*Journal of Steroid Biochemistry*, 1975, Vol. 6, pp. 1151–1155. Pergamon Press. Printed in Great Britain

# IODINE LABELLED LIGANDS IN THE RADIOIMMUNOASSAY OF SYNTHETIC PROGESTINS

R. J. WARREN and K. FOTHERBY

Royal Postgraduate Medical School, Ducane Road, London, W.12, England

(*Received* 2 *September* 1974)

SUMMARY

Labelled ligands with specific activities of 120–160 Ci/mmol were produced by iodination of the tyrosine methyl esters of norgestrel and norethisterone 3-oximes. The syn and anti isomers of the steroid oxime tyrosine methyl esters could be separated and interacted with an antiserum raised against the steroid 3-carboxymethyl oxime to a similar extent. Some diiodinated steroid tyrosine methyl ester was also produced and this also interacted with the antiserum to the same extent as the monoiodinated derivatives. The specificity of the interaction with the antiserum depends on the labelled ligand used. Greater specificity was obtained with a [$^3$H]-ligand than with a [$^{125}$I]-ligand but use of the latter enabled the antiserum to be used for measuring metabolites of norgestrel and norethisterone. Plasma extracts after, but not before, chromatography on Sephadex gave inhibition curves parallel to those of the standard.

## INTRODUCTION

The use of iodine labelled ligands in the radioimmunoassay of steroids has been described by Midgley *et al.*[1, 2] and by Jeffcoate *et al.*[3]. Labelling of either the protein moeity of the steroid-protein conjugate or the aromatic ring of conjugates of the steroid with tyrosine or histamine have been employed. As pointed out by the above investigators, the advantages of the high specific activity ligands obtained by iodine labelling are the higher sensitivities that may be achieved, the speed and precision of counting and the use of more dilute antisera. One other major advantage of iodine labelling is that it enables radioimmunoassays to be developed for compounds for which no tritiated preparation with a suitably high specific activity for use as labelled ligand exists. Thus 3-O-succinyl digitoxigenin tyrosine methyl ester labelled with $^{125}$I was used as labelled ligand in a radioimmunoassay for digitoxin [4]. Recently Warren and Fotherby[5] used the iodinated tyrosine methyl esters (TME) of the 3-(O-carboxymethyl) oximes of norgestrel (DL-13$\beta$-ethyl-17$\alpha$-ethynyl-17$\beta$-hydroxygon-4-en-3-one) and norethisterone (17$\alpha$-ethynyl-19-nortestosterone) for the development of radioimmunoassays for these synthetic progestogens. The present paper elaborates on some parts of the methodology, particularly the iodination, which have recently been further studied.

## MATERIALS AND METHODS

The raising of antisera against norgestrel and norethisterone and the radioimmunoassay of these progestogens has been described [5]. The [$^3$H]-norethisterone had a S.A. of 20 Ci/mmol. Norethisterone-17$\beta$-hemisuccinate was prepared as described by Thorneycroft *et al.*[6] and coupled to BSA [7]. Analysis showed the presence of only 12 molecules of norethisterone per molecule of BSA. The antigen was injected into female New Zealand white rabbits as described previously [5] and after six months one of two rabbits injected showed an antibody titre which gave 50% binding of [$^3$H]-norethisterone at a dilution of 1:6000.

Thin-layer chromatography was carried out on aluminium-backed silica gel plates GF254 (Merck) and developed in the solvent system toluene–ethyl acetate (1:2, v/v). Sephadex LH 20 was preswollen in benzene–methanol (95:5, v/v) and columns (approximately 10 × 0·4 cm) packed in benzene–methanol solution. Elution of the steroid was controlled by using the dye azobenzene [8]. Norgestrel was eluted in the 1 ml fraction immediately after the elution of azobenzene and tetrahydronorgestrel in the next 2 ml fraction. Norethisterone was eluted in the fraction 0·5–1·5 ml after elution of the dye and tetrahydronorethisterone in the following 2 ml fraction. The flow rate of the columns was about 8 ml/h.

