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PROGRAMMING AND DIFFERENTIATION OF RAT LIVER ENZYMES

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SUMMARY

At birth testicular androgens irreversibly programme hypothalamic centres involved in hypothalamo-pituitary control of hepatic sex-dependent steroid and drug metabolism. This imprinting process results in activation of a hypothalamic 'feminostatin'—secreting centre that is turned on just before puberty. Feminostatin inhibits pituitary secretion of 'feminotropin', a novel pituitary hormone that feminizes the basal type of metabolism characterizing the autonomous liver, i.e. the liver of hypophysectomized and gonadectomized rats. Consequently, female rats that are devoid of feminostatin will secrete feminotropin from the pituitary, leading to a feminine type of hepatic metabolism. Male rats, on the other hand, have an active feminostatin-secreting centre and the inhibition of pituitary feminotropin release results in an autonomous type of liver metabolism. Full 'masculinity' of hepatic metabolism in male rats is induced by testicular androgens that act on the hypothalamo-pituitary axis to stimulate release of a pituitary 'masculinizing factor'. Female rats show a relative androgen unresponsiveness and seem incapable of releasing masculinizing factor after treatment with androgens. A possible explanation for this is the absence of androgen receptor proteins in female brain as contrasted to the presence of androgen receptors in male pituitary, hypothalamus and pineal gland. It seems reasonable to assume on the basis of present knowledge that the level of androgen receptors in the central nervous system is programmed at the time of birth by testicular androgens.

The hypothesis presented above has been based on results obtained from experiments involving hypophysectomy, pituitary transplants under the kidney capsule, electrothermic lesions in the hypothalamus, neonatal and postpubertal castrations, treatment with androgens and estrogens at various times during development and transplantations with different lines of pituitary tumour cells. The novel pituitary hormone, feminotropin, has also been studied *in vitro* using hepatoma (HTC) cells or isolated hepatocytes in tissue culture. Feminotropin increases the apparent 5α-reductase activity in HTC cells at subsaturation concentrations of substrate (androstenedione) by decreasing the K_m value of the enzyme. Furthermore, feminotropin increases the apparent 5α/16α ratio in isolated hepatocytes, i.e. the ratio between total 5α-reduced and 16α-hydroxylated metabolites formed from androstenedione. Feminotropin has been shown to be different from any of the known pituitary hormones. It is stored in pituitary granules with a density of 1.13–1.17 g/cm^3 and has a molecular weight of about 20,000 daltons. Feminotropin is produced by isolated pituitary cells in primary culture and by certain lines of pituitary tumour cells, e.g. $C_8$11RAP cells. Tissue culture medium from cultures of this cell line serves as source in our purification programme for feminotropin.

INTRODUCTION

The development of a male phenotype in mammals starts with the formation of testes from the mesonephros. Most likely this is a direct expression of the Y chromosome in the gonad cell. Further development of male or female phenotypical characteristics depends on whether or not the fetus is exposed to testicular secretion products. Genotypically male and female fetuses are equally responsive to these factors. In the absence of testicular products, there is an inherent trend of the tissues to develop into a female phenotype [1].

During a first fetal period testicular factors differentiate the genital tract tissues into male internal and external genitalia. Testosterone administration substitutes all the effects caused by testicular secretion with the exception of the regression of the Müllerian ducts. A hormonal factor, probably a protein, has been postulated to inhibit the development of the Müllerian ducts [2]. The second period of male differentiation has mostly been studied in rats since it occurs neonatally in this species. In other species the differentiation takes place during the late prenatal or early postnatal period [3]. This differentiation is not expressed until puberty. All known effects of the testes during this period can be substituted by the administration of testosterone.

The period of highest sensitivity to androgen in the rat has been reported to occur immediately after birth [4]. Neonatal androgen exposure modifies the pattern of gonadotropin release during adult life; a high dose of androgen given neonatally prevents the occurrence of estrus cyclicity in the female rat and a constant estrus syndrome characterized by polycystic ovaries, anovulation and persistent cornification of vaginal epithelium develops [4, 5]. Furthermore, adult sexual

behaviour is programmed at birth [4, 6], but it has been proposed that different mechanisms are involved in androgen differentiation of the adult sexual behaviour and of the adult pattern of gonadotropic hormones [7, 8]. Thirdly, the metabolism of steroid hormones in the liver and the androgen responsiveness of the liver is sexually differentiated at birth [9–13].

The first report on sexual differences in liver metabolism of steroids in the rat was published in 1953 by Hübener and Amelung [14]. Further reports were soon published [15–18]. It was found that hydroxylation of steroids was more efficient in male than in female animals [17–18]. The 5α-reductase activity, on the other hand, was found to be higher in female rats [30]. The dependence of continuous androgen exposure from birth up to and during adult life for the maintenance of male metabolic characteristics was first described by Yates in 1958 [30]. DeMoor and Denef, and Gustafsson and Stenberg then showed that the adult masculine type of metabolism was dependent on neonatal androgen exposure [9–12]. However, no sexual differences were detected in the rat until 30 days of age [19–23]. Consequently, the neonatal effect of androgen is not expressed until around 30 days of age, whereas the effect of testosterone in the adult animal is observed within a few days [20]. In addition to the sexual differences characterizing liver metabolism of steroid hormones, in the rat, differences also exist with respect to the activities of other hepatic enzyme systems [24].

In addition to these findings on programming of liver enzymes at birth, Gustafsson and Stenberg have shown that the rat liver is imprinted by androgens during a short critical postnatal period so that it displays a higher degree of androgen responsiveness in adult life, as measured by changed activities of sex-dependent hepatic enzymes after androgen administration [13]. This probably explains why adult male rat liver enzymes are androgen-sensitive whereas the adult female rat liver is practically unresponsive towards androgens [13, 20, 25]. Neonatal programming of androgen responsiveness in androgen target tissues seems to be a general phenomenon. Guinea pigs, treated with cyproterone acetate pre- and postnatally and castrated early in life, developed a relative androgen unresponsiveness with respect to testosterone stimulation of prostate, seminal vesicle and preputial gland growth as compared to postpubertally castrated control animals [26]. A mutant strain of male rats carrying vestigial testes, during both the pre- and postnatal period, responded much less following androgen administration with regard to stimulation of growth of external genitalia than postpubertally castrated normal littermate rats [27]. Insufficient testicular biosynthesis of androgens during the neonatal period has been proposed to be of etiological significance in androgen unresponsiveness in the adult individual both in man and in animals [27].

In order to understand the mechanism of androgen-mediated sexual differentiation of liver metabolism we have investigated both the possibility of direct androgen effects on the liver cell, involving the formation of a steroid–receptor complex in analogy with what has been shown in other androgen-sensitive tissues [28] and to the possibility of central control of hepatic enzyme activities, mediated *via* the hypothalamo-pituitary axis.

RESULTS AND DISCUSSION

5α-Reduction of androgen in liver nuclei. Reduction of testosterone to 5α-dihydrotestosterone has been regarded as an essential step in the mechanism of action of testosterone in prostate tissue [29, 30]. Furthermore, end organ insensitivity to androgen in certain forms of male pseudohermaphroditism in man has been explained on the basis of 5α-reductase deficiency [27]. In view of these facts it was important to investigate whether a low level of nuclear 5α-reductase in female rat liver could explain the androgen unresponsiveness of this tissue. The kinetic characteristics and substrate specificity of hepatic nuclear 5α-reductase in male and female rats were studied using a highly purified nuclear preparation [31]. The enzyme was shown to have a pH optimum about 6.5, to have an absolute requirement for NADPH and to reduce androstenedione, testosterone, progesterone and deoxycorticosterone (K_M values in the range of $4–27 \times 10^{-6}$ M) but not corticosterone or cholestenone. The substrate specificity of hepatic nuclear 5α-reductase is different from that of microsomal 5α-reductase that can convert corticosterone, indicating that the nuclear and microsomal enzymes are not identical. Of great interest was the finding that nuclear 5α-reductase in female rats was about thirty times more active than in male rats (114.0 pmol as compared to 3.8 pmol of substrate converted per 10^6 nuclei per min). In view of the androgen unresponsiveness of female rat liver, this result is difficult to reconcile with a possible role of 5α-dihydrotestosterone as mediator of testosterone action in rat liver. In addition, the regulatory mechanisms involved in control of nuclear 5α-reductase were studied. The activity of the enzyme in liver of male rats increased after castration but did not normalize following subsequent androgen administration [32]. Again, these results are not in agreement with a similar role of the hepatic nuclear 5α-reductase as the androgen-dependent prostate nuclear enzyme [33], i.e. production of a physiologically active androgen, 5α-dihydrotestosterone. On the basis of the available data it could rather be suggested that hepatic nuclear 5α-reduction is involved in inactivation of testosterone since a higher capacity for 5α-reduction was found in female than in the androgen-responsive male rats.

