DETECTION OF 3α -, 3β - AND 17β -HYDROXY- 5β - STEROID DEHYDROGENASES IN EPIDIDYMAL SPERMATOZOA OF HOLSTEIN BULLS

G. J. KILLIAN AND R. P. AMANN

*Dairy Breeding Research Center, The Pennsylvania State University,
University Park, Pa. 16802, U.S.A.*

(Received 24th May 1973)

Summary. Hydroxysteroid dehydrogenases (HSD) were evaluated histochemically in tissues representing seven regions of the epididymides from twenty-five neopubertal and three adult bulls. The epithelium of the epididymides was devoid of HSD activity against each of twenty steroid substrates tested; only $3\beta,16\beta$ -dihydroxy-5-androsten 3-methyl ester was dehydrogenated. In luminal spermatozoa, however, 3α -, 3β -, 16β - and 17β -HSD activities were detected in sections from most neopubertal and all adult bull epididymides incubated with 5β -androsterone, 5β -epiandrosterone, $3\beta,16\beta$ -dihydroxy-5-androsten 3-methyl ester and 5β -dihydrotestosterone. All other substrate incubations were negative. There was no age-associated difference in demonstrable HSD activity of epididymal spermatozoa. In smears of unwashed testicular spermatozoa from adult bulls, 3α -HSD activity was most conspicuous on the sperm head and cytoplasmic droplet, 3β -HSD activity was most prevalent in the mid-piece and the 17β -HSD activity predominated in the mid-piece and the cytoplasmic droplet.

INTRODUCTION

Androgens present within the epididymis are apparently essential for the acquisition of fertility by spermatozoa (Blaquier, Cameo & Burgos, 1972; Orgebin-Crist, Dyson & Davies, 1972). Both the epididymal epithelium and the spermatozoa are capable of steroid interconversions (Scott, Baggett & White, 1963; Seamark & White, 1964; Inano, Machino & Tamaoki, 1969; Gloyna & Wilson, 1969; Frankel & Eik-Nes, 1970; Vreeburg & Aafjes, 1971) and the enzymes which catalyse these conversions may, therefore, play an important rôle in determining the androgenic environment within the epididymis. Although all the enzymes of interest cannot be studied, histochemical techniques (Baillie, Ferguson & Hart, 1966) have aided the localization and identification of hydroxysteroid dehydrogenases (HSD) in the epididymal epithelium and spermatozoa of hamsters (McGadey, Baillie & Ferguson, 1966) and rats (Moniem, 1972).

The objective of this study was to determine the types of HSD present in the

bovine epididymis and to determine if this activity is altered during the first 30 weeks after puberty.

MATERIALS AND METHODS

Epididymides were obtained from twenty-five young Holstein bulls maintained on a weekly ejaculation regimen with planned sexual preparation (Killian & Amann, 1972). Five bulls had been assigned to each of five groups and were killed at 34 weeks of age, at puberty, or at 10, 20 or 30 weeks after puberty. Puberty was defined as the age when the first ejaculate was obtained which contained a minimum of 50×10^6 spermatozoa with at least 10% showing progressive motility (Wolf, Almquist & Hale, 1965). The mean age $\pm$ S.D. at which puberty occurred was 41.1 ± 4 weeks ($N = 20$). Epididymides from three adult Holsteins, aged 5, 6 and 7 years, were also evaluated.

Tissues representing the efferent ducts; proximal, mid- and distal caput; proximal and distal corpus; and proximal and distal cauda epididymidis were quenched in Genetron-23 (Rebhun, 1965) and immersed in liquid nitrogen (-196°C) for storage until evaluated 14 to 32 months later. Length of storage in liquid nitrogen should not have affected tissue histochemistry (Melnick, 1965). The HSD activity in cryostat tissue sections (8 μm nominal thickness) was evaluated using slight modifications of the method described by Baillie *et al.* (1966). Tissue sections mounted on coded slides were incubated at pH 7.5 in 0.15 M-sodium-potassium phosphate buffer (Humason, 1967) to which 0.5 mg of nitro blue tetrazolium/ml and 2 mg β -nicotinamide adenine dinucleotide/ml were added just before use. The substrate steroid was dissolved in dimethyl formamide (5 mg/ml) and added to give 0.5 mg/ml in the final reaction mixture. Control incubations used a similar medium lacking substrate but including dimethyl formamide. After incubation for 60 min, the sections were fixed for 15 min in 10% neutral formalin and rinsed briefly in distilled water. The cover-glasses were mounted using an aqueous 25% glycerol solution.

