THE EFFECTS OF KEPONE ON URINARY LEVELS OF ESTRONE, ESTRADIOL-17B, AND ESTRIOL IN THE LABORATORY MOUSE

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
1965

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ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. George W. Ware, Department of Zoology and Entomology, The Ohio State University, for his cooperation, encouragement and extreme patience while serving as my adviser. I also wish to thank him for the advice and understanding given me outside the realm of the classroom these past seven years. I wish also to thank Dr. Frank Fisk, my co-adviser, for the cooperation and assistance shown me throughout my college career.

I am especially indebted to Dr. Keith Brown of the Department of Poultry Science, The Ohio Agricultural Experiment Station, for the use of his laboratory facilities, for his guidance and for his understanding in the two-week period I spent in his laboratory that somehow stretched to six months.

I wish also to thank my colleague, Dave Bajpayee, of the Poultry Science Department, whose help and suggestions are greatly appreciated.

Recognition is due also to Allied Chemical Corporation, Morristown, New Jersey, for their cooperation in supplying the technical grade Kepone used in this study.

To my friends, both in Wooster and in Columbus, I wish to express my deep appreciation for their encouragement and assistance.

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FIELDS OF STUDY

Major Field: Entomology

Studies of insect toxicology. Professors George W. Ware, and Frank W. Fisk.

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INTRODUCTION

The real and potential hazards associated with pesticides have aroused an increasing concern over their use for the control of insects, weeds, other pests, and plant diseases. One area of pesticide research in which this concern has become manifest is on the physiological effects of sub-lethal dosages of insecticides on the reproductive success of non-target organisms including man.

Investigations concerning the influence of insecticides on the reproductive success of mammals has, in the past, dealt largely with a comparison of the fertility or fecundity of the organisms tested and the control organisms. There is only a smattering of information available on the actual physiological effects of insecticide consumption on the reproductive system.

The first study of pesticide influence on reproduction in mammals was reported by Thienes et al. (1946). In this study, rats were injected daily with subconvulsive doses of 2 mg/kg of nicotine. It was found that there was an increase in the number of non-fertile pairs and a decrease in the number of litters produced. It was further observed that the progeny of the treated parents were less fertile than the controls. A little insight into the physiological aspects of reproductive failure in this case was made by the observation that at this dosage, the occurrence of estrus was decreased, while at smaller dosages there was no interference with the estrous cycle. Borgman (1952) and Treon and Cleveland (1955) observed that aldrin at dietary

levels exceeding 10 ppm reduced the number of pregnancies in rats, but had no effect on litter size. In the later study, dieldrin at 2.5 ppm had the same effect, but aldrin at 2.5 ppm and DDT at 25 ppm had no effect on reproduction.

Ball, Kingsley and Sinclair (1953) demonstrated that aldrin influenced the estrous cycle of rats. His data showed that at 20 ppm aldrin caused a significant estrus depression, while at 5 ppm and 10 ppm the occurrence of constant estrus was higher than for either the control groups or for groups maintained at higher aldrin concentrations.

Records on the reproductive success of mice fed Kepone at dietary levels of 17.5, 25, 30 and 37.5 ppm show that significant effects on reproduction are apparent. Based on 100% success for the control mice, the reproductive success for the above Kepone concentrations are 85.3%, 50.2%, 6.5%, and 7.6% respectively (Good, Miller and Ware, 1965). In another test they found that at 10 ppm Kepone in the diet there was a 53.5% reduction in the reproductive rate due to the production of fewer litters by the treated mice.

The results of tests by Bernard and Gaertner (1964) indicate that the fertility of mice declines with exposure to increased concentrations of DDT. At 200 and 300 ppm dietary DDT levels for a 70 day test period, the number of young per litter was the same as for the controls, but the number of females that produced no young was significantly greater in the 300 ppm DDT-fed group than in the control and 200 ppm groups.

One of the most significant works dealing with the physiological effects of an insecticide on reproduction is that of Huber (1963). In

this study it was determined that female mice fed a diet containing 40 ppm Kepone had the tendency to go into constant estrus or prolonged estrus, and that after two months on the diet ovulation was inhibited. The average estrous cycle was 7.7 days in the treated mice compared with 4.8 days in the controls. In this same study he found that the level of luteinizing hormone (LH) was reduced by approximately 25%, while the level of follicle stimulating hormone (FSH) remained normal. However, histological and vaginal smear data suggested that the Kepone-fed females were under a more constant stimulation of FSH than the controls. At the 10, 30, and 37.5 ppm dietary levels of Kepone, the number of young was reduced by 23.9%, 79.3% and 87.0% respectively, with second litters born or produced only in the control and 10 ppm mice. No young were born to females fed 40 ppm. Histological studies showed the pituitary and thymus glands to be normal. Uteri appeared normal except for those in the estrus phase, which showed definite cystic hyperplasia of the endometrium. He ascribed this condition to prolonged or abnormal estrogen stimulation. The ovaries showed inhibition or severe reduction of ovulation and corpora lutea formation.

Excellent reviews pertaining to the estrous cycle and the hormonal interactions involved are given by Huber (1963), Snell (1941) and Turner (1960). Thus no literature review is presented here.

Huber (1963) has proposed an hypothesis concerning the effects of Kepone on reproduction in laboratory mice. Essentially he suggested that the rapid accumulation and persistence of Kepone within the brain results in an interference in the permeability of the cell membranes

thus preventing normal functioning. As a result, a constant stimulation of an inhibitory center blocking the rhythmic release of LH, or of an activating center prolonging FSH release, may develop. This blocking of LH secretion would prevent ovulation and formation of corpora lutea, upsetting the normal feedback oscillations of the adenohypophysial-ovarian system. Thus, the continuous or prolonged secretion of FSH with small amounts of LH would result in a continuous secretion of estrogens from the vesicular follicles producing a constant estrus and endometrial hyperplasia of the uterus. He further stated that the amount of LH secreted may be sufficient to act with FSH to cause estrogen secretion, but insufficient for ovulation.