Metabolism, protein binding and nuclear uptake of androgen in liver. Since it was not considered likely that 5α-dihydrotestosterone is the mediator of androgen action in liver, it was essential to investigate

the nature of the physiologically active form of testosterone in rat liver. Surprisingly, intraperitoneal administration of [1,2,6,7-^{3}H]-testosterone to castrated male and female rats results in selective nuclear uptake of [^{3}H]-androstenedione [34]. Androstenedione has not previously been considered as a mediator of androgen action [35] and does not compete well with 5α-dihydrotestosterone for binding to the β-protein in prostate [36]. The amount of [^{3}H]-androstenedione taken up in liver nuclei was similar in rats of both sexes when calculated per mg of DNA. Both *in vivo* and *in vitro* studies demonstrated the occurrence of two androstenedione-binding proteins in male and female rat liver cytosol that were separable by gel filtration on Sephadex G-100 [37]. The protein eluted at 0.40 column vols had a sedimentation coefficient of 10 *s* both in 0.01 and 0.4 M KCl, was stable towards treatment with dextrancoated charcoal, was unaffected by incubation with DNase and RNase, but was destroyed following incubation with proteolytic enzymes. Saturation of this protein with androstenedione *in vitro* was obtained at a substrate concentration of about 1×10^{-7} M and the concentration of binding sites was calculated to be about 1.6×10^{-16} mol/mg of cytosol protein. The second androstenedione-binding protein was eluted from Sephadex G-100 at 0.55 column vols, sedimented at 3.3 *s* and had an isoelectric point of 5.1. Extraction of liver nuclei of castrated male rats with 0.4 M KCl following intraperitoneal administration of [^{3}H]-testosterone resulted in release of an androstenedione-protein complex with a pI of 5.1. Reconstitution experiments *in vitro* indicated specific and saturable nuclear uptake of the second androstenedione-binding protein (pI 5.1) from liver cytosol (Fig. 1). The exact relationship between the 3.3 *s* and 10 *s* androstenedione-binding proteins is not clear at the present time although the following explanations are possible. Androgen receptor proteins are known to aggregate under certain experimental conditions [38] and it is possible that the 10 *s* protein represents an aggregated form of the 3.3 *s* protein. It may also be speculated that the two proteins are not structurally related but have related functions; the 10 *s* protein may have a ligand protective and/or transport role limited to the cytosol, serving to deliver

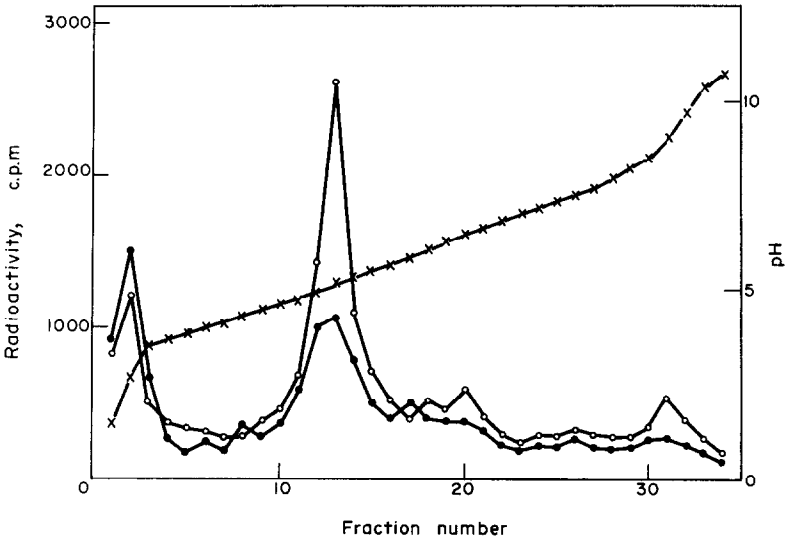

Fig. 1. Reconstitution experiments with radioactively labelled cytosol from liver of castrated male rats given [1,2,6,7-^{3}H]-testosterone and unlabelled liver nuclei from castrated male rats. Isoelectric focusing of (a) dextran-coated charcoal-treated labelled cytosol heated at 37°C for 30 min (control experiment; radioactivity O—O) and (b) dextran-coated charcoal-treated labelled cytosol incubated with unlabelled liver nuclei at 37°C for 30 min (radioactivity, ●—●). The concentration of nuclei was 2×10^7 per ml of cytosol.

Eight-week-old male Sprague–Dawley rats were castrated under ether anesthesia 14–16 h before the experiment. Each rat was given an intraperitoneal injection of 250 μCi of [1,2,6,7-^{3}H]-testosterone (specific radioactivity, 84 Ci per mmol; Radiochemical Centre, Amersham, England) in 120 μl of acetone. Thirty min later the animals were killed by a blow on the head. The livers were perfused with cold physiological saline and cytosol and liver nuclei were prepared as described by Gustafsson and Pousette [31]. The cytosol preparation was chromatographed on a Sephadex G-25 column, equilibrated in TKE-buffer (0.01 M Tris–HCl, pH 7.4–0.01 M KCl–0.001 M EDTA), and the void vol. (containing protein-bound radioactivity) was treated twice with dextran-coated charcoal and analyzed by isoelectric focusing. Ten-ml columns were fractionated into counting vials and 0.5 ml of double-distilled water was added prior to measurement of pH. Radioactivity measurements were performed using Instagel (Packard Instrument Co., Inc. Warrenville, Downers Grove, Ill., U.S.A.) as scintillator liquid. Ferritin (pI 5.0) and hemoglobin (pI 7.2 and 7.6) were purchased from Sigma Chemical Co. (St. Louis, MO., U.S.A.) and used as standards.

unmetabolized androstenedione to the 3.3 *s* protein that is responsible for the intranuclear androgen transport.

Based on these studies it may be suggested that androstenedione is a physiologically active form of testosterone in male and female rat liver. This finding is in line with the conclusion drawn from the studies on hepatic nuclear 5α-reductase that 5α-dihydrotestosterone probably is not involved in androgenic regulation of sex-dependent hepatic enzymes. Furthermore, similar uptake of radioactive androstenedione and testosterone in liver nuclei of male and female rats following systemic administration of [^{3}H]-testosterone indicates that nuclear 5α-reductase does not play any significant role in regulation of intranuclear concentration of 3-oxo-4-ene-androgens. The finding of similar hepatic intranuclear concentration of androgens in rats of both sexes and the presence of androstenedione-binding proteins in liver of both male and female rats makes it less likely that the androgen unresponsiveness of female rat liver can be explained on the basis of an impaired androgen receptor function. The possible role of androstenedione as mediator of androgen action in regulation of sex-dependent liver enzymes therefore appears doubtful although this androgen metabolite may well have a function in other aspects of androgenic control of liver physiology. Although it may be argued that the androgen unresponsiveness of the female liver may be due to deficiencies in later stages of androgen action, e.g. impaired binding to chromatin, the results presented call for another approach to elucidate the mechanisms behind the androgen unresponsiveness of the female rat liver.