The following steroids (Sigma Chemical Co. or Schwartz/Mann Biochemicals) which were used are subsequently referred to by their trivial names: testosterone = 17 β -hydroxy-4-androsten-3-one; androstenedione = 4-androsten-3,17-dione; 5 α -androstanedione = 5 α -androstan-3,17-dione; 5 β -androstanedione = 5 β -androstan-3,17-dione; 5 α -dihydrotestosterone = 17 β -hydroxy-5 α -androstan-3-one; 5 β -dihydrotestosterone = 17 β -hydroxy-5 β -androstan-3-one; 5 α -androsterone = 3 α -hydroxy-5 α -androstan-17-one; 5 β androsterone = 3 α -hydroxy-5 β -androstan-17-one; 5 α -epiandrosterone = 3 β -hydroxy-5 α -androstan-17-one; 5 β -epiandrosterone = 3 β -hydroxy-5 β -androstan-17-one; cortisone = 17 α ,21-dihydroxy-4-pregnene-3,11,20-trione; hydrocortisone = 11 β ,17 α ,21-tri-hydroxy-4-pregnene-3,11,20-trione-21-acetate; oestradiol = 1,3,5(10)-oestratriene-3,17 β -diol.

Testosterone, 5 α -dihydrotestosterone, 5 α -androsterone, 5 β -androsterone, 5 α -epiandrosterone and 5 β -epiandrosterone were incubated with tissues from all neopubertal and adult bulls. In addition, a complete set of epididymal tissue sections from at least one adult bull was incubated with 3 α ,17 β -dihydroxy-5 α -androstan-3-one, 3 α ,17 β -dihydroxy-5 β -androstan-3-one, 3 β ,17 β -dihydroxy-5 α -androstan-3-one,

5 β -dihydrotestosterone, 3 β -hydroxy-5-androsten-17-one, 3 β ,16 β -dihydroxy-5-androsten 3-methyl ester, androstenedione, 5 α -androstanedione, 5 β -androstanedione, 11 β -hydroxy-4-androsten-3,17-dione, cortisone, hydrocortisone, oestradiol, 16 α -hydroxyoestrone, and 16 β -hydroxyoestrone diacetate.

To monitor the efficacy of the HSD-staining technique, sections of bull testis were periodically processed with the experimental tissues. Positive reactions were obtained for bull testicular tissue incubated with 5 β -androsterone, 5 α -epiandrosterone, 5 β -epiandrosterone and 5 β -dihydrotestosterone. Hamster epididymal tissue gave results similar to those reported by McGadey *et al.* (1966) including positive reactions with testosterone, 5 α -androsterone, 5 β -androsterone and 5 β -epiandrosterone. After incubation with reduced β -nicotinamide adenine dinucleotide, intense diaphorase activity was observed in both the epithelium and luminal spermatozoa throughout the epididymis, using tissue sections from one adult bull. The negative results reported were, therefore, not due to an absence of the diaphorase needed (Baillie *et al.*, 1966) for the HSD reaction.

RESULTS

Spermatozoa in all control tissues incubated without substrate were negative, but slight deposits of the formazan reaction product were often present in the epithelium (Pl. 1, Fig. 1). Washing of tissue sections in 0.15 M-phosphate buffer (pH 7.4) for 10 min before incubation only slightly diminished this non-specific reaction.

No HSD activity was detected in the epithelium of the efferent ducts and epididymis (Pl. 1, Figs 2 to 4) of any neopubertal bull (six substrates). For the twenty-one steroids incubated with adult epididymal tissue, only 3 β ,16 β -dihydroxy-5-androsten 3-methyl ester was dehydrogenated by the epithelium. This substrate, tested with tissues from two adult bulls, showed a weak positive reaction in the epithelium of the efferent ducts and that of the ductus epididymidis in the mid- and distal caput and in the cauda regions (Pl. 1, Fig. 5).

