CHAPTER-7

SIGNIFICANCE OF HISTOCHEMICAL VARIATIONS IN 3α, 3β AND 17β-HYDROXYSTEROID DEHYDROGENASE ACTIVITIES OF SUBMANDIBULAR GLAND OF RAT UNDER THE INFLUENCE OF ESTROUS CYCLICITY.

Since long it has been realized that the salivary glands of laboratory rodents and some other mammals show sex dimorphism (Lacassagne, 1940a; Baldi and Charreu, 1972 and Sawada and Tetsuo, 1993). Further, age dependent dimorphism has also been documented (Katsukawa *et al.*, 1980; Katsukawa *et al.*,1983; Sawada and Tetsuo, 1993). Though the salivary glands of rats and mice, particularly submandibular glands, have been shown to handle metabolically not only androgens (Blom *et al.*, 1993) but also estrogenic and gestagenic compounds (El Attar, 1974; Laine and Ojanotko-Harri, 1990 and Blom *et al.*, 1993). Somehow these have been regarded more as androgen target organs (Baldi and Charreu, 1972; Coffey, 1973b; Booth, 1977). However, presence of estradiol (Evans and Stewart, 1980; Choe *et al.*, 1983) and progesterone (Luisi *et al.*, 1981; Walker *et al.*, 1981; Evans, 1986; Lentone *et al.*, 1988; Adenkunlin *et al.*, 1989) in the human saliva has been studied by several workers to gain understanding about the assessment of ovarian functions on one hand and for predicting the time of ovulation on the other hand. Activities of steroid metabolising enzymes 17β-, 3α and 3β-hydroxysteroid dehydrogenase in human salivary glands have been studied histochemically by Sirigu *et al.*(1982). More biochemical work has been done on rats and mice in this respect by several workers (Rosner, 1965 and 1969; Baldi and Charreu, 1972; Charreu *et al.*,1976; Ferguson and Bannon, 1983; Kyakumoto *et al.*, 1986; Poteat *et al.*, 1986; Furuyama, 1989; Furuyama *et al.*, 1990; Sawada and Tetsuo, 1993). Kyakumoto (1986) and Furuyama (1989) have analysed the differential cytosolic and nuclear receptor dependent capacities of submandibular glands in mouse and rat respectively. However, most of the work cited so far does not take into account the variations due to estrous cyclicity along these lines in the

submandibular gland of female rodents. It was, therefore, thought desirable to study histochemically possible alterations in the submandibular gland of rat during different stages of the estrous cycle as far as its steroid metabolizing capacity is concerned. The idea was to test the hypothesis that during different stages of estrous cycle sensitivity of metabolic machinary of this gland is likely to differ in this respect. With this view in mind some key enzymes of steroid metabolism *viz.*- 3α -, 3β- and 17β-hydroxysteroid dehydrogenase (HSDH) activities were studied histochemically.

MATERIAL AND METHODS

For the study of normal cyclic variations, only those females which had normal 4-day estrous cycles were sacrificed at 09:00 am at each stage of estrous cycle. The left submandibular gland from each rat was quickly excised after decapitation under mild ether anaesthesia and transfered to a cryostat microtome maintained at -20^0 C. Fresh frozen sections of 15 μ thickness were taken on a clean slide and finger thawed. 17β– hydroxysteroid dehydrogenase was localized employing the method of Kellogg and Glenner (1966), using testosterone and estradiol as substrates. 3β-hydroxysteroid dehydrogenase was localized according to the method of Wattenberg (1958) using dehydroepiandrosterone and pregnenolone as the substrates, while 3α -hydroxysteroid dehydrogenase was demonstrated histochemically as per the method of Balough (1966) using androsterone as the substrate. Incubation of the sections was carried out at 37^0 C. NAD was used as a coenzyme while Nitro blue tetrazolium salt was used as hydrogen acceptor. Stained sections were washed thoroughly in distilled water and post- fixed in 10% neutral formalin for 15 minutes, washed again in distilled water mounted in glycerine jelly. Control sections for the enzyme were incubated in media devoid of the respective substrates.

