# THE SEXUAL ORIGIN OF BLOODSTAINS: SPECIFIC DETERMINATION OF TESTOSTERONE USING RADIOIMMUNOASSAY

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

FRANK EDWARD SCHEHR

1976

# THE SEXUAL ORIGIN OF BLOODSTAINS: SPECIFIC DETERMINATION OF TESTOSTERONE USING RADIOIMMUNOASSAY

by

Frank E. Schehr

AN ABSTRACT

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

School of Criminal Justice

1976

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ABSTRACT

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Three experiments were performed using radioimmunoassay (RIA) for the determination of the sexual origin of bloodstains. It was also necessary to measure a second determinant in a bloodstain to determine the volume of blood represented by the stain so that testosterone could be expressed per unit volume of blood. The first experiment dealt with the RIA of testosterone concentrations in whole blood. Values for the concentrations of testosterone found closely approximated the volumes found in the literature for serum or plasma. This indicated that differences between males and females might be feasible when whole blood is shed and then found in the dried condition.

The second experiment dealt with the application of the RIA technique on dried bloodstains. Extraction efficiencies calculated from the recoveries found in the

radioactive tracer stains were low and postulated to be an error in preparation of the radioactive tracers onto the stains. Overlap between males and females in this experiment initiated a change in procedure.

The third experiment included not only the improved extraction technique, but also a blind study trial. Extraction efficiency was enhanced by preparing radioactive tracers in the fluid whole blood stage before drying the blood onto the substrate material. Results of the assays showed a significant difference between male and female origin of bloodstains. The blind trial study was conducted on eighteen bloodstains cut from cotton fabric, and this investigator's judgment was 90 percent correct for discrimination between male and female origins.

The determination of hemoglobin by use of cyanmethemoglobin method was studied to determine the amount of blood present in an unknown stain. No correlation was found between the measurement of hemoglobin extracted from a stain and the amount of testosterone extracted from the same stain. Difference in sexual origin were still detected, however, when the stain approximated the size of a 0.1 ml stain of whole blood.

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## ACKNOWLEDGMENTS

The author would like to extend special thanks first to Detective First Lieutenant Donald Bennett for affording the time to complete the laboratory portion of this work.

Appreciation is extended to Professor Ralph Turner for the personal interest and guidance so freely given while preparing for this degree.

Sincerest appreciation to Dr. Harold Hafs, James W. Schwalm, and Ms. Jean Horshuh of the Dept. of Dairy Science, Michigan State University, for their continued guidance and technical assistance in the procedural details of this research, the use of the Dairy Physiology facility, and the constructive review of this manuscript.

Special acknowledgment also to Dr. Laurence Simson, Pathologist at E. W. Sparrow Hospital, for the generous time given by his laboratory in the collection of blood samples.

The author wishes to extend his deepest and most sincere gratitude to his wife Jeanne for her encouragement and assistance during the research and preparation of this manuscript.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

# Chapter 1

## INTRODUCTION

In recent years, the determination of the sexual origin of bloodstains has occupied much of the research in forensic serology. This research has included studies of the differences in chromosomes between males and females, the counting of female Barr bodies, and the identification of menstrual blood. The research presented in this paper centers upon the fact that males and females differ substantially in the amounts of androgens (sex hormones) in the bloodstream, and presents the method of radioimmunoassay (RIA) for detecting these differences.

The choice of an analytical procedure for the detailed laboratory evaluation and interpretation for comparative purposes of a particular item of evidence depends on many factors, including the nature of the particular crime and likely identifying characteristics. Y-chromosome detection, Barr body counting, and menstrual blood identification, suffer from the absence of clearly defined and distinguishable standards, either from test samples, reference collections, or statistical background data. Radioimmunoassay, however, lends itself readily to

quantitative study, especially for steroid hormones. Much background data is available on the differences in the concentration of androgens in blood from males and females, and in recent years most of this data comes from the technique of radioimmunoassay.

A major hormone difference between the sexes is testosterone. Therefore this research centers upon the quantitation of testosterone through radioimmunoassay and the applicability of this testing to dried bloodstains. This research describes an attempt to differentiate a bloodstain with regard to the sex of the person depositing it. The first phase of the work involved detecting the level of the hormone in whole blood extracts, principally because much of the background work involved serum or plasma. This was undertaken for the reason that one does not lose only serum or plasma from a wound or cut, but whole blood. The second phase of the research involved applying this method to dried bloodstains, both with known amounts of blood on a stain and a blind trial on unknown amounts. Since this is a quantitative study, the amount of blood present in a stain is important. Therefore the third phase of the research deals with a method of estimating the amount of blood in a stain, using hemoglobin determination as the internal standard.

It is expected that this paper will produce other avenues of research for the application of radioimmunoassay to forensic serology, and suggest solutions for overcoming difficulties arising with the application of clinical methods to forensic science.

# Chapter 2

## REVIEW OF THE LITERATURE

For purposes of review, this chapter is separated into three divisions: (1) sex determination on dried bloodstains, (2) principle of radioimmunoassay, and (3) testosterone concentration in humans.

## SEX DETERMINATION ON DRIED BLOODSTAINS

Methods of nuclear sex determination by means of staining techniques are based on the structural differences of the sex chromosomes. In normal females these chromosomes occur as an homologous pair called the X-chromosomes. These sex chromosomes take a less active part in cell division and their constituent chromatin (heterochromatin) shows a greater degree of simplification with respect to the DNA base pairs than does the euchromatin of the remaining chromosomes. The Y-chromosome is composed of a large number of consecutive adenine-thymine pairs (1); this forms the basis of the differential staining behavior of the Y-heterochromatin of the male cells. The sluggish behavior of the X-chromosomes during cell division led to its discovery and subsequently to the discovery by Barr (2) of the sex chromatin or Barr body (a derivative

of one or more X-chromosome displaying positive heteropyknosis). This is the basis for the several attempts at sex determination on dried bloodstains utilizing Y-chromosome flourescense and Barr body counting (3,4,5).

Two staining techniques are employed in the detection of the characteristics of the heterochromatins. The first is based on the affinity of quinacrine dihydrochloride for adenine-thymine pairs. This is a flourescent stain and its combination with the higher concentration of these pairs results in a higher degree of flourenscence of the Y-chromosome (3). This appears as a bright spot in the nucleus of the cell; the second is based on the presense of a denser area of fused X-chromatin taking up an aceto-orcein dye and appearing as a dark red spot on the inside of the nuclear membrane in cells from females.

Philips and Webster (4) simplified the method devised for Y-chromosome flourescence for all types of cells by introducing magnesium ions to the staining procedure. These ions have been shown to cause the DNA helix to supercoil, bringing the sites into closer proximity and thus increasing the level of flourescence.

Philips and Gitsham (7) applied this new method in a series of blind trials and evaluated the results. The percentage of negative nuclei (not exhibiting

Y-chromosome flourescence) was plotted against the percentage of positive nuclei (exhibiting flourescence) summed with half the positive-negative score. In each trial, the combined positive and positive-negative scores of male stains cover a wide range, often overlapping with the female region. The differentiation between male and female stains was no better if negative nuclei were plotted against positive nuclei alone. The trials were investigated by both researchers, with Philips' score significantly higher than Gitsham. Clearly, each person's judgment needs to be "calibrated," and different minimal scores for the varying degrees of certainty of maleness would be needed. It was also unfortunate that the complementary test for femaleness, which would have been provided by the identification of the Barr body in polymorphonuclear lymphocytes, proved unreliable in their hands (8). Other studies performed on these drumsticks or Barr bodies showed exceptions to the fact that one chromatin body can be found in normal females and none in normal males(6). Low counts of the chromatins, affecting the reliability of the differentiation, are common when working with stains. Many factors contribute to this effect, but the age of the specimen and its general condition may be important. Due to the good stability of steroid hormones, it is hoped that this

factor will be negated in the tests with radioimmunoassay for testosterone.

Methods presently used for the identification of menstrual blood are the assay of soluble fibrinogen in bloodstain extracts and the characterization of lactic dehydrogenase isoenzymes in stains (9,10,11). The demonstration that a bloodstain is of menstrual origin may be of great importance in forensic investigations of sexual assault. One of the characteristics of menstrual blood is that it is fluid (12) and cannot be made to clot;[1] the fluidity of menstrual blood has formed the basis for a number of earlier suggestions for the identification of menstrual bloodstains. Popielski states that the absence of fibrin threads in a bloodstain is consistent with it being due to menstrual blood (13). This method, however, is satisfactory only when heavy bloodstains, not soaked into the substratum, are studied. Other investigators have tried to utilize the high fibrinolytic activity of menstrual blood to breakdown "fresh fibrin obtained by defibrination" and to study the products by paper electrophoresis (14) but with limited success. The work by Whitehead and Divall (9,10) utilized a hemagglutination

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[1]Clot-like material in menstrual discharge consists of red cells aggregations to mucoproteins and glycogen, and is not composed of fibrin.

inhibition technique to measure soluble fibrinogen.[2] Their results were consistent with finding the presence of high proportions of fibrin degradation products coinciding with present theories on menstrual bleeding. However, they also found that the fluidity and noncoagulability of menstrual blood is a property shared with certain postmortem bloods (15). On this basis alone it is not possible to distinguish between menstrual bloodstains and postmortem bloodstains. The circumstances of a case can, however, exclude the possible contamination of bloodstained clothing, etc. with postmortem blood.

This same problem also arises with the method of lactate dehydrogenase (LDH) determination. Using electrophoretic methods, Asano.Oya, and Hayagawa (11) relied on the concentration of the LDH-4 and LDH-5 bands[3] of this enzyme, for the identification of menstrual blood. Comparison was made between these isoenzymes in menstrual blood, normal venous blood, cadaveric blood, and blood shed during partuition. The marked increase of the bands LDH-4 and LDH-5 (measuring by densitometry) was used for differentiation of menstrual blood from other "types of

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[2]"Soluble fibrinogen" is a collective term for fibrinogen and fibrin degradation products.

[3]Lactate Dehydrogenase (LDH) is separated into five bands on electrophoresis: LDH-1, LDH-2, LDH-3, LDH-4, and LDH-5. The enzyme patterns are characteristic for each organ and tissue of the body.

blood." Increases in cadaveric blood and blood during partuition were also noted. Time studies on LDH isoenzymes in menstrual bloodstains showed a slight decrease in comparison with the fresh stains used in earlier studies. The data indicate that this method can differentiate menstrual blood stains if examined within one week.

## PRINCIPLE OF RADIOIMMUNOASSAY

The principle of radioimmunoassay has been applied to many organic compounds of biologic interest. One of the requirements for the application of radioimmunoassay to the measurement of a compound is that this compound must be antigenic and must elicit high affinity antibodies. The smallest compound which has been shown to be immunogenic is the hormone vasopressin (molecular weight 1080) (16). Usually compounds with molecular weight below 1000 are not immunogenic per se. Since steroids have molecular weights much below 1000, they are not immunogenic. However, when coupled to protein carriers, they become immunogenic. Reviews covering this subject can be found (17,18,19,20).

All RIA procedures are based on the original observation by Berson and Yalow (21) that low concentrations of antibodies to the antigenic hormone, insulin, could be detected by their ability to bind radiolabeled insulin. Unknown concentrations of antigen may be

determined by taking advantage of the observation that the radiolabeled hormone molecules, or tracers (Ag*I) compete physiochemically with the nonlabeled hormone molecules (AgII) for binding sites on the antibodies (AbIII) (see Fig. 1). Moreover, the assay requires that behavior between the standard and unknown antigen be identical in their ability to displace labeled antigens from a labeled antigen-antibody immune complex (Ag*AbIV), but not identical behavior between the tracer and the unknown or standard antigen. When increasing amounts of unlabeled antigen (AgII) are added to the assay, the limited binding sites of the antibody ABIII are progressively saturated and the antibody can bind less of the radiolabeled antigen (Ag*I). The antibody solution or antiserum is diluted to allow about 50% of the tracer dose of Ag*I to be bound in the absence of unlabeled standard or unknown antigen. If there is no nonspecific inhibition of the immunochemical reaction, a diminished binding of labeled antigen offers evidence for the presence of unlabeled antigen. After an incubation of the three essential components (Ag*I, AgII, and AbIII), the antigen-antibody complexes, or bound antigens (Ag*AbIV and AgAbVI) are separated from the free antigens (AgV and Ag*VII) and the radioactivity of either or both are measured.

| | Bound | Free |
|---|---|---|
| | Ag*Ab | Ag |
| | IV | V |
| Ag* + Ag + Ab | | |
| I II III | | |
| | Ag Ab | Ag* |
| | VI | VII |

| | | |
|---|---|---|
| I | Ag* | Labeled Antigen |
| II | Ag | Unlabeled Antigen |
| III | Ab | Antibody to Ag (and Ag*) |
| IV | Ag*Ab | Labeled Antigen Bound to Antibody |
| V | Ag | Free Unlabeled Antigen |
| VI | AgAb | Unlabeled Antigen Bound to Antibody |
| VII | Ag* | Free Labeled Antigen |

Source: Yalow, R. S., and Berson, S. A. Assay of plasma insulin in human subjects by immunological methods. Nature 184, 164 (1959).

