannot be explained by a model of simple diffusion.

Evidence supporting the existence of a carrier mediated process for steroid translocation within the plasma membrane of rat hepatocytes has been presented. Using initial velocity measurements at 15°C, Rao et al. (1977) showed that the total uptake of estrone and 17β-estradiol by isolated cells was saturable in the micromolar range and inhibited by sulfhydryl reagents. Uptake was reduced by approximately 30% by the action of enzymes that disrupt membrane integrity, i.e. β-glucosidase, β-glucuronidase and phospholipase A; whereas neuraminidase, pronase and phospholipase D did not alter estrogen migration. Involvement of metabolic energy was indicated by the reduced transport upon treatment with 2,4-dinitrophenol (20nmolar) and Antimycin A. Although the specificity of uptake was not investigated in this system, the authors postulate the existence of a membrane component that facilitates the uptake of estrogen from the plasma and delivers it to the cell interior.

Denisov, Sergeev, Saksonov and Shutko (1980) found that the binding of 17β-estradiol to liver plasma membranes from female albino rat was temperature dependent and inhibited

noncompetitively by cortisol in a one hundred fold excess. A saturable component for 17ß-estradiol was detected, however at physiological concentrations practically all the 17ß-estradiol interacted with the unsaturable binding system.

3. Steroid transport relationships

3.1 Steroid transport and mechanism of action

Estrophilic binding sites have been detected on the plasma membrane of some target cells. The approaches used to demonstrate these binding sites include immunofluorescent detection of poly-estradiol binding sites and immobilization of whole cells on estrogen derivatized supports. The use of these probes that are not membrane permeable allows the measurement of cell surface interaction exclusive of true cytoplasmic receptor binding and nuclear translocation.

Nenci, Fabris, Marchetti and Marzola (1980) demonstrated that the outer surface of breast cancer cells binds a fluorescent macromolecular estradiol derivative (17ß-estradiol-6-carboxymethyloxime-bovine serum albumin fluorescein isothiocyanate). Fluorescent binding was abolished by preincubation of the cells with an antibody to

polyestradiol. The uniform membrane fluorescence was absent from mouse fibroblasts (non target cells) and could also be abolished from target cells by pre- or co-incubation of the latter with the non fluoreiscenated estradiol derivative.

Pietras and Szego (1977) examined the affinity of isolated endometrial, liver and intestinal cells from female rats to estrogen immobilized by covalent linkage to nylon monofilaments. They observed that the number of surface binding sites correlated with the capacity of the cells from these various tissues to accumulate and retain estrogen. In liver and endometrium the interaction was also temperature dependent, undergoing a four fold decrease when the temperature was lowered from 22°C to 4°C. The binding was due to the 17β-estradiol moiety of the fibers, as it was inhibited by prior incubation of the cells with 17β-estradiol and could be displaced by 17β-estradiol but not 17α-estradiol. Further studies by Pietras and Szego (1979b) showed that not all liver cells from immature rats were homogeneous with respect to their estrogen binding properties. Also, the cultured hepatocytes 'eluted' from the derivatized fibers appeared to be more responsive, as a group, to 17β-estradiol and the hepatocarcinogen dibutylnitrosamine than their nonbinding counterparts.

Jackson and Chalkey (1974) postulated that the membrane binding site is actually a precursor of the cytoplasmic

receptor, or the cytoplasmic receptor itself residing in the plasma membrane. Upon estrogen receptor complex formation, the entity can then undergo translocation and conversion to a form with high affinity for the nuclear chromatin. This model was initially refuted by a large number of investigators on the basis of insensitivity of the putative membrane 'receptor' to proteolytic enzymes and to certain sulfhydryl reagents (Williams and Gorski 1973); the failure of DES to compete efficiently with 17β-estradiol for surface binding (Nenci et al. 1980; Terayama, Okamura and Suyemtzu 1976; Rao et al. 1977) and on the stringent dependence of surface binding on ambient temperature (Rao et al. 1977). However, in a recent review Szego (1984) attributes the discrepancies in interpretation of the data to the uncontrolled disruption of tissues and cells during preparation of estrogen binding molecules. The concencus is that the plasma membranes of some target cells contain components which bind steroids with high affinity and specificity. However it has not been ascertained whether these binding sites play a role in hormone transit into cells. The other possible roles that could be attributed to these sites are that they act as an exchange intermediate with the external environment or as steroid metabolizing enzymes. They could also serve as a channel in the intracellular transfer to the nucleus or function in cell to cell recognition (Giorgi and Stein 1981).

3.2 Steroid transport and metabolism

i) uptake of conjugates

In general, conjugated steroids carry a charge at physiological pH and cross the membrane more slowly than free steroids, however their apparent permeability coefficients are much smaller than would be expected from their solubility in organic solvents (Giorgi and Stein 1981).

Holinka and Gurpide (1980) carried out studies with estrone and its sulfoconjugate. Upon injection of the radiolabelled compounds into mature female rabbits, label distribution indicated that estrone sulfate reaching hepatic tissues was directly utilized. The uterus, metabolized estrogens *in vitro* in the same manner as the liver, however it did not appear capable of utilizing the blood borne conjugate. Estrone sulfate in the circulation is strongly bound to serum albumin, and the fenestrations in liver sinusoidal epithelium may offer an easier access to ionized or bulky molecules than uterine epithelium.

Tseng *et al.* (1972) perfused slices of human placenta with radiolabelled estrone, 17β-estradiol, estrone sulfate and 17β-estradiol 3-sulfate, and observed that steroid uptake did not exhibit saturation at concentrations up to 1 micromolar. The sulfate derivatives entered term placental slices as readily as estrone and estradiol, a finding not

expected if simple diffusion was occurring.

In isolated rat liver cells, estrone sulfate is taken up, in the absence of measurable sulfatase activity, by a process that is saturable, (apparent Km of 0.8±0.17μM), exhibits its pH optimum in the physiological range, is temperature dependent, and is sensitive to sulfhydryl reagents and metabolic inhibitors. The uptake of estrone sulfate is sodium dependent and the steroid competes for uptake with sodium taurocholate. Thus, the uptake of estrone sulfate by rat hepatocytes exhibits all the hallmarks of an energy requiring, carrier-mediated transport system (Lopez Del Pino 1978, Schwenk and Lopez Del Pino 1980).

ii) metabolism-linked transport

The only direct evidence for metabolism-linked steroid transport has been presented by Lefebvre, Shultz, Groman and Watanabe (1979a,b). Their study involved the uptake of testosterone into membrane vesicles from Pseudomonas testosteroni. The behavior of the uptake process with respect to pH optimum, specificity, cofactor requirements, effect of sulfhydryl reagents and interaction with specific antibodies indicated that a surface bound NAD-dependent 3(17)β-hydroxysteroid dehydrogenase was involved in the uptake process and during transport, testosterone was oxidized to androstenedione.

iii) enterohepatic circulation

In most mammals the major route of excretion of estrogens is the urine. For example, in rats only 5 to 10% of administered estrogens are recovered in the feces. However there is considerable evidence for the enterohepatic circulation of estrogens and other steroids. Natural and synthetic estrogens and progestins are excreted to a considerable extent in bile, principally as their glucuronide derivatives (Dada and Martins, 1983). These steroids may be absorbed from the intestine and re-enter the circulation. In some cases steroid metabolism also occurs in the intestine.

The absorption of estrogens is most likely controlled by their structure, concentration and their interaction with other gut contents. In an early study by Smith, Tapley and Ross (1963), it was determined that the in vitro transfer of radiolabelled estrone or 17β-estradiol from rat mucosa to serosa occurred equally well along the entire small intestine. The transport of labelled 17β-estradiol was carried out in everted sacs at 37°C for two hours and resulted in the accumulation of a water soluble conjugate of estrone on the serosal side. The mucosal medium contained estrone and a small amount of the conjugate, all of the estradiol having been oxidized.

Intestinal metabolism may affect the bioavailability of exogenous as well as endogenous estrogens. The effect of certain drugs on intestinal metabolism with respect to estrogen transport was investigated by Dada and Martins (1983). Incubation of acetylsalicylic acid or phenobarbitone with isolated rat intestine sacs appeared to alter the intestinal estrogen glucuronyl transferase and estrogen-3-sulfatase resulting in an inhibition of 17β-estradiol and estrone sulfate transport by up to 50% of control values. Conversely, chloroquine phosphate stimulated both the rate and net absorption of the steroids. Ethanol, in concentrations of 40 to 400mM inhibited conjugate transport but enhanced free 17β-estradiol uptake.

In general estrogen conjugates are hydrolyzed prior to uptake of the steroid by intestinal mucosa and microbial inhabitants of the gastro-intestinal tract are a major source of the hydrolytic enzymes (Back, Breckenridge, Chapman, Crawford, May, Orme and Rowe 1981; Huijghebaert, Sim, Back and Eyssen 1984). The supply of enzymes may be altered by antibiotics as reflected in a decrease in plasma and urinary estriol levels within 2-4 days of the administration of therapeutic oral doses of antibiotics to pregnant women (Dada and Martins 1983). Neomycin and lincomycin action in the gastro-intestinal tract of rabbits has been linked to an increased plasma clearance of

ethinylestradiol and in rats the suppression of gut microflora leads to an increase in faecal excretion of conjugated estrogens (Back, Breckenridge, Cross, Orme and Thomas, 1982).

4. Objectives of thesis research

The objective of this thesis was to study the uptake of estrogens by rabbit tissues and to determine the role of this transport process in the metabolism and biological action of estrogens. The small intestine was chosen for initial studies because this tissue does not have appreciable amounts of the soluble estrogen receptor (Tong, Layne, Dostaler and Williamson 1983) which could interfere with the measurement of estrogen transport. However, the extent to which estrogens undergo enterohepatic circulation in the rabbit (Quamme, Layne and Williamson 1971b) indicates that this tissue is active in estrogen transport.

The preliminary studies were carried out with everted sacs, an *in vitro* system close to the *in vivo* situation with the absorptive surface exposed and cell-cell junctions intact. The everted sacs were replaced by isolated epithelial cells with which *in vitro* parameters are more easily controlled and interpreted, and ultimately, binding

to membrane fractions was investigated. Comparison of uptake of both free and conjugated estrogens was undertaken in order to assign a role to the endogenously formed glycosides. Uptake of estrogens by liver tissue and hepatocytes was examined to show the similarities in transport of estrogens by target and nontarget tissues.

The rabbit was chosen as the experimental model since estrogen metabolism in this species is well defined and involves the formation of conjugated derivatives (Layne 1970). By the 'classical' definition of a hormone, 17β-estradiol is the most potent estrogen in the rabbit, 17α-estradiol being an inactive product of estrogen metabolism (Williams, Henry, Collins and Layne 1968, Williamson and Layne 1970). Interconversion of these two forms is carried out by distinct 17β- and 17α-hydroxysteroid dehydrogenases present primarily in the liver and kidney. The major metabolite excreted in urine when either estrone, 17α-estradiol or 17β-estradiol is administered to rabbits is 17α-estradiol-3GA-17NAG (Layne, Sheth and Kirdani 1964; Layne 1965; Gwilliam, Paquet, Williamson and Layne 1974). This conjugate is formed by the sequential addition of the sugars to the estrogen nucleus. Glucuronic acid is transferred from UDPGA to the 3-hydroxyl position of estrone, 17α-estradiol or 17β-estradiol. Transfer of N-acetyl-glucosamine from UDPNAG occurs only to the 17α-hydroxyl group of 17α-estradiol 3-glucuronide

(Layne, Labow and Williamson 1975). Sulfate conjugates have not been detected in rabbit excreta although rabbit liver can synthesize estrogen sulfate in vitro (Layne et al. 1964; Collins, Williams and Layne 1967; Collins, Jirku and Layne 1968; Quamme, Layne and Williamson 1971a).

The tissue distribution of the NAG transferase parallels that of the glucuronyl transferase. Both enzymes have been detected in the microsomal fraction of rabbit intestine, liver and kidney. The relative abundance of the two activities in the latter tissues relates to the strict sequence of metabolic conjugation. The supply of both enzymes is more abundant in liver than in kidney, however, in rabbit kidney the neutral sugar transferase has the higher measurable activity, preparing the monoglucuronide for excretion in the urine as the double conjugate (Layne 1970). Two other membrane associated transferase activites have been demonstrated in rabbit liver. The first transfers glucose from UDPG to the phenolic hydroxyl of estrone, 17α-estradiol or 17β-estradiol; the second transfers the sugar moiety from the donor nucleotide to the 17α-hydroxyl of 17α-estradiol-3-glucuronide (Comerton, Layne and Williamson 1978; Labow, Williamson and Layne 1973, Labow, Williamson, Layne and Collins 1975). 17α-Estradiol-3-glucuronide-17-glucoside is a minor excretory product in urine of rabbits injected with ^{3}H-estrone (Layne, Labow, Paquet and Williamson 1976).

However, 3-glucoside formation has only been demonstrated under *in vitro* conditions.

Heteroglycosidases having a high affinity for estrogen glycosides are widespread in many species. For example, a steroid β N-acetyl-glucosaminidase which effectively removes the 17NAG from 17α-estradiol-3GA-17NAG is present in rabbit liver, kidney, spleen, plasma, large intestine, small intestine, uterus, ovary, epididymis and testis (Whittemore and Layne 1965). The predominant glycolytic activity in metabolic tissues is generally β-glucuronidase. However some rabbit tissues also have a soluble steroid β-D-glucosidase. The enzyme, detected in kidney, small intestine and liver has a very high affinity for the phenolic glucosides of estrone, 17β-estradiol and 17α-estradiol. (Mellor and Layne 1974).

In the rabbit, 85% of an injected dose of 17β-^{3}H-estradiol is recovered in urine as 17α-estradiol-3-glucuronide 17N-acetyl-glucosaminide (the double conjugate). However, during the first 24 hours post injection, 20-30% of the label can be detected in bile as the double conjugate (≈80%), the monoglucuronide of 17α-estradiol and 17β-estradiol and as free steroid (Quamme *et al.* 1971b). The fact that up to 75% of the metabolites excreted in the bile are finally excreted in the urine,

indicates that steroid reabsorption occurs in the intestine.

CHAPTER II MATERIALS AND METHODS

1. MATERIALS

Unlabelled steroids were purchased from the Sigma Chemical Company, St. Louis, Missouri, U.S.A. [6,7-^{3}H]17β-Estradiol, specific activity 44 Ci/mmole; [2,4,6,7-^{3}H]17β-estradiol, specific activity 108 Ci/mmole; [6,7-^{3}H]estrone, specific activity; 47.9 Ci/mmole and Inulin-carboxyl, (carboxyl-^{14}C), specific activity 2.0 mCi/g were obtained from the New England Nuclear Corporation, Boston, Massachussetts. Uridine Diphospho-D-(U^{14}C) glucuronic acid, ammonium salt, specific activity 283 mCi/mmole was purchased from Amersham Corporation, Oakville Ontario.

[6,7-^{3}H]17β-Estradiol 3-glucuronoside (E_2β3GA) was prepared by incubating [6,7-^{3}H]17β-estradiol (E_2β) (108 Ci/mmole) with 0.5ml of a preparation of rabbit liver microsomes and 0.5 μmol uridine diphospho-D-glucuronic acid (UDPGA). Incubation conditions and extraction of the reaction product were carried out as described by Collins et al. (1968). Purification of E_2β3GA was carried out by preparative thin layer chromatography on Silica Gel N in the solvent system: chloroform: isopropanol: formic acid (5:3:1). [6,7-^{3}H]17β-estradiol 3-glucoside was prepared from [6,7-^{3}H]17β-estradiol 3-glucuronide as described by

Collins, Williamson and Layne (1970). The glucoside was then purified by preparative thin layer chromatography on silica gel N using the solvent system: chloroform: ethanol (4:1) (Williamson, Collins, Layne, Conrow an Bernstein 1969). The conjugates of estradiol labelled with ^{14}C in the glycoside moiety were prepared by the method outlined above. In this case, the estrogen was unlabelled ($E_2\beta$) and the sugar nucleotide was uridine diphospho-D-($U^{14}C$) glucuronic acid (283mCi/mmole). [6,7]-^{3}H-17α-Estradiol 3-glucuronide 17-N-acetylglucosaminide ($E_2\alpha$-3GA-17NAG) was isolated from the urine of female rabbits that had been injected with [6,7-^{3}H]estrone (E_1). The conjugate was adsorbed on Amberlite XAD-2 and eluted in methanol (Bradlow, 1968). The conjugate was further purified by preparative thin layer chromatography in chloroform : isopropanol: formic acid (5:3:1).

Nylon monofilament bolting cloth (Nitex) was obtained from B & SH Tompson & Co. Ltd., Montreal, Canada. Trypan blue was obtained from Gibco, Grand Island, New York. Bio Rad Protein Assay Kit was from Bio Rad Laboratories (Canada) Ltd., Mississauga, Ont. Amberlite XAD-2 resin lot 348611 was purchased from BDH Laboratories, Montreal, Quebec. β-Glucuronidase from beef liver (Ketodase),was from General Diagnostics, Morris Plains, New Jersey. Silicone oils AR20 and AR200 were from Wacker Chemie, Germany. Silica Gel N was obtained from Macherey Nagel and Co., Germany.

