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# The Determination of the Sex of an Individual from a Dried Bloodstain Using Radioimmunoassay of Testosterone, Progesterone, and Estradiol-17$\beta$

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**ABSTRACT:** A radioimmunoassay (RIA) technique was developed for the purpose of determining the sex of an individual by measuring the steroids testosterone (T), progesterone (P), and estradiol-17$\beta$ ($E_2$) in dried bloodstains. The steroid values from a single bloodstain are reported as ratios T/P, T/$E_2$, and P/$E_2$. The results reported include data on the validation of this technique, results of analysis of 146 duplicate bloodstains representing 112 samples from females and 34 samples from males as controls, and results of analysis of 114 duplicate bloodstains representing 60 from females and 54 from males that were submitted as a blind study to test the accuracy of this RIA technique in determining the sex of an individual from a dried bloodstain.

**KEYWORDS:** pathology and biology, human identification, radioimmunoassay, sex determination, bloodstains, testosterone, progesterone, estradiol-17$\beta$

A technique for the biological classification of an individual as male or female from a dried bloodstain has long been sought by the forensic scientist. The usefulness of such a technique is obvious in cases where both suspect and victim are of the same blood type but are of opposite sexes. The differences between male and female are both genotypic and phenotypic. The genetic differences between male and female are evident in the somatic tissue as the sex chromatin material. The phenotypic differences are, in part, a manifestation of the endocrine differences between male and female. The ability to determine genotypic and endocrine differences between male and female is available in biomedical and clinical investigative techniques. The visualization of sex chromatin material [*1,2*] has been applied to dried bloodstains for forensic science applications [*3*] but is limited to identifying bloodstains from males by detecting the Y chromosome. A bloodstain could not, with any degree of confidence, be considered female in origin solely because of an inability to detect the Y chromosome.

The endocrine differences between the normal male and female are quantitative (with the exception of hormones associated with pregnancy). The techniques to detect these dif-

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ferences are now extremely sensitive ($10^{-15}$ g) in contrast to the older bioassay and instrumental techniques ($10^{-3}$ to $10^{-9}$ g) that were lacking in sensitivity and thus limited in forensic science applications. Radioimmunoassay (RIA) is one of these sensitive techniques; it was first introduced by Yalow and Berson [*4*] to measure insulin. There are now RIAs for a large variety of endogenous and exogenous compounds [*5, 6*].

Three steroid hormones, testosterone (T), progesterone (P), and estradiol-17$\beta$ ($E_2$) (nomenclature in Table 1), elaborated principally by the gonad, account for some of the endocrine differences between male and female. Numerous investigators report steroid hormone values in plasma or serum using RIA [*7–9*]. Testosterone, considered a male sex steroid hormone, averages 5000 pg/mL of plasma in the male and 200 pg/mL of plasma in the female. Progesterone and estradiol-17$\beta$, considered female sex steroids, average 100 to 15 000 and 40 to 300 pg/mL of plasma, respectively, in the female and 100 and 25 pg/mL of plasma, respectively, in the male.

I describe in this paper a method for determining the sex of an individual from a bloodstain by measuring testosterone, progesterone, and estradiol-17$\beta$ by using a modified RIA technique. I reported the preliminary work on this project in 1976 [*10*] and 1977 [*11*]. This method involves a simple extraction of a solubilized bloodstain followed by celite column chromatography, collection of the steroids as three separate fractions from one column, and quantification of the steroids by the appropriate radiolabeled ligand assay. Results of steroid RIAs are expressed in picogram amounts per bloodstain and then calculated into ratios, one steroid to another, T/P, T/$E_2$, and P/$E_2$. Steroid ratios were determined in bloodstain samples from control male and female volunteers and from samples analyzed as part of a blind study to test the accuracy of this technique in predicting the sex of individuals from their bloodstains.

TABLE 1—*Specificity of antiserum.*

| Steroid Tested | Percent Cross-Reactivity[a] | | |
|---|---|---|---|
| | TAb[b] | PAb[c] | $E_2$Ab[d] |
| Testosterone[e](T)—17$\beta$-hydroxy-4-androstene-3-one[f] | 100.00 | <0.01 | <0.01 |
| Progesterone[e](P)—4-pregnene-3,20-dione[f] | <0.01 | 100.00 | <0.01 |
| Estradiol-17$\beta$[e]($E_2$)—1,3,5(10)-estratriene-3,17$\beta$-diol[f] | 0.60 | <0.01 | 100.00 |
| Dihydrotestosterone[e](DHT)—17$\beta$-hydroxy-5$\alpha$-androstan-3-one[f] | 18.72 | 0.33 | <0.01 |
| 17$\alpha$-hydroxyprogesterone[e]—17$\alpha$-hydroxy-4-pregnene-3,20-dione[f] | <0.01 | <0.01 | <0.01 |
| Estradiol-17$\alpha$[e]—1,3,5(10)-estratriene-3,17$\alpha$-diol[f] | <0.01 | <0.01 | 10.00 |
| Estrone[e]—3-hydroxy-1,3,5(10)-estratriene-17-one[f] | <0.01 | 0.10 | 8.00 |
| Estriol[e]—1,3,5(10)-estratriene-3,16$\alpha$,17$\beta$-triol[f] | <0.01 | <0.01 | 3.03 |
| Androstenediol[e]—5-androstene-3$\beta$,17$\beta$-diol[f] | 3.55 | <0.01 | <0.01 |
| Cortisone[e]—17$\alpha$,21-dihydroxy-4-pregnene-3,11,20-trione[f] | <0.01 | <0.01 | <0.01 |
| Androstenedione[e]—4-androstene-3,17-dione[f] | 3.14 | 0.10 | <0.01 |
| Dihydroandrosterone[e]—5$\alpha$-androstan-3$\alpha$,17$\beta$-diol[f] | 44.00 | <0.01 | <0.01 |
| Cortisol[e]—11$\beta$,17$\alpha$,21-trihydroxy-4-pregnene-3,20-dione[f] | <0.01 | <0.01 | <0.01 |
| 5$\alpha$-androstan-3$\beta$,17$\beta$-diol[f] | 7.33 | <0.01 | <0.01 |
| Corticosterone[e]—11$\beta$,21-dihydroxy-4-pregnene-3,20-dione[f] | <0.01 | 1.43 | <0.01 |

[a] Cross-reactivity read at 50% of initial binding [*14*].