In view of the above considerations, the objectives of this study were to determine whether the urinary estrogen levels in the Kepone-fed, constant estrus mice were higher, lower, or of the same magnitude as normally estrus mice, and to establish the estrogen secretion pattern of normal mice throughout their estrous cycle.

Twelve methods employing the use of chloroacetyl derivatives of the estrogens for detection by electron-capture gas chromatography were tried. Although a few of the methods gave promising results for the analysis of estrone, estradiol-17B and estriol, the sensitivity required could not be attained. A major problem, as evidenced by thin layer chromatography, was in obtaining good yields of the derivatives. In the cases of estradiol-17B and estriol, more than one chloracetyl derivative was formed making analysis the more complex. This method was abandoned in favor of the fluorometric method since its sensitivity

as shown by a number of methods is a thousand times greater than colorimetry and that unlike most methods employing radioisotopes it determines endogenous steroid metabolites directly.

To adapt fluorometric techniques to this problem, it was first necessary to modify existing quantitative techniques for measuring estrogens of very low titer in 24 hour samples of mouse urine. A review of the literature revealed that no methods had been developed for such estimations and that no data were available on the levels to be expected. Most existing methods deal with the fluorometric measurement of human urinary estrogens where the amounts of urine are quite large compared to those of mice, but where the levels of estrogens that can be determined are in the submicrogram range.

Using the fluorometric method, a number of hydrolysis, extraction, and chromatographic systems were used without success. The method reported employs those conditions that gave the best results.

Contemporary methodology of estrogen analysis has its basis in work introduced 15 to 35 years ago by such researchers as Marrian (1930) who first observed fluorescence of estrogens in the presence of concentrated sulfuric acid, Kober (1931), whose color reaction, a series of color and fluorescence changes induced by heating and diluting sulfuric acid solutions of estrogens, remains the basis for most modern day fluorescence techniques, and Cohen and Marrian (1934) whose method of estrogen separation from biological sources still serves as the basis for extraction and purification. Great advances in estrogen analysis have been developed in recent years by McAnally and Hausman

(1954), Nakao and Aizawa (1956), Ittrich (1958, 1960), Preedy and Aitken (1961), and Barlow (1963). It is from these works that the following procedures for microfluorometric estrogen analysis have largely been taken or modified.

METHODS AND MATERIALS

Rearing facilities and maintenance

Female white mice of the BALB/cJaxGnMc strain, produced by sibling matings for more than 100 generations, were used in this study. The animals were confined in stainless steel, standard pair cages with not more than six per cage. Litter and water were changed weekly. A standardized mouse food[1] was used throughout the study. The mice were maintained in an air conditioned, constant temperature rearing room held between 70-74°F. and a relative humidity of 20-40% throughout most of the study. During a part of the study, it was necessary to maintain the mice in facilities that could not be controlled for conditions other than ambient for temperature and relative humidity.

Tests were conducted with females on diets impregnated with 0, 15 and 30 ppm Kepone[2], (Decachlorooctahydro-1,3,4-metheno-2H-cyclobuta(CD) pentalen-2-one). Four replicates of six mice each were fed at each dietary level.

The food pellets, as supplied by the manufacturer, were ground by means of a burr mill to a fine powder. Each concentration of Kepone was dissolved in acetone to give the final desired concentration when applied at 25 ml per kg of chow. The acetone solutions were added by

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[1]Purina Mouse Chow, Ralston Purina Company, Checkerboard Square, St. Louis, Missouri.

[2]Supplied and manufactured by Allied Chemical Corporation, Morristown, New Jersey.

pipette to the chow which was constantly stirred in a 10 gallon rotary battery mixer. Approximately 7% moisture was added to facilitate pelletizing and the impregnated chow was repelletized in a California Model CL Laboratory Pellet Mill[3] to 3/8 x 1 inch cylindrical pellets. The desired concentrations of Kepone were prepared from a 93.6% technical grade material.

Vaginal smear technique

After receiving the Kepone diet for two months, vaginal smears were taken to determine whether any change from the normal estrous pattern could be determined.

The method used for obtaining the vaginal smears was the medicine dropper or lavage method (Snell, 1941) since this method was shown to be the least irritating with little disturbance of the estrous cycle (Emery and Schwabe, 1936). A fine-pointed medicine dropper containing a few drops of physiological saline solution was inserted into the vagina and the liquid was quickly forced out and then withdrawn back into the dropper. This fluid was then placed on a glass slide and a drop of methylene blue, which aided in distinguishing between cell types, was added together with a cover slip. The smear was then examined microscopically at 430X to determine what stage of the estrous cycle was represented. Vaginal smears were taken once each day from five mice of each group (0, 15 and 30 ppm) for a two week period.

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[3]Manufactured by California Pellet Mill Co., Crawfordsville, Indiana.

Collection of urine

Twenty-four hour urine collections were made by placing a mouse in a six-inch funnel fitted with a four inch floor of one-fourth inch screening and a collection vial at its tip. A continous source of water was provided for the animals. Fecal material was removed from the funnel and collection vial every two to four hours for the first twelve hours of urine collection. At the end of the 24-hour period, any additional fecal pellets were removed from the collection vials and the urine samples stored in a deep-freeze until analysis could be performed. Vaginal smears were taken at the beginning and at the end of each collection period to determine the stage of estrous represented.

Cleaning of glassware for steroid work

Clean glassware is essential in fluorometric work. The cleaning method that appeared to be the most satisfactory in this laboratory consisted of the following, rather involved, procedure.

When possible, new glassware was used and checked for scratches or other imperfections that might cause steroids to adhere to the etched glass. Those pieces that showed imperfections were discarded. All glassware was washed with detergent and thoroughly rinsed with water. The glassware was then boiled for at least one hour in a 1:1 mixture of concentrated sulfuric acid: concentrated nitric acid and rinsed with tap water at least ten times. Then, each piece was rinsed one time with a 4:1 ethanol:acetic acid mixture, five times with distilled water and finally, one time with 95% ethanol containing a trace (1 to 2 drops/liter) of acetic acid. Throughout this procedure it was

essential that those portions which contacted solvents were not touched with the fingers or fluorescent contaminants were likely to be left. The glassware was then stored in a dust free place, and when necessary placed on cheesecloth to drain. The cheesecloth was replaced at each washing. The use of paper toweling was avoided since fluorescent materials are usually present. No lubrication of any kind was used for stopcocks and other ground glass fittings. When possible, glassware containing acid was washed immediately after use, thus avoiding the necessity of going through the acid bath.

