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IN VITRO METABOLISM OF TESTOSTERONE IN THE RAT BRAIN DURING SEXUAL MATURATION—III: STUDIES OF THE FORMATION OF MAIN ANDROSTANE-DIOLS AND ANDROSTENE-DIOLS

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SUMMARY

Tissue fragments obtained from hypothalamus, hypophysis, prostate and cerebral cortex of male rats were incubated in the presence of [1,2-^{3}H]-testosterone. The incubation media were subsequently extracted, and the following compounds: 5α-androstan-3α,17β-diol; 5α-androstan-3β,17β-diol; 5β-androstan-3α,17β-diol; 5β-androstan-3β,17β-diol; 4-androsten-3α,17β-diol; 4-androsten-3β,17β-diol, were identified and compared with testosterone. The largest amounts of 5α-androstan-3α,17β-diol were produced by the prostate and the hypophysis and of 4-androsten-3α,17β-diol by the hypothalamus. A peak of hypophyseal 5α-androstan-3α,17β-diol was observed in the 6-week-old rats; this phenomenon could be related to the peak of gonadotropin which has been demonstrated in the serum of rats at this age.

INTRODUCTION

In its target tissues, such as prostate and seminal vesicles, testosterone is metabolized into 5α-androstan-17β-ol-3 one† (5α-dihydrotestosterone, DHT), and 5α-androstan-3α,17β-diol (A3α) [1]. The presence of these compounds in the cell seems to be closely related to its androgenic activity [1, 2]. Other metabolites derived from the reduction of testosterone have also been demonstrated and shown to possess some biological activity [2–4]. It thus appeared interesting to study their formation in fragments of hypophysis and of hypothalamus obtained from rats of different age groups, and to correlate the variations of these concentrations to the sexual development and maturation in this species.

MATERIALS AND METHODS

I. *Techniques*

(a) *Animals.* Sprague–Dawley male rats were divided into four age groups, 3 weeks (immature), 4 weeks (prepubertal), 6 weeks (pubertal) and 9 weeks (adults) [5]. All animals were castrated 48 h before the experiments.

(b) *Steroids.* Nonradioactive steroids (T, DHT, A3α, A3β, E3α, E3β, 4-ene,3α,4-ene,3β) were supplied by Steraloids Inc., Pawling, New York [4-^{14}C]-labelled steroids (testosterone, 5α-dihydrotestosterone, 4-androsten-3,17 dione) with a S.A. of 50 mCi/mmol (C.E.A. Saclay) were used as tracer for recovery purposes.

[4-^{14}C]-androstanedione was obtained by oxidation of [4-^{14}C]-DHT using chromic anhydride.

The labelled standards were purified on silica gel thin layer plates in the system $CHCl_3$: ether (90:10 v/v).

Radioactive steroid samples were counted in an automated 3-channel Packard Tri-Carb scintillation spectrometer (model 3003). Each sample was counted in 10 ml of toluene containing 4·5 g/l Permablend III Packard (1% error on the counts).

(c) *Incubations.* The rats were decapitated and the tissue fragments immediately excised from the hypothalamus, the hypophysis, the frontal cortex (control tissue) and the ventral lobe of the prostate (typical target tissue). For each age group, the same amount of tissue from each organ was used: 80–100 mg of hypophysis, 180–210 mg of hypothalamus or cerebral cortex, 300–400 mg of prostate, obtained from 10 to 20 rats depending upon their size. A single incubation was performed for each type of organ and for each age group.

Tissue samples were placed in Krebs bicarbonate buffer pH 7·4 [7] (1 ml per 25 mg of tissue) containing

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† *Abbreviations and trivial names*: T = testosterone = 4-17β-hydroxy-4-androsten-3-one; DHT = dihydrotestosterone = 5α-androstan-17β-hydroxy-3-one; *Androstanediols*; A3α = 5α-androstan-3α,17β-diol; A3β = 5α-androstan-3β,17β-diol; 5β *Androstanediols*: E3α = 5β-androstan-3β,17β-diol; E3β = 5β-androstan-3β,17β-diol; *Androstenediols*: 4-ene, 3α = 4-androstene-3α,17β-diol; 4-ene, 3β = 4-androstene-3α,17β-diol; *Diones*: Androstenedione = 4-androstene-3,17-dione; Androstanedione = 5α-androstane-3,17-dione; 5β-androstanedione = 5β-androstane-3,17-dione.

