Radio-enzymic Assay of Oxo Steroids by their Reduction with the Tritiated Form of Reduced Nicotinamide-Adenine Dinucleotide

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A method of analysis is proposed based on the enzyme-catalysed transfer of tritium from [^3H]NADH to suitable substrates. Its practicability is demonstrated on the examples of oestrone and progesterone with the respective use of the $3\beta,17\beta$ - and $3\alpha,20\beta$ -hydroxy-steroid oxidoreductase. Specificity is tested by application to the analysis of the plasma of pregnant women and measurement of the $^3\text{H}/^{14}\text{C}$ ratios on purification of the enzymic reduction products.

In the reversible enzyme-catalysed oxidation of alcohols by NAD^+ the spectrophotometric measurement of the change in the concentration of NADH (Warburg & Christian, 1936) and the fluorimetric measurement of NAD^+ (Lowry *et al.*, 1957) form the basis for the enzymic determination of ketones and alcohols. In the steroid field, Hurlock & Talalay (1956, 1957) used the 3α -hydroxy steroid oxidoreductase (EC 1.1.1.50) from *Pseudomonas testosteroni* to assay 3α -ols and 3-ones of the androstane and pregnane series and the $3\beta,17\beta$ -hydroxy steroid oxidoreductase (EC 1.1.1.51) from the same source to assay 3β - and 17β -ols and the corresponding ketones of the androstane series. Hübener's 20β -hydroxy steroid oxidoreductase (EC 1.1.1.53) from *Streptomyces hydrogenans* (Hübener & Lehmann, 1958; Hübener *et al.*, 1959; Hübener & Sahrholz, 1960), later shown to catalyse also the interconversion of 3α -ols and 3-ones (Pocklington & Jeffery, 1967; Gibb & Jeffery, 1972, 1973a), has been used to assay 20-oxo- and 20β -hydroxypregnanes (Henning & Zander, 1962; Hübener, 1962; Rick, 1962).

In early work on the steric course of NADH action [$4\text{-}^2\text{H}$]NADH was used and it was then shown that the oxidoreductases of ethanol, L-lactate and L-malate catalysed the transfer of ^2H from and to the same side (side I or α or A) of the pyridine ring of NAD (Fisher *et al.*, 1953; Loewus *et al.*, 1953, 1955). In contrast, the reduction of 4-androstene-3,17-dione in the presence of the $3\beta,17\beta$ -oxidoreductase was shown to be due to the transfer of the $4\text{B-}^2\text{H}$ (Talalay *et al.*, 1955). Similar work has been carried out using [$4\text{A-}^3\text{H}$]- and [$4\text{B-}^3\text{H}$]NADH (Jaraback & Talalay, 1960; Betz & Warren, 1968; Björkhem & Danielsson, 1970; Gibb & Jeffery, 1973b).

The enzyme-catalysed transfer of ^3H from [$4\text{-}^3\text{H}$]NADH to a suitable substrate suggests itself as an analytical procedure since the amount of the

substrate is given by the radioactivity of the product. The present work aims to demonstrate the practicability of this analytical concept on the examples of oestrone and progesterone with the respective use of the $3\beta,17\beta$ - and $3\alpha,20\beta$ -hydroxy steroid oxidoreductase. For the determination of progesterone, [$4\text{B-}^3\text{H}$]NADH was prepared by the enzymic reduction of NAD^+ with a phenolic ^3H donor, [$17\text{-}^3\text{H}$]oestradiol, so that any trace of the donor remaining in the [$4\text{B-}^3\text{H}$]NADH preparation could be readily removed from the formed [$20\text{-}^3\text{H}$] 20β -hydroxy-4-pregnen-3-one. Similarly, a neutral tritium donor (a mixture of products obtained by the reduction of 5α -androstane-3,17-dione with NaB^3H_4) was used in the determination of oestrone. Hurlock & Talalay (1957) used NAD^+ nucleosidase (NAD^+ glycohydrolase; EC 3.2.2.5) from *Neurospora* to destroy NAD^+ and thus to ensure complete reduction of the ketonic substrate. For the same purpose we used the commercially available calf spleen NAD^+ nucleosidase.

Experimental

General

Reagents were of analytical grade. All solvents were distilled, benzene and methanol after having been refluxed over CaH_2 . Water was distilled twice in an all-glass apparatus.