The glass wool used as plugs for the chromatography columns was placed in conc. H_2SO_4 for one hour, washed with distilled water until neutral to litmus and then washed with absolute ethanol. It was then air dried, heated to 125-130°C in a forced air oven and stored in a glass jar.

Reagents

1. Hydrochloric acid, concentrated, Baker's reagent grade.
2. Potassium hydroxide, 10%, for fluorometric use, obtained from the Hartman-Leddon Co., Philadelphia, Pa. Item no. 1464a.
3. Benzene, reagent grade. Purify by shaking with several changes of conc. H_2SO_4 (1/20 volume) until the acid layer reaches a stable fluorescence. It is necessary to use the same filter system and fluorometer settings used for estrogen quantitation. Wash the benzene layer three times with distilled water, or until water layer is neutral to litmus. After drying over anhydrous Na_2SO_4, distil twice in an all-glass apparatus,

rejecting suitable fractions (10-20%) at the beginning and end of the distillation. Store in a dark glass bottle.

4. Diethyl ether, reagent grade. Purify by shaking for approximately three minutes with 1/10 volume of 0.3M $FeSO_4$ in 0.4M H_2SO_4 (100ml/l) followed by brief shaking with three times 1/10 volume of distilled water. Distil once in an all-glass apparatus, rejecting suitable fractions (10-20%) at beginning and end. The ether is used within six hours (Hobkirk and Metcalf-Gibson, 1963).
5. Sodium bicarbonate solution, saturated, for fluorometric use, obtained from the Hartman-Leddon Co., Philadelphia, Pa. Item no. 2850.
6. Methanol, absolute. Purify by refluxing one hour with 2,4-dinitrophenyl-hydrazine (5 g/l) and concentrated HCl (10ml/l). Cool, filter and distil twice in all glass apparatus (Bush, 1961).
7. Ethanol, absolute and 95%. Rossville Gold Shield Alcohol, obtained from Commercial Solvents Corporation, Terre Haute, Indiana. Needs no purification.
8. Sulfuric acid. Baker's reagent grade or DuPont reagent are recommended as having the lowest background fluorescence.
9. 90% sulfuric acid. Carefully add 450 ml of 96% reagent grade H_2SO_4 to 30 ml distilled water.
10. 65% sulfuric acid. Carefully add 325 ml of 96% reagent grade H_2SO_4 to 155 ml distilled water.

11. Aluminum oxide, activated, neutral for chromatography. Obtained from Brinkmann Instrument Co., Great Neck Long Island, N.Y. Heat to 200-250°C for 4-6 hours prior to using and store in closed bottles.

12. Estrogen stock solutions. Dissolve 10 mg each of estrone[3], estradiol-17β[3] and estriol[4] in 10 ml of absolute ethanol. Working standards are prepared from these stock solutions. All solutions are stored in the refrigerator and new working standards are prepared every two weeks.

Estrogen standard curves

Standard solutions containing one nanogram (1×10^{-9} gram) per microliter (1ng/ul) of estrone, estradiol-17β and estriol were prepared and standard curves were determined (Fig. 2). A 50 ul syringe was used to deliver the desired quantities of each estrogen. Fluorescence was determined by the method to be discussed. Three replicates of each compound were tested each day for ten days. After initial tests, using estrogen concentrations of 0 to 50 ng in 10 ng increments to establish the straight-line relationship, a concentration of 20 ng was run with each test to establish the reproducibility of the curves.

Hydrolysis and extraction

The procedures used for the hydrolysis of the estrogen conjugates and for their subsequent extraction were modifications of the methods described by Nakao and Aizawa (1956), Hobkirk and Metcalf-Gibson (1963), and McAnally and Hausman (1954).

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[3]The Upjohn Company, Kalamazoo, Michigan.
[4]Sigma Chemical Company, St. Louis, Missouri.

A 0.5 ml aliquot of the 24 hour urine sample was brought to an 8.5 ml working volume in glass stoppered tubes with distilled water and 1.5 ml of concentrated HCl was added and mixed thoroughly. The tubes were loosely stoppered and placed in a boiling water bath for 30 minutes to hydrolyze the estrogen conjugates. The samples were cooled in an ice bath and the contents transferred as completely as possible to 60 ml separatory funnels. Each tube was rinsed three times with 2 ml portions of diethyl ether which was added to the separatory funnels. Four ml of ether were added, and the contents gently swirled before stoppering the funnels. The funnel was gently shaken and the stopper was loosened frequently until pressure ceased to develop. The funnel was then vigorously shaken for one minute to effect extraction. This procedure was repeated twice with 10 ml portions of ether. The aqueous layer was then discarded. The combined ether extract was next washed with 2 ml saturated $NaHCO_3$ and then with two 5 ml portions of distilled water. The ether layer was then extracted twice with 10 ml portions of 10% aqueous KOH. The combined KOH extract was adjusted to pH 8.0-9.0 with 65% H_2SO_4 using indicator paper and cooled to room temperature or below in running water and extracted three times with 10 ml portions of ether. The ether extract was washed three times with 5 ml portions of distilled water and evaporated to dryness in a vacuum dessicator. The residue was taken up with three 2 ml portions of benzene and submitted to chromatography.

Ten replicates each of the standard estrogen solutions containing 50 nanograms were extracted by this method to determine the recovery.

Chromatography

The chromatographic system used for the separation of the three estrogens was a modification of that used by Nakao and Aizawa (1956). Pipettes (20 ml volumetric) with the tops removed and with treated glass wool plugs inserted into the tips were used as chromatography columns. Ten ml of benzene were added to the columns and one gram of activated neutral alumina was immediately poured into the benzene and allowed to settle. After the benzene had passed through the column, the urine extract was quantitatively transferred and allowed to penetrate completely onto the column. The estrone, estradiol-17B and estriol were eluted from the columns with 15 ml of 1% and 10 ml each of 5% and 30% methanol in benzene, respectively, using compressed oxygen to maintain a flow rate of 15-20 drops per minute. Chromatography was carried out at 30°C. The eluates were collected in 5 ml fractions in 13 x 100 mm culture tubes and evaporated to dryness at 80°C in a forced air constant temperature oven.