Microfuge tubes were 400 µl polyethylene from Sarstedt, St. Laurent Quebec. Glass fiber filters were Reeve-Angel 934-AH from a local supplier.

All other chemicals used were of analytical grade and obtained from commercial suppliers.

2. METHODS

2.1 Isolation and identification of estrogen metabolites

Two methods were employed to isolate and identify estrogen metabolites formed after incubation of estrogen substrates with tissue or isolated cell preparations. In the first method, estrogens present in the incubation medium were adsorbed to Amberlite XAD-2 and eluted from the resin with methanol (Bradlow, 1968). The second method involved the differential extraction of the estrogen metabolites from the incubation medium as described by Collins et al. (1968) Both methods gave very similar results. Identification of metabolites present in the eluates or extracts was achieved by cochromatographing the labelled steroids on 0.25 mm silica gel N with authentic standards. The free estrogens estrone, 17α- and 17β- estradiol were resolved by chromatography in the solvent system benzene:acetone (4:1). Estrogen glucosides were identified by chromatography in the

solvent system chloroform:ethanol (4:1). Estrogen glucuronides and the double conjugate 17α-estradiol-3GA-17NAG were identified by chromatography in the solvent system benzene:methyl ethyl ketone:ethanol:water (3:3:3:1) or chloroform:isopropanol:formic acid (5:3:1).

2.2 Liquid Scintillation Counting

Radioactivity was measured in a Searle Mark III 6880 liquid scintillation counter. Nonpolar samples were counted in 10 ml of a toluene-based cocktail containing 4.0 g/l PPO (2,5-diphenyl-oxazole). Polar samples were counted in 10 ml of cocktail containing a 1:1 ratio of Aquasol-2 (New England Nuclear Corp., Boston Mass) and xylene. Samples containing cell and membrane protein were counted in 10 ml Aquasol-2 or a cocktail consisting of 100 µl Protosol (New England Nuclear) and 10 ml Aquasol-2.

2.3 In vitro studies with tissue preparations

2.3 i) preparation of tissues

a) intestine

Tissue sections of small intestine consisted of everted sacs. Clean sections of small intestine, approximately 2cm in length, were everted and secured at both ends with surgical thread to avoid direct contact of the incubation medium with the connective tissue. The weight of these

everted pieces varied between 0.9 and 1.1g.

b) liver

Liver tissue was kept on ice and slices approximately 0.5mm thick were prepared with a Stadie Riggs apparatus. The number of slices giving an initial wet weight between 0.7 and 0.9g (usually 5 slices) was transferred into the incubation medium.

2.3 ii) incubation procedures

The liver tissue slices and everted intestine sections were incubated in 5ml of suspension buffer containing 0.1 nmol of the radiolabelled steroid. Incubations were carried out in 25ml beakers on ice or in a water bath at 37°C for the specified time. At the end of the incubation period the medium was removed with a Pasteur pipet and extracted with 5ml of benzene to extract the unconjugated estrogens and arrest any enzymatic activity. The benzene layer and the medium were stored at -20°C until analysed as described in section 2.1. The tissues were immediately flushed three times with 10ml of ice cold suspension buffer and the washes were discarded.

The liver tissue was homogenized in 10 mls of methanol with a Polytron homogenizer. For small intestine, the mucosal cells were scraped and washed into the methanol

prior to homogenization. The resulting suspension from each tissue was transferred to a 15ml COREX centrifuge tube and spun at 5,000 rpm in the SS-34 rotor of a Sorvall refrigerated centrifuge, to sediment the insoluble particles. The pellet was washed with an additional 5ml of methanol and the two methanol fractions combined. An aliquot of this methanol extract was reserved for liquid scintillation counting to estimate tissue-associated label and the remainder was taken to dryness by evaporation under a stream of nitrogen. The dried residue was redissolved in 2ml of distilled water and analysed for estrogen metabolites as described in section 2.1.

2.4 In vitro studies with isolated cell preparations

2.4 i) preparation of intestinal epithelial cells.

Epithelial cells of rabbit small intestine were isolated as described by Kamac, Siedlecki and Zmudzka 1977. Female rabbits (average age 3 months, weight 2kg) were killed by cervical dislocation. The small intestine was dissected and the adhering mesenterum removed. The intestine was sectioned into pieces 15cm in length. Each intestine section was rinsed thoroughly with an ice cold solution of 0.15M NaCl containing 1mM dithiothreitol (DTT), and kept on ice in this solution until everted.

The intestine 'sections' were everted with the aid of a

fine teflon pestle. Both ends of the tissue were secured with surgical thread to avoid contact of buffers with the connective tissue. These everted sections were extensively rinsed in ice cold 0.15M NaCl, containing 0.5mM DTT and kept on ice until ready to be incubated (less than 30 minutes).

The everted sections were first incubation in 250ml of citrate dissociating medium [NaCl, 8.3 g/l; KCl, 0.5g/l; HEPES, 2.4 g/l and $Na_3C_6H_5O_7.2H_2O$, 7.94 g/l dissolved in distilled water and titrated with 1M NaOH to pH 7.4 at 25°C]. The incubation was carried out in a shaking water bath at 37°C for 15 min. The everted intestine was then rinsed by immersing in a further 75ml of warmed citrate dissociating buffer. The rinse and incubation media were discarded as they contained dead cells and debris. The small intestine pieces were then transferred to 75ml of EDTA medium [NaCl, 8.3 g/l; KCl, 0.5 g/l; HEPES, 2.4 g/l and EDTA, disodium salt, dihydrate, 0.56 g/l dissolved in distilled water and titrated with 1M NaOH to pH 7.0 at 25°C]; and incubated for 30 minutes at 37°C. The tissue was then removed and rinsed in a further 50ml of warmed EDTA medium. The incubation procedure was repeated a total of three times, so that the total incubation time in EDTA buffer was 90min. The incubation media and rinses were combined, cooled on ice for 5 min and filtered through two layers of Nitex (250μ /100μ). The filtrate was centrifuged at 750 rpm in a Sorvall HS-4 rotor for 5min and the

supernatant discarded. The cell pellet was washed three times by suspending the pellet in EDTA medium followed by centrifugation at 750 rpm. The final cell pellet was suspended in ice cold suspension buffer [NaCl, 4.0 g/l; KCl, 0.4 g/l; $CaCl_2 .2H_2O$, 0.18 g/l; $MgCl.6H_2O$, 0.13 g/l; KH_2PO_4, 0.15 g/l; Na_2SO_4, 0.1 g/l; HEPES, 7.2 g/l; TES, 6.9 g/l and Tricine, 6.5 g/l; dissolved in distilled water at room temperature and titrated to pH 7.6 with 1M NaOH] and filtered through one layer of Nitex (61μ)

Viability of the epithelial cells was estimated by exclusion of the vital dye Trypan Blue (Baur, Kasperek and Pfaff, 1975, Schreiber and Schreiber 1973). Cell concentration and viability were estimated by counting two different dilutions of cells in a Neubauer counting chamber under the 10X objective of an optical microscope. Protein content of the cell suspension was determined by the method of Bradford (1976).

2.4 ii) Isolation of hepatocytes from rabbit liver.

Rabbit hepatocytes were prepared by the method of P.O. Seglen (1972). The liver of a female rabbit anaesthetized with methoxyflurane was perfused <u>in situ</u> from the portal vein with isotonic EDTA medium (as with intestine cells) at 37°C, for 30 minutes. Without interrupting the perfusion, the liver was dissected and the EDTA medium was replaced by

a solution containing 0.5g% collagenase and 0.1g% hyaluronidase. The liver was transferred to a buchner funnel that sat on top of a 500 ml erlenmeyer flask kept at 37°C. Recirculating perfusion of the liver enabled the use of only 250ml of enzyme solution, and was continued for 15 min or until enzymatic digestion of the capillaries inhibited further perfusion. The cells could then be isolated by gently scraping the distended liver with a spatula. The collected cells and tissue debris were then incubated at 37°C for 30 min to allow completion of the collagenase action. This was followed by rapid cooling and subsequent manipulations were carried out on ice. The cell suspension was filtered through two layers of Nitex (250μ/150μ), and the filtrate was centrifuged at 300rpm in a Sorvall HS-4 rotor for a total of 5min. The cell pellet was washed three times by gentle ressuspension in cell suspension buffer(section 2.4i). Cell viability and concentration was estimated as described for the intestinal epithelial cells. In studies on steroid uptake, cells were used within 6 hours of preparation.

2.4 iii) Estrogen uptake by isolated intestinal epithelial cells and hepatocytes

Freshly prepared cells in suspension buffer were equilibrated for five minutes at the assay temperature, Incubations were initiated by the addition of the

radiolabelled estrogen in ethanol to the cell suspension to give the desired final steroid concentration. A typical incubation consisted of: 247.5µl of cell suspension (1 mg protein/ml) to which was added 2.5µl of the radiolabelled steroid solution (5pmol). In experiments designed to compare uptake of various estrogens, the specific activitiy of the starting material was adjusted to be in the range of 32.4 to 40.3 Ci/mmol. The incubation was carried out in 15ml test tubes in order to provide a large air-buffer interface. After the incubation, the cells and incubation medium were separated by one of two methods. In initial studies, separation was achieved using a glass fibre filter as described by Rao et al. (1977). An aliquot of the incubation mixture was pipetted into 1 ml of ice cold 0.15M NaCl atop a 2.4cm glass fiber filter supported in a Millipore apparatus. The medium was then removed by suction and the filter washed twice with 5ml of ice cold 0.15M NaCl. The filter paper was then transferred to a scintillation vial and 1 ml of ethyl acetate, followed by 10 ml of the scintillation cocktail 'Aquasol-2' were added. Control incubations to measure free steroid trapped in the glass fiber filter consisted of suspension buffer plus radioactive steroid in the appropriate proportions, carried through the same steps. Values for these controls did not exceed 0.8% of total dpm applied to the filter. If time studies were undertaken, where the sampling number was greater, the volume of the suspension was increased, the steroid

concentration being kept constant, and aliquots were removed at the indicated time intervals and filtered as described above.

An alternate method involving centrifugation through a layer of silicone oil (Klingenberg and Pfaff, 1967; Anwer, Kroher and Hegner, 1976) was employed for the rapid separation of cells from incubation medium. 200µl of a 1:1 mixture of silicone fluids AR 20 and AR 200 (from Wacker Chemie, Munich) was pipetted into 400µl polyethylene microfuge tubes. A 50 or 100µl aliquot of the incubation suspension to be analysed was transferred to the top of the silicone oil. The tubes were then centrifuged for 60 seconds in a Beckman model 152 microfuge. This procedure enabled the separate analysis of both the medium and cell pellet. The medium was removed with a pasteur pipet and transferred to a test tube containing 500µl of methanol. This fraction was taken to dryness under a stream of nitrogen, and subjected to thin layer chromatography with standards to determine the metabolites present in the medium as described in section 2.1. Radioactivity in the cell pellet was measured to determine steroid uptake. In some incubations, the cell pellet was extracted with absolute methanol and the methanol extract was analysed for steroid metabolites. A 1:1 mixture of the silicone oils was found to give optimal separation of cells from medium as monitored by the exclusion from the cell pellet of ^{14}C-carboxy-inulin,

a large molecular weight carbohydrate (Anwer et al. 1976, Conway and Downey 1950). Blank values for cell-free incubations led to the recovery, in the bottom of the tube, of less than 0.9% of the total dpm layered on the silicone oil.

2.4 iv) Efflux of steroids from small intestine epithelial cells

In order to study efflux, steroids were first incorporated into cells by incubating radioactive [6,7-^{3}H]17β-estradiol ($1X10^{6}$ dpm, 102 Ci/mmol) with 5ml of a cell suspension (4mg protein/ml) for 3 hours on ice. This incubation temperature was found suitable because preliminary results indicated a lack of measurable steroid metabolism under these conditions. At the end of the three hour incubation period, the cells were diluted with 10 ml of ice cold suspension buffer and centrifuged at 1000 rpm in the SS34 rotor of a Sorvall refrigerated centrifuge. The cell pellet was then gently resuspended in 10 ml of fresh, ice cold suspension buffer and recentrifuged. This washing procedure was repeated once again and the final cell pellet was taken up in 1 ml of the suspension buffer. The radioactivity associated with the cells was determined by layering 0.1 ml of the cell suspension on top of 200µl of the silicone oil mixture (as in 2.4 iii) and centrifuging for 60 seconds in the Beckman microfuge. The cell pellet

was then counted in 10 ml of Aquasol-2.

Portions (0.5 ml) of the cell suspension were then incubated at 4°C and at 37°C. Aliquots were removed at the time intervals and cell associated radioactivity was determined as described above. The steroid metabolites present in the medium and in the cell pellet were determined as in the uptake experiments.

2.4 v) Enzyme assays

For purposes of comparison of uptake and metabolism data, all assays for enzyme activity were performed using the conditions employed to measure steroid uptake. The 17α-and 17β-hydroxysteroid dehydrogenase activities were measured as described by Lau, Layne and Williamson (1982). Incubations contained 3nmol of steroid substrate, 0.5μmol of the cofactor (NAD or NADP) and enzyme source in a final volume of 3.0 ml. The incubation was carried out for 30 min at 37°C and the reaction terminated by extracting the steroids with 5ml of benzene. The benzene layer was taken to dryness under a stream of nitrogen and analysed as in section 2.1.

Glucosyl and glucuronyl transferase activities were measured as described by Collins, Williamson and Layne (1970). Transfer was effected by incubating the tissue

fraction in the presence of 5 pmol [6,7-^{3}H]-17α-estradiol or [6,7-^{3}H]-17α-estradiol 3-glucuronide and 0.5μmol of the sugar nucleotide uridine diphosphoglucose or uridine diphosphoglucuronic acid in a final volume of 3.0 ml. Incubations were carried out in a shaking water bath at 37°C for 60 min. The steroid aglycone was then extracted into benzene. The glucoside was recovered by extraction of the incubate with ethyl acetate. In the case of the glucuronide, acidification of the medium to pH 2 was carried out prior to ethyl acetate extraction.

Hydrolysis of estrogen glycosides was monitored by quantitation of the steroid aglycone that was liberated by the enzyme action (Mellor and Layne 1971, 1974). A typical assay consisted of the labelled glucoside substrate (0.5nmol), enzyme sample and suspension buffer to a total volume of 1 ml. Times and temperatures paralleled conditions used for uptake studies. Reactions were terminated by the addition of benzene (1 ml) to extract the estrogen aglycone which was then quantified by liquid scintillation counting.

