

## ON THE PRESENCE OF TESTOSTERONE IN PERIPHERAL BLOOD FROM NORMAL MEN

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SUMMARY

By criteria of repeated thin-layer chromatography and gas-liquid chromatography, ultraviolet and infrared spectrometry and isotope dilution, the presence of testosterone in peripheral blood of normal human male adults is highly probable.

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Since the isolation and identification of testosterone<sup>1</sup>, evidence has been provided that this steroid may be secreted by the human testis<sup>2,3</sup> and ovary<sup>4,5</sup>. There are also indications that testosterone might be a secretory product of the human adrenal gland<sup>6,7</sup>.

In the human, testosterone has been identified in testicular vein plasma<sup>2,3</sup>, in peripheral plasma of normal males following administration of high doses of chorionic gonadotrophin<sup>8</sup> and in urine<sup>9,10</sup>. Efforts to identify testosterone in ovarian vein plasma<sup>3,4</sup> and in peripheral plasma of normal human adults<sup>8</sup> have thus far been unsuccessful.

During the last few years several methods for the estimation of testosterone in peripheral human blood have been reported<sup>11,14</sup>. The specificity of these methods leaves little doubt that the "testosterone values" obtained for plasma of normal male adult (0.50–1.00  $\mu\text{g}/100\text{ ml}$ ) mainly represent this steroid hormone. The specificity of the "testosterone values" for plasma of adult females; 0.02–0.26  $\mu\text{g}/100\text{ ml}$  (ref. 11) 0.05–0.31  $\mu\text{g}/100\text{ ml}$  (ref. 12) and 0.033–0.10  $\mu\text{g}/100\text{ ml}$  (ref. 13) is, however, open for discussion.

Isolation and identification of testosterone from normal peripheral blood by classical chemical criteria has, however, not been published. In this report the isolation and characterization of testosterone from a pool of peripheral blood from normal male adults will be described.

Moreover, a limited number of techniques for obtaining infrared spectra of microgram amounts of substances have hitherto appeared<sup>15–19</sup>. The application of these techniques to the identification of small amounts of material isolated from biological sources has often been hampered by interfering impurities that are either

present in the isolated samples or might be introduced in preparing such samples for infrared spectroscopic analysis. One of us<sup>20</sup> has developed a technique for the incorporation of small amounts of material into micro potassium pellets; this technique may have distinct advantages over the ones previously described as far as the introduction of extraneous material (from solvent, glassware, etc.) is concerned and may give reasonable infrared spectra of pure steroids when from 0.5–1.0  $\mu\text{g}$  is incorporated in the micro pellet.

## MATERIALS AND METHODS

*Solvents* were redistilled before use. Special care had to be taken in the purification of *benzene*; this was repeatedly washed with concentrated sulfuric acid and water, followed by distillation (twice) through a 100-cm Vigreux column.

*Glassware* contacting samples for infrared spectrometry was cleaned as described previously<sup>21</sup>.

[1,2-<sup>3</sup>H] *Testosterone* (17 C/mmol) was obtained from the Radiochemical Centre (Amersham) and purified by repeated paper and thin-layer chromatography in several systems. The purity was checked by reverse isotope dilution. After mixing with pure authentic unlabeled testosterone, the mixture was taken through several purification steps (chromatography and recrystallization). The specific activity remained constant in these procedures, indicating the purity of the [1,2-<sup>3</sup>H] *testosterone*.

*Blood* was obtained from 4 normal healthy male adults (aged: 28, 31, 33 and 36 years respectively) and collected in heparinized bottles. The blood was immediately centrifuged and the plasma separated from the red cells. The plasma was processed immediately (see RESULTS).

*Thin-layer chromatography* was done on silica gel GF<sub>254</sub> (Merck, according to Stahl, containing a UV fluorescent indicator). 30 g silica gel was mixed with 60 ml distilled water and the resulting slurry was sufficient to prepare 5 plates (20 × 20 cm) of 0.3 mm thickness.