In contrast to results for the epididymal epithelium, HSD activity was detected in epididymal spermatozoa. In tissue sections from most neopubertal (eighteen of twenty-one with epididymal spermatozoa) and all adult bull epididymides, 3 α - and 3 β -hydroxysteroid dehydrogenases were detectable in luminal spermatozoa using 5 β -androsterone and 5 β -epiandrosterone as substrates (Pl. 1, Figs 2 and 3). All incubations of companion sections with 5 α -androsterone, 5 α -epiandrosterone, testosterone and 5 α -dihydrotestosterone, however, were negative.

The intensity of the reaction for 3 α - and 3 β -hydroxy-5 β -steroid dehydrogenases in epididymal spermatozoa varied among bulls of the same post-pubertal age and there were inconsistencies within epididymides. In most bulls, 3 α - and 3 β -HSD activities were detected in spermatozoa located in most, but not all regions of the same epididymis. No pattern to this variation was evident. A slight increase in the intensity of the HSD reaction over the mass of spermatozoa in the lumen of the ductus epididymidis, which appeared to be

associated with increasing sperm concentration, was observed in bulls killed after puberty.

Weak to moderate 17β -HSD activity was present in spermatozoa found in epididymal tissue sections from all three adult bulls incubated with 5β -dihydrotestosterone (Pl. 1, Fig. 4). This 17β -HSD activity was later tested, using tissue from the distal corpus epididymidis, for all neopubertal bulls and was detected in luminal spermatozoa. Formazan deposition was abundant on epididymal spermatozoa in tissues from two adult bulls incubated with $3\beta,16\beta$ -dihydroxy-5-androsten 3-methyl ester (Pl. 1, Fig. 5). A positive, but much less intense reaction, however, also resulted from incubation with 16β -hydroxyoestrone diacetate. No positive reaction was found in epididymal spermatozoa or tissue with any of the other steroid substrates.

The presence of 3α -, 3β - and 17β -hydroxy- 5β -steroid dehydrogenases was confirmed in smears prepared from unwashed testicular spermatozoa (Pl. 1, Figs 6 to 9) recovered after cannulation of the rete testis (Voglmayr, Kavanaugh, Griel & Amann, 1972). Detailed examination of these smears, representing three different samples and two adult bulls, provided evidence of enzyme localization within spermatozoa leaving the testis. The 3α -HSD activity was most conspicuous on the head and cytoplasmic droplet of the spermatozoa (Pl. 1, Fig. 7); the 3β -HSD activity was most prevalent in the mid-piece (Pl. 1, Fig. 8); and the 17β -HSD activity predominated in the mid-piece and the cytoplasmic droplet (Pl. 1, Fig. 9).

DISCUSSION

In the epithelium of the bull epididymis, HSD activity was demonstrated only with $3\beta,16\beta$ -dihydroxy-5-androsten 3-methyl ester. Because of the 3-methyl group, presumably the 16β -hydroxyl group was dehydrogenated. Traces of

EXPLANATION OF PLATE 1

FIG. 1. Control preparation of the distal cauda epididymidis of a bull killed 30 weeks after puberty. CT, connective tissue; E, epithelium; S, spermatozoa. $\times 250$.

FIG. 2. Preparation showing 3α -HSD activity in luminal spermatozoa with 5β -androsterone substrate. The distal cauda epididymidis of a bull killed 30 weeks after puberty. $\times 250$.

FIG. 3. Preparation showing 3β -HSD activity in spermatozoa with 5β -epiandrosterone substrate. Efferent ducts of a bull killed 10 weeks after puberty. $\times 410$.

FIG. 4. Preparation showing 17β -HSD activity in spermatozoa with 5β -dihydrotestosterone substrate. The mid-caput epididymidis of an adult bull. $\times 250$.

FIG. 5. Preparation showing HSD activity in the epithelium and spermatozoa with $3\beta,16\beta$ -dihydroxy-5-androsten 3-methyl ester. The mid-caput epididymidis of an adult bull. $\times 250$.

FIG. 6. Control preparation of rete testis spermatozoa from an adult bull in a smear incubated without substrate. Spermatozoa in smears shown in Figs 6 to 9 were obtained through a cannula from the rete testis. $\times 825$.