[b] Testosterone antibody made against testosterone-3-0-carboxymethyloxime/human serum albumin in the rabbit.

[c] Progesterone antibody made against 11$\alpha$-hydroxy-4-pregnene-3,20-dione-hemi-succinate/bovine serum albumin in the rabbit.

[d] Estradiol-17$\beta$ antibody made against estradiol-17$\beta$-succinyl/bovine serum albumin in the sheep.

[e] Trivial name.

[f] Systematic name using nomenclature approved by International Union of Pure and Applied Chemistry and International Union of Biochemistry [*15, 16*].

## Experimental Procedures

Although the measurement by RIA of steroids in serum and plasma has been routine [*8, 9*], the quantitation of steroids in whole blood in the liquid or dried form is rare; an exception is the microfilter paper method of Pang et al [*12*] for the measurement of 17α-hydroxyprogesterone. Additionally, the wide variety of samples that is routinely received in a forensic science laboratory imposes many unknowns. For these reasons a partial purification of the steroids and adaptation of the column chromatography techniques of Parker et al [*8*] and Anderson et al [*9*] were incorporated.

### *Steroids*

Radioactive steroids, testosterone 1,2,6,7$^3$H ($^3$HT), progesterone 1,2,6,7$^3$H ($^3$HP), and estradiol-17β 2,4,6,7$^3$H ($^3HE_2$) were purchased from New England Nuclear and were purified monthly by ascending chromatography (Chromar GF, Mallinckrodt). The solvent system employed for $^3$HT and $^3$HP was benzene/ethyl acetate 9:1 (v/v), whereas $^3HE_2$ was purified in benzene/ethanol 97:3 (v/v). Following chromatography, the $^3$H steroid areas corresponding to authentic nonlabeled steroids were first eluted with 10 mL of benzene/ethanol 9:1 (v/v) by the Osdova method [*13*]. The solvent was then evaporated under a stream of nitrogen. Finally, the $^3$H steroid was reconstituted in either benzene ($^3$HP) or benzene/ethanol 9:1 (v/v) ($^3$HT and $^3HE_2$). Nonradioactive steroids were purchased from Steraloids and were purified by recrystallization in three solvent systems (methanol/water, ethanol/water, and ethyl acetate/hexane). Their melting points were checked after each recrystallization, and stock solutions (1 mg/mL) were stored at 4°C in either methanol ($E_2$) or benzene (T and P).

### *Reagents*

Solvents were used without purification unless otherwise indicated. Anhydrous ethyl ether (AR), SpectrAR®-grade 2,2,4-trimethylpentane (iso-octane) and Nanograde®-quality benzene, ethyl acetate, hexane, and methanol were from Mallinckrodt. Scintillation-grade toluene and Triton X-100 were from Eastman Organics. Deionized water (minimum 10-MΩ resistance) was obtained from a Milli Q (Millipore) water purification system after treatment with a Milli-RO (Millipore). Propylene glycol (Mallinckrodt) and ethanol (Fisher Scientific) were redistilled prior to use. Celite 545 (Fisher Scientific) was heated 18 h at 450°C prior to column preparation.

### *Samples*

The bloodstains analyzed represented two types of samples from three different sources. The first type was from healthy male and female volunteers in the laboratory and was used to form a data base for known males and females. The bloodstains were collected on washed sheeting by either finger stab or venipuncture and allowed to air-dry. The second type was used to test the accuracy of this technique in determining the sex of an individual from a bloodstain in a blind study. The bloodstains were from individuals whose sexual identification was unknown to me but known by the individuals preparing the bloodstains for the blind study. These bloodstains were either actual case work samples submitted for serological examination or had been prepared on washed cotton sheeting from liquid whole blood samples (ethylenediaminetetraacetic acid [EDTA] used as the anticoagulant) provided by a local hospital.

*Extraction*

Dried bloodstains (400 $mm^2$ size) were placed in 16- by 125-mm screw-capped (lined with Teflon®) culture tubes and were first soaked in 1.5 mL of deionized water for 2 h. Then, 5 mL of ethyl ether at −10°C were added. Tubes were capped and extracted by vigorously agitating for 30 s. The samples were then centrifuged for 5 min at 600*g* and the aqueous layer was frozen. The ether layer was decanted into disposable, flint glass 12- by 75-mm test tubes and dried under a stream of nitrogen. Fresh ethyl ether (5 mL) was then added to the extraction tube, and extraction, centrifugation, and freezing of the aqueous layer was repeated with the second ether extract combined with the first ether extract. The ether was evaporated under a stream of nitrogen, and the sample extract was stored at 4°C in benzene until the chromatography step.

*Column Chromatography*

Isolation of the steroids of interest (T, P, and $E_2$) was performed on disposable mini-celite columns using the modified procedures of Parker et al [8] and Anderson et al [9]. The procedure uses a gradient elution with iso-octane the principle solvent and an increasing percentage of ethyl acetate for each fraction run through the column. Columns (disposable glass 5-mL graduated pipettes, Corning), stoppered with a 4-mm glass bead, were packed tightly with 1.2 g of celite/propylene glycol 2:1 (w/v) to a height of 55 mm; the top of the celite/propylene glycol was then layered with 3 mm of washed silica (fine granular silica sand from Fisher Scientific washed ten times with deionized water, then five times with methanol, and dried at 100°C). Packed columns were then washed two times with 3.5 mL each iso-octane under positive nitrogen pressure. Reconstituted sample was then layered over the silica in 0.2 mL of iso-octane/methanol (97.5:2.5, v/v). The sample tube was rinsed two times separately with 0.2 mL of iso-octane/methanol, 97.5:2.5 (v/v), and the separate rinses were applied to the silica layer on the column. Gradient elution was then carried out with iso-octane/ethyl acetate mixtures increasing by 5% ethyl acetate for each 4-mL fraction eluted through the column until 32 mL of solvent had been eluted. Fractions were iso-octane/ethyl acetate 100:0, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, and 65:35 (v/v). Fractions were collected into 12- by 75-mm flint glass test tubes, and solvent fractions were evaporated under a stream of nitrogen at 45°C for the P fraction and at 55°C for the T and $E_2$ fractions. Steroid fractions were stored in benzene at 4°C until assay.