*Gas-liquid chromatography.* A Barber Colman 10 chromatograph was used for purification of plasma testosterone. The samples were chromatographed on a 6-ft. 2% SE-30 column (on Gas Chrom P, 80–100 mesh) operated at 220° with an Argon inlet pressure of 25 psi (1.8 kg/cm<sup>2</sup>). This column had been conditioned and used for a long time at a temperature high enough to avoid any bleeding of stationary phase at the temperature of operation in this experiment. The Argon ionization detector was maintained at 245°, while the temperature of the flash heater was 250°. The method for the collection and condensation of effluent gas coming off the column has been described previously<sup>22</sup>. The sensitivity setting of the Argon ionization detector in this experiment was such that 1  $\mu\text{g}$  authentic progesterone gave a peak area of 1.75 cm<sup>2</sup>.

An F and M model 400 Biomedical Analyzer was used for the quantitation of free testosterone and testosterone chloroacetate in the determinations of testosterone specific activity.

For the quantitation of free testosterone a 6-ft. 1% XE-60 column (on Gas Chrom P 80–100 mesh) was used in combination with a flame ionization detector; the column temperature was kept at 210° with the flash heater and the detector at 250°.

Pure nitrogen was used as carrier gas with an (outlet) velocity of 75 ml/min. Androstenedione was added to all samples as internal standard for correction of losses during transference onto the column and losses during gas chromatography. The sensitivity setting of the flame ionization detector was such that 0.1  $\mu$ g authentic testosterone gave a peak area of 7.1  $\text{cm}^2$ .

For the quantitation of testosterone chloroacetate a 3-ft. 1% XE-60 column (on Gas Chrom P 80-100 mesh) was used in combination with an electron capture detector; the column temperature was kept at 220° with the detector at 200° and the flash heater at 250°. Pure nitrogen was used as carrier gas with an (outlet) velocity of 75 ml/min; Argon, containing 10% methane (outlet velocity 225 ml/min), was added as a purge gas to the carrier gas stream just prior to entering the electron capture detector. The electron capture detector was operated with a pulsating potential gradient (53 V/cm) of 0.75  $\mu$ sec duration and a pulse interval of 150  $\mu$ sec.

20 $\beta$ -Hydroxypregn-4-en-3-one chloroacetate was used as an internal standard. The sensitivity setting of the electron capture detector was such that 0.01  $\mu$ g testosterone chloroacetate gave a peak area of 10.4  $\text{cm}^2$ .

*Radioactivity* was measured in a Nuclear Chicago model 725 Liquid Scintillation Spectrometer. Samples were evaporated in glass vials and dissolved in 10 ml scintillation fluid (4 g 2,5-diphenyloxazole and 40 mg 1,4-bis-2-(5-phenyloxazolyl)-benzene in 1 l toluene). Counting efficiency for  $^3\text{H}$  was approximately 35%.

*UV spectra* were measured with a Zeiss Spektral photometer using a M4Q monochromator.

*Infrared spectra* were recorded with a Perkin-Elmer model 21 infrared spectrophotometer using the Perkin-Elmer beam condensing unit in the sample beam only.

The samples were incorporated into a 0.5-mm circular potassium bromide pellet using a Perkin-Elmer micro die assembly. The 0.5-mm diameter hole in the sample disc was filled with potassium bromide (Merck, infrared quality). The sample, preferably concentrated in the tip of a small tube (2 ml conical centrifuge tube), was dissolved in 10  $\mu$ l of a not too volatile solvent (benzene, cyclohexane). The solution was transferred to a micro test tube (internal diameter: 1-2 mm; capacity: 20-50  $\mu$ l) and the volume reduced to approximately 0.1  $\mu$ l by allowing the solvent to evaporate at room temperature. The concentrated solution was transferred with a 0.5  $\mu$ l (Hamilton, model 7000.5N) syringe onto the KBr in the sample disc. After allowing the solvent to evaporate, the complete transference procedure was repeated once more. Finally the pellet holder was placed in the disc holder and the pellet was prepared in the usual way by applying vacuum to the micro disc. A detailed description of sample preparation and its incorporation into the potassium bromide pellet has been published elsewhere<sup>20</sup>.

