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THE EFFECTS OF PHARMACOLOGICALLY-INDUCED REVERSIBLE ASPERMATOGENESIS AND CRYPTORCHIDISM ON SERUM ESTROGENS OF THE MALE RAT

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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* * * * *

The Ohio State University

1974

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PUBLICATIONS

W. R. Gomes, R. W. Hall and L. R. Boots (1970) "Radioimmunoassay of Serum Gonadotrophins in Aspermatogenic Rats". Proc. 3rd Ann. Mtg. Society for the Study of Reproduction, p. 10.

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FIELDS OF STUDY

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INTRODUCTION

Little is known and less is understood concerning the mechanism by which testicular elements (germinal cells) control the secretion of FSH by the enterior pituitary in the male mammal. The results of several studies since the 1930's (see 19 for references) suggest that elements within the seminiferous tubules may regulate FSH secretion. Many studies have shown an increase in serum or urinary FSH levels in conditions which affect spermatogenesis; e.g. cryptorchidism, immune aspermatogenesis, pharmacologically induced reversible aspermatogenesis or poorly defined clinical conditions (see 19). These studies have led to the conclusion that the testis produces a hormone, "inhibin", which is responsible for feedback inhibition of FSH secretion. The nature of this "inhibin" as well as the source are unknown; however, several hypotheses have been proposed.

Estrogens, perhaps produced by the tubular elements, have been suggested as the agent regulating FSH production and/or release. The cytoplasmic droplet of immature sperm has also been suggested as a source of the feedback agent and others have suggested that a lack of binding, metabolism or utilization of FSH may lead to the higher levels of FSH in the previously mentioned conditions (see 19). A role for the Sertoli cell (29) in FSH regulation has been proposed but the function of this cell type has never been delineated.

Previous work in this laboratory (19) has indicated that reversible, pharmacologically-induced aspermatogenesis results in increased serum FSH levels, no change in serum LH levels, and no change or a slight decline in serum testosterone levels of the treated animals. The level of FSH was highest when spermatogonia were the only germinal cells present in the testes of treated animals. As much has been said concerning the possible role of estrogens in FSH regulation, this study was undertaken to determine the types and levels of estrogens present in the serum of adult male rats and the effect of conditions which deleteriously affect the spermatogenic elements and give rise to increased serum FSH levels, on the levels of the estrogens circulating in the adult male rat.

REVIEW OF LITERATURE

FSH and "inhibin". A recent review by Steinberger (40) points out the basic lack of knowledge concerning the role of FSH in spermatogenic function. It is the current opinion that FSH is active in the maturation of the developing spermatids. The role of the testes, and mainly the spermatogenic elements, in controlling FSH secretion by the anterior pituitary is the subject of much speculation.

A reciprocal relationship appears to exist between spermatogenic function and serum FSH levels, based on both clinical and more carefully controlled experimental evidence (19). Franchimont (14), using patients with azoospermia or oligospermia, noted higher FSH levels when gametogenesis was arrested prior to spermatid formation, with normal levels appearing when spermatids were present. Amatayakul et al. (1) found rapid marked increases in serum FSH and LH levels in adult male castrate rats, while cryptorchid rats showed similar, but less marked, increases in serum FSH and LH. Swerdloff et al. (42, 44) found that FSH levels in immature rats were 2-3 times higher than adult levels, being maximal at 35 days of age, and declined with the appearance of mature sperm in the tubules. The decline in FSH and the development of spermatogenesis were prevented by cryptorchidism. Gomes et al. (19) found increased FSH levels following administration of two antispermatogenic drugs and concluded that at least

partial control of FSH secretion must lie in the seminiferous tubules. Debeljuk et al. (9) found increased serum FSH in male rats following the administration of the antispermatogenic drug busulfan.

Two general hypotheses have been proposed to explain the relationship between FSH and spermatogenesis -- the "non-utilization" theory, and the "inhibin" theory. Heller (see 32) suggested that the germinal epithelium metabolized FSH during the action of the hormone on the tissue; if spermatogenic elements were lost, non-utilization of FSH would lead to increased serum levels and increased urinary excretion. Increases in pituitary FSH, which occur in aspermatogenic animals (20), presumably could be explained by a short-loop feedback of FSH. However, Dufau et al. (13) found that human chorionic gonadotropin was not inactivated during binding to testicular receptors; unless FSH behaves differently, these data offer good evidence to refute Heller's non-utilization theory.

Many workers have suggested that the tubule produces a substance, generally referred to as "inhibin", that regulates circulating levels of FSH. Swerdloff et al. (42) report that the feedback action of spermatogenesis on serum FSH begins between 35 and 50 days of age in rats. At this time, serum FSH begins to decrease from its highest levels (35 days) and continues to decrease until adult levels are reached at about 63 days of age (42). The fall in FSH was prevented by cryptorchidism, leading them to propose the elaboration of an FSH inhibitor as sperm are produced. Although other workers have found

an increase in rat FSH between 40 and 60 days of age, and FSH levels do not decline in men during sexual maturation, the inverse relationship between full spermatogenesis appears valid for adult men as well as rats (see 20 for references).

A general lack of information concerning the actual compounds involved in FSH regulation has prevented this field from advancing much beyond the speculative stage. The classes of compounds which are usually included in such speculation include androgens, estrogens and a nonsteroidal inhibin. For a number of years, testicular testosterone was considered sufficiently inhibitory to explain FSH relationships. Since several workers had found that testosterone inhibited FSH, but less effectively than it inhibited LH (7, 27, 43), a small decrease in testosterone production by aspermatogenic testes could lead to increased FSH with continued depression of LH. A number of workers have indicated decreased testosterone production by aspermatogenic testes (see 20), but others have shown no consistent effect on, or have recorded, in cryptorchid testes, increased testosterone production (20). Single doses of testosterone were ineffective in inhibiting FSH in castrate male rats (16, 25), while daily treatment reduced FSH and LH equally (17). Walsh et al. (47) treated intact male rats with the antiandrogen, cyproterone, to decrease feedback effects of androgens on pituitary gonadotropins; levels of cyproterone which increased serum LH 25-50% had no affect on FSH levels.

Effects of androgens other than testosterone have been noted (20), and the results suggest that one of these compounds, 5α-androstanediol, may selectively suppress LH in aspermatogenic rats with no effect on FSH. Although 5α-androstanediol was markedly more effective than testosterone or dihydrotestosterone in depressing LH in castrated male rats, it had no significant effect on FSH levels. If a compound such as 5α-androstanediol were increased, at the expense of testosterone, in aspermatogenic animals, FSH might be allowed to rise with a concomitant suppression of LH. The production of 5α-reduced metabolites in the testis may be of minor importance, since large amounts of these compounds appear to be produced by peripheral conversion of testosterone (20, 27).

Among the several authors who have proposed that the FSH-inhibiting substance from the testis is an estrogen, Johnsen (23) and Lacy (29) have proposed the most refined hypothetical models. They propose that spermiogenesis somehow increases the ability of the Sertoli cell to produce estrogens which, in turn, suppress FSH production and/or release. Lacy (29) has reported the presence of Sertoli cell estrogens in the rat, Longscope et al. (31) and Baird et al. (3) have shown that the human testis secretes estrone and estradiol-17β, and Kaivola and Johnson (see 20 for reference) report decreased urinary excretion of estrone by men with sperm abnormalities. Kulin and Reiter (28) reported that, in men, FSH was decreased by low doses of ethinyl estradiol, but LH was not affected, lending support to the estrogen feedback hypothesis. Kalra et al. (25) found that

estrogen injections into castrated male rats depressed plasma LH, but increased circulating FSH and Naftolin et al. (33) demonstrated that hypothalamic tissues of the rat can convert androgens to estrogens, even though this aromatization step is apparently unnecessary for androgenic feedback activity (43).

Although the above studies document both ends in the hypothetical chain of events necessary to support the hypothesis that inhibin is an estrogen (i.e., the testis can produce estrogens and estrogens can inhibit FSH), the apparent weakness comes in the intermediate links (20). Peripheral aromatization is extensive (3) and it has been suggested that this can account for all of the circulating estradiol and most of the estrone in men (31). However, Baird et al. (3) calculate that the testis contributes approximately 21% of the total circulating estrogens. It would appear, that due to the low level of estrogens contributed to the circulation by the testis, any slight changes in testicular production would be overwhelmed by peripheral androgen conversion. If this were so, changes in testicular estrogens might not be of sufficient magnitude to perform a feedback function.

If inhibin exists, and if it is nonsteroidal in nature, as suggested some forty years ago (see 19 for references), an entirely new approach to the search for inhibin appears necessary. Perhaps the first step in this direction was shown by Setchell and Sirinathsinghji (39) who found antigonadotropic activity in rete testis fluid from rams and boars and suggested that this heat-labile activity might represent inhibin. If this activity were lost in

aspermatogenic animals, confirming its inhibin-like properties, further investigation of the rete testis fluid would be needed to identify the compound(s) involved.

Regardless of the nature of the feedback of spermatogenesis on FSH, several factors must be considered in studies on inhibin. Although destruction of the spermatogenic elements in the testis generally results in a selective increase in FSH, this increase never reaches the levels attained after castration, leading several authors to suggest that a second level of control over FSH must exist in the testis. Whether this is testicular androgen, a Sertoli cell product or merely the residual production of inhibin, diminished but not destroyed by aspermatogenesis, must be resolved (20).

Estrogen levels in the male mammal. Until recently, little was known of the estrogenic steroids present in male mammals. Estrogens have been detected and levels measured in the testicular tissue of fetal sheep, of the horse, of man (3) and the rat (10) and the synthesis of estrogens by equine testis *in vitro* has been well documented. Conversion of precursors to estrogens *in vitro* has also been recorded for man, the rat and the dog (see 10).

DeJong *et al.* (10) reported estradiol-17β levels of 2.0 ± 0.9 pg/ml and 17.5 ± 8.4 pg/ml in the peripheral and testicular venous blood, respectively, of the rat. These workers calculated that the testicular secretion of estradiol-17β was 11 ng/day. They (10) also found no effect of HCG or FSH on circulating estrogen levels which they attributed to compartmentation of steroid production.

Baird et al. (3) determined the concentrations of estrone and estradiol-17β in the spermatic and peripheral venous plasma of men. They reported the concentrations of estrone (360 ± 50 pg/ml) and estradiol (2080 ± 280 pg/ml) to be higher in the testicular venous plasma than in the peripheral circulation (estrone-74 ± 15 and estradiol-32 ± 5 pg/ml) and conclude that this confirms that in men, the testis secretes both estrone and estradiol. They (3) calculate, based on a 21 ml/min blood flow, an estradiol production rate of 30 g/day by the testis.

Estrogen identification methods. Methods for the identification of estrogens have been extensively reviewed by Preedy (37); the reader is referred to this article for a more complete coverage of the topics excerpted here.

The absolute identification of estrogens, as with other substances is difficult to achieve; however, by employing several procedures, one can more firmly establish the identity of a given estrogen. Basically, one compares the physicochemical characteristics of the unknown substance to those of a known standard estrogen and if these are found to be the same for a number of different tests, then one can, for practical purposes, consider the unknown and known to be identical.

There are a number of identification procedures one may use. These include: superimposition of radioactive standard estrogen and unknown curves by column chromatography; isotope and reverse istope dilutions; repreated crystallization to a constant specific

activity; multiple chromatography of derivatives; infrared spectroscopy; and bioassay of the isolated material compared with bioassay of a standard known estrogen.

Multiple chromatography, using one or several types of chromatography and several different solvent systems to compare the migration of the unknown with the migration of a standard estrogen may be used. One should, if the compounds are identical, get identical migration patterns of the known and unknown compounds. One can also, using chromatography and a quantative assay, such as RIA or fluorometric analysis, superimpose the chromatographic patterns of the unknown upon the migration of radioactive standard by quantitating the levels of the unknown in each fraction collected and the radioactivity in each fraction.

Following the separation of the unknown, one can make derivatives with unlabeled and labeled compounds such as monobenzoates, 3-methyl esters, or acetates. The migrations of these derivatives, using multiple chrometography are compared with those of authentic steroid derivatives, by superimposing the elution patterns of the two compounds.

As the estrogens absorb light in the infrared region, due to specific groupings on the molecule, one can fingerprint the steroid due to the characteristic absorption pattern of each estrogen. However, this method requires amounts of hormone much higher than that found in most biological samples and therefore has found little use in estrogen identification.

Bioassay also requires large amounts of isolated steroid and many animals upon which to run the assay. With large amounts of estrogen, one can inject graded doses into castrate female rats or mice and compare the proportion showing vaginal cornification to that induced by a standard estrogen preparation and obtain some evidence concerning the nature of the unknown.

Isotope and reverse isotope dilution can also be used for identifying and quantitating estrogens. With isotope dilution, one desires to determine the level of a non-radioactive hormone, while with reverse isotope dilution, one desires to determine the level of labeled steroid contained in a sample. In the former case, one adds a known amount of labeled hormone to the unknown sample, chromatography is done, and one measures the mass of steroid and level of radioactivity present in the peak tubes of the curve. In the latter case, one wants to know the level of radioactive material present in the original sample. A known amount of cold steroid is added to the sample and the above steps repeated. By setting up a simple proportion, one can determine the amount of labeled or unlabeled hormone present in the original sample.