Standard solutions of the estrogens were chromatographed separately and in combination to determine recovery. Fortified urine samples and standard samples were also taken through the hydrolysis, extraction and chromatography steps to determine total recovery (Tables 4 and 5).

Development of fluorescence

Bates (1954) has pointed out that the absorption spectra of all the acid products of the estrogens exhibit maxima with broad peaks ranging from 410 to 450 mu with the resulting fluorescence being a broad band with a peak near 500 mu. Udenfriend (1962) states that

because of the broad absorption peaks, it is desirable to utilize the 436 mu mercury line with filter instruments.

Detailed investigations of the absorption and fluoroscence characteristics of the estrogen acid products by Bauld et al. (1960) have shown that the three estrogens under consideration in this paper have similar spectra with an absorption maximum at 453 to 456 mu and a fluorescence maximum at 479 to 484 mu (Fig. 1). Because of the marked overlapping of the absorption and fluorescence spectra, excitation cannot by carried out at the peak, but must be done at some point further removed from the fluorescence. This was the reason for choosing the 436 mu mercury line (Udenfriend, 1962).

In this study, the Turner Model 110 Fluorometer equipped with a high sensitivity conversion kit was used. The primary combination of the Wratten 2A and 47B filters to isolate the 436 mu mercury line and the Wratten 4 secondary filter covering the emission peaks of the three estrogens were utilized in consideration of the above discussion as was the #110-851 lamp which has practically no emission between the 436 mu and 546 mu mercury lines.

The method used for the development of fluorescence was a modification of those developed by McAnally and Hausman (1954), Preedy and Aitken (1961) and Bauld et al. (1960).

The dried residue was redissolved in 0.1 ml of absolute ethanol with repeated shaking and warming at 37°C in a water bath. One ml of 90% H_2SO_4 was added and the sample heated in a water bath at 80°C for exactly 10 minutes. The samples were immediately cooled to below room

temperature in an ice bath for at least 5 minutes and 5 ml of 65% H_2SO_4 was added. The samples were mixed by pouring back and forth from the culture tubes to cuvettes at least 5 times and the samples were read fluorometrically between 60 and 100 minutes after mixing.

RESULTS

Vaginal smears

In general, the results of the vaginal smear tests follow closely those reported by Huber (1963). The control mice showed typical and distinct smears for all stages of the estrous cycle. The average length of the estrous cycle in these control females was 4.7 days as compared to the 4.8 days reported by Huber (1963).

Smears taken from the mice fed 15 ppm Kepone showed a fluctuation between the estrus and the metestrus phases only. No other phase of the estrous cycle was observed. Except for an occasional fluctuation between the estrus and metestrus condition, the 30 ppm Kepone treated mice were all in constant estrus.

Unlike the results obtained by Huber (1963), no cases of interrupted estrus were observed in the 15 ppm or the 30 ppm Kepone-treated mice. An explanation of this might lie in the make-up of the Kepone-treated food. In this study, a much more refined procedure was followed to insure that the food was uniformly fortified. In Huber's (1963) work such a procedure was not feasible, so in all likelihood his concentrations from pellet to pellet were not homogenous.

The implications of the above results in conjunction with the studies of Good, Miller and Ware (1965) and Huber (1963) in which the reproductive success of mice declined with increased dietary Kepone concentrations, is that this insecticide has a cumulative effect on the mechanisms governing estrous.

Standard curves and fluorescence

The primary purposes for determining the standard curves of estrone, estradiol-17B and estriol were to test whether linearity could be established at such low levels of estrogen concentration and to test the technique described for the development of fluorescence.

There was a linear relationship between fluorescence intensity and the amount of estrogen between 0 and 50 nanograms, the lower and upper limits tested.

The regression lines depicted in Figure 2 show the average results of 15-30 samples of each estrogen at concentrations ranging from 0 to 50 ng quantities in 10 ng increments. Under the conditions of fluorometry set forth in the text, the standard deviations ranged from 0.55% to 2.51% (Table 1).

As in the study of Preedy and Aitken (1961), fluorescence intensity was observed to increase with time, with a leveling off of the curve between 60 and 100 minutes. Fluorescence measurements were therefore taken between these times.

One major problem that was met when this portion of the study was started was in obtaining consistent results between replicates. After considerable effort, it was found that the purification of the reagents was an essential undertaking in order to bring their fluorescence levels below those of the estrogen levels used and within the sensitivity limits of the fluorometer. The necessity of having absolutely clean glassware was also determined. Minute rubber particles, contaminating fluorescence from fingers, paper toweling, Tygon tubing and

insufficient washing techniques were all shown to be major factors contributing to the inconsistency of results.

Another step that proved most critical in obtaining consistent result was that of quickly chilling the samples at the end of the 10 minute period of fluorescence development. Immediate submersion in an ice water bath or immediate transfer to a deep freeze for at least a 5 minute period resulted in extremely uniform results. Prior to rapid cooling of the samples they were immersed in a bath of running tap water, the temperature of which fluctuated from ambient to a few degrees below ambient. The results obtained using this tap water cooling technique fluctuated widely from replicate to replicate. Evidently, the rate at which fluorescence development is inhibited depends on temperature. Unless the samples are quickly chilled, fluorescence development continues until the samples reach a temperature that is sufficiently cool to inhibit such development. With the tap water cooling, it is presumed that unequal cooling took place at a rate slow enough to permit continued fluorescence development in some samples thus resulting in inconsistent values. Rapid cooling in the ice bath or in the freezer was apparently fast enough to inhibit fluorescence development in all samples simultaneously.

Hydrolysis and extraction

Virtually all estrogens in human urine, and presumably in mouse urine, are in the form of conjugates (sulfates and glucosiduronates) making hydrolysis a necessary preliminary step since methods are not available for the direct estimation of estrogen conjugates.