Regulation of liver enzymes. General principles. Figure 2 illustrates the large sexual differences characterizing hepatic metabolism of androstenedione and 5α-androstane-3α,17β-diol in the rat. Important steps towards the understanding of sexual differentiation of liver metabolism were taken when Gustafsson and Stenberg found that hypophysectomy abolished sexual differences in hepatic metabolism [39] and when Gustafsson *et al.* demonstrated that male and female rats have different hypothalamic control of the pituitary factors regulating liver metabolism [40]. In order to understand the regulation of sex-dependent enzymes in rat liver it is helpful to define four principal types of steroid metabolism in adult rats, as shown in Table 1. The development into the different metabolic patterns depends on whether and when the

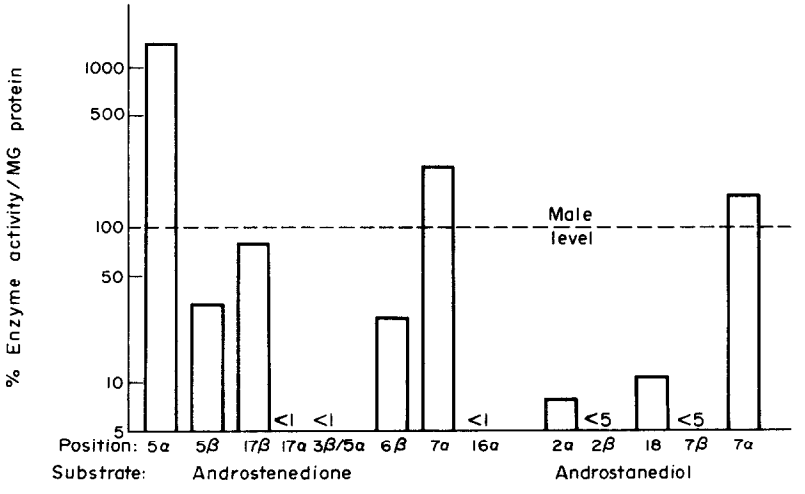

Fig. 2. Enzyme activities in female rat liver compared to those in male liver. Microsomal and cytosol fractions were incubated with androstenedione (reduction in positions 5α, 5β, 17β, 17α and 3β, hydroxylations in positions 6β, 7α and 16α) and 5α-androstane-3α,17β-diol (hydroxylations in positions 2α, 2β, 18,7β and 7α).

Eight-week-old Sprague–Dawley rats were killed by cervical dislocation, the liver was excised, and subcellular fractions were prepared at 0– +4°C in a modified Bucher medium [10, 12, 75]. A Potter–Elvehjem homogenizer was used for the preparation of tissue homogenate. Microsomes and cytosol were isolated by differential centrifugation. Microsomes were resuspended in Bucher medium. An NADPH-regenerating system was added prior to incubation in sufficient amounts to ensure maximal enzyme activities. After preheating aliquots of the preparations to 37°C the incubations were started by adding [^{14}C]-labelled substrates dissolved in acetone. Linear conversions with time and enzyme concentration were obtained. Steroid were extracted into organic solvents. The products were partly isolated by thin-layer chromatography. Radioautographic localization of the steroids was followed by extraction of radioactive compounds from the different zones. An aliquot was taken for measurement of radioactivity in a liquid scintillation counter. Resolution of products that did not separate by t.l.c. was obtained by gas–liquid chromatography of the silyl ethers using SE-30 as stationary phase. Radio-gas chromatography was used for relative quantitation of metabolites. Identifications of metabolites were performed by gas chromatography–mass spectrometry using LKB 2091 and 9000 instruments. Enzyme activities were related to the amount of protein in each incubation.

Table 1. Four different patterns of hepatic steroid metabolism in rats.

Type of metabolism	Etiology	Occurrence
OO	No exposure to androgen at birth or during adult life.	Normal females. Ovariectomized females. Neonatally castrated males.
TO	Exposure to androgen at birth (imprinting), but not later in life.	Males castrated more than 14 days after birth. Animals castrated and treated with androgen at birth.
OT	No exposure to androgen at birth but continuous exposure during adult life.	Type OO animals treated continuously with androgen during adult life.
TT	Exposure to androgen both at birth (imprinting) and continuously during adult life.	Normal males. Type TO animals treated continuously with androgen during adult life.

animal is exposed to androgen, but the patterns are not directly affected by the genotype of the animal.

In view of the superior control of sex-dependent liver enzymes by the hypothalamo-pituitary axis [40] it is reasonable to assume that the neonatal androgenic imprinting of liver metabolism occurs as an irreversible programming of a hypothalamic centre as has been hypothesized in case of neonatal programming of sexual behaviour [7] and gonadotropin release [8]. Also androgenic regulation of hepatic steroid metabolism in adult rats seems to take place mainly *via* androgenic action on the hypothalamo-pituitary axis since hypophysectomized male rats are unresponsive to androgens in terms of liver enzyme activities [39, 41].

Specificity of neonatal androgenic programming of liver metabolism. Physiological neonatal programming of enzyme levels in the liver of adult rats was studied after castration at different ages. Although an extensive imprinting was seen in animals castrated at 7 days of age, complete imprinting required presence of testes for more than 14 days after birth. In attempts to duplicate the neonatal effects of testes on programming of hepatic enzyme levels, two different dosages of testosterone propionate were used. It was found that a single injection of 0.145 μmol of testosterone propionate on the second day after birth was practically without effect, whereas 1.45 μmol gave a complete programming of hepatic enzyme activities in the adult rat [12]. The hepatic enzyme levels were not assayed until about 4 months after the time of programming. Therefore, even delayed effects of neonatal androgen exposure, as has been described for the programming of the pattern of gonadotropin release in adult rats [4], would have been seen. The smaller dosage of androgen given would have been enough to induce the development of a constant estrus syndrome [42]. Since this dosage was not enough to programme the hepatic enzyme activities, it may be suggested that different mechanisms are involved in programming the liver enzyme levels and the pattern of pituitary release of gonadotropins.

Table 2 shows the results obtained after neonatal administration of different substances to castrated rats [43]. It can be seen that in addition to testosterone propionate, estradiol benzoate and dihydrotestos-

Table 2. Specificity in programming of Type TO metabolism after administration of different substances subcutaneously in one injection 24–48 h after birth or in three injections on days 2, 3 and 4 after birth to neonatally castrated male rats. The metabolic pattern of the liver was studied 60 days after birth

Substance	Amount administered	Type of metabolism in adult rats
Vehicle 1 (propylene glycol)	1 × 20 μl	OO
Vehicle 2 (20% (v/v) ethanol in propylene glycol)	3 × 24 μl	OO
Testosterone propionate in vehicle 1	1 × 1.45 μmol	TO
Estradiol benzoate in vehicle 1	1 × 1.45 μmol	TO
5α-Dihydrotestosterone propionate in vehicle 1	1 × 1.45 μmol	TO
Etiocholanolone propionate in vehicle 1	1 × 1.45 μmol	OO
Epitestosterone propionate in vehicle 1	1 × 1.45 μmol	OO
op'-DDT in vehicle 2	3 × 2.82 μmol	OO

terone propionate were also efficient in inducing a Type TO metabolism in the adult animals, i.e. these agents were capable of programming the postulated hypothalamic liver metabolism centre. On the other hand, etiocholanolone propionate, epitestosterone propionate and *op'*-DDT were without effect in this respect. When given neonatally, testosterone propionate, estradiol benzoate and op'-DDT have been reported to affect the pattern of gonadotropin release in adult rats [4, 42, 44]. Furthermore, it has been described that 5α-dihydrotestosterone propionate does not induce the masculine type of gonadotropin release, nor can it imprint masculine behavioural patterns [7, 8, 45, 46]. These results give further indications for the existence of two separate hypothalamic programming centres for hepatic enzyme levels and pattern of gonadotropin release.