*Radioimmunoassay*

The three steroids of interest, T, P, and $E_2$, were quantified with antisera generously supplied by Drs. E. D. Plotka, Marshfield Clinic Foundation, Marshfield, Wisc. (P and $E_2$), and H. D. Hafs, Michigan State University, East Lansing (T). Table 1 contains the data on the specificity for the three antisera. The assay buffer consisted of 0.1% gelatin (unflavored Knox gelatin) in 0.1*M* phosphate-buffered saline (PBS) at pH 7.0 (0.39*M* $NaH_2PO_4 \cdot 1\ H_2O$ [monobasic sodium phosphate monohydrate], 0.061*M* $Na_2HPO_4 \cdot 7\ H_2O$ [dibasic sodium phosphate heptahydrate], 0.154*M* sodium chloride, and 0.0154*M* sodium azide in deionized water. The charcoal solution containing 650 mg of charcoal (Fisher Scientific Norit A washed ten times in deionized water and oven-dried at 100°C) and 65 mg of Dextran T-70 (Pharmacia) in 0.1 L assay buffer was kept in suspension with constant stirring at 4°C.

All sample and standard tubes containing either dried purified extract or steroid had assay buffer, antisera, and $^3H$ antigen added in volumes listed in Table 2. Briefly, assay buffer was added to all tubes, which were then gently and briefly mixed. Dilute antisera was

TABLE 2—*Volume of reagents in assay.*

| Tube Identification | Added at Start of Incubation | | | Added After Incubation | | |
|---|---|---|---|---|---|---|
| | PBS[a] | Ab in PBS[a] | $^3$H Ag in PBS[a] | PBS[b], 0.5% | PBS[a] | Charcoal Solution |
| Total counts | 0.5 | 0.1 | 0.1 | 0.1 | 0.2 | ... |
| Nonspecific binding | 0.6 | ... | 0.1 | 0.1 | ... | 0.2 |
| 0[c] | 0.5 | 0.1 | 0.1 | 0.1 | ... | 0.2 |
| 10[d] | 0.5 | 0.1 | 0.1 | 0.1 | ... | 0.2 |
| 25 | 0.5 | 0.1 | 0.1 | 0.1 | ... | 0.2 |
| 50 | 0.5 | 0.1 | 0.1 | 0.1 | ... | 0.2 |
| 75[e] | 0.5 | 0.1 | 0.1 | 0.1 | ... | 0.2 |
| 100 | 0.5 | 0.1 | 0.1 | 0.1 | ... | 0.2 |
| 175 | 0.5 | 0.1 | 0.1 | 0.1 | ... | 0.2 |
| 250 | 0.5 | 0.1 | 0.1 | 0.1 | ... | 0.2 |
| 375 | 0.5 | 0.1 | 0.1 | 0.1 | ... | 0.2 |
| 500[f] | 0.5 | 0.1 | 0.1 | 0.1 | ... | 0.2 |
| 1000[g] | 0.5 | 0.1 | 0.1 | 0.1 | ... | 0.2 |
| Unknown | 0.5 | 0.1 | 0.1 | 0.1 | ... | 0.2 |

[a] PBS = phosphate-buffered saline, 0.1*M*, pH 7.0, with 0.1% gelatin; Ab = antibody and Ag = antigen.
[b] PBS with 0.5% gelatin.
[c] Zero steroid standard tube.
[d] 10 to 1000 pg steroid for standard curve.
[e] Only present in $E_2$ standard curve.
[f] Last point on $E_2$ standard curve.
[g] Last point on P and T standard curve.

then added with initial dilutions 1:4000, 1:20 000, and 1:15 000 for P, T, and $E_2$, respectively, to achieve 50% binding. The tubes again were briefly and gently mixed before adding the final incubation reagent, $^3$H-labeled antigen containing 50 pg of steroid (approximately 20 000 disintegrations per minute [dpm]). All tubes were briefly and gently mixed, covered with aluminum foil, and incubated 12 to 18 h at 4°C. After incubation, all tubes and remaining reagents were kept at 4°C for the completion of the antibody-antigen separation step. A volume of 0.1 mL (Table 2) 0.5% gelatin in PBS was added to all tubes. The tubes were gently and briefly mixed, and then 0.2 mL of charcoal solution were added to all tubes (except total counts); the tubes were gently mixed for 5 s. After a 20-min incubation at 4°C, the tubes were centrifuged at 1000*g* for 10 min at 4°C. The supernatant was then decanted into liquid scintillation vials containing 10 mL of counting cocktail (1 L of toluene, 0.5 L of Triton X-100, and 5.5 g of Permablend II from Packard Instrument Co.).

Samples were counted twice at 10 min each in a Packard Scintillation Spectrometer, Model 3330, and calculations were performed using the log-logit transformation described by Rodbard et al [*17*]. The quantities of steroids (T, P, and $E_2$) in each bloodstain were expressed as the ratio of steroids to each other (T/P, T/$E_2$, and P/$E_2$).

## Results

Before this technique could be applied to the problem of determining the sex of an individual from a dried bloodstain by measuring steroids therein, I first validated the technique using established criteria [*5, 18-20*]. One group of analyzed bloodstains represented samples from known males and females to form a data base. A second group of bloodstains consisted of a blind study to determine the accuracy of this technique.