RESULTS

From the results obtained it was observed that during the estrous stage the submandibular salivary gland of female rat exhibited general positive reaction as far as 3β- and 17β-HSDH activities are concerned. 3α -HSDH activity was nearly absent (Fig. 3). The 3β-HSDH enzyme activity could be demonstrated with both pregnenolone and DHEA as substrates, however, between the two the former was apparently preferred one (Fig.s 4 & 5). As far as 17β-HSDH activity is concerned it was noticed that the glandular tissue could not handle testosterone as substrate (Fig.s 1 & 2).

During the metestrous stage estradiol was the preferred substrate of 17β-HSDH activity and not the testosterone (Fig.s 7 & 8). Stronger enzymic action was observed with estradiol as the substrate in the ductal and acinar regions 3β-HSDH activity was more or less similar to that under estrous condition with pregenolone as substrate but it became milder with DHEA (Fig.s 9 & 10). During this stage of the estrous cycle 3α -HSDH activity was demonstrable. However, it was very mild and of diffuse type in acinar regions, but comparatively clearly noticeable in the ductal system of the gland (Fig. 8).

Diestrous stage was found to induce general positive reactions with all the substrates used for the 3 different steroid dehydrogenases, (Fig.s 11-14). That of 3β-HSDH was comparatively stronger with pregnenolone as substrate (Fig. 15) 17β-HSDH enzyme activity with testosterone as substrate was observed to be localized only in the larger collecting ducts of the glands.

Proestrous stage was characterized by stronger ductal enzymic reaction with the exception of 3β-HSDH on DHEA when it was very mild (Fig. 21). The acinar parts of the gland exhibited a general diffuse (all the three) enzyme activities. Comparatively the 3α -HSDH activity was maximum in its intensity in the acinar as well as ductal region than during any other stages of the estrous cycle (Fig. 19).

PLATE - 1

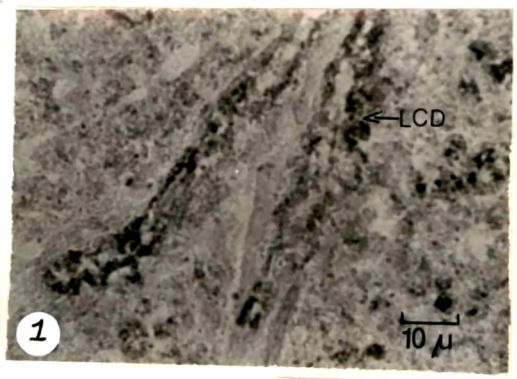
Histochemical reactions in L.S. of submandibular gland of female rat in **estrous** stage.

Fig.s 1 and 2 . Demonstrate 17β - HSDH activity with estradiol and testosterone as substrates, respectively.

Fig. 3. Shows 3α - HSDH activity which is almost negligible.

Fig.s 4 and 5. Demonstrate 3β – HSDH activity with pregnenolone and DHEA as the substrates, respectively. Note higher granular density in granular ducts and milder intensity in acinar regions (AC) in Fig. 4 and comparatively hazy colouration in acinar region of Fig. 5.

Abbreviations used:-
AC - Acinar region
GD - Granular duct
LCD - Large collecting duct
SCD - Small collecting duct
ICD - Intercalated duct



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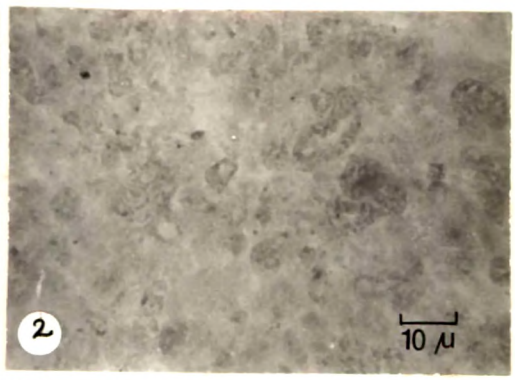