Sex-dependent metabolism and protein binding of androgen in brain. In view of the difficulties in explaining the different androgen responsiveness of male and female rat liver as being due to differences in the direct action of androgen on the liver (see above) and in view of the superior control of the hypothalamo-pituitary axis in regulation of hepatic sex-dependent enzymes it was logical to search for sex differences in androgen action in adult rat brain.

Significant sex differences were seen following intraperitoneal administration of [1,2,6,7-^{3}H]-testosterone in the pattern of androgen metabolites recovered from several brain regions, including the pituitary gland, the pineal gland and hypothalamus. The predominant metabolites in male brain were [^{3}H]-testosterone and [^{3}H]-androstenedione whereas the quantitatively important metabolites in female brain were 5α-[^{3}H]-androstane-3α,17β-diol, 5α-[^{3}H]-androstane-3β,17β-diol, [^{3}H]-epitestosterone and 5α-[^{3}H]-dihydroepitestosterone [47]. It may be suggested that one contributing factor for the androgen unresponsiveness in female rats is the faster metabolism of testosterone to inactive compounds in female brain.

Experiments both *in vivo* and *in vitro* revealed the occurrence of high-affinity, low-capacity binding sites for testosterone in the male pituitary, the pineal gland and the hypothalamus (K_D values in the range $1 \times 10^{-10} - 1 \times 10^{-9}$ M and number of binding sites 1.0–1.4 $\times 10^{-14}$ mol/mg of protein) (Fig. 3). The steroid–protein complexes were heat-labile and sensitive to protease, were excluded from Sephadex G-200 and had isoelectric points of about 5.1. The ligand specificity, as determined from competition experiments, was (given in decreasing order of affinity): testosterone, 5α-dihydrotestosterone and estradiol, androstenedione; corticosterone did not compete significantly for binding sites. Of great interest was that no androgen-binding proteins of similar nature were found in the pituitary, the pineal gland or hypothalamus from adult female rats [47]. Absence of receptor protein from androgen target tissues, a situation characteristic of male pseudo-hermaphroditic rodents displaying the testicular feminization syndrome, is combined with unresponsiveness to androgen [27]. On the basis of the presented experimental data one would therefore predict that female rats may display unresponsiveness towards androgen, at least with regard to certain aspects of brainmediated androgen action. It is tempting to suggest that deficiency of androgen receptor protein in female brain is an important factor behind the androgen unresponsiveness of female rat liver. In agreement with this hypothesis, 28-day-old female rats that have an androgen-dependent liver enzyme pattern [25], were shown to contain androgen receptor protein in the brain. It seems reasonable to assume that the presence of androgen receptor protein in male hypothalamus is related to neonatal imprinting by testicular androgen. Whether androgen receptor levels in other regions of the brain are controlled by the hypothalamus or by direct imprinting at birth remains to be elucidated.

Pituitary control of hepatic enzyme activities. As stated above, the sexual differences in liver metabolism disappear after hypophysectomy [39, 48]. The hepatic metabolism of hypophysectomized rats was of Type TO according to the classification given in Table 1 and therefore similar to the metabolism in postpubertally castrated male rats. These results indicate that the pituitary is responsible for the maintenance of a Type OO metabolism in adult female rats. Probably this influence begins at about 30 days of age in the female, since, from about this age the Type TO metabolism is changed to the Type OO metabolism seen in adult females. We have called the mediator of this effect 'feminizing factor' or, in accordance with the IUPAC-IUB Commission on Biochemical Nomenclature Recommendations (1974) [49], 'feminotropin'. The existence of a hypophyseal

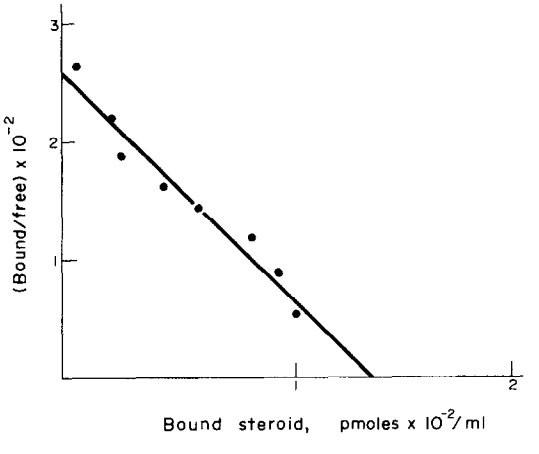

Fig. 3. Scatchard plot of [1,2,6,7-^{3}H]-testosterone binding in cytosol from male rat pituitary. Pituitary cytosol from castrated male rats was incubated with [1,2,6,7-^{3}H]-testosterone for 30 min at 37°C. Protein-bound radioactivity was determined using Sephadex G-25 chromatography. The amount of nonspecific androgen binding in pituitary cytosol was negligible. The dissociation constant (K_D) was calculated to 0.9 $\times 10^{-9}$ M and the number of binding sites to 1.2 $\times 10^{-14}$ mol per mg of protein.

feminizing factor has recently been confirmed by Denef [50].

When a pituitary of male or female origin was implanted under the renal capsule of an adult hypophysectomized and castrated rat a feminine (Type OO) hepatic steroid metabolism developed [41]. This indicates that the pituitary is able to release feminotropin autonomously. In agreement with this concept, the masculine type of hepatic steroid metabolism (Type TT) in male rats of the Fisher strain was changed into a feminine type (Type OO) following transplantation of MtT/F_4 pituitary tumour cells [51]. As expected, liver metabolism of steroid hormones in female rats was relatively unaffected following transplantation of pituitary tumour cells. This line of research, employing pituitary tumour cell lines (which is carried out in collaboration with Dr. Carlos Sonnenschein, Boston) has been followed up in an investigation of the effect of four different clonal pituitary tumours on hepatic steroid metabolism [52]. Transplanted tumours derived from the cell line denoted by $C_8$11RAP caused an alteration of the steroid metabolism to a completely female pattern in male rats whereas it had little effect on metabolism in female rats. This tumour cell line was the only one that appeared to secrete prolactin according to the observed level of this hormone in host serum [52]. The other cell lines tested ($C_2$9RAP, $C_1$3RAP and $C_3$11RAP) did not appear to secrete prolactin but had general effects on the hepatic steroid metabolism of both sexes that could not be interpreted as changes towards a female or a male pattern of metabolism [52]. Since the $C_8$11RAP alone is originally derived from a single cell [53] and since this cell line also produces prolactin it seems possible that feminotropin is produced in a similar type of cell (the mammotroph) as prolactin even in the normal pituitary. Interestingly, mammotrophs occur in much greater quantities in the female than in the male pituitary [54].

In order to investigate the possible effects of already known pituitary hormones on hepatic steroid metabolism, lutropin, follitropin and prolactin were administered to castrated rats of both sexes. No significant effects on the metabolic pattern were seen when 0.2 mg of lutropin or prolactin was given daily to animals with Type OO or Type TO metabolism [55, 56]. However, when the same dose of follitropin was given to castrated female rats (Type OO) a masculinization was seen (a change to a Type TO metabolism) [55]. Male rats have been reported to have higher levels of follitropin in serum than females [57] and it is therefore possible that some of the sexual differences in hepatic metabolism in rats could be explained on the basis of a follitropin-induced masculinization in male rats.