*Validation*

*Chromatography*—There are steroid radioimmunoassay techniques that do not use chromatography after the organic solvent extraction of serum or plasma [*14, 21, 22*]; others use some type of chromatography [*7–9*] to purify the steroids after extraction, prior to measurement. I used a column chromatography technique that would allow for the simultaneous separation of the steroids of interest (T, P, and $E_2$) from a single bloodstain, allow removal of substances that might interfere with the measurement of steroids in the assay because of the myriad materials that bloodstains are found on in the forensic science laboratory, and separate the steroids of interest (T, P, and $E_2$) from any other steroids present in blood in appreciable amounts that might significantly cross-react with the appropriate antibody (Table 1). A very satisfactory separation of the three steroids, T, P, and $E_2$, was achieved with the celite/propylene glycol, 2:1 (w/v) columns, as is depicted in Fig. 1. There is one steroid present in peripheral blood in appreciable amounts that significantly cross-reacts, and this is dihydrotestosterone (DHT) (Table 1). The DHT concentration in male blood averages 600 pg/mL and averages between 70 and 150 pg/mL in the female [*8*]. Radiolabeled $^{14}C$ DHT appears in the 11- to 14-mL fractions, demonstrating that the column separation technique adequately removes this possible contaminant.

Although the column chromatography technique separates the $^{3}H$ steroids, T, P, and $E_2$, as shown in Fig. 1, this separation does not assure that the immunoreactive, non-$^{3}H$-labeled steroids appear in the same fractions as the $^{3}H$-labeled steroids. Consequently, $^{3}H$ T, P, and $E_2$ (50 000 dpm each) were added to separate 1-mL aliquots of liquid male blood and allowed to equilibrate; 0.1 mL of radioactively labeled blood was then pipetted onto washed cotton sheeting. After the blood spots were allowed to air-dry they were extracted and chromatographed as previously described, except that 1-mL fractions were collected. The fractions were dried and reconstituted in 1 mL of benzene, and 0.5 mL were removed for counting of radioactivity, while the remaining 0.5-mL aliquot in the 12- by 75-mm test tube was dried and assayed for immunoreactivity in the appropriate radiolabeled ligand assay. The profiles of immunoreactive and radioactive steroids as eluted from the columns are plotted in Figs. 2, 3, and 4 and, as illustrated, the radioactive and immunoreactive peaks coincide.

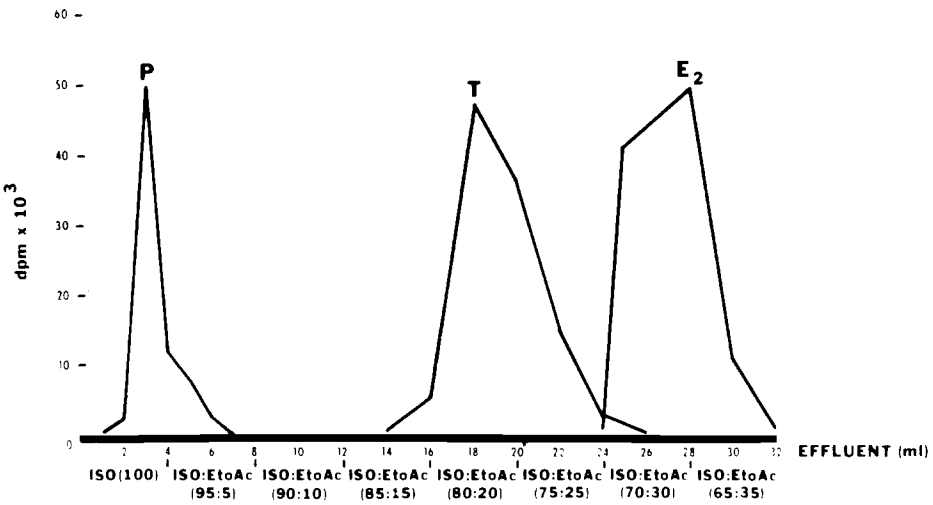


FIG. 1—*Fractionation of progesterone* (P), *testosterone* (T), *and estradiol* ($E_2$) *on celite/propylene glycol 2:1 (w/v) stationary phase and* isooctane *(ISO)/ethyl acetate (EtoAc) (v/v) mobile phase.*

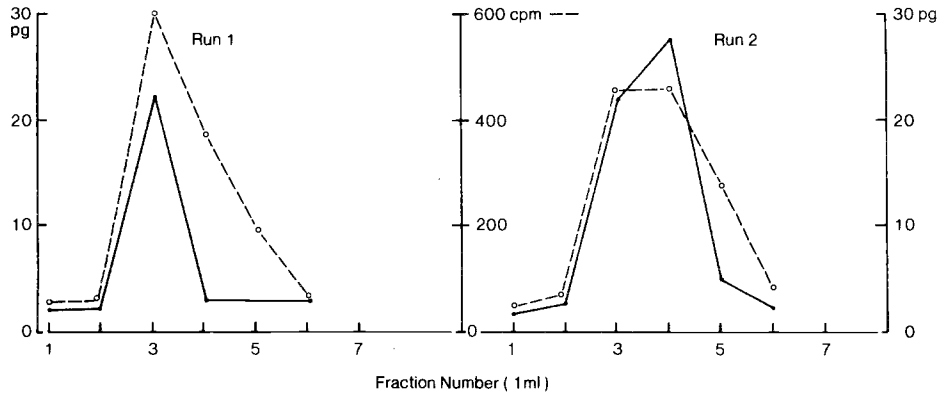


FIG. 2—*Duplicate profiles of immunoreactive* (——) *and radioactive* (------) *progesterone.*