Assay Methods

Many procedures have been devised for the quantative estimation of estrogens following extensive purification procedures. These fall into several categories: colorimetric, fluorometric, enzymic and competitive protein-binding assays (37).

Colorimetric. Of the colorimetric assays, the Kober reaction is the best known. The color producing agents are a 2% quinol solution in varying concentrations of sulfuric acid. After heating, the color intensity is measured by absorption at 430, 516, and 550 nm and compared to a standard curve of increasing levels of a known standard estrogen solution. This method has a sensitivity of approximately 0.2 μg estrogen in 2.5 ml.

Fluorometric. Estrogen fluoresces strongly when heated with phosphoric or sulfuric acids. Fluorometric analysis is more sensitive than the Kober reaction, measuring 5 ng of estrone with ease and accuracy.

Enzymatic assays. Enzymatic assays depend on the measurement of NADH formed or depleted by the interconversion of hydroxy- and keto-steroids by NAD-linked hydroxysteroid dehydrogenases. The sensitivity of the method is limited by the photometric determination of NADH and has a lower limit of about 300 ng.

Competitive protein-binding assays. These assays fall into two categories: protein-binding assays, which use a steroid binding protein from blood plasma or receptor proteins found in steroid target tissues; and the radioimmunoassay (RIA), which uses antibodies against steroids bound to a protein.

The assay method using plasma steroid binding proteins has been described in detail in the book by Odell and Daughaday (34), and have three basic problems: (1) the availability of the binding protein; (2) separation of bound and free steroids; and (3) the removal of

other binding proteins that may interfere with the assay. Even though these assays are almost as sensitive as the radioimmunoassay for most biological samples, the extreme specificity of the antibodies, plus the methodological problems of the protein-binding assays, have relegated the protein-binding assay to a minor role in steroid quantitation. Some of the methodological problems of the protein-binding assay are: (1) competition between the low capacity, highly specific binding proteins and the ever-present low sensitivity-high capacity serum albumins; (2) the availability of third trimester pregnant human females from whom the sex steroid binding protein is obrained; (3) the stability of the protein; and (4) the day-to-day variations in laboratory atmosphere which affect the reliability of these assays.

Radioimmunoassay (RIA). As the method of estrogen quantitation used in this research deals with RIA, a more complete review of this method and its history will be given. However, for a more complete review of the theory, methodology and statistical analysis of the RIA, the reader is referred to the excellent books on the subject (34, 35).

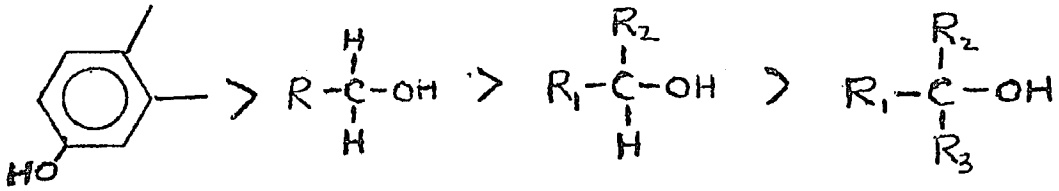
Steroids and other low molecular weight compounds are not inherently antigenic. Landsteiner demonstrated in the early 1900's that antibodies could be produced against low molecular weight compounds if they are conjugated to a substance that is antigenic and the low molecular weight compound will function as a hapten (26).

The first antibodies to steroids were produced by Sachs and coworkers in 1925 using cholesterol and lecithin mixed with pig serum.

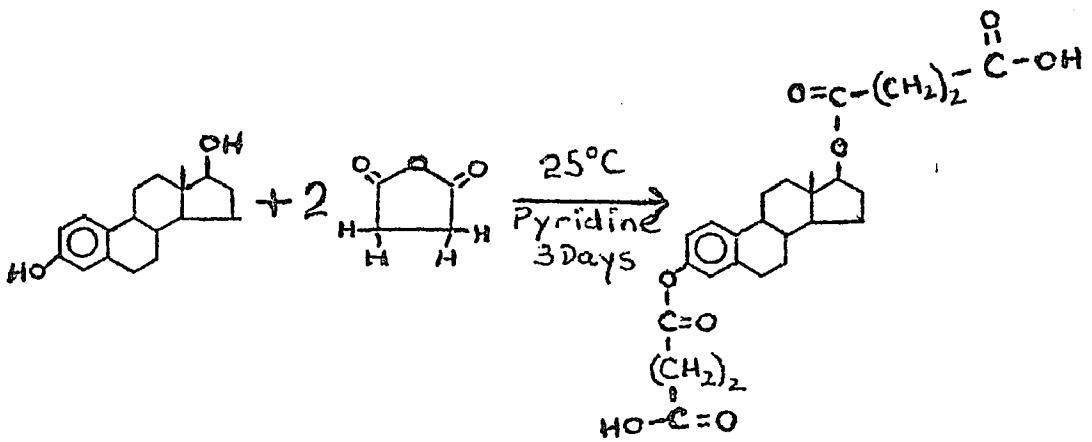
Sprunt et al. produced antibodies to estradiol-17β conjugated to albumin of various species. Much more work was done in the 1950's and 60's by several groups by forming o-carboxymethyl-hydroxylamine derivatives with ketone functions and hemisuccinate or chlorocarbonate derivatives with steroid hydroxyl functions and conjugating them to proteins (see 34).

Production of steroid-protein conjugates. The antibody used in this study was produced by Dr. Burton Caldwell (see 35) using estradiol-17β -17-hemisuccinate-bovine-serum-albumin.

Estradiol-17β contains 2 functional hydroxyl groups: a phenolic hydroxyl at C-3 and a secondary alcohol at C-17. The order of reactivity of these functional groups is phenol > primary alcohol > secondary alcohol > tertiary alcohol. Schematically (33): where R ≠ H.

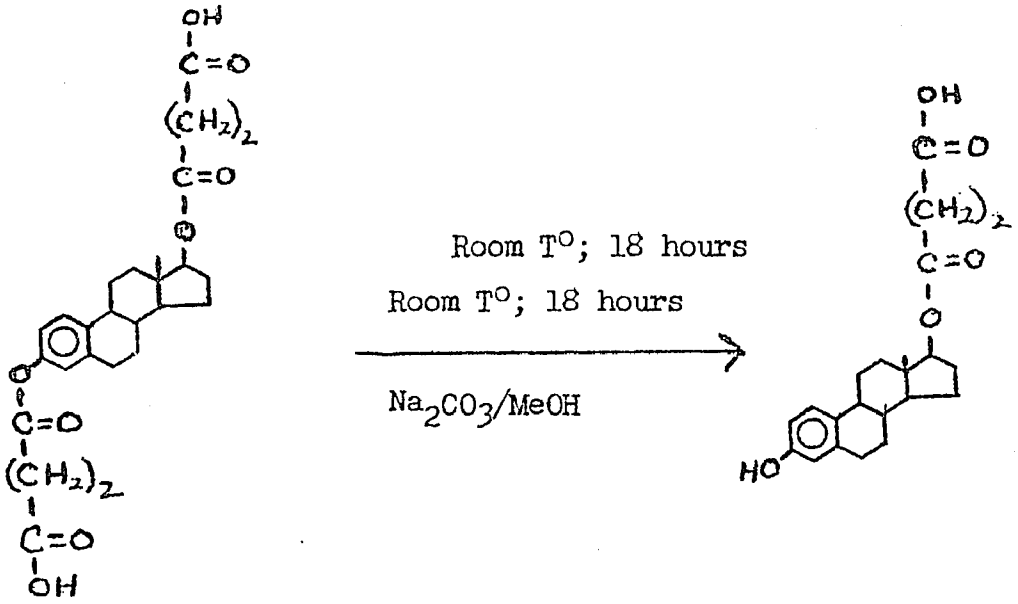


To prepare the 17-monosuccinate derivative of estradiol-17β, one must first prepare the 3,17-disuccinate derivative by the following reaction (34):

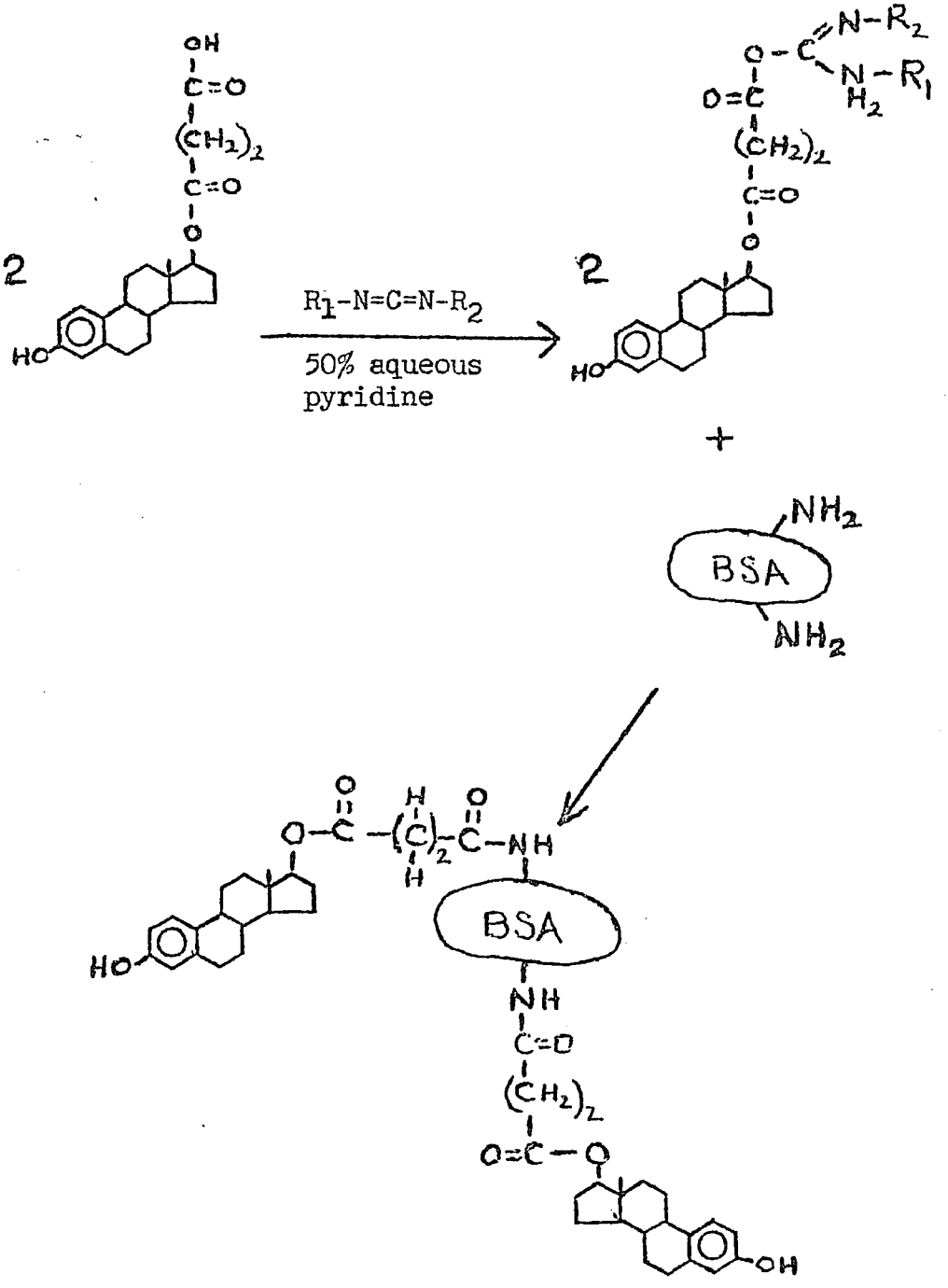


Estradiol-17β + 2 Succinic anhydride ⟶ Estradiol-17β-disuccinate

The 17-monosuccinate derivative is produced by selective hydrolysis of the C-3 ester (34):



The following reaction is used to conjugate the 17-monosuccinate derivative to the ε-amino groups of the lysine residues of the bovine serum albumin (BSA;34).



Antibody production. In a typical method of producing antibodies in large animals, the antigen is mixed with an adjuvant in a water-oil emulsion for injection. Using a sheep, the injections are placed in the area of the base of the neck to provide maximal stimulation to the regional lymph nodes. Generally, weekly injections are given for 6 weeks, followed, starting at 2 months, by monthly booster injections. Animals are bled periodically and the antibody titer checked using serial dilutions of the plasma and determining the dilution at which 50% of the labeled steroid is bound to the antibody. The titer generally plateaus at 8-9 months following the initial injection.