Figure 1.--Diagram showing the various components of RIA.

The criteria for acceptance of an assay includes reliability and practicability. The reliability of an assay depends on its specificity, sensitivity, accuracy, and precision (22). All of these criteria were judged at different points of this research, and will be presented in the appropriate discussion chapter.

## HUMAN TESTOSTERONE CONCENTRATIONS

The purpose of this section is to review briefly a fairly substantial body of knowledge about the concentration of testosterone in human plasma or serum, in normal and abnormal conditions. For the most part, this review will be directed to testosterone in the male; however, testosterone concentration in the female will be discussed, particularly when it may be appropriate to compare and contrast them between the two sexes.

In the normal, sexually mature, adult male, there is good agreement that the concentration of testosterone in peripheral plasma is between 3.5 and 10.5 ng/ml. These data, which are not generally disputed, have been derived from many different laboratories using for the most part RIA (see Table 1). In round numbers, the values for males are about 10 times those found in the female. Plasma concentrations of testosterone were measured in oligospermic and azoospermic males (45), range 20-51 years of age. The only significant difference was that

Table 1

Mean Serum or Plasma Testosterone Levels in Normal Women and Men.

| Method | ng/ml | Female | Male | Ref. |
|---|---|---|---|---|
| RIA[a] | range | .22-.45 | 3.65-8.15 | |
| | mean | .32±.07 | 5.90±1.49 | 23 |
| | n(age) | 12(25-40) | 13(25-40) | |
| RIA | range | .25-.80 | 4.50-11.00 | |
| | mean | .54±.22 | 7.90±1.43 | 24 |
| | N9age) | 22(25-40) | 30(25-40) | |
| RIA | range | .18-.51 | 3.18-8.10 | |
| | mean | .31±.10 | 5.14±1.19 | 27 |
| | n(age) | 10(21-65) | 20(21-65) | |
| RIA | range | .30-.85 | 3.80-9.65 | |
| | mean | .57±.17 | 6.86±1.96 | 26 |
| | n(age) | 20(21-65) | 24(21-65) | |
| RIA | range | .09-.25 | 2.50-8.80 | |
| | mean | .22±.07 | 4.90±1.60 | 25 |
| | n(age) | 18(19-55) | 19(21-60) | |
| RIA | range | .09-.51 | 1.67-10.89 | |
| | mean | .26±.15 | 4.62±1.99 | 28 |
| | n(age) | 11(20-32) | 22(19-35) | |
| RIA | range | .10-.81 | 3.10-8.21 | |
| | mean | .35±.12 | 5.31±1.70 | 29 |
| | n(age) | 53(20-30) | 14(20-28) | |
| RIA | range | .10-.58 | .15-.90 | |
| | mean | .28±.15 | .46±.21 | 29 |
| | n(age) | 14(58-79) | 11(61-80) | |
| DID[b] | range | .20-1.20 | 4.40-13.00 | |
| | mean | .70±.30 | 7.40±2.60 | 30 |
| | n(age) | 10(21-55) | 15(19-40) | |
| DID | range | .04-.70 | 2.80-14.40 | |
| | mean | .35±.22 | 6.70±2.30 | 31 |
| | n(age) | 20(21-65) | 21(21-35) | |

[a]RIA Radioimmunoassay.

[b]DID Double isotope derivative technique.

testosterone was lower in the oligospermic male, but that this concentration never fell to that of the female.

Blood concentrations of testosterone reflect the momentary balance between entry into and removal from the blood. In the male, about 1000 liters of plasma are cleared of testosterone each day (32). Although the liver is undoubtedly the principal organ responsible for the metabolic removal of testosterone from plasma, extra hepatic metabolism of testosterone certainly occurs (33,34), but the precise contribution from skin, muscle, and other tissue has not been precisely determined. With plasma concentration of testosterone between 3.5 and 10.5 ng/ml., and clearance rates of between 900 and 1200 liters/day, the rate of entry of testosterone into the blood may be calculated, assuming a steady state, to be between 3 and 13 milligrams/day. But, is the state steady, and is secretion synonymous with rate of entry?

It seems likely that the state is not entirely steady. There has been some controversy as to whether the level of this steroid changes within the day or between days (34,36,37). The weight of evidence would now suggest that within the day there is a variation, with higher morning and lower evening values. Plasma testosterone was measured by a competitive protein binding procedure at 10-20 minute intervals in eight normal adult men (37). Testosterone appeared to be released episodically with

increasing blood concentrations throughout sleep and appeared to relate to other hormone increases. Testosterone ranged from approximately 7-14 ng/ml of plasma throughout the time period, with no consistent rhythms across eight subjects for any particular of time. However, investigators found little evidence that there is such systematic variation in plasma concentration of testosterone over a period of five weeks; in fact, values were found to range widely in an apparently random fashion (38). Even with this variation though, concentration of testosterone in males always exceeds that in females (39).

Infancy, Childhood, Adolescence, and Old Age

At the extremes of life there are distinct differences from the normal adult male pattern of testosterone secretion. In the immediate neonatal period, plasma testosterone is elevated in both male and female infants, and there is no significant sex difference (39,40). Immediately after birth plasma testosterone falls to values that are at, or below, the normal female range, and remain at this level until puberty. Umbilical cord plasma concentrations of testosterone have been measured by radioimmunoassay in both male and female fetuses at 12 to 22 weeks of gestation and at term (39,40). Testosterone concentration from 12 to 18 weeks average 249 ng/100ml and 29 ng/100ml for male and female fetuses,

respectively. But at term there were no significant differences in testosterone concentrations between sexes, averaging 84 ng/100 ml and 77 ng/100 ml for male and female fetuses, respectively. Increased testosterone concentration in male fetuses paralleled leydig cell hyperplasia and the presence of 3 B-hydroxysteroid dehydrogenase in human fetal testis (39).

Frazier and Horton (41) have shown that during this period the ratio of plasma levels of androstenedione to testosterone ratio is the order of two. It should be noted that as much as 5% of peripheral testosterone is derived as a conversion product mainly from androstenedione (42). This other steroid hormone, androstenedione, is secreted from the leydig cells and is represented in Figure 2. This progression and activity in the body is important for this analysis, and each steroid is cross-checked with the testosterone antibody for possible interference or cross-reactivity. During this time before puberty, about 40% of testosterone is derived from androstenedione by conversion.

As puberty approaches in the male there is a gradual increase in the concentration of testosterone in plasma. These measurable chemical changes precede by many months the pubertal anatomical changes. Plasma testosterone concentrations in male children through puberty have been determined with several methods yielding

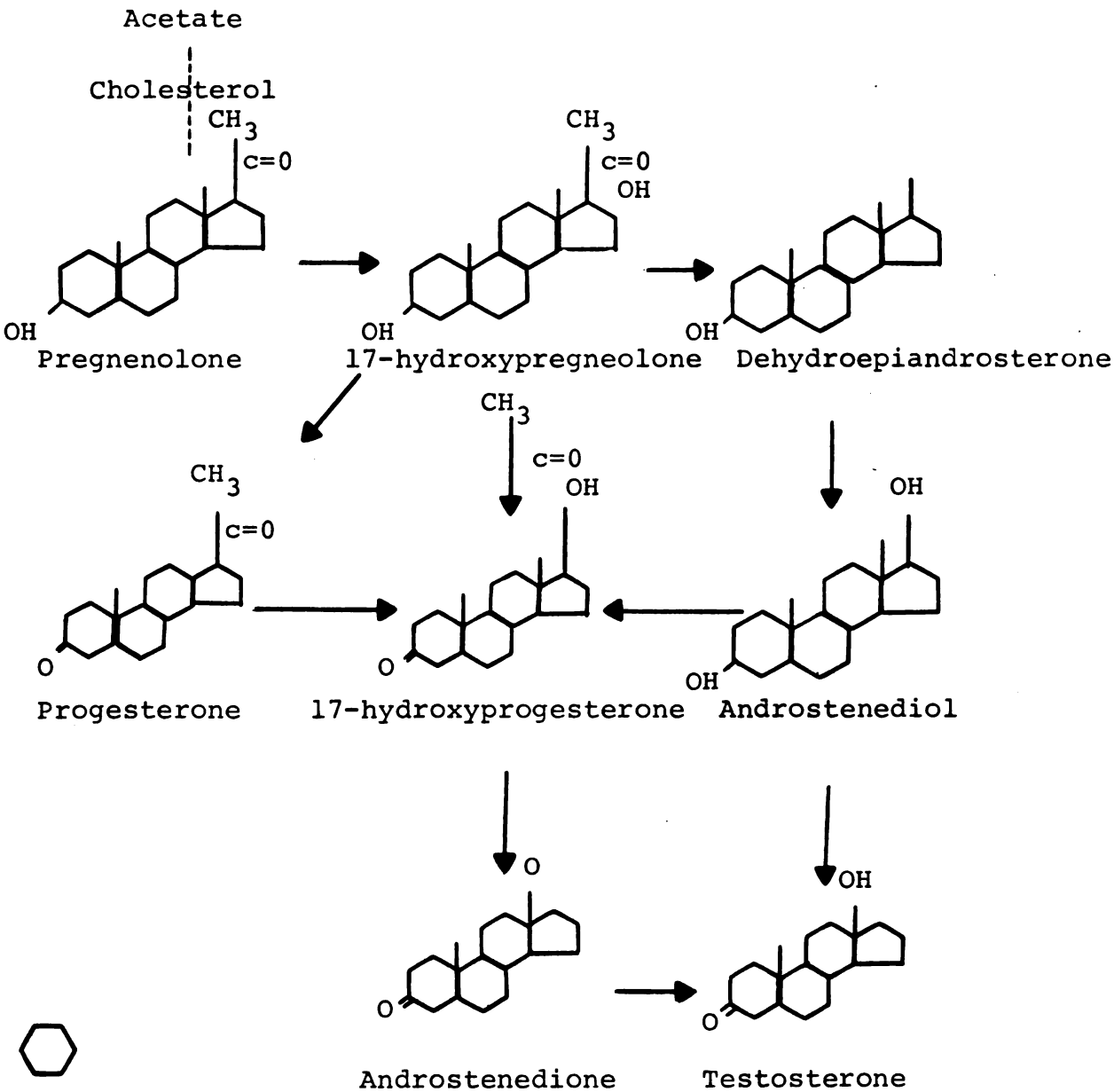


Figure 2.--Pathways of testosterone biosynthesis in the leydig cell.

similar results. Testosterone measured by a double isotope derivative technique in boys from 7 to 14 years showed an increase from 5.4 to 19 ng/100 ml up to 250 to 482 ng/100 ml (43). Adult male concentrations were reported to be 625 ± 104 ng/100 ml (25,26). Serum testosterone measured by competitive protein binding showed an increase from 21 ng/100 ml for 8 years olds to 52 ng/100 ml for 16-year-old girls. Plasma testosterone in girls measured by RIA ranged from 5.9 to 25.5 ng/100 ml for ages 6 and 13 respectively (43). The finding of plasma testosterone in young males above the prepupertal level but below the adult male range usually indicates that puberty is likely to proceed normally (43).

At the other extreme of life, an interesting study by Kent and Acone (44) showed that the clearance of testosterone from blood fell progressively with advanced years; mean plasma testosterone was lower in older men, than the normal adult values for males.

Abnormalities of Testosterone Secretion

In the male, an apparent deficiency of androgen secretion is more commonly encountered clinically than androgen excess. Low plasma testosterone can result from disorders that primarily and directly affect the testis and Leydig cell function.

Following castration, the concentration of testosterone in plasma falls to low levels within two

or three days, and although testosterone can be measured in the plasma of these individuals, there is reason to believe that it is derived by conversion from androstenedione (45) (see Figure 2).

Numerous studies have been made on the concentration of plasma testosterone in Klinefelter's syndrome, the most common cause of male hypogonadism (46,47,48). These have shown that a fairly wide range of values may exist among people with this disease, from those found in the normal female to those observed in the adult male. Attempts by a number of workers to correlate testosterone concentration with chromatin patterns have not yielded meaningful results. Hudson et al. (49) attempted to correlate plasma concentration with the number of interstitial cells, but did not find this to be helpful. Paulsen et al. also pointed out the difficulty of correlating plasma levels of testosterone with the degree of secondary sexual development (50). It is of interest in the few studies that have been made, plasma androstenedione appears to be normal in Klinefelter's syndrome.

It would seem relevant to refer to a number of studies that have been undertaken on males with an XYY chromosomal complement. Although these subjects may show distinctive anatomical and psychological features, abnormalities of testicular function do not appear to

be present. Hudson et al. investigated several such subjects and found blood testosterone concentration to be normal (51,52).

The testes may fail to mature because of primary gonadotropic hormone failure or following surgical removal of the gonadotropic stimulus before puberty. In such individuals plasma testosterone is usually lower than normal, the ultimate level depending upon the extent and duration of the failure of the gonadotropic secretion (53).