Various procedures for the hydrolysis of estrogen conjugates in biological fluids have been described by Brown and Blair (1958), Preedy and Aitken (1961) and by Nakao and Aizawa (1956). All are essentially alike in that a strong acid such as hydrochloric or sulfuric is added to the fluid to be hydrolyzed. The procedures differ only by the amounts of acid added and the duration of hydrolysis. Some destruction of the estrogens occurs in all methods. In view of these considerations, the method of Nakao and Aizawa (1956) was adapted to this study as the one most likely to give the best recovery of the estrogens with the least amount of destruction. This decision was made in light of the fact that a 0.5 ml quantity of urine was employed containing only nanogram quantities of estrogen. It was felt that if longer hydrolysis times were used, even if the rate of destruction was a small constant fraction, the fraction would be more significant due to the more dilute nature of the sample. On the nanogram level this would constitute a much larger error of the true estrogen levels present as compared with equivalent losses on the microgram level, since they would be magnified by 1000 times.

Numerous extraction procedures are available for estrogens, and most are based on the procedures of Engel et al. (1950) and Brown (1955). That of Nakao and Aizawa (1956) was used in this study with but one major change. In the procedure of Nakao and Aizawa (1956), the pH of the alkaline extract was adjusted to slightly acid, whereas in the procedure outlined here, the pH of the alkaline extract was adjusted to 8.0-9.0. The reason for this was to bring about purification of

the estrogen fraction and leave contaminating materials in the aqueous phase. At a pH above 9.0 or below 8.0, much of the contaminating materials present in urine will also be extracted into the ether fraction. Between pH 8.0 and 9.0, a quantitative, almost contaminant free estrogen extract is obtained.

Another important consideration is the use of peroxide-free ether. Hobkirk and Metcalf-Gibson (1963) state that peroxides have a destructive effect on estrogens and that it is important to clean and use the ether in a six hour period. Since the amounts of estrogens were of such minute quantities in this study, it was felt that the ether should be used as soon as possible after purification to prevent estrogen destruction. Therefore, the ether was used as it was distilling, and fresh ether was purified by the method outlined under the reagents section just prior to being distilled. The ether was not tested for the absence of peroxides, so the low recoveries may very well be due to estrogen destruction in part. Table 2 depicts the recoveries and the standard deviations for each of the estrogens.

Chromatography

Alumina has been used successfully in the methods of Brown (1955), Eberlein, Bongiovanni, and Francis (1958) and Nakao and Aizawa (1956) for the separation of estrone, estradiol-17β and estriol. Although the method employed by Nakao and Aizawa (1956) appears to be the simplest and least time consuming, it is not without its disadvantages. The standardization of the alumina is often difficult, and one sample may vary greatly from another depending on the moisture content, as a

small percentage variation may greatly alter the characteristics of the adsorbant. In this study, it was found necessary to heat the alumina to 200-250°C for 2-4 hours, let it cool, then store in closed bottles. All samples were chromatographed using the same batch of alumina, or by testing subsequent batches until one could be found with the same properties. Standardization from batch to batch was not attainable, and results could not be duplicated to any degree of accuracy in the majority of cases.

It was necessary to use one gram of alumina to effect separation of the three estrogens in this study as compared to the 0.3 gram used by Nakao and Aizawa (1956). Using the lesser amount, estrone and estradiol-17B were both eluted with the 1% methanol:benzene mixture, whereas, using one gram of alumina, almost complete separation was achieved, although there was some carry-over of estrone into the estradiol fraction and vice versa. The amount of carry-over varied from 0 to about 7% making correction factors impractical. Column recovery data are presented in Table 3.

Since fluorescence showed a linear relationship between the estrogen levels tested (Fig. 2) and since there were widely variable results between the measurements of fluorescence in the hydrolysis-extraction, chromatography and total estrogen recoveries (Table 2-4), calculations for these tables were based on the average of the fluorescence values obtained with standards carried through these techniques. Thus, the ratio of the sample reading to the average readings of the samples was multiplied by the concentration of the standards added:

$$\text{Sample Concentration} = \frac{\text{Sample Fluor.}}{\text{Avg. of Fluor. for this technique}} \times \text{Standard concentration (Amount added)}$$

Calculations of recovery rates for the fortified urine samples (Table 5) were performed in much the same manner:

$$\text{Fluor. of fortified urine sample} - \text{Average Fluor. of urine samples} = \text{Fluor. due to Amount added}$$

$$\text{Amount Recovered} = \frac{\text{Fluor. due to amt. added}}{\text{Avg. of estrogen sample Fluor. for total recovery}} \times \text{Standard concentration}$$

$$\frac{\text{Amount recovered}}{\text{Amount added}} \times 100 = \% \text{ recovery}$$

Urine analysis

An analysis of variance was performed on the estrone, estradiol-17B, estriol, and total estrogen data for each of the estrous phases of the control mice and for the 15 and 30 ppm Kepone-treated mice. The data were calculated both on the basis of the 0.5 ml urine aliquots (Tables 6-9) and for the total 24 hour urine samples (Tables 11-14). In all cases the analysis of variance indicated that there were significant differences among the means of the above groups. A mean separation test, L.S.D. (least square difference), was applied to the data from each group; the results of these tests are shown in Tables 10 and 15.

The mean separation test showed that there was no difference between the amounts of the three estrogens excreted by the control mice in the estrus phase and the 15 ppm and 30 ppm Kepone-treated mice which were in constant estrus. This was true on both the 0.5 ml aliquot and total 24 hour urine sample basis. These results, along with the vaginal smear data, lend support to the findings of Huber (1963) who believed that the occurrence of constant estrus was due to a hormone imbalance directly related to the insecticide Kepone. These findings also tend

to support his hypothesis that certain portions of the brain are affected in such a manner that FSH secretion is prolonged or that LH secretion is inhibited, resulting in a continuous secretion of estrogen.