The relative amounts of mono- and di-iodinated derivatives formed on iodination of the steroid tyrosine methyl esters were determined as described by Massaglia *et al.*[9]. Preparation of the steroid 3-(O-carboxymethyl) oxime tyrosine methyl esters and their iodination were carried out as described by Warren and Fotherby[5].

## RESULTS

### *Iodination of steroid tyrosine methyl esters*

During preparation of the steroid oximes, two isomers, syn and anti, are produced. As shown by Ismail and Love[10] for testosterone, the tyrosine methyl esters of the two isomers can be separated by thin-layer chromatography. After iodination of the

methyl esters [11] and chromatography of the reaction mixture on silica gel G, the isomeric iodinated tyrosine methyl esters could be separated from each other and from chloramine T and free iodide [5]. The $R_F$ values for the iodinated isomers of norgestrel-3(-O-carboxymethyl) oxime TME were 0·34 and 0·38 and of norethisterone-3(-O-carboxymethyl) oxime TME 0·27 and 0·33. The labelled esters were eluted with methanol from the thin-layer plates with a recovery of about 95%.

In preliminary experiments the amount of $^{125}$I incorporated into the isomeric steroid tyrosine methyl esters increased up to a reaction time of about 2·5 min. The amount of radioactivity incorporated into the two isomers varied from occasion to occasion even with a constant reaction time of 2·5 min. However the amount of label incorporated into the less polar isomer was always greater that that incorporated into the more polar one, suggesting that the less polar isomer was more readily iodinated or that in forming the oximes the two isomers were not produced in equal amounts. The latter possibility seemed more likely to be correct. If it is assumed that the molar extinction coefficients of the two isomers are similar, the ratio of more polar:less polar isomer as determined from U.V. spectra was 2:3 and if this mass difference is taken into account the specific activities of the two isomers are similar. For five separate labellings of norgestrel oxime TME the mean (±S.D.) S.A. of the more polar isomer was 120 ± 13 Ci/mmol and that of the less polar isomer was 124 ± 23 Ci/mmol. For seven separate labellings of norethisterone oxime TME, the specific activity of the more polar isomer was 156 ± 33 Ci/mmol and for the less polar isomer 153 ± 17 Ci/mmol.

In further experiments the iodination reaction mixture using norgestrel oxime TME was examined by t.l.c. and scanning the chromatograms for radioactivity. Four areas were detected which contained $^{125}$I; one at the origin corresponded to free $^{125}$I, a second ($R_F$ 0·32–0·42) included the isomeric steroid oxime tyrosine methyl esters, a third had a similar $R_F$ value (0·46–0·50) to chloramine T while the fourth was less polar than chloramine T ($R_F$ 0·6–0·8). The amount of radioactivity incorporated into each of these areas after varying times of iodination is shown in Table 1. The amount of iodine incorporated into the steroid tyrosine methyl esters appeared to be maximum between 1·5 and 2·25 min.

During iodination of oestrogens [3] a large amount of iodine is incorporated into the phenolic ring but little if any substitution into the nucleus of neutral steroids occurred. Under our conditions of iodination Warren and Fotherby[5] no significant amount of $^{125}$I was incorporated into norethisterone or norgestrel themselves so that no iodination of the 17α-ethynyl group occurred.

*Interaction of iodinated products with antiserum*

To determine whether one isomer was superior to the other for use as labelled ligand, their interaction and that of the other iodinated products, with the antiserum was investigated. When solutions of the two labelled isomers of norgestrel oxime TME were used with equal counting rates (11,000 c.p.m.) with the antiserum against norgestrel at a dilution of 1:28,000, standard curves over the 0–200 pg range were parallel (Fig. 1). In the labelling of this particular batch of norgestrel oxime TME ester, 170 μCi of iodine were incorporated into the less polar isomer and a 100 μCi into the more polar isomer. With no unlabelled steroid added to the assay tubes, 65% of the less polar isomer was bound compared with 50% of the more polar isomer. The labelled material less polar than chloramine T after elution from thin-layer chromatograms did not interact with the antiserum although the labelled material with an $R_F$ value of 0·46–0·50 gave a curve similar to those obtained with the labelled norgestrel oxime tyrosine methyl esters. This latter finding suggested the possibility that both mono- and di-iodinated norgestrel oxime TME might have been produced during the labelling procedure. Accordingly each of the labelled areas eluted from the t.l.c. was hydrolysed [9] and the iodinated tyro-