Plasma testosterone was abnormal in a variety of disorders that are not normally regarded as testicular in origin. Plasma levels of testosterone have been found to be low in perhaps the best recognized of these disorders, liver cirrhosis, in which alcohol seems to play a part (54). Other studies showed this same result, but as an increase in testosterone metabolism in the liver, and as the data indicated there is no compensatory increase in the biosynthesis of testosterone under these conditions (55). Kolodny has extended research into the effects of Marihuana on testosterone. Continued use can interfere with the production of this hormone, suppressing the supply of testosterone. According to his results, concentration of testosterone had decreased by an average of one-third (56).

In females, serum androgens vary randomly during individual menstrual cycles. When the cycle was divided into three equal periods, androstenedione and testosterone were highest during the middle period (1932 and 342 pg/ml, respectively) as compared to the first (1689 and 289 pg/ml, respectively) or the last third (1553 and 252 pg/ml, respectively) of the cycle (43).

Recent animal experiments have suggested that vasectomy may be followed by transitory testicular dysfunction and transitory reductions in serum testosterone levels (57). Studies by Smith et al. and Kobrinsky et al. (58); however, showed that mean hormone levels stayed within normal adult male ranges. Plasma samples were obtained from 50 men 1 month before and 1 month after vasectomy. Both studies found significant changes in individual values, but the new values were within the normal range of variation for adult males.

# Chapter 3

## RESEARCH DESIGN AND PROCEDURES

The background study and subsequent research was conducted at the Dairy Physiology Laboratory, Dairy Science Department, in Anthony Hall, Michigan State University. All materials and methods were those of this laboratory. There were some slight modifications introduced. For clarity, this chapter is separated into four divisions under the headings of (1) materials, (2) experiment 1: whole blood testosterone assays, (3) experiment 2 & 3: bloodstain testosterone assays, and (4) hemoglobin determination.

## MATERIALS

Solvents and reagents. Benzene (Nanograde, Fisher Scientific Co.) was twice distilled. Hexane (Fisher) was glass distilled commercially. A solution of Benxene: Hexane (1:2) was prepared and stored at room temperature in a dark bottle. To separate bound and free testosterone, 0.5 ml of 1.0% dextran T-70 (Pharmacia, Uppsula, Sweden) and a 0.50% carbon decolorizing neutral norit (washed twice with methanol, Fisher Scientific Co.) in glass distilled water was kept at 4°C.

Glassware used in routine analysis was disposable.

Steroids and antiserum. Nonlabeled standard testosterone was obtained from Sigma Chemical Co. $^{3}$H-1, 2,6,7-testosterone repurified by column chromatography using Sephadex LH-20 (Pharmacia Co.), 85 c/mM, was obtained from the New England Nuclear Co., Boston, Mass. Anti-testosterone (MSU-74) was prepared in rabbits against 3-oxime testosterone conjugated to human serum albumin. Antibody was diluted to 1/50,000 phosphate (0.01 M) buffered (pH 7.4) Saline with 0.1% gelatin (Knox Gelatin) added.

Instruments. Counting of radioactive bound antigens was performed utilizing Nuclear Chicago's Mark I liquid scintillation spectrometer or their ISOCAP/300 liquid scintillation system. Liquid scintillation fluid (3a70B Preblend scintillation cocktail, Research Products International Corp., Illinois) was used to dilute the supernatant solution to 5 ml before placing in the instrument.

Materials for analysis. Blood samples were drawn from apparently normal males and females, ranging from age 17 years to 79 years. Samples were drawn under the authority of Dr. Laurence Simson, Pathologist, at E. W. Sparrow Hospital, Lansing, Michigan, into heparinized tubes to prevent coagulation. Portions of the samples were frozen to lyse the cells for the whole blood

determinations. Another portion of the sample was used for the bloodstain preparation. These were made on cotton weave fabric (cleaning cloths).

## WHOLE BLOOD TESTOSTERONE ASSAYS[4]

Experiment 1

Duplicate aliquants of whole blood (o.1 ml) were placed in disposable culture tubes. To account for procedural losses, 2700 cpm of $^{3}$H-1,2,6,7-testosterone was added to a third aliquant from a representative number (6-8 within each assay) of unknowns. These samples of whole blood plus the $^{3}$H-1,2,6,7-testosterone (tracer) were vortexed for 5 seconds and endogenous and labelled hormone are allowed to equilibrate. The tracer is used for recovery analysis of testosterone. This then is compared to the total counts obtained from the radioactive hormone itself. Comparison gives the extraction efficiency > for the unknown sample, be it fluid blood or dried stain. The duplicate samples of whole blood were extracted by vortexing with 2.0 ml of Benzene:hexane (1:2) for 30 seconds. Tubes were then stored at -20°C for at least 1 hour to freeze the whole blood. With precautions taken to avoid thawing the whole blood, the organic solvent extracts destined for radioimmunoassay

[4]Procedure of J. Mapes and H. D. Hafs, Jan. 1975. See also Reference 59 (Smith and Hafs).

were decanted into 12 X 75 mm disposable test tubes and those for procedural losses were decanted into scintillation vials. Radioactivity of these extracts was averaged to determine a single correction factor to account for procedural losses of testosterone in all unknowns (59).

Standard testosterone was pipetted from a stock solution of 10 ng/ml and at least two sets (0.0,0.01,0.02, 0.05,0.1,0.25,0.50,1.0,1.5, and 2.0 ng/ml) were included in each assay. Standard testosterone and the whole blood extracts were dried under a stream of air.

Antibody (0.2 ml) in 0.01 M phosphate buffered saline containing gelatin was added to each tube. Then, $^{3}$H-1,2,6,7-testosterone (13000 cpm), in 0.2 ml of 0.1% gelatin and 0.01 M phosphate buffered saline, was added to each tube. The contents were vortexed for 5 seconds and incubated at 4°C for 12-16 hours.

To separate bound and free testosterone, 0.5 ml of 1.0% dextran T-70 and 0.50% carbon decolorizing neutral norit in glass distilled water was added to each tube. This technique is performed as quickly as possible to avoid the stripping action of the bound testosterone by dextran. The contents were vortexed for 5 seconds and then centrifuged at 2,900 g for 15 minutes. After centrifugation, a 0.5 ml aliguant of the supernatant fluid was diluted to 5 ml with the liquid scintillation fluid for quantification in the instrument.

## BLOODSTAIN TESTOSTERONE ASSAYS

### Experiment 2

Bloodstains were prepared on cotton weave fabric and on wool fabric. To achieve this, 0.1 ml from the samples of whole blood was pipetted onto the substrate material. For this study, extraction of testosterone from the stains for assay was performed between 36-48 hours after preparation.

The testosterone radioimmunoassay procedure was essentially that used for the whole blood assays with these modifications. Duplicate samples of the stained substrate material were placed in 16 X 125 mm disposable culture tubes. A third sample was prepared by drying 0.01 ml of $^{3}$H-1,2-testosterone onto the stained substratum, and placed into a culture tube for procedural losses. At this time, 1.0 ml of glass distilled water was added to each unknown and the tracers. The stains were allowed to extract in the distilled water for 1 hour. To each tube was added 5 ml of benzene:hexane (1:2), and the samples extracted by vortexing for 30 seconds. An emulsion was created in the tube by the presence of the stained substrate material, and to overcome this problem, the tubes were centrifuged at 2500 g for 10 minutes, stored at -20°C for 1 hour to freeze the aqueous layer, and then recentrifuged for 10 minutes at 2500 g. The samples

were again stored at -20°C for 1 hour, to freeze the aqueous phase for ease of decanting. Extracts destined for radioimmunoassay were decanted into 12 X 75 mm disposable culture tubes and those tracers for procedural losses into scintillation vials. The procedure then followed that of the whole bloods.

Experiment 3

This experiment was conducted to improve the extraction efficiency of the entire assay on bloodstains and to introduce some time saving into the extraction process.

Stains were prepared by adding .1 ml of the whole blood samples to cotton weave fabric. A blind trial was also run using stains collected from casework samples collected over the past nine months. The stains from casework with an unknown amount of blood deposited on them were also submitted to hemoglobin determinations, to give an estimate of the concentration of blood dried onto the substrate.

The procedure changed slightly from that outlined in the 2nd Experiment, for purposes of better extraction efficiency the tracer stains for procedural losses were prepared by first adding the $^{3}$H-1,2,6,7-testosterone ($^{3}$H-1,2,6,7-testosterone) to the whole blood sample before drying on the fabric. This would allow the endogenous

and labelled testosterone in the sample to equilibrate before drying. The samples were extracted in distilled water to extract the bloodstain. The solvent was added, the tubes were stored at -50°C, for one-half hour in a freezer, then centrifuged for 10 minutes at 2500 g. This step essentially removed the emulsion present in the tube after vortexing. The tubes were then refrozen at this temperature for one-half hour before decanting. It was found that the lower temperature broke the emulsion better, thus eliminating the need for extra centrifuging. The procedure then followed the prescribed assay (see Figure 3).

The standard testosterone amounts were also changed for this study. Two sets of the following amounts were pipetted for the standard curve points: 0.01,0.02,0.05, 0.08,0.1,0.25,0.5,0.8,1.0, and 1.5 ng/ml. This was included because of the extracted amounts found previously for bloodstains, which were found to be lower than the amount for whole blood assay in Example 1.

## HEMOGLOBIN DETERMINATION

Since the amount of blood on the substrate material may vary with the individual, the rate of bleeding, and the conditions of drying it was necessary to measure a second determinant in the stain to determine the volume of blood represented by the stain so testosterone could

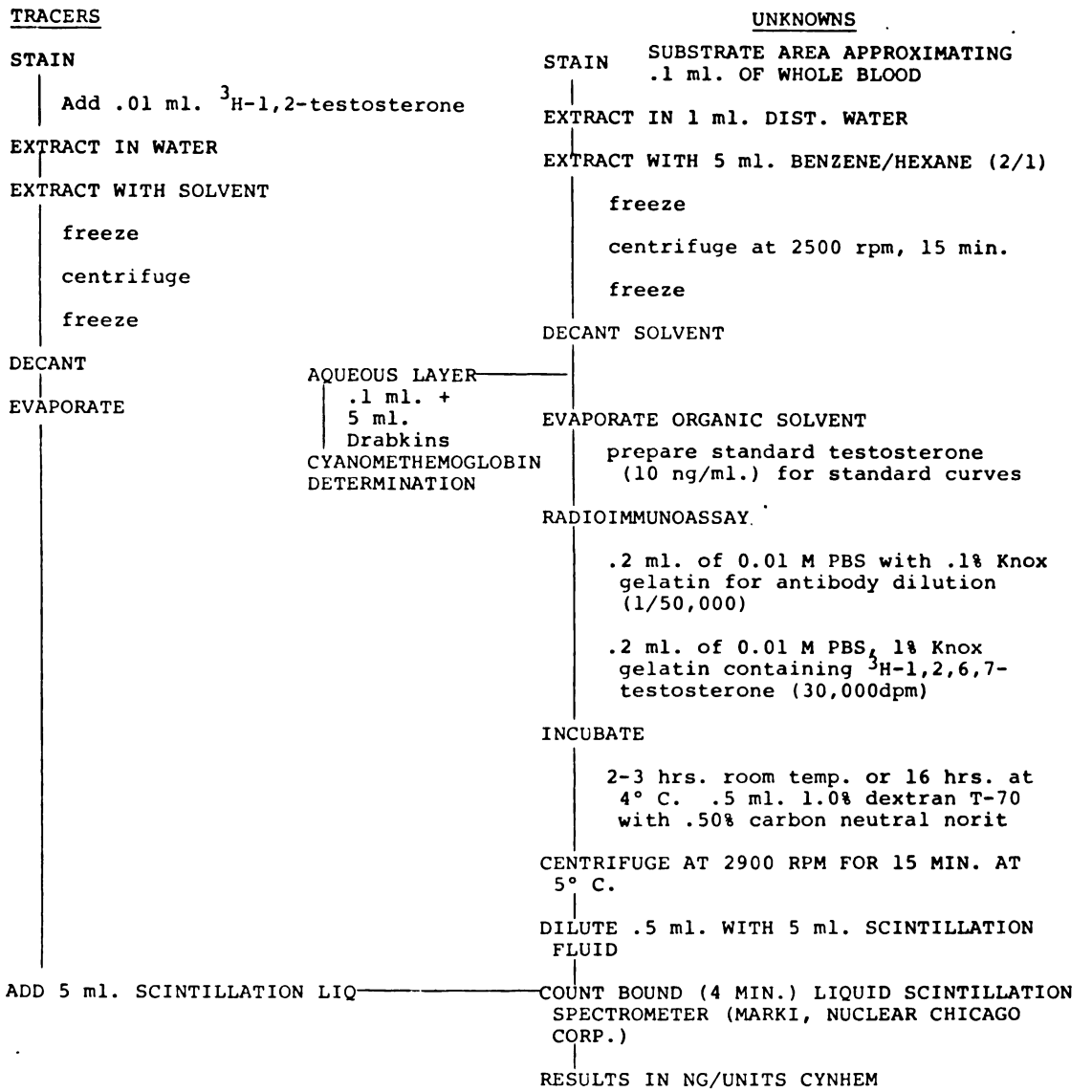


Figure 3.--Flowsheet for radioimmunoassay of testosterone in bloodstains.

be expressed per unit volume of blood. Since hemoglobin is relatively constant in the bloodstream, and only varys slightly in normal adults (values between 11-16 grams/deciliter), it was felt this might be an adequate internal standard.