20 ng of [1,2-^{3}H]-testosterone per ml (S.A., 42 Ci/mmol). Incubations were carried out at 37°C for 2 h in an atmosphere containing 95% O_2, in a Dubnoff type metabolic shaker. The incubations were terminated by separating the media from the fragments and all fractions were stored frozen at −20°C until analyzed.

II. *Extraction of the fraction of higher polarity than testosterone*

After addition of cold and radioactive carriers (listed in Fig. 1), all steroids were extracted from the incubation media with chloroform; subsequently, after a phenolic partition, they were separated by paper chromatography (Whatman 3 MM) in a hexane–methanol–water system (100:90:10, v/v). The area situated from the origin to the testosterone (detected by the use of a standard) was eluted. This fraction containing testosterone and all the compounds of lesser mobility in this first system were then chromatographed on silica gel thin layer plates using a system chloroform–ethanol (95:5 v/v) (Fig. 1). Two fractions were obtained: (a) one corresponding to testosterone and (b) one of the less mobile compounds, *i.e.* more polar than testosterone.

The fraction migrating as testosterone was then eluted and re-chromatographed on Whatman n° 2 paper using a system hexane–benzene–methanol–water (666:333:800:200 by sol). This system was able to separate testosterone from 5β-androstan-3β,17β-diol and from epitestosterone.

The compounds less mobile than testosterone obtained in both silica thin layer plates and Whatman n° 2 paper systems were then pooled for further analysis (Fig. 1).

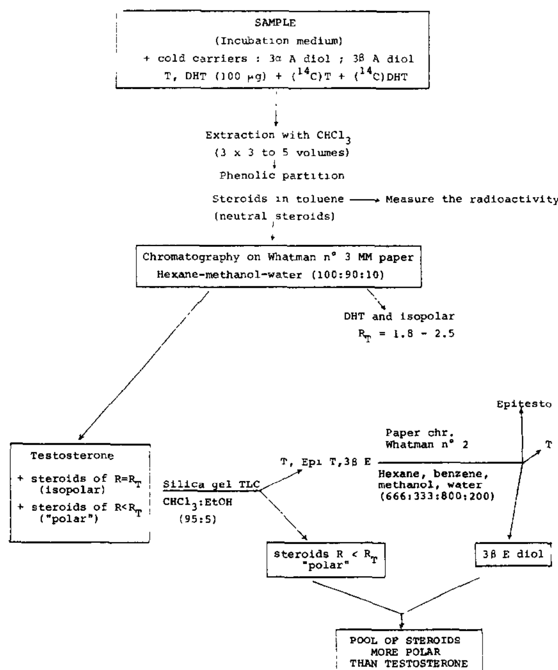

Fig. 1. Method of obtaining the polar metabolites of testosterone.

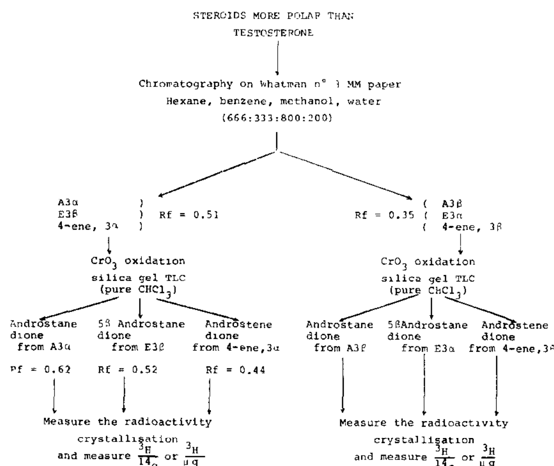

Fig. 2. Method of separation of the androstane and androstenediols (A3α, A3β, E3α, E3β, 4-ene, 3α, 4-ene, 3β).

III. *Separation and characterization of the diols* (Fig. 2)

To our knowledge, there is not, at the present time, a system offering a good resolution for the separation of the six diols that we have studied, i.e. A3α, A3β, E3α, E3β, 4-ene-3α and 4-ene 3β. Savard[8] has also shown that attempts to separate androstane(5α),3α-ol from androstane(5β),3β-ol were not successful.

Alternatively, we have been able to separate the oxide derivatives of these compounds from one another, namely androstanedione from 5β-androstanedione and this latter from androstenedione.