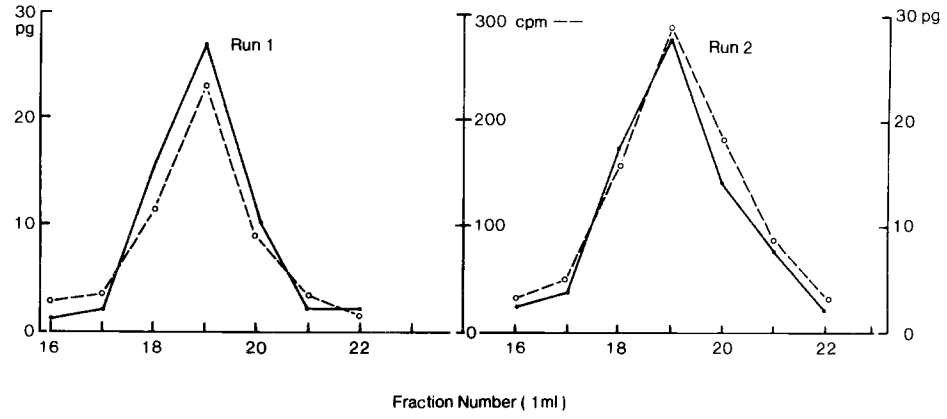


FIG. 3—*Duplicate profiles of immunoreactive* (——) *and radioactive* (------) *testosterone.*

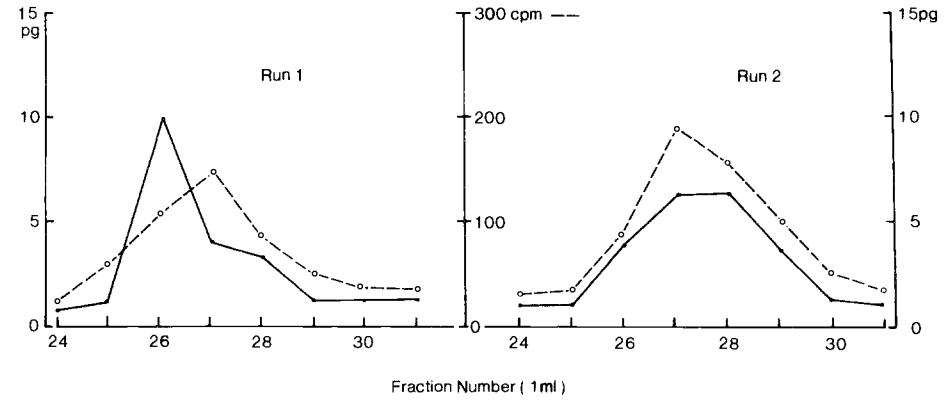


FIG. 4—*Duplicate profiles of immunoreactive* (——) *and radioactive* (------) *estradiol-17β.*

*Extraction*—Ether was the solvent of choice as it extracted in excess of 90% of $^3$H-labeled T, P, and $E_2$ that had been added to liquid blood subsequently spotted on cloth and allowed to dry. Additionally, ether removed a minimum amount of pigment from colored cloth (compared to solvents such as ethyl acetate) and was not destructive to those fabrics tested.

*Accuracy*—Known amounts of steroid were added to saline and applied to washed cotton sheeting in 0.1-mL aliquots. The cloth was allowed to air-dry and left undisturbed for three weeks, after which time the area corresponding to the 0.1-mL saline stain was extracted, chromatographed, and assayed by the appropriate radiolabeled ligand assay. As seen in Table 3, 86% of the testosterone, 94% of the progesterone, and 90% of estradiol-17$\beta$ were recovered from cloth.

The next step was to determine what effect aging of the bloodstain would have on recovery and measurement of the three steroids. From 20 mL of blood removed by venipuncture, 0.1-mL aliquots were pipetted onto cloth that was allowed to air dry; I extracted five stains immediately after the bloodstains dried. Five additional stains were extracted weekly for eight weeks. All extracts were chromatographed and then assayed simultaneously, and the data representing the zero and eight-week-old bloodstains are depicted in Table 4. There was no difference in the amount of testosterone assayed in the fresh stain at zero weeks (238 $\pm$ 4 pg) and in eight-week-old bloodstains (232 $\pm$ 16 pg). Likewise, there was no difference in either progesterone assayed at zero weeks (140 $\pm$ 5 pg) and eight weeks (154 $\pm$ 10 pg) or estradiol-17$\beta$ assayed at zero weeks (16 $\pm$ 3 pg) and eight weeks (17 $\pm$ 2 pg).

*Sensitivity and Blanks*—The lowest point on each standard curve was at 10 pg, and the blanks (clean cloth area equivalent to the size of the bloodstain) were consistently less than 10 pg.

*Precision*—The precision of steroid assays, expressed as percent coefficient of variation, is shown in Table 5. On 26 duplicate determinations performed in the same assay, the percent coefficient of variation for the ratios T/P, T/$E_2$, and P/$E_2$ were 9.0, 8.2, and 10.5, respectively. In ten duplicate determinations in two different assays, the percent coefficient of variation for the ratios T/P, T/$E_2$, and P/$E_2$ were 13.8, 16.4, and 16.0, respectively.

### *Steroid Ratios in Known Males and Females*

The results of analysis of 112 duplicate bloodstains from female and 34 duplicate bloodstains from male volunteers in this laboratory are shown in Table 6 and are expressed as the mean plus or minus the standard deviation. As noted in Table 6 the T/P ratio mean in the female was 0.37 $\pm$ 0.24 compared to 3.51 $\pm$ 1.31 for the male with a significant difference

TABLE 3—*Recovery of known amounts of steroid from cotton cloth.*

| Steroid | Added[a] | Recovered[b] | % Recovered |
|---|---|---|---|
| Testosterone | 200 | 172[c] $\pm$23[d] | 86 |
| Progesterone | 100 | 94 $\pm$17 | 94 |
| Estradiol-17$\beta$ | 50 | 45 $\pm$8 | 90 |

[a] Picograms of steroid in 0.1 mL saline placed onto cotton cloth and allowed to dry for three weeks.
[b] Picograms recovered.
[c] Values represent the mean of twelve samples.
[d] Standard deviation.