The cyanmethemoglobin method was chosen for determining the hemoglobin level of a particular stain. This method measures the sum of oxyhemoglobin, methemoglobin, and carboxyhemoglobin present in a sample. On bleeding and drying, as when a bloodstain is formed, the oxygenation of the blood proceeds virtually to completion and then another and much slower process intervenes. This process is the oxidation by atmospheric oxygen of the iron atom in the hemoglobin molecule from the ferrous to the ferric state, to form the compound methemoglobin (58). Since the method involves this measuring of methemoglobin, it would be included in the total absorption for cyanmethemoglobin at 540 nanometers on a spectrometer.

Accordingly, a Drabkin's solution was prepared from the following reagent grade chemicals: Sodium bicarbonate--2 grams; Potassium cyanide--.05 grams; Potassium ferricyanide--.2 grams; and brought up to a volume of 1 liter with distilled water. Hycel Cyanmethomoglobin standard (80 mg/ml, Hycel Co., Houston, Texas) was used for the standard curve.

The following amounts of cyanmethemoglobin standard and Drabkin's solution were pipetted into 13 mm X 105 mm matched cuvettes, and mixed by inversion:

| | | | | | | |
|---|---|---|---|---|---|---|
| Standard (ML) | 0.5 | 1.0 | 1.5 | 2.5 | 3.0 | 3.5 |
| Drabkin's (ML) | 5.5 | 5.0 | 4.5 | 3.5 | 3.0 | 2.5 |

The % transmittance (measured at 540 nm) of each tube was recorded against a blank of Drabkin's solution using a Bausch and Lomb Spectronic 70 spectrometer. These values were plotted on semi-log paper as % tramittance vs. concentration in grams/deciliter of hemoglobin.

The equivalents for the hemoglobin standard used in this determination were calculated from the factor received from the amount of extract used in this determination. The factor in this study was 1/501 volume, and the equivalents of hemoglobin was ascertained at 40 grams/deciliter.

The procedure for the bloodstain extracts was as follows. 0.1 ml of the aqueous layer left over from the benzene:hexane extraction was added to the 13 mm X 105 mm cuvettes, and then 5 ml of the Drabkin's solution was added and let stand for 5 minutes. The cuvette was then placed in the spectrometer and the % transmittance read at 540 nm. Percent transmittance was then converted to grams/deciliter by use of the standard scale (60) (see Figure 4). This calculation was then compared to the assayed value of

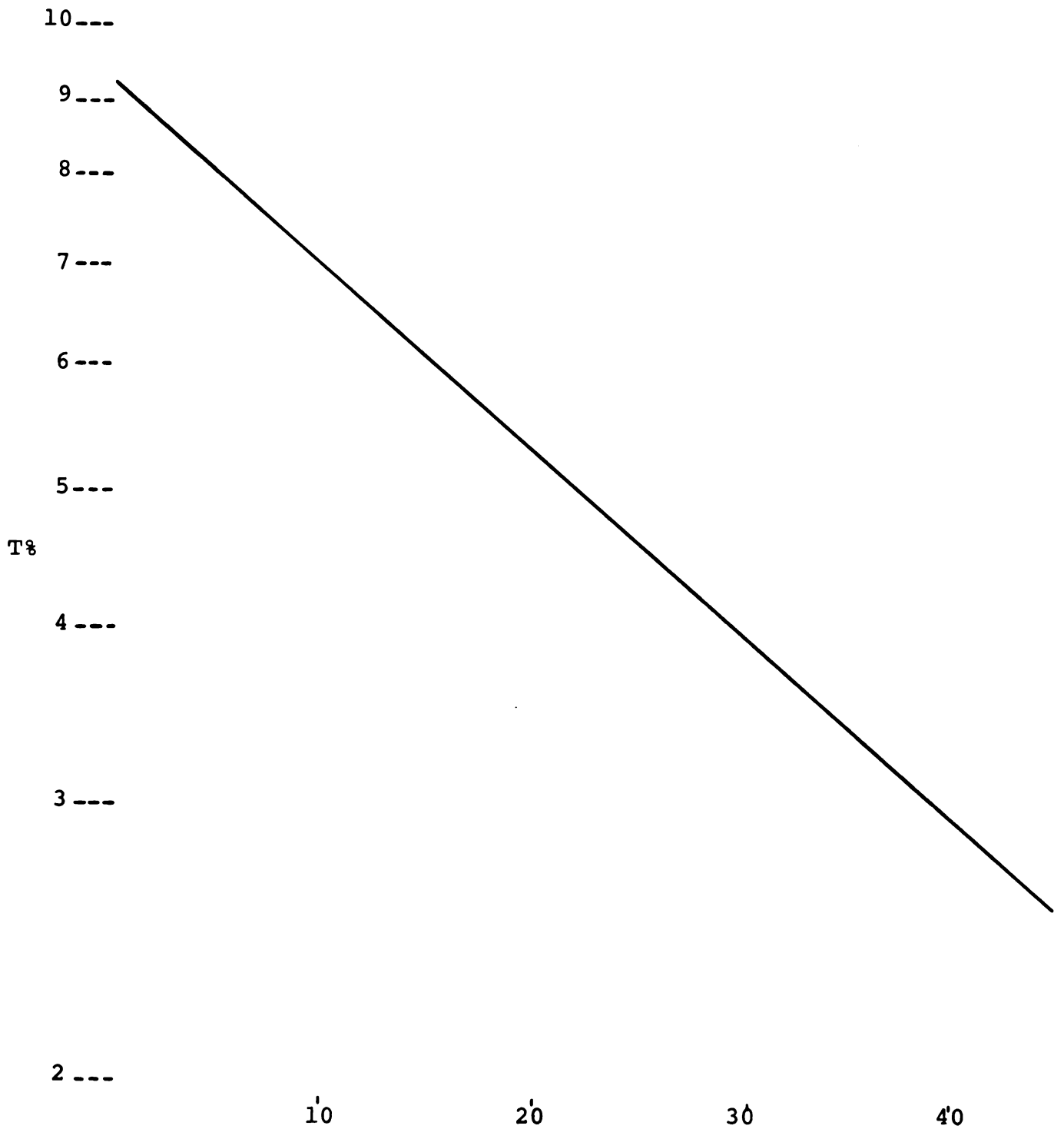


Figure 4.--Semilog plot of standard curve for determination of hemoglobin.

testosterone and comparisons made on the amount of testosterone in nanograms per units of hemoglobin.

# Chapter 4

## ANALYSIS OF THE DATA

### CALCULATIONS

Counting data for samples and testosterone standards were analyzed by computer programs developed by James W. Schwalm at MSU (Dept. of Dairy Science) for calculations of the radioimmunoassay data. The use of such programs facilitated computation of assay data, enabling corrections for recovery, sample aliquants, and nonspecific counts to be rapidly performed, and giving duplicate estimates for each sample. In this system, the standard curve is linearized by log-log transformation; calculated by fitting data to a polynomial equation via multiple regression analysis procedures; and yields a correlation of all duplicate points on the curve (see Figures 5 and 6).

### RELIABILITY CRITERIA OF THE ASSAYS

#### Standard Curve

The standard curve transformation data is shown in Figure 5 for assays performed in Experiment 2. The regression analysis accounted for 99% of the variation

| SAMPLE | REP | TIME (MIN) | GROSS COUNTS | CPM CORRECTED FOR BACKGROUND | TIME ADJUSTED TO 10000 CPM | MEAN ADJUSTED COUNTING TIME |
|---|---|---|---|---|---|---|
| STANDARD CURVE FOR DIRECT EXTRACTION ASSAY ---- | | | | | | |
| COUNTER BKG. | 1 | 4.00 | 162. | 0.0 | 0.0000 | |
| | 2 | 4.00 | 164. | 0.0 | 0.0000 | |
| | 3 | 4.00 | 159. | 0.0 | 0.0000 | |
| | | | | 0.0 | | 0.0000 |
| STANDARD NO. 1 < .010 NG / ML> | 1 | 4.00 | 5087. | 1231.4 | 8.1207 | |
| | 2 | 4.00 | 5214. | 1263.2 | 7.9166 | |
| | 3 | 4.00 | 5407. | 1311.4 | 7.6253 | |
| | | | | 1268.7 | | 7.8875 |
| STANDARD NO. 2 < .020 NG / ML> | 1 | 4.00 | 5011. | 1212.4 | 8.2480 | |
| | 2 | 4.00 | 4819. | 1164.4 | 8.5880 | |
| | 3 | 4.00 | 5153. | 1249.2 | 8.0053 | |
| | | | | 1208.7 | | 8.2736 |
| STANDARD NO. 3 < .050 NG / ML> | 1 | 4.00 | 4605. | 1110.9 | 9.0016 | |
| | 2 | 4.00 | 4724. | 1140.7 | 8.7663 | |
| | 3 | 4.00 | 4296. | 1033.7 | 9.6743 | |
| | | | | 1095.1 | | 9.1317 |
| STANDARD NO. 4 < .080 NG / ML> | 1 | 4.00 | 4002. | 960.2 | 10.4149 | |
| | 2 | 4.00 | 3990. | 957.2 | 10.4475 | |
| | 3 | 4.00 | 3954. | 948.2 | 10.5467 | |
| | | | | 955.2 | | 10.4694 |
| STANDARD NO. 5 < .100 NG / ML> | 1 | 4.00 | 3633. | 867.9 | 11.5213 | |
| | 2 | 4.00 | 3619. | 864.4 | 11.5685 | |
| | 3 | 4.00 | 3779. | 904.4 | 11.0569 | |
| | | | | 878.9 | | 11.3776 |
| STANDARD NO. 6 < .250 NG / ML> | 1 | 4.00 | 3731. | 639.9 | 15.6270 | |
| | 2 | 4.00 | 3429. | 566.9 | 17.6393 | |
| | 3 | 4.00 | 3490. | 582.2 | 17.1772 | |
| | | | | 596.3 | | 16.7691 |
| STANDARD NO. 7 < .500 NG / ML> | 1 | 4.00 | 1690. | 332.2 | 26.1666 | |
| | 2 | 4.00 | 1445. | 320.4 | 31.2094 | |
| | 3 | 4.00 | 1762. | 400.2 | 24.9896 | |
| | | | | 357.6 | | 27.2047 |
| STANDARD NO. 8 < .800 NG / ML> | 1 | 4.00 | 1385. | 305.9 | 32.6886 | |
| | 2 | 4.00 | 1306. | 261.2 | 38.2897 | |
| | 3 | 4.00 | 1268. | 276.7 | 36.1446 | |
| | | | | 281.3 | | 35.5556 |
| STANDARD NO. 9 < 1.000 NG / ML> | 1 | 4.00 | 1064. | 225.7 | 44.3131 | |
| | 2 | 4.00 | 1034. | 230.7 | 43.3526 | |
| | 3 | 4.00 | 1017. | 213.9 | 46.7472 | |
| | | | | 223.4 | | 44.7594 |
| STANDARD NO. 10 < 1.500 NG / ML> | 1 | 4.00 | 967. | 201.4 | 49.6483 | |
| | 2 | 4.00 | 849. | 171.9 | 58.1677 | |
| | 3 | 4.00 | 947. | 196.4 | 50.9122 | |
| | | | | 189.9 | | 52.6547 |

Figure 5.--Log transformation of data for RIA standard curve.

```
•9
R SQRD =  .990
IND VAR   REGRESSION COEFFICIENTS
    1          .5028084E+02
    2         -.3494339E+02
    3          .8231944E+01
INTERCEPT -   -.6479982E+02
```

FIGURE 6.LOG-LOG TRANSFORMATION OF DATA FOR GOODNESS OF FIT OF STANDARD CURVE.

| STD CURVE PT. | ACTUAL Y | LOG ACTUAL Y | ACTUAL X | LOG ACTUAL X | ESTIMATED X | X - ESTIMATED X |
|---|---|---|---|---|---|---|
| 1 | 7.8823 | .8967 | .0100 | -2.0000 | .013363 | -.003363 |
| 2 | 8.2736 | .9177 | .0200 | -1.6990 | .018945 | .001055 |
| 3 | 9.1317 | .9606 | .0500 | -1.3010 | .035736 | .014264 |
| 4 | 10.4694 | 1.0199 | .0800 | -1.0969 | .073720 | .006280 |
| 5 | 11.3776 | 1.0561 | .1000 | -1.0000 | .105839 | -.005839 |
| 6 | 16.7691 | 1.2245 | .2500 | -.6021 | .308844 | -.058844 |
| 7 | 25.5646 | 1.4076 | .5000 | -.3010 | .499269 | .000731 |
| 8 | 35.5556 | 1.5509 | .8000 | -.0969 | .689024 | .110976 |
| 9 | 44.7594 | 1.6509 | 1.0000 | 0.0000 | 1.030854 | -.030854 |
| 10 | 52.6547 | 1.7214 | 1.5000 | .1761 | 1.571153 | -.071153 |

```
IF A PLOT OF STANDARD CURVE IS DESIRED, TYPE --1--. IF NOT TYPE --0--.
•1

THIS IS A LOG-LOG PLOT OF TIME (Y AXIS) VS. CONC. (X AXIS).
PLEASE NOTE, --0.00-- CONC. VALUES ARE NOT PLOTTED.
```