2.5 Studies with brush border and basolateral membranes.

2.5 i) Preparation of plasma membrane fractions from rabbit small intestine

The small intestine from a female rabbit was thoroughly rinsed with ice cold 0.9% NaCl and everted. The mucosa was scraped with a glass slide and the cells obtained were homogenized in 75 volumes of ice cold 5mM EDTA, pH 7.5 for 20 seconds in a Sorvall omnimixer at setting 9. Enriched brush border and basolateral membrane fractions were prepared from this homogenate by the methods of Forstner, Sabesin and Isselbacher (1968), Parkinson, Ebel, DiBona and Sharp (1972) and Wilson and Treanor (1977). The procedure is described in Figure 1. The following solutions were prepared:

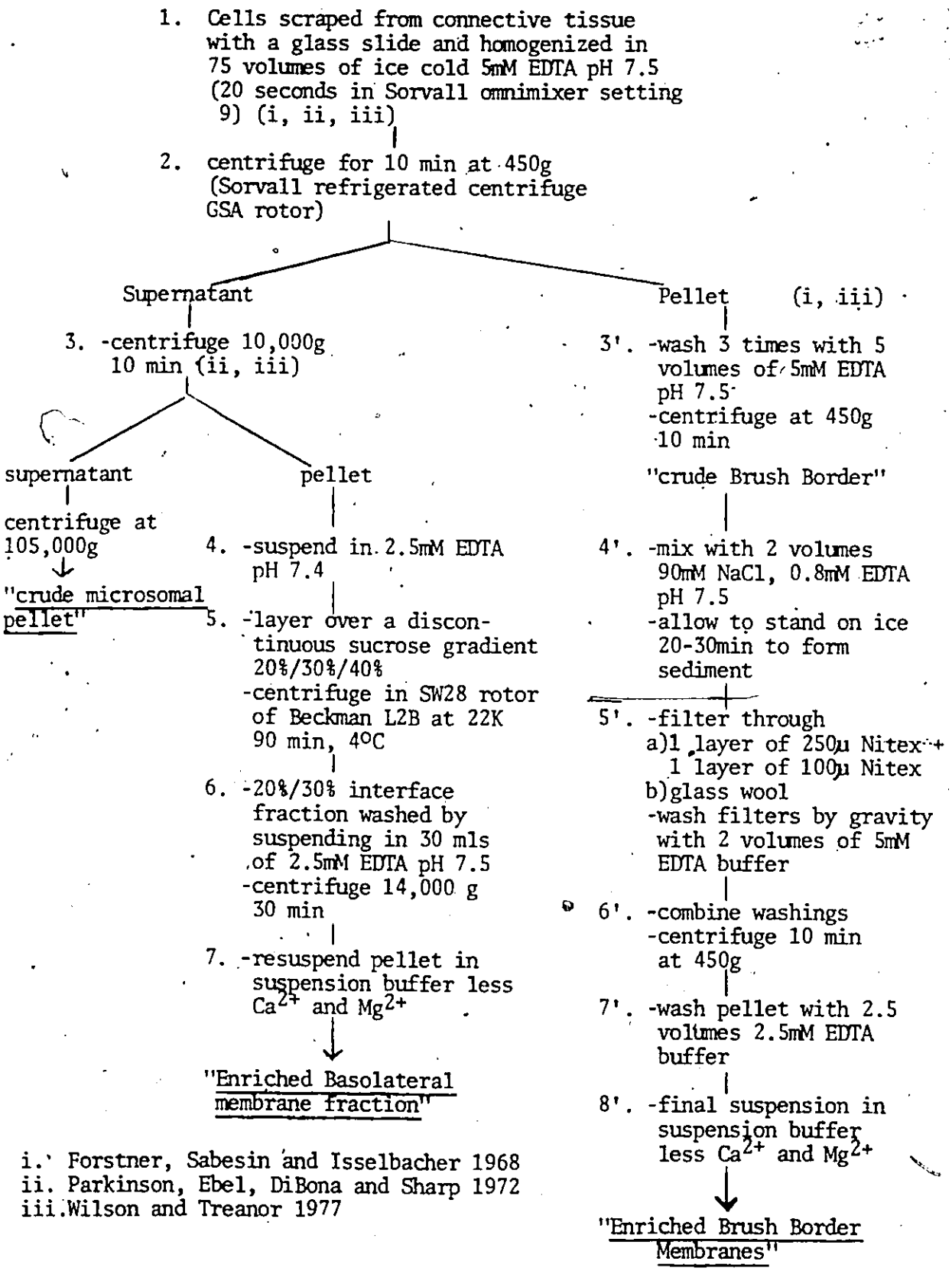
1) 10mM EDTA: 3.72 g of disodium EDTA dihydrate was titrated to pH 7.4 with 1M NaOH and brought to a final volume of 1 l. This stock solution was then used to prepare 5mM and 2.5mM EDTA.

2) 0.8mM EDTA 90 mM NaCl: 149mg of disodium EDTA dihydrate and 2.63 g NaCl in distilled water brought to pH 7.4 with 1M NaOH and to a final volume of 0.5 l.

3)Sucrose solutions for discontinuous gradients were percent weight per volume at 25°C.

2.5 ii) Preparation of membrane fractions for Electron Microscopy

Fig 1. Preparation of membrane fractions from small intestine



i. Forstner, Sabesin and Isselbacher 1968
ii. Parkinson, Ebel, DiBona and Sharp 1972
iii. Wilson and Treanor 1977

Samples of basolateral and brush border membranes were obtained by the method described in section 2.5i. The pellets (approximately 15mg protein) were suspended in 1ml of 0.9% NaCl. The membranes were fixed for one hour in 5ml of 4% glutaraldehyde in 0.02M cacodylate buffer, pH 7.0. The brush border specimen was centrifuged at 600g for 45 minutes to form a compact pellet. Basolateral membranes were centrifuged at 12,000g in the SS 34 rotor of a Sorvall refrigerated centrifuge for one hour. The glutaraldehyde was then removed with a pasteur pipet and replaced with 3ml of an osmium tetraoxide (OsO_4) solution (2% OsO_4 in 0.02M cacodylate buffer, pH 7.0). The pellets were teased into small fragments with a glass rod and allowed to stand for one hour. The solution was then replaced by fresh OsO_4 and the fragments again allowed to stand for one hour, after which, 1.5ml of the solution was removed.

The samples were dehydrated by a graded series of ethanol washings: 50%, 70%, 95% to a final one hour wash in 100% ethanol. The fixed, dehydrated membrane fragments were cleared with 3ml of styrene, which is miscible with both the ethanol and the embedding solution Vestopal W. The styrene was finally replaced by Vestopal W, a polyester resin to which is added a peroxide initiator and activator. The embedding solution containing fixed membrane fragments was mixed at room temperature for 48 hours on a rotator, then transferred to dried gelatin capsules and stored in an oven

at 60°C for 4 days prior to sectioning.

2.5 iii) Steroid binding to isolated membrane fractions

Membrane fractions were suspended in suspension buffer to give a protein concentration of 1mg/ml and 250µl of this suspension was incubated with radiolabelled estrogen. The estrogen concentration was $2X10^{-8}$M unless otherwise stated. Incubations were carried out in duplicate on ice with occasional shaking or at 37°C in a gently shaking water bath. After incubation, 100µl of the incubation mixture was assayed for radioactivity and 100µl was pipetted into 1ml of ice cold 0.15M NaCl atop a 0.9cm glass fiber filter in a Millipore apparatus and filtered with suction. The filter was subsequently washed with 0.15M NaCl (2X5ml) and transferred to a scintillation vial. Ethyl acetate (1 ml) was first added and after 15 min 10ml of Aquasol-2 was added for liquid scintillation counting. Control incubations containing labelled substrate in suspension buffer were carried out to determine nonspecific binding of steroid to the filter.

Results

1. Uptake and metabolism of estrogens by small intestine everted sacs

Experiments were undertaken with everted segments of small intestine, an *in vitro* model that does not involve extensive disruption of cell barriers. The protocol for measuring estrogen uptake by tissue segments is described in section 2.3.ii of Methods. The uptake of 17β-estradiol by small intestine everted sacs is shown in Fig. 2. The data indicate that the uptake is both temperature and time dependent. The pattern of metabolites present in the tissue and medium is also shown in Fig. 2. At 4°C the predominant estrogen present in both the tissue and medium after 5 and 30 minutes of incubation is 17β-estradiol. However, at 37°C the glucuronide derivative is the major metabolite present in the tissues. The glucuronide metabolite is also present in the medium, particularly after 30 minutes of incubation. After 30 min incubation, there is also a significant amount of the double conjugate 17α-estradiol-3-GA-17-NAG present in both tissue and medium.

The results obtained when small intestine everted sacs were incubated with 17α-estradiol or estrone were similar to those shown for 17β-estradiol in Fig 2. Further analysis of

Figure 2.

Uptake and metabolism of unconjugated estrogen by small intestine everted sacs

2 cm sections (wet weight 0.9-1.1 g) of everted intestine were incubated in 5ml of suspension buffer containing 0.1×10^{-9} moles of radiolabelled estrogen.
The incubation medium was removed and analysed as in Methods section 2.1
The washed sacs were scraped into methanol and homogenized.
The methanol extract was analysed as for the medium

total estrogen

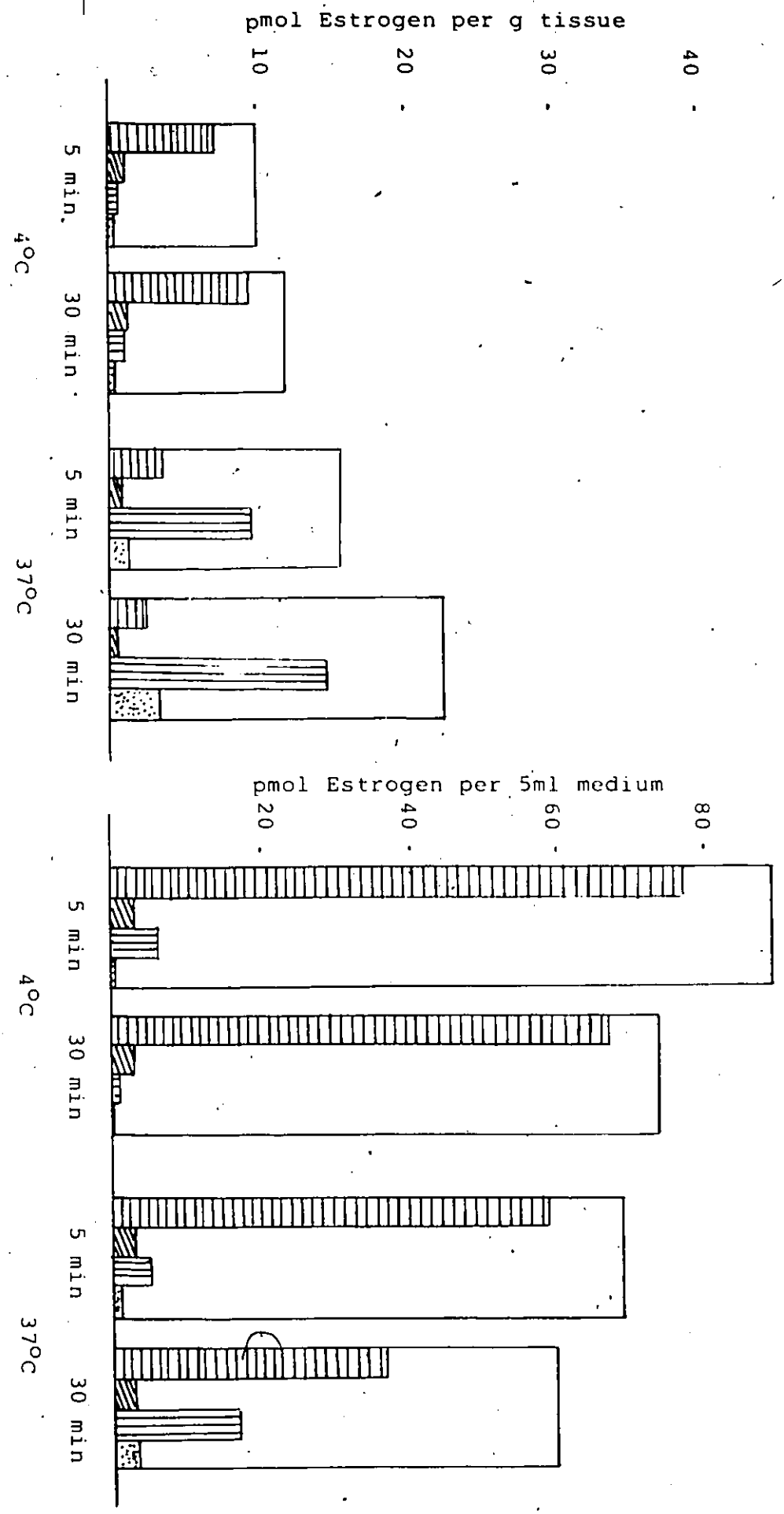
contribution of unconjugated estrogen to total

contribution of 3-glucoside to total

contribution of 3-glucuronide to total

contribution of the double conjugate to total (17α-estradiol-3-glucuronide-17-N-acetylglucosaminide)

Further analysis of free estrogen fraction extracted from tissue and incubation medium showed that there was only slight metabolism of the 17-oxy position, and most of the estrogen recovered was the starting material.


pmol Estrogen per g tissue
10
20
30
40
5 min.
30 min
5 min
30 min
4°C
37°C
pmol Estrogen per 5ml medium
20
40
60
80
5 min
30 min
5 min
30 min
4°C
37°C

the free estrogen fraction extracted from tissue and incubation medium when estrone and 17α-estradiol were incubated showed that most of the estrogen recovered was the starting material.

Uptake of 17α-estradiol 3-glucuronide-17-glucoside, 17β-estradiol 3-glucoside and 17α-estradiol 3-glucuronide was also measured. Of these, only incubations with the glucoside produced appreciable cell associated label by a process that was markedly time and temperature dependent. Analysis of the estrogens present in the medium revealed that the glucose moiety was being removed, even at 4°C and the free steroid was being metabolised to the glucuronide derivative within the tissue. (data not shown)

The hydrolysis of 17β-estradiol 3-glucoside in the medium and the appearance of an estrogen glucuronide derivative prompted the assay for β-glucosidase, β-glucuronidase and glucuronyl transferase activities in the medium. Enzyme activities shown in Fig. 3 are a percent of the total activity present in a 20% homogenate of the scrapings of a 1.0± 0.1g everted sac. The β-glucosidase is the predominant activity recovered in the incubation medium. The amount released increases with time to 25% of the total available after 60 minutes incubation. The β-glucuronidase is less available and does not increase significantly with time. No transferase activity was detected, even in the

Figure 3.

Enzyme release from small intestine everted sacs

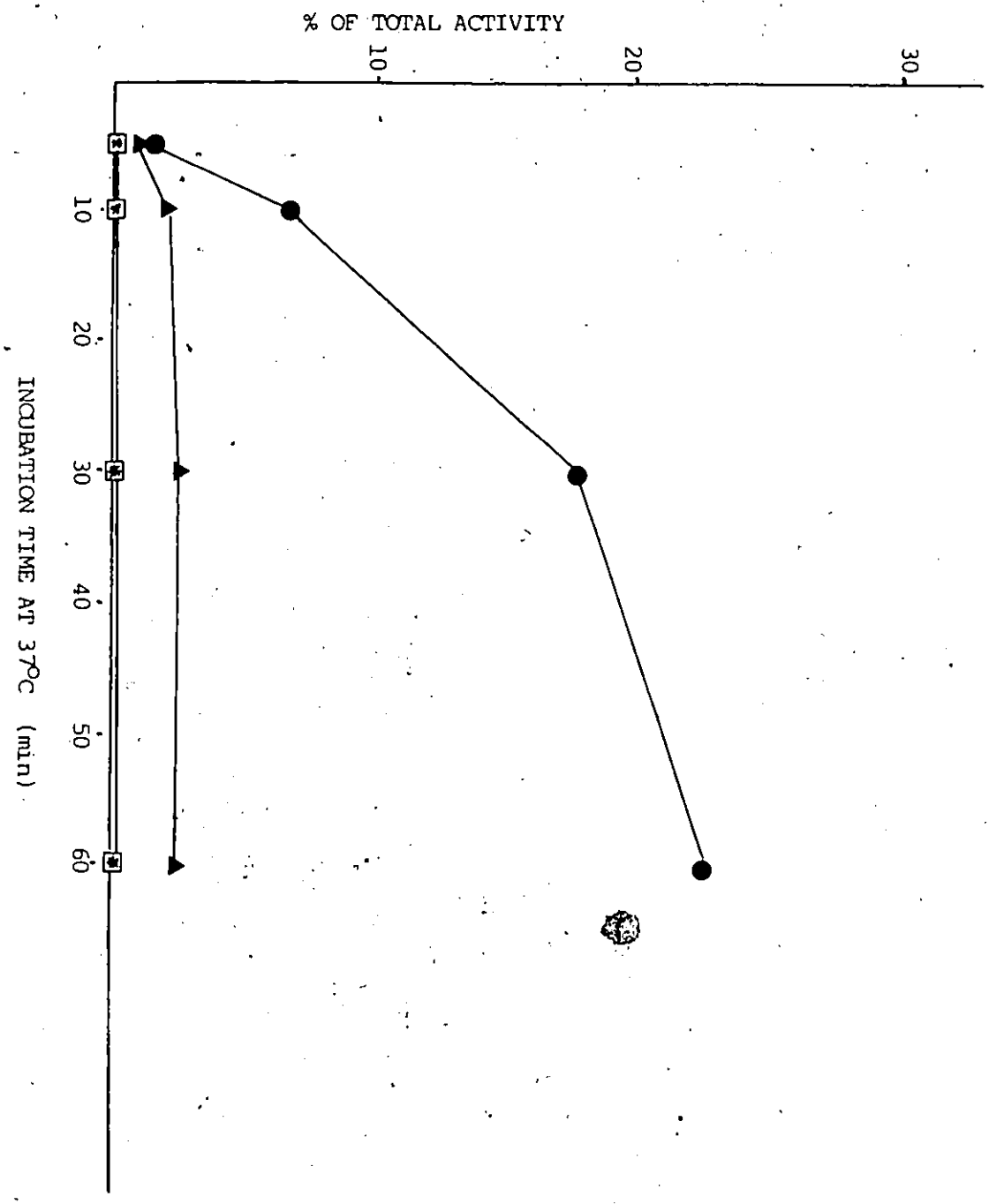
All enzyme activities were assayed in suspension buffer pH 7.6, the incubation buffer for uptake experiments at a steroid substrate concentration of 0.5 µM. Enzyme activity is expressed as a percent of the total activity in 1ml of a 20% homogenate (w/v) of scrapings of a 1.0 ± 0.1 g everted sac. Glucuronyl transferase activity was assayed with and without the addition of 0.5 µmol of UDPGA.

● β-glucosidase (total activity: 1104 pmol/ml 60 min)

▲ β-glucuronidase (total activity: 374 pmol/ml 60 min)

□ glucuronyl transferase - no cofactor (total activity: 316 pmol/ml 60 min)

✱ glucuronyl transferase + UDPGA 0.5 µmol/ml (total activity: 1516 pmol/ml 60 min)


% OF TOTAL ACTIVITY
10
20
30
INCUBATION TIME AT 37°C (min)
10
20
30
40
50
60

presence of added cofactor.