TABLE 4—*Picograms of steroid recovered from aged bloodstains.*[a]

| Weeks | Testosterone | Progesterone | Estradiol-17β |
|---|---|---|---|
| 0 | 238 | 140 | 16 |
| | ±4 | ±5 | ±3 |
| 8 | 232 | 154 | 17 |
| | ±16 | ±10 | ±2 |

[a] Each value represents the mean plus or minus the standard deviation of five bloodstains (0.1 mL each).

TABLE 5—*Precision of steroid assays.*

| | Steroid Ratios | | |
|---|---|---|---|
| | T/P | $T/E_2$ | $P/E_2$ |
| Coefficient of variation, % | | | |
| Within assay[a] | 9.0 | 8.2 | 10.5 |
| Between assay[b] | 13.8 | 16.4 | 16.0 |

[a] $n = 26$ duplicate bloodstains.
[b] $n = 10$ duplicate bloodstains.

TABLE 6—*Comparison between female and male mean ratios of steroids in bloodstains and levels of significance.*

| Steroid Ratio | Type of Samples | $n$[a] | $\bar{X}$[b] | $SD$[c] | Level of Significance[d] |
|---|---|---|---|---|---|
| | | CONTROLS[e] | | | |
| T/P | female | 112 | 0.37 | 0.24 | $P < 0.001$ |
| | male | 34 | 3.51 | 1.31 | |
| $T/E_2$ | female | 112 | 1.05 | 0.52 | $P < 0.001$ |
| | male | 34 | 5.46 | 2.84 | |
| $P/E_2$ | female | 112 | 6.64 | 7.96 | $P < 0.001$ |
| | male | 34 | 1.65 | 0.75 | |
| | | UNKNOWNS[f] | | | |
| T/P | female | 40 | 0.51 | 0.40 | $P < 0.001$ |
| | male | 36 | 4.78 | 3.73 | |
| $T/E_2$ | female | 40 | 1.53 | 1.48 | $P < 0.001$ |
| | male | 36 | 10.84 | 12.10 | |
| $P/E_2$ | female | 40 | 3.88 | 4.46 | $P < 0.05$ |
| | male | 36 | 2.23 | 1.44 | |

[a] Number of samples analyzed in duplicate.
[b] Mean.
[c] Standard deviation.
[d] Differences analyzed using one way analysis of variance.
[e] Control samples used to form data base for known males and females.
[f] Those samples identified as either male or female in blind studies.

($P < 0.001$) between the means. The $T/E_2$ ratio mean from females was 1.05 ± 0.52 compared to 5.46 ± 2.84 in the samples from males. The difference between means for the males and females was significant ($P < 0.001$). The $P/E_2$ ratio mean for the female control samples was 6.64 ± 7.96, whereas the $P/E_2$ ratio mean for males was 1.65 ± 0.75. Al-

though there was a large standard deviation for the female samples, the difference between means was significant ($P < 0.001$).

The data from each sample from an individual of known sex were then examined as if the sexual identity of the individual were unknown. This was done to establish criteria for predicting the sex of an individual from bloodstains when sexual identity is unknown.

Therefore, to determine whether a bloodstain originated from a female, an upper range for the T/P and $T/E_2$ ratios was calculated. The female upper range for T/P was, simply, the mean of the T/P ratios plus one standard deviation or $0.37 + 0.24 = 0.61$. The upper range for the female $T/E_2$ ratio was calculated in the same manner. Therefore, any $T/E_2$ value below 1.57 was considered to be from a female. The ratios for both T/P and $T/E_2$ from unknown bloodstains would have to be below the respective upper range for the unknown bloodstain to be considered female in origin.

Similarily, to determine whether a bloodstain originated from a male, a lower range for the T/P and $T/E_2$ ratios was calculated. The lower T/P range for males was the mean of the T/P ratios minus one standard deviation of $3.51 - 1.31 = 2.20$. Therefore, any T/P ratio above 2.20 was considered to be from a male. The lower range for the male $T/E_2$ ratio was calculated in the same manner ($5.46 - 2.84 = 2.62$). Therefore, any $T/E_2$ ratios above 2.62 were considered to be from a male. The ratios for both T/P and $T/E_2$ from unknown bloodstains would have to be above these respective lower ranges to be considered male in origin.

The T/P and $T/E_2$ ratios of bloodstains that were above the upper range acceptable for females but below the lower range acceptable for males were considered too atypical to be called either male or female, and the results were therefore inconclusive. Additionally, results were considered inconclusive if the T/P and $T/E_2$ ratios from duplicate analyses were not in close agreement.

The number of bloodstains from individuals whose sexual identity was known (the control group) with T/P and $T/E_2$ ratios within 1, 1.5, 2, 2.5, and 3 standard deviations of the respective ratio mean appear in Figs. 5 (T/P) and 6 ($T/E_2$). Ninety-three of the 112 bloodstains from the control females were below the T/P ratio mean plus one standard deviation (below a T/P ratio of 0.61). Virtually the same number, 92 of 112, of bloodstains were also below the $T/E_2$ ratio mean plus one standard deviation (below a $T/E_2$ ratio of 1.57) for the control female data. A total of 31 of 34 bloodstains from the control males was above both the T/P and $T/E_2$ ratio means minus one standard deviation for each (above 2.20 for T/P and 2.62 for $T/E_2$). The percentages of bloodstains categorized as originating from a female or male using this criterion (within one standard deviation) were 82 and 91, respectively. The remaining bloodstains were not assigned a sex as to origin, and the results were therefore considered inconclusive. It is evident from examination of Figs. 5 and 6 that the number and, consequently, percentage of bloodstains that could be categorized as originating from either male or female would be increased by using 1.5 or even 2 standard deviations of the ratio mean in the population studied without sacrificing accuracy.