*Extraction and preliminary purification.* The plasma pool obtained from normal adult males (total 1120 ml, *plasma I*) and a pool of 1 l blood bank plasma to which was added 10  $\mu$ g authentic testosterone (*plasma II*), were treated in the same way. The different procedures used in the present investigation for the isolation and identification of testosterone in these plasma pools are depicted in Table I. To the plasma samples were added NaOH pellets to give a final concentration of 0.5% NaOH. The alkaline plasma was extracted with 3  $\times$  2 vol. ethyl ether; the combined ether extract was washed with 0.1 vol. 0.05 N HCl and twice with 1/3 vol. distilled water. The washed ether extract was filtered over anhydrous sodium sulphate and evaporat-

ed to dryness in a rotating vacuum evaporator. To remove extraneous lipid material, the residue was dissolved in  $3 \times 10$  ml hexane and subsequently extracted with  $6 \times 10$  ml 70% methanol. Following evaporation of the combined 70% methanol extracts in a rotating vacuum evaporator, the residues appeared still rather fatty; thus the defatting procedure was repeated.

TABLE I

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FLWSHEET: IDENTIFICATION OF TESTOSTERONE IN MALE PLASMA

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Ether extraction of alkalinized plasma.
Evaporation of ether extract.
Hexane/70% methanol partition of residue.
Evaporation of 70% methanol extract.
Hexane/70% methanol partition of residue.
Evaporation of 70% methanol extract.
Thin-layer chromatography of residue in the solvent system ethylacetate-cyclohexane 1:1.
U.V. spectrometry of the extracted testosterone area.
Thin-layer chromatography in solvent system ethylacetate-cyclohexane 1:1.
U.V. spectrometry of the extracted testosterone area.
Thin-layer chromatography in solvent system benzene-ethylacetate 4:1 (plate developed twice).
U.V. spectrometry of the extracted testosterone area.
Gas chromatography on a 6-ft. 2% SE-30 column.
Collection of effluent gas which should contain testosterone.
Thin-layer chromatography in solvent system benzene-ethylacetate 4:1.
Benzene-water partition of extracted testosterone area.
Incorporation of testosterone fractions in micro potassium bromide pellets.
Infrared spectrometry of potassium bromide pellets (see: Fig. 1).
Isotope dilution with authentic [1,2- <sup>3</sup> H] testosterone (Table II).
(a) estimation of specific radioactivity of plasma testosterone following infrared spectrometry (potassium bromide pellets extracted and [1,2- <sup>3</sup> H] testosterone added to extract).
(b) estimation of specific radioactivity following thin-layer chromatography in solvent system benzene-ethylacetate 4:1.
(c) estimation of specific radioactivity after chloroacetylation of plasma testosterone and thin layer chromatography in solvent system benzene-ethylacetate 6:1.

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*Purification by thin-layer chromatography.* Following the second defatting, the residues were chromatographed on a thin-layer plate<sup>21</sup>; 5 and 10  $\mu$ g authentic testosterone were applied on separate lanes. The plate was developed in the system ethyl acetate-cyclohexane 1:1 (pure testosterone ran approximately 6 cm in this system). The plasma samples did not show a distinct UV-absorbing spot in the testosterone area, but showed UV absorption all over the length of the plate. A 2.5-cm area of the sample lane which should contain testosterone was eluted with 95% methanol. After evaporation of the methanol, the residues were rechromatographed on a thin-layer plate in the system ethyl acetate-cyclohexane 1:1. This time a more distinct UV spot, which behaved chromatographically like authentic testosterone, could be observed in the plasma samples. These areas were eluted, the eluate evaporated and the residue dissolved in vacuum distilled methanol. This solution, however, did not show any reasonable UV-absorption spectrum between 200 and 300  $m\mu$  when read against a proper silica gel blank. All samples were quantitatively recovered from the methanol solutions and applied onto a thin-layer plate. The plates were developed in benzene-ethyl acetate 4:1; pure testosterone ran 3.5 cm in this solvent system. After allowing the solvent to evaporate off the plate, the plate was redeveloped in benzene-ethyl acetate 4:1. Following this chromatography the plasma samples I and II

showed a very distinct and condensed UV spot (approximately 1 cm in diameter) at the same distance from the origin (8.5 cm) as authentic testosterone. The testosterone areas were eluted from the silica gel and the UV absorption of the residues dissolved in vacuum distilled methanol was measured against a silica gel blank. The plasma samples showed this time a distinct absorption peak at 241 m $\mu$ . Following recording of the UV spectra, the samples were concentrated in the tips of conical 2-ml centrifuge tubes.