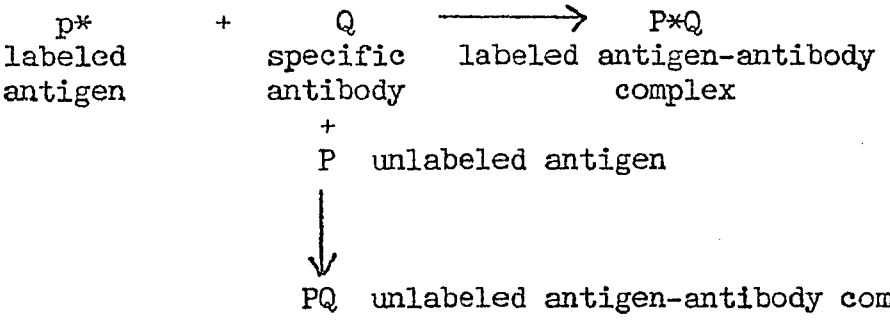
In order to separate the antibodies, which are gamma globulins, from other proteins in the serum, some of which will also bind steroids, one adds rivanol (2-ethoxy-6,9-diaminoacridine lactate) in 0.4% aqueous solution to the serum (4 parts rivanol to 1 part serum). The rivanol precipitate is removed by centrifugation and excess rivanol is removed by the use of charcoal and centrifugation, leaving a serum free of cross-reacting proteins with a globulin dilution of 1:5 (35).

Following rivanol purification, a titer check and specificity check are made. To determine specificity, one determines the binding of steroids other than the one against which the antibody was made. Percent cross-reactions are determined by the amount of unlabeled steroid which will displace 50% of the bound labeled steroid against which the antibody is most specific. For example:

mass of estradiol which displaces 50% of the bound labeled estradiol = x. Mass of other steroid required to displace 50% of the bound labeled estradiol = y. Therefore: $(y/x) \cdot 100$ = percent cross-reaction.

For specific antibodies directed against steroids to be practical for radioimmunoassay they should be (1) relatively easy to produce, (2) of high enough titer for wide-spread or long-term use, (3) sufficiently stable to allow for long-term storage or transportation, (4) as specific as the binding proteins from target tissues or plasma and (5) of high enough affinity to yield an assay with a sensitivity equal to other available methods of determination.

Assay theory. The competitive inhibition principle of radioimmunoassay can be summarized as shown.



The unlabeled antigen competes against labeled antigen for binding to the antibody and thereby diminishes the binding of the labeled antigen. For determination of the concentration of an unknown amount of antigen, the degree of competitive inhibition observed in the unknown is compared to that obtained in known standard solutions. The validity of the assay depends on identical behavior of the standard antigen and the antigen in the unknown (34).

For an excellent review of the mathematical theory of radioimmunoassay, the reader is referred to the chapter by Feldman and Rodbard in the book by Odell and Daughaday (34). As stated by these authors, the basic assumptions of the radioimmunoassay are:

(1) the antigen is present in one homogeneous form.

(2) the antibody is present in one homogeneous form.

(3) one molecule of antigen reacts with one molecule of antibody.

(4) no allosteric or cooperative effects exist, i.e., the antigen and antibody react according to the first order mass-action law.

(5) radioactively-labeled and unlabeled antigen have the same physico-chemical properties.

(6) the antigen and antibody react until equilibrium is reached.

(7) bound and free forms of antigen can be separated perfectly without perturbing equilibrium.

(8) the ratio of bound to free antigen or the ratio of bound to total antigen can be measured perfectly.

One can portray mathematically the radioimmunoassay as follows:

$$(1) \quad P + Q \underset{k'_1}{\overset{k_1}{\rightleftharpoons}} PQ$$

(2) $P^* + Q \underset{k'_2}{\overset{k_2}{\rightleftharpoons}} P^*Q$

where P is unlabeled antigen; P* is labeled antigen; Q is the antibody; and PQ and P*Q the unlabeled and labeled antigen-antibody complexes, respectively; k_1 and k_2 are the association rates, and k'_1 and k'_2 are the dissociation rates.

At equilibrium, according to the first order mass-action law:

(3) $K = k_1/k'_1 = [PQ] / [P] \cdot [Q]$

(4) $K^* = k_2/k'_2 = [P^*Q] / [P^*] \cdot [Q]$

K and K* are affinity constants. By assumption 5, $K = K^*$.

We define the bound to free ratio for labeled antigen as

(5) $R = [P^*Q] / [P^*]$

the total concentration of bound antigen as

(6) $B = [PQ] + [P^*Q]$

and the found to total ratio as

(7) $B/T = R/1 + R = [P^*Q]/p^* = [PQ]/p.$

(8) where $p = [P] + [PQ]$, and

(9) $p^* = [P^*] + [P^*Q]$.

p* = the total concentration of labeled antigen and p = the total concentration of unlabeled antigen.

The total concentration of antibody, q, is:

(10) $q = [Q] + [P^*Q] + [PQ]$

The concentrations are expressed as moles/liter and the K values as liters/mole.

Combining equations 3-10 gives

(11) $R = K(q - B)$

and indicates a linear relationship between the bound-to-free ratio (R) and the total concentration of bound antigen, (B).

By plotting R vs. B one obtains a Scatchgard plot enabling one to estimate K from the slope, and q by the horizontal axis intercept. The vertical axis intercept is Kq. By plotting real data and fitting a straight line, one can obtain an estimate of K and q for any real assay system (34).

MATERIALS AND METHODS

Reagents and Supplies

Sources of supplies. Radioactive estrone (6,7-^{3}H-estrone, s.a. 147 mci/mg) and estradiol-17β (2,4,6,7-^{3}H-estradiol-17 , s.a. 390mci/mg) and Liquifluor were obtained from New England Nuclear Corporation, Boston, Mass. and used without further purification.

Crystalline estrone (E_1) and estradiol-17β (E_2) were purchased from Sigma Chemical Company, St. Louis, Mo.

Reagent grade benzene, methanol, toluene and sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$; M. W. 138) were obtained from J. R. Baker Chemical Co., Phillipsburg, N.J. Diethyl ether and sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$; M. W. 268) were purchased from Mallinckrodt Chemical Works, St. Louis, Mo. Biosolve BBS #3 was produced by Beckman Instrument Co., Fullerton, Calif. All solvents were used without further purification.

Other materials and chemicals used were activated charcoal (Norit-A, Coleman, Matheson and Bell), Sephadex LH-20 and Dextran T-70 (Pharmacia Fine Chemicals, Uppsala, Sweden), sodium azide (NaN_3, Fisher Scientific Co.) and unflavored gelatin (General Chemical Co.).

E_2 antiserum was produced by Dr. Burton Caldwell by immunizing sheep with E_2-17-hemisuccinate-bovine-serum-albumin. The sheep plasma was treated with rivanol and charcoal and supplied as a 1:5 dilution of the original plasma.

Reagents. The basic buffer solution for E_1 and E_2 radioimmunoassay was prepared by mixing 32.7 g sodium phosphate dibasic heptahydrate, 10.8 g sodium phosphate monobasic monohydrate, 2.0 g sodium azide and 8.0 g sodium chloride with double-distilled, double-deionized water to a volume of 2 liters. The unadjusted pH was 7.0 + 0.1. Unflavored gelatin was added to the buffer to a concentration of 0.1% and the solution stored at 4°C.

A dextran-coated charcoal suspension was prepared monthly by mixing 250 mg. Norit-A, 25 mg. Dextran T-70 in 100 ml assay buffer containing 0.1% gelatin. The contents were shaken vigorously for 30 seconds and stored at 4°C. Both the charcoal and assay buffer solutions were freshly made monthly.

Tritium labeled E_2 was stored at 4°C in benzene:methanol (9:1, V/V). For use in the radioimmunoassay, sufficient steroid was transferred to a clean flask, dried under nitrogen and dissolved in assay buffer (0.1% gelatin) to give a final radioactivity of 4×10^4cpm (ca. 1×10^5 dpm; ca. 150 pg) per ml. of buffer solution.

Unlabeled E_1 and E_2 were prepared in benzene:methanol (85:15) to give a final concentration of 100 pg/ml. Antiserum to E_2 was stored in a stock solution of 1:5 dilution. For E_2 assay, the antiserum was diluted to 1:35,000 with assay buffer (0.1% gelatin). For E_1 assay, the antiserum was diluted to 1:10,000.

Scintillation fluid was prepared by mixing 40 ml. Liquifluor and 155 ml. Biosolve BBS #3 with 1 liter of toluene.

Columns for chromatography were prepared by soaking Sephadex LH-20 in benzene:methanol (85:15, V/V) overnight, washing with fresh solvent to remove the fine particles and packing 9 mm (i.d) glass columns to a height of 30 cm. Columns were washed with 200-300 ml of solvent, and standardized with ^{3}H-E_1 and -E_2. One ml fractions were collected and counted in a Packard Scintillation Counter.

Experimental Procedures

Blood Samples. Samples obtained from adult male rats (250-400 g.) treated with WIN 18446, and busulfan or vehicle alone, and previously analyzed for FSH, LH and testosterone concentrations, were pooled (2-3 animals per pool) within the weekly treatments groups, to a volume of 2 ml per pool (see reference 19 for treatment groups and references concerning the drugs used). Samples were stored at -20°C until thawed for extraction.

Surgery: Bilateral cryptorchidism was produced by making a midventral incision in the ether-anesthetized rat. Both testes were retracted into the abdominal cavity and retained there by a loose ligature. Sham controls were handled as above, but testes were replaced in the scrotum before closing the incision. At 0.5, 1, 2, 4, 8, and 16 days following the operations, five cryptorchid and sham controls were lightly anesthetized, bodyweights were recorded, and rats were exsanguinated via cardiac or aortic puncture. Testes, seminal vesicles and ventral prostates were removed, trimmed and weighed.

Blood was allowed to clot and the serum was removed after contrifugation (4°C, 6000 rpm, 15 minutes). Serum was frozen and stored at -20°C until thawed for testosterone and estrogen assays.

Testosterone assay was performed on diethyl ether extracts of serum (0.05 ml) without further purification. Details of the testosterone assay have been published (19).

Radioimmunoassay. Pooled serum (2 ml.) and serum from cryptorchid rats was extracted (3 x 3 ml.) with diethyl ether. Ether extracts were dried under nitrogen in a water bath (35°C) and the residue dissolved in 1.0 ml. of benzene:methanol (85:15). Duplicate 0.25 ml. aliquots (equivalent to 0.5 ml of serum) of the extracts were applied to Sephadex LH-20 columns and estrone and estradiol were separated. The E_1 and E_2 fractions were dried under nitrogen, and 0.1 ml of antiserum (1:35,000 for E_2; 1:10,000 for E_1) was added to each tube and vortexed gently; 0.1 ml of 2,4,6,7-^{3}H-E_2 (4000 cpm; 15 pg) was added and mixed. Following overnight incubation at 4°C, 0.5 ml of charcoal suspension was added, mixed and the tubes incubated on ice for 20 minutes. The tubes were centrifuged at 3090 x g for 20 minutes and the supernatant decanted into scintillation vials containing 10 ml of biosolve-liquifluor scintillation fluid. Each sample was counted for 1 minute, a standard curve (0-90 pg E_1 or E_2) was run with each group of samples. The standard curve constructed and each unknown sample quantitated against the appropriate standard curve. E_2 values were corrected for procedural losses. E_1 values were corrected for procedural losses and for the lower cross-reactivity of estrone with

the estradiol antibody in the heterogeneous assay system used. The characteristics of the antibody have been published (48); the percent cross-reactivity of E_1 with the E_2 antibody is 63.7%.

Steroid identification. Serum from male rats and rams was extracted with deithyl ether and dried under nitrogen. The residues were taken up in benzene:methanol (85:15). Aliquots were removed from the extracts, labeled E_1 and E_2 were added and the samples were chromatographed on Sephadex LH-20 columns. One half of each 1 ml fraction was removed and counted for the presence of label. The remaining 1/2 ml was dried in assay tubes and radioimmunoassay done on each fraction. The resulting radioactive and radioimmunoassay curves were superimposed.

Due to the low levels of E_1 and E_2 encountered in the rat serum, and the similarity in the elution patterns (label and RIA) of the rat and ram serum, ram serum was used for the following procedure.

Ram serum (1 l. volumes) was extracted and the extracts chromatographed to separate estrone and estradiol. The separated steroids were dried in conical tubes and acetylated. Pyridine (0.1 ml) and acetic anhydride-1-C^{14} (ca. 5 ci) were added to each tube. ^3H-E_1 and ^3H-E_2 were dried in separate conical tubes and 0.1 ml pyridine and 0.1 ml unlabeled acetic anhydride were added. Acetylation was done at room temperature in a darkened container for 24 hours. Following the incubation period, 1.0 ml of distilled water was added to each tube to stop the reaction, and each mixture was extracted (4 x 2 ml) with ethyl acetate. The extracts were dried under

nitrogen and the residues taken up in benzene:methanol and the extracts chromotagraphed. An aliquot of C^{14} labeled acetaye was added to an aliquot of ^{3}H labeled acetate and the mixture chromatographed. One ml fractions were collected in scintillation vials, counted and the ^{3}H and C^{14} elution curves superimposed.

