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Müllerian-Inhibiting Activity of Calf Fetal Testes: Relationship to Testosterone and Protein Synthesis

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ABSTRACT

Müllerium-inhibiting activity was detected in incubation media of calf fetal testicular tissue. Dialysis of the medium did not modify its biological activity, but testosterone content of dialyzed medium was diminished by 92 percent. Four-fold concentration of incubation medium greatly enhanced its biological activity, which was destroyed by heat and blockage of sulphydryl groups by 0.5 M iodoacetic acid. Addition of cycloheximide to the medium during incubation decreased its biological activity, indicating that part of the activity is due to hormone newly synthesized during the incubation. These results prove that the anti-müllerian hormone is a macromolecule and suggest that it is probably a protein.

INTRODUCTION

The biochemical nature of the hormone responsible for the regression of the müllerian ducts of the male fetus has not yet been elucidated. Since the work of Jost, who in 1947, showed in fetal rabbits that the müllerian inhibitor is different from testosterone, progress in this direction has been slow, limited to negative findings indicating that neither the 17-hydroxylated metabolites of testosterone (Josso, 1971) nor prostaglandins E_1, E_2, $F_2\alpha$ (Jost et al., 1973) A_2 and B_1 (Josso, 1974) can be identified with the mammalian anti-müllerian hormone (A.M.H.). The macromolecular nature of this hormone was suggested by the inability of fetal testicular tissue to inhibit its target-organ *in vitro* when separated from it by a dialysis membrane (Josso, 1972a).

Further progress was limited by the experimental conditions used up to now, in which hormone secretion and bioassay take place simultaneously. This is suitable for the physiological study of the anti-müllerian activity of tissues but, to investigate the biochemical nature of A.M.H. it is necessary to obtain the active hormone in a liquid medium. We have found that müllerian-inhibiting activity can be detected in incubation media of calf fetal testes and we have studied its response to various treatments designed to alter protein synthesis, concentration or configuration.

MATERIALS AND METHODS

Testicular tissue was obtained at a local slaughterhouse from 384 fetal calves, 6 to 65 cm in crown-rump length, and incubated 4 h at 37 C in a Dubnoff metabolic shaker, set at 80 oscillations per min, in a O_2-CO_2 (95:5) atmosphere. U ^{14}C L-leucine (sp act 297 mci/mM) purchased from the Centre de l'Energie Atomique (Saclay, France) was added at a 1 μ ci/ml concentration to Eagle's minimum essential medium deprived of leucine, but containing 0.5 percent fetal calf serum. Five ml of this incubation medium was used per g of tissue. Incubations were performed twice in the presence of cycloheximide (0.035 M) and once in the presence of actinomycin D (0.008 M).

At the end of the incubation, medium was collected by centrifugation at 12,000 g for 10 min at +4 C, and frozen. Media were pooled, usually in batches of 50 to 100 ml and dialyzed against non-radioactive Eagle's medium Sample volume was not significantly altered by dialysis. The pools were then concentrated four-fold by ultrafiltration on an Amicon ®UM 20 E membrane (mol. cutoff 20,000 according to manufacturer). In each of the 11 pools, before and after dialysis and after concentration, radioactivity was monitored in an Intertechnique SL 30 spectrometer (Plaisir, France) after addition of 5 ml Instagel® emulsifier solution (Packard Instruments Co.) to the sample. Quenching was determined by external standardization and appropriate corrections were made. Testosterone content was determined by radioimmunoassay (Forest et al., 1973). The specificity of the method was checked by measuring the testosterone content of one pool, before and after dialysis, by the double isotope derivative method (Rivarola and Migeon, 1966). The ^{14}C/^{3}H ratio obtained at the end of the procedure remained constant throughout 5 successive recrystallizations.

In some cases, after the incubation, the medium was subjected to procedures designed to alter protein

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configuration: exposure to heat (80 C during 30 min), to dithioerythritol 0.001, 0.002, 0.005 M, and to sulfhydryl-blocking agents (iodoacetic acid 0.5 M and N-ethylmaleimide 0.001 and 0.05 M). Alkylation was allowed to proceed during 4 h at +4 C and pH 8, and the excess reagent was then removed by dialysis.