Table 1. Variation of incorporation of $^{125}$I into different fractions of reaction mixture with time

| Time of iodination (seconds) | % Radioactivity found in each fraction of chromatogram | | | |
|---|---|---|---|---|
| | $R_F$ 0 | $R_F$ 0·32–0·42 | $R_F$ 0·46–0·50 | $R_F$ 0·6–0·8 |
| 15 | 25 | 27 | 28 | 20 |
| 30 | 29 | 29 | 28 | 14 |
| 50 | 34 | 30 | 24 | 12 |
| 70 | 29 | 30 | 27 | 14 |
| 90 | 30 | 36 | 23 | 11 |
| 135 | 31 | 34 | 25 | 10 |
| 165 | 30 | 30 | 30 | 10 |

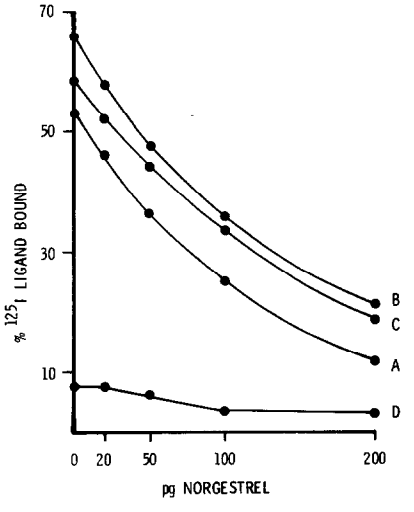


Fig. 1. Binding of different $^{125}$I labelled fractions from thin-layer chromatogram with Rivanol-treated norgestrel antiserum at a dilution of 1:28,000. Curve A, more polar isomer of norgestrel oxime TME; curve B, less polar isomer; curve C, fraction with $R_F$ 0·46–0·50 subsequently shown to be the di-iodo derivative of norgestrel oxime TME; curve D, fraction with $R_F$ 0·6–0·8.

Table 2. Cross-section of various steroids with antisera against norethisterone using [$^3$H]-norethisterone or [$^{125}$I]-norethisterone as labelled ligand

| Steroid tested | % Relative activity Net-3-oxime antiserum [$^{125}$I]-Net | [$^3$H]-Net | Net-17-succinate antiserum [$^3$H]-Net |
|---|---|---|---|
| Norethisterone (Net) | 100 | 100 | 100 |
| Net-3-oxime | 110 | 100 | 0·1 |
| Net-17$\beta$-succinate | 0·02 | 0·02 | 105 |
| 3$\alpha$,5$\beta$-TetrahydroNet | 103 | 35 | 0·2 |
| 5$\beta$-DihydroNet | 100 | 55 | 31 |
| Net acetate | 100 | 34 | 30 |
| Net oenanthate | 5·1 | 9·1 | 22 |
| Ethynyloestradiol | 8 | 1·3 | 0·3 |
| Testosterone | 0·2 | 0·001 | 0·2 |
| Oestradiol | 0·01 | <0·001 | <0·01 |
| Cortisol | 0·001 | <0·001 | <0·01 |
| DHA | 0·01 | <0·001 | <0·01 |

$$\% \text{ Relative activity} = \frac{\text{pg of norethisterone to produce 50\% binding} \times 100}{\text{pg of steroid under test to produce 50\% binding}}$$

sine released was examined by paper chromatography. From the areas of the t.l.c. containing the more and less polar isomers of norgestrel oxime TME only mono-iodotyrosine was detected. From the area with an $R_F$ of 0·46–0·50 only di-iodotyrosine was observed and from the less polar area neither mono- nor di-iodotyrosine was produced.