A comparison of the mean amounts of estrogen excreted during the proestrus, estrus, metestrus and diestrus phases (Tables 10 and 15) of the estrous cycle in the control mice shows that the concentrations of all three estrogens increase from the proestrus to the estrus phase and then decrease in the metestrus phase, reaching a low in the diestrus phase. Although a comparison of the L.S.D.'s does not show significant differences among all of the stages, it is assumed that such estrogen fluctuations have occurred. To justify this assumption, consideration has to be given to the method of urine collection. As stated in the methods and materials section, vaginal smears were taken at the beginning and end of each 24 hour collection period to determine the boundaries of each sample in respect to the estrous stage represented. Those samples that were proestrus at the beginning and estrus at the end of the collection period were termed estrus samples, those that were estrus at the beginning and metestrus at the end were likewise termed estrus samples, and so on. It becomes apparent that a proestrus-estrus sample may better represent the proestrus condition than the estrus condition, depending on whether the smear was taken near the beginning or end of the proestrus phase. This same reasoning holds for all of the samples that represent more than one phase. It also becomes apparent that the estrogen concentration can vary widely because of this, giving readings that are at best only indications as to the true levels to be found at any particular stage of the estrous cycle.

DISCUSSION

The procedures for hydrolysis, extraction, chromatography, and fluorometry embodied in this method have been modified from those previously reported in the estimation of estrogens in biological fluids. Although the methods do not give the consistent results desired in quantitative analytical techniques, as shown by the large standard deviations, it should be born in mind that the highest levels of estrogen concentrations employed in this study are as low as or lower than the lowest levels employed in other studies. It is evident, therefore, that the methods of hydrolysis, extraction and chromatography would be more critical and lead to greater variability due to loss at these lower levels of estrogens than would the employment of larger amounts.

If a great degree of accuracy is desired, more critical standardization has to be established for the reagents used and for the apparatus employed. Microglassware, fraction collectors, and more critical control of temperature, relative humidity and pH measurements are desirable.

Adsorption of the estrogens on glassware throughout the procedure is a consideration that should not go unmentioned. Udenfriend (1962) states that the loss of organic substances by adsorption onto surfaces becomes particularly troublesome at or below the microgram level. To combat this, the amount of glass surface should be minimized. Thus, microglassware may help in obtaining more consistent and higher yields when working with such low estrogen levels. He further states that

procedures developed for the recovery of 50 ug of a compound will frequently not serve for 0.05 to 0.5 ug of the same compound, and that the best conditions for a given isolation are often found by trial and error.

Another point not to be overlooked is the possibility of urinary contaminants being measured as estrogens, thus giving false readings. As stated earlier, the pH of the alkaline extract is very critical in eliminating these contaminants. If contaminants still remain in the chromatography step, some may be passed through the column with the estrogens, and measured as such. Best results would be obtained if the eluates were collected in 0.5 ml increments instead of the 5 ml fractions employed in this study, and fluorescence developed in each of the 70 resulting fractions to show a truer picture of the eluted materials. In this way the procedure is constantly monitored and doubtful estrogen curves can be recognized.

Even with the drawbacks inherent in this method, differences have been shown in mouse urine estrogen levels, therefore quantitation on such a micro scale seems feasible.

Since many aspects of fluorescence assay are still in the trial and error stage, it is felt that this study has contributed at least a few significant steps toward the determination of estrogens at the submicrogram level.

CONCLUSIONS

1. Continuous estrus in female laboratory mice is an indirect result of prolonged dietary consumption of the insecticide Kepone at 15 and 30 ppm.

2. Kepone may have a cumulative effect on the mechanisms governing estrous.

3. Urinary estrogens can be measured quantitatively in 0.5 ml of mouse urine using the fluorometric procedure.

4. Significant differences can be demonstrated among the various stages of the estrous cycle in normal female mice by fluorometric determinations of urinary estrogen levels.

5. There are no significant differences in the amounts of estrone, estradiol-17B and estriol excreted by control mice in the estrus phase and Kepone-treated mice in continuous estrus.

TABLE 1. Average fluorescence values and their standard deviations obtained with estrone, estradiol-17B and estriol using the fluorescence development procedure.

	Estrone					Estradiol-17B					Estriol				
Number of observations	15	30	15	15	15	15	30	15	15	15	15	30	15	15	15
Estrogen added (ng)	10	20	30	40	50	10	20	30	40	50	10	20	30	40	50
Average fluorescence	17.8	35.5	53.8	65.0	89.6	15.1	29.7	45.0	59.9	75.8	8.9	18.5	27.2	36.9	45.7
Standard deviation	0.68	1.01	1.79	1.47	1.99	0.64	0.55	0.76	1.64	2.51	1.33	1.50	1.29	0.96	1.58

TABLE 2. Recovery obtained for estrone, estradiol-17B and estriol using the hydrolysis and extraction method.

		Estrone		Estradiol-17B		Estriol	
Number of observations		10		10		10	
Estrogen added (ng)		50		50		50	
		Fluor.	ng	Fluor.	ng	Fluor.	ng
Recovery	Mean	34.6	49.99	28.3	49.99	13.4	55.59
	Range	30-42	43.35-60.99	24-32	42.40-56.53	13-18	48.50-67.16
Standard deviation		3.72	5.37	2.50	5.17	1.87	6.92

TABLE 3. Recovery obtained for estrone, estradiol-17B and estriol using the column chromatography procedure.

		Estrone		Estradiol-17B		Estriol	
Number of observations		10		10		10	
Estrogen added (ng)		20		20		20	
		Fluor.	ng	Fluor.	ng	Fluor.	ng
Recovery	Mean	27.9	19.99	24.7	19.99	12.5	20.00
	Range	25-31	17.92-22.22	20-32	16.19-25.91	10-14	16.00-22.40
Standard deviation		2.02	1.45	3.67	2.83	1.45	2.30

TABLE 4. Recovery obtained for estrone, estradiol-17B and estriol taken through the hydrolysis, extraction and chromatography procedures.

		Estrone		Estradiol-17B		Estriol	
Number of observations		10		10		10	
Estrogen added (ng)		50		50		50	
		Fluor.	ng	Fluor.	ng	Fluor.	ng
Recovery	Mean	27.2	49.99	27.0	49.99	17.3	49.80
	Range	21-36	38.60-62.50	17-41	31.48-75.92	7-26	20.23-74.28
Standard deviation		5.01	9.20	6.93	12.83	5.25	15.02

TABLE 5. Recovery rates of fortified urine samples taken through the hydrolysis, extraction and chromatography procedures.