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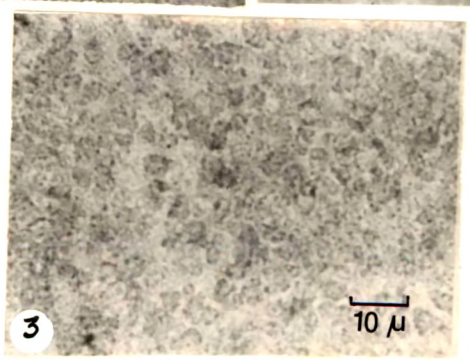

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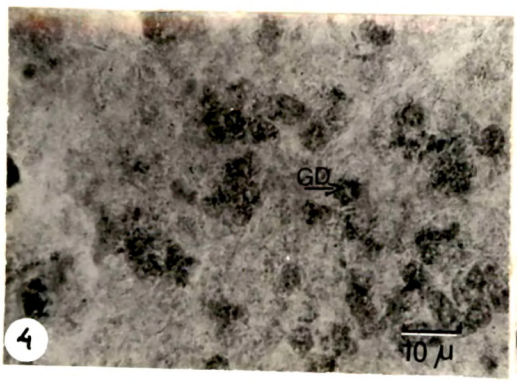

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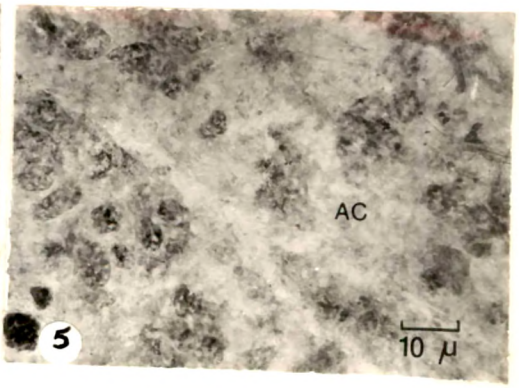


5

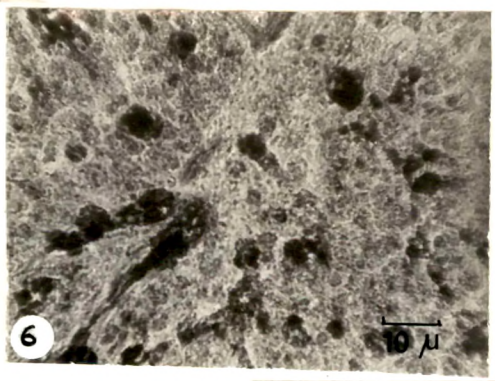
PLATE - 2

Histochemical reactions in L.S. of submandibular gland of female rat in **metestrous** stage.

Fig.s 6 and 7. Represent 17β - HSDH activity with estradiol and testosterone as the substrates, respectively. Here also estradiol is the preferred substrate. With testosterone, mild enzyme activity can be noted in the granular duct system. Larger collecting ducts (LCD), however, show strong activity That in Fig. 6 is much stronger in the duct system and also clearly noticeable in the acinar regions.

Fig. 8. Very diffuse mild reaction of 3α -HSDH can be noted in general but comparatively more enzyme activity is noticeable in the ductal system.