2. Small intestine epithelial cells

Isolated epithelial cells were obtained by the method of Kamac et al. The yield from a starting wet weight of 50 g of small intestine was $1.3X10^9$ cells. The mean viability was 83.8±2.9%, and 1mg cell protein was obtained from $2.9X10^6$ cells.

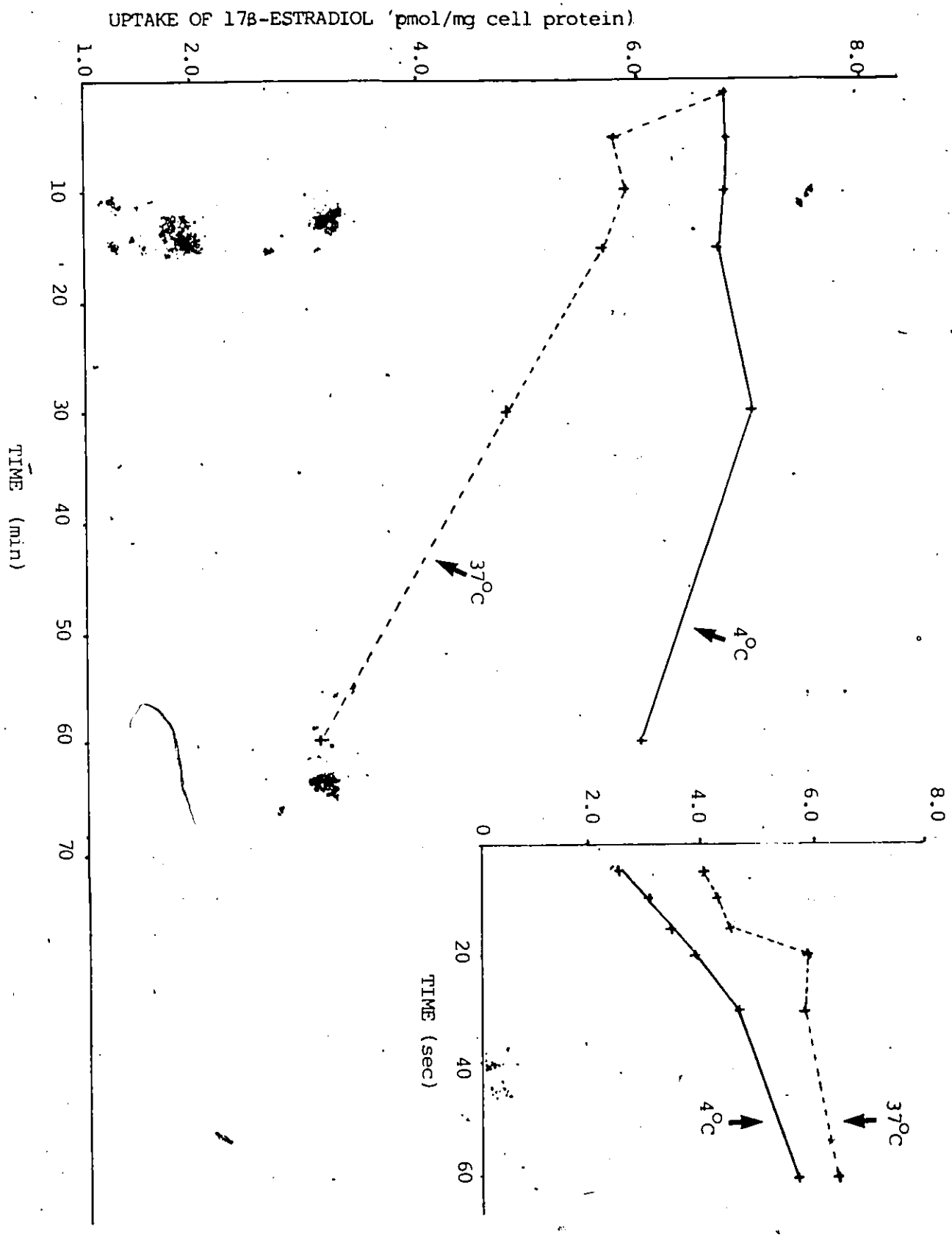
2.1 Uptake and metabolism of free estrogen

Preliminary experiments were carried out to establish the optimal conditions for measuring estrogen uptake by isolated epithelial cells. Uptake of 17β-estradiol (10^{-7}M) by isolated cells was linear with respect to cell protein concentration up to 4.2mg/ml at 4°C and 37°C. In incubations with 1mg/ml cell protein the uptake of 17β-estradiol was proportional to steroid concentration over the range 10^{-11} to $5X10^{-6}$M (data not shown). The uptake of 17β-estradiol as a function of incubation time and temperature is shown in Fig. 4. Uptake of 17β-estradiol by epithelial cells is very rapid. The rate of association of label with cells is marginally faster at 37°C, but at both temperatures, near maximum uptake is reached by 60 seconds. At 4°C, the cell associated label remains constant for at least 30 minutes with a slight decrease noted at 60 minutes,

Figure 4.

Uptake of 17β-estradiol by isolated epithelial cells of small intestine

Cells (final concentration 1mg/ml protein) were incubated in the presence of (6,7-^{3}H)-17β-estradiol at a final concentration of 2 X 10^{-8}M in suspension buffer pH 7.6 (volume 250 µl). Cell associated label was determined after centrifugation through silicone oil, as described in the methods. Inset shows early sampling times (less than one minute).


UPTAKE OF 17β-ESTRADIOL (pmol/mg cell protein)
1.0
2.0
4.0
6.0
8.0
10
20
30
40
50
60
70
TIME (min)
37°C
4°C
0
2.0
4.0
6.0
8.0
20
40
60
TIME (sec)
4°C
37°C

whereas at the higher temperature, there is a progressive decrease in cell associated label from the maximum value at 60 seconds as the incubation time is extended.

The effect of medium pH on uptake of 17β-estradiol was also investigated. The pH of suspension buffer was varied between 6.0 and 8.5 and the percent cell associated label determined after a fifteen minute incubation at 37°C. Maximal uptake of 17β-estradiol was observed at pH 7.0 with a rapid decrease below this pH (Fig. 5). The decrease in estrogen uptake was more gradual in alkaline medium. The viability of the isolated cells was reduced under these conditions by 7.5% at both pH extremes.

It has been shown that a large proportion of the circulating steroid is bound to a plasma protein (Munck and Wira 1975). This state enhances the solubility of the steroid and allows for the attainment of a greater overall concentration. Therefore the effect of bovine serum albumin (BSA) on the uptake of 17β-estradiol by isolated epithelial cells was evaluated and the results are shown in Fig. 6. When BSA was included in the incubation medium at a concentration greater than 5 mg/ml there was a considerable decrease in cell associated label. 30% of the maximum uptake was obtained in the presence of 25 mg/ml BSA. At BSA concentrations of 0 to 5 mg/ml, there was a slight

Figure 5.

Effect of pH on uptake of 17β-estradiol by small intestine epithelial cells.

Final cell protein concentration was 1.0 mg/ml and that of (6,7-^{3}H)-17β-estradiol was 1 X 10^{-9} M in suspension buffer pH 6.0 - 9.0. 250µl incubations were carried out at 37°C for 15 min.

Points are mean ± standard deviation of 4 determinations.

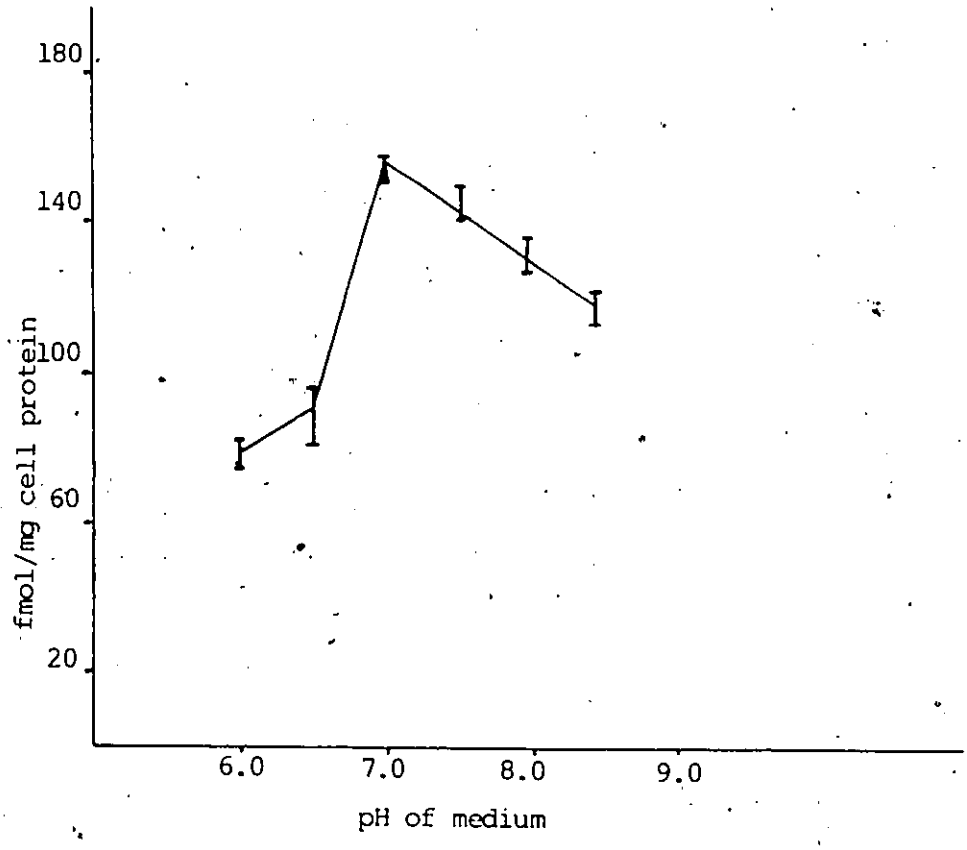
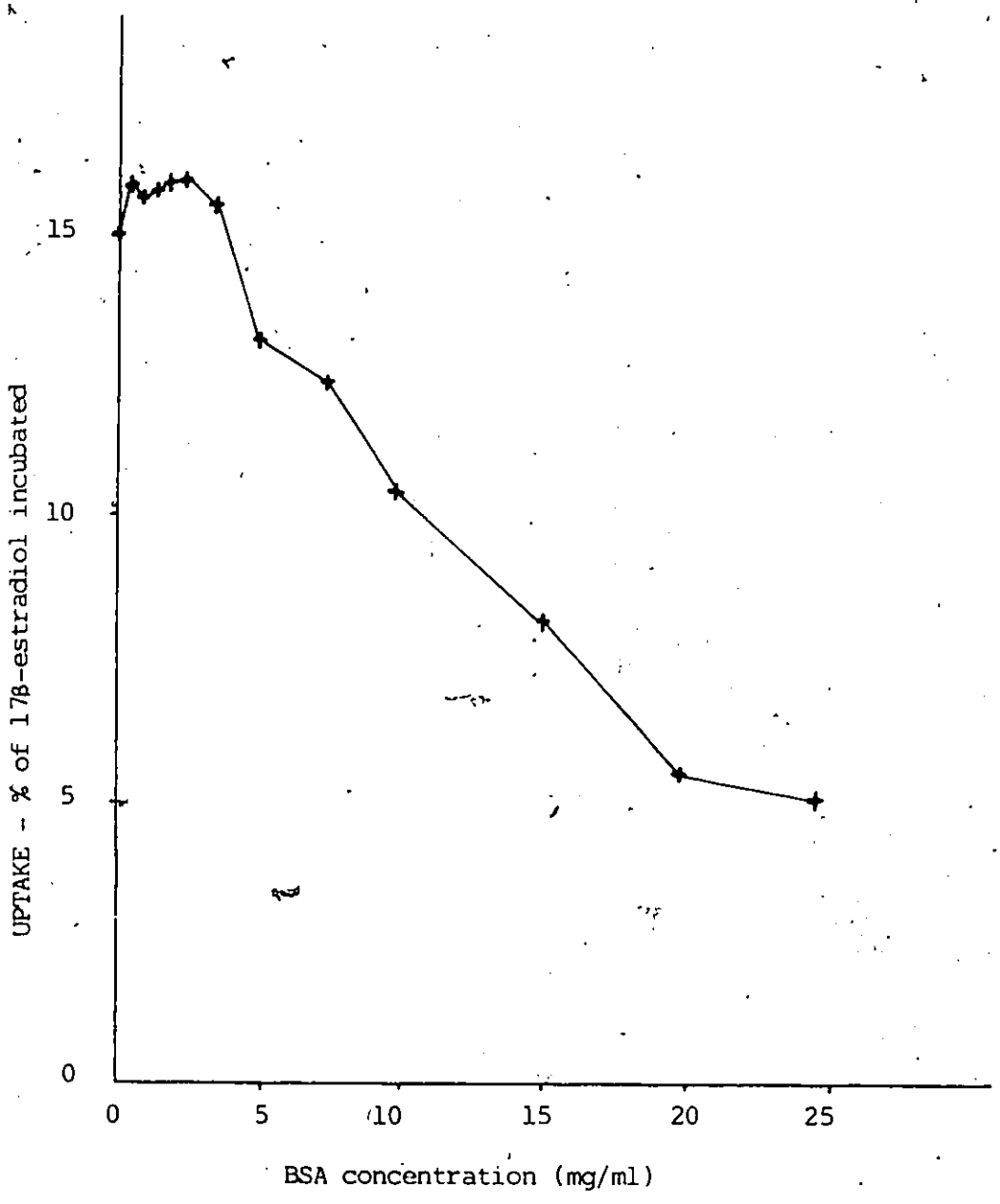

180
140
100
60
20
fmol/mg cell protein
6.0
7.0
8.0
9.0
pH of medium

Figure 6.

Effect of serum albumin on the uptake of ^{3}H-17β-estradiol by small intestine epithelial cells

The incubation medium contained small intestine epithelial cells at a final concentration of 1mg protein/ml in suspension buffer.
The ^{3}H-17β-estradiol concentration was 5 X 10^{-9}M and bovine serum albumin (BSA) was added to a concentration of 0 to 25 mg/ml in a final volume of 500µl.
The incubations were carried out for 10 min at 37°C. Cell associated label was determined by centrifugation of the incubation contents through a layer of silicone oil as described in Methods.
The results are the average of three separate experiments.


UPTAKE - % of 17β-estradiol incubated
0
5
10
15
0
5
10
15
20
25
BSA concentration (mg/ml)

enhancement of uptake.

The metabolism of [6,7-^{3}H]17β-estradiol that occurs during incubation with isolated epithelial cells is shown in Fig. 7. 17β-Estradiol was recovered unchanged from both the medium and the cell fraction when it was incubated with epithelial cells at 0-4°C. However at 37°C there was a loss of 17β-estradiol from the medium accompanied by the appearance of estrone and estrone-glucuronide. Estrone was the predominant steroid in the cell pellet. The amount of this metabolite present in the cell pellet increased slightly during the initial incubation period (0-10 min) and then decreased with longer incubation times. The other major metabolite present in the cell was estrone glucuronide, its level remained essentially constant throughout the experiment.

2.2 Uptake and metabolism of conjugates

Uptake of 17β-estradiol 3-glucoside by cells occurs at 37°C but not to an appreciable extent at 0-4°C (Fig 8). At the higher temperature there is an initial increase in the uptake of radioactivity but prolonged incubation results in a decrease in cell associated radioactivity. 17β-Estradiol 3-glucuronide is not taken up to a significant extent by

Figure 7.

Metabolism of 17β-estradiol during uptake by epithelial cells of female rabbit small intestine

1mg/ml cell protein in suspension buffer was incubated on ice and at 37°C in the presence of 2×10^{-8} M ^{3}H-17β-estradiol. At the indicated times, cell and medium were separated by centrifugation through a layer of silicone oil and analysed as in section 2.1.