Statistics. Data were analyzed by the use of Student's t test and correlations were computed as outlined by Snedecor and Cochran (40).

RESULTS

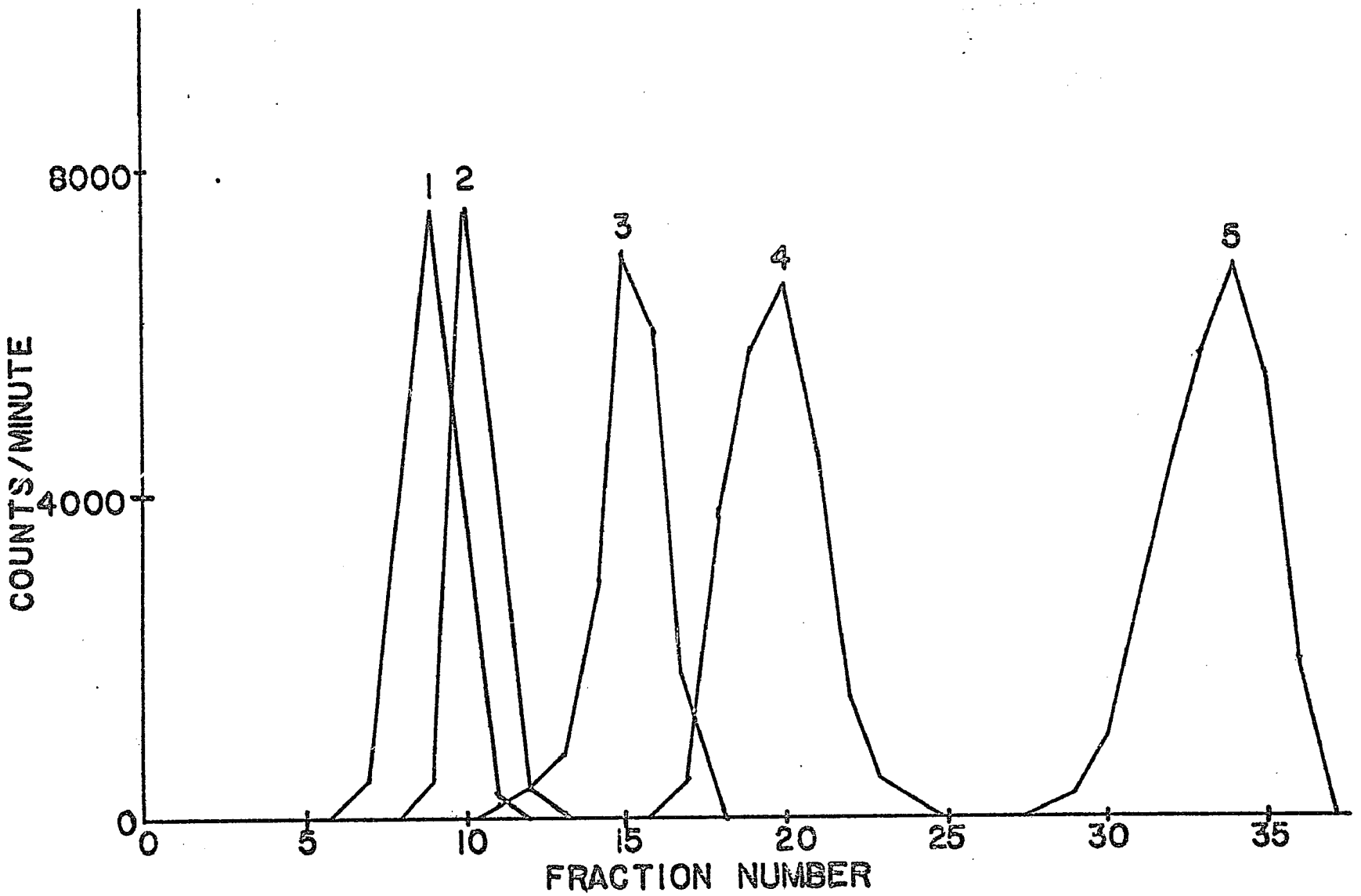
Steroid identification: Typical elution patterns of the steroids and steroid derivatives of interest in this study are shown in figure 1.

Superimposition of labeled E_1 and labeled E_2 profiles on the RIA pattern of rat and ram serum are shown in figures 2 and 3. Peaks corresponding to E_1 and E_2 are evident in both and a third peak, of unknown identity, appears in the RIA pattern midway between the E_1 and E_2 peaks. Elution and RIA curves of equilin and equilinen, estrogens having an unsaturated B ring, were run. Both steroids migrated similarly to E_1 but neither cross-reacted well with the anti-E_2 antiserum (Equilin = 5.9%; Equilinen = 2.1%). Equilin and equilinen differ from E_1 in that the former has a single unsaturation in the B ring while the latter has a double unsaturation in the B ring. The dihydro-derivatives of equilin and equilinen, which differ only in B unsaturations from E_2 were not run; the identity of the third peak remains unknown.

Elution of acetates: Presumptive E_1 and E_2, separated by column chromatography from ram serum, were acetylated with acetic anhydride-1-C^{14}. Tritiated E_1 and E_2 were acetylated with unlabeled acetic anhydride and aliquots of each were handled as previously described. The tritium and C^{14} - labeled acetates were mixed and applied to the Sephadex - LH20 columns and the elution carried out using benzene: methanol (85:15). The results are presented in figure 4 with the

Figure 1. Chromatographic pattern of steroids eluted from Sephadex LH-20 columns

Peak
1. Estrone acetate
2. Estradiol acetate, testosterone acetate, dihydrotestosterone acetate
3. Testosterone
4. Estrone
5. Estradiol-17β


STEROID ELUTION PATTERN
8000
4000
0
COUNTS/MINUTE
1
2
3
4
5
0
5
10
15
20
25
30
35
FRACTION NUMBER

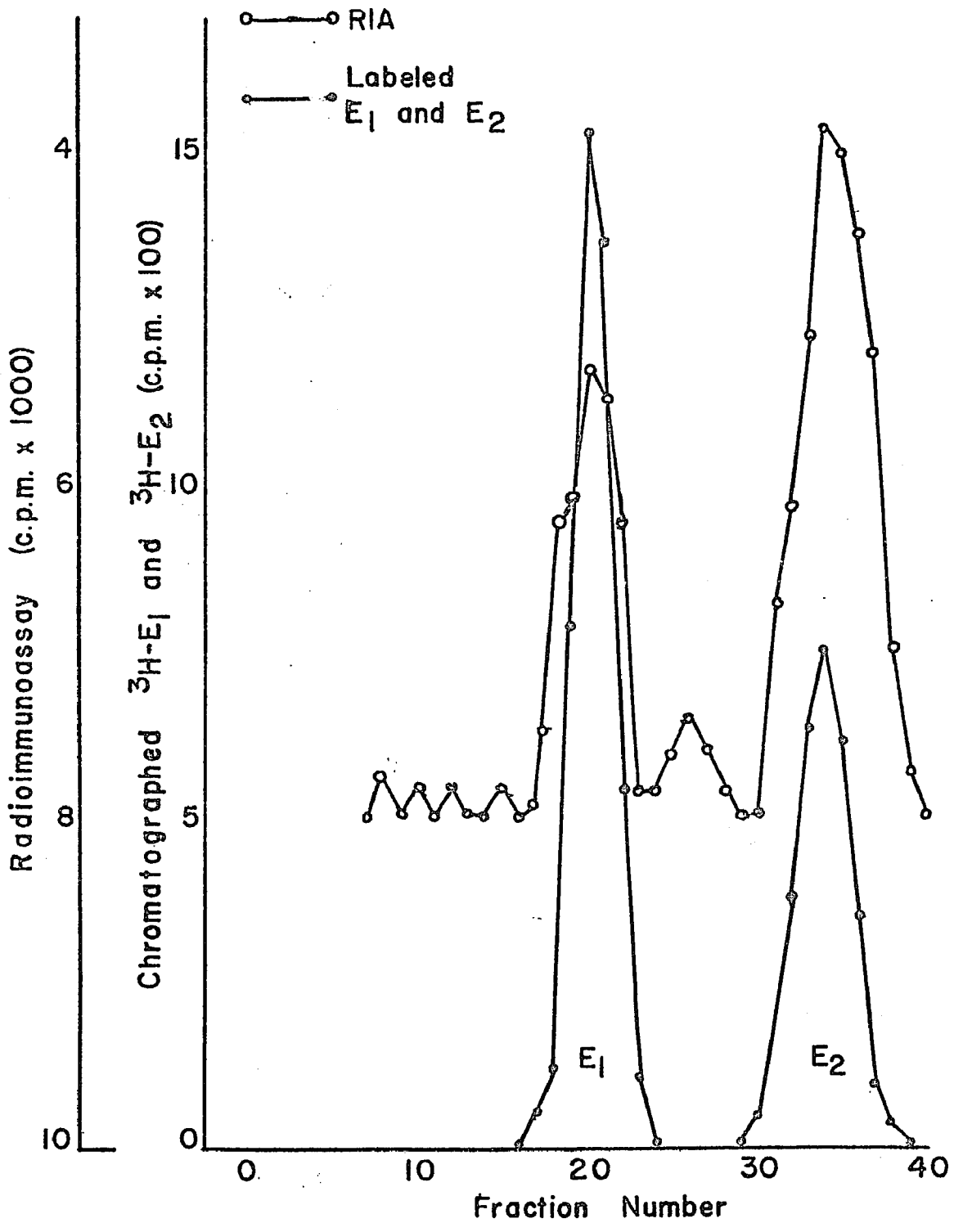


Figure 2. Chromatographic pattern of labeled E_1 and E_2 superimposed upon the pattern determined by RIA of rat serum extracts.

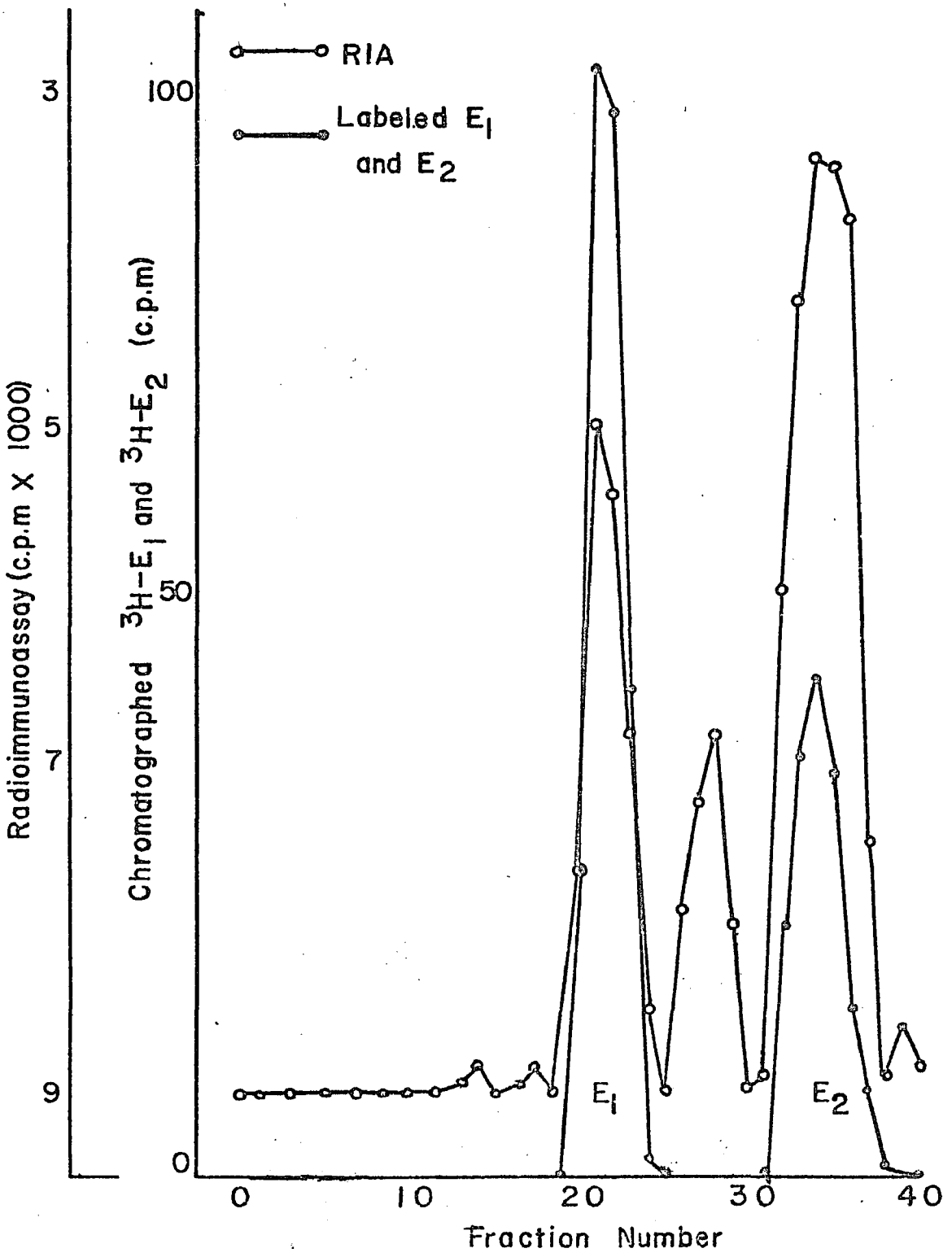


Figure 3. Chromatography pattern of labeled E_1 and E_2 and pattern determined by RIA of ram serum extracts.