It thus appeared desirable to separate first the six diols into two groups which would be then separately oxidized prior to silica gel thin layer chromatography. Each of these two groups should include one of the 5α-androstane (3α or 3β ol, 17β ol), one of the 5β-androstane (3α or 3β ol, 17β ol) and one of the 4-ene-androstenediols (3α or 3β ol, 17β ol) since their oxide derivatives can be separated on the silica gel system. It has been demonstrated [9] that using paper chromatography on Whatman n° 2 with the hexane–benzene–methanol–water (666:333:800:200 by vol.) system it is possible to separate a group of compounds of lesser mobility containing the 3β (5α) and the 3α(5β). Consequently this procedure was used to separate the pool of the six diols into two fractions of $R_f = 0{\cdot}51$ and $R_f = 0{\cdot}35$ respectively (Fig. 2). Both fractions were then separately oxidized according to the following procedure. The dry extracts were dissolved in 0·2 ml of acetic acid, and 0·1 ml of a 2% chromic anhydride solution in water was added to the samples which were then allowed to stand for 2 h at room temperature in darkness. At the end of the oxidation, the mixture was neutralized with 1 *N* NaOH, the steroids extracted with ether and finally chromatographed on silica gel thin layer plates in order to separate the diones (Fig. 2).

Recovery was determined by measuring by the Zimmerman method, in the oxide derivative extracts, the remaining fraction of a known amount of non

Table 1. Testosterone and its more polar metabolites extracted from incubation medium of rat hypophysis, prostate, hypothalamus and cerebral cortex

Hypophysis	AGE OF RATS (weeks)			
	3	4	6	9
Testosterone	70.5	65.2	62.7	79.1
Polar	17.8	-	25.4	13.3
Total diols	5.7	4.2	12.0	3.8
N.I.	12.1	-	13.4	9.5

Prostate	AGE OF RATS (weeks)			
	3	4	6	9
Testosterone	36.8	54.7	40.2	60.6
Polar	19.8	-	42.4	19.3
Total diols	5.5	2.3	10.5	1.7
N.I.	14.3	-	31.9	20.0

Hypothalamus	AGE OF RATS (weeks)			
	3	4	6	9
Testosterone	90.0	96.3	84.1	85.2
Polar	5.9	-	21.9	13.1
Total diols	2.0	2.2	3.4	2.0
N.I.	3.9	-	18.5	11.1

Cerebral cortex	AGE OF RATS (weeks)			
	3	4	6	9
Testosterone	88.7	94.8	79.5	92.9
Polar	8.7	-	22.0	4.7
Total diols	2.6	2.0	1.2	1.2
N.I.	6.1	-	20.8	3.2

Percentage of the total radioactivity extracted by toluene. The non-identified fraction (N.I.) is the polar fraction less the total diols.

radioactive steroids which had been added to the medium prior to the chloroform extraction. It was considered that all steroids of both groups, extracted by the same procedure, had the same recovery.

Purity was checked in two groups, for androstanediones and androstenediones after successive recrystallizations in the presence of ^{14}C-androstanedione and ^{14}C-androstenedione until the ratio ^{3}H/^{14}C remained constant; and for 5β-androstanediones by determination of the S.A. (^{3}H/μg of added cold steroids) in both crystals and mother liquors. Complete disappearance of ^{3}H in the crystals was expressed in terms of non detectable (ND) amounts in the tables. In addition the minimal significant ^{3}H radioactivity which could be safely counted in the samples and which corresponded to five times the background was 0·1% of the starting radioactive material. Any value found between "non detectable" and 0·1% is listed <0·1 in the tables.

RESULTS

Results given in Tables 1 and 2 are expressed in percent of the total activity. The total activity is the

Table 2. The polar metabolites of testosterone extracted from the incubation medium

Hypophysis	AGE OF RATS (weeks)			
	3	4	6	9
DHT	6.9	3.4	2.3	1.9
A3α	4.0	2.0	8.6	1.2
A3β	0.3	0.4	1.7	0.2
E3α	N.D	<0.1	<0.1	0.2
E3β	0.5	<0.1	<0.1	0.1
4-ene,3α	0.3	0.7	1.3	1.3
4-ene,3β	0.5	1.1	0.3	0.8