	Estrone	Estradiol-17B	Estriol
Number of observations	4	4	4
Estrogen added (ng)	50	50	50
Fluorescence (avg.)	39.50	35.25	24.00
Fluor. due to urine (avg.)	17.4	14.8	10.00
Fluor. due to amount added (avg.)	22.1	20.45	14.00
ng Recovered (avg.)	40.62	37.86	40.46
% Recovered (avg.)	81.24	75.72	80.92
Standard Deviation (%)	14.23	8.21	10.55

TABLE 6. Analysis of variance of estrone in mice based on 0.5 ml aliquots of urine.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Between estrous phases	3,483.800	6	580.63	6.16**
Error	4,898.407	52	94.20	--
Total	8,382.207	58	--	--

**At .01 level, F = 3.17 for df of 6 and 52.

TABLE 7. Analysis of variance of estradiol-17β in mice based on 0.5 ml aliquots of urine.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Between estrous phases	3,333.823	6	555.63	2.78*
Error	10,363.315	52	199.29	--
Total	13,697.138	58	--	--

*At .05 level, F = 2.28 for df of 6 and 52.

TABLE 8. Analysis of variance of estriol in mice based on 0.5 ml aliquots of urine.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Between estrous phases	15,807.065	6	2,634.51	11.10**
Error	12,336.739	52	237.24	--
Total	13,697.138	58	--	--

**At .01 level, F = 3.17 for df of 6 and 52.

TABLE 9. Analysis of variance of total estrogens in mice based on 0.5 ml aliquots of urine.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Between estrous phases	53,441.064	6	8.906.84	8.29**
Error	55,841.229	52	1,073.86	--
Total	109,282.29	58	--	--

**At .01 level, F = 3.17 for df of 6 and 52.

TABLE 10. Estrogen content in 0.5 ml aliquots of 24 hour samples of mouse urine.

Sample	[illegible] ng) *		Estradiol-17B (mean ng) *		Estriol (mean ng) *		Total Estrogens (mean ng) *	
Control Mice								
Proestrus-Proestrus	15.38	A	20.31	AC	13.34	A	49.04	A
Proestrus-Estrus	31.75	B	28.41	A	38.61	C	98.78	B
Estrus-Metestrus	32.77	B	31.65	A	50.05	BC	114.48	B
Metestrus-Diestrus	17.42	AC	19.24	AC	20.02	AC	56.69	A
Diestrus-Diestrus	6.77	A	4.70	BC	13.01	A	24.48	A
Kepone Treated Mice								
15 ppm Const. Estrus	30.26	B	29.70	A	53.72	B	113.35	B
30 ppm Const. Estrus	25.37	BC	30.08	A	53.59	B	109.01	B

*Any two means covered by the same letter in each column are alike.

TABLE 11. Analysis of variance of estrone in mice based on 24 hour urine samples.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Between estrous phases	8,224.805	6	1,370.800	4.408*
Error	16,170.050	52	310.962	--
Total	24,394.855	58	--	--

*At .05 level, F = 2.28 for df of 6 and 52.

TABLE 12. Analysis of variance of estradiol-17B in mice based on 24 hour urine samples.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Between estrous phases	7,197.845	6	1,199.640	2.221*
Error	28,078.173	52	539.96	--
Total	35,276.018	58	--	--

*At .05 level, F = 2.28 for df of 6 and 52.

TABLE 13. Analysis of variance of estriol in mice based on 24 hour urine samples.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Between estrous phases	37,370.086	6	6,228.347	6.85*.
Error	47,225.752	52	908.187	-
Total	84,595.839	58	--	--

*At .05 level, F = 2.28 for df of 6 and 52.

TABLE 14. Analysis of variance of total estrogens in mice based on 24 hour urine samples.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F
Between estrous phases	125,102.22	6	20,850.370	5.31*
Error	204.033.31	52	3,923.717	--
Total	329,135.54	58	--	--

*At .05 level, F = 2.28 for df of 6 and 52.

TABLE 15. Estrogen content in 24 hour samples of mouse urine.

Sample	Estrone (mean ng)	*	Estradiol-17B (mean ng)	*	Estriol (mean ng)	*	Total Estrogens (mean ng)	*
Control Mice								
Proestrus-Proestrus	19.08	A	25.58	AB	16.67	A	61.34	A
Proestrus-Estrus	44.66	B	39.65	B	51.48	BC	135.80	B
Estrus-Metestrus	43.77	B	42.54	B	67.06	BC	153.37	B
Metestrus-Diestrus	23.73	AB	26.41	AB	22.77	ACD	72.91	AB
Diestrus-Diestrus	9.25	A	6.49	A	18.21	AD	32.36	A
Kepone Treated Mice								
15 ppm Const. Estrus	46.07	B	42.62	B	79.54	B	168.25	B
30 ppm Const. Estrus	34.49	B	43.18	B	77.79	BC	155.70	B

*Any two means covered by the same letter in each column are alike.

