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TESTOSTERONE "RECEPTORS" IN PURIFIED NUCLEI OF RAT ANTERIOR HYPOPHYSIS

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SUMMARY

In this work, the presence of two macromolecular associations of [^{3}H]-testosterone in purified nuclei of rat anterior hypophysis was demonstrated.

After incubation of slices of hypophyseal tissue with [^{3}H]-testosterone purified nuclei were obtained free from cytoplasmic contamination. After extraction with M NaCl and gel filtration on Sephadex G 200, two peaks of bound radioactivity were obtained. The first, with a concentration of $0{\cdot}6 \times 10^{-13}$ mol by mg of proteins is excluded from gel Sephadex G 200, the second with a concentration of $2{\cdot}1 \times 10^{-13}$ mol by mg proteins is retained on this gel. There is no binding with histones. The radioactive material bound to the nuclear "receptors" is mainly testosterone.

The amount of dihydrotestosterone is small. Again, we have noted the presence of a non polar steroid. They represent respectively 66, 7 and 7% of the radioactivity of the chromatogram.

INTRODUCTION

WE PREVIOUSLY demonstrated the presence of a macromolecular association of (^{3}H)testosterone in the cytoplasm of the anterior hypophysis and the hypothalamus of normal and castrated rats[1, 2]. We studied several characteristics of these "receptors", and determined the nature of androgens bound to cytoplasmic macromolecules[3, 4]. Continuing our research we sought to find a macromolecular binding of the hormone in the purified nuclei of anterior hypophysis of normal rats.

MATERIAL AND METHODS

Biological material

Male wistar rats 70 days old were used. They were killed by decapitation without anaesthesia.

[^{3}H]-*testosterone*. Supplied by C.E.N. (Belgium). (Specific Activity 42Ci/mmole). The original solution; Benzene–ethanol (90 : 10v/v) was evaporated to dryness under vacuum and the dry residue was dissolved in ethanol at 95°C. This solution was then diluted by a 0·05 mol/l phosphate buffer at pH 7·4 and used for incubating with slices of hypophysis.

The radiochemical purity of (^{3}H)testosterone was checked by paper chromatography using the Kochakian and Stidworthy solvent system[5].

Method of incubation

During each experiment 12–24 rat anterior hypophysis were rapidly collected, weighed and each divided in two. They were incubated in 3 ml of Krebs–Ringer bicarbonate-glucose buffer containing 75 ng of tritiated testosterone (24×10^6 dpm).

Incubations were performed at 37°C under O_2 shaking for 30 min. After this,

slices were removed from the medium, washed with cold Krebs solution and used for isolating the nuclei.

Preparation of the nuclei

Nuclei were prepared by a method based on that of Widnell and Tata[6] modified by Dutton and Mahler[7]. All operations were performed at 4°C. At lower temperatures a change in the viscosity of sucrose results in an important loss of nuclei. The slices of hypophysis were homogenized with a Dounce homogenizer in 0·32 M sucrose which contained 3 mM $MgCl_2$(pH 6·5). A 25% (w/v) homogenate was obtained with 20 strokes of the pestle to crush the tissue without raising the temperature. This homogenate was diluted with a similar amount of the same sucrose solution, and distilled water added to lower the final concentration of sucrose to 0·25 M. The homogenate was then layered on 0·6 volume of 0·32 M sucrose and centrifuged at 600 *g* for 6 min. The crude nuclear pellet was suspended again in 0·8 ml of 0·32 M sucrose. After adding to the suspension enough 2·4 M sucrose, containing 1 mM $MgCl_2$(pH 6·5), to reach 4·5 ml, it was centrifuged at 4°C for 75 min at 53,500 *g* in an SW 50 Spinco Rotor. The purified nuclei were deposited on the bottom of the centrifugation tube. If the nuclei must be kept, they are suspended in 0·25 M sucrose. Their structure and the absence of cytoplasmic contamination were verified by optical and electron microscopy and by measuring the RNA/DNA ratio in the original homogenate and the final pellet.