Prostate	AGE OF RATS (weeks)			
	3	4	6	9
DHT	30.8	4.6	11.8	16.9
A3α	4.5	1.3	4.6	0.2
A3β	0.4	0.2	1.4	0.1
E3α	N.D	<0.1	0.1	N.D
E3β	N.D	<0.1	0.5	0.7
4-ene,3α	0.6	0.4	3.9	0.5
4-ene,3β	0.1	0.4	0.3	0.2

Hypothalamus	AGE OF RATS (weeks)			
	3	4	6	9
DHT	1.8	0.3	1.3	0.9
A3α	<0.1	0.4	1.3	0.3
A3β	N.D	0.1	<0.1	N.D
E3α	N.D	<0.1	<0.1	<0.1
E3β	0.1	<0.1	0.1	<0.1
4-ene,3α	1.9	0.5	1.8	1.5
4-ene,3β	<0.1	1.2	0.2	0.1

Cerebral cortex	AGE OF RATS (weeks)			
	3	4	6	9
DHT	0.4	<0.1	0.6	0.3
A3α	0.1	0.2	<0.1	0.2
A3β	<0.1	<0.1	<0.1	0.1
E3α	0.1	<0.1	<0.1	<0.1
E3β	<0.1	<0.1	<0.1	0.4
4-ene,3α	1.0	0.8	0.6	0.2
4-ene,3β	1.4	0.9	0.7	0.3

The values are given as percentages of the total radioactivity extracted in toluene after phenolic partition and corrected for the recovery.
The results of DHT are taken from Loras *et al.*[6] for comparison.
All steroids have been recrystallized.
N.D.: non detectable.

one measured in the media extracted by toluene after phenolic partition. The values obtained for the compounds more polar than testosterone (listed on "polar" in the tables) are higher when measured just after their separation from testosterone than the ones (called total diols) calculated by addition of the numbers found for each diol after the separation of their oxide derivatives. The difference is called "non identified" (N.I.) steroids. The presence of such non identified steroids could be detected in all media obtained from the incubation of all tissues including the cerebral cortex (Table 1). The nature and the role of these compounds was not elucidated in the present study.

(a) *Total diols* (Table 1). All six diols studied were recrystallized. Their content, expressed in per cent of the starting radioactivity, could thus be determined with accuracy.

Values obtained with the hypophysis and the prostate were higher than the ones found in the other tissues regardless of the age group.

In addition, in the hypophysis, the hypothalamus and the prostate, their level was higher for the six-week-old rats than for the three- and nine-week-old ones, whilst in the cerebral cortex, used as tissue control, this level was relatively low and remained constant.

(b) *Individual diols* (Table 2). There was practically no 5β-androstane-3α/3β, 17β diol in any of the four tissues. In the hypophysis and the prostate, the overall analysis showed that the tissues produced mainly A3α and DHT. In the prostate, the A3α produced was the same at 3 weeks and 6 weeks whereas in the hypophysis it was clearly higher at 6 weeks than at 3, 4 and 9 weeks. The production of A3β was identical for each age group in the hypophysis and the prostate. DHT and A3α production followed a similar evolution in relation to age and were proportional except in two instances, namely: in the 6 week rat hypophysis the peak of A3α was very sharp, and in adult prostate there was no longer a production of A3α.

In the hypothalamus and the cortex, the overall analysis showed that these tissues were not very active in metabolizing testosterone but they elicited a different metabolic pattern. The hypophysis and the prostate mainly formed androstanediols, whilst the cortex and the hypothalamus mainly formed androstenediols. However there is no apparent relationship between the rate of production of these metabolites and the age of the rats.

DISCUSSION

The nature of the components more polar than testosterone is open to debate. Produced by the metabolism of testosterone, they could be oestrogens or polyhydroxylated compounds or dihydroxylated compounds, *i.e.* 5-ene-diols, diols in which the 17 OH group is in the α position and diols in which the 17 OH group is in the β position. Their oestrogenic nature can be eliminated since oestrogens are separated from polyhydroxylated and dihydroxylated steroids by phenolic partition. Also, it is very unlikely that during the incubations, 5-ene-diols could have been obtained from testosterone since the reaction 5-ene$\rightarrow$4-ene has been shown to be irreversible [10]. Since no epitestosterone could be detected in the incubation media, the 17 hydroxylated steroids present were likely to be hydroxylated in the β and not in the α position. Polyhydroxylated steroids were probably separated from the diols by chromatography, for only the zone opposite to diols and oxidized standards was eluted. The non identified polar steroid fraction was probably composed of these products.