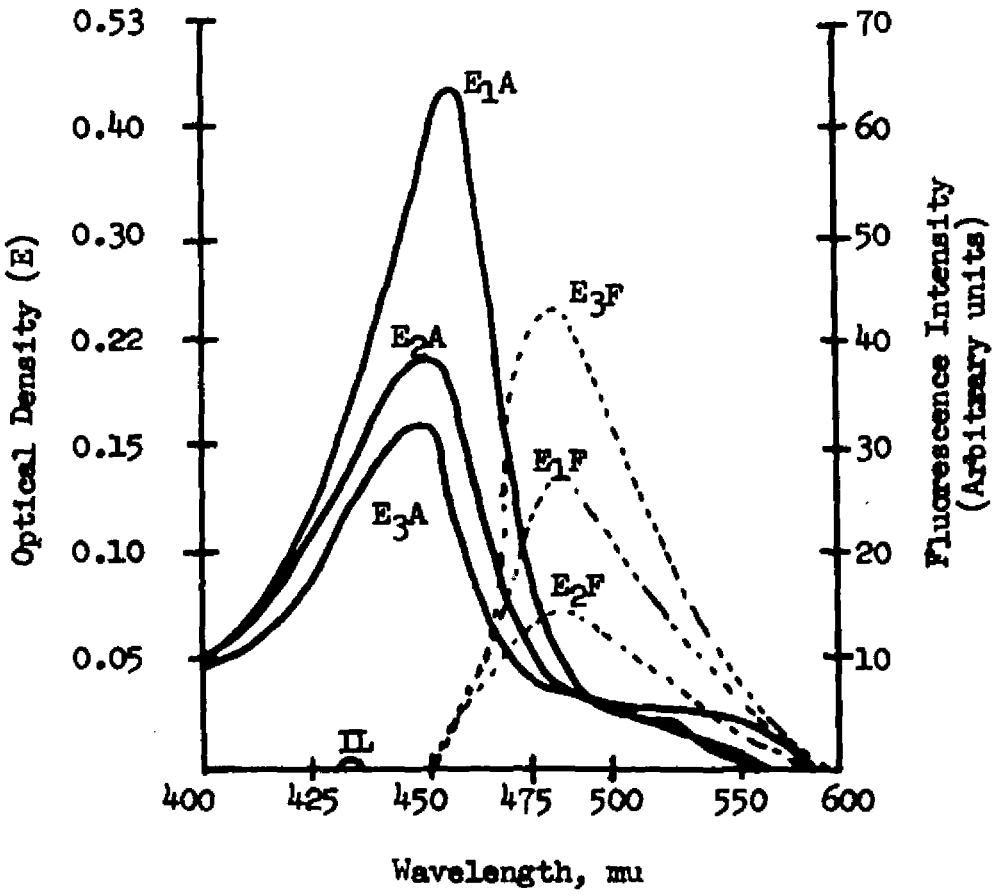

Figure 1. Absorption (A) and fluorescence (F) spectra of estrone (E_1), estradiol-17B (E_2) and estriol (E_3) in 75% (v/v) H_2SO_4. IL = incident light (after Bauld et al., 1960).

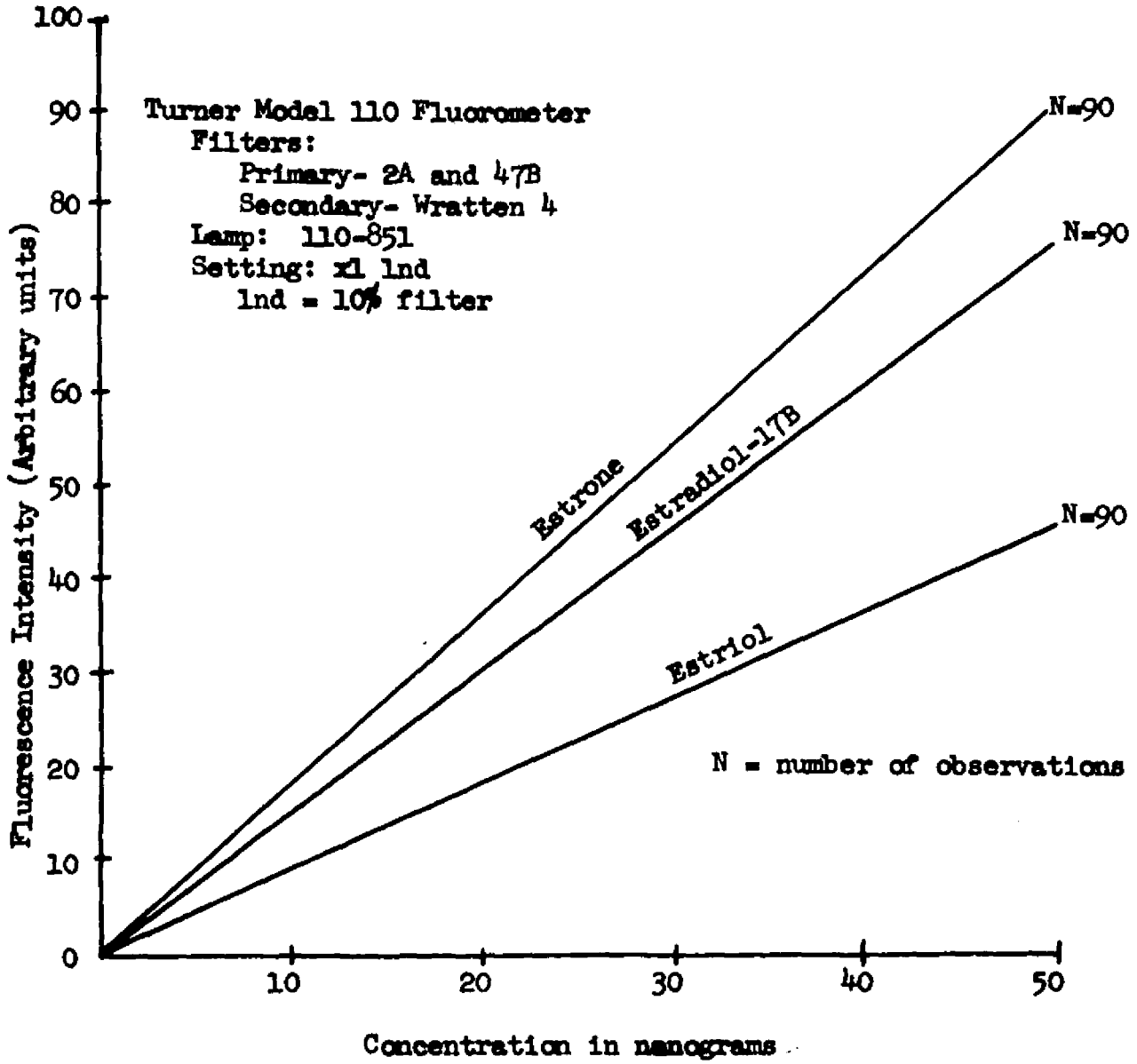

Figure 2. Standard curves of estrone, estradiol-17B and estriol that were run for purposes of testing linearity at low estrogen levels and to test the development of fluorescence technique.

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