Hypothalamic control of hepatic enzyme activities. In order to investigate the possible hypothalamic control of feminotropin release from the rat pituitary, hepatic steroid metabolism was studied in male and female rats following electrothermic lesion of the hypothalamus including the median eminence [40]. Seven days after operation, hepatic steroid metabolism in male rats was generally feminized, whereas hepatic metabolism in female rats remained essentially unchanged. These results support the hypothesis presented above that the secretion of feminotropin from the female pituitary is largely independent of hypothalamic control. Furthermore, the inability of the male pituitary to secrete feminotropin seems to be due to a release-inhibiting influence from the hypothalamus. In accordance with the IUPAC-IUB nomenclature we would like to propose the name feminostatin for this hypothalamic factor. The existence of a feminostatin-secreting centre in the hypothalamus offers an explanation for the mechanism of neonatal androgenic programming of hepatic metabolism. It may be suggested that androgen exposure during the critical neonatal period results in the turning-on of the secretion of feminostatin from the hypothalamus. Interference with this androgenic imprinting process, as in male pseudohermaphroditic rats that are androgen-insensitive due to lack of androgen receptor protein [27], or in pre- and postnatally cyproterone acetate-treated male rats that are androgen-insensitive due to occupancy of androgen receptor protein by exogenous ligand [58], leads to a 'silent' feminostatin centre and thus an autonomous pituitary secreting feminotropin which in turn feminizes the basic Type TO liver metabolism to a Type OO metabolism [59–63]. An exciting task now is to localize the hypothalamic centre involved in regulation of hepatic metabolism.

Another aspect of hypothalamo-pituitary regulation of hepatic metabolism is estrogenic regulation. Administration of estradiol benzoate to intact or castrated male rats leads to feminization of liver metabolism, i.e. metabolic patterns Type TT or TO are changed to Type OO [20, 64, 65]. The liver metabolism in female rats, however, is not changed following administration of estrogen [20, 64, 65]. When hypophysectomized rats (hepatic metabolism Type TO) were given estradiol benzoate, no effects were seen on liver metabolism [39, 41]. These results indicate that an intact pituitary is a prerequisite for estrogen-induced feminization of liver metabolism in male rats and it may be suggested that estradiol benzoate-treatment activates feminotropin release from the male pituitary. It cannot be stated yet whether estrogen acts directly on the pituitary or whether it acts on the feminostatin centre in the hypothalamus by turning off feminostatin secretion. Receptor proteins for estradiol have been described both in the pituitary [66] and the hypothalamus [67] and it is possible that both mechanisms may operate in parallel.

Our present concept of hypothalamo-pituitary regulation of sex-dependent hepatic metabolism is outlined in Fig. 4.

Studies on feminotropin activity using the steroid metabolism of HTC cells. In order to study the

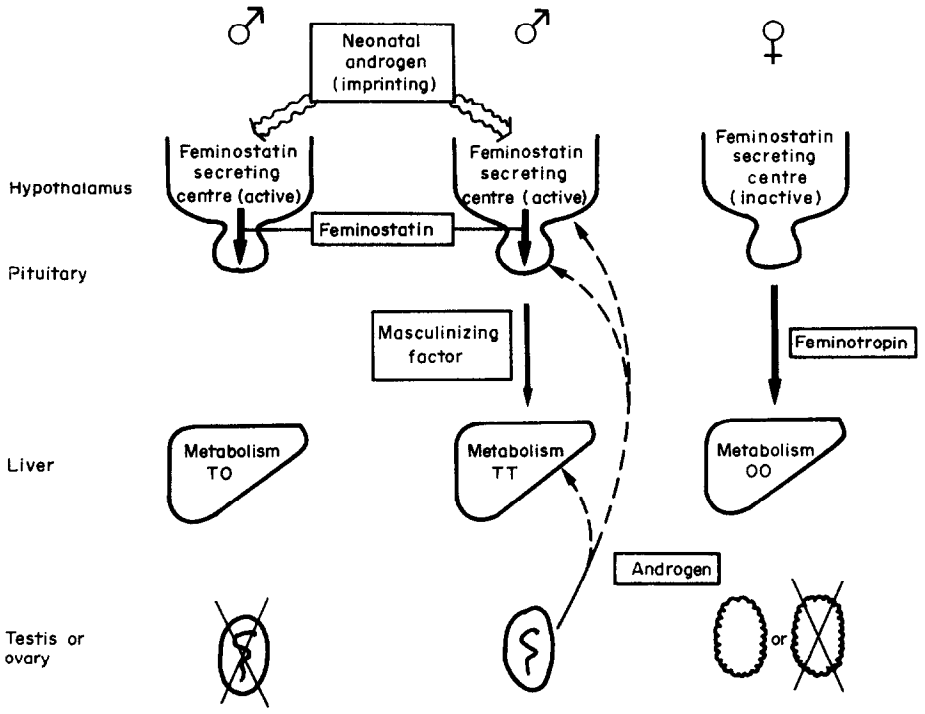

Fig. 4. Present concept of hypothalamo-pituitary control of hepatic steroid metabolism in rats. In male rats, neonatal androgen imprinting at birth programmes the hypothalamus so that a few weeks after birth, a centre starts secreting feminostatin that inhibits feminotropin secretion from the pituitary. In postpubertally castrated male rats, hepatic metabolism is essentially free from pituitary control (metabolism Type TO). In intact male rats, the basal, autonomous hepatic metabolism (Type TO) is masculinized (to Type TT) due to androgen-induced release of a pituitary masculinizing factor that either causes the TO → TT transformation itself or makes the liver sensitive to androgens that are directly responsible for the TO → TT transformation. In female rats, the feminostatin-secreting centre in the hypothalamus has never been activated and the pituitary is uninhibited in its secretion of feminotropin, that feminizes hepatic metabolism (TO → OO transformation). The secretion of feminotropin is not significantly influenced by the ovary.

characteristics of feminotropin, an assay system has been developed using the steroid-metabolizing capacity of a hepatoma cell line in culture (HTC cells). This cell line was originally isolated from a chemically-induced hepatoma (No. 7288 C) produced in a rat of the Buffalo strain and has been shown to carry at least one liver-specific marker enzyme, a glucocorticoid-inducible tyrosine aminotransferase [68]. Using androstenedione as the steroid substrate, the major enzyme activity in the HTC cells was found to be 5α-reductase. The activity of this enzyme is sex-dependent in the intact liver, the apparent reaction velocity being higher in female than in male rats [10, 12]. The high molecular weight fraction of homogenized female pituitary glands (separated using gel chromatography on Sephadex G-25) increased the 5α-reductase activity in HTC cell homogenates at subsaturation concentrations of the substrate whereas the corresponding male extract was without effect (Table 3) [69]. The response of the cells with respect to a log dose of pituitary extract was linear over a wide range of concentrations of female pituitary extract. Therefore, the increase in activity of 5α-reductase was taken as a measure of the activity of feminotropin. Kinetic studies showed that the increased enzyme activity at subsaturation concentrations of androstenedione was due to a decreased apparent K_M value and not to an increased apparent V_{max} value (Fig. 5). This agrees well with the kinetic differences of 5α-reductases in gonadectomized male and female rats (metabolic patterns Type TO and Type OO, respectively), the increased 5α-reductase activity in female rats being due to a lower K_M [69].

Table 3 also shows that a variety of combinations of purified pituitary hormones were without feminotropin-like effect on the 5α-reductase activity when added to HTC cells. Preincubation of the cells with certain hormones increased the apparent activity of 5α-reductase in the homogenates, but based on the concentrations of these hormones reported in pituitary tissue (prolactin [70], follitropin and lutropin [71], thyrotropin [72], somatotropin [73]), none of the standard hormone preparations exceeded a potency 15% of that of the female pituitary extract itself. These results indicate that feminotropin is a novel pituitary hormone, the effect of which cannot

Table 3. Effects of crude and partially prufied hypophyseal extracts, of purified rat hypophyseal hormones and of steroid hormones on 5α-reductase activity in homogenates of HTC cells

