Journal of Steroid Biochemistry, 1973, Vol. 4, pp. 85–87. Pergamon Press. Printed in Great Britain

SHORT COMMUNICATION

ELIMINATION OF TRITIUM FROM METABOLITES OF [16-^{3}H]-PREGNENOLONE

H. W. A. DE BRUIJN and H. J. VAN DER MOLEN
Department of Biochemistry, Division of Chemical Endocrinology, Medical Faculty at Rotterdam, Rotterdam, The Netherlands

(*Received* 7 *August* 1972)

PREGNENOLONE plays a key role in the biosynthesis of steroid hormones and radioactively labelled pregnenolone is a commonly used substrate for *in vitro* and *in vivo* studies. The newly available [16-^{3}H]-pregnenolone is a useful substrate in those experiments which require a substrate of high specific radioactivity and in which the [7α-^{3}H]-pregnenolone cannot be used. The [7α-^{3}H] label is lost for example when 5-ene-3β-hydroxysteroids, such as pregnenolone and dehydroepiandrosterone are oxidised to the corresponding 4-ene-3,6-diketosteroids, 4-pregnene-3,6,20-trione and 4-androstene-3,6,17-trione. This conversion has been used for quantitative estimation of nanogram amounts of 5-ene-3β-hydroxysteroids by gas chromatography with electron capture detection [1]. Generally the stability of tritium next to an oxo group is questionable [2, 3].

During a study on testosterone biosynthesis in testis, rabbit testes were perfused *in vitro*, with a continuous infusion of [16-^{3}H]-pregnenolone into testicular arterial blood. Specific radioactivities of the steroid intermediates in spermatic vein blood were measured in order to gain information on the testosterone production [4]. These specific activities indicated that tritium was eliminated from some of the steroids. This loss of [^{3}H] label must have occurred either during metabolism or during the isolation procedures. Because it was of importance to know whether the loss of [16-^{3}H] occurred during a metabolic conversion, a mixture of [16-^{3}H]- and [4-^{14}C]-pregnenolone was infused. Elimination of tritium would then result in a decrease of the [^{3}H]/[^{14}C] ratio in the individual steroids, as compared to the [^{3}H]/[^{14}C] ratio in pregnenolone. We have observed that alkaline treatment eliminated the [16-^{3}H] label from metabolites with a 17-oxo group probably due to a base catalysed enolisation of this 17-oxo group. During *in vitro* metabolism of pregnenolone to C_{19}-steroids the [16-^{3}H] label remained unaffected.

EXPERIMENTAL

[16-^{3}H]-Pregnenolone (S.A. 21 Ci/mmol) was purchased from C.E.N., Mol, Belgium. The manufacturer specified that more than 98% of the tritium was attached to carbon atom 16, although its steric location could not be determined [5]. The specific activity of [4-^{14}C]-pregnenolone was 55·7 mCi/mmol. Rabbit testes were perfused as described by Van de Mark and Ewing [6] with a continuous infusion of [16-^{3}H]- and [4-^{14}C]-pregnenolone into testicular arterial blood. The isolation and purification of steroids was essentially the same as described previously [4]. Testosterone was measured as the 17β-monochloroacetate [7].

Androstenedione was reduced enzymatically to testosterone [8] and also measured as the chloroacetate. Dehydroepiandrosterone was purified by t.l.c. as the 3β-acetate, then hydrolysed in 1 ml of methanol, containing 0·2 ml 0·5 M NaOH, for 45 min at 50°C, and subsequently oxidised by 0·1 ml of CrO_3 in 90% acetic acid (5 mg/ml) for 10 min [1]. The resulting 4-androstene-3,6,17-trione was purified by t.l.c. and estimated with electron capture detection after g.l.c. [1].

17α-Hydroxypregnenolone and 5-androstene-3β,17β-diol were separated by t.l.c. after acetylation, followed by hydrolysis of the acetates as described above. 17α-Hydroxypregnenolone was further reduced with $NaBH_4$ [4] and subjected to the CrO_3-oxidation, resulting in 4-androstene-3,6,17-trione. 5-Androstene-3β, 17β-diol was also oxidised and measured as 4-androstene-3,6,17-trione [1].