*Specificity of interaction with antisera*

The ability of steroids closely related to norethisterone and of some other steroids commonly found in blood to displace the $^{125}$I or $^3$H ligands from antisera raised against norethisterone-3-oxime or norethisterone-17$\beta$-hemisuccinate are shown in Table 2. The interaction of naturally occurring steroids not containing a 17$\alpha$-ethynyl group with either antisera irrespective of the labelled ligand used was negligible. For the remainder of the steroids tested there appeared to be a much greater degree of cross-reaction with the $^{125}$I ligand than with the $^3$H ligand. Using the antiserum raised against norethisterone-3-oxime and the $^{125}$I ligand, norethisterone acetate and the 5$\beta$-dihydro and 5$\beta$-tetrahydro derivatives of norethisterone interacted to a similar extent to norethisterone and norethisterone-3-oxime themselves. Using [$^3$H]-norethisterone as ligand, norethisterone-3-oxime again interacted to an equal extent as norethisterone but the interaction of dihydro- and tetrahydronorethisterone and norethisterone acetate was greatly reduced. Using the antisera to norethisterone-17$\beta$-succinate and [$^3$H]-norethisterone, both norethisterone and norethisterone-17$\beta$-succinate interacted to the same extent and significant interaction was found with norethisterone acetate and oenanthate as well as with dihydronorethisterone. Further reduction to tetrahydronorethisterone eliminated completely the cross-section.

*Binding of iodine labelled and tritium labelled ligands to antiserum*

Values for the binding of the iodinated or tritium labelled ligands with varying dilutions of the antiserum against norethisterone are shown in Fig. 2. Both for the untreated and the Rivanol-treated antisera, 50% binding of ligand was achieved at higher dilutions of antiserum using the iodine labelled then

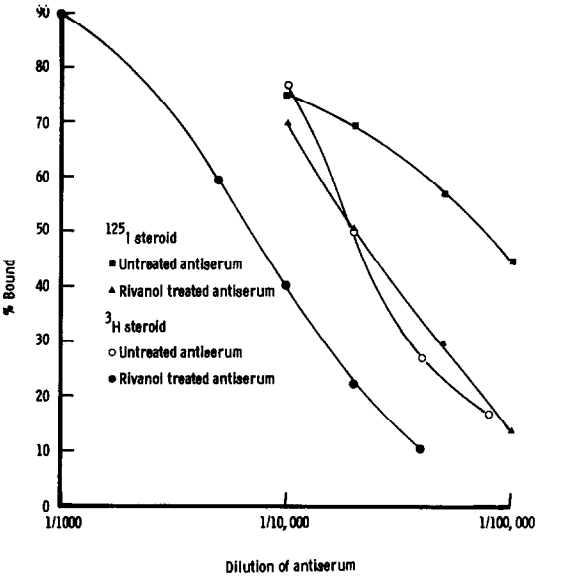


Fig. 2. Dilution curve for antiserum before and after Rivanol treatment with $^{125}$I norethisterone oxime TME (■——■, untreated antiserum; ▲——▲, Rivanol treated antiserum) or [$^3$H]-norethisterone (○——○, untreated antiserum ●——●, Rivanol treated antiserum).