*Purification by gas-liquid chromatography.* The residues in the tubes were dissolved in toluene and applied onto the 6-ft 2% SE-30 gas chromatography column under the conditions described. The effluent gas was collected and condensed during the time that testosterone was supposed to come off the column. From the gas chromatographic tracings it was evident that the plasma samples contained almost exclusively a substance with the retention time of authentic testosterone. In the plasma samples, however, a small peak was observed with a retention time slightly shorter than that of testosterone; this peak was not collected in the "testosterone" fractions. After evaporation of the toluene in which the effluent gas was collected<sup>22</sup>, the residues were once more chromatographed on thin-layer plates in the solvent system benzene-ethyl acetate 4:1. The plasma samples showed again a very distinct UV-absorbing spot in the testosterone area. These spots were eluted from the silica gel using a benzene-water partition: to silica gel scraped off the plate was added 1 ml benzene. After addition of 0.2 ml water and mixing, the benzene and water layers were separated by centrifugation (3000 rev./min), thus giving a benzene layer completely free of silica gel particles. The benzene layer was transferred to a 2-ml conical centrifuge tube and evaporated to dryness. This extraction procedure was repeated three times, the extracts combined, evaporated and concentrated in the tip of the small tube. The final residue was incorporated into a micro potassium bromide pellet as described.

*Isotope dilution with [1,2-<sup>3</sup>H] testosterone.* Following infrared spectrometry, the potassium bromide pellets of plasma samples I and II and 10  $\mu$ g authentic testosterone used for infrared spectrometry, were each dissolved in 1 ml distilled water and extracted with 1 ml benzene three times. The combined extracts from each sample were evaporated to dryness. To each residue was added 100000 disintegrations/min (approximately 0.00085  $\mu$ g) [1,2-<sup>3</sup>H] testosterone dissolved in 1 ml methanol. After evaporation of the methanol, 1 ml of a methanol solution containing 5  $\mu$ g androstenedione was added to each sample, the contents of the tubes were thoroughly mixed and from each sample duplicate (0.1 ml) aliquots were taken for counting of radioactivity. The remainder of the sample was evaporated to dryness, the residues dissolved in 100  $\mu$ l hexane and aliquots (1, 2 or 3  $\mu$ l) were injected onto the 6-ft. 1% XE-60 column under the conditions described. By comparison of the peak areas measured by the flame ionization detector with those of standard amounts of pure testosterone and androstenedione, the amount of testosterone in each sample was estimated. The total testosterone content of the 10  $\mu$ g reference sample, sample I and plasma sample II was 8.1, 2.8 and 4.0  $\mu$ g respectively.

The remainder of the hexane solution was evaporated to dryness and the residue subjected to thin-layer chromatography in the system benzene-ethyl acetate 4:1. The areas on the sample lanes corresponding in chromatographic mobility to authentic testosterone were scraped off the plate and extracted with 95% methanol. Following evaporation of the methanol extract, the residue was dissolved in 1 ml of a

methanol solution containing 5  $\mu\text{g}$  androstenedione. The content of the tubes was thoroughly mixed and from each sample two 0.1 ml aliquots were removed for counting of radioactivity. The remainder of the sample was evaporated to dryness, the residue dissolved in 100  $\mu\text{l}$  hexane and aliquots were injected onto the 6-ft. 1% XE-60 column to estimate the testosterone mass as described before.