- ○ 17β-estradiol
- ● estrone
- △ 17β-estradiol-3-glucuronide
- ▲ estrone-3-glucuronide

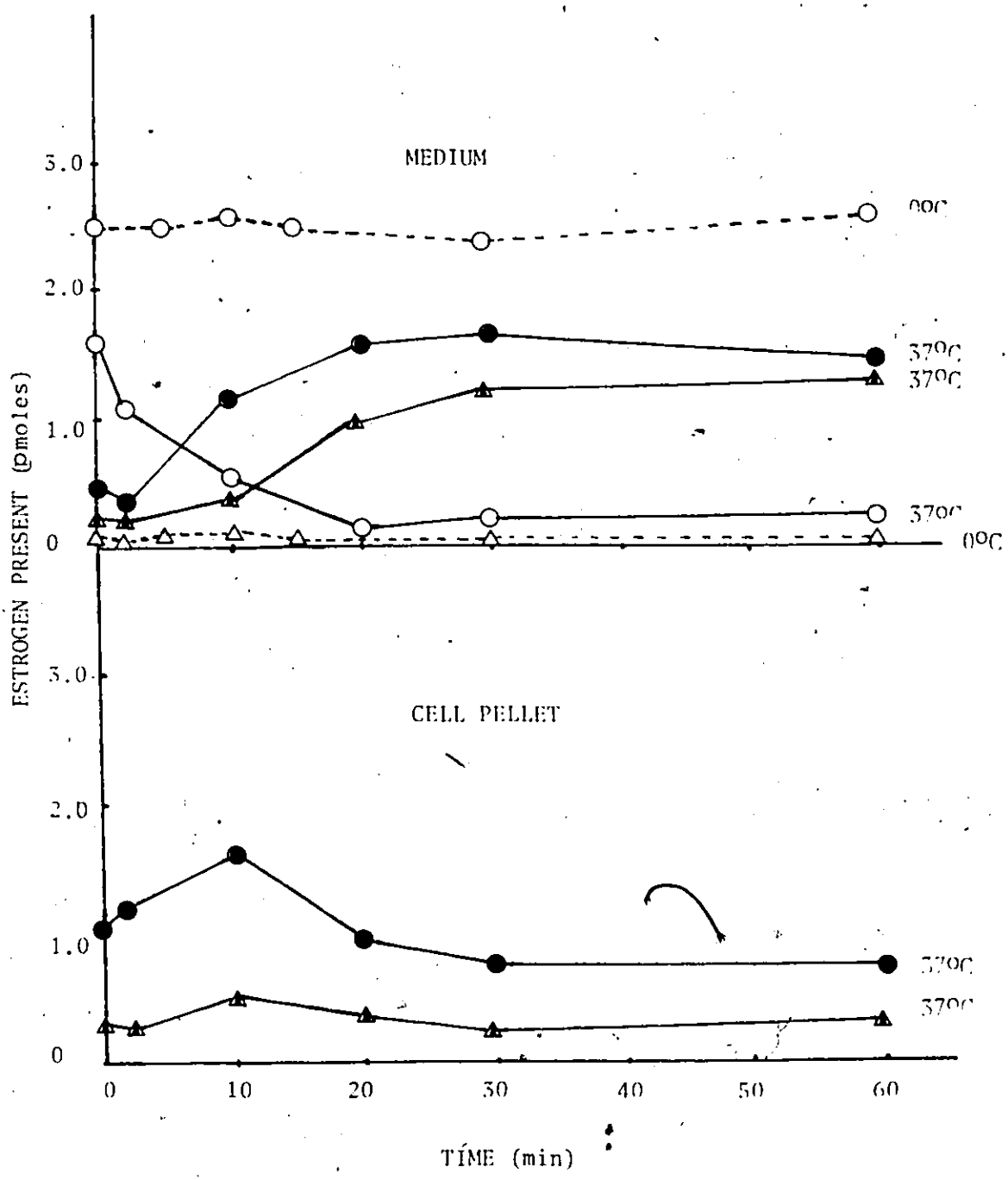

MEDIUM
3.0
2.0
1.0
0
0°C
37°C
37°C
37°C
0°C
ESTROGEN PRESENT (pmoles)
CELL PELLET
3.0
2.0
1.0
0
37°C
37°C
0
10
20
30
40
50
60
TIME (min)

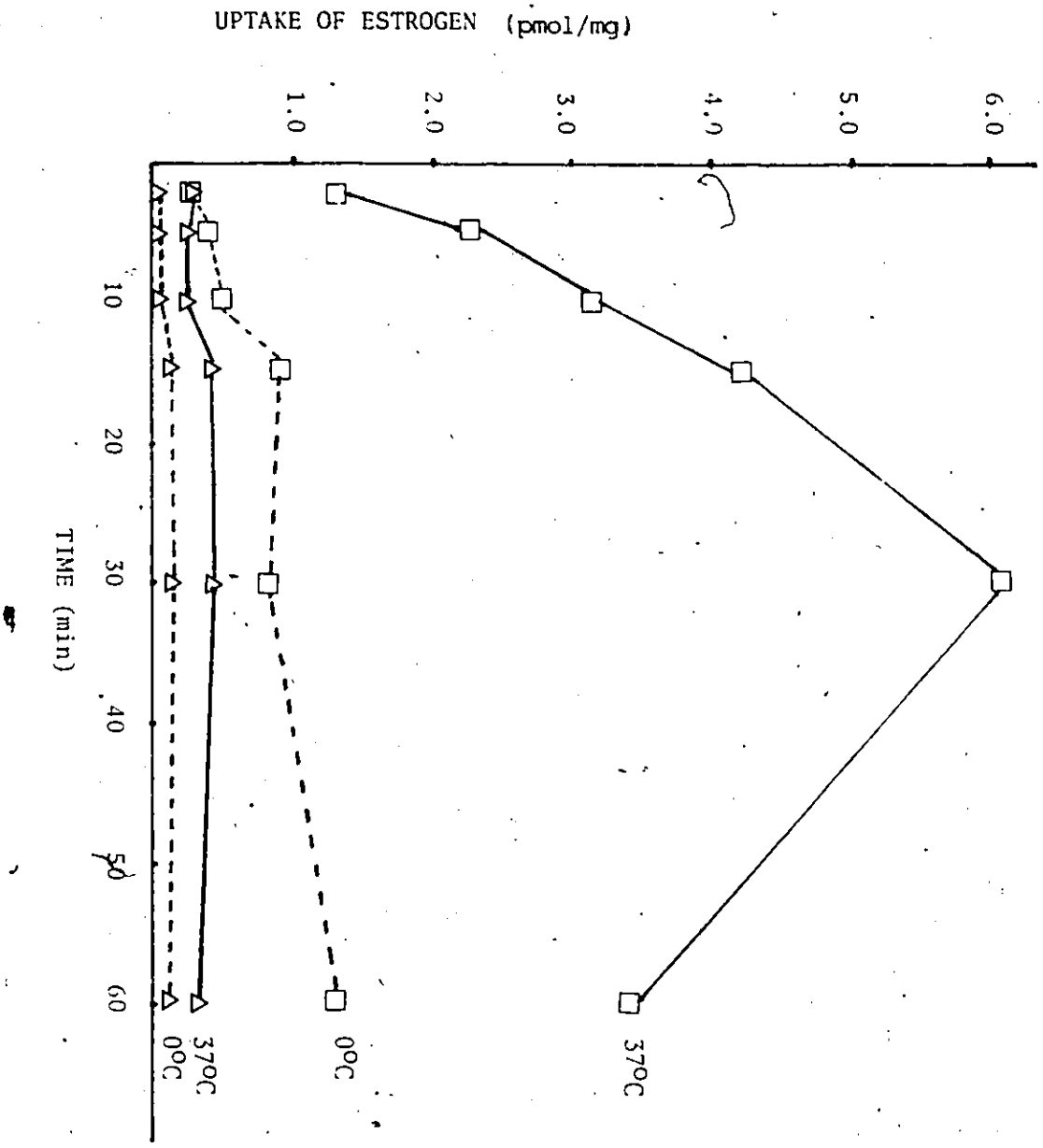
Figure 8.

Uptake of 17β-estradiol 3-glucoside and 17β-estradiol 3-glucuronide by epithelial cells of female rabbit small intestine

1 mg/ml cell protein was incubated in the presence of 2×10^{-8} M radiolabelled steroid in suspension buffer. The total volume of the incubation mixture was 250 µl. The uptake was determined by counting the cell pellet after centrifuging the incubation mixture through a layer of silicone oil as described in the Methods (2.4ii).

□ uptake with 17β-estradiol 3-glucoside

△ uptake with 17β-estradiol 3-glucuronide


UPTAKE OF ESTROGEN (pmol/mg)
1.0
2.0
3.0
4.0
5.0
6.0
TIME (min)
10
20
30
40
50
60
37°C
0°C
0°C
37°C

epithelial cells at either temperature. Incubation of 17α-estradiol 3-glucuronide-17-N-acetyl-glucosaminide gave results identical to those obtained with the glucuronide (data not shown).

The metabolism of 17β-estradiol 3-glucoside under the conditions employed for uptake was also determined and the results are shown in Fig 9. Metabolism at 4°C is not significant. However, at 37°C there is a rapid loss of the glucoside from the medium with a corresponding increase in 17β-estradiol and estrone 3-glucuronide.

Uptake of radioactivity from 17β-estradiol 3-glucoside labelled with ^{14}C in the sugar moiety was compared to the uptake of radioactivity from this conjugate labelled with ^{3}H in the steroid moiety (Fig 10). The uptake of radioactivity with the ^{14}C-labelled conjugate did not follow the pattern obtained with the tritium labelled compound, the ^{14}C-label being taken up by cells at a much slower rate. Identification of the cell associated ^{14}C compound was not undertaken.

Uptake studies with 17β-estradiol and its 3-glucoside derivative demonstrated the presence of metabolites of these estrogens in the incubation media (Figs 7 and 9). It was possible that the enzymes responsible for this metabolism were released from cells under the conditions used for the

Figure 9.

17β-Estradiol 3-glucoside metabolites present in the medium during uptake by epithelial cells of female rabbit small intestine

Small intestine epithelial cells at a concentration of 1 mg/ml cell protein in suspension buffer were incubated with an initial concentration of 2×10^{-8} M of (6,7-^{3}H)-17β-estradiol 3-glucoside. Uptake was determined by counting the cell pellet after centrifuging the incubation medium with cells through a layer of silicone oil as in Methods . The cell-free medium was analysed for labelled metabolites at the indicated times.

○-○ 17β-estradiol, 0°C

○—○ 17β-estradiol, 37°C

□-□ 17β-estradiol 3-glucoside, 0°C

□—□ 17β-estradiol 3-glucoside, 37°C

▲—▲ estrone 3-glucuronide, 37°C

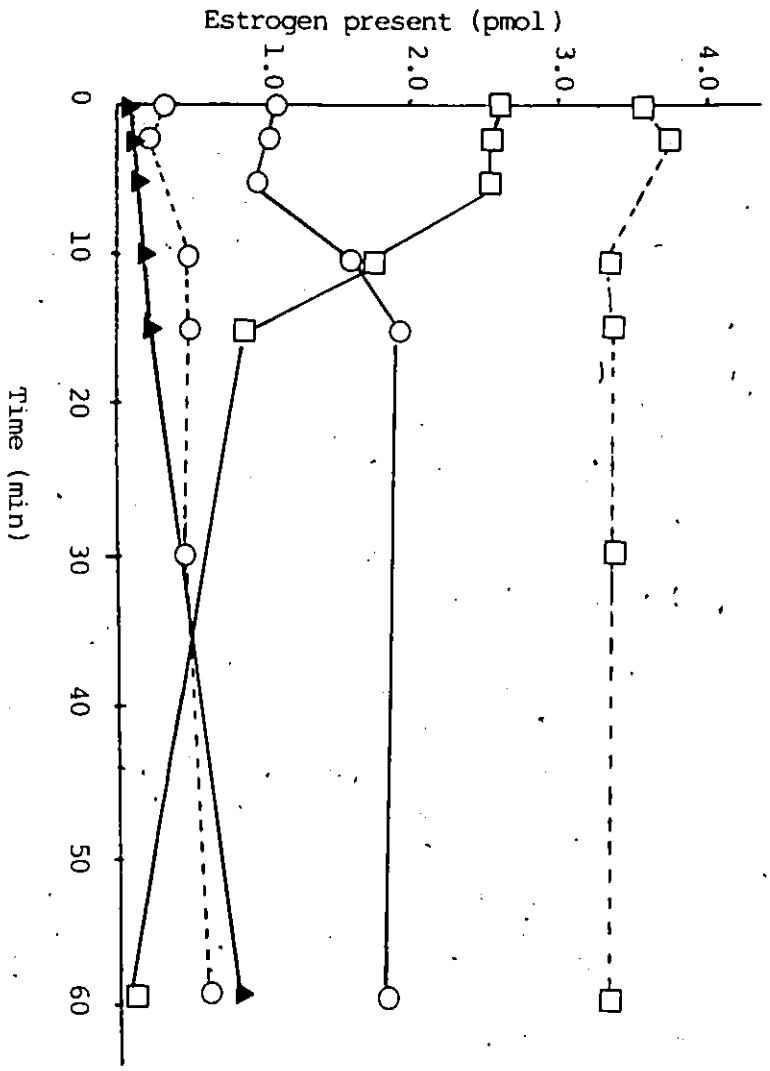

Estrogen present (pmol)
1.0
2.0
3.0
4.0
0
10
20
30
40
50
60
Time (min)

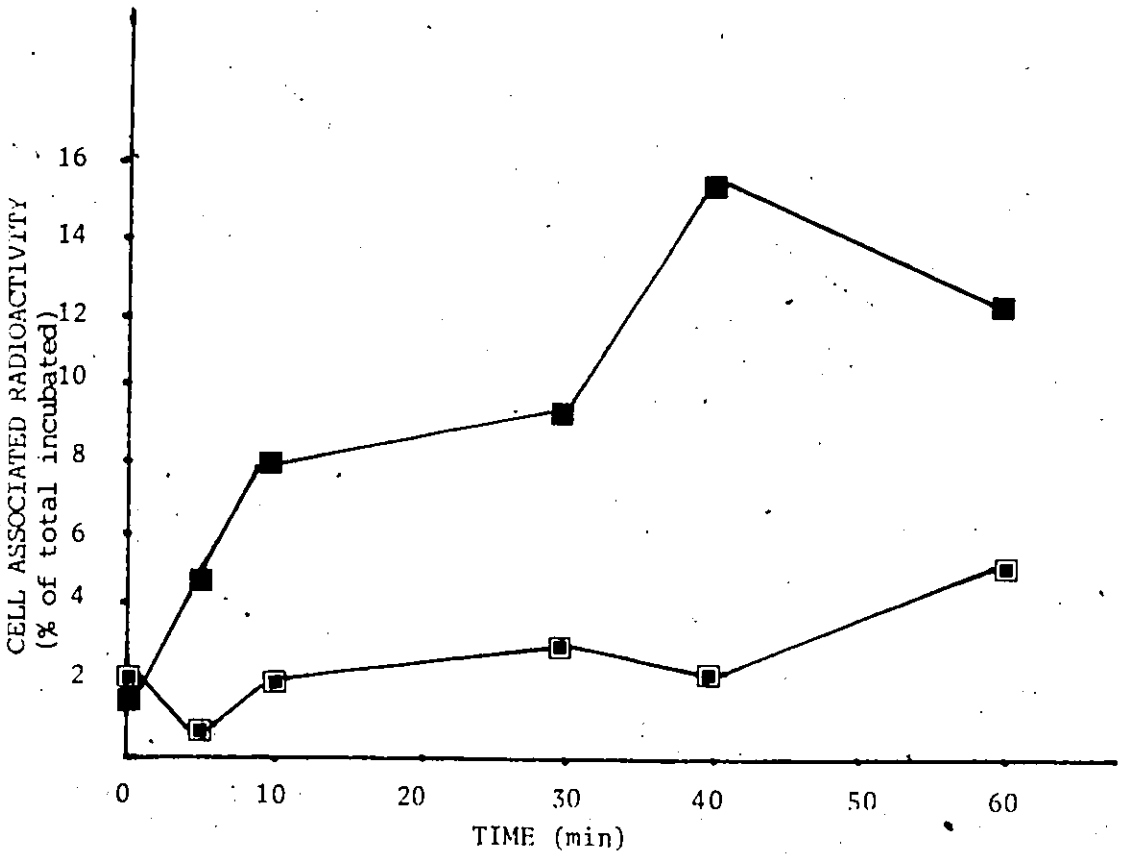
Figure 10.

Uptake of ^{3}H-17β-estradiol 3-glucoside and 17β-estradiol 3-[^{14}C]-glucoside by small intestine epithelial cells at 37°C

250µl of cells at a final concentration of 1 mg/ml (protein) were incubated in suspension buffer pH 7.6 in the presence of steroid at a concentration of 2 X 10^{-8} M. Cell associated radioactivity was determined at the indicated times by centrifugation through a layer of silicone oil as described in Methods.

■ uptake with ^{3}H-17β-estradiol 3-glucoside

▣ uptake with 17β-estradiol 3-[^{14}C]-glucoside


CELL ASSOCIATED RADIOACTIVITY
(% of total incubated)
16
14
12
10
8
6
4
2
0
10
20
30
40
50
60
TIME (min)

uptake studies. Therefore the cell-free medium, obtained under the experimental conditions used for uptake, was monitored for the presence of these enzymes and the results are shown in Table 1. The only steroid metabolizing enzyme activities detected in the medium were steroid β-glucosidase and the 17β-hydroxysteroid dehydrogenase. These activities increased in the medium as a function of time. It is noteworthy that in the absence of added cofactor, no dehydrogenase activity could be detected even at one hour.

2.3. Efflux of estrogens from epithelial cells

Epithelial cells containing [6,7-^{3}H]17β-estradiol were prepared as described in the methods (section 2.4) and steroid efflux was measured by the loss of radioactivity from cells incubated in a steroid-free medium at 4°C and at 37°C. Cell associated label was determined at various times and the results are shown in Fig 11. The amount of label retained by the cells was constant at incubation times up to 60 minutes at 4°C In incubations at 37°C, 48% of the cell associated radioactivity was lost after 60 minutes. The major estrogen metabolite appearing in the medium at 37°C was estrone glucuronide (data not shown).