C^{14} and ^{3}H counts contained in each fraction superimposed. ^{3}H- and C^{14}-E_1 acetates migrated together indicating that the compounds are identical. ^{3}H- and C^{14}-E_2 acetates also appear to migrate together but yield a biphasic peak. It is likely that the biphasic pattern represents mono- and diacetates of E_2 due to incomplete acetylation of some of the E_2 molecules. This may be due to differences in reactivity of the phenolic hydroxyl present at the 3 carbon and the secondary alcohol at carbon 17 of the estradiol-17β molecule. In addition, the conditions of acetylation may not have yielded a good pyridine: acetic anhydride ratio for complete acetylation of both hydroxyls on the estradiol-17β molecule, as pointed out by Dominguez, et. al. (11).

From the results of the two experiments, one can reasonably assume that both estrone and estradiol-17β are present in the peripheral serum of the rat and ram, along with a minor, unidentified estrogen.

Busulfan & WIN 18446: Changes in serum E_1 levels after chemical induction of aspermatogenesis are shown in table 1 and figure 5. Estrone levels did not differ from control values except at week 20 when the WIN 18446 group was higher ($p<0.05$) than 20-week control levels.

Serum E_2 levels (table 1, figure 6) did not differ from control values over the 20-week period except for the week 16 WIN 18446 levels which were higher ($p<0.05$) than control lvels for the same period.

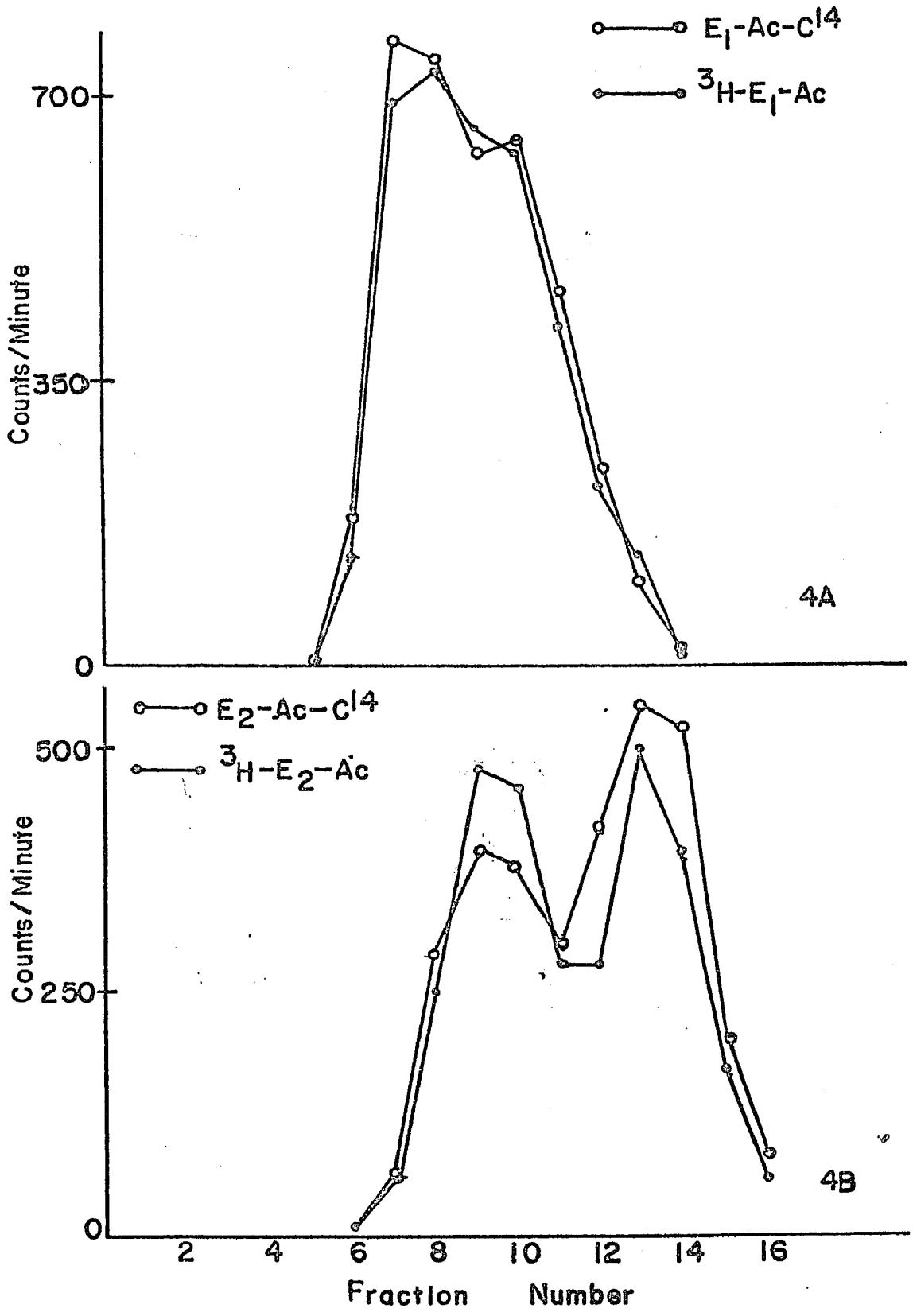


Figure 4. Chromatographic pattern of ^{3}H- and C^{14} Labeled estrone and estradiol acetates.

TABLE 1
WEEKLY CHANGES IN SERUM ESTRONE, ESTRADIOL-17
AND TOTAL ESTROGENS FOLLOWING TREATMENT WITH
WIN 18446 AND BUSULFAN.

Treatment Week	Control[a]	Busulfan[a]	WIN 18446[a]
	Estrone (pg/ml)		
1	11.5 ± 2.0(4)[b]	19.5 ± 0.5(2)	45.0 ± 26.7(2)
2	35.7 ± 8.7(6)	38.7 ± 9.2(4)	19.0 ± 2.0(2)
3	161.0 ± 63.9(2)	30.0 ± 6.7(4)	62.0 ± 21.8(8)
4	162.7 ± 17.3(3)	29.0 ± 21.0(2)	39.8 ± 14.0(8)
6	48.5 ± 14.7(4)	45.8 ± 11.3(4)	58.8 ± 24.7(6)
8	31.5 ± 0.5(2)	107.2 ± 37.5(4)	59.8 ± 17.7(6)
10	34.0 ± 5.2(10)	37.0 ± 14.4(4)	63.8 ± 23.6(6)
12	55.5 ± 16.5(6)	32.4 ± 7.5(5)	74.0 ± 34.9(4)
16	14.0 ± 1.0(2)	19.5 ± 2.4(4)	106.5 ± 73.5(2)
20	23.8 ± 4.8(4)	37.5 ± 18.4(2)	93.5 ± 15.8(6)*
	Estradiol-17β (pg/ml)		
1	37.8 ± 27.5(4)	23.7 ± 7.2(3)	125.0 ± 55.0(2)
2	67.5 ± 39.2(4)	58.0 ± 38.0(2)	105.0 ± 74.8(2)
3	20.7 ± 8.8(4)	48.5 ± 26.5(2)	33.5 ± 14.4(8)
4	40.0 ± 28.0(4)	128.5 ± 8.5(2)	11.4 ± 4.4(8)
6	13.5 ± 1.5(2)	116.3 ± 37.4(4)	90.0 ± 21.7(6)
8	47.0 ± 10.1(4)	26.6 ± 6.5(8)	17.3 ± 11.3(8)
10	40.0 ± 13.9(2)	57.5 ± 33.5(2)	84.6 ± 24.2(8)
12	29.5 ± 13.5(4)	40.8 ± 18.1(4)	56.0 ± 41.4(4)
16	26.5 ± 5.5(2)	33.5 ± 20.5(2)	51.5 ± 5.5(2)*
20	78.5 ± 34.2(4)	28.5 ± 5.0(4)	78.3 ± 8.2(6)
	Total Estrogens (E_1 + E_2; pg/ml)		
1	49.3 ± 4.3(8)	43.2 ± 10.9(5)	170.0 ± 49.0(4)*
2	103.2 ± 16.5(10)	96.7 ± 12.1(6)	124.0 ± 8.0(4)
3	181.7 ± 26.6(6)	78.5 ± 8.9(6)*	95.5 ± 13.2(16)**
4	202.7 ± 29.7(7)	157.5 ± 28.8(4)	51.2 ± 8.0(16)**
6	62.0 ± 11.9(6)	162.1 ± 7.1(8)**	148.8 ± 15.9(12)**
8	78.5 ± 7.2(6)	133.8 ± 19.0(12)*	77.1 ± 8.0(14)
10	74.0 ± 4.7(12)	94.5 ± 12.2(6)*	148.4 ± 13.9(14)**
12	85.0 ± 11.5(10)	73.2 ± 8.5(9)	130.0 ± 25.4(8)
16	40.5 ± 4.3(4)	53.0 ± 6.2(6)	158.0 ± 34.0(4)*
20	102.0 ± 19.0(8)	66.0 ± 6.0(6)	171.8 ± 15.7(12)*

a. Values are $\bar{X}$ ± S. E.
b. Number in parentheses are the number of determinations per group.
* Significantly different from control value (P 0.05)
** Significantly different from control value (P 0.01)

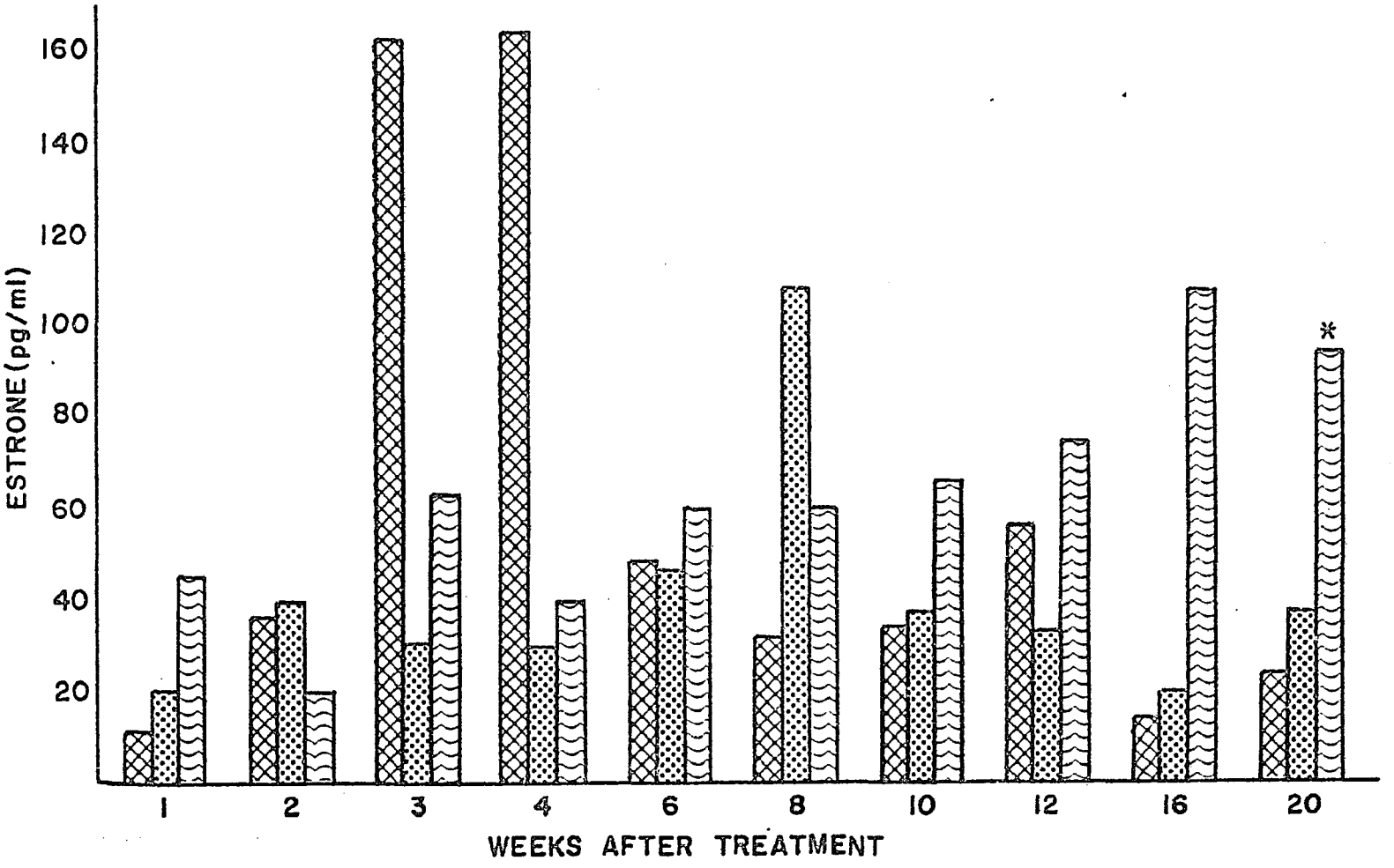


FIGURE 5. Weekly changes in serum estrone levels following treatment with busulfan and WIN 18446