The rest of the hexane solution was evaporated and dried in a vacuum desiccator. To the dried residue was added 0.5 ml of a solution of monochloroacetic anhydride in tetrahydrofuran (10 mg/ml) and 0.1 ml pyridine. Reaction was carried out overnight in a desiccator. After addition of 1 ml distilled water the chloroacetate was extracted with 1 ml ethyl acetate three times. The combined ethyl acetate extracts were washed once with 1 ml 6 *N* HCl, twice with 1 ml distilled water and subsequently evaporated to dryness<sup>14</sup>. The residue was chromatographed on thin-layer plates in the solvent system benzene-ethyl acetate 6:1. The areas on the sample lanes corresponding in chromatographic mobility to authentic testosterone chloroacetate were scraped off the plate and extracted using the benzene-water partition as described before. After evaporation of the benzene extracts, 1 ml of a freshly prepared methanol solution containing 2  $\mu\text{g}$  20 $\beta$ -hydroxypregn-4-en-3-one chloroacetate was added to each sample, the contents of the tubes were thoroughly mixed and aliquots were taken for counting of radioactivity. The remainder of the sample was evaporated to dryness, the residue dissolved in 500  $\mu\text{l}$  benzene and aliquots (1-10  $\mu\text{l}$ ) were injected onto the 3-ft. 1% XE-60 column under the conditions described, using an electron capture detector for quantitative estimation of testosterone chloroacetate<sup>14</sup>.

## RESULTS

The infrared spectra of the micro potassium bromide pellets are shown in Fig. 1 and the data on specific radioactivity of the isolated testosterone are given in Table II. The gas chromatographic tracings obtained during the mass estimation of plasma testosterone showed for all samples, besides the peaks of the internal standards, only peaks corresponding in retention times with those of pure testosterone and testosterone chloroacetate.

TABLE II

### SPECIFIC ACTIVITY OF TESTOSTERONE

Following infrared spectrometry the KBr pellet was extracted and 100000 disintegrations/min [<sup>3</sup>H] testosterone was added to the extract. Specific activities were measured as described in the text of this paper. All data are given as disintegrations/min <sup>3</sup>H  $\cdot$  10<sup>3</sup>/ $\mu\text{mol}$  of steroid.

Purification process	Testosterone standard (10 $\mu\text{g}$ )	Plasma II	Plasma I
After infrared spectrometry and before thin-layer chromatography	302	602	866
After thin-layer chromatography as the free compound	327	587	867
After chloroacetylation and thin-layer chromatography	299	595	851

## DISCUSSION

The main aim of this work was, on the one hand, to investigate the possibilities of the described technique to obtain infrared spectra of microgram amounts of