Prostate, hypophysis and nervous tissues showed a decreasing capacity to metabolize testosterone confirming our earlier studies [6]. In the four tissues studied, 5β reductase activity was present but always low. The presence of DHT, A3α, and A3β in the prostate, hypophysis and hypothalamus was detected as in other reports [11, 12]. The metabolic pathway of testosterone is the same in the hypophysis and in the prostate. However, although the values obtained for A3α were equivalent in the hypophysis and the prostate, the amount of DHT produced by the hypophysis was much lower than the one found with the prostate. According to the classical sequence T$\rightarrow$DHT$\rightarrow$A3α, it seems possible that in the prostate the metabolism of T is essentially into DHT whilst in the hypophysis DHT is actively transformed into A3α.

The age group corresponding to sexual maturity was characterized by a higher production by the hypophysis and the prostate of the compounds reduced in the 3 position; this result suggests a possible activation of the C_3 reductases during puberty.

In our data, the relatively large amount of A3α found in the incubation medium of prostatic tissue can be explained by a regulation mechanism as the one suggested by Bruchovsky and Wilson[13]. They attributed to the compound A3α a role as inactive metabolite, which would prevent an excessive accumulation of the active hormone. However in organotypic cultures of prostate it appeared that A3α was not completely devoid of biological activity [11].

From the present study of steroid metabolism in the hypophysis it appears that A3α might play an important role in the hypothalamo-pituitary-gonadal regulation as suggested by the increase of this compound in the incubation media of 6-week-old rats. Although a single incubation for each age of rats was performed, in all of them, pooled tissues from 10 to 20 rats were used; in addition, we measured other testosterone metabolites in several incubations for each age group [6] and found a reasonable reproductibility. For these reasons, it seemed justified to think that spontaneous variations between animals were minimized and that the observed differences were related to ages. It should also be noted that only the media of incubation were studied in the present report. The data obtained concern only the steroids

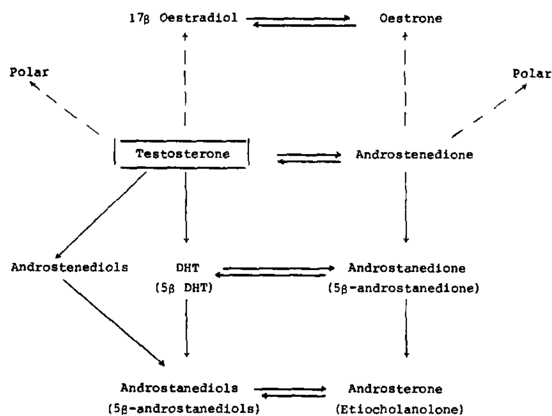

Fig. 3. Testosterone metabolism, after 21 and 22, with modifications.

released in the media regardless of the amount which could still be bound to the cells at the end of the incubation. The fluctuations of A3α observed in the incubation media of the hypophysis do not seem to correlate with the increase of LH plasma concentration reported during the course of maturation [16]. However it is interesting to note that the increase of A3α that we have found around the 40th day coincides with the fall of hypophyseal LH observed by Lisk[17] in male rats and also to the liberation of LH *in vitro* reported by Spona *et al.*[18]. However other experimental data are somehow contradictory; while the present study was in progress, Zanisi *et al.*[14] reported that in adult male castrated rats there is a decreasing inhibitory action on LH release by the following compounds: A3α > A3β > DHT > Testosterone. These data were obtained with high doses of steroids (0·75 to 3 mg/rat/day). Moreover Sar and Stumpf[15] have shown a selective incorporation of ^{3}H-testosterone in the LH and FSH cells of the pituitary gland of immature male and female rats and in adult castrated male rats. Recently, Swerdloff[15] has described a peak of FSH in the serum of 6 week old rats while we were able to demonstrate a rise in the production of A3α by the hypophysis of the rats of the same age group.

Androstenediols have been reported by Dorfman *et al.*[19] and by Liao and Fang[3] to be biologically active. In the hypothalamus and the cortex, the metabolic pathway, T → androstenediols, acting as a bypass for DHT, predominates over the pathway T → DHT: the role of these androstenediols in the nervous tissues has not yet been elucidated. However, this mechanism could possibly lead to the formation of aromatic compounds, since (a) only minute amounts of diols are produced and (b) it is known that the hypothalamus is capable of aromatization [20]. These two steroids could both play a role in the cortex maturation as their levels decrease progressively from 3 to 9 weeks.