Fig.s 9 and 10. Demonstrate 3 β - HSDH activity with pregnenolone and DHEA as the substrates respectively. It is clear that the former is the preferred substrate.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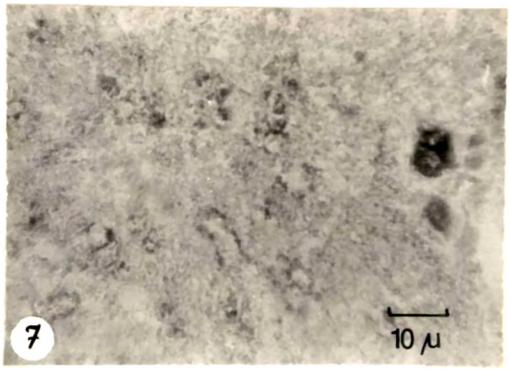
10 µ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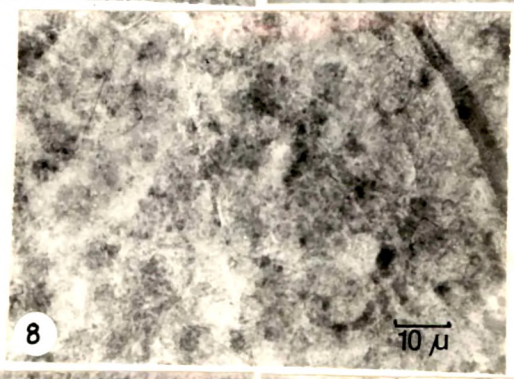
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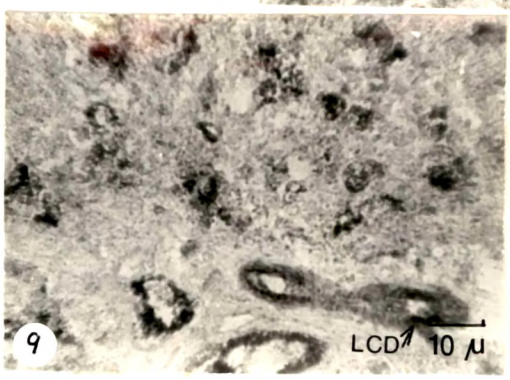

9
LCD
10 µ

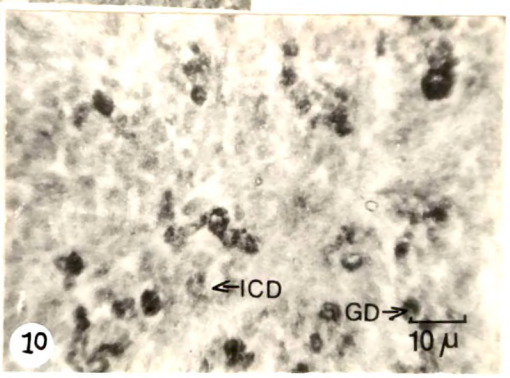

ICD
GD
10
10 µ

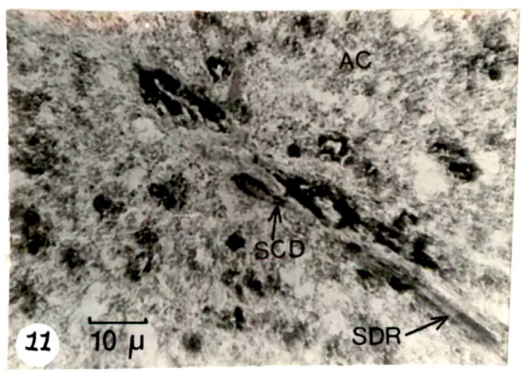
PLATE - 3

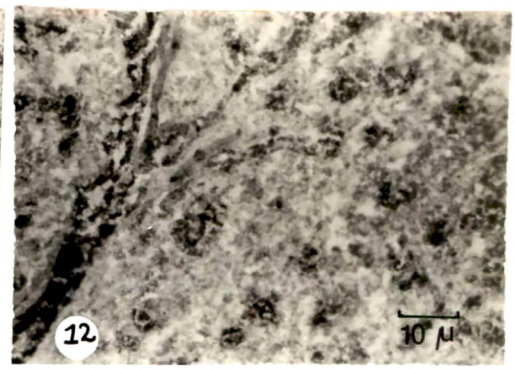
Histochemical reactions in L.S. of submandibular gland of female rat in **diestrous** stage.

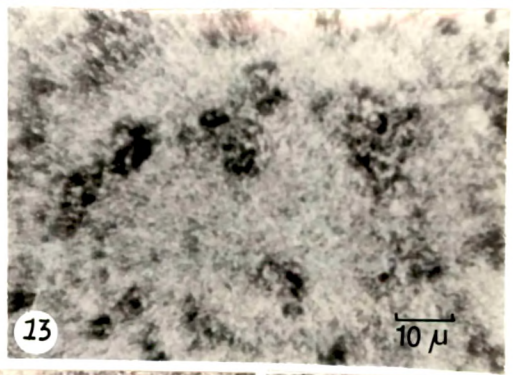
Fig.s 11 and 12. Demonstrate 17β - HSDH activity with estradiol and testosterone as the substrates. Note the stronger activity in the larger collecting ducts.