3. Membrane fractions from small intestine epithelial cells

Table 1

Release of steroid metabolizing enzymes from female rabbit intestine epithelial cells

Epithelial cells were incubated at 37°C under the conditions employed for the uptake studies but in the absence of added estrogens. At the indicated times, the cells were removed by centrifugation and the cell-free medium was assayed for steroid metabolizing enzymes under the conditions of uptake as described in Methods. Steroid glucuronyl transferase activity and 17β-hydroxysteroid dehydrogenase activity were measured both in the presence and absence of added cofactor (UDPGA or NADP)

TABLE 1 RELEASE OF STEROID METABOLIZING ENZYMES FROM FEMALE RABBIT INTESTINE EPITHELIAL CELLS

ENZYME ACTIVITY IN 1ML OF CELL-FREE INCUBATION MEDIUM

INCUBATION TIME(MIN)	STEROID β-GLUCURONIDASE	STEROID β-GLUCOSIDASE	GLUCURONYL TRANSFERASE	GLUCURONYL TRANSFERASE +UDPGA	17β-HYDROXYSTEROID DEHYDROGENASE	17β-HYDROXYSTEROID DEHYDROGENASE +NADP
	pmol/60min	nmol/60min	pmol/60min	pmol/60min	nmol/30min	nmol/30min
0	0.5	2.8	0.5	0.5	0.0	4.4
5	0.5	5.0	0.5	1.5	0.0	3.8
15	0.5	7.0	0.5	1.5	0.0	3.3
30	0.5	9.5	0.5	1.5	0.0	5.4
60	0.5	12.2	0.5	1.5	0.0	8.2

Total enzyme activity was from a homogenate of isolated epithelial cells (1mg protein/ml)

Total activity in 1ml of homogenate was as follows:

Steroid β-glucuronidase: 12 pmol/60min

Steroid β-glucosidase: 14 nmol/60min

Glucuronyl transferase: 38 pmol/60min

Glucuronyl transferase (+UDPGA) : 1300 pmol/60min

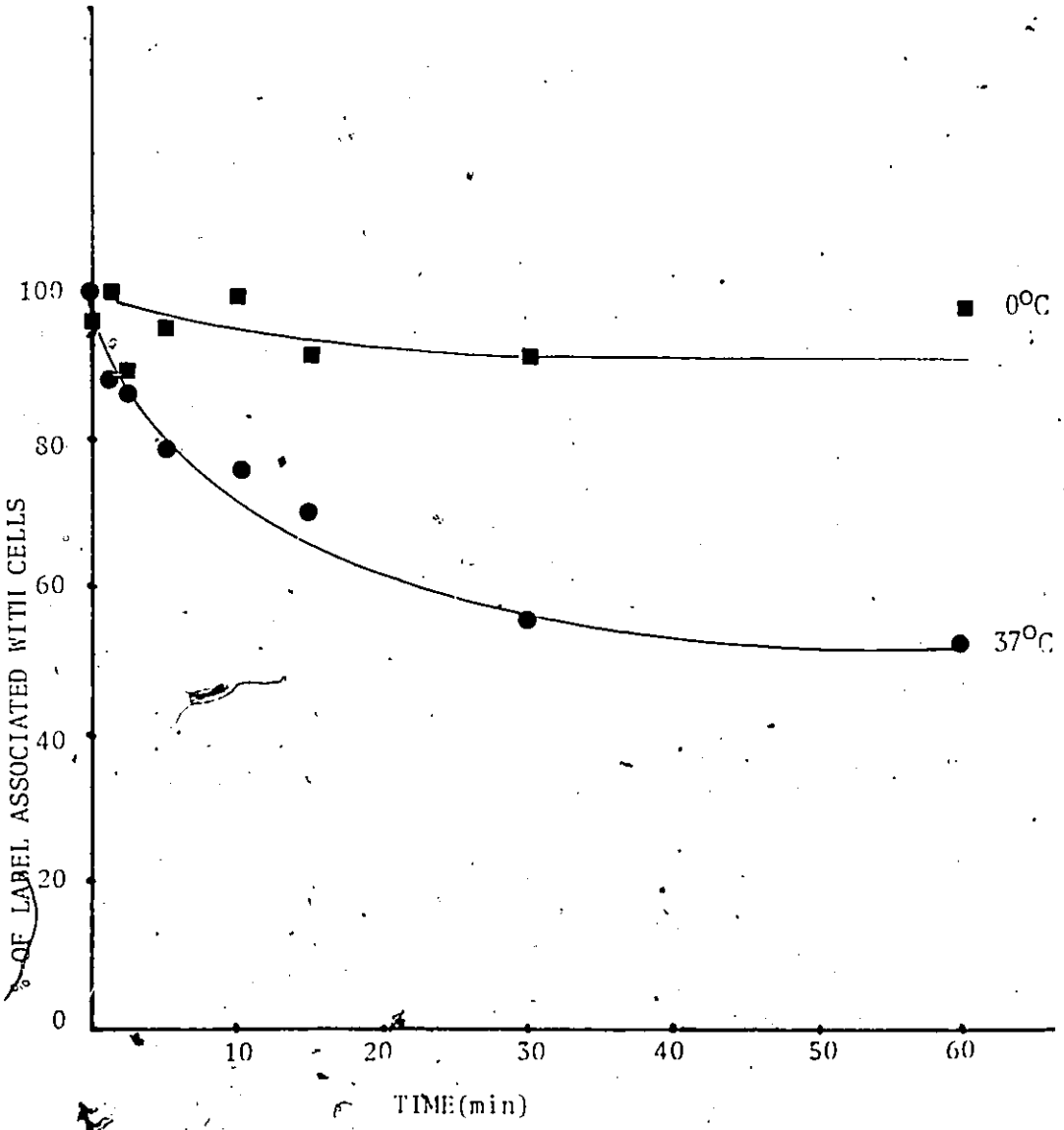
17β-hydroxysteroid dehydrogenase: 2.95 nmol/30min

17β-hydroxysteroid dehydrogenase (+NADP): 10.5 nmol/30min

Figure 11

Release of estrogens from epithelial cells of female rabbit small intestine

Epithelial cells (20 mg protein) in 5 ml of suspension buffer were incubated with 1 X 10^6 dpm of [6,7-^{3}H]-17β-estradiol for 3 hours at 0°C. After washing the cells with 3 X 10 ml of ice cold suspension buffer, the final pellet was resuspended in 1 ml of the buffer. One aliquot (0.5 ml) was incubated on ice and the second aliquot (0.5 ml) was incubated at 37°C. Cell associated label was determined as for the uptake experiments.


100
80
60
40
20
0
% OF LABEL ASSOCIATED WITH CELLS
10
20
30
40
50
60
TIME(min)
0°C
37°C

Membrane fractions were obtained from fresh female rabbit small intestine as described in Methods section 2.5ii. Two enriched fractions were derived from this preparation. The brush border fraction, or the membranes that may be responsible for sequestering the estrogens that present themselves in the gut; and the basolateral membranes that conceivably lead away from the gastro-intestinal tract to the portal system. These membranes were prepared in order to assay for specific binding proteins.

Transmission electron micrographs of the preparations show the brush border membranes to be a fairly homogeneous population (Fig. 12a), whereas the basolateral membranes appear to be more fragmented (Fig. 12b). A micrograph of a crude microsomal preparation is included for comparison (Fig. 12c).

The data obtained on the uptake of 17β-estradiol 3-glucoside indicated that uptake was preceded by hydrolysis of the glucoside (Figs. 8 and 9). Glucosidase activity was released from the cells into the medium during the incubation (Table 1). It was therefore of interest to determine if this enzyme activity was also associated with a specific membrane component. The results are presented in Table 2. Loss of glucosidase activity was evident as purification of both brush border and basolateral membranes

Figure 12

Transmission electron micrographs of membrane fractions from small intestine

a) brush border

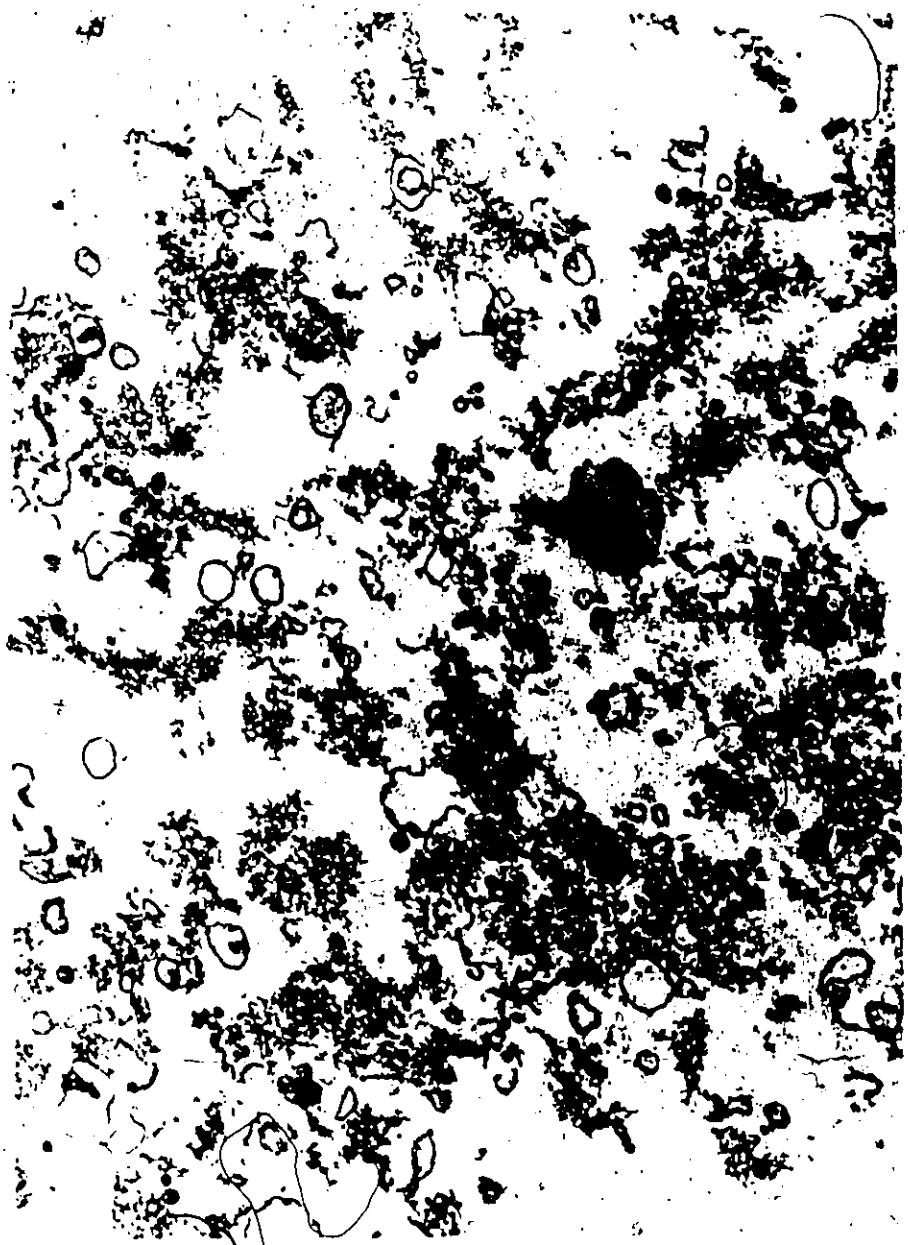
b) basolateral

c) crude microsomal

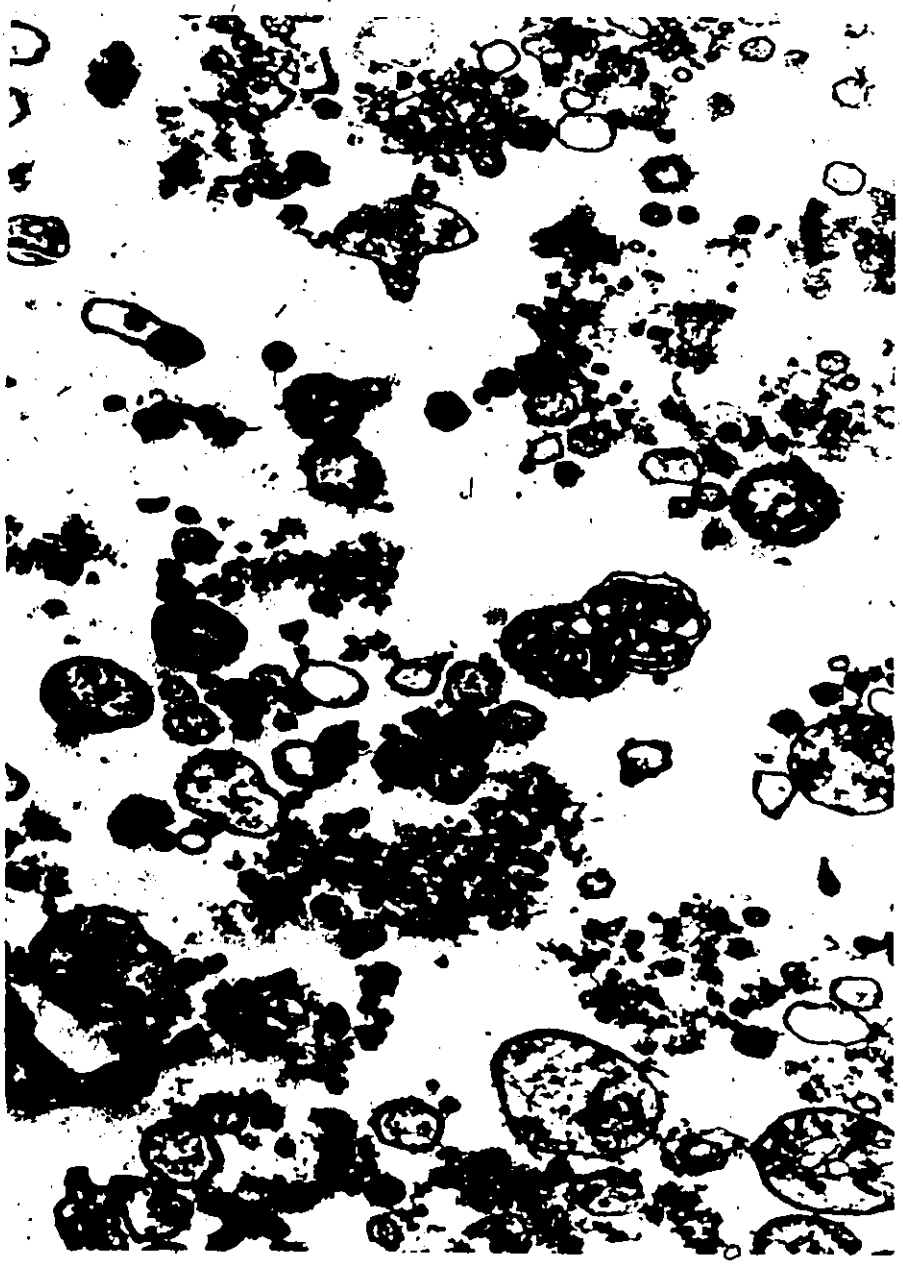
Membrane fractions were isolated as described in the methods (Fig. 1).

Magnification: 21,000X

BRUSH BORDER



BASOLATERAL



CRUDE MICROSOMAL

TABLE 2 β-GLUCOSIDASE ACTIVITY ASSOCIATED WITH SMALL INTESTINE MEMBRANE FRACTIONS

FRACTION	VOLUME (ml)	PROTEIN (mg/ml)	SPECIFIC ACTIVITY nmol/mg protein/60min
Homogenate	3700	1.51	13.22
W-1 (400Xg ppt)	300	1.85	3.74
W-2	275	1.36	0.73
W-3	275	1.22	0.30
W-4	250	0.82	0.30
W-5	60	2.05	0.21
Brush Border	26	3.90	0.17
10,000Xg ppt	15	30.00	1.05
Basolateral W-1	100	0.48	0.84
Basolateral	23	1.69	0.22

The tissue was homogenized in 75 volumes of 5mM EDTA buffer. Enzyme activity was assayed in the suspension buffer (pH 7.6) at 37°C. W designates a wash cycle.

progressed, indicating that the enzyme activity responsible for hydrolysis of 17β-estradiol 3-glucoside is not a component of these membranes. There is, however residual contaminating activity that may affect uptake studies with membrane fractions.

The membrane fractions were also assayed for glucosyl and glucuronyl transferase activities. No enrichment of glucosyl transferase was apparent in any membrane fraction, but enrichment of glucuronyl transferase to three fold the amount assayable in crude homogenate was evident in the basolateral fraction. By far, the highest glucuronyl transferase activity resided in the crude microsomal pellet (data not shown).