Extraction of proteins and radioactivity

Two methods were used: (1) Successive extractions by 0·05 M phosphate-buffer, pH 7·4 and M NaCl. The purified nuclear pellet was suspended in 2 ml of 0·05 M phosphate buffer and homogenized with a glass pestle. After being centrifuged at 1000 *g* for 10 min at 4°C, the supernatant fraction containing soluble nucleoplasmic proteins was submitted to chromatography on a column of Sephadex G 25 or G 200.

Next 2 ml of a M NaCl was added to the pellet from the preceeding centrifugation. After 2 h of magnetic stirring in a cold room at 7°C, the solution obtained was centrifuged at 20,000 *g* for 20 min. The supernatant was examined by chromatography on a gel Sephadex G 25 or G 200 column. The final pellet was suspended in 2 ml of a N NaOH solution and after complete dissolution the residual proteins and radioactivity were measured.

(2) Direct extraction by M NaCl. The purified nuclear pellet was suspended in 3 ml of a MNaCl. After 2 h of magnetic stirring in a cold room at 7°C, the homogenate was centrifuged at 20,000 *g* for 20 min. The supernatant was then analysed by gel filtration on Sephadex G 25 or G 200. The pellet obtained from this centrifugation was used to determine the level of residual proteins and radioactivity.

Gel filtration

The filtrations on Sephadex gel were performed in a cold room at 7°C. We used columns of 27 cm × 1·5 cm containing 10 g of Sephadex G 25 fine equilibrated in 0·05 M phosphate buffer or in M NaCl. The void volume of these columns was 16 ml. The proteins are eluted between 16 and 26 ml, the free steroids begin at 50 ml. Some chromatography was performed on columns of Sephadex gel G 200,

equilibrated either in 0·05 M phosphate buffer or in M NaCl. These columns had a height of 75 cm and a dia. of 1·5 cm, and contained 5 g of Sephadex. Column was operated under a pressure of 7·5 cm and the output was 7 ml/h. The void volume was 38 ml.

Chromatography of steroids

The steroids bound to the nuclear macromolecules were extracted from the eluates of the Sephadex column by a mixture of chloroform-methanol 2:1 (v/v) following the technique of Folch *et al.*[8]. They were then separated by paper chromatography in the solvent system of Kochakian and Stidworthy[5] after the addition of internal standards to the extracts. After development for 4–5 h at 30°C, the chromatogram was cut into bands 1 cm wide and the radioactivity counted.

Methods of measurement

The proteins were measured following the technique of Lowry *et al.*[9]. DNA following that of Ceriotti[10] and RNA that of Mejbaum[11].

The radioactivity of the aqueous eluates from the gel filtration was measured by liquid scintillation in the Bray mixture[12]. That of the chromatograms was counted after the bands of paper were immersed in 10 ml of a solution containing 5 g of PPO 0·1 g of dimethyl POPOP for 1 l of toluene. The measurements were taken with the help of a Mark I counter. External standardization was used to correct for quench if necessary.

RESULTS

(1) *Purity of anterior hypophysis nuclei*

This was examined by optical microscopy after staining the nuclei by toluidine blue and by electron microscopy. From these observations we concluded that the nuclei were in a satisfactorily pure state (Fig. 1). The electron microscope examination confirmed the integrity of the nuclear membranes and the notable absence of cytoplasmic contamination (Fig. 1). The reduction of the RNA/DNA ratio from 1·05 in the original homogenate to 0·2 in RNA/DNA final pellet demonstrated that a satisfactory degree of purification has been achieved.

The technique used permitted us to recover 30–40% of the nuclei of the anterior hypophysis.