Fig. 13. Shows the positive 3β – HSDH activity with higher enzyme activity in the ductal portions.

Fig.s 14 and 15. Demonstrate 3β - HSDH activity with DHEA and pregnenolone as the substrates.


AC
SCD
SDR
11
10 μ


12
10 μ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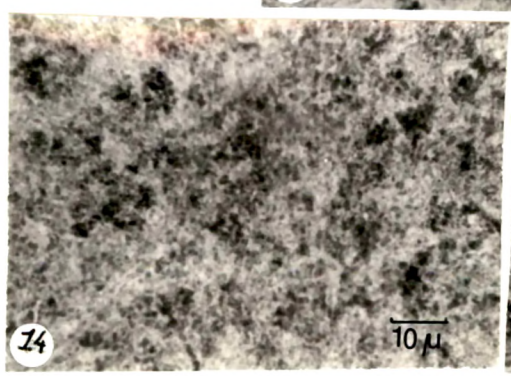
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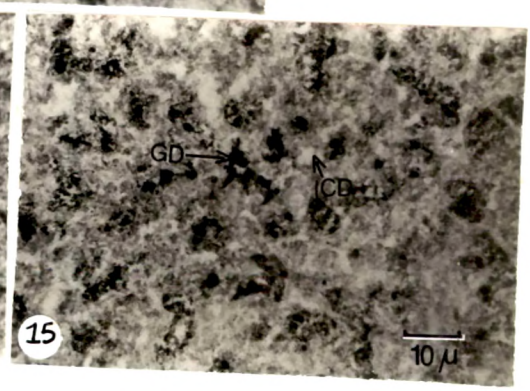

GD
CD
15
10 μ

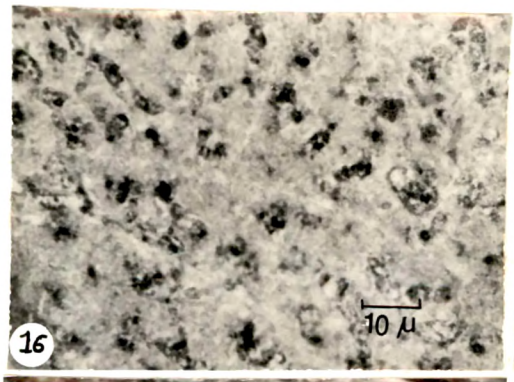
PLATE - 4

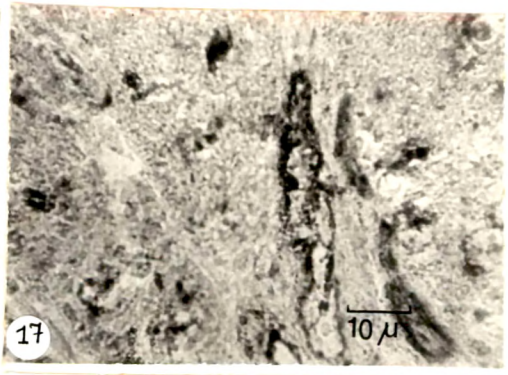
Histochemical reactions in L.S. of submandibular gland of female rat in **proestrous** stage.

Fig.s 16 and 17. Show 17β - HSDH activity with estradiol as the substrate. Fig 17 is a view to show particularly the stronger enzyme activity in the larger ducts of the gland.

Fig. 18. Depicts the 17β - HSDH activity with testosterone as the substrate.

Fig. 19. Shows the 3α - HSDH activity. The distribution pattern of this is similar to that seen in Fig. 17. at this stage this enzyme activity was at its maximum.