Radioactivity was measured in a Nuclear Chicago Mark I liquid scintillation counter. The samples were counted in a toluene solution containing 4 g diphenyloxazole (PPO) and 40 mg 1,4-bis-2-(5-phenyloxazoyl) benzene (POPOP) per 1.

RESULTS

The [^{3}H]/[^{14}C] ratios of the isolated steroids, as given in Table 1 are similar in the precursor pregnenolone and its metabolites. This proves that tritium was not eliminated during the metabolism of pregnenolone and that [16-^{3}H] and [4-^{14}C]-pregnenolone were metabolised in the same way.

Table 1. [^{3}H]/[^{14}C] ratios in steroids isolated from the testicular venous blood after simultaneous infusion of [16-^{3}H]- and [4-^{14}C]-pregnenolone into the testicular artery (mean and S.D. of six estimations are given)

Steroid isolated	Estimated as	[^{3}H]/[^{14}C]
Pregnenolone	Pregnenolone acetate	31·9 ± 0·9
Testosterone	Testosterone chloroacetate	31·7 ± 0·1
Androstenedione	Testosterone chloroacetate	30·4 ± 0·6
5α-androstane-3β,17β-diol	5α-androstane-3β,17β- diol dichloroacetate	33·9 ± 1·0
5-androstene-3β,17β-diol	4-androstene-3,6,17-trione	33·9 ± 2·9
17α-hydroxy pregnenolone	4-androstene-3,6,17-trione	34·5 ± 3·3

However, after alkaline hydrolysis of dehydroepiandrosterone acetate the [^{3}H]/[^{14}C] ratio was 2·2 ± 0·1, thus 93% of the [16-^{3}H] label was eliminated. When in a separate experiment dehydroepiandrosterone was isolated and purified without acetylation and alkaline hydrolysis no elimination of tritium was found ([^{3}H]/[^{14}C] in dehydroepiandrosterone was 29·2 ± 0·2, in pregnenolone 27·5 ± 2·5, 4 estimations). When the 4-androstene-3,6,17-trione, obtained from 5-androstene-3β, 17β-diol was further purified by ether-alkali partition [9] the ratio dropped from 33·9 to 3·3, indicating a 90% loss of tritium. More than 80% of this tritium was present in the acidified water phase. In a control experiment the isolated and purified androstenedione was exposed to the methanolic alkali solution as used for hydrolysis. This resulted in a tritium loss of 90·4 ± 4·1% (7 estimations).

Acetates of 17β-hydroxysteroids could be subjected to alkaline hydrolysis without loss of the [16-^{3}H]-label. The acetic acid, used in the oxidations, did not promote the loss of the tritium at carbon atom 16. Base-catalysed enolisation of the 17-oxo group offers a suitable explanation for these observations. These results are in agreement with those of Fishman [2], who demonstrated that both

the [16α-^{3}H] and [16β-^{3}H] label are removed from estrone benzoate by enolisation of the 17-oxo group.

From these results it can be concluded that [16-^{3}H]-pregnenolone can successfully be used in investigating steroid metabolism only if alkaline treatment of the metabolites with a 17-oxo group is avoided.

REFERENCES

1. de Jong F. H. and van der Molen H. J.: *J. Endocrinol.* **53** (1972) 461.
2. Fishman J.: *J. Org. Chem.* **31** (1966) 520.
3. Pierrepoint C. G., Galley J. McI., Griffiths K. and Grant J. K.: *J. Endocrinol.* **38** (1967) 61.
4. van der Molen H. J. and Eik-Nes K. B.: *Biochem. biophys. Acta* **248** (1972) 343.
5. Personnal communication with CEN, Mol, Belgium.
6. Van de Mark N. L. and Ewing L. L.: *J. Reprod. Fert.* **6** (1963) 1.
7. Brownie A. C., van der Molen H. J., Nishizawa E. E. and Eik-Nes K. B.: *J. clin. Endocr.* **24** (1964) 1091.
8. Bardin W. C. and Lipsett H. B.: *Steroids* **9** (1967) 71.
9. Menini E. and Norymberski J. K.: *Biochem. J.* **84** (1962) 195.