[$4\text{-}^{14}\text{C}$]Oestrone (58 mCi/mmol), [$4\text{-}^{14}\text{C}$]oestradiol (58 mCi/mmol), [$4\text{-}^{14}\text{C}$]progesterone (60 mCi/mmol), [$1,2,6,7\text{-}^3\text{H}$]progesterone (107 Ci/mmol), [$17\text{-}^3\text{H}$]oestradiol (approx. 9 Ci/mmol) and the crude product (approx. 20 Ci/mmol) of the reduction of 5α -androstane-3,17-dione by NaB^3H_4 were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. The last material was purified as outlined below. All other labelled compounds were purified before use by t.l.c. 20β -Hydroxy-4-[$4\text{-}^{14}\text{C}$]pregnen-3-one

was prepared by the enzymic reduction of [4-¹⁴C]-progesterone (Henning & Zander, 1962). NAD, NAD⁺ nucleosidase and the crude hydroxy steroid oxidoreductase from *Ps. testosteroni* were supplied by Sigma (London) Chemical Co., Kingston-upon-Thames KT2 7BH, U.K. 3 α ,20 β -Hydroxy steroid oxidoreductase was supplied by Boehringer Corp. (London) Ltd., London W5 2TZ, U.K.

Radioactivity on chromatograms was detected with a Packard 7200 scanner. It was counted with a Packard Tri-Carb liquid-scintillation Spectrometer (model 3375), samples being dissolved in a solution of PPO (2,5-diphenyloxazole; 0.4%, w/v) and POPOP [1,4-bis-(phenyloxazol-2-yl)benzene; 0.004%, w/v] in toluene containing ethanol (1%, v/v).

Gelman strips (type SA) (with systems A and B) or Whatman SG 81 paper (with systems D and E) were used for chromatography. The solvent systems were (by vol.): (A) chloroform; (B) *n*-hexane-ethyl acetate (17:3); (C) 2,2,4-trimethylpentane-2-methylpropan-2-ol (19:1); (D) toluene-methanol (49:1); (E) *n*-hexane-ethyl acetate (3:1). With system B chromatography was by the descending technique overnight.

Acetylation was carried out by depositing the alcohol on a piece (1 cm²) of Whatman 3MM paper and then exposing it to the vapours of acetic anhydride and pyridine (Norymberski & Riondel, 1967). Surplus reagents were removed *in vacuo* and the acetate was then eluted with methanol.

Solutions were taken to dryness by evaporation of the solvent in a stream of N₂ at 37°C.

Purification of the NaB³H₄ reduction product of 5 α -androstane-3,17-dione

In preliminary experiments it was found that the crude material supplied by The Radiochemical Centre contained labile ³H. To remove it, a portion (approx. 1 mCi) was acetylated and the diacetate formed was then hydrolysed in 0.3 ml of M-NaOH in 80% aqueous ethanol overnight at room temperature. Water (3 ml) was added and the mixture was extracted with ethyl acetate (6 ml). The extracted material was chromatographed in system A. Scanning revealed two overlapping radioactive areas; they were eluted with methanol (5 ml) and the solvent was then evaporated. The eluted material is presumably a mixture of [3,17-³H]5 α -androstane-3 β ,17 β -diol and its 3-epimer.

Partial purification of NAD⁺ nucleosidase

To solubilize the commercial material, NAD⁺ nucleosidase (300 mg) was triturated with a solution of sodium taurocholate (30 mg) in 0.1 M-sodium phosphate buffer (1.0 ml) of pH 7.0. The resultant suspension was centrifuged at 500 g for 5 min and the supernatant was then applied to a Sephadex G-50 column (30 cm $\times$ 1 cm) to remove the bile salt. Elution was with a 0.04 M-sodium phosphate buffer, pH 7.0,

and 2.0 ml fractions were collected. Fractions 5 and 6 contained the bulk of NAD⁺ nucleosidase activity; they were combined and stored at -18°C for up to 6 months without loss of activity.

*Partial purification of the 3 β ,17 β -hydroxy steroid oxidoreductase from *Ps. testosteroni**

An acetone-dried powder of the crude enzyme was prepared according to the method of Stempfeler & Sidbury (1964). Further purification involved sonication, treatment with streptomycin sulphate and a simple (NH₄)₂SO₄ fractionation (Delin *et al.*, 1964). The protein pellet obtained by increasing the (NH₄)₂SO₄ concentration from 25 to 50%, and derived from 5 g of the crude commercial preparation, was taken up in 0.1 M-sodium phosphate buffer (pH 7.0; 15 ml) and the solution was dialysed overnight at 4°C against the same buffer (250 ml). The activity of the non-diffusible enzyme preparation was 0.19 unit/ml (0.18 unit/mg of protein), one unit bringing about the oxidation of 1 μ mol of oestradiol per min in the presence of NAD⁺ at pH 9.5 and at 23°C.