(2) *Study of the variations in the concentration of nuclei "receptors" as a function of the length of incubation of the hypophysis*

After incubation of the slices from 12 hypophysis in the presence of 75 ng of (^{3}H)testosterone during 5–180 min, the purified nuclei were extracted by M NaCl and the extract filtered on Sephadex gel G 25, in order to determine the specific activity of the bound radioactivity. This experiment demonstrated the rapid diffusion of the androgen in the nucleus because after 5 min of incubation, bound androgen appears, with a concentration of $0{\cdot}13 \times 10^{-13}$ mol per mg of proteins. It seems that after 60 min of incubation a maximal concentration is reached ($0{\cdot}69 \times 10^{-13}$ mol/mg).

(3) *Extraction of nuclear proteins and radioactivity*

(a) *Successive extractions by* 0·05 M *phosphate buffer and* M NaCl. The phosphate buffer extracts about 11% of the nuclear proteins and 66% of the radio-

Table 1. Variations of the nuclear "receptors" of rat anterior hypophysis as a function of the length of incubation of the slices in the presence of (^{3}H)testosterone. Nuclei were extracted by M NaCl and the extract filtered on Sephadex G 25.

	5 min	30 min	60 min	120 min	180 min
Bound proteins					
μg by organ	15·7	24·5	18·5	15·8	15·2
% of total proteins	55	66	62	50	38
Radioactivity in dpm by organ					
bound	21	146	135	104	85
free	767	2021	1562	1874	2539
Specific activity (dpm by mg of proteins)	1440	5929	7297	6030	6160
Concentration of androgens by mg of proteins ($\times 10^{-3}$ mol)	0·13	0·55	0·69	0·59	0·59

activity. Gel filtration on Sephadex G 25 permits us to ascertain that a part of the extracted radioactivity is bound to macromolecules, the other part being free (Fig. 2). The specific activity of the binding is 7250 d.p.m. per mg of proteins. This extract contains soluble nucleoplasmic proteins as well as certain enzymes and ribosomes.

Gel filtration on Sephdex G 200 in 0·05 M phosphate buffer permitted us to separate several protein fractions. The first was eluted between 40 and 60 ml, the others between 72 and 88 ml (Fig. 3).

Treatment with M NaCl of the pellet of nuclei, previously extracted with phosphate buffer, extracts an additional 28% of the residual proteins and 51% of the residual radioactivity. The gel filtration of this extract, on a Sephadex G 25 column in M NaCl demonstrated the existence of a fraction of bound radioactivity (Fig. 2). The specific activity of this bound material is 4925 d.p.m. per mg of proteins. M NaCl dissolves a large part of the histones, the DNA and the non histone proteins of the chromatine.

The filtration of this extract prepared in M NaCl on Sephadex gel G 200 permitted us to isolate many protein fractions (Fig. 3). The first was collected between 36 and 64 ml, the following between 64 and 120 ml. This latter fraction includes histones.

In the cases of gel filtration on Sephadex G 200, the bound radioactive material is eluted with the first protein fraction.

(b) *Extraction of purified nuclei by* M NaCl *alone*. The direct treatment of purified nuclei with M NaCl permitted the extraction of 61% of the nuclear proteins, 93% of DNA and 73% of the nuclear radioactivity. Filtration on Sephadex gel G 25 separates the radioactive fraction bound to the macromolecules from the free radioactive fraction. The amount of bound steroid is $0{\cdot}6 \times 10^{-13}$ mol per mg of proteins.

By gel filtration on Sephadex G 200, equilibrated in M NaCl it is possible to identify two major protein peaks. One is eluted from 40 to 60 ml, the other from 61 to 112 ml (Fig. 4). The bound radioactivity is dissociated into two fractions; the first is eluted between 40 and 56 ml, the second between 56 and 82 ml (Fig. 4). The histones were collected in the second protein fraction and were retained on