Müllerian-inhibiting activity was studied by the method of Picon (1969) in all pools after concentration, in 4 before and in 5 after dialysis. After passage through a 0.45 μ Millipore filter, the medium was placed in the well of a Falcon® organ culture dish, and 4 to 6 reproductive tracts of 14.5-day-old fetal rats of both sexes were explanted on its grid after removal of the gonads. After 3 days, the explants were fixed and serially sectioned. One section out of ten, representing a total of 25 to 35 sections for each explant, was mounted and histologically examined. The correspondence between slide number and experimental procedure was not known to the observer at this time.

The degree of müllerian duct regression was scored, on each individual section of the slides, according to the following criteria: (1) complete regression: the duct epithelium is replaced by a fibrous whorl (Figs. 1 and 2); (2) partial regression: duct epithelium is still visible but its height is decreased, and it rarely contains dividing cells. The lumen is narrow, surrounded by a fibroblastic ring (Fig. 3); (3) no regression: müllerian and wolffian ducts have approximately the same width, müllerian epithelium is normal and shows frequent mitotic figures. Cell density is increased around the duct but the cells do not have the elongated morphology characteristic of fibroblasts (Figs. 4, 5, 6).

The scores attributed to the müllerian duct in each section are used to draw a reconstruction of the müllerian system of a given reproductive tract. This reconstruction serves to assess the degree of müllerian inhibition in the reproductive tract, considered as a whole.

Tracts showing no alteration of the müllerian duct on either side are considered *normal* (Figs. 5 and 6).

Total inhibition is defined as complete regression of the müllerian duct on both sides and on the whole length of the tract, except in the immediate vicinity of the urogenital sinus (Figs. 1 and 2).

Incomplete inhibition is said to occur when müllerian regression is heterogeneous, asymetrical, and/or limited to the anterior segment of the ducts, more sensitive to A.M.H. at this stage of development (Figs. 3 and 4). Because the same concentration of A.M.H. does not always evoke an identical response in individual fetal rat reproductive tracts (Josso, 1972b), we did not attempt to recognize degrees in the incomplete type of inhibition of the müllerian duct system, and therefore we cannot measure small variations of müllerian-inhibiting activity.

RESULTS

Testosterone Concentration

Mean testosterone concentration in media fell from 174.3 ± 44.6 ng/ml before dialysis to 14.4 ± 7.8 ng/ml after dialysis; i.e. 8 percent of the initial amount. Ultrafiltration produced a slight rise in the testosterone concentration of the retentate, with a mean of 24.2 ± 12.2 ng/ml, which represents 13 percent of the initial figure. The difference is probably due to binding of testosterone to proteins present in the medium. When cycloheximide was added during incubation, testosterone content of the dilute, non-dialyzed medium was 160 and 121.6 ng/ml on two separate occasions. Cooke et al. (1974) have reported that cycloheximide does not affect basal testosterone secretion by isolated interstitial tissue. After addition of actinomycin D to the medium its testosterone content was 84.5 ng/ml before dialysis.

Radioactivity

Mean radioactivity due to ^{14}C L-leucine expressed as 10^2 d/min/ml, was 7,906 ± 1,749 before dialysis and 162 ± 53 after this procedure. The latter figure is a reflection of the amount of newly synthesized proteins released into the medium. After four-fold concentration, ^{14}C counts increase to 542 ± 133 × 10^2 d/min/ml. Protein synthesis was severely limited by cycloheximide, with only 17 × 10^2 and 20 × 10^2 d/min/ml of dialyzed and concentrated medium but was not significantly affected by actinomycin D, with 360 × 10^2 d/min/ml of dialyzed and concentrated medium.

Müllerian-inhibiting Activity

The effect of dialysis, concentration and various forms of protein denaturation on the müllerian-inhibiting activity of the medium is shown on Table 1. Dialysis did not modify the biological activity, which was dramatically enhanced after four-fold concentration. Biological activity was destroyed by heat and concentrated iodoacetic acid, diminished by N-ethylmaleimide 0.05 M, and cycloheximide, and unaffected by N-ethylmaleimide 0.001 M and dithioerythritol 0.001 and 0.002 M. Similar concentrations of these agents added to plain Eagle's medium had no effect upon the müllerian duct. Actinomycin 0.008 M and higher concentration of dithioerythritol resulted in necrosis of the explants.