Estrogen binding to the isolated membrane fractions was measured and the results are presented in Table 3. Binding of estrogens to both membrane fractions decreased as the polarity of the estrogen increased. The brush border membrane bound slightly more free estradiol than did the basolateral membranes, the opposite was true for the conjugates. The statistical significance of these differences was not evaluated.

The effect of incubation time and temperature on the binding of 17β-estradiol and 17β-estradiol 3-glucuronide to isolated brush border membranes is shown in Fig 13. The

TABLE 3 BINDING OF ESTROGENS BY MEMBRANE FRACTIONS

ESTROGEN	BRUSH BORDER	BASOLATERAL
17β-estradiol	49	41
17β-estradiol 3-glucoside	4.6	8
17β-estradiol 3-glucuronide	3.4	5.6
17α-estradiol 3-glucuronide	0.6	1.8
17-N-acetyl glucosaminide		

Values are expressed as fmol/mg membrane protein. Incubations were carried out at 37°C for 30 min with an estrogen concentration of 2.0×10^{-8} M, membrane protein concentration of 1.1 mg/ml in a total volume of 250µl.

Figure 13.

Uptake of 17β-estradiol and 17β-estradiol 3-glucuronide by small intestine brush border membrane fraction

Incubations were carried out using membrane fraction (0.05mg) and initial steroid concentrations of 10^{-9} M in suspension buffer pH 7.6 (250µl final volume). Binding was determined as in Methods section 2.5 iii.

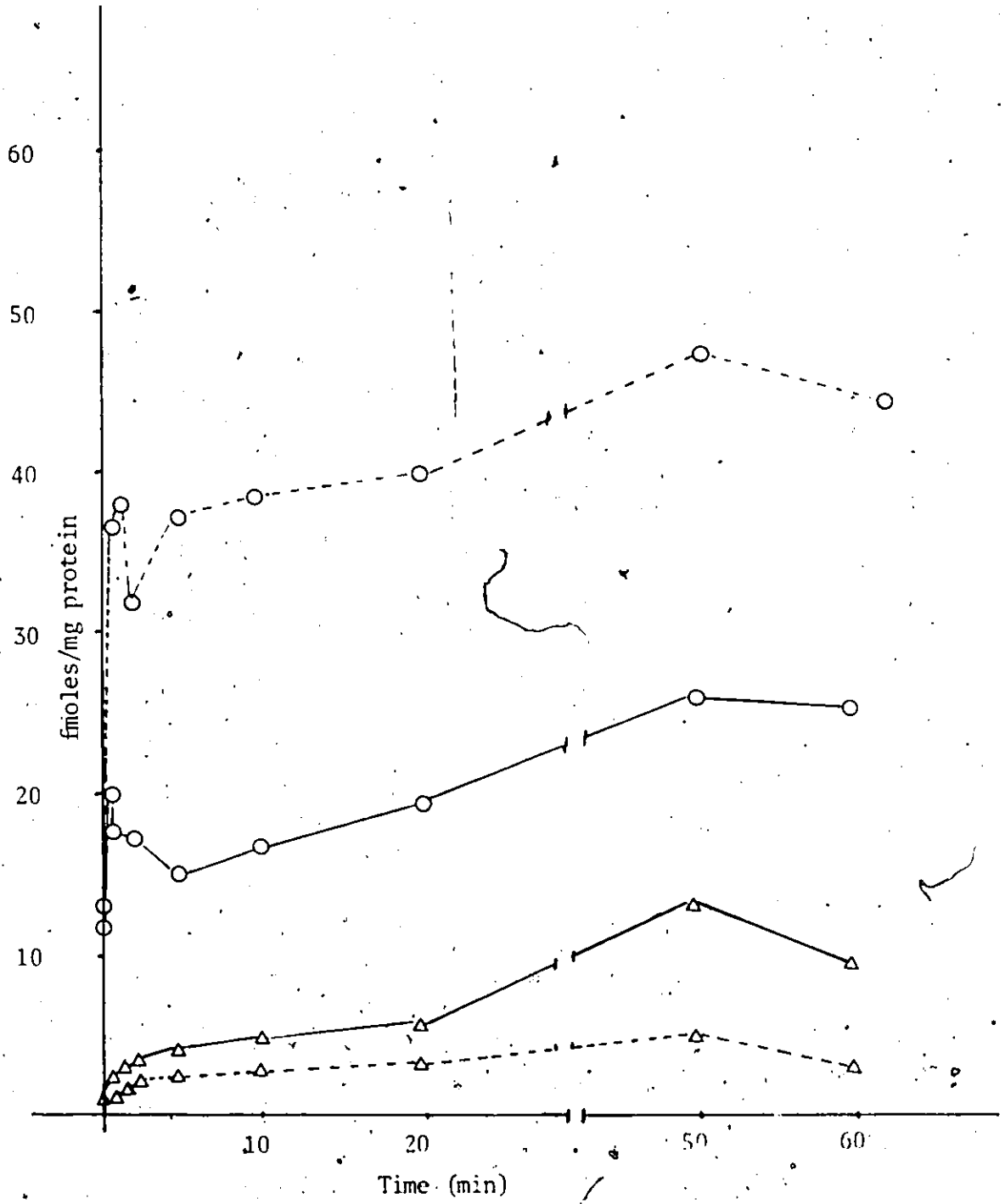
Each point is the average of three determinations and corrected for binding of labelled steroid to the glass fiber filter.

○···○ 17β-estradiol 4°C

○—○ 17β-estradiol 37°C

△···△ 17β-estradiol 3-glucuronide 4°C

△—△ 17β-estradiol 3-glucuronide 37°C


60
50
40
30
20
10
fmoles/mg protein
10
20
50
60
Time (min)

binding of 17β-estradiol is very rapid and is increased only slightly by prolonged incubation. The extent of binding is decreased when membranes are incubated at 37°C. 17β-estradiol 3-gluturonide binding is substantially lower than the free estrogen. The binding of the glucuronide is also affected by incubation time and temperature. The binding of 17β-estradiol 3-glucoside to the brush border membranes was similar to that observed with the glucuronide derivative (data not shown).

4. Estrogen transport in the liver

The liver plays a primary role in estrogen metabolism. This tissue also has estrogen receptors and appears to be a target tissue for this hormone. Therefore estrogen transport was measured in this tissue for comparison to transport in the intestine, a nontarget tissue.

4.1 Studies with liver slices

Preliminary studies carried out with tissue slices showed that the entry of 17β-estradiol into liver tissue was not temperature dependent. The uptake of 17β-estradiol 3-glucoside increased from 5% to 20% of the estrogen incubated when the incubation temperature was increased from 4°C to 37°C while uptake of 17β-estradiol 3-glucuronide

showed a slight increase from 3.6 to 6.5%. There was essentially no entry of 17α-estradiol-3GA-17NAG at either temperature. The pattern of metabolites recovered for all incubations was similar the double conjugate being the major metabolite. The glucuronide derivative and, when 17β-estradiol or 17β-estradiol 3-glucoside were the steroids incubated, free estrogen was also present. Trace amounts of glucoside were also detected in the latter case, however quantities did not avail themselves for positive identification.

4.2 Preliminary studies with hepatocytes

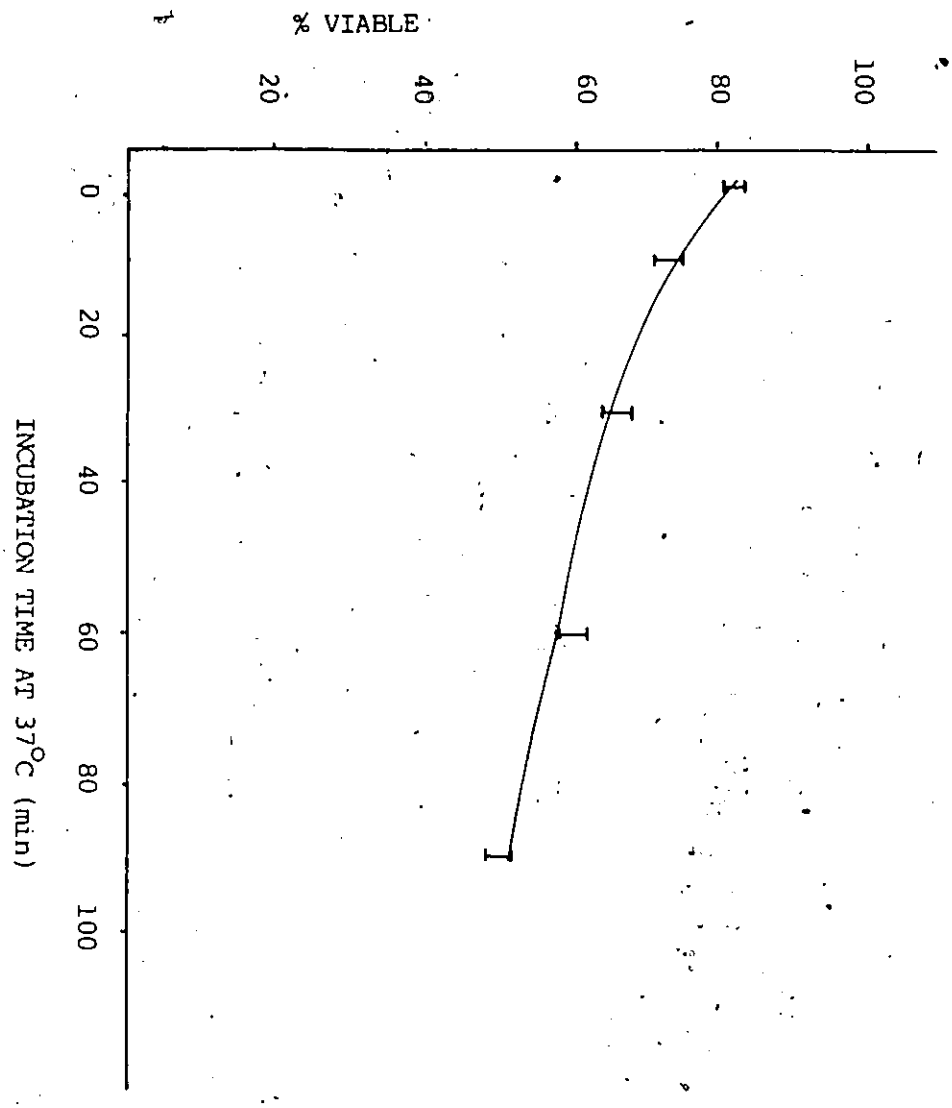
Viability of rabbit hepatocytes isolated by the method of P.O. Seglen (1972) was estimated at 71± 9% by exclusion of the vital dye Trypan Blue. The yield from 1 g of liver was $44X10^6$ cells. The cells were suspended in medium to give a concentration of $8X10^5$ cells/ml which corresponded to a protein concentration of 1mg/ml.

Hepatocytes were used the day of isolation. Freshly prepared washed hepatocytes could be kept on ice for up to 6 hours without a loss of cell viability. Because incubations were also to be carried out at physiological temperature, the effect of the incubation alone on the cell viability was determined and the results are shown in Fig. 14.

Figure 14.

Viability of isolated hepatocytes on incubation at 37°C

Cells (1 mg protein/ml) were incubated in suspension buffer pH 7.6 at 37°C for the indicated times. The cells were then cooled on ice and mixed with an equal volume of 0.4% (w/v) Trypan Blue, and examined under the microscope. Viability was expressed as the percent of cells that excluded the vital dye.


% VIABLE
20
40
60
80
100
0
20
40
60
80
100
INCUBATION TIME AT 37°C (min)

Incubation of isolated hepatocytes in isotonic suspension buffer at 37°C for 30 minutes decreased the viability from 81% to less than 60%. Room temperature was less harsh, therefore incubations were carried out at 24°C.

Uptake of estrogens by rabbit hepatocytes in vitro is shown in Fig. 15. The uptake of 17β-estradiol at 24°C is rapid, reaching a maximal value within 5 min of incubation. There is a slow loss of cell associated radioactivity subsequent to this time. Uptake of 17β-estradiol 3-glucoside reaches a maximum after 30 minutes of incubation under these conditions. The more polar glucuronide derivative is taken up only to a limited extent under these conditions. Data similar to that shown in Fig 15 was obtained when 17α-estradiol and its 3-glucoside derivative were incubated with hepatocytes.

In vitro uptake of 17β-estradiol was linear with respect to cell concentration up to 2.3×10^6 cells/ml. Uptake of estradiol was also found to be proportional to the initial steroid concentration over the range of 5×10^{-10} to 5×10^{-6} M.

Fig. 16 shows the pattern of metabolites present in the incubation medium during the uptake of 17β-estradiol by hepatocytes. There is a rapid loss of 17β-estradiol with a corresponding increase in the 3-glucuronide derivative.

Figure 15.

Uptake of estrogens by rabbit hepatocytes in vitro

Isolated hepatocytes (1 mg protein/ml) in suspension buffer pH 7.6 were incubated with estrogens (final concentration 2×10^{-9} M) in a total volume of 250µl. Incubation temperature was 24°C.
Binding was determined by centrifugation of cells through a layer of silicone oil and is expressed as fmol/mg cell protein. Each point is the average of three determinations.

○ 17β-estradiol

▷ 17β-estradiol 3-glucoside

▷ 17β-estradiol 3-glucuronide

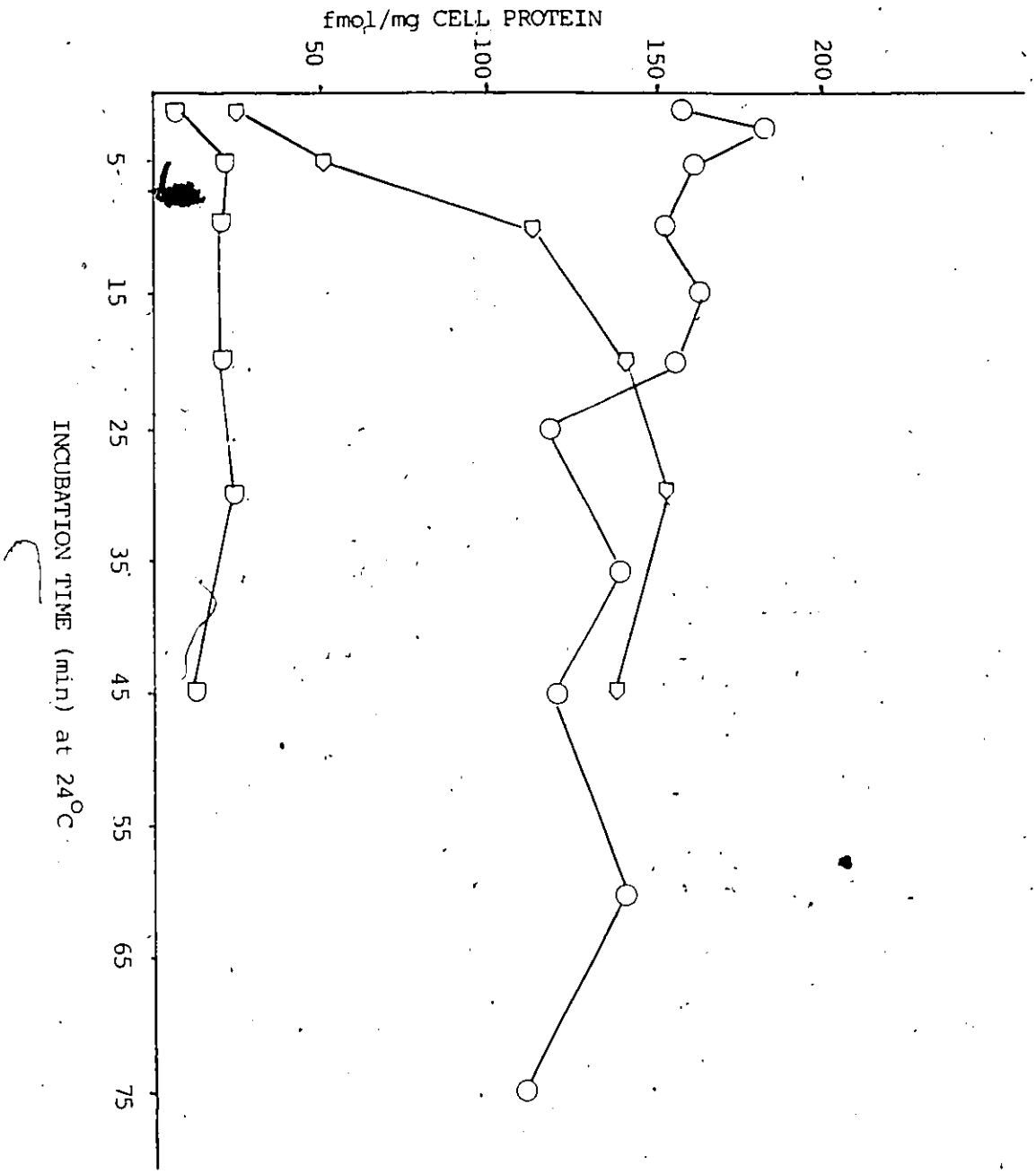

fmol/mg CELL PROTEIN
50
100
150
200
5
15
25
35
45
55
65
75
INCUBATION TIME (min) at 24°C

Figure 16.