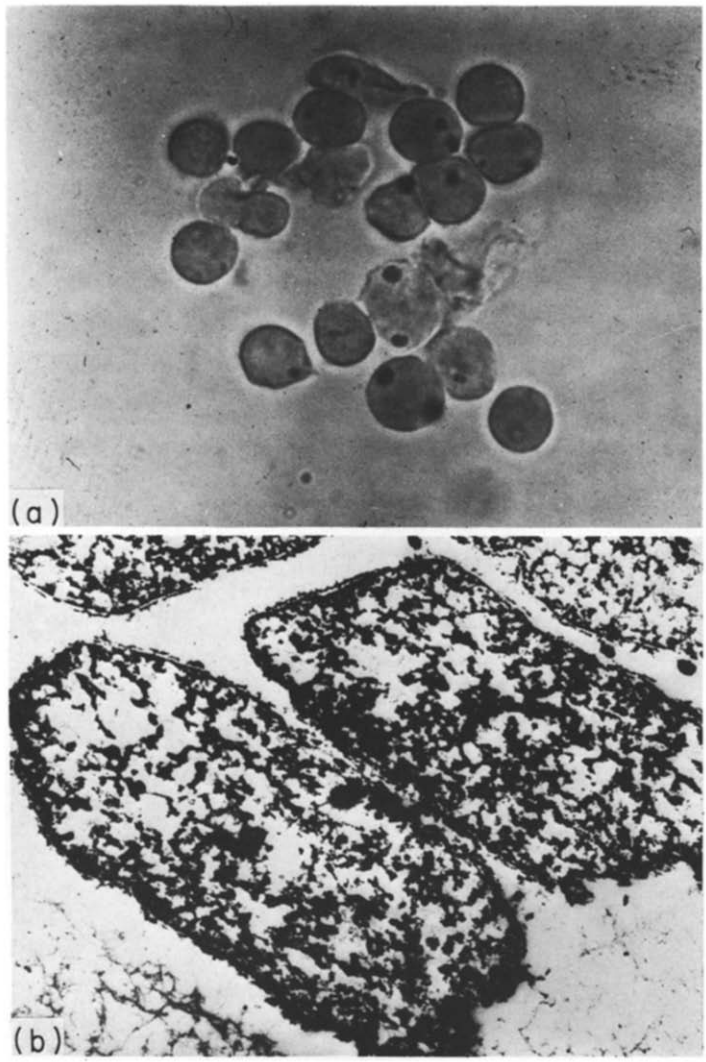

Fig. 1. Purified nuclei examined by optical microscopy after coloration by toluidine blue and by electron microscopy. These purified nuclei were prepared after incubation of slices of anterior hypophysis during 30 min at 37°C, in Krebs buffer.

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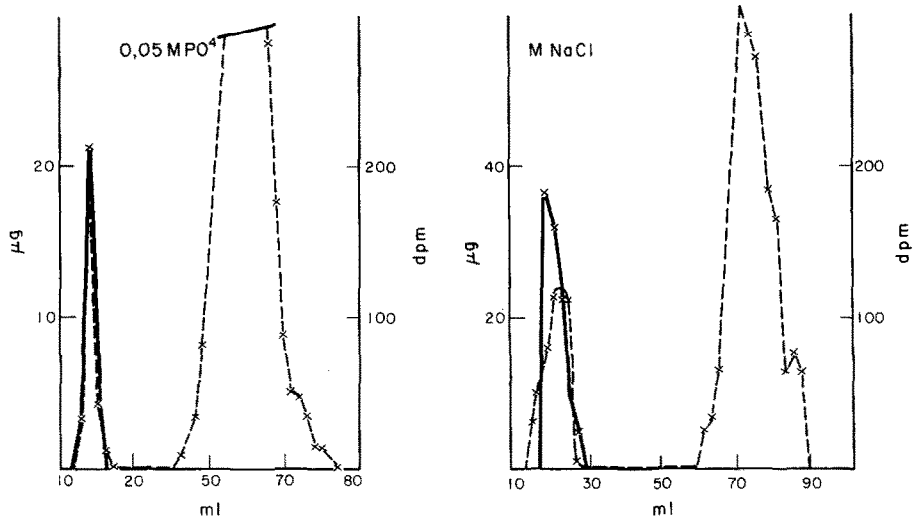

Fig. 2. Gel filtration on Sephadex G 25 of nuclear extracts obtained after extraction by 0·05 M phosphate buffer and then by a solution of M NaCl. ——— elution of proteins, - - - - - - elution of radioactivity.