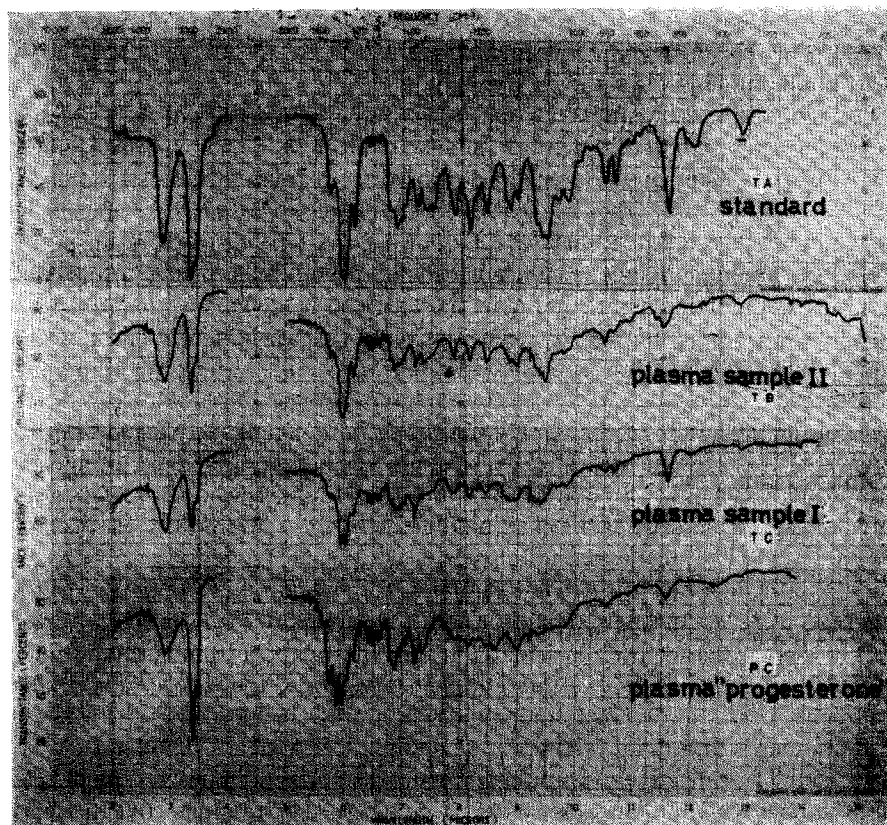


Fig. 1. Infrared spectra of: TA: 10  $\mu$ g authentic testosterone; TB: residue of plasma pool II (10  $\mu$ g testosterone added prior to extraction and purification); TC: residue of plasma pool I (peripheral male plasma); PC: residue of a plasma pool believed to contain progesterone.

steroids isolated from biological sources and, on the other hand, to obtain infrared data that might establish the presence of testosterone in male peripheral blood. The entire purification of the plasma sample is best visualized in light of this objective.

In preliminary experiments<sup>21</sup> we observed that final purification of a "plasma" steroid by gas-liquid chromatography alone does not result in fractions clean enough to obtain adequate spectra of small amounts of steroids. Though infrared spectra obtained from the residue of pure solvents taken through the method for testosterone isolation showed almost negligible "background" absorption, the spectra obtained from gas chromatography effluent fractions of extensively purified plasma steroids exhibited broad diffuse absorption bands that made comparison of such spectra with those of standard substances impossible. In order to avoid these impurities we have found that the additional purification of the condensed gas from the gas chromatographic column by thin-layer chromatography and by a water-benzene partition were most useful.

Even while the infrared spectra do not exhibit strong absorption peaks, it is remarkable how little unspecific absorptions the background of the spectra show. This is in great contrast with the blank spectra obtained from freeze-dried or lyophilized

potassium bromide powder<sup>15,16</sup>; these spectra always show a more or less pronounced diffuse absorption between 7 and 10  $\mu$  making the use of this technique difficult when applied to small amounts of unknown compounds.

The negligible background of our spectra is probably the result of the technique used for the incorporation of samples into potassium bromide. The unspecific infrared absorptions in potassium bromide pellets prepared from freeze-dried or lyophilized KBr<sup>16,17</sup> presumably result from impurities from the glassware or the atmosphere, adhering to the highly active absorbing KBr powder. As a result of these unspecific absorptions the amount of substance needed in the pellets to recognize an infrared spectrum of a well defined compound is in the order of 5  $\mu$ g. This we feel has been overcome in the present method by using untreated potassium bromide powder. With the present technique we have obtained distinguishable spectra with amounts of steroids as small as 0.5–1.0  $\mu$ g. Losses due to transference and handling limit the sensitivity of the technique to 2–3  $\mu$ g. During the purification of the plasma extracts the presence of testosterone was already strongly suggested by criteria of specific retention during gas-liquid chromatography and by UV absorption. By comparison of the infrared spectrum (TC) of the compound isolated from plasma I (male plasma) with the spectrum (TB) of the extract of plasma II (plasma + added testosterone), it appears that the over-all pictures are similar and show many of the details of the spectrum (TA) of 10  $\mu$ g authentic testosterone. If we compare for example the spectrum (PC) of a progesterone fraction isolated from a plasma pool from normal women with that (TC) of the testosterone fraction of male plasma, there is no doubt that these represent spectra of different compounds. From the comparison of the infrared spectra of standards and unknowns it is our opinion that plasma samples I and II (spectra TC and TB respectively) contain testosterone. It would, however, have been impossible to uncover an unknown structure from these spectra alone. In retrospect it might be possible to assign certain absorption peaks in these spectra to characteristic functional groups, but trying to do so without any additional information about the compound under investigation would still have lead to erroneous conclusions due to small amounts of impurities present (*cf.* spectra TA, TC and TB). The existing methods of micro infrared analysis are therefore of limited value for identifying steroids in small amounts.