DISCUSSION

Our results show that müllerian-inhibiting activity is released into the incubation medium by calf fetal testicular tissue. Biological activity is not affected by dialysis, which reduces testosterone concentration by 92 percent. The

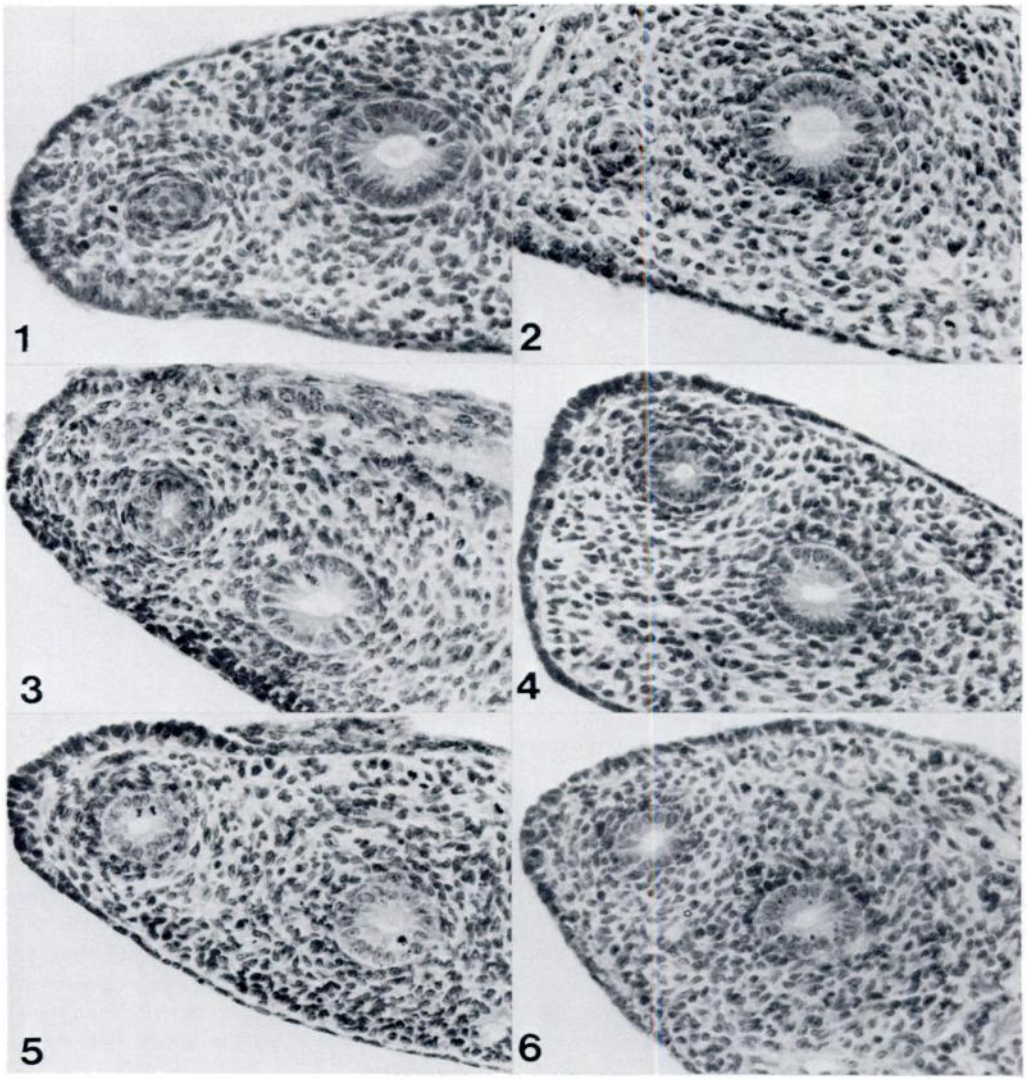

FIG. 1. Anterior segment of a 14.5-day-old fetal rat reproductive tract cultured in the presence of dialyzed and X 4 concentrated medium (pool 261: testosterone content 28.8 ng/ml, radioactivity 594 X 10^2 d/min/ml): note complete regression of mullerian duct.

FIG. 2. Same tract, middle segment: regression of the mullerian duct persists.

FIG. 3. Anterior segment of a 14.5-day-old fetal rat reproductive tract, cultured in the presence of pool 261 after X 4 concentration and treatment by N-ethylmaleimide 50 mM: The mullerian duct is narrow, the epithelial cells are shrunken, surrounded by a fibroblastic ring.

FIG. 4. Same tract, middle segment. Mullerian duct is normal.