16
10 µ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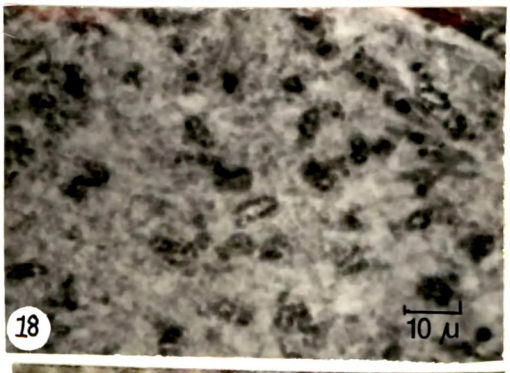
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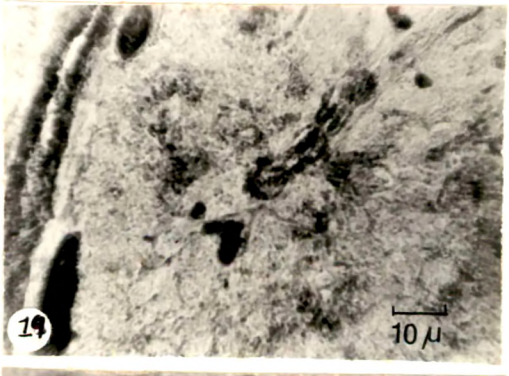
10 µ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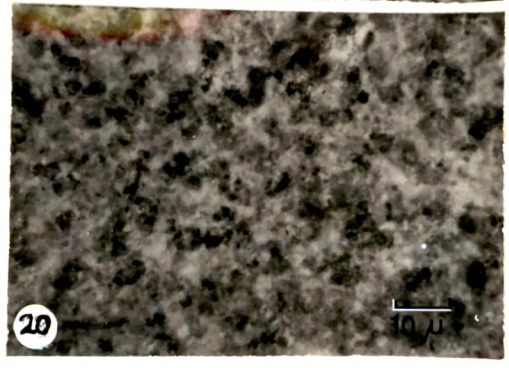
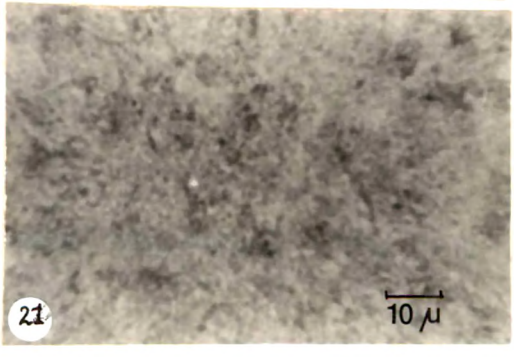

20
10 µ


21
10 µ

On the other hand, DHEA was noticed to be not a preferred substrate with respect to 3β-HSDH In this stage other enzymic reactions were seen, in general, to be localized mostly in acinar regions and smaller ducts.

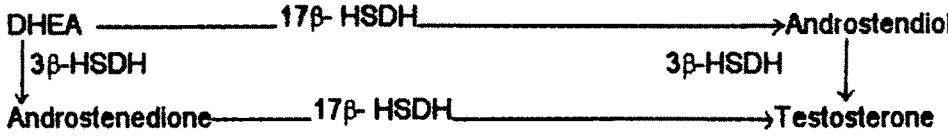
DISCUSSION

The three hydroxysteroid dehydrogenases under investigation, which have also been studied mostly under *in vitro* conditions and histochemically by several workers (Baldi and Charreu, 1972; Charreu, *et al.*, 1976; Katsukawa, *et al.*, 1980; Sirigu *et al.*, 1982; Kataukawa *et al.*, 1983; Furuyama *et al.*, 1990), did exhibited noteworthy variations in their localization as well as intensities according to the stages of the estrous cycle in rat. Most of the studies cited earlier have not referred to stage-wise differences, except for a gross classification as follicular and/or luteal phase in case of human females only. So much so that Sirigu *et al.* (1982) stated that 3α-HSDH was not detectable histochemically in human salivary gland. However, Furuyama *et al.*(1990) succeeded in isolating 3α -HSDH from cytosol of rat submandibular gland employing DHT $\rightleftharpoons$ 3α –diol coversion with a molecular weight of 30 KD. From the results presented it appears that this enzymic activity gets initiated during metestrous stage to increase progressively through diestrous stage, reaching maximum intensity during proestrous, indicating time-bound sensitivity of submandibular gland of female rats during which period, in all probability, a more potent androgenic steroid *viz.*-DHT is generated possibly proving its androgen-dependence of the gland even in case of females. However, this situation lasted only during proestrous stage, when it has been pointed out (Chapter-2 and Chapter-6) that glandular sialic acid as well as protein concentrations drop and the saliva becomes less viscous. It is, therfore, suggested that the said androgenic influence may be related to glandular sialic acid concentration Lowering of total protein concentration has been related to catabolic influence of high titres of estrogens during proestrous stage (Chapter-2). Now histochemical evidence supports these contentions through enhanced titre of estrogen (Butcher *et al.*, 1974) 17β-HSDH and conversion of androgens to DHT