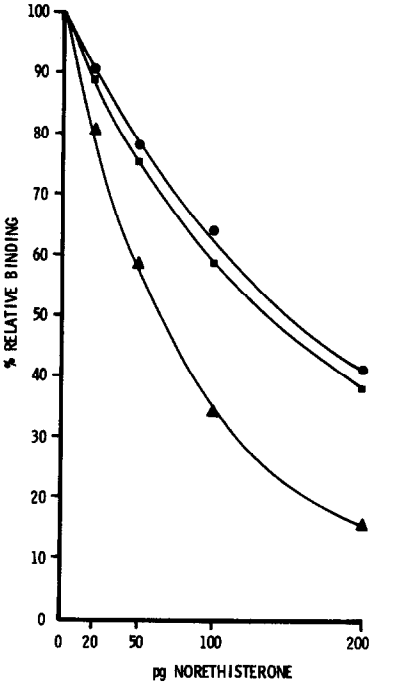


Fig. 3. Binding of labelled ligands with Rivanol-treated norethisterone antiserum. ▲——▲ $^{125}$I norethisterone oxime TME, S.A. 150 Ci/mmol, antiserum dilution 1:16,000; ●——● $^{125}$I norethisterone oxime TME, S.A. 20 Ci/mmol, antiserum dilution 1:5000; ■——■ [$^3$H]-norethisterone, S.A. 20 Ci/mmol, antiserum dilution 1:6000.

the tritium labelled steroid; for Rivanol-treated antisera the dilutions were 1:20,000 and 1:7000 respectively. The effect on sensitivity is shown in Fig. 3. With dilutions of antisera similar to those required for 50% binding, the amount of unlabelled norethisterone required to reduce the relative binding of the iodine-labelled ligand to 50% was 60 pg whereas twice as much norethisterone (125 pg) was required to decrease the relative binding of the tritium-labelled ligand by this amount. These differences are almost entirely due to differences in the S.A. of the labelled ligand. When the iodinated ligand was diluted with unlabelled ligand to achieve the same S.A. as the [$^3$H]-norethisterone, identical binding curves were obtained (Fig. 3).

*Loss of labelled steroid in polythene tubes*

Some labelled ligand may be lost by adhering to the polythene tubes used in the assay. Assay tubes were set up containing the labelled ligand but without the addition of antiserum and carried through the incubation procedure; the solution was removed from the tubes which were then washed twice with 1 ml water. For 5 samples investigated 31% of the radioactivity originally added to the tube remained in the tube after the washing process. Only part of the label adhering to the tube could be removed by the addition of Dextran-charcoal solution. If after incubation, the tubes were treated with Dextran-charcoal solution as in the assay, the contents of the tube removed and the tube washed once with 1 ml water, even under these conditions 18% of the radioactivity originally added to the tube remained behind. The amount of labelled ligand adhering to the tubes was reduced when antiserum was also added prior to incubation. Under these conditions removal of the supernatant after incubation left only 21% of the original label in the tube and after treatment with Dextran-charcoal, a further 6% of the radioactivity was removed from the tube. Addition of charcoal therefore does remove small amounts of radioactivity adhering to the tube. These results emphasize that re-use of the tubes should be avoided. In establishing dilution curves the maximum binding of $^{125}$I ligand to the antiserum varied from 80–85% and this may be partially accounted for by some of the labelled ligand adhering to the tube. Since the assays are carried out in a liquid phase system and always under the same conditions the amount of labelled ligand sticking to the tube should remain constant and will not affect the assay.

*Blank values*

Warren and Fotherby[5] found the blank values when 1 ml plasma from male or female subjects not receiving either norgestrel or norethisterone was processed corresponded to a mean value of 5·3 pg for the norgestrel assay and 8·0 pg for the norethisterone assay and only rarely did the blank values exceed 20 pg. Although valid results can be obtained by the direct assay of extracts of plasma, in practice in order to eliminate interference from contaminating substances and also to ensure separation of the biologically active progestogens from their metabolites, extracts of plasma are chromatographed on Sephadex LH 20. The effect of such chromatography on standard curves is shown in Fig. 4. There was no significant difference between binding of the standard alone and the standard to which samples of a chromatographed plasma extract were added.