Determination of oestrone

The mixture of the epimeric [3,17-³H]5 α -androstane-3,17 β -diols (approx. 0.5 mCi) was mixed with methanol (0.005 ml), water (0.3 ml), 0.2 M-Tris buffer (pH 9.5; 0.6 ml), 0.004 M-NAD⁺ (0.15 ml) and the preparation of the 3 β ,17 β -hydroxy steroid oxidoreductase (0.03 ml). After 1 h at 20°C the pH was adjusted to 7.0 by the addition of 1 M-sodium phosphate buffer (0.21 ml) of pH 5.6 and the mixture was first extracted with ethyl acetate (4 $\times$ 5.0 ml) and then with *n*-hexane (2 $\times$ 5.0 ml). (Extraction with *n*-hexane is necessary to remove residual ethyl acetate, which interferes with the subsequent enzymic reduction.) To the aqueous phase was added water (0.1 ml), 0.1 M-sodium phosphate buffer (pH 7.0; 0.3 ml), the NAD⁺ nucleosidase preparation (0.2 ml) and the 3 β ,17 β -hydroxy steroid oxidoreductase preparation (0.3 ml). Portions (0.1 ml each) of this mixture were dispensed into tubes (60 $\times$ 6 mm) containing different amounts of oestrone in 1,3-propanediol (0.005 ml) or, for the determination of blanks, the solvent alone. The tubes were flushed with N₂ and then stoppered. After 16 h at 37°C the content of each tube was diluted with water (0.2 ml) and the mixture extracted with dichloromethane (1.0 ml). After the extract was washed with water (0.25 ml), oestradiol (10 μ g) was added and the solvent was then evaporated. The residue was partitioned between 2,2,4-trimethylpentane-benzene (1:1, v/v; 0.75 ml) and 1 M-NaOH (0.75 ml). Each phase was back-extracted with an equal volume of the other phase. The organic phases were discarded. The aqueous phases were combined, adjusted to pH 10 by the addition of NaHCO₃ (0.15 g) and the mixture then extracted with benzene (2 $\times$ 2.0 ml).

The extracts were washed with a carbonate buffer of pH 10 made up from *m*-NaOH (2.0 ml) and NaHCO_3 (0.2 g). The combined extracts were taken to dryness. A portion (usually 0.4) of the residue was taken for the counting of ^3H radioactivity. Occasionally, the remainder was chromatographed in system A.

Determination of progesterone

The oxidation mixture for the generation of $[4\text{-}^3\text{H}]\text{NADH}$ consisted of $[17\text{-}^3\text{H}]\text{oestradiol}$ (approx. 0.5 mCi) as ^3H donor and the other constituents used in the preceding experiment except that a smaller volume (0.05 ml) of 0.004 M- NAD^+ was used. The reaction and subsequent extraction were carried out as before. Water (0.3 ml), 0.1 M-phosphate buffer (pH 7.0; 0.3 ml), NAD^+ nucleosidase (0.2 ml) and $3\alpha,20\beta$ -hydroxy steroid oxidoreductase (0.01 ml of a 1 mg/ml solution) were added. All the following operations were as in the determination of oestrone except that after the partition step the

aqueous phases were discarded whilst the organic phases were combined. 20β -Hydroxy-4-pregnen-3-one (10 μg) was the carrier.

Specificity tests

$[4\text{-}^{14}\text{C}]\text{Oestrone}$ (2.0 ng; 940 d.p.m.) or $[4\text{-}^{14}\text{C}]\text{progesterone}$ (2.0 ng; 840 d.p.m.) was added to human pregnancy plasma (1.0 ml). After 30 min at room temperature the plasma was diluted with water (4.0 ml), and extracted with a mixture of 2,2,4-trimethylpentane and ethyl acetate (7:3, v/v; 3×5 ml). The extract was taken to dryness. Phenolic and neutral fractions were separated as described above.

For the determination of progesterone, the neutral fraction was chromatographed in system A and progesterone was then located by scanning for radioactivity. The area was eluted with ethanol (5 ml) and the eluate, or a portion of it, was enzymically reduced as outlined above. For the determination of oestrone, the phenolic fraction was enzymically reduced without any preparatory treatment. After reduction of either oestrone or progesterone, a portion of the product was taken for the determination of the $^3\text{H}/^{14}\text{C}$ ratio. The remainder was treated in different ways to establish the constancy of the isotope ratio. Details are given with results for their better understanding.