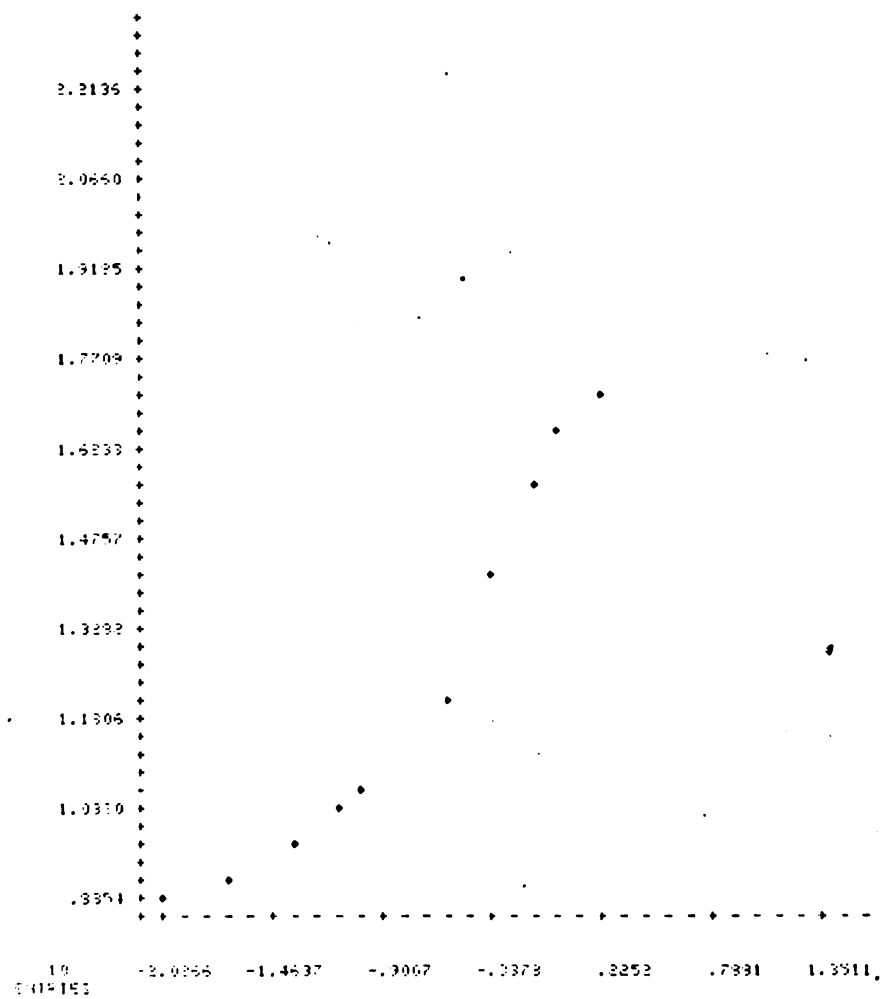


Figure 6.--Log-Log transformation of data and regression analysis for fitting the standard curve.

($R^2$ = 0.990). The between assay variation of the standard curve points was significant (r = .988) for each point on the standard curve. A comparison was made between the log-log transformation of five replicate standard curves of assays run on different days.

Recovery and Precision

The mean recovery of tritiated ($^3$H-testosterone) from the whole blood samples of Experiment 1 was 86 ± 4.8 (S.D.) %. The mean recovery of triated testosterone for Experiment 2 of the bloodstain assays was 61 ± 8.0 (S.D.) %. The mean recovery for Experiment 3 of the bloodstain assays was 84 ± 6.9 (S.D.) %. The accuracy and precision of the assay were estimated by repeated analysis of pooled whole blood from males and pooled stains from females. The interassay precision was 5-9% (coefficient of variation) for the pooled whole blood and 8-11% (coefficient of variation) for the pooled stains.

Specificity

The specificity of the method was determined by that of the antibody employed in the assay. It was tested by determining cross-reactions with various steroids and sterols, and by the comparison of RIA of testosterone in direct extracts of whole blood with that of testosterone isolated from the same extracts by column chromatography on Sephadex LH-20.

The chromatographic validation was performed to verify the specificity of the antibody used to determine testosterone in human whole blood. Ten male and ten female whole bloods were selected. The testosterone concentration of each was determined by the RIA procedure outlined in the methods chapter (see page 24) on direct extracts. In addition, on extracts of each of the samples chromatography was performed on Sephadex LH-20. In this procedure, 2½ cc. Glaspak mini-columns were packed with Sephadex. After evaporation of the organic solvent extract, 0.2 ml of benzene:methanol (90:10) was added to each extract, and this was placed on the respective column. Benzene:methanol (90:10) was then eluted through the column, letting pass the first 0.8 ml of solvent and collecting the next 2.0 ml. This fraction of the eluent containing testosterone was then submitted to the RIA procedure. Table 2 shows the comparison of the direct extraction of whole blood with those isolated by chromatography. As the results show, the values are correlated (r=0.988) for the separate techniques.

The antibody used (MSU-74) was tested against other steroids and sterols found in human blood; it cross-reacted significantly only with dihydrotestosterone (28%) (see Table 3). Since the concentration of dihydrotestosterone in males and females is only 0.65 ± 0.24

Table 2

Comparison of Testosterone Levels for Direct Extraction and Column Purification of Whole Blood Extract.

| Sex | Age | Direct ng/ml | Column ng/ml |
|---|---|---|---|
| Male | 30 | 4.067 | 5.782 |
| Male | 34 | 4.064 | 3.823 |
| Male | 54 | 7.430 | 7.299 |
| Male | 49 | 4.628 | 4.967 |
| Male | 62 | 3.645 | 2.306 |
| Female | 44 | .321 | .386 |
| Female | 28 | 1.214 | .574 |
| Female | 55 | .691 | .868 |
| Female | 20 | .671 | .631 |
| Female | 34 | .205 | .220 |
| Male | 20 | 5.174 | 4.668 |
| Male | 48 | 7.169 | 7.743 |
| Male | 45 | 5.818 | 7.648 |
| Male | 68 | .643 | .654 |
| Male | 24 | 6.662 | 6.167 |
| Female | 27 | .399 | .408 |
| Female | 45 | .489 | .301 |
| Famale | 76 | .303 | .342 |
| Female | 42 | 1.270 | 1.325 |
| Female | 55 | .205 | .226 |

Table 3

Percent Binding, Sensitivity, and Crossreactions of MSU-74 Rabbit Antitestiosterone Sera.[a]

| Steroid | % Crossreaction[c] |
|---|---|
| Dihydrotestosterone | 28 |
| Androstenedione | 0.9 |
| Androstadiene-3,17-dione | 0.04 |
| Epiandrosterone | 0.03 |
| Dihydroepiandrosterone | 0.02 |
| Estradiol | 0.02 |
| Estrial | 0.02 |
| Estrone | 0.02 |
| Progesterone | 0.02 |
| 17-a-hydroxyprogesterone | 0.01 |
| Cortisol | 0.01 |
| Cholesterol | 0.001 |
| % Binding in zero tube | 39[b] |
| % Binding reduction by .01 ng. | 26[b] |

[a]1/50,000 dilution.

[b]Based on three determinations.

[c]Pooled data from antibodies 2,15,17,19 and 21 comprising MSU-74.

(S.D.) ng/ml and 0.29 ± .09 (S.D.) ng/ml respectively, these amounts would not seem to interfere in this type of study.

Sensitivity

Sensitivity was estimated by including background tubes in the counter, and by including zero tubes (no standard testosterone added) in with the assays. In the bloodstain assays, blanks were controlled by including unstained substrate material throughout the determination.

## EXPERIMENT 1--WHOLE BLOOD ASSAY RESULTS

Two hundred whole blood samples were obtained for testosterone analysis. Of these, a portion of 170 were frozen for RIA analysis. Twenty-six aberrant observations were found in the study, mainly from errors in the technique. Of these, several whole bloods were unsuitable for preparation into a dried stain, either from lysing due to storage problems or contamination. Table 4 shows the data for the 144 samples. Figure 7 shows the distribution of the samples over the range of testosterone measured.

Although the values for males are somewhat lower than the values reported in the literature for serum or plasma, the age-range in the Table 4 is much larger. In this particular study of whole blood, for males in the

Table 4

Mean Whole Blood Testosterone Levels in Males and Females--Experiment 1. (ng/ml)

| Age | | 17-60 | 61-79 |
|---|---|---|---|
| Males | Range | .727-11.27 | .313-4.95 |
| | Mean+S.D. | 3.75 ± 2.48 | 1.39 ± 1.26 |
| | n | 63 | 16 |
| Females | Range | .113-1.274 | .103 - .691 |
| | Mean+S.D. | .421 ± .22 | .361 ± .18 |
| | n | 53 | 14 |

age-range 22-45, it was shown that the testosterone concentration was 1.136 - 10.126 ng/ml, Mean+S.D. to be 4.37 ± 2.20 ng/ml. This is consistent with the values reported in the literature for this age range.

Since the sample used was whole blood, the cellular constituents of blood were not separated as they would be in the plasma or serum testosterone estimates. This could be compared to the levels of glucagon in the blood, when measurements with whole blood as compared to serum or plasma were different to 30 percent, based on the hematocrit or red cell count in the body, which comprises approximately 30 percent of venous blood. This study indicates that the steroid hormone levels do not seem to be dependent on this observation.

Differences between males and females were studied statistically using a paired t-test, and significant levels

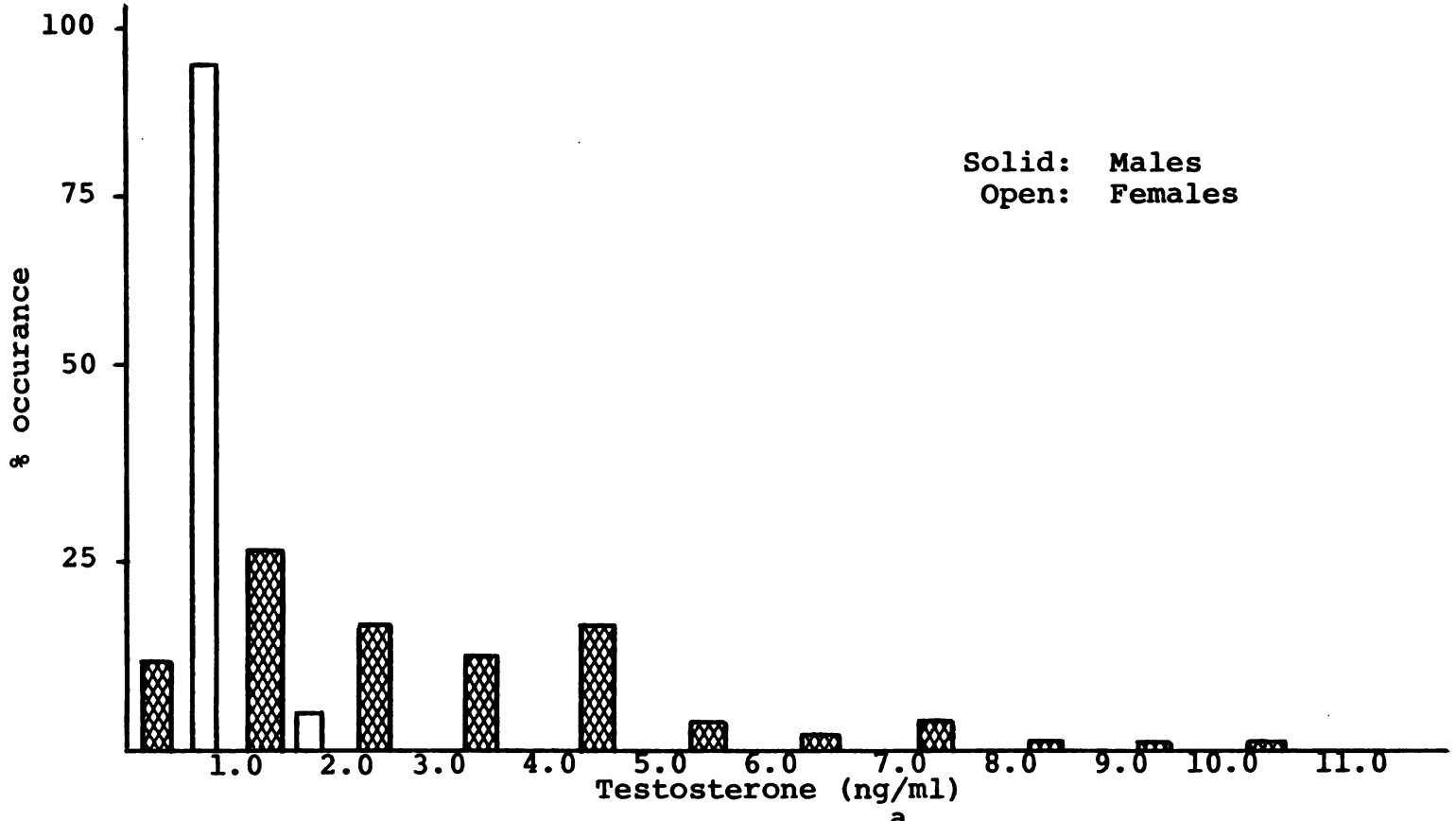


Figure 7.--Percent occurrence in males and females[a] of concentrations of testosterone.[b] Whole blood assays: Experiment 1.