Addition to tissue culture medium	5α-reductase activity (% of control)
None (control)	100 (6.3 pmol/min · mg protein)
Lutropin (2 μg/ml)	129
Follitropin (2 μg/ml)	208
Thyrotropin (2 μg/ml)	178
Somatotropin (2 μg/ml)	108
Prolactin (2 μg/ml)	153
Prolactin + somatotropin (0.2 μg/ml of each)	97
Prolactin + thyrotropin (0.2 μg/ml of each)	94
Prolactin + follitropin (0.2 μg/ml of each)	91
Prolactin + lutropin (0.2 μg/ml of each)	109
Somatotropin + thyrotropin (0.2 μg/ml of each)	109
Somatotropin + follitropin (0.2 μg/ml of each)	91
Somatotropin + lutropin (0.2 μg/ml of each)	99
Thyrotropin + follitropin (0.2 μg/ml of each)	102
Thyrotropin + lutropin (0.2 μg/ml of each)	95
Follitropin + lutropin (0.2 μg/ml of each)	90
Prolactin + somatotropin + thyrotropin (0.2 μg/ml of each)	100
Prolactin + somatotropin + follitropin (0.2 μg/ml of each)	108
Prolactin + somatotropin + lutropin (0.2 μg/ml of each)	108
Prolactin + thyrotropin + follitropin (0.2 μg/ml of each)	101
Prolactin + thyrotropin + lutropin (0.2 μg/ml of each)	111
Prolactin + follitropin + lutropin (0.2 μg/ml of each)	86
Somatotropin + thyrotropin + follitropin (0.2 μg/ml of each)	96
Somatotropin + thyrotropin + lutropin (0.2 μg/ml of each)	103
Somatrotropin + follitropin + lutropin (0.2 μg/ml of each)	96
Thyrotropin + follitropin + lutropin (0.2 μg/ml of each)	94
Vasopressin (0.1 U/ml)	88
Oxytocin (2 U/ml)	69
Corticotropin (0.1 U/ml)	101
Estradiol benzoate (12 nM)	84
Testosterone propionate (11 nM)	71
Dexamethasone (1 nM)	131
Female hypophyseal extract*(macromolecular fraction from Sephadex G-25 chromatography) (equivalent to 50 μg of hypophyseal tissue/ml)	302
Male hypophyseal extract* (dito)	102
Extract from $C_8$11RAP pituitary tumour† (equivalent to 1 mg of tumour tissue/ml)	560
$C_8$11RAP pituitary tumour cells (coculture)‡	430
Extract from $C_1$3RAP pituitary tumour† (equivalent to 1 mg of tumour tissue/ml)	117
Extract from $C_3$11RAP pituitary tumour† (equivalent to 1 mg of tumour tissue/ml)	143
Partially purified feminotropin (0.1 μg/ml)§	312

Pituitaries were homogenized in ice-cold phosphate-buffered saline (1 mg/ml of tissue). The homogenate was centrifuged at 2000 *g* for 10 min. The supernatant was further centrifuged at 20,000 *g* for 30 min and the supernatant was chromatographed on Sephadex G-25. The macromolecular fraction was sterilized by filtration through Millipore filters (pore size, 0.2 μm) (Millipore Corp., Bedford, MA.) prior to addition to the cellular culture medium.

† The pituitary tumour tissue was liberated from necrotic material and homogenized in ice-cold phosphate-buffered saline (1 ml/mg of tissue). The homogenate was centrifuged at 2000 *g* for 10 min. The supernatant was used for addition to the cellular culture medium after sterilization by filtration through millipore filters.

‡ 10^3 cells of the $C_8$11RAP pituitary tumour cell line were added to each dish containing HTC cells and coculture performed for three days prior to assay for steroid metabolism in the HTC cells. The loosely attached pituitary tumour cells were washed away prior to harvesting the HTC cells.

§ Purified according to the schedule discussed in the text.

The cells were grown in Eagle's minimal essential medium (with Earle's salts) supplemented with 5% calf serum, 5% fetal calf serum, penicillin (200 i.u./ml) and streptomycin (125 μg/ml) in 60 mm petri dishes. Each dish, containing 5 ml of medium, was seeded with 5×10^4 cells. After 4 days the medium was changed and the extracts or hormones to be tested were added. A total of 10 petri dishes were used for each addition to be tested. The cells were maintained for a further 3 days in the new medium and then harvested, washed free of medium with phosphate-buffered saline, pH 7.4, and immediately analyzed for enzyme activity. Homogenates were prepared in a modified Bucher medium [75], pH 7.4, using an all-glass Potter–Elevehjem homogenizer. The steroid substrate used was [4-^{14}C]-androstenedione (1.17 μM) and the incubations were performed at 37°C for 30 min using an NADPH-regenerating system [12]. Duplicate incubations were performed in all cases and the results are expressed as pmol of product formed per min per mg of protein. Steroid metabolites were measured with established methods [10, 12] including thin-layer chromatography and radioactivity measurement.

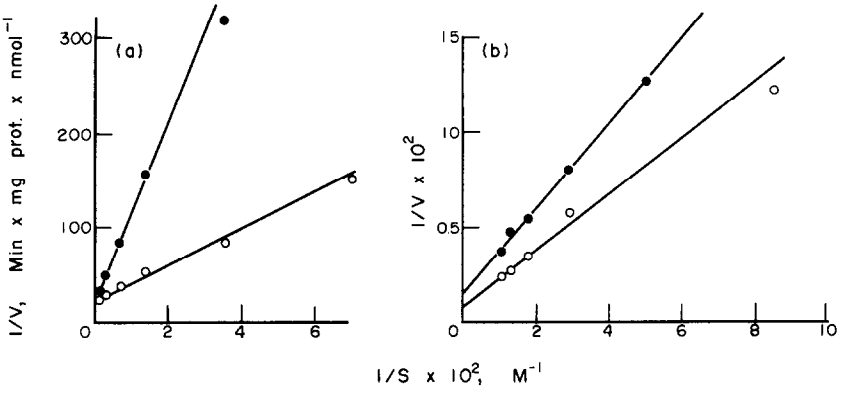

Fig. 5. Effect of substrate concentration on the activity of 5α-reductase in homogenates of (A) HTC cells and (B) primary cultures of rat hepatocytes with (○—○) and without (●—●) pretreatment with a feminotropin preparation. The feminotropin preparation (supernatant fraction from homogenized female rat pituitaries in the case of the HTC cells (see Table 3 for experimental details) and a granular fraction from rat pituitaries in the case of the hepatocytes (see legend to Fig. 6 for experimental details) was added to the cells in their respective medium one to three days before analysis of the homogenates for enzyme activity. The substrate used was [4-^{14}C]-androstenedione and incubation conditions were as described in Table 3 and in the legend to Fig. 6. The 5α-reductase activity, measured as the total production of 5α-reduced metabolites (5α-androstane-3,17-dione and 3α- and 3β-hydroxy-5α-androstan-17-one) was expressed in nmol of product per min per mg of protein and plotted on a double reciprocal Lineweaver–Burk plot. The results obtained from such treatment is as follows; (A) HTC cells ($n = 3$): $K_M = 667 \pm 120\,\mu M$, $V_{max} = 0.07 \pm 0.01$ (without pretreatment), $K_m = 99 \pm 4\,\mu M$***, V_{max} 0.05 ± 0.01 (with pretreatment); (B) hepatocytes ($n = 5$): $K_M = 278 \pm 44\,\mu M$, $V_{max} = 1.4 \pm 0.2$ (without pretreatment), $K_M = 154 \pm 9\,\mu M$*, $V_{max} = 1.3 \pm 0.3$ (with pretreatment). The results are expressed as mean ± 1 standard deviation. Statistical significance was tested using Student's t-test and the level of significance set at 0.05.* = $P < 0.05$; *** = $P < 0.001$.

be explained on the basis of combinations of the already known hormones.

In good agreement with the *in vivo* results discussed above [52], extract from $C_8$11RAP pituitary tumour cells were found to contain feminotropin-like activity when tested in the HTC cell system (Table 3). An increased 5α-reductase activity was also seen following coculture of HTC cells with $C_8$11RAP pituitary tumour cells. On the other hand, extracts from $C_1$3RAP or $C_3$11RAP pituitary tumour cells had no significant feminotropin-like activity on HTC cell 5α-reductase. This is also in excellent agreement with findings *in vivo* since $C_1$3RAP or $C_3$11RAP pituitary tumours did not feminize hepatic steroid metabolism when transplanted to male rats [52].