The accuracy of the estimation of specific activities (Table II) using gas-liquid chromatography for mass estimation of testosterone is approximately 5%. The specific activity of the isolated plasma testosterone changed little following repeated purification by different physical methods. Such data provide additional evidence that the plasma compound from which the infrared spectrum was obtained, was testosterone. Moreover, the observation that the gas chromatographic tracings of all samples of the male plasma pool (sample I), besides the peak of the internal standards, only exhibited peaks with the retention time of pure testosterone and testosterone chloroacetate adds strength to this conclusion.

#### REFERENCES

- 1 K. DAVID, E. DINGEMANSE, J. FREUD AND E. LAQUEUR, *Z. Physiol. Chem.*, 233 (1935) 218.
- 2 W. M. LUCAS, W. F. WHITMORE, JR. AND C. D. WEST, *J. Clin. Endocrinol. Metab.*, 17 (1957) 465.
- 3 N. HOLLANDER AND W. P. HOLLANDER, *J. Clin. Endocrinol. Metab.*, 18 (1958) 966.
- 4 V. B. MAHESH, R. B. GREENBLATT, C. K. AYDAR AND S. ROY, *Fertility Sterility* 13 (1962) 513.



- 5 G. MIKHAIL, J. ZANDER AND W. M. ALLEN, *J. Clin. Endocrinol. Metab.*, 23 (1963) 1267.
- 6 H. G. BURGER, J. B. KENT AND A. E. KELLIE, *J. Clin. Endocrinol. Metab.*, 24 (1964) 432.
- 7 R. L. VAN DE WIELE, P. C. MACDONALD, E. BOLTÉ AND S. LIEBERMAN, *J. Clin. Endocrinol. Metab.*, 22 (1962) 1207.
- 8 G. W. OERTEL AND K. B. EIK-NES, *Proc. Soc. Exptl. Biol. Med.*, 102 (1959) 553.
- 9 K. SCHUBERT AND K. WEHRBERGER, *Naturwissenschaften*, 12 (1960) 280.
- 10 A. M. CAMACHO AND C. J. MIGEON, *J. Clin. Endocrinol. Metab.*, 23 (1963) 301.
- 11 M. FINKELSTEIN, E. FORCHIELLI AND R. I. DORFMAN, *J. Clin. Endocrinol. Metab.*, 21 (1961) 98.
- 12 B. HUDSON, J. COGHLAN, A. DULMANIS, M. WINTOUR AND I. EKKEL, *Australian J. Exptl. Biol. Med. Sci.*, 41 (1963) 235.
- 13 A. RIONDEL, J. F. TAIT, M. GUT, S. A. S. TAIT, E. JOACHIM AND B. LITTLE, *J. Clin. Endocrinol. Metab.*, 23 (1963) 620.
- 14 A. C. BROWNIE, H. J. VAN DER MOLEN, E. E. NISHIZAWA AND K. B. EIK-NES, *J. Clin. Endocrinol. Metab.*, 24 (1964) 1091.
- 15 W. B. MASON, *Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy*, March, 1958.
- 16 W. B. MASON, *Anal. Chem.*, 34 (1962) 342.
- 17 H. P. SCHWARZ, R. C. CHILDS, L. DREISDACH, S. V. MASTRANGELO AND A. KLESCHICK, *Appl. Spectry*, 12 (1958) 35.
- 18 M. SPARAGANA AND W. B. MASON, *Steroids*, 2 (1963) 245.
- 19 H. J. VAN DER MOLEN, *Ph. D. Thesis*, University of Utrecht, 1961.
- 20 J. H. VAN DER MAAS, *Ph. D. Thesis*, University of Utrecht, 1965.
- 21 H. J. VAN DER MOLEN, B. RÜNNEBAUM, E. E. NISHIZAWA, E. KRISTENSEN, T. KIRSCHBAUM, W. G. WIEST AND K. B. EIK-NES, *J. Clin. Endocrinol. Metab.*, 25 (1965) 170.
- 22 E. E. NISHIZAWA AND K. B. EIK-NES, *Biochim. Biophys. Acta*, 86 (1964) 810.