The outlined procedure of extraction and fractionation permits the concurrent assay of oestrone and progesterone in one plasma sample. When $[^{14}\text{C}]\text{oestrone}$ and $[^3\text{H}]\text{progesterone}$ were added to a plasma sample and the sample then processed as above, no ^3H could be detected in the phenolic fraction and no ^{14}C in the neutral fraction.

When only progesterone (or any other neutral steroid) is to be assayed, the outlined procedure could be greatly simplified.

Discussion

Blanks and standards

Results are summarized in Table 1 and in Fig. 1. The large s.d. values of the mean blank values arise

Fig. 1. Calibration curves

For details, see the text. ●, Progesterone; $y = 24\,300x + 820$ ($r = 0.998$). ■, Oestrone; $y = 11\,250x + 890$ ($r = 0.967$).

Table 1. Determination of oestrone and progesterone

For details see the Experimental section. All values are rounded off.

	Blanks (d.p.m.; mean $\pm$ s.d.)	Standards	
		d.p.m./ng (mean $\pm$ s.d.)	s.d. between duplicates (d.p.m.)
Oestrone	8500 $\pm$ 3600 ($n = 19$)	29 000 $\pm$ 4250 ($n = 24^*$)	$\pm$ 2700 (0.5–4.0 ng) ($n = 24^*$)
Progesterone	18 800 $\pm$ 9000 ($n = 8$)	51 000 $\pm$ 4250 ($n = 15^*$)	$\pm$ 3600 (0.3–4.4 ng) ($n = 15^*$)

* Number of duplicate pairs.

Table 2. *Constancy of $^3\text{H}/^{14}\text{C}$ ratios after dilution of the tritiated reduction products of plasma oestrone and progesterone with the respective ^{14}C -labelled compounds*

For details see the Experimental section.

	³ H/ ¹⁴ C ratio				Mean
	Initial	After acetylation and sequential chromatography			
Oestrone					
Mean (<i>n</i> = 4)	4.00	3.89	3.75	3.66	3.82
±s.d.	0.17	0.08	0.17	0.18	0.15
Progesterone					
Mean (<i>n</i> = 4)	7.66	7.30	7.92	7.73	7.65
±s.d.	0.23	0.10	0.13	0.16	0.26

from differences between results obtained on different days. Four concurrent determinations of the blank in the oestrone assay had a coefficient of variation of 10.0%. The same explanation applies to the large variations of the d.p.m./ng values. When, after enzymic reduction, one paper-chromatographic or t.l.c. step was introduced, the blank values were approximately 65% lower. The s.d. values between duplicate assays (Snedecor, 1952) correspond to ± 0.09 ng of oestrone and ± 0.07 ng of progesterone respectively.

Specificity

To test specificity, three different experimental designs were used.

(i) Plasma from a woman in her sixth week of pregnancy was analysed for oestrone and for progesterone in quadruplicate. After enzymic reduction of the phenolic and neutral fractions, the reaction products were severally chromatographed in system B. The separated $[17\text{-}^3\text{H}]\text{oestradiol}$ was mixed with $[4\text{-}^{14}\text{C}]\text{oestradiol}$ and $[20\text{-}^3\text{H}]\text{20}\beta\text{-hydroxypregn-4-en-3-one}$ with $20\beta\text{-hydroxy-4-[4-}^{14}\text{C}]\text{pregnen-3-one}$. Each mixture was acetylated and each product was sequentially chromatographed in systems C, D and E. The $^3\text{H}/^{14}\text{C}$ ratios were determined before acetylation and then after each chromatographic step. The results (Table 2) show that after one chromatographic step the reduction products of endogenous oestrone and progesterone were essentially pure.

(ii) Plasma samples from the same woman were analysed for progesterone in duplicate. (They were taken at the 15th, 16th, 20th and 22nd week of pregnancy and contained 85/56, 53/53, 154/166 and 75/79 ng of progesterone/ml respectively; a fall of the progesterone concentration in the 22nd week of pregnancy is not exceptional.) A portion (0.2 of the total) of each reduced neutral plasma extract was mixed with $20\beta\text{-hydroxy-4-[4-}^{14}\text{C}]\text{pregnen-3-one}$. The initial $^3\text{H}/^{14}\text{C}$ ratios were within 6.0–8.5. Each

sample was chromatographed in system A. It was then acetylated and the acetate sequentially chromatographed in the systems C and D. The mean changes ($\pm$ s.d.) of the $^3\text{H}/^{14}\text{C}$ ratios after each successive purification step were $-17.3 \pm 4.4\%$, $-8.3 \pm 7.0\%$ and $+1.6 \pm 4.5\%$ respectively. Thus, radiochemical purity was attained in all instances only after the second chromatographic step. It is possible that the first chromatographic system was less efficacious than that used in the experiments described in (i). The mean ($\pm$ s.d.) recovery of ^{14}C (from $[4\text{-}^{14}\text{C}]\text{progesterone}$ added to plasma) before chromatography of the reduction product was $73.4 \pm 2.0\%$.