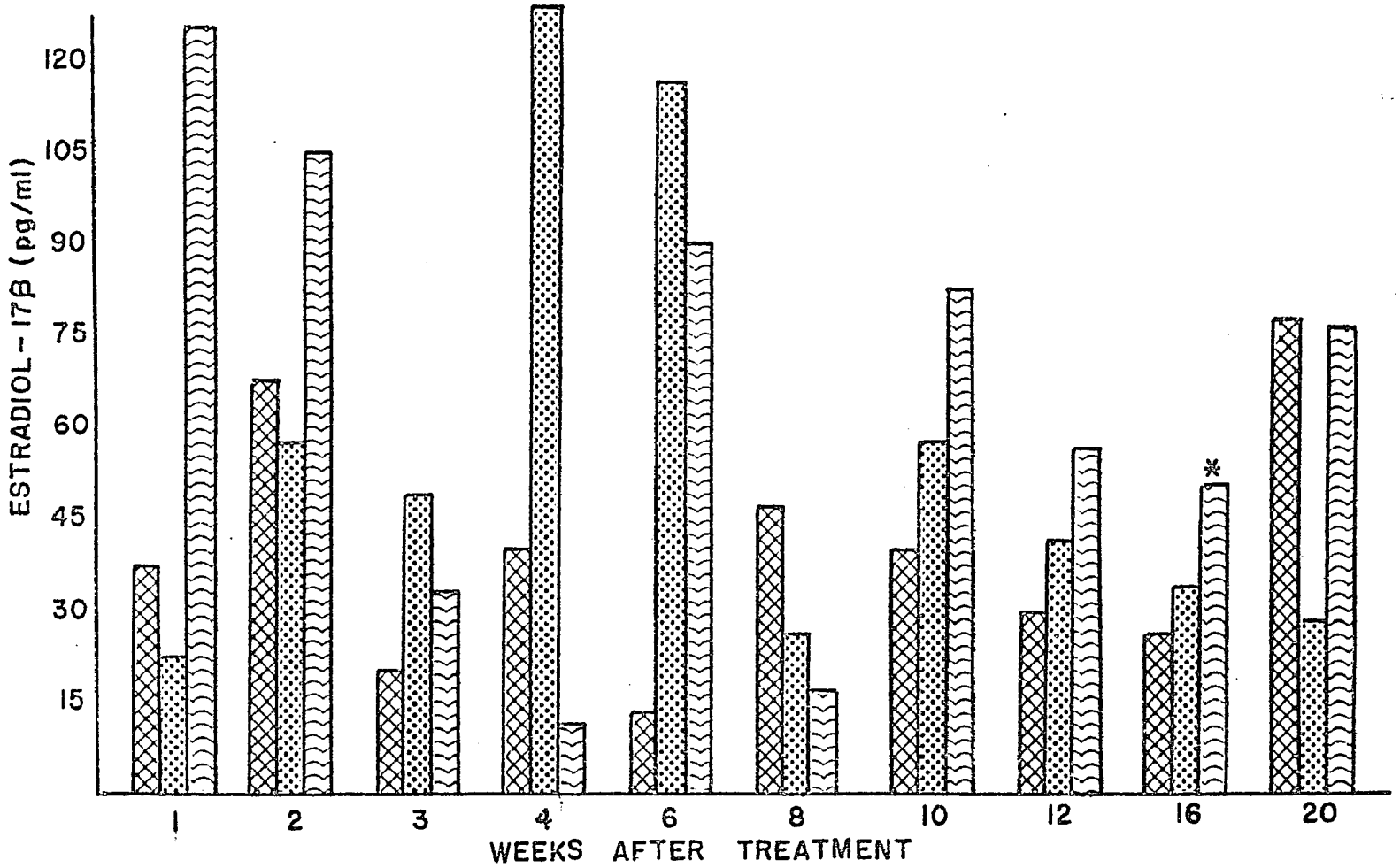


FIGURE 6. Weekly changes in serum estradiol-17β levels following treatment with busulfan and WIN 18446

Weekly changes in total (E_1 + E_2) estrogen levels (table 1, figure 7) showed more consistent changes than either hormone considered alone. Total estrogen levels of the busulfan-treated rats increased through week-6 and declined to control levels through 20 weeks after treatment. Total estrogen levels were higher than control values (p 0.05) at weeks 6, 8 and 10, and lower (p 0.05) than control levels at weeks 3 and 12. Total estrogen levels for the WIN 18446-treated rats declined to a minimum level ($p<0.05$) 4 weeks after treatment and tended to increase thereafter. Total estrogen levels for the WIN 18446 group were higher than control values ($p<0.05$) at weeks 1, 6, 10, 16, and 20 and lower ($p<0.05$) at weeks 3 and 4 post-treatment.

Cryptorchid Rats. Changes in body and organ weights of the rats in the cryptorchid study are presented in table 2. Body weights were not different between the two groups except at day 1 when the cryptorchid group was heavier ($p<0.05$) than controls. This is probably not related to the surgical procedures used.

Seminal vesicle weights did not differ between groups throughout the treatment period. Ventral prostate weights (mg/100g body weight) were heavier than controls at day 1 ($p < 0.05$) and lighter than controls at days 4 and 8 ($p < 0.05$). Testes (g) were heavier than controls at day 1, and lighter at days 2, 8 and 16 ($p < 0.05$). When adjusted for body weight, testes (mg/100g body weight) were less than controls at days 8 and 16 ($p < 0.01$).

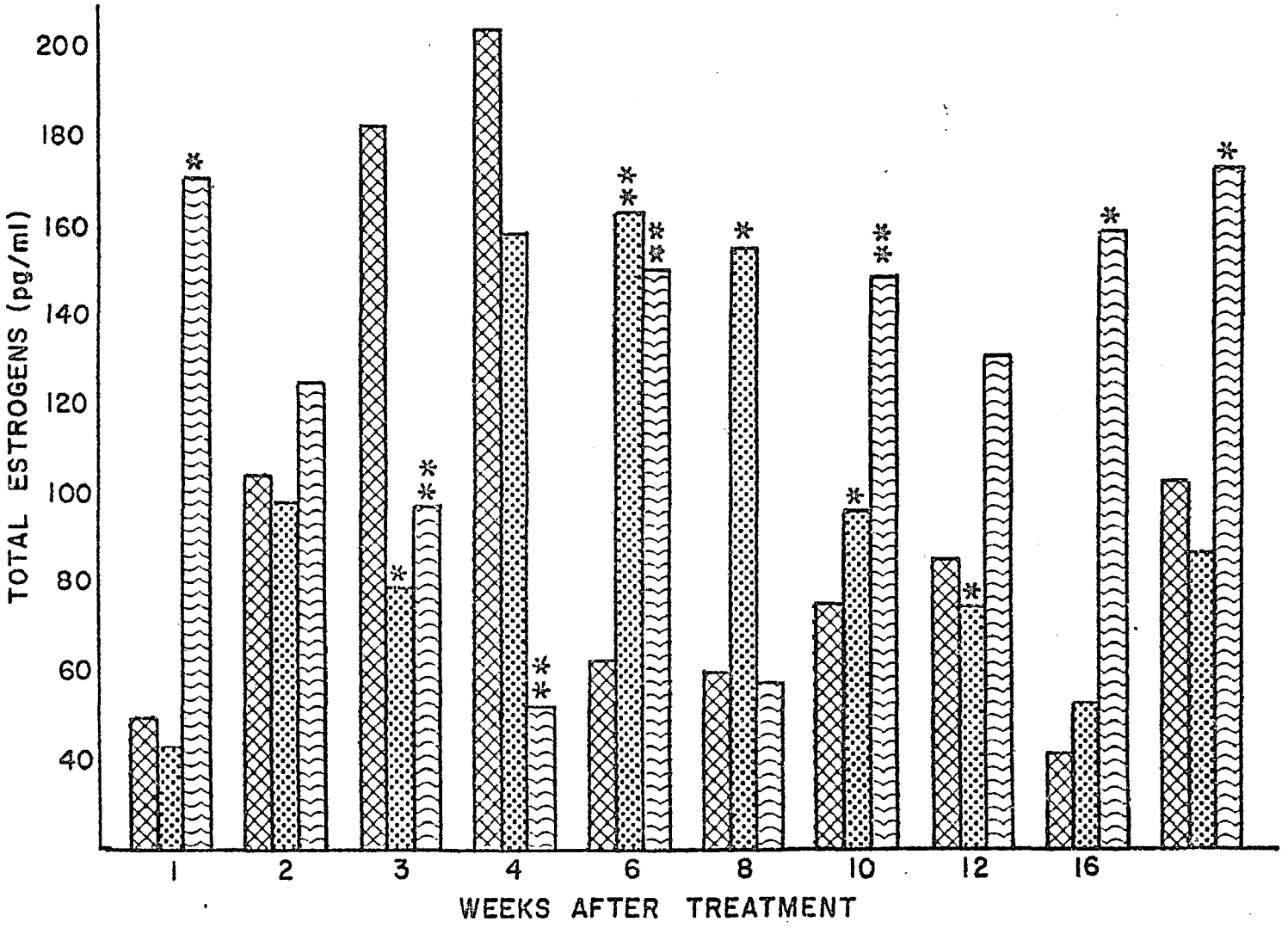


FIGURE 7. Weekly changes in serum total estrogen levels following treatment with busulfan and WIN 18446

TABLE 2
CHANGES IN RAT ORGAN WEIGHTS
FOLLOWING ARTIFICIAL CRYPTORCHIDISM

Time (days)	Control $\bar{X} \pm$ S. E.	n	Cryptorchid $\bar{X} \pm$ S. E.
	Body Weight[a]		
0.5	272.9 ± 12.9	5	263.5 ± 27.5
1	227.6 ± 8.0	5	270.5 ± 9.1**
2	304.1 ± 34.4	5	257.4 ± 15.4
4	267.1 ± 10.3	5	263.8 ± 22.5
8	284.8 ± 15.4	5	261.1 ± 20.7
16	335.1 ± 14.7	5	302.3 ± 17.3
	Seminal Vesicles[b]		
0.5	217.4 ± 20.7	5	178.2 ± 10.8
1	151.2 ± 6.9	5	183.1 ± 24.2
2	166.7 ± 23.1	5	163.2 ± 16.0
4	183.9 ± 6.7	5	152.6 ± 15.5
8	172.2 ± 22.0	5	132.2 ± 1.7
16	154.8 ± 8.9	5	157.8 ± 18.8
	Ventral Prostate[b]		
0.5	118.2 ± 14.4	5	99.2 ± 22.9
1	87.5 ± 5.3	5	108.9 ± 5.9*
2	99.0 ± 12.4	5	102.7 ± 14.7
4	124.3 ± 7.6	5	91.8 ± 3.5**
8	111.6 ± 16.4	5	80.5 ± 2.9*
16	138.6 ± 8.1	5	126.3 ± 12.1
	Testes[a]		
0.5	1.407 ± 0.159	10	1.392 ± 0.059
1	1.500 ± 0.048	10	1.930 ± 0.130**
2	1.678 ± 0.087	10	1.558 ± 0.053*
4	1.472 ± 0.033	10	1.548 ± 0.062
8	1.638 ± 0.063	10	0.782 ± 0.028**
16	1.648 ± 0.040	10	0.750 ± 0.060**
	Testes[b]		
0.5	575.0 ± 27.4	10	541.3 ± 28.9
1	660.3 ± 19.8	10	710.0 ± 38.0
2	562.7 ± 18.8	10	610.1 ± 20.7
4	552.0 ± 10.3	10	587.0 ± 21.2
8	577.7 ± 15.9	10	306.7 ± 19.7**
16	494.7 ± 12.1	10	248.9 ± 19.3**

a. Values presented in grams.
b. Values presented in g/100 g. body weight.
* Significantly different from control value ($p < 0.05$)
** Significantly different from control value ($p < 0.01$)

Hormone levels are shown in table 3. Serum testosterone (ng/ml serum) tended to be lower than controls (except at day 8) throughout the treatment period, but were significantly lower only at days 4 and 16 ($p \leqslant 0.01$). Serum E_2 levels tended to be lower throughout the treatment period except at day 1 when E_2 was higher (NS) than controls. E_2 was significantly lower than controls at days 4 and 16 ($p \leqslant 0.05$).

Serum E_1 levels were higher than controls at days 0.5 ($p \leqslant 0.05$), 1, 2 and 8 and lower than controls at days 4 and 16.