[a]Males (n=79) Females (n=67)

[b]Includes concentrations for males and females, ages 61-79 years.

of difference were found at the p=.01 level, $d_f$=51. However, males aged 61-79 had testosterone concentrations equivalent to that in females 17-60 ($d_f$ = 15). Therefore, testosterone alone does not differentiate between males and females of these age groups.

## EXPERIMENT 2--BLOODSTAIN ASSAY RESULTS

Seventy-one stains were studied in this experiment over several different assays. All stains were prepared in the laboratory and were tested 36-48 hours after drying. The results of these assays are shown in Table 5 and in Figure 8.

Table 5

Mean Bloodstain Testosterone Levels in Males and Females--Experiment 2. (ng/ml)

| Age | | 17-60 | 61-79 |
|---|---|---|---|
| Males | Range | 2.04-7.325 | 2.11-4.67 |
| | Mean±S.D. | 3.61 ± 1.17 | 3.43 ± .70 |
| | n | 28 | 10 |
| Females | Range | 1.85-3.67 | .92-2.81 |
| | Mean±S.D. | 2.57 ± .48 | 2.18 ± .54 |
| | n | 22 | 11 |

Extraction efficiency based on the tracer stains prepared was in the range of 47-56 percent. The extraction efficiency is used to give a correction factor to approximate

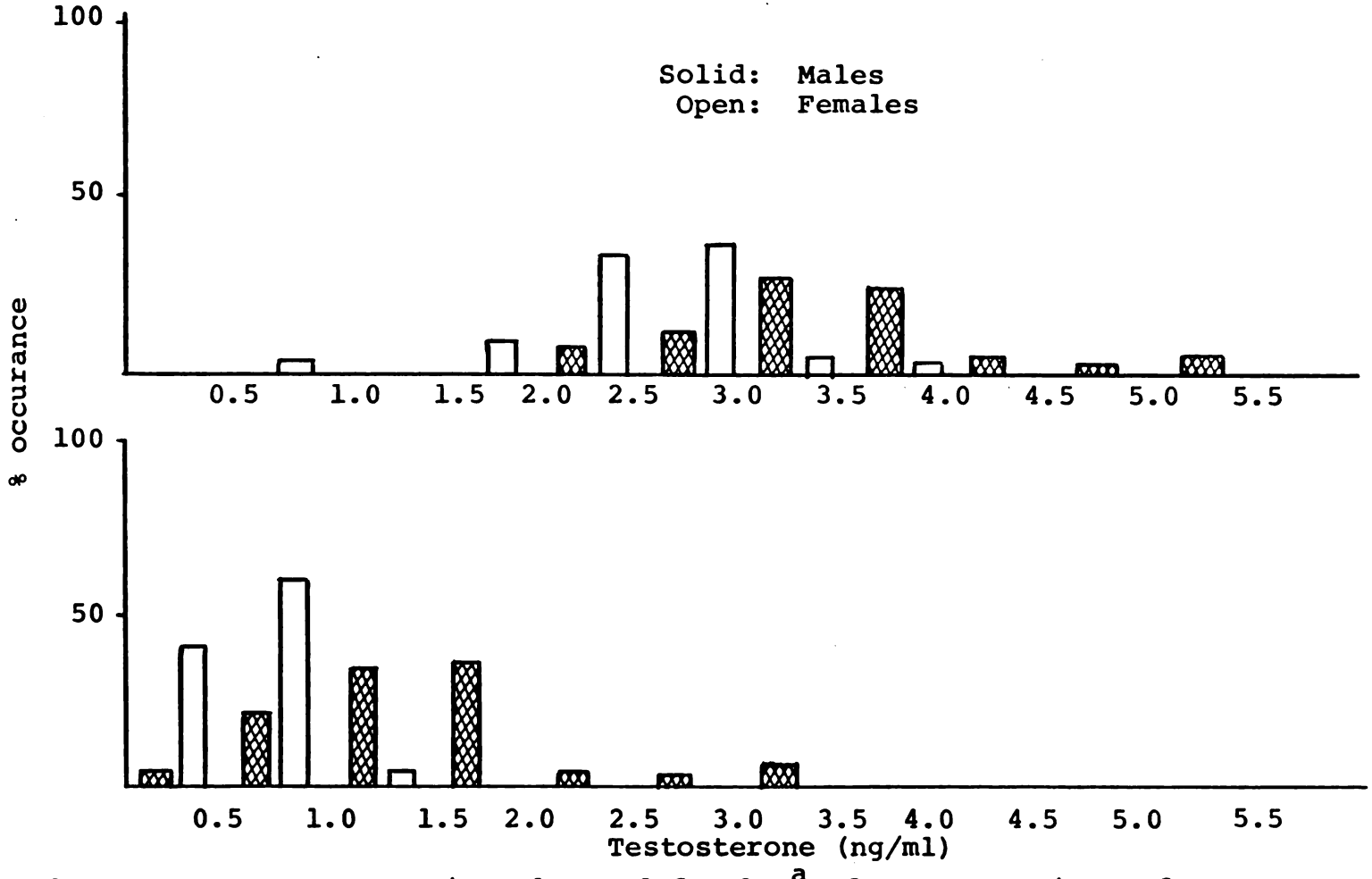


Figure 8.--Percent occurrence in males and females[a] of concentrations of testosterone. Bloodstain assays: (top) Experiment 2, (bottom) Experiment 3.

[a]Includes concentrations of males and females, ages 61-79 years.

the expected values received in the whole blood assays. As can be seen from the results, the correction factor only increased the overall values for female concentrations, which could be misleading. Although the difference between male and female stains was significant using a paired t-test, a different method was incorporated to increase the extraction of testosterone from dried blood. Experiment 3 was conducted to look at this problem.

## EXPERIMENT 3--BLOODSTAIN ASSAY RESULTS

Two assays consisting of 60 stains were utilized for this experiment, as outlined in the design and procedure chapter. The results are shown in Table 5 and distribution for the total assay are shown in Figure 8.

Table 6

Mean Bloodstain Testosterone Levels in Males and Females--Experiment 3. (ng/ml)

| Age | | 17-60 | 61-79 |
|---|---|---|---|
| Males | Range | .775-3.275 | .539-1.116 |
| | Mean±S.D. | 1.68 ± .54 | .769 ± .25 |
| | n | 27 | 8 |
| Females | Range | .439-1.281 | .313-.724 |
| | Mean±S.D. | .590 ± .17 | .550 ± .19 |
| | n | 29 | 6 |

The extraction efficiency for this experiment (84 ± 69 [S.D.] %) gave a lower correlation factor (11.0) than in Experiment 2 (61 ± 8.0% extraction efficiency) (17.2 correction factor). The results show a lowered value for male testosterone concentration, even with the correction factor, which suggests a systematic error. One explanation is the drying process onto cotton substrate material. The results may be explained by postulating that during the drying process of a normal bloodstain, i.e., venous blood, clotting proceeds and fibrin threads are formed that become attached to the material substratum and are not soluble under the conditions of extraction reported here. Besides fiber adhering to the substratum, loss of sensitivity is because crusts have not completely dissolved to obtain a complete suspension of blood in solution. Structurally, the cellular constituents of blood are composed of a lipoprotein envelope enclosing a red, iron-containing protein known as hemoglobin.

Since most steroids are bound to plasma proteins (61), solvent extraction is less effective if the plasma proteins bind the steroids with high affinity. This binding of the steroid could be enhanced by the affinity of the protein to the material substratum. Therefore, if extraction is carried out soon after addition of radioactive tracer (one-half hour in this experiment),

the added tracers are still unbound and extract easily and efficiently, whereas the steroids already present (up to 48 hours on the substrate material) are more tightly bound and resist extraction. The end result is a higher recovery of tracers than of endogenous steroids and when correction for recovery is made, the endogenous level of these samples is under estimated.

If plasma and cellular proteins also have high affinity for the substrate material, as has been postulated for fibrin, then the endogenous steriods also would be more tightly bound to the substrate material. However, this does not explain the higher extraction efficiency in Experiment 3 than Experiment 2. If anything, the radioactive tracer being dried onto the already prepared stain (as was done in Experiment 2) would be bound only to the outside crust of blood on the substrate. Possibly, this layer of crusted dried blood on the outside of the stain was undissolved in the aqueous layer, causing it to be untouched by the solvent extraction.[6]

The results between males and females were compared using a paired t-test and found to be significantly

---

[6]One test was carried out where the stains were not extracted first in distilled water, Benzene:hexane was added directly to the stained substrate material. The extraction efficiency was calculated at .6%. The drying process therefore must encapsulate in someway the constituents of blood, making them impervious to organic solvents. This has been found to be true in extracting with other solvents except those of a very polar nature, close to that of water.

different at the p=.05 level, $d_f$=26. However, no difference was found between males 61-79 and females 18-60.

Blind Trial Study

Eighteen stains were used for this study and extraction was performed as in Experiment 3. The stains were prepared from casework whole bloods received up to fourteen months earlier in the lab. The aqueous layer was submitted to the cyanmethemoglobin determination after extracting with the organic solvent. Prior to the blind study trial, the 0.1 ml stains prepared for Experiments 2 and 3 were submitted to this determination. These bloodstains fell within the range of 65-75 percent transmittance (4.6 - 7.5 gms/dl). Although age affects the amount of hemoglobin detected (62), it was felt that the amount present in the size of cut substrate would be fairly consistent, regardless of the age of the dried stain. The results of the study are shown in Table 7 and the distribution across the concentration of testosterone measured is shown in Figure 8.

As the results point out, there seems to be no relationship between the amount of testosterone present, the hemoglobin level after organic solvent extraction, and the age of the stain. Stains No. 2, 4, and 5 illustrate this. All stains cut from cotton fabric approximated the size of a 0.1 ml stain. The stains fell within two

Table 7

Results of Blind Trial Study.

| Stain # | Sex[a] | Testosterone Detected ng/ml | Hgb gms/dl | Age of Stain (months) |
|---|---|---|---|---|
| 1 | F | .043 | 35.2 | 2 |
| 2 | M | .914 | 35.8 | 4 |
| 3 | F | .353 | 4.8 | 7 |
| 4 | M | 3.046 | 17.6 | 5 |
| 5 | M | 1.584 | 18.6 | 6 |
| 6 | M | 1.239 | 9.3 | 9 |
| 7 | M | .980 | 2.0 | 13 |
| 8 | F | .122 | 2.3 | 11 |
| 9 | M | .849 | 15.8 | 3 |
| 10 | F | .066 | 20.0 | 1 |
| 11 | M | .237 | 4.8 | 14 |
| 12 | F | .081 | 7.4 | 5 |
| 13 | F | .137 | 7.2 | 4 |
| 14 | M | 1.868 | 7.0 | 2 |
| 15 | M | 1.322 | 7.4 | 15 |
| 16 | F | .063 | 11.8 | 5 |
| 17 | M | 1.306 | 9.3 | 5 |
| 18 | F | .033 | 6.3 | 4 |

[a]The unknown stains used were received from caseload whole bloods in the laboratory. Age range for these reported were between 22 and 61 years.

months of being prepared, but the hemoglobin level in grams per deciliter calculated from the cyanmethemoglobin method were not consistent with the concentration of testosterone. Differences do exist for males and females regardless of the hemoglobin level, but the spurious results detract from the overall method--to express testosterone concentration in a particular volume of blood in a stain.

It is known that during the drying process, especially on absorbent material, that cellular components dry in the center of a stain while serum migrates to the periphery of the stain (63). One explanation for the inconsistent results could be the portion of a dried stain that is used for analysis. It was found that there were undissolved crusts in the aqueous solution, which might prohibit the complete dissolving of hemoglobin in the suspension. In this regard, the presence of cellular constituents from the center of a stain could "blanket" the cyanmethemoglobin spectrum along with coextracted degradation products from the denaturation of blood during the drying process.

However, it must be noted that differences in males and females were detected, and this investigator judged sixteen correct out of the eighteen stains tested, a 90 percent proficiency. Stain No. 11 was a male stain, age 61, which could account for the lowered testosterone

concentration. If one were to make 1.25 ng/ml the discriminating level, where concentration above this certain amount in ng/ml would mean the stain was of male origin, then for this study six of the ten male stains would have been judged correctly. This investigator though allowed for the results of the entire assay to enter into the judgment. This observation strengthens the idea that proper controls and reference stains should be included within every assay attempted.

The results do show that some other method for relating testosterone to the volume of blood present in a stain will have to be investigated.