Studies on feminotropin activity using the steroid metabolism of isolated hepatocytes. Since steroid metabolism in HTC cells deviates from that in normal liver we have considered it essential to ascertain that feminotropin also feminizes steroid metabolism in isolated hepatocytes in tissue culture. Hepatocytes were isolated using perfusion technique (for details, see legend to Fig. 6) and were kept in monolayer in Leibowitz L-15 medium containing 1% male rat serum. Incubations of cell homogenates were performed using subsaturation concentrations of [4-^{14}C]androstenedione. Feminization of steroid metabolism in male hepatocytes was measured by calculating the ratio between 5α-reduced and 16α-hydroxylated products (5α/16α ratio) which were measured with established methods [10, 12]. Generally, feminization of steroid metabolism in isolated male hepatocytes in tissue culture is obtained by purified preparations of feminotropin but not by unfractionated pituitary extracts. This indicates that isolated male hepatocytes are more sensitive *in vitro* to pituitary factors that counteract the feminizing activity of feminotropin than is the case with HTC cells. Figure 5 shows that the feminotropin-induced, apparent increase in 5α-reductase activity in isolated hepatocytes using subsaturation concentrations of substrates is mainly due to a decrease in the K_M of the enzyme, as was also the case with HTC cell 5α-reductase.

Studies on granular storage of feminotropin. Figure 6 shows a separation of the male and female pituitary granules by sucrose-gradient centrifugation. Feminotropin activity has been assayed using both the hepatocyte- and the HTC-cell-systems and it can be seen that results obtained with both methods indicate that feminotropin is stored in two types of granules with densities of 1.17 and 1.13 g/cm^3, again separating it from lutropin and follitropin that are stored in much denser granules. Prolactin was stored in granules of a similar density to those containing feminotropin. The results shown in the figure also indicate the presence of feminotropin granules in male pituitary. Thus, feminotropin seems to be stored in granule form in both female and male pituitaries but appears to exist in soluble, extragranular form only in female pituitaries. The reason why feminotropin is only detected

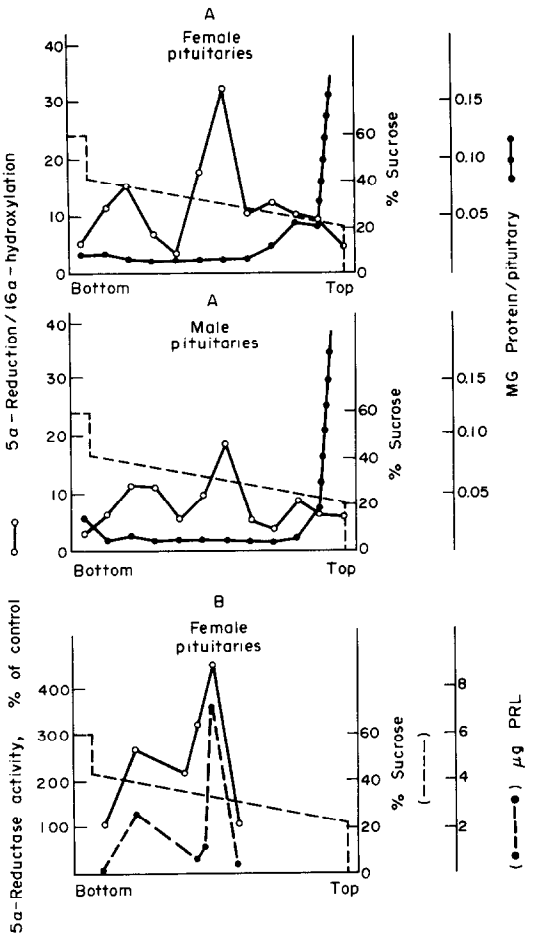

Fig. 6. Distribution of feminotropin-containing pituitary granules on a 20–40% (w/v) sucrose density gradient after centrifugation at 100,000 *g* for 1 h. Pituitaries were homogenized in ice-cold phosphate-buffered saline (1 ml/mg of tissue). The homogenate was centrifuged at 1,000 *g* for 10 min and 0.2 ml of the supernatant was layered on top of a 10 ml 20–40% sucrose density gradient. Centrifugation was performed in an SW 50.1 rotor. The centrifuge tubes were punctured in the bottom and the fractions (each of which corresponding to the contents in 1/20 pituitary) were added to isolated hepatocytes kept in primary monolayer culture (Fig. 6A; see below) or to HTC cells (Fig. 6B; see legend to Table 3).

Hepatocytes were isolated and kept in culture as follows: Adult male rat livers were perfused *in situ via* the portal vein. A calcium-free buffer (144 mM NaCl–7.0 mM KCl–10.1 mM HEPES–11 mM glucose–5.1 mM sodium pyruvate–5.8 mM sodium fumarate–4.7 mM sodium glutamate, pH 7.4) was infused for 5 min (total vol. 250 ml) followed by infusion of a collagenase buffer (Eagle's minimum essential medium with 2.0 mM $CaCl_2$, 26.6 mM HEPES, 26.4 mM TES, 31.8 mM Tricine and 50 mg/100 ml of collagenase Type I (Sigma Chemical Co.)) that was collected from the inferior vena cava and recycled. After 10 min the liver was taken out and dispersed mechanically. The suspended cells were filtered through cotton gauze, cooled to 0–4°C and washed 2–3 times by centrifugation in buffer with collagenase omitted. About 5×10^5 cells per ml of medium were kept as a monolayer in Leibowitz L-15 medium supplemented with 10 mM glucose, 1.0 mM disodium succinate, 60 mg/100 ml benzyl penicillin and 1% (v/v) male rat serum. The medium was changed the day after isolation of hepatocytes. After the medium change in female hypophyseal extracts when the hypophyseal homogenate is centrifuged at 2,000 *g* and filtered through Millipore filters (*cf.* above) may be that the hormone-containing granules do not penetrate the filter during this procedure. Preliminary results indicate that male pituitaries are devoid of a factor present in female pituitaries that promote the release of feminotropin from the granules.

Figure 7 shows the effects of increasing amounts of feminotropin-containing granules on the 5α/16α ratio in tissue cultured hepatocytes. Initially, the ratio increases in a linear fashion, but with increasing concentration of feminotropin granules, the ratio drops markedly. An explanation for this is offered by the results (also shown in Fig. 7) obtained following addition of prolactin to tissue cultured hepatocytes; prolactin gives a decreased 5α/16α ratio, an effect that tends to be more pronounced with increasing concentrations of prolactin. Therefore, it may be suggested that the marked drop in the 5α/16α ratio seen with higher concentration of feminotropin granules is the result of the presence of prolactin in the same granules.

Physical characteristics and purification of feminotropin. Isoelectric focusing of female pituitary extracts indicated that feminotropin is a single protein with

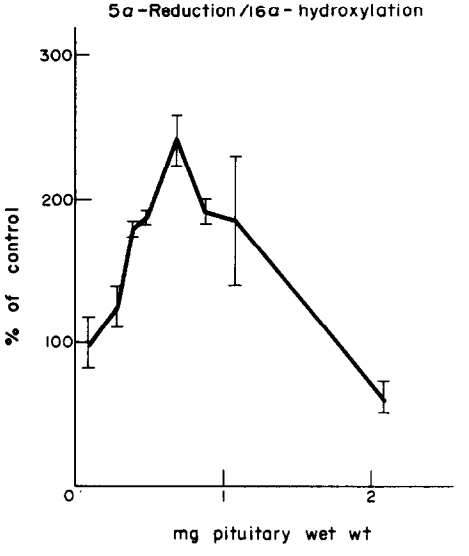

Fig. 7. Feminization response of isolated hepatocytes in primary monolayer culture after addition of increasing amounts of pituitary granules sedimenting between 20.5 and 60% (w/v) sucrose after centrifugation of a 1000 *g* pituitary homogenate supernatant at 100,000 *g* for 1 h. For experimental details, see legend to Fig. 6.