TABLE 3
CHANGES IN SERUM ESTROGEN AND TESTOSTERONE LEVELS FOLLOWING ARTIFICIAL CRYPTORCHIDISM

Time (days)	Control X ± S. E.	n	Cryptorchid X ± S.E.
	Estradiol-17β (pg/ml)		
0.5	5.6 ± 3.9	5	12.0 ± 3.7
1	40.0 ± 59.0	5	81.2 ± 13.0
2	N.D.(a)	5	5.6 ± 3.6
4	6.2 ± 1.8	5	2.2 ± 1.0*
8	23.6 ± 10.0	5	7.2 ± 3.7
16	6.8 ± 1.0	5	2.0 ± 1.0**
	Estrone (pg/ml)		
0.5	6.0 ± 2.2	5	39.6 ± 17.6*
1	138.8 ± 19.0	5	122.0 ± 16.5
2	7.6 ± 2.6	5	3.6 ± 2.2
4	109.6 ± 22.1	5	83.6 ± 7.7
8	130.0 ± 20.6	5	140.0 ± 17.5
16	110.0 ± 16.3	5	105.2 ± 19.7
	Testosterone (ng/ml)		
0.5	2.38 ± 0.56	5	0.98 ± 0.21
1	3.18 ± 1.26	5	1.78 ± 0.42
2	6.48 ± 2.16	5	2.60 ± 0.17
4	3.80 ± 0.30	5	1.30 ± 0.50**
8	2.58 ± 0.53	5	4.74 ± 1.66
16	4.54 ± 0.44	5	1.68 ± 0.29**

* Significantly different from control value (P 0.05)
** Significantly different from control value (P 0.01)
a Nondetectable levels

DISCUSSION

Results on parameters previously studied (19) using busulfan and WIN 18446 are shown in figures 8 - 11. Male rats weighing 250-350g were treated with a single i.p. injection of the antispermatogenic agents busulfan (10 mg/kg body weight) or WIN 18446 (125 mg/kg body weight) or vehicle alone. Groups of control, busulfan- and WIN 18446-treated rats were sacrificed periodically between 1 and 20 weeks after treatment. Testis weights (figure 8) decreased rapidly during 3-6 weeks after treatment. Thereafter, testis weights increased, but remained below ($p \leqslant 0.05$) control levels 20 weeks after treatment. Testis weights (figure 8) decreased rapidly during 3-6 weeks after treatment, reaching minimal levels ($p \leqslant 0.01$) by 8 weeks after treatment. Thereafter, testis weights increased, but remained below ($p \leqslant 0.05$) control levels 20 weeks after treatment. Histological loss of spermatogenic elements, paralleling weight loss was seen; by 8 weeks after treatment, seminiferous tubules contained only basal spermatagonia. Tubules became repopulated at 10 weeks and quantitatively normal spermatogenesis was reestablished by 20 weeks. Concentrations of serum LH (figure 9) did not differ over the 20-week period, but serum FSH (figure 10) increased significantly by 3 weeks in both groups, reaching peak levels in busulfan-treated rats at 10 weeks and 12 weeks in WIN 18446 treated rats. FSH levels were highest, in the treated rats, when testis weight and spermatogenic activity were minimal and returned to control levels with the

Weekly changes in hormone and testis weights following treatment with WIN18446 and Busulfan (Busulfan ——— ; WIN18446 -----; controls, shaded area).

Figure 8 Testis weights

Figure 9 Serum LH (NIAMD LH-RP-1)

Figure 10 Serum FSH (NIAMD FSH-RP-1)

Figure 11 Serum testosterone

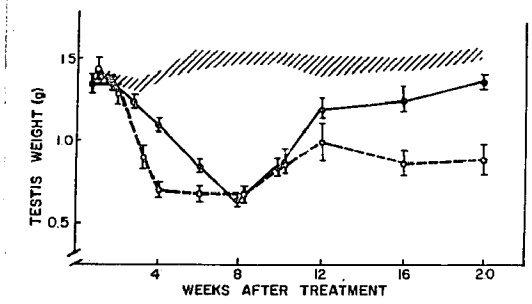


Figure 8

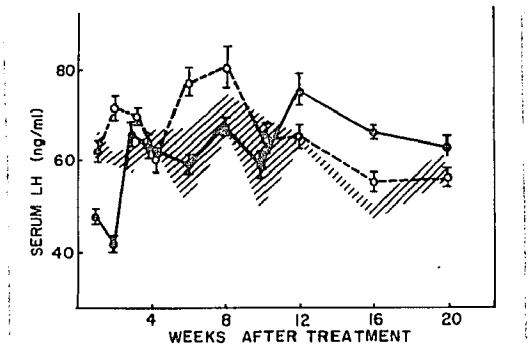


Figure 9

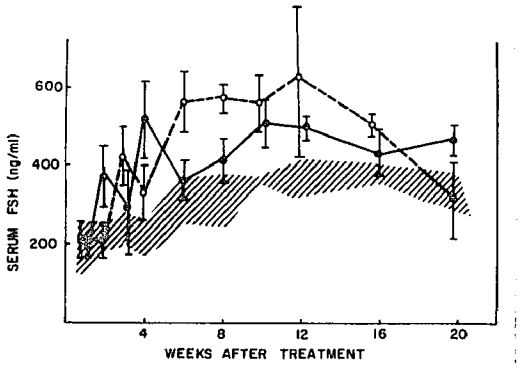


Figure 10

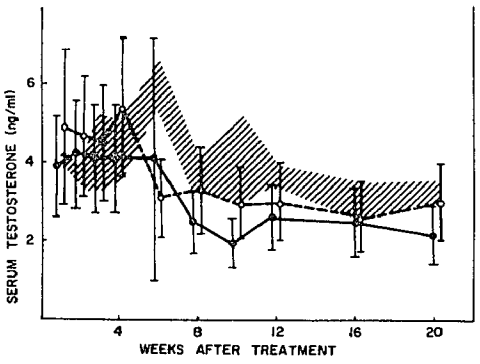


Figure 11

recurrence of spermatogenesis. Testosterone levels (figure 11) tended to decline in treated animals, particularly during the period of aspermatogenesis (19).

These results tended to support the concept of an inhibitory effect of spermatogenesis on circulating levels of FSH in rats (14) and was the basis for the selection of these particular samples for estrogen analysis.

A number of workers have, over the past 40 years, noted and attempted to describe the reciprocal relationship that appears to exist between spermatogenesis and gonadotropin secretion in males by the use of clinical studies on aspermia and oligospermia (14, 23, 29), cryptorchidism, (1, 42, 44) and drug treatments (9, 19), to produce aspermatogenic animals. These studies have led to the suggestion that the germinal epithelium secretes a hormone, "inhibin", which is responsible for the feedback regulation of FSH secretion. Increases in pituitary FSH levels have been reported in autoimmune aspermotogenic guinea pigs (see 19), and pituitary FSH levels have been reported to decline in male rats during busulfan-induced loss of germinal elements (8). Both groups suggested these effects were due to increased production and release of FSH during germinal cell loss.

In a carefully controlled study relating changes in serum gonadotropins and histologically apparent germinal cell loss, due to the actions of two different antispermatogenic drugs, Gomes et al. (19) confirmed the relationship between spermatogenesis and serum FSH

levels. A consistant relationship between degenerative and regenerative changes and testis weight changes existed, as did a significant reciprocal relationship between serum FSH and the testis weight changes, indicating a cause and effect relationship.

As noted by Gomes and Vandemark (20), a general lack of knowledge concerning the actual compounds involved in FSH regulation has prevented work concerning FSH regulation from progressing beyond pure speculation.

Johnsen (23) and Lacy (29) have proposed that the FSH inhibiting substance from the testis is an estrogen, and further that the process of spermiogenesis, somehow increases the ability of the Sertoli cell to produce estrogens, which regulate FSH production and/or resease.

The superimposition of the column chromatographic elution of labeled estrogens and the RIA of eluted extracts from ram and rat serum and the superimposition of the elution patterns of ^{3}H- and C^{14}- labeled estrone (E_1) and estradiol (E_2) indicated that estrogens are produced by the males of the two species. The serum levels of E_1 and E_2, in the control animals were a range of 11.5-162.7 pg/ml (estrone) and 20.7-78.5 pg/ml for estradiol-17β. DeJong et al. (10) reported levels of 2.0 ± 0.9 pg/ml for E_2 in the male rats weighing 200-250g. The levels reported here are much higher and may reflect the difference in bodyweights and ages of the animals in the two studies. Baird, et al. (3) reported levels of 74 ± 15 pg/ml of E_1 and 32 ± 5 pg/ml of E_2 in the peripheral plasma of men. In their study (3) it appears that E_1 levels are approximately 2 times higher

than E_2 levels, a condition that is seen in the rats used in this study. In man, E_2 levels in the testicular venous effluent are about 5.8 times higher than the levels of E_1 secreted by the testis, indicating that peripheral conversion of androgens (3) to E_1 may be higher than peripheral conversion to E_2 or that the oxidation of E_2 to E_1 is extensive, peripherally, in the male (5). Free and Tillson (15), while not separating estrone and estradiol, reported levels of an "estrone-like" compound in the testicular and peripheral venous blood of male rats. They reported that the testicular venous level of this "estrone-like" compound was 200 ± 114 pg/ml, with peripheral levels of 120 ± 11 pg/ml. The peripheral level of the "estrone-like" material falls within the range (40.5 - 202.7 pg/ml) of the total estrogen levels found in this study. The level in testicular venous blood (200 ± 114 pg/ml; anesthetized rats) is 17 times higher than the levels reported by De Jong (19) for E_2 concentration of the testicular venous plasma (17.5 ± 8.4 pg/ml), indicating a clear discrepancy in the results found in these studies.

The studies of De Jong (10) and Free and Tillson (15) indicate that the rat testis is capable of synthesizing and secreting estrogens.

In the present study, no clear pattern appears to exist between theand the levels of serum E_1 and E_2 (figs. 5, 6). Serum E_1 levels are and the levels of serum E_1 and E_2 (figs. 5, 6). Serum E_1 levels are higher (p 0.05) at week 20 following treatment with WIN 18446, but appear no different from control levels in the busulfan-treated group.

Serum E_2 levels in the WIN 18446 appear to decline through week 4, increase at week 6, decline again at week 8 and appear to be higher than control levels at weeks 10-20. E_2 levels in the busulfan-treated group increase through week 4 and decline in weeks 6-8 and thereafter remain at a fairly constant level, similar to those of the control rats. Control levels remain constant through week 16 and increase at week 20.

Total estrogen (E_1 + E_2) levels show a pattern similar to that exhibited by the serum E_2 levels. Total estrogen levels decline through week 4, and tend to show an increase through weeks 6-20 for the WIN 18446-treated animals. The levels for the busulfan-treated group increase through week 6, and decline to about control levels thereafter. Points at which significant differences between control and treated levels exist are shown in figure 7 and table 1. Control levels remain fairly constant throughout the study period, except for the inordinately high values at weeks 3 and 4, which appear to reflect the high E_1 levels found at these two periods.

Changes in the testis weights in the cryptorchid animals generally reflect the previous findings of Amatayakul et al. (1) and Gomes (unpublished data). The increased weights at day 1 reflect the 50g difference in body weights observed between the control and treated groups. The changes in seminal vesicle weights appear to not be reflected in changes in serum testosterone levels. However, the possibility that the changes may be reflected in changes in levels of other androgenic steroids, not measured in this study, such as

Δ^4- androstenedione (2) can not be ruled out. Inano and Tamaoki (22) have shown that defective steroid biosynthesis, (decreased testosterone production, but normal Δ^4- androstenedione) occurs in the cryptorchid rat testis. The decreased ventral prostate weights at days 4, 8 and 16 appear to follow the decreased levels of testosterone observed at these times.

Serum testosterone levels are generally lower in the bilateral cryptorchid rats as noted by Amatayakul et al. (1) but Gomes (unpublished data) and Lloyd (see reference 20) found serum testosterone levels in cryptorchid rats to be higher than controls at 4-32 days after cryptorchidism. The reason for the discrepancy in these results is unknown, but the results reported here tend to support the observations of Inano and Tamaoki (22) and Amatayakul et al. (1) that testosterone synthesis and/or release may be defective in cryptorchid rats.

Serum E_2 in the cryptorchid rats levels are generally closer to those of De Jong et al. (10) than those reported in the drug study reported here. The difference between the results of the two studies is unknown. Serum E_1 levels are higher than those reported in the drug study. In an early study, Takewaki (45) found that lower doses of estrogen were needed for cornification of vaginal grafts in cryptorchid male rats as opposed to the amount of estrogen needed for cornification of grafts in normal males. This suggested that this was the result of higher estrogen levels in the cryptorchid males. Hill (21) demonstrated that ovaries grafted into the ears of castrate

male mice secreted androgens sufficient to maintain normal seminal vesicle and prostate weights. Cooling the grafts to 22°C increased androgen production while heating to 33°C removed any evidence of androgen production, suggesting that the higher temperature (near body temperature) was necessary for the conversion of androgens to estrogens in the ovary. Both of these studies tend to suggest that the temperature optimum for aromatization of androgens in both testes and ovaries may be higher than scrotal temperature. The results presented here tend to not support this conclusion, at least for testes placed at a higher temperature.