# Chapter 5

## DISCUSSION

The purpose of this study was to devise a radioimmunological method for the specific determination of testosterone in bloodstains. Fortunately, there was available a selective antisera with carefully checked specificities. Results from the validation study between direct extracts of known levels of testosterone in whole blood against column chromatographic separations of testosterone showed the antisera to be suitable for human studies. The correlation coefficient between the direct extraction and the chromatographic separation was significant (r=.986) (see Table 3). The properties of the antibody had been characterized in the Dairy Physiology Laboratory, Michigan State University, and the specificity obtained from the chromatographic validation gave considerable reliability to the data.

With regard to the cross-reactivity of dihydrotestosterone, a number of reports have appeared in recent years on its concentration in the serum of normal men and women (64,65). There is a considerable variation in the values reported in different studies, probably because there are no anti-dihydrotestosterone antibodies, as of

yet, and measurement is usually achieved in samples with the use of anti-testosterone itself. Therefore, different antibodies of testosterone would react with variable results in these studies.

The difference in serum dihydrotestosterone concentration between males and females is highly significant (P=.001). The ratio of testosterone to dihydrotestosterone is much lower in serum from females than from males, being 1.4 and 5.2 respectively. Moreover, the concentration of dihydrotestosterone in serum is higher in the luteal than in the follicular phase of the menstrual cycle. This is analagous to serum testosterone concentrations in the female (66), and might reflect a secretion of dihydrotestosterone by the corpus luteum, or an increase of its precursor testosterone during the latter half of the menstrual cycle. One female in this study with the whole blood assays, and included in the validation study, was found to have 1.23 ng/ml testosterone in direct extraction assays, while the separation on Sephadex LH-20 showed a testosterone concentration of 0.57 ng/ml. Because of the lowered amounts of the male values in the bloodstain assays, this type of cross-reaction might be a difficulty in differentiation probabilities. Without the use of chromatography though, the false increases in apparent serum or plasma testosterone would be approximately 10-15 percent in men, but

closer to 30-40 percent in women, with the use of this antisera. To alleviate this problem of the female during the menstrual cycle and the cross reactivity of dihydrotestosterone, a chromatographic separation should be introduced into the procedure.

Testosterone data from the whole blood assays of Experiment 1 were consistent with those in the literature for blood serum or plasma. Therefore, the samples used seemed not to be endocrinologically abnormal. The twenty-six samples discarded were not suitable for bloodstain preparation because of storage problems of the whole blood causing the sample to lyse.

The bloodstain analysis of Experiment 2 illustrated the problem with faulty preparation of the tracer stains for procedural losses. The extraction difficulties encountered with the tracer stains may be explained by the drying process. Adding the labelled testosterone suspended in an organic solvent to a crust of dried blood on a particular substrate did not allow the endogenous steroids in the stain to equilibrate with the added steroid. Fixation of stains by organic solvents is a routine practice in forensic science, for it prevents the dissolving of hemoglobin and other water soluble material. This phenomenon is consistent with the lower extraction efficiency of Experiment 2. Unfortunately, the causes of the particular behavior of dried blood are not completely

clear at this time. Possibly, the lowering of sensitivity is because the crusts have not completely dissolved in distilled water to obtain a complete suspension of stroma. Perhaps a slight change of pH of the aqueous solution, similar to the use of ammoniacal extracts developed for aged bloodstains will offer a solution to this problem.

Extraction efficiency in Experiment 3 was higher than that in Experiment 2. Lower values for males were observed, with no significant change in the female concentration as compared to the whole blood results of Experiment 1. Lower values would also be expected in the females, and this adds to the reason for introducing a chromatographic separation to reduce cross-reacting steroids. Moreover, when dealing with a lower concentration of testosterone, as in females, the standard curve used should be changed. Since the values fall on the low end of the curve, sensitivity is not as great as for the male values. Possibly, this could only be alleviated when, once a female is indicated, that a second RIA procedure be run with a lower concentration of testosterone used for the standard curve.

But the lower values for males (although distinguishable from the females) is an inherent problem in the technique. The higher extraction recovery in the tracer stain was contrary to the expected extraction recovery in the unknowns. Perhaps the use of a bloodstain

tracer prepared one-half hour before extraction caused this problem. The equilibrating for only a half-hour of endogenous and labeled testosterone may not have bound the tracer to the products of the whole blood as much as the testosterone already present. One solution might be the storage of tracer whole bloods or tracer stains over several different periods, so that if a stain were to be analyzed, the tracer used would be more approximate in age to the stain being tested. Extraction efficiency then would more closely approximate the extraction of endogenous steroid. It might also be possible to eliminate the need for tracer analysis altogether, since differentiation of the sexual origin of stains is dependent only on the level or concentration of a particular steroid.

The differentiation between sexes was the ultimate goal of this research. Figure 9 shows the distribution of testosterone levels of bloodstains analyzed in Experiment 3. The graph shows that approximately 90 percent of the male testosterone concentrations fall above the level of 1.25 ng/ml. For forensic purposes, this level would be the differentiation point for determining whether or not a stain was in fact of male origin. At this time, the measurement of testosterone levels below this point would be inconclusive, as was shown in the t-test for differentiating males of age 61-79 years and

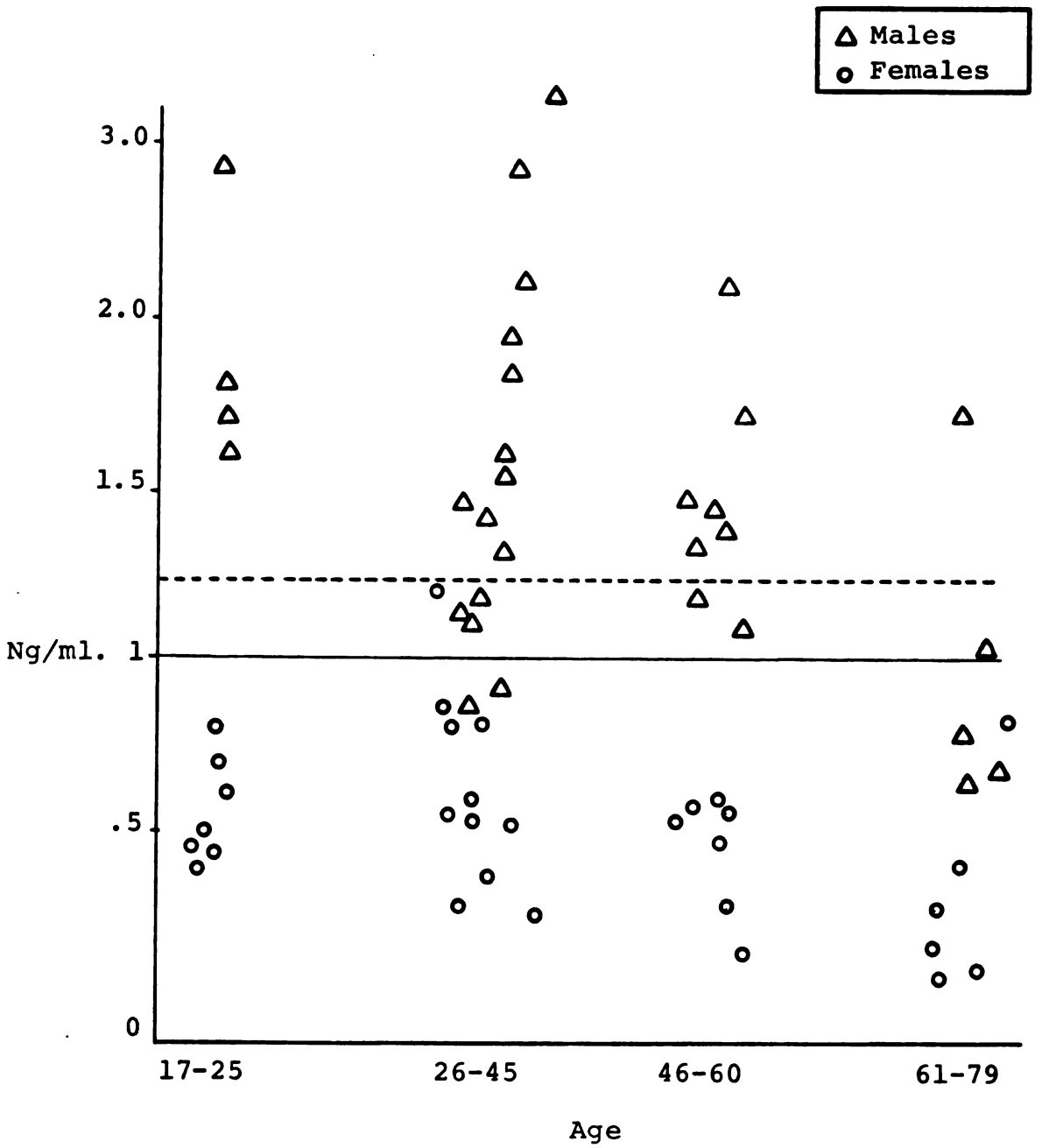


Figure 9.--Distribution of testosterone levels in males and females of different age groups. Results of bloodstain assay--Experiment 2.

females of age 17-60 years. The differences in levels of older males and older females are discrete, but the probability of differentiation at these levels is impractical.

The results of the blind study trial indicate that this method of radioimmunoassay is a valid technique, even for unknown amounts of blood in a stain. The 90 percent proficiency would be acceptable in lieu of the points in Figure 9. On a practical basis, the statement that data points below 1.25 ng/ml would be inconclusive, increases the validity of such a determination. It is unfortunate that the level of testosterone present could not be correlated with the hemoglobin content calculated with the cyanmethemoglobin method. The method may have several drawbacks. In addition to the age of a stain, the portion of a stain that is tested seems to influence the measurement. It is felt that some other method of measuring the amount of blood present in a stain should be studied. This study also indicated no difference in analytical characteristics in testosterone levels in fresh stains and those up to fourteen months in age.

At the time that this research was undertaken, other investigators at the FBI laboratory and in Pittsburgh were looking at the problem of the amount of blood present in a stain as an index to the testosterone concentration. This work is not completed, but preliminary

studies show results for testosterone levels consistent with those presented in this paper.

Brown and associates at the FBI Laboratory suggest a different approach to the amount of blood present in a stain (67). Their method includes the measurement of estradiol and progesterone in the stain and comparing these with the concentration of testosterone in the same stain. Testosterone to estradiol ratios would be higher in the male, and both would increase on a commensurate basis with the amount of blood present on the substrate. Progesterone would aid as an index to the female in the late luteal phase of the menstrual cycle, which would account for a higher testosterone estimate.

Shaler, et al. (68) at the University of Pittsburgh are also looking into the sexual origin of bloodstains at this time. Besides measuring estradiol as the internal standard against testosterone, they are attempting to fractionate the organic solvent extract from the bloodstain in order to identify the enzyme Peptidase A (an isoenzyme of peptidase present in blood which has a racial population frequency) and to separate another fraction for toxicological analysis. This type of analysis should find merit in the area of forensic science.

For the present, the relative ease of the RIA assay of testosterone using specific antibody gives a fairly reliable indication of the sexual origin of a

bloodstain. Although the amount of blood present in a stain for relating back to testosterone concentrations without the use of another assay presents difficulties, it appears that radioimmunoassay of steroid hormones offers the convienience of practicability and objective interpretation that other methods of sex determination on bloodstains lack. The RIA method for testosterone is seemingly neither dependent on the age of a stain nor hopefully on the conditions of drying, i.e., bacterial contamination, temperature, or putrefaction. Again, this is based on the relative stability of the steroid hormones. These conditions should be given considerable attention, though, by investigators.

# Chapter 6

## SUMMARY

Radioimmunoassay of testosterone in whole blood and bloodstain extracts was applied for the development of a technique for the determination of the sexual origin of a bloodstain. To account for the amount of blood present on a stained substrate material, a cyanmethemoglobin method was utilized for hemoglobin determinations as an internal standard to relate to the testosterone concentration. Testosterone in whole blood for males and females averaged 3.75 ± 2.48 (S.D.) and 0.421 ± 0.22 (S.D.) ng/ml respectively for the age range 17-60 years. For the age range 61-79 years, the values were 1.39 ± 1.2 (S.D.) and 0.361 ± 0.18 (S.D.) ng/ml for males and females respectively.

An improved extraction technique for bloodstains indicated average testosterone concentrations of 1.68 ± 0.54 (S.D.) ng/ml for males and 0.590 ± 0.17 (S.D.) ng/ml for females over the age range 17-60 years. Values for older males (0.769 ± 0.25 [S.D.] ng/ml) and older females (0.550 ± 0.19 [S.D.] ng/ml) were also noted. Males aged 17-60 had higher (p=.05) testosterone concentrations than females, ages 17-60, but no significant difference was

found between females 17-60 years and males 61-79 years. The discriminating probability of the two latter groups would then approach random error (p=.5).

A blind study trial showed 90 percent proficiency for this investigator, judging the sexual origin on 16 out of 18 unknown bloodstains. However, no correlation was found between the amount of blood present in a stain as determined by the cyanomethemoglobin method and the concentration of testosterone. However, the differentiation of males from females on a stain approximating 0.1 ml of dried blood is possible, regardless of the amount of blood present.

The major conclusion of this study is that radioimmunoassay is a reliable and viable technique for determining the sexual origin of bloodstains in the analysis of physical evidence for forensic purposes.