*Blind Study*

The mean and standard deviation for the blind study samples correctly identified are also in Table 6. The T/P ratio mean for the female samples was $0.51 \pm 0.40$, and for the male samples identified it was $4.78 \pm 3.73$; difference between the means was significant ($P < 0.001$). The $T/E_2$ ratio mean for the identified female samples was $1.53 \pm 1.48$, whereas the $T/E_2$ ratio mean for the identified male samples was $10.84 \pm 12.10$ with, again, the difference between means significant ($P < 0.001$). The difference between the $P/E_2$ ratio mean female samples ($3.88 \pm 4.46$) and the $P/E_2$ ratio mean for male samples ($2.23 \pm 1.44$) was also significant ($P < 0.05$), although the standard deviation for the identified female samples was larger than the mean, as was the case in the control samples.

Once the samples were identified as male, female, or inconclusive, the prediction rate and

NUMBER OF SAMPLES WITHIN[a] OR NOT WITHIN[b] THE INDICATED STANDARD DEVIATION (SD) OF THE MEAN TESTOSTERONE/PROGESTERONE (T/P) RATIOS FOR THE APPROPRIATE SEX

| SEX | N[c] | WITHIN SD | | | | | NOT WITHIN SD | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 1.5 | 2 | 2.5 | 3 | 1 | 1.5 | 2 | 2.5 | 3 |
| | | CONTROLS | | | | | | | | | |
| ♀ | 112 | 93 | 106 | 111 | 112 | | 19 | 6 | 1 | 0 | |
| ♂ | 34 | 31 | 34 | | | | 3 | 0 | | | |
| | | BLIND STUDY SAMPLES IDENTIFIED | | | | | | | | | |
| ♀ | 40 | 34 | 37 | 39 | 39 | 39 | 6 | 3 | 1 | 1 | 1 |
| ♂ | 36 | 36 | | | | | 0 | | | | |
| | | CONTROLS PLUS BLIND SAMPLES IDENTIFIED | | | | | | | | | |
| ♀ | 152 | 127 | 143 | 150 | 151 | 151 | 25 | 9 | 2 | 1 | 1 |
| ♂ | 70 | 67 | 70 | | | | 3 | 0 | | | |
| | | ALL SAMPLES ANALYZED | | | | | | | | | |
| ♀ | 172 | 141 | 158 | 168 | 170 | 170 | 31 | 14 | 4 | 2 | 2 |
| ♂ | 88 | 81 | 88 | | | | 7 | 0 | | | |

[a] THOSE NUMBER OF SAMPLES BELOW (♀) OR ABOVE (♂) THE MEAN T/P RATIO PLUS (♀) OR MINUS (♂) THE INDICATED SD

[b] THOSE NUMBER OF SAMPLES ABOVE (♀) OR BELOW (♂) THE MEAN T/P RATIO PLUS (♀) OR MINUS (♂) THE INDICATED SD

[c] NUMBER OF SAMPLES ANALYZED IN DUPLICATE

FIG. 5—*Analysis of* T/P *results.*

accuracy rate (as percentages) were calculated by using the criteria just discussed, within one standard deviation of the T/P and $T/E_2$ ratio mean. A total of 40 of the 60 bloodstains from females were identified as female for a 66.7% prediction rate, and the remaining 20 were called inconclusive. A total of 36 of 54 bloodstains from males were identified as male for a 66.7% prediction rate, and the remaining 18 were called inconclusive. The accuracy rate for predicting the sex from bloodstains from this population was 100%.

The data were then examined to determine whether the use of one standard deviation of the mean was too conservative in predicting sex. As with the data from the control group, this set of data, the blind study, was characterized as falling within 1, 1.5, 2, 2.5, or 3 standard deviations of the mean for the T/P and $T/E_2$ ratio means. The number of blood-

NUMBER OF SAMPLES WITHIN[a] OR NOT WITHIN[b] THE INDICATED STANDARD DEVIATION (SD) OF THE MEAN TESTOSTERONE/ESTRADIOL ($T/E_2$) RATIOS FOR THE APPROPRIATE SEX

| SEX | N[c] | WITHIN SD | | | | | NOT WITHIN SD | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 1.5 | 2 | 2.5 | 3 | 1 | 1.5 | 2 | 2.5 | 3 |
| | | CONTROLS | | | | | | | | | |
| ♀ | 112 | 92 | 105 | 110 | 111 | 112 | 20 | 7 | 2 | 1 | 0 |
| ♂ | 34 | 31 | 34 | | | | 3 | 0 | | | |
| | | BLIND STUDY SAMPLES IDENTIFIED | | | | | | | | | |
| ♀ | 40 | 30 | 33 | 38 | 40 | | 10 | 7 | 2 | 0 | |
| ♂ | 36 | 36 | | | | | 0 | | | | |
| | | CONTROLS PLUS BLIND SAMPLES IDENTIFIED | | | | | | | | | |
| ♀ | 152 | 122 | 138 | 138 | 151 | 152 | 30 | 14 | 14 | 1 | 0 |
| ♂ | 70 | 67 | 70 | | | | 3 | 0 | | | |
| | | ALL SAMPLES ANALYZED | | | | | | | | | |
| ♀ | 172 | 140 | 157 | 168 | 171 | 172 | 32 | 15 | 4 | 1 | 0 |
| ♂ | 88 | 85 | 88 | | | | 3 | 0 | | | |

[a] THOSE NUMBER OF SAMPLES BELOW (♀) OR ABOVE (♂) THE MEAN $T/E_2$ RATIO PLUS (♀) OR MINUS (♂) THE INDICATED SD

[b] THOSE NUMBER OF SAMPLES ABOVE (♀) OR BELOW (♂) THE MEAN $T/E_2$ RATIO PLUS (♀) OR MINUS (♂) THE INDICATED SD

[c] NUMBER OF SAMPLES ANALYZED IN DUPLICATE

FIG. 6—*Analysis of* $T/E_2$ *results.*

stains within the standard deviations just mentioned for the T/P ratio appear in Fig. 5, whereas those for the $T/E_2$ ratio appear in Fig. 6.