The results obtained from the present study can be reconciled in a diagram describing some aspects of the metabolism of testosterone in nervous tissues (Fig. 3). This scheme parallels those suggested by Samuel in liver [21] and Eberlein in such non endocrine tissues as liver, skin and skeletal muscles [22].

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Note added in proof

Tissues from two groups of fifteen 6-week-old male rats, castrated for 24 h and 48 h respectively were subsequently incubated as described. The results agreed with the data already obtained for this age group.

Tissue	Hypophysis		Hypothalamus		Cerebral cortex	
Castrated for	24 h	48 h	24 h	48 h	24 h	48 h
Testosterone	55·1	60·2	62·5	70·5	81·3	77·3
D.H.T.	10·0	6·5	—	2·8	0·5	0·8
Total diols	13·4	7·4	11·1	8·2	6·3	6·7
A 3α	9·5	5·9	5·9	3·9	1·5	2·9
A 3β	1·2	0·8	1·8	0·7	0·5	0·6
E 3α	<0·1	ND	<0·1	<0·1	<0·1	<0·1
E 3β	<0·1	<0·1	<0·1	ND	ND	ND
4-ene,3α	0·9	0·4	0·5	0·7	0·6	0·6
4-ene,3β	1·8	0·4	2·9	2·8	3·6	2·5

REFERENCES

1. Wilson J. D. and Gloyna R. E.: *Recent Prog. Horm. Res.* **26** (1970) 309–336.
2. Robel P., Lasnitzki I. and Baulieu E. E.: *Biochimie* **53** (1971) 81–96.
3. Liao S. and Fang S.: *Vitam. Horm.* **27** (1969) 17–90.
4. Eckstein B., Golan R. and Shani J.: *Endocrinology* **92** (1973) 941–945.
5. Monbon M., Loras B., Reboud J. P. and Bertrand J.: *J. steroid Biochem.* **5** (1974) 417–423.
6. Loras B., Genot A., Monbon M., Buecher F., Reboud J. P. and Bertrand J.: *J. steroid Biochem.* **5** (1974) 425–431.
7. Krebs H. A.: *Biochim. biophys. Acta* **4** (1960) 249–269.
8. Savard K.: *Recent Prog. Horm. Res.* **9** (1954) 186–211.
9. Kochakian C. D. and Stidworthy G.: *J. biol. Chem.* **199** (1952) 607–612.
10. Talalay P. and Wang V. S.: *Biochim. biophys. Acta* **18** (1955) 300–301.
11. Robel P.: *Acta endocr., Copenh.* Suppl. 153 (1971) 279–294.
12. Massa R., Stupnicka E., Kniewald Z. and Martini L.: *J. steroid Biochem.* **3** (1972) 385–399.
13. Bruchovsky N. and Wilson J. D.: *J. biol. Chem.* **243** (1968) 2012–2021.
14. Zanisi M., Motta M. and Martini L.: *J. Endocr.* **56** (1973) 315–316.
15. Sar M. and Stumpf W. E.: *Endocrinology* **92** (1973) 631–635.
16. Swerdloff R. S., Jacobs H. S. and Odell W. D.: In *Gonadotropins* (Edited by B. B. Saxena, C. G. Beling and H. M. Gandy). Wiley Interscience, New York (1972) pp. 546–566.
17. Lisk R. D.: *Neuroendocrinology* **3** (1968) 18–24.
18. Spona J. and Luger O.: *Fedn Eur. Biochem. Soc. Lett.* **32** (1973) 49–51.
19. Dorfman R. I., Dorfman A. S. and Gut M.: *Acta endocr., Copenh.* **40** (1962) 565–570.
20. Naftolin F., Ryan K. J. and Petro Z.: *Endocrinology* **90** (1972) 295–298.
21. Samuels L. T.: In *Metabolic Pathways* (Edited by D. M. Greenberg). Academic Press, New York (1960) pp. 431–480.
22. Eberlein W. R., Winter J. and Rosenfield L.: In *Hormones in Blood* (Edited by C. H. Gray and A. L. Bacharach). Academic Press, New York, Vol. II, pp. 187–220.