Serum FSH levels in crytorchid rats (Gomes, unpublished data; 1) rise to a peak level 16-20 days post-surgery and remain at this high level through at least 40 days. Serum LH increases also, perhaps reflecting the decline in serum T levels. Serum E_2 levels are lower throughout the treatment period, while serum E_1 levels appear to be higher than controls through day 2, then decline through day 16. Thus, it appears, in this study that a reciprocal relationship may exist in these two groups.

If, as Johnsen (23) and Lacy (29) suggest, estrogens produced by the testis regulate FSH production and/or secretion, one should find a reciprocal pattern for estrogen and FSH levels in the male. The changes in serum E_2 and total estrogen levels seen in the WIN 18446- treated group decline rapidly through week 4 as did the testis weight (fig. 8) of the animals in this group. Serum FSH levels (fig. 10) increased through week 12 and declined thereafter, while

serum E_2 levels declined through week 4 and generally increased through week 20.

In the busulfan-treated group, E_2 levels increase through week 4 and decline to control levels at week 8 and remain near control levels for the remainder of the study period. Testis weight (figure 8) declines through week 8 and increases thereafter. Serum FSH levels increased through 10 weeks and declined thereafter. In this group, no reciprocal relationship between testis weight or serum FSH levels and serum E_2 or total estrogen levels appears to exist.

Perhaps the different responses of the serum estradiol levels may reflect some of the biochemical changes present in the testes of the drug-treated rats. Johnson et al. (24) reported an increase in sterol esters of rats treated with WIN 18446, and Reddy and Svoboda (38) noted that hyperplasia of the smooth endoplasmic reticulum occurred following treatment of rats with WIN 18446. Kar et al. (26) noted a decrease in sterol esters in the testes of busulfan-treated rats. If cholesterol ester stores in the testes, reflect changes in steroid production as suggested by Johnson (24) and Bartke (4) for the testis and Armstrong (2) for the ovary perhaps the changes noted above might be reflected in testicular estrogen production. If, as suggested, by Bartke (4), a decline in cholesterol ester results in maintenance of free cholesterol levels for continuous steroid production, a decline in sterol esters observed in the testes of busulfan-treated rats could mean that estrogen synthesis was increased concomitant with a lesion in the esterification of

cholesterol. If this indeed occurred, one might expect the estrogen levels in the busulfan-treated rats to increase, if the esterification lesion were of sufficient duration show a decline. In the WIN 18446-treated rats, estrogens would decline if esters were formed at the expense of free cholesterol, and then increase if free cholesterol increased. De Jong (10) reported that administration of FSH caused no increase in testicular estrogen secretion, so the increases in serum estradiol levels are probably not due to increased estrogen production stimulated by the high levels of FSH noted in the previous study (19).

Alternatively, one could propose that increased peripheral aromatization of testosterone resulted in the increased estrogen levels. If the decreases in serum testosterone levels (see fig. 11) were due to changes in testosterone synthesis and/or secretion by the testes, the increases in serum estrogen levels might well reflect changes in the availability of testosterone for peripheral aromatization. A number of workers (see 5 for review) in the late 1930's, reported on the conversion of testosterone to estrogens in castrate male rats and concluded that the male is capable of converting excess tertosterone to substances possessing estrogenic activity. Serum testosterone levels (19) of the busulfan treated males appeared to decline through week 10 (4 ng to 1.94 ng/ml and increased to control levels at week 20. In the WIN 18446 treated group testosterone levels increased through week 4 (4.9 to 5.4 ng/ml), declined to their lowest value at 6 weeks, and increased to control levels

thereafter. If only a portion of the amount of testosterone were converted to estrogens (2000 pg/ml decline in the busulfan group and the 500 pg increase in the WIN group) were convertible to estrogens, the changes in serum estrogen levels could well be reflected by the changes in serum testosterone levels.

No significant correlations between serum testosterone levels and serum E_1 or E_2 levels were found in the cryptorchid study. This may refute the previously proposed argument that changes in serum estrogen levels may be reflected by changes in the amount of testosterone available for peripheral aromatization, and suggest that the changes may be due to decreased production of E_2 by the testis. Perhaps estrone production by the testis is increased at the expense of E_2 production, and then both are decreased as the time of cryptorchidism increases.

Inano and Tamaoki (22) suggest that Δ^4- androstenedione (Δ^4-A) production by the testis is unaltered by cryptorchidism, and Breuer (5) suggests that Δ^4-A is the preferred precursor for estrogen production. In equine testis the ratio of estradiol:estrone production is 20:1 (5), indicating that a C18-17β -hydroxysteroid dehydrogenase is present, with the equilibrium shifted in favor of E_2 production. One might suspect that some shift in this equilibrium toward E_1 might yield the results found. In the male testosterone may be converted to E_2 via two routes: (1) directly ($T \rightarrow E_2$), or (2), indirectly ($T \rightarrow \Delta^4\text{-A} \rightarrow E_1 \rightarrow E_2$). As testosterone levels decline in the serum, and the production of A remains unaltered (21), less T

would be available for direct conversion to E_2, thereby decreasing E_2 output, even though Δ^4-A levels may remain constant. If one postulates that the equilibrium of the $E_1 \rightarrow E_2$ reaction shifts toward E_1, due to the effect of increased temperature on the 17β -hydroxy-steroid dehydrogenase, either due to heat lability of the enzyme or a decrease in available NADH (NADPH) due to a heat effect on the electron transport system, one might see an increased output of E_1. If one or both of these events should occur, one should see an increase in E_1 production by the testis. The decline in serum E_1 levels might be due to the increased time of exposure of the testis to abdominal temperature affecting some point in the Δ^4-A E_1 sequence. As pointed out by Baird et al. (3), the testis contributes approximately 21% of the total circulating estrogens in men. As the proportion of this 21% contributed by testicular conversion of T to E_1 and E_2 is not known at this time, one cannot speculate on the real effect of a decline in testicular conversion of T to E_1 and E_2 on serum levels and its possible affect on serum FSH levels. It would seem that one must determine the rate of conversion of T to E_1 and E_2 and Δ^4-A to E_1 and E_2 in normal and treated animals to determine whether the proposed mechanism is tenable.

Two of the studies performed lend credence to the possibility that estrogens, particularly E_2, may inhibit FSH secretion, due to the apparent reciprocal relationship between serum E_2 and FSH levels. Studies done with busulfan indicate that this supposed relationship does not exist. It is difficult to reconcile the fact that 3 treat-

ments which result in aspermatogenesis, and the reciprocal relationship which exists between FSH and the spermatogenic process (9, 19), should have different effects on the relationship between serum estrogens and FSH if "inhibin" is an estrogen. It is obvious that further experimentation is required to determine: the relationship of testicular estrogen production and aspermatogenic conditions; whether estrogen levels decline in castrate animals; and whether estrogens in physiological doses can depress serum FSH in cryptorchids. Could transplanting ovaries into castrate male rats, which, if successful, should become highly follicular with high estrogen secretion, yield any evidence to the solution of the enigma of FSH control? If "inhibin" is nonsteroidal, as suggested by Setchell and Sirinathsinghji (39), does the level of this compound change in aspermatogenic conditions?

Due to the lack of consistent changes in the serum estrogen levels in both treatment groups, little can be said concerning the role estrogens play in FSH regulation. It would appear that the changes in serum estrogens may be accounted for by changes in serum testosterone and/or the availability of testosterone for peripheral or testicular testosterone aromatization. Thus the estrogens may not be the long sought after "inhibin". However, further studies must be conducted, using other treatments which affect both spermatogenesis and serum FSH levels before definitive conclusions may be reached. Perhaps the elusive "inhibin" is not an estrogen or androgen, leading one to conclude that the search must be pointed in other directions,

perhaps to, as expressed by Setchell and Sirinathsinghji (39), their first real candidate for the elusive "inhibin".

SUMMARY AND CONCLUSIONS

Little is known and less is understood concerning the mechanism by which testicular elements control the secretion of FSH by the anterior pituitary in the male mammal. The results of many studies since the 1930's have shown an increase in serum or urinary FSH levels in conditions which affect spermatogenesis. These studies have led to the conclusion that the testis produces a hormone, "inhibin", which is responsible for feedback inhibition of FSH secretion. The nature of this "inhibin", as well as the source are unknown, however estrogens, possibly produced by the tubular elements, have been proposed as a possible candidate for "inhibin".

This study was undertaken to determine the types and levels of estrogens present in the serum of the adult male rat and the effect of conditions which deleteriously affect the spermatogenic elements and give rise to increased serum FSH levels, on the levels, on the levels of the estrogens circulating in the adult male rat.

The presence of estrone (E_1) and estradiol-17β (E_2) were confirmed by superimposition of labeled and RIA chromatography curves, and superimposition of ^{3}H-E-Ac and extracted, acetylated presumptive E-Ac-1-C^{14} curves. In both cases, the curves coincided leading to the reasonable conclusion that E_1 and E_2 are present in the peripheral serum of the rat.

Pooled serum samples from adult male rats treated with a single i. p. injection of Busulfan (B, 10 mg/kg body weight) or WIN 18446 (W, 125 mg/kg body weight) and previously analyzed for the levels of

FSH, LH and testosterone (T) were analyzed for the levels of E_1 and E_2 for a period covering weeks 1-20 post-injection. Adult males were surgically cryptorchidized (Cr) and animals sacrificed at 0.5, 1, 2, 4, 8, and 16 days after surgery. All treated values were compared to the values obtained from vehicle-injected or sham-operated controls (C) by the Student's t test.

Previous work has shown that the drug treatments resulted in an increase in serum FSH, to peak levels at the time of maximum testicular regression (10 weeks) followed by a decline to control levels at 20 weeks. Serum T declined (N.S.) while serum LH remained unchanged over the 20 week period.

Serum E_1 levels in the B group (range: 19.5 $\pm$ 0.5-107.2 $\pm$ 37.5 pg/ml) and the W group (range: 19.0 $\pm$ 26.7 pg/ml) were not significantly different from control values (range: 11.5 $\pm$ 2.0-106.5 $\pm$ 73.5 pg/ml) over the 20 week period, except for the week 20 W value, which was higher ($p < 0.05$) than the corresponding control value. Serum E_2 values in the B group rose from a level of 23.7 $\pm$ 7.2 pg/ml at week 1 to a peak value of 128.5 $\pm$ 8.5 pg/ml at week 4 and declined thereafter. None of the B values differed significantly from control levels. In the W group, serum E_2 levels declined from a level of 125.0 $\pm$ 55.0 pg/ml at week 1 to a low level of 11.4 $\pm$ 4.4 pg/ml at week 4 and generally increased thereafter. Serum E_2 levels were significantly higher than control values at week 16 W. Total estrogens ($E_1 + E_2$) showed changes similar to those of the serum E_2 levels. Serum $E_1 + E_2$ levels were significantly lower at week 4 ($p < 0.05$) and

significantly higher than control levels at weeks 6 ($p < 0.01$), 8 ($p < 0.05$) and 10 ($p < 0.05$) for the B group. Values for the W group were significantly higher than controls at weeks 1 ($p < 0.05$), 6 ($p < 0.05$), 10 ($p < 0.01$), 16 ($p < 0.05$) and 20 ($p < 0.05$) and significantly lower at weeks 3 and 4 ($p < 0.01$).

Serum E_1 levels for cryptorchid animals were significantly higher than controls (C = 6.0 ± 2.2 pg/ml) at day 0.5 (Cr = 39.6 ± 17.6; $p < 0.05$). Values at the other days did not differ from control levels. Serum E_2 levels generally declined in cryptorchid animals and were significantly ($p < 0.05$) lower at days 4 (2.2 ± 1.0 pg/ml) and 16 (2.0 ± 1.0 pg/ml) than the corresponding control levels (day 4:6.2 ± 1.8 pg/ml; day 16: 6.8 ± 1.0 pg/ml).

Serum testosterone (T) levels increased through day 2 but were not significantly different from control levels. Serum T levels were lower than controls at days 4 and 16 ($p < 0.01$). Serum T levels ranged from 2.38 ± 0.56 ng/ml to 6.48 ± 2.16 ng/ml and 0.98 ± 0.21 ng/ml to 4.74 ± 1.66 ng/ml for the control and treated groups, respectively.

If estrogens were "inhibin", one should find a reciprocal relationship existing between the E_1 and/or E_2 levels and the serum FSH levels. Two of the studies (WIN 18446 and cryptorchid) suggest that estrogens, particularly E_2, may be inversely related to serum FSH levels. However, the busulfan studies do not support this observation. Perhaps the testicular contribution to the total serum estrogen concentration is insufficient to be taken as anything other than a normal

variation in serum estrogen levels. Further studies must be undertaken to obtain more concrete evidence for any relationship between estrogens and serum FSH. The results of these studies show an inconsistent relationship between the two hormones, and little can be said concerning the role estrogens play in FSH regulation; thus, the estrogens may not be the long sought "inhibin".

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