FIG. 7. Preparation showing 3α -HSD activity of rete testis spermatozoa from an adult bull in a smear incubated with 5β -androsterone substrate. $\times 825$.

FIG. 8. Preparation showing 3β -HSD activity of rete testis spermatozoa from an adult bull in a smear incubated with 5β -epiandrosterone substrate. $\times 825$.

FIG. 9. Preparation showing 17β -HSD activity of rete testis spermatozoa from an adult bull incubated with 5β -dihydrotestosterone. $\times 825$.

PLATE 1

(Facing p. 62)

16 β -HSD activity have been detected in the hamster epididymal epithelium with this substrate and also with 16 β -hydroxyoestrone (McGadey *et al.*, 1966).

The 3 α -, 3 β - and 17 β -HSD, of physiological importance in many tissues (Baillie *et al.*, 1966), are apparently absent or at low levels in the bovine epididymal epithelium. The inability to demonstrate these activities was apparently not a deficiency of the technique, the efficacy of which was demonstrated by the positive controls described under 'Materials and Methods'.

The report of 3 α -hydroxy-5 β -steroid dehydrogenase in the epithelium of the hamster cauda epididymidis (McGadey *et al.*, 1966) was confirmed for the single hamster epididymis examined in the present study. In addition, 3 β -hydroxy-5 β -steroid dehydrogenase activity was detected in the epithelium of the corpus and cauda epididymidis. McGadey *et al.* (1966) did not find 3 β -HSD activity in the epithelium of the hamster epididymis using the same 5 β -epiandrosterone substrate. For rat epididymis, however, 3 β -HSD activity is predominantly in the epithelium of the corpus, although activity was detected in the epithelium of the caput and cauda (Moniem, 1972). Data for one rabbit epididymis (G. J. Killian, unpublished observations) would suggest a species similarity with the bull; 3 α - and 3 β -HSD activities were detected in luminal spermatozoa but not in the epithelium.

Biochemical studies with ejaculated spermatozoa from several species including bulls have demonstrated 17 β -HSD by the conversion of testosterone to androstenedione (Scott *et al.*, 1963; Seamark & White, 1964). This enzyme has also been demonstrated histochemically in hamsters by McGadey *et al.* (1966) and, in the present study, with testosterone substrate; only spermatozoa in the cauda epididymidis gave a positive reaction. With testosterone as the substrate, however, this 17 β -HSD activity was not found in the present study in bull epididymal or testicular spermatozoa. These apparent inconsistencies in 17 β -HSD activity between testicular or epididymal spermatozoa and ejaculated bovine spermatozoa may reflect differences in their functional development. Alternatively, the histochemical technique for detection of HSD may be less sensitive than biochemical analysis.

The 3 α -, 3 β - and 17 β -HSD activities in epididymal spermatozoa were consistently detected with monohydroxy-5 β -steroids but not with the corresponding 5 α -steroids. This fact, and the negative results with 3 β -hydroxy-5 α -androstene-17-one using tissues from one adult bull, implies that in epididymal spermatozoa, HSD are specific for 5 β -steroids. The specificity and apparent localization of 3 α -, 3 β - and 17 β -HSD at specific sites on spermatozoa may be of physiological significance. These enzymes catalyse interconversions among 5 β -androsterone, 5 β -epiandrosterone, 5 β -dihydrotestosterone and 5 β -androstenedione. Interestingly, Voglmayr (1971) found that 5 β -androsterone, 5 β -epiandrosterone and 5 β -androstenedione were among the most effective of eleven androgens in altering the metabolic patterns *in vitro* of washed bull testicular and ejaculated spermatozoa. Thus, although 5 β -dihydrotestosterone was not tested by Voglmayr (1971), the 3 α - and 3 β -hydroxy-5 β -steroid dehydrogenases localized in spermatozoa by the present study are involved in interconversions of androgens known to alter glucose and lipid metabolism by bovine spermatozoa.

ACKNOWLEDGMENTS

Authorized for publication on 16th May 1973 as paper No. 4448 in the journal series of the Pennsylvania Agricultural Experiment Station.