Of the bloodstains identified as originating from females in the blind study and being within one standard deviation of the steroid ratio mean, 82% (33 of 40) were from the T/P ratio data in Fig. 5 and 75% (30 of 40) were from the $T/E_2$ ratio data in Fig. 6, whereas greater than 95% of the bloodstains were within two standard deviations for both the T/P and $T/E_2$ steroid ratio means. In contrast, all of the bloodstains identified as originating from males in the blind study were within one standard deviation of the steroid ratio means for both the T/P and $T/E_2$ ratios.

It is apparent from perusal of the remaining data in Figs. 5 and 6, those bloodstains identified in the blind study plus the control bloodstains and then all of the bloodstains analyzed in this study, that 80% of these bloodstains were within one standard deviation of both steroid ratio means, T/P and $T/E_2$, for both sexes. Furthermore, this population fits a normal (Gaussian) distribution as evidenced by the fact that greater than 66.7% of steroid ratios from both females and males are within one standard deviation of their respective means.

## Discussion

This report is, to my knowledge, the first one published that used radioimmunoassay of steroids as a means of determining the sex of an individual from his or her bloodstain. The first published reports of RIA techniques being used to determine sex were by Czekala and Lasley [*23*] and Bercovitz et al [*24*], who measured estrogens in bird feces. There have been, however, four presentations at Annual Meetings of the American Academy of Forensic Sciences on the measurement of steroids with RIA in an attempt to determine the sex of an individual from his or her bloodstain.

I gave two of these presentations during the early stages of development of the technique herein discussed: the first at the 28th Annual Meeting in 1976 [*10*] and the second at the 29th Annual Meeting in 1977 [*11*]. The first of the other two reports was given at the 28th Annual Meeting by R. C. Shaler et al [*25*] and included a discussion on the usefulness of predicting the sex of an individual by measuring two steroids, testosterone and estradiol, in bloodstains and reporting the results as a ratio of these two steroids. The measurement of testosterone by RIA, compared to hemoglobin concentrations in bloodstains, was discussed by F. E. Schehr at the 29th Annual Meeting [*26*] as a means of predicting an individual's sex from his or her bloodstain.

At this juncture I would like to make the first of two cautionary notes regarding predicting the sex of individuals by measuring steroids in their bloodstains. The measurement of steroids in bloodstains is technically feasible, as I have presented in the Results section of this paper. The interpretation of the data obtained by this technique as to what sex an individual would be is an entirely different matter. The testosterone, progesterone, and estradiol-17$\beta$ levels in bloodstains composing the control data were from healthy male and female volunteers in this laboratory in an age group (19 to 35 years) reflecting active gonadal steroidogenesis. This is evident in the steroid ratios in Table 6 and in Figs. 5 and 6 where the majority (90% or greater) of known males and females were within two standard deviations of the population mean.

The levels of steroids in individuals with normal and other than normal gonadal steroidogenesis are well documented [*8, 9, 27-29*]. In determining the sex of an individual for forensic science purposes, I am more confident utilizing two steroid ratios, T/P and $T/E_2$, obtained from analyzing steroids in bloodstains, rather than relying on simply the $T/E_2$ ratio, as does the method of Shaler et al [*25*]. Likewise, I am more confident in relying on the ratios of steroids when the efficiency of extraction for all three steroids is similar, as reported earlier in this paper, rather than on values derived after subjecting two unrelated compounds, testosterone and hemoglobin, to extraction methods of differing efficiency. Moreover, it is noted that levels of testosterone may be inadvertently spurious as a function of variation in blood hemoglobin concentrations resulting from differences in sex and states of health [*30*].

In point of fact the test samples analyzed were from females and males of unknown status regarding health. Since these samples were from a hospital, it is likely that not all donors were healthy, although many causes for hospitalization have no impact at all on reproductive health. In fact, despite the blind study representing ages from birth to 80 years in the female population analyzed and from 16 to 78 years in the male population, thus covering the range

of hormone levels anticipated and reported in the literature [*8, 9, 27-29*], no incorrect conclusions were made. Indeed, using one standard deviation of the mean to predict the sex for females may be too conservative.

The second cautionary note I want to make is relative to the selection of the bloodstain to be analyzed by this technique. It is anticipated that as this technique is applied to a larger number of samples than I have analyzed in this study and to samples more typical of the varied type of samples received in the forensic science laboratory, some limitations regarding the types of bloodstains selected to be analyzed will become evident. One obvious limitation is sample size. I have been analyzing bloodstains in the 400 $mm^2$ size range with reliable results but have not as yet determined the minimal sample size required for reliable results. The bloodstain must be solubilized to extract the steroids, and this extraction of steroids could be inhibited by the type of material that the bloodstain is on or the age of the bloodstain. As I discussed earlier, bloodstains aged at room temperature for eight weeks yield reliable results (Table 4), and bloodstains analyzed after storage for five years at $-30°C$ also furnish steroid levels consistent with the originating sex. The type of material could influence the levels of steroids in instances where the material previously had these steroids naturally occurring. As an example, clothing manufactured from materials that are animal in origin could have steroids in the very fiber. Since the antibody does not distinguish between progesterone originating from either a human's or sheep's gonad, the resultant progesterone levels might not truly reflect the level of progesterone in the bloodstain alone.

The capability of measuring steroids in a bloodstain by this technique and then predicting the sex of the individual that furnished the bloodstain is now available. I have based this technique on measuring three steroids found in peripheral blood that reflect the biological status of that individual, in this case the reproductive state. Measurement of additional hormones that reflect differences in the reproductive status, such as human chorionic gonadotropin in the pregnant female, will provide additional information about the individual's biological state at the time the blood was obtained and thus provide additional markers as to that individual's identity.

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