(iii) Single samples ($n = 12$) of plasma from four pregnant women were analysed for oestrone. Concentrations found (in ng/ml; weeks of pregnancy in parentheses) were: no. 1, 6.4, 7.7, 12.0 (14, 22, 26); no. 2, 3.7, 3.0, 5.6 (13, 17, 25); no. 3, 1.0, 3.0, 3.5 (11, 15, 19); no. 4, 3.7, 3.0, 6.5 (6, 8, 10). After chromatography in system A, the mean change ($\pm$ s.d.) of the $^3\text{H}/^{14}\text{C}$ ratio was $+4.0 \pm 12.5\%$. In contrast with the experiments described in (i) and (ii), no ^{14}C -labelled product was added after reduction. Consequently the $^3\text{H}/^{14}\text{C}$ ratios were very high and the spill-over of tritium into the ^{14}C -channel was significant. It is possible that this impaired the precision of the measurement to an extent which would account for the large s.d., particularly since the mean change ($+1.3\%$) of the isotope ratio for oestrone standards (1.7–8.0 ng; $n = 10$) had a s.d. of the same order of magnitude ($\pm 15.2\%$). This means that the chromatographic purification of the material obtained from the reduction of the phenolic fractions of different plasma extracts did not lead to statistically significant changes of the $^3\text{H}/^{14}\text{C}$ ratios. It does not necessarily follow that $[17\text{-}^3\text{H}]\text{oestradiol}$ was in all instances the sole ^3H -labelled reduction product. Until this point is clarified, the chromatographic separation of the $[17\text{-}^3\text{H}]\text{oestradiol}$ formed would seem advisable.

The mean recovery of ^{14}C was $60.6 \pm 34.8\%$. The large variation reflects mainly differences between the recoveries of oestrone on extraction of plasma

samples from different persons rather than a large systematic error.

Conclusions

The results demonstrate the feasibility of a new analytical approach based on the enzyme-catalysed transfer of ^3H from $[4\text{-}^3\text{H}]\text{NADH}$ to a suitable substrate. This was the sole aim of the present work. It was extraneous to that aim to establish the optimal conditions for the assay of progesterone or oestrone; the two compounds merely served as convenient models. It is therefore not possible, and in the context irrelevant, to compare the radio-enzymic assays of progesterone and oestrone with their established routine assays.

The assay of oestrone did not require its initial separation from the phenolic fraction of the plasma extract. It is possible that the chromatographic separation of the $[17\text{-}^3\text{H}]\text{oestradiol}$ formed is also unnecessary. Chromatographic separation of progesterone is necessary because of the presence of many other 20-oxosteroids in human plasma. This was not done efficaciously, as the reduction product has to be submitted to at least one chromatographic step before radiochemical purity was attained. Chromatography of the reduction product is in any case desirable since it drastically decreases the blank values and may be necessary for the assay of very low concentrations of the substrate. These examples suggest that, in general, the radio-enzymic method is much simpler to perform than existing isotope derivative methods.

The radio-enzymic method can be used as a double-isotope derivative method. It has, therefore, a built-in means of checking the accuracy and specificity of each assay, a distinct advantage over methods such as, for instance, radioimmunoassay methods, which lack that property.

The relative simplicity of the present method is due to the specific introduction of the ^3H label. The degree of specificity of that reaction depends on the substrate specificity of the enzyme used. We used two commercially available hydroxy steroid oxidoreductases, both of bacterial origin. Enzymes of animal origin are usually more substrate-specific; it is conceivable that their use could very considerably simplify the analytical procedure.

The method is applicable to all ketones reducible by NADH or NADPH in the presence of the appropriate enzyme. Its scope of application could be extended to the corresponding alcohols by their initial oxidation. The enzymic reduction of double bonds provides another group of suitable substrates; an example is the reduction of steroidal 4-en-3-ones to

3-oxo $[5\beta\text{-}^3\text{H}]\text{steroids}$ by $[4\text{A-}^3\text{H}]\text{NADPH}$ (Björkhem & Danielsson, 1970).

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