Additions were made of the preparations to be tested for feminotropin activity and enzyme activities were assayed about 20 h later. 0.5–1.0 × 10^6 cells were homogenized and incubated with subsaturation concentrations of [4-^{14}C]-androstenedione in the presence of an NADPH-regenerating system. Steroid metabolite transformation was assayed according to established procedures [10, 12].

an isoelectric point of 8.3 [74]. This further distinguishes feminotropin from the known pituitary hormones which generally have isoelectric points below 8.0 (with the exception of lutropin that had an isoelectric point of 9.0 in our experiments).

Currently, work in our laboratory is concentrated on the purification of feminotropin. Three sources of starting material are used: normal female rat pituitary glands, growth medium from cultures of pituitary tumour cells (cell line $C_8$11RAP [53]) and transplanted pituitary tumour tissue (cell line $C_8$11RAP). The latter two sources are employed to yield a greater quantity of feminotropin for further work while the normal tissue is used as a control that the feminotropin produced by these tumour cells act similarly to the 'normal' hormone in the various assays.

The purification scheme includes chromatography on DEAE-Sephadex A-50, ammonium sulphate fractionation and chromatography on Sephadex G-75. The feminotropin purified in this way has been shown to be active in both HTC- and hepatocyte- (see below) assay systems. The elution pattern from Sephadex G-75 indicates a molecular weight of about 20,000 daltons and this figure is further indicated by gel electrophoresis in the presence of sodium dodecyl sulphate (SDS).

Figure 8 illustrates the low sedimentation coefficient (about 2 *s*) of feminotropin when assayed with the hepatocyte system. The low *s*-value is in agreement with the findings obtained with the HTC-cell-assay system.

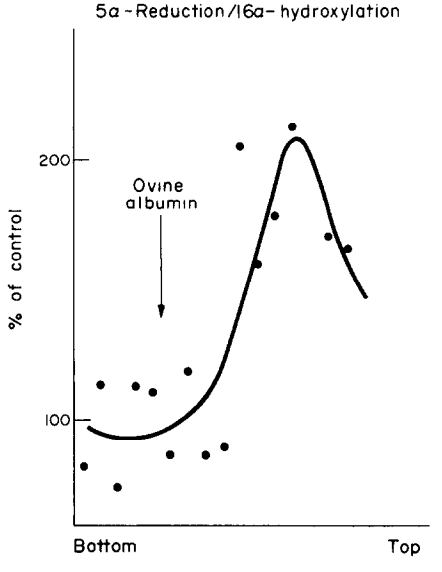

Fig. 8. Sucrose density gradient centrifugation of feminotropin. Pituitary granules prepared as described in legends to Fig. 6 and 7 were heated to 37°C for 30 min to liberate feminotropin. 0.30 ml solution was applied on top of a 5–20% (w/v) sucrose gradient and centrifuged at 190,000 *g* for 24 h in an SW 50.1 rotor. Ovine prolactin (3.7 *s*) was used as an external standard. Feminotropin activity was assayed using the isolated hepatocyte system (see legend to Fig. 5). Each fraction corresponded to the material present in 1/12 of a pituitary.

Concluding remarks

Hypothalamo-pituitary control of sexually differentiated liver metabolism in rats is mediated *via* a feminizing factor, feminotropin, and a masculinizing factor. Estrogenic and androgenic effects on the post-pubertal liver are exerted *via* feminotropin and the masculinizing factor and absence of the pituitary is combined with unresponsiveness of the liver to sex steroids. Reports are beginning to appear in literature on hypothalamo-pituitary control of classical sex steroid target tissues [*cf.* 76] and the question may be raised whether pituitary factors are also obligatory for full responsiveness of at least some of these target tissues to sex hormones. Friesen and collaborators have suggested that the pituitary secretes a factor that induces prolactin receptors in liver cells [77] and it may be speculated that other pituitary factors are involved in regulating levels of receptors for sex steroids in their target organs. The results presented in this paper that demonstrate an obligatory role of the pituitary in sex hormonal action in the liver, call for intensified efforts to clarify the significance of the pituitary in sex hormonal action in other mammalian target tissues.

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DISCUSSION

Dörner. We have observed a significant increase of life span in male rats castrated on the first day of life, but not on the 21st day of life. On the other hand, we have seen a significant decrease of life span in females treated with androgens perinatally. Therefore, I would like to ask if you think that such effects may also be mediated, at least in part, by "feminostatin".

Gustafsson. They may well be. It is possible that this novel factor (feminotropin) also has functions other than just regulating liver metabolism.

Dörner. A second question, please. Dr. Gustafsson, could you be so kind as to explain more exactly the localization of this "feminostatin producing region" in the brain?

Gustafsson. I am afraid I cannot do that. We have performed experiments to see whether the centre that is imprinted at birth in our experiments is identical to the centre that regulates cyclicity of gonadotropin patterns and male sexual behaviour. We have investigated possible imprinting effects on liver enzymes of dihydrotestosterone propionate, estradiol benzoate, testosterone propionate, epitestosterone

propionate and etiocholanolone propionate. With epitestosterone propionate and etiocholanolone propionate no imprinting is obtained. However, we observe imprinting both with testosterone propionate, dihydrotestosterone propionate and estradiol benzoate (Gustafsson J.-Å. and Stenberg Å.: Specificity of neonatal androgen-induced imprinting of hepatic steroid metabolism in rats, *Science* **191** (1976) 203–204). In contrast to these findings, male sexual behaviour and gonadotropin patterns have been reported not to be imprinted by dihydrotestosterone propionate but only by testosterone propionate and estradiol benzoate. These results may indicate that another centre is involved in imprinting of liver enzymes than that involved in regulation of male sexual behaviour and gonadotropin patterns. We are working on the exact localization of this centre. As I told you we have indications that there is an area in the periventricular region of the male brain where well defined lesions result in feminization of liver enzymes.

Naftolin. Can you be any more specific about the periventricular area?

Gustafsson. Not at the present time, I am afraid.

De Moor. Let me comment about the receptor studies in the hypothalamus and in the pituitary. Some time ago we have been looking for testosterone receptors in rat prostate and could not find them using sucrose gradient ultracentrifugation. However, when we used ammonium sulphate precipitation or gel filtration, i.e. separation techniques of short duration, we could clearly demonstrate that there was a testosterone receptor not only in rat prostate but also in rat uterus. Sucrose gradient ultracentrifugation takes about 18 h and during this long period, even at 4°, most of the added testosterone is metabolized.

Gustafsson. When working with steroid receptors I think it is essential not only to use *in vitro* techniques; you also have to use *in vivo* experiments. The results I presented to you have been obtained both from *in vitro* experiments and from *in vivo* experiments. In other words we have administered radioactive testosterone to castrated male and female rats and we have taken out various brain regions. We have then studied both radioactive metabolites of testosterone and labelled androgen-receptor complexes present in these regions, and even using this experimental approach we cannot show the presence of androgen receptor proteins in female rats. So it seems likely that during *in vivo* conditions the receptors in female rats are less significant than the receptors in male rats. Concerning the metabolism of androgens, our results are in good agreement with yours. From the *in vivo* studies it is quite evident that there is a much smaller metabolism of testosterone in the male rat brain than in the female rat brain and this may also help to explain the relative androgen unresponsiveness of the female rat brain.

De Moor. I agree, but may I ask you a second question. How did you add your hormones to the *in vitro* systems you used? Indeed, your negative results could be explained by the short half-life of the added hormones. You say that you added quite a lot of hormones to your *in vitro* system. But, how did you add them? Did you add them in superfusion, or once, or every two or three hours?

Gustafsson. Well, in most experiments they have been added at once. However, under the experimental conditions we used for our binding assays, there was negligible metabolism of added ligands, both in cytosol from male and female brain.