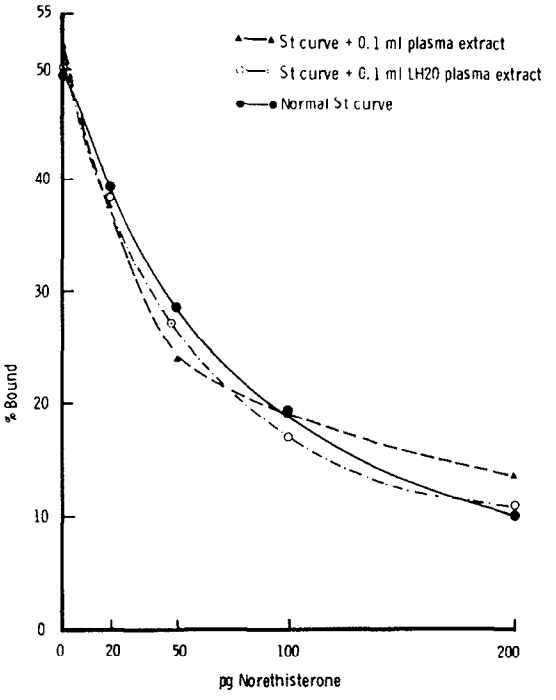


Fig. 4. Effect of plasma extracts on standard curve of norethisterone. ●—●, Normal standard curve; ▲—▲, standard curve after addition of extract of 0·1 ml plasma to each assay tube; ○—○, standard curve after addition of column fraction from Sephadex LH 20 chromatography of extract of 0·1 ml plasma.

## DISCUSSION

Some general points concerning the use of iodine-labelled ligands have been considered by Hunter[12] and such ligands have been shown to be useful in the radioimmunoassay of steroids [1, 3, 5, 13, 14]. Although the tyrosine methyl esters of the steroids have been used for labelling, little investigation of the iodination of these derivatives has been carried out. Ismail and Love[10] who described the separation of the *cis* and *trans* isomers of testosterone-3-oxime-tyrosine methyl ester found that there was no difference in the antiserum specificity between the two isomers. This is confirmed by our results. Although the two isomers of the steroid oxime may not be formed in equal amounts their tyrosine methyl esters appear to be iodinated to the same extent as shown by the similarity in S.A. There is however a slight difference in their extent of interaction with the antiserum, possibly as a result of a different titre of antibodies in the antiserum to the isomeric antigens. Under the same conditions, the antiserum against nor-

gestrel bound 50% of the more polar isomer compared with 65% of the less polar one. Apart from this difference in the relative binding of the two isomers, the values found for the affinity constant of the two isomers, calculated from Scatchard plots of values obtained from standard curves, were similar ($4{\cdot}3 \times 10^9$ l/mol for the less polar isomer and $3{\cdot}8 \times 10^9$ l/mol for the more polar isomer). These values are similar to the values for iodine-labelled conjugates reported by Midgley *et al.*[2] and Cameron *et al.*[15]. However the values reported by these investigators refer to untreated antiserum whereas our values relate to Rivanol-treated antiserum. It is obvious from binding curves described previously [5] that untreated antiserum will bind the ligand more avidly than treated antiserum, the avidity for untreated antiserum being so great that the standard curves obtained are much less sensitive than when treated antiserum is used.

Preparations of the iodinated steroid oxime tyrosine methyl esters can be produced with specific activities of 120–150 Ci/mmol. Although these values are lower than those which can be obtained by iodination of steroid-protein conjugates, they are higher than the S.A. obtained by incorporation of four atoms of tritium into the steroid molecule. Our results agree with previous ones [2] that inhibition curves obtained with iodinated steroid oxime tyrosine methyl esters are more sensitive than those obtained with tritiated steroids and that the greater sensitivity obtained with the iodinated tyrosine methyl esters is due to the high S.A. of these compounds.

Our investigations show that the iodination of the tyrosine methyl esters is a rapid reaction and that increasing the reaction time beyond $2\frac{1}{2}$ min does not lead to a greater incorporation of iodine into the esters. Calculation shows that under our conditions only about 0·05 mol of radioiodine is incorporated per mol of steroid oxime TME; a value similar to that found by Midgley *et al.*[2]. Thus there is plenty of scope for increasing the amount of $^{125}$I incorporated per molecule and producing a much higher S.A. ligand. Some di-iodinated derivative of the steroid oxime TME is produced during iodination and this seems to interact with the antiserum to a similar extent as the mono-iodinated esters. Small amounts of the di-iodinated form were also found by Massaglia *et al.*[90] under their conditions of iodination and they found the di-iodinated derivatives to be more unstable than the mono-iodinated ones.