This research was supported by U.S.P.H.S. Research Grant HD-01356, Contract NIH-NICHD-69-2137 and funds allocated to the Pennsylvania Agricultural Experiment Station. Mrs Frances Kordek provided skilled assistance.

REFERENCES

- BAILLIE, A. H., FERGUSON, M. M. & HART, D. M. (1966) *Developments in Steroid Histochemistry*, p. 8. Academic Press, London.
- BLAQUIER, J. A., CAMEO, M. S. & BURGOS, M. H. (1972) The role of androgens in the maturation of epididymal spermatozoa in the guinea pig. *Endocrinology*, **90**, 839.
- FRANKEL, A. I. & EIK-NES, K. B. (1970) Metabolism of steroids in the rabbit epididymis. *Endocrinology*, **87**, 646.
- GLOYNA, R. E. & WILSON, J. D. (1969) A comparative study of the conversion of testosterone to 17β -hydroxy- 5α -androstan-3-one (dihydrotestosterone) by prostate and epididymis. *J. clin. Endocr.* **29**, 970.
- HUMASON, G. L. (1967) *Animal Tissue Techniques*, p. 503. Freeman, San Francisco.
- INANO, H., MACHINO, A. & TAMAOKI, B. (1969) *In vitro* metabolism of steroid hormones by cell-free homogenates of epididymides of adult rats. *Endocrinology*, **84**, 997.
- KILLIAN, G. J. & AMANN, R. P. (1972) Reproductive capacity of dairy bulls. IX. Changes in reproductive organ weights and semen characteristics of Holstein bulls during the first thirty weeks after puberty. *J. Dairy Sci.* **55**, 1631.
- MCGADEY, J., BAILLIE, A. H. & FERGUSON, M. M. (1966) Histo-chemical utilization of hydroxysteroids by the hamster epididymis. *Histochemie*, **7**, 211.
- MELNICK, P. J. (1965) Effect of freezing rate on the histo-chemical identification of enzyme activity. *Fedn Proc. Fedn Am. Socs exp. Biol.*, Suppl. **15**, s259.
- MONIEM, K. A. (1972) Histochemical localization of 3β -hydroxysteroid dehydrogenases in the rat epididymis. *J. Reprod. Fert.* **28**, 461.
- ORGBIN-CRIST, M. C., DYSON, A. L. M. & DAVIES, J. (1972) Contribution a l'étude de la maturation des spermatozoïdes: contrôle hormonal. In *Fécondité et Stérilité du Mâle. Acquisitions Récentes*, p. 227. Ed. C. Thibault. Masson, Paris.
- REBHUN, L. I. (1965) Freeze substitution: fine structure as a function of water concentration in cells. *Fedn Proc. Fedn Am. Socs exp. Biol.*, Suppl. **15**, s217.
- SEAMARK, R. F. & WHITE, I. G. (1964) The metabolism of steroid hormones in semen. *J. Endocr.* **30**, 307.
- SCOTT, T. W., BAGGETT, B. & WHITE, I. G. (1963) Metabolism of testosterone by semen and the effect of testosterone on oxidative metabolism of spermatozoa. *Aust. J. exp. Biol. med. Sci.* **41**, 363.
- VOGLMAYR, J. K. (1971) The effect of testosterone and related steroids on the oxidative and glycolytic activity and on lipid synthesis of testicular and ejaculated bull spermatozoa. *Acta endocr., Copenh.* **68**, 793.
- VOGLMAYR, J. K., KAVANAUGH, J. F., GRIEL, L. C., JR & AMANN, R. P. (1972) A modified technique for cannulating the rete testis of the bull. *J. Reprod. Fert.* **31**, 291.
- VREEBURG, J. T. M. & AAFJES, J. H. (1971) Dihydrotestosterone (5α -androstan- 17β -ol-3-one) in the epididymis of rats. In *Current Problems in Fertility*, p. 203. Eds. A. Ingelman-Sundberg and N. O. Lunell. Plenum Press, New York.
- WOLF, F. R., ALMQUIST, J. O. & HALE, E. B. (1965) Prepuberal behavior and puberal characteristics of beef bulls on high nutrient allowance. *J. Anim. Sci.* **24**, 761.