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# APPENDIX

The following computer printouts are compilations of the raw data from the assays outlined in the Procedures chapter. The computer program is written in BASIC language. The original data sheets are indexed and will be kept in the library at the Scientific Laboratory Section V, Michigan State Police Headquarters, 714 S. Harrison Ave., East Lansing, Michigan.

STAND ALONE STATISTICS PROGRAM

```
PUNCH

READY PUNCH; CR TO START
10 PRINT "STATISTICAL PROGRAM"
20 PRINT "ENTER DATA AND TERMINATE WITH 0"
25 LET N=0
30 INPUT X
40 IF X=0 THEN 90
50 LET S=S+X
60 LET S2=S2+(X*X)
65 LET N=N+1
70 LET M=S/N
80 GOTO 30
90 LET V=(N*S2-S*S)/N/(N-1)
100 LET D=SQR(V)
110 LET P=.6745*D
120 LET E=SQR(V/N)
130 LET C=D/M
135 PRINT
136 PRINT
137 PRINT
140PRINT "NUMBER","SUM","SUM-SQUARES"
150 PRINT N,S,S2
160 PRINT
170 PRINT "MEAN","VARIANCE","STD. DEV."
180 PRINT M,V,D
190 PRINT
200 PRINT "PROB. ERR.","STD. DEV. MN.","COEFF. VAR."
210 PRINT P,E,C
220 END
TYPE

*
```

```
*LIST 10,270

10 DIM X(100),Y(25)
20 PRINT"ENTER PROG. #"
30 PRINT"1=STAT."
31 PRINT"2=LST. SQ."
32 PRINT"3=GLASS"
40 INPUT Z
50 ON Z GO TO 60,70,1000,1900
58 PRINT "NO SUCH OPTION"
59 GO TO 20
60 PRINT"ENTER X'S, 0=END"
61 FOR I=1 TO100
62 INPUT X(I)
63 IF X(I)=0 THEN 65
64 NEXT I
65 GOSUB 100
66 END
70 PRINT"HOW MANY PAIRS?";
71 INPUT N1
72 PRINT"ENTER X,Y"
73 FOR I=1 TO N1
74 INPUT X(I),Y(I)
75 NEXT I
76 GOSUB 300
77 END
100 PRINT
115 PRINT "STATISTICS PROGRAM"
116 PRINT
120 LET I8=1
130 LET S=S2=0
140 FOR I=1 TO 100
150 IF X(I)=0 THEN 200
160 LET S=S+X(I)
170 LET S2=S2+X(I)*X(I)
180 LET M=S/I
190 NEXT I
200 LET I=I-1
210 LET V=(I*S2-S*S)/I/(I-1)
220 LET D=SQR(V)
230 LET E=SQR(V/I)
240 LET C=D/M
245 IF I8=2 THEN 270
250 PRINT"NO.","SUM","MEAN","STD. DEV."
260 PRINT I,S,M,D
270 RETURN

*
```

```
STATISTICAL PROGRAM
ENTER DATA AND TERMINATE WITH 0
?.974
?.951
?.206
?.223
?.453
?.454
?.428
?.307
?.383
?.382
?.373
?.295
?.210
?.567
?.368
?.558
?.545
?.617
?.956
?.410
?.619
?.197
?.415
?.266
?.291
?.320
?.363
?.235
?.691
?.113
?.242
?.187
?.454
?.393
?.806
?.378
?.361
?.701
?.350
?.219
?.345
?.146
?.208
?.701
?.543
?.155
?.141
?.472
?.961
?.417
?.325
?.467
?.194
?0
```

WHOLE BLOOD - EXP 1

FEMALES

17 - 60

NG/ML

| NUMBER | SUM | SUM-SQUARES |
|---|---|---|
| 53 | 22.336 | 11.9883 |
| **MEAN** | **VARIANCE** | **STD. DEV.** |
| 0.421433 | 4.95218E-02 | 0.222535 |
| **PROB. ERR.** | **STD. DEV. MN.** | **COEFF. VAR.** |
| 0.1501 | 3.05675E-02 | 0.528043 |

```
*
```

```
 RUN

STATISTICAL PROGRAM
ENTER DATA AND TERMINATE WITH 0
?.960
?8.496
?8.232
?2.193
?1.482
?.-5.488
?7.621
?4.940
?7.521
?7.220
?5.701
?3.338
?5.046
?2.916
?1.996
?4.778
?1.378
?4.061
?3.794
?5.022
?2.455
?1.136
?2.288
?2.204
?1.591
?2.208
?3.449
?4.181
?1.100
?1.269
?2.141
?1.423
?2.073
?1.751
?1.700
?1.529
?10.126
?11.271
?1.008
?5.481
?6.533
?1.463
?4.218
?4.210
?3.078
?4.090
?1.997
?1.918
?1.892
?4.864
?4.719
?2.703
?1.113
?3.851
?8.959
?.727
?1.184
?2.542
?7.377
?4.893
?4.648
?4.046
?2.915
?0

NUMBER          SUM             SUM-SQUARES
 63              236.507         1269.01

MEAN            VARIANCE        STD. DEV.
 3.75408         6.14758         2.47943

PROB. ERR.      STD. DEV. MN.   COEFF. VAR.
 1.67238         0.312379        0.660464

*
```

WHOLE BLOOD - EXP 1

MALES

17 - 60

NG/ML

```
 RUN

STATISTICAL PROGRAM
ENTER DATA AND TERMINATE WITH 0
?2.494
?.421
?3.051
?.456
?.776
?4.951
?2.059
?1.031
?1.539
?.478
?.446
?.313
?1.351
?1.787
?.660
?.4-506
?0
```

WHOLE BLOOD - EXP 1

MALES

61 - 79

NG/ML

| NUMBER | SUM | SUM-SQUARES |
|---|---|---|
| 16 | 22.319 | 54.9349 |
| **MEAN** | **VARIANCE** | **STD. DEV.** |
| 1.39494 | 1.58675 | 1.25966 |
| **PROB. ERR.** | **STD. DEV. MN.** | **COEFF. VAR.** |
| 0.849644 | 0.314916 | 0.903026 |

*

```
 RUN

STATISTICAL PROGRAM
ENTER DATA AND TERMINATE WITH 0
?.373
?.415
?.183
?.306
?.569
?.034
?.620
?.691
?.387
?.411
?.440
?.272
?.199
?.162
?0
```

WHOLE BLOOD - EXP 1

FEMALES

61 - 79

NG/ML

| NUMBER | SUM | SUM-SQUARES |
|---|---|---|
| 14 | 5.062 | 2.2774 |
| **MEAN** | **VARIANCE** | **STD. DEV.** |
| 0.361571 | 3.4394E-02 | 0.185456 |
| **PROB. ERR.** | **STD. DEV. MN.** | **COEFF. VAR.** |
| 0.12509 | 4.95652E-02 | 0.512917 |

```
*
```

```
STATISTICAL PROGRAM
ENTER DATA AND TERMINATE WITH 0
?5.266
?3.475
?2.861
?3.843
?2.368
?4.368
?3.312
?3.396
?3.090
?3.200
?3.874
?3.433
?3.533
?5.213
?3.789
?2.795
?2.776
?2.382
?3.677
?2.045
?3.126
?3.899
?3.377
?2.138
?2.615
?4.039
?7.325
?5.933
?0
```

BLOODSTAINS - EXP 2

MALES

17 - 60

NG/ML

| NUMBER | SUM | SUM-SQUARES |
|---|---|---|
| 28 | 101.148 | 402.396 |

| MEAN | VARIANCE | STD. DEV. |
|---|---|---|
| 3.61243 | 1.37061 | 1.17073 |

| PROB. ERR. | STD. DEV. MN. | COEFF. VAR. |
|---|---|---|
| 0.789659 | 0.221248 | 0.324085 |

```
*RUN
```

```
STATISTICAL PROGRAM
ENTER DATA AND TERMINATE WITH 0
?3.475
?2.903
?2.973
?2.897
?2.903
?3.032
?2.513
?2.724
?2.634
?2.427
?2.266
?2.408
?2.238
?2.048
?2.065
?2.089
?1.855
?1.899
?2.321
?2.848
?2.454
?3.677
?0
```

BLOODSTAINS - EXP 2

FEMALES

17 - 60

NG/ML

| NUMBER | SUM | SUM-SQUARES |
|---|---|---|
| 22 | 56.649 | 150.771 |
| **MEAN** | **VARIANCE** | **STD. DEV.** |
| 2.57495 | 0.233429 | 0.483145 |
| **PROB. ERR.** | **STD. DEV. MN.** | **COEFF. VAR.** |
| 0.325881 | 0.103007 | 0.187632 |

```
*RUN
```

```
STATISTICAL PROGRAM
ENTER DATA AND TERMINATE WITH 0
?2.713
?3.291
?4.673
?3.414
?3.280
?3.235
?2.119
?3.641
?3.939
?3.997
?0
```

BLOODSTAINS - EXP 2

MALES

61 - 79

NG/ML

| NUMBER | SUM | SUM-SQUARES |
|---|---|---|
| 10 | 34.302 | 122.146 |

| MEAN | VARIANCE | STD. DEV. |
|---|---|---|
| 3.4302 | 0.498116 | 0.705773 |

| PROB. ERR. | STD. DEV. MN. | COEFF. VAR. |
|---|---|---|
| 0.476044 | 0.223185 | 0.205753 |

```
*RUN
```

```
STATISTICAL PROGRAM
ENTER DATA AND TERMINATE WITH 0
?2.465
?2.814
?2.716
?1.835
?2.354
?2.717
?2.115
?2.104
?1.794
?.923
?02.218
?0
```

BLOODSTAINS - EXP 2

FEMALES

61 - 79

NG/ML

| NUMBER | SUM | SUM-SQUARES |
|---|---|---|
| 11 | 24.055 | 55.552 |

| MEAN | VARIANCE | STD. DEV. |
|---|---|---|
| 2.18682 | 0.294813 | 0.542967 |

| PROB. ERR. | STD. DEV. MN. | COEFF. VAR. |
|---|---|---|
| 0.366231 | 0.163711 | 0.248291 |

*

```
*RUN

STATISTICAL PROGRAM
ENTER DATA AND TERMINATE WITH 0
?1.861
?1.478
?2.740
?3.275
?1.409
?1.942
?1.398
?1.782
?1.597
?1.846
?1.437
?1.637
?1.877
?1.468
?1.299
?1.236
?2.133
?1.649
?1.232
?2.806
?1.798
?1.532
?.775
?1.301
?1.355
?1.590
?.949
?0
```

BLOODSTAINS - EXP 3

MALES

17 - 60

NG/ML

| NUMBER | SUM | SUM-SQUARES |
|---|---|---|
| 27 | 45.402 | 84.1618 |
| **MEAN** | **VARIANCE** | **STD. DEV.** |
| 1.68155 | 0.300613 | 0.548282 |
| **PROB. ERR.** | **STD. DEV. MN.** | **COEFF. VAR.** |
| 0.369816 | 0.105517 | 0.326056 |

```
*RUN

STATISTICAL PROGRAM
ENTER DATA AND TERMINATE WITH 0
?.504
?.751
?.748
?.436
?.744
?.508
?.586
?.458
?.639
?1.281
?.644
?.550
?.568
?.638
?.563
?.536
?.475
?.741
?.524
?.601
?.443
?.689
?.578
?.439
?.399
?.613
?.634
?.381
?.462
?0
```

BLOODSTAINS - EXP 3

FEMALES

17 - 60

NG/ML

| NUMBER | SUM | SUM-SQUARES |
|---|---|---|
| 29 | 17.133 | 10.9366 |
| **MEAN** | **VARIANCE** | **STD. DEV.** |
| 0.590793 | 2.90916E-02 | 0.170562 |
| **PROB. ERR.** | **STD. DEV. MN.** | **COEFF. VAR.** |
| 0.115044 | 3.16727E-02 | 0.288701 |

BLOODSTAINS - EXP 3

MALES

61 - 79

NG/ML

```
 RUN

STATISTICAL PROGRAM
ENTER DATA AND TERMINATE WITH 0
?.539
?.661
?1.116
?.762
?.686
?.724
?.468
?.446
?0
```

| NUMBER | SUM | SUM-SQUARES |
|---|---|---|
| 8 | 5.402 | 3.96625 |

| MEAN | VARIANCE | STD. DEV. |
|---|---|---|
| 0.67525 | 4.55077E-02 | 0.213325 |

| PROB. ERR. | STD. DEV. MN. | COEFF. VAR. |
|---|---|---|
| 0.143888 | 7.54219E-02 | 0.31592 |

*

```
 RUN

STATISTICAL PROGRAM
ENTER DATA AND TERMINATE WITH 0
?.274
?.199
?.162
?.724
?.478
?.446
?.686
?0


NUMBER          SUM             SUM-SQUARES
 7               2.969           1.56309

MEAN            VARIANCE        STD. DEV.
 0.424143        5.06355E-02     0.225023

PROB. ERR.      STD. DEV. MN.   COEFF. VAR.
 0.151778        8.50507E-02     0.530536

*
```

BLOODSTAINS - EXP 3

FEMALES

61 - 79

NG/ML