through agency of 3α-HSDH, mainly in ductal region than the acinar region. Many workers have suggested that there is rapid transepithelial transference of ovarian hormones from blood to saliva (Walker *et al.*, 1979; Evans and Stewart, 1980; Read *et al.*,1984; Tho *et al.*, 1985; Wang and Knyba, 1985; Evans, 1986; Vuorento *et al.*, 1989; Blom *et al.*, 1990) and hence steroid metabolising capacity of the submandibular gland is not of much consequence. This may appear true in case of salivary levels of progesterone, testosterone and estradiol, but what the present investigation points to is that acinar portions do show these enzyme activities and the significance of these is to be viewed from their influences on modifying certain metabolic patterns.

A noteworthy point is that, generally these enzyme activities are more commonly as well as intensely localized in granular ducts, whenever present, than in acinar regions, as has been pointed out by others(Sirigu *et al.*, 1982; Blom *et al.*, 1993) The major exceptions were lack of 3α- and 17β-HSDH (with testosterone as substrate) activities only during estrous and metestrous stages. The first point is, in all probability, due to very obvious influence of higher estrogen level on overall stimulation of metabolic activity, particularly in the granular duct regions. This is indicated by rapid conversion and availability of DHT, the potent androgen to counter balance of first situation .

The second point about lack of preference for testosterone and androsterone e as substrates by 17β- and 3α - HSDH enzyme activities, in all probablity, point to lesser degree of androgen sensitivity during estrous and metestrous stages However, as metestrous sets in, greater enzymic affinities for estradiol and dihydroepiandrostenedion as substrates become apparent more in granular ducts, which probably is due to the rising progesterone level. The 3β- and 17β-HSDH enzymic activities in metestrous, by virtue of their actions as depicted :—



may reinitiate androgenic *milieu* subtantiating androgen dependence. Here it may be recalled that the saliva becomes more watery due to sialic acid accumulation in the gland during this stage of the cycle, in consonance with the licking behaviour of the animal (Chapter-6).

Alternatively, the above enzyme activity patterns may also lead to conversion of involved metabolites to elaboration of estrogenic compounds, but this situation is not likely as has been shown by Butcher *et al.*(1974) that minimum hormonal titres are encountered during diestrous stage. Under the circumstances, it has been recorded that many enzyme activities and metabolites were low with glaring exceptions of high protein and sialic acid concentrations. So it appears that at minimal ovarian hormone levels the submandibular gland metabolism apparently gets geared to synthetic machinery.

Another aspect of supportive nature comes from the works of Kyakumoto *et al.* (1986) and Furuyama (1989) that there are sex-dependent differences in the distribution of cytosolic and nuclear androgen-receptors of mouse and rat submandibular glands, respectively. It is known that the hormone receptor populations are influenced by female sex hormones (Janne *et al.*, 1978; Clark and Peck, 1979; Robel *et al.*, 1981; West *et al.*, 1983). This is the probable basis of cyclic variations in distribution and intensities of the hydroxysteroid dehydrogenases studied here.

From the foregoing account it becomes apparent that normal cyclic fluctuation of different ovarian hormones do influence not only the steroid metabolising capacities of the submandibular gland of female rats, but also bring about

alterations of metabolic patterns of these glands. The recent literature on hormone receptors, their influence on steroid metabolizing enzymes, regulation of receptor populations and cellular distributions; all add to corroborative information. It would not be out of place to mention here that the hypothesis suggested in the initial stage seems to be a working one, and further extensive work may prove it conclusively.