The steroid oxime tyrosine methyl esters appear to be stable in the solid state and in our experience are also stable when stored in methanol solution at 4°C for at least 6 months. After this time, some deterioration may occur and on occasions the antiserum dilution required for 50% binding falls considerably. This fall in antibody titre occurs with untreated antiserum as well as with the rivanol-treated antiserum and that purified by precipitation with ammonium sulphate. In these instances, when some of the original tyrosine methyl ester stored in the solid state has been iodinated and tested, the original titre of antisera for 50% binding was obtained. The nature of this change in the tyrosine methyl ester is not known. Chromatography of the individual $^{125}$I labelled isomers prepared from the steroid oxime TME stored in solution for 6 months showed only one major radioactive peak corresponding with the authentic isomer and there was no indication of free iodine being liberated. With labelled ligands prepared from steroid oxime TME which had shown deterioration, there was a marked increase in the sensitivity of the standard curve but such standard curves were unsuitable for assay purposes since the blank value increased considerably and the working range of the standard curve was markedly reduced. The effective life of the iodinated steroid oxime TME stored in methanol at 4°C was about eight weeks.

It is of interest that, as reported by others [3, 14], the degree of cross-reaction for any particular antiserum varies with the labelled ligand which is used. In general, the specificity appears to be lower when $^{125}$I labelled ligands are used compared to tritiated ligands. This has also been shown to be true for the assay of cortisol using a competitive protein-binding method [16]. The lower specificity obtained with an iodine-labelled ligand may not be a disadvantage since it provides, as in the case of the synthetic progestins studied here, an easy way of measuring metabolites of the biologically active steroids.

*Acknowledgements*—This work was supported by a grant from the Medical Research Council. [$^3$H]-Norethisterone was a gift from Dr. E. H. Cameron, Cardiff.

## REFERENCES

1. Midgley A. R., Niswender G. D. and Ram J. S.: *Steroids* **13** (1969) 731–737.
2. Midgley A. R., Niswender G. D., Gay V. L. and Reichert L. E.: *Recent Prog. Horm. Res.* **27** (1971) 235–301.
3. Jeffcoate S. L., Gilby E. D. and Edwards R.: *Clin. chim. Acta* **43** (1973) 343–349.
4. Oliver G. C., Parker B. M., Brasfield D. L. and Parker C. W.: *J. clin. Invest.* **47** (1968) 1035–1042.
5. Warren R. J. and Fotherby K.: *J. Endocr.* **62** (1974) 605–618.
6. Thorneycroft I. H. and Stone S. C.: *Contraception* **5** (1972) 129–146.
7. Erlanger B. F.: In *Methods in Immunology and Immunochemistry* (Edited by Williams C. A. and Chase M. W.). Academic Press, New York (1967) pp. 144–150.
8. Collins W. P., Mansfield M. D., Alladina N. S. and Sommerville I. F.: *J. steroid Biochem.* **3** (1972) 333–348.
9. Massaglia A., Barbieri U., Siri-Upathum C. and Vitali R.: *Int. J. appl. Radiat. Isotopes* **24** (1973) 455–462.
10. Ismail A. A. A. and Love D. N.: *J. Endocr.* **55** (1972) xiii.
11. Hunter W. M. and Greenwood F. C.: *Nature* **194** (1962) 495–496.
12. Hunter W. M.: *Brit. med. Bull.* **30** (1974) 18–23.
13. Gilby E. D., Jeffcoate S. L. and Edwards R.: *J. Endocr.* **58** (1973) xx.
14. Cameron E. H. D., Morris S. E., Scarisbrick J. J. and Hillier S. G.: *Biochem. Soc. Trans.* **1** (1973) 1115–1117.
15. Cameron E. H. D., Morris S. E. and Nieuweboer B.: *J. Endocr.* **61** (1974) xxix–xxxx.
16. Malvano R., Dotti C. and Grosso P.: *Clin. chim. Acta* **47** (1973) 167–173.