FIG. 5 and 6. Anterior and middle segment of a 14.5-day-old rat fetal productive tract, cultured in the presence of pool 261 after X 4 concentration and heating at 80 C for 30 min. Müllerian duct is normal: Note that the cells surrounding the epithelium though their density is increased, are not spindle shaped as in Fig. 3.

lack of correlation demonstrated between testosterone and müllerian-inhibiting activity is probably also true for other steroids, since the likelihood for the concentration of a small molecule to be higher after dialysis and ultrafiltration than it was initially, is small. This confirms our previous conclusions regarding the macromolecular nature of mammalian A.M.H. (Josso, 1972a), and contradicts the opinion of Price and Ortiz (1965) who believed that androgen is responsible for the müllerian inhibition seen in male fetal rats and guinea-pigs. The relationship between the mechanism of müllerian inhibition in mammals and in birds is still

TABLE 1. Influence of dialysis, concentration and protein denaturing agents on müllerian-inhibiting activity of incubation media.

Treatment of medium	Number of rat reproductive tracts studied	Degree of müllerian inhibition		
		Complete	Incomplete	None
Dilute medium[a]				
Not dialyzed	20	0	9	11
Dialyzed	20	0	11	9
× *4 Concentrated*[b] *medium*				
Control	66	59	7	0
80 C 30 min	6	0	0	6
Iodoacetic acid .5 M[c]	6	0	0	6
NEM[d] .001 M	6	6	0	0
NEM .05 M	12	0	8	4
DTE[e] .001 M	18	16	2	0
DTE .002 M	6	6	0	0
Cycloheximide .035 M[f]	12	5	7	0

[a] 5 ml per g of tissue.

[b] after dialysis.

[c] added after incubation and before dialysis.

[d] N-ethylmaleimide, added after incubation and before dialysis.

[e] Dithioerythritol, added after incubation and dialysis.

[f] added during incubation and before dialysis.

open to discussion since Wolff et al. (1952), and more recently Stoll et al. (1972) and Lutz-Ostertag (1974) have used testosterone to produce selective lysis of avian müllerian ducts *in vitro*.

In view of the suggested correlation between müllerian-inhibiting activity and the concentration of newly synthesized proteins in the medium, we studied the effect on the former of inhibitors of protein synthesis. The deleterious effect of actinomycin D on the survival of the explants did not allow us to evaluate its influence on the müllerian-inhibiting activity of the medium. Cycloheximide decreased biological activity, indicating that part of it at least is due to hormone newly synthesized during incubation.

In order to further support the hypothesis of the protein nature of A.M.H. we studied the effect on müllerian-inhibiting activity of various protein denaturing agents. Heating, and blockage of thiol groups by concentrated iodoacetic acid, destroyed biological activity, but another sulphydryl reagent, N-ethylmaleimide, was less effective at the lower concentrations generally used. As the experiment was not performed on purified A.M.H., other substances present in the medium may have interfered in the alkylation reaction. For example, Matthews and Williams (1974) have reported that NAD protects an active thiol group of pig heart lipoamide dehydrogenase against 0.1 M iodoacetamide. Conclusions regarding the role of disulphide bonds cannot be reached at the present time since biological activity was not affected by low concentrations of dithioerythritol, and higher amounts of the reducing agent were toxic in our bioassay.

A.M.H. is produced by fetal Sertoli cells (Josso, 1973; Blanchard and Josso, 1974) which have received less attention than their post-natal counterparts. For instance, adult Sertoli cells are thought to secrete specific proteins into testicular fluid (Setchell, 1968). The best known of these, androgen-binding protein (ABP) is detected in the rat epididymis 3 weeks after birth (Hansson et al., 1973), shortly after the development of the blood-testis permeability barrier (Vitale et al., 1973), whereas the rat testis loses its anti-müllerian activity 4 days post-natally (Picon, 1970). Wilson (1973) has produced evidence for the presence of an evanescent testosterone-binding protein in the ducts and mesonephros of fetal rabbits but since no sex difference was found, it probably bears no relationship to A.M.H.

In conclusion, the thermolability and partial sulphydryl dependence of the macro-molecule responsible for müllerian duct inhibition in the male fetus, indicate that A.M.H. is probably a protein. Definite proof of its identity and deeper insight into its structure-activity relationship will be possible only when a quantitative assay of its biological activity becomes available and when the hormone can be studied in a purified form.

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