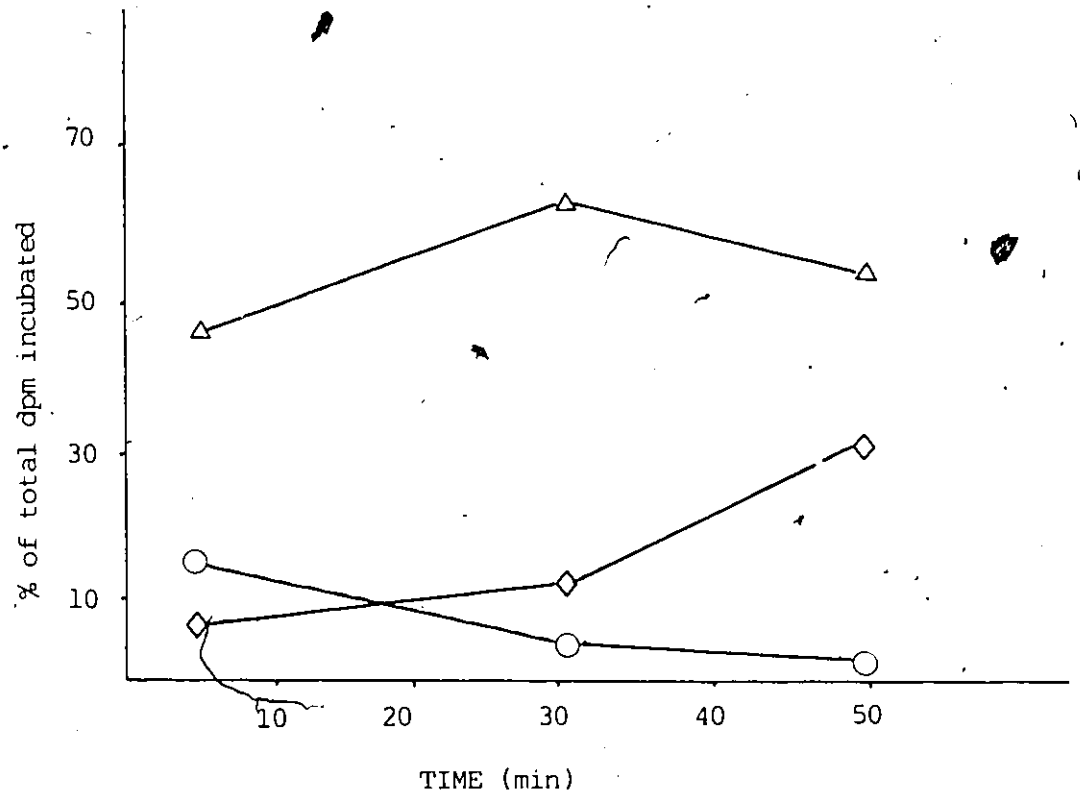
Metabolites of 17β-estradiol present in medium after incubation with isolated hepatocytes

The initial concentration of 17β-estradiol was 2×10^{-8}M, and the cell concentration was equivalent to 1 mg cell protein/ml. The incubation medium was separated from the cell pellet by centrifuging through a layer of silicone oil as described in the methods section and analysed by thin layer chromatography as in section 2.1.

○ free steroid

△ glucuronide

◇ double conjugate (17α-estradiol 3-glucuronide 17-NAG)


70
50
30
10
% of total dpm incubated
10
20
30
40
50
TIME (min)

There is also a progressive increase in the double conjugate 17α-estradiol 3-glucuronide 17-NAG.

The results shown in Fig. 15 indicate that there is a limited but measurable uptake of 17β-estradiol 3-glucuronide by isolated hepatocytes. In order to establish whether this uptake involved the intact estrogen glucuronide or was a result of hydrolysis of the glucuronide and subsequent uptake of the aglycone, 17β-estradiol 3-glucuronide labelled with ^{14}C in the sugar moiety was prepared. Incubation of this ^{14}C-glucuronide was carried out at 4° and 37°C. At the lower temperature, uptake after 30 minutes reached a maximum of 1.3% of the total incubated. At 37°C uptake increased slowly from 2.0% at 1 min to a maximum of 4.1% after 30 min of incubation. The same profile was observed when this glucuronide labelled with tritium in the steroid moiety was incubated with hepatocytes. Further analysis of the metabolites within the medium by differential extraction followed by thin layer chromatography revealed very small amounts of free ^{3}H estrogen (<0.5% of total counts), the majority of the ^{3}H and ^{14}C label resided with the glucuronide fraction. Both radioactive labels were also present in the double conjugate 17α-estradiol 3-glucuronide-17-N-acetyl-glucosamine.

DISCUSSION

Steroid hormones, as all other extracellular compounds that act intracellularly must penetrate the plasma membrane barrier. This is achieved by diffusion through the lipid moiety or by specialized transport requiring interaction with membrane components and/or the participation of energy providing substances.

Studies with intestine

The intestine is not itself a target organ for estrogens. It is however the main route of entry of exogenous and 'bile borne' estrogens and is intimately related to the liver via the portal vein. In the rabbit, the intestine harbors a large number of assayable estrogen metabolizing activities which may participate in determining the fate of these compounds.

The studies described in this thesis have shown that the unconjugated estrogens readily associate with everted small intestine. The uptake is rapid and increases with time and temperature of incubation. 17α-Estradiol, 17β-estradiol and estrone are taken up equally well, an indication that the transport process in small intestine is not affected by the functional group at C-17 of the estrogen.

In studies carried out with isolated epithelial cells, uptake of free estrogens was very rapid, with a temperature effect seen only at early times (Fig 4). The rate of association of hormone to cells was marginally greater at 37°C but had reached near maximal levels by 60 seconds even at 4°C. The uptake was also proportional to the initial steroid concentration in the range of 5×10^{-10} to 5×10^{-5} M. The in vitro uptake by small intestine was not eliminated by a pH shift of ±1 unit around physiologic pH, and the observed decrease in estrogen-cell interaction at lower pH may be due to alteration in membrane fluidity. Uptake of 17β-estradiol by both everted intestine sacs and isolated epithelial cells fulfills the criteria for simple diffusion. The reports dealing with transport of estrogens and other steroid hormones by tissues are numerous (see review by E.P. Giorgi 1980). Most studies indicate that passive diffusion is the mode of entry of steroids. However, data in support of mediated diffusion and specific interaction of steroids with membrane components of target tissues has also been presented (Allera, Rao and Breuer 1980; Nenci *et al.* 1980; Pietras and Szego 1979a; Rao *et al.* 1977).

There is an ongoing temperature dependent process that alters the equilibrium between cell and medium associated estrogen, expressed as a loss of cell associated

radioactivity with time (Fig 6). Under these experimental conditions, incubated 17β-estradiol is converted to estrone glucuronide. The rate of synthesis and the distribution of this metabolite between cell and medium suggests that the decrease in cell associated radioactivity results from formation of the estrone glucuronide and its release into the medium. Assay for glucuronyl transferase in intestine tissue and isolated cell uptake systems indicates that the activity is not released to the medium, but is either associated with the plasma membrane or intracellular. These observations agree with the data of Smith et al. (1963) and Dada and Martins (1983) who reported the accumulation in the serosal compartment of estrone 3-glucuronide, upon incubation of estradiol or estrone with everted rat intestine.

The majority of circulating steroids are bound to serum proteins, and it is likely that only the unbound molecule enters the cell (Munck and Wira 1975). Figure 7 shows a decrease in cell associated label when BSA in increasing concentrations is included in the incubation medium. This indicates that the uptake of estrogen by cells competes with the binding of estrogen by serum protein. The interaction of the serum protein with 17β-estradiol could reduce the partitionning or affinity of the steroid for the cell membrane. The small intestine is devoid of soluble receptors (Tong et al. 1983a), which may explain in part the discrepancy with the observations of Pardridge and Mietus

(1979) studying *in vivo* unidirectional influx of labelled steroids in liver. They injected radiolabelled 17β-estradiol in sera containing varying amounts of sex hormone binding globulin and albumin into the portal vein of anesthetized rats. From the analysis of radioactivity sequestered by the tissue, the estradiol that entered the liver was more than could be accounted for by the unbound fraction. They concluded that protein bound hormone is readily available to the liver by disassociation of the steroid from binding proteins and free diffusion into the membrane.

Estrogens are rendered less lipophillic by conjugation. Giorgi and Stein (1981) reported that conjugated steroids cross membranes much more slowly than their unconjugated counterparts, but their permeability coefficients are much faster than would be expected by their solubility in organic solvents. The uptake process is more complex than simple diffusion.

Uptake of the 3-glucuronide of 17β-estradiol or estrone occurs to a very limited extent in small intestine (Fig 8). The hydrophilic nature of the conjugate may exclude it from the plasma membrane.

Uptake of 17β-estradiol 3-glucoside by small intestine (cells and tissue) is temperature dependent. At 37°C, the

uptake is also time dependent (Fig 9). Uptake of 17β-estradiol 3-^{14}C-glucoside by intestine cells occurred to a very limited extent (Fig 10) and hydrolysis of the glucoside followed by transport of ^{14}C-glucose could not be ruled out. Therefore, if uptake of intact glucoside is occurring under the experimental conditions employed, the contribution to the overall process is minimal. It appears that the major uptake of the estrogen occurs subsequent to the action of a β-glucosidase. The intestine is a rich source of steroid β-glucosidase (Mellon and Layne 1971, 1974) and the activity is abundant in the incubation medium under conditions employed for uptake studies. The enzyme activity likely originates from epithelial cells as the protocol for cell isolation does not favor co-purification of microorganisms. It is possible that this enzyme is released into the medium as a result of cell damage. Measurement of β-glucosidase activity during the isolation of brush border and basolateral membranes indicates that this enzyme is not a component of these plasma membranes (Table 2).

Evidence for metabolism-linked efflux

In the intestine the transport of small molecules is generally unidirectional, from the mucosal to the serosal side of the epithelial cell layer. Given the tight

junctions of the small intestine epithelium, very little crosses from the mucosa to the serosa intercellularly. The present studies suggest that the uptake of estradiol by isolated cells is not linked to a cell process. However, the temperature and time dependent appearance of conjugates in the incubation medium suggests a metabolism linked export process for estrogens.

At 4°C, only a small amount of radioactivity was lost from cells that had been labelled with ^{3}H-17β-estradiol and that which was released was unconjugated estrogen. This is a sign that there was little or no ongoing metabolism at the lower temperature. 17β-estradiol permeates the plasma membrane even at 4°C. However, once sequestered does not readily diffuse out of the epithelial cells. A possible explanation for this observation is non specific binding to high capacity, non specific intracellular proteins or membrane components, which may reduce the apparent concentration gradient of steroid thus slowing the outward diffusion process. It could also be attributed to membrane asymetry: in the absence of an active transport of free estrogen out of the cell, the inner surface of the membrane may be less compatible than the outer surface with diffusion or solubility of steroid , amplified at reduced temperature. Conversely, when the labelled cells were warmed to 37°C, there was a decrease in cell associated radioactivity with time. Under these conditions estrone 3-glucuronide was the

major estrogen identified in the medium. These data suggest that 17β-estradiol is metabolized to estrone glucuronide intracellularly or during its transit into or out of the cell. Under the experimental conditions employed the release of glucuronyl transferase into the medium was negligible (Table 1). Glucuronyl transferase activity could be measured in basolateral membranes but not in brush border membranes indicating that the basolateral membranes might be involved in estrogen metabolism and that the membrane glucuronyl transferase might participate in the transport process.

Role of plasma membranes

Pietras and Szego (1979a) report that intact uterine cells bind to estrogen immobilized to an inert support, whereas small intestine cells do not. However the present studies have shown that the plasma membranes of rabbit small intestine can bind estrogens (Table 3). The binding of 17β-estradiol and its 3-glucuronide derivative to small intestine membranes was proportional to initial concentrations in the range of 10^{-6} to 10^{-9}M. The slight decrease in binding of 17β-estradiol observed at 37°C could indicate that higher temperatures lead to faster dissociation of steroid-protein complexes (Giorgi, 1980).

The binding of 17β-estradiol 3-glucuronide occurred to a

greater extent to basolateral membranes than to brush border membranes however the statistical significance of this higher binding was not established. The data is consistent with the involvement of the basolateral membrane in export of the steroid, as its glucurnide derivative.

Studies with liver

The liver is the major metabolic organ in the system. It receives blood borne estrogens via the portal vein and the hepatic artery. The liver processes these hormones for excretion in the bile or can re-direct them to the general circulation. Yet another alternative is the interaction of estrogen with the soluble receptor in this non classical target tissue.

The uptake of free estrogens by liver slices was very rapid and differed from intestine only in that no significant temperature dependence on uptake was observed. In this case, the sampling times were restricted and the very high turnover of free estrogen, that was probably occurring even at 4°C, may have reduced the concentration of substrate available for uptake. With isolated hepatocytes, the uptake was also very rapid.

The liver was not unlike the small intestine with

respect to its management of 17β-estradiol 3-glucoside. The uptake profile was time and temperature dependent which can be correlated with the availability of the soluble β-glucosidase activity. Uptake of 17β-estradiol 3-glucuronide by hepatocytes was more pronounced than in intestine. Uptake of 17β-estradiol 3-^{14}C-glucuronide followed the same profile as uptake of ^{3}H-17β-estradiol 3-glucuronide, and radioactive tracer also migrated with the double conjugate on thin layer chromatography. This data indicates that the monoconjugate may be taken up intact by the isolated hepatocytes and conjugated with N-acetyl-glucosamine. Another possibility, is that a membrane associated NAG-transferase is accessible in the damaged cells which amount to approximately 30% of the total, and that the 'trapping' observed is enzyme mediated.

Schwenk, Lopez Del Pino and Bolt (1979) provide a precedent for conjugate uptake by target tissues. Uptake of estrone sulfate by isolated rat hepatocytes is much slower than uptake of estrone. The authors do not carry out an assay for sulfatase, but analysis of intracellular and extracellular metabolites at various times during the incubation support the conclusion that deconjugation follows uptake of the sulfate. Estrogen sulfates were not assayed for in our system as they are not a major metabolite in rabbit, though liver <u>in vitro</u> can synthesize them (Layne 1970).

reported by Tong, Seper, Layne and Williamson (1983b). The process is very rapid at 0°C and 30°C and equilibrates within 60 seconds. Not unlike the data obtained with isolated hepatocytes, the unconjugated estrogen is taken up to the greatest extent followed by 17β-estradiol 3-glucoside and ultimately 17β-estradiol 3-glucuronide. The sequestered 17β-estradiol and the 3-glucuronide derivative are unchanged but the 3-glucoside derivative is metabolized to 17β-estradiol by a nuclear β-glucosidase at 30°C.

General conclusions

The intestine is the first and main route of entry of foreign compounds. Endogenous estrogens excreted in the bile are also available for uptake and undergo hydrolysis and reductive metabolism in the gut. The factors affecting the uptake by transforming (or lack of) the steroid are varied ex.: dietary status, gut microflora and drug interactions. Understanding the enterohepatic recirculation of steroids may provide an answer to the question of why some compounds are orally active and others, though structurally similar, are not. The transport of estrogens across the intestine may even give rise to a "reactivation" and may be relevant in regulating the availability of the hormones (Adlecreutz and Jarvenpaa 1982).

hormones (Adlecreutz and Jarvenpaa 1982).

Classically, estrogen target tissues were distinguished by the presence of high affinity binding proteins residing in the cytosol. After binding, the estrogen-receptor complex could be detected in the nuclear fraction of the cell. A recent review by Gorski et al. (1984) proposes a revised estrogen receptor model where the receptor protein is located in the nuclear compartment of the cell even in the absence of estradiol. Szego (1984) suggests an analogy between the mechanism of action of peptide and steroid hormones from the primary recognition site on the plasmalemma to concentration of hormones into organelles and other vesicular components during transit to a nuclear binding site in the target cell. These intermediate organelles may contain enzymes and play a role in activation or inactivation of the effector. This model involves transport through the plasma membrane, to the vesicular bodies, through the nuclear envelope and ultimately transport out of the cell for disposal or reactivation.

Uptake cannot be dissociated from metabolism and interaction with receptor proteins in target tissues. For example, in the rabbit 17β-estradiol is the most active estrogen and is the template for measuring receptor content. Its conversion to 17α-estradiol and conjugation with glucuronic acid and N-acetyl-glucosamine destines the

hormone for excretion by the kidney. The fate of the monoconjugates is not as clear. If these metabolites are taken up intact by cells, it is by a minor component or by a process that is not conserved in vitro. However, the 3-glucoside and 3-glucuronide of 17β-estradiol were observed to interact with the estradiol receptor of rabbit liver cytosol (Tong et al. 1983a), and like 17β-estradiol, were taken up by a non saturable process, in the absence of released hydrolases, by isolated liver nuclei (Tong et al 1983b) The role of these endogenous conjugates in the mechanism of estrogen action and in estrogen transport merits further investigation.

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