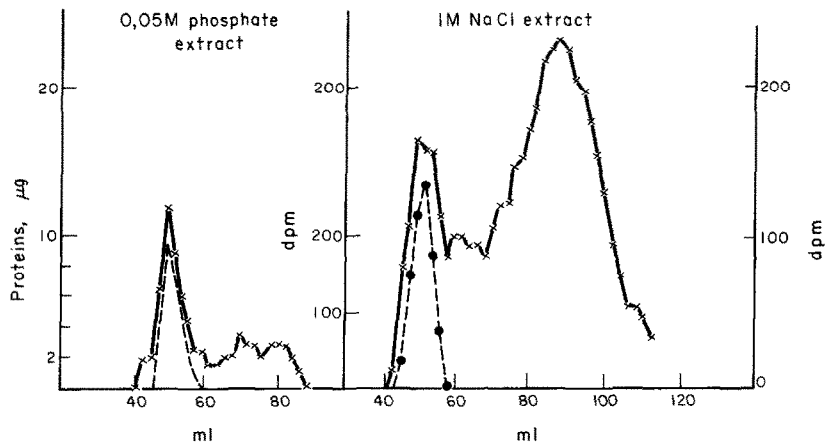

Fig. 3. Gel filtration on Sephadex G 200 of nuclear extracts. On the left, extracts obtained by extraction with 0·05 M phosphate buffer, on the right, extracts obtained by extraction with M NaCl. ——— elution of proteins, - - - - - - elution of radioactivity.

the gel. The DNA is excluded from the gel and is collected at the same time as the first protein peak (Fig. 4).

The concentration of the first radioactive fraction is $0{\cdot}6 \times 10^{-13}$ mol per mg of protein, the second would be $2{\cdot}1 \times 10^{-13}$ mol per mg.

(4) *Nature of the androgens bound to the nuclear "receptors" of the hypophysis*

After gel filtration on Sephadex G 25, the bound radioactivity was extracted by the technique of Folch *et al.* [8], and the steroids were separated by paper chromatography in the Kochakian and Stidworthy solvent system [5]. The nature of the steroids bound to the nuclear receptors was tentatively identified in the

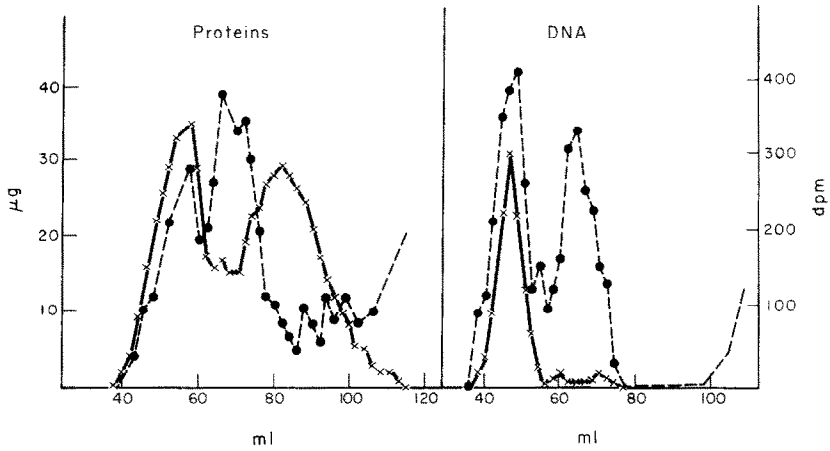

Fig. 4 Gel filtration on Sephadex G 200 of purified nuclei directly extracted by M NaCl. ——— elution of proteins (on the left), elution of DNA (on the right) - - - - - - elution of radioactivity-

phosphate extract, in the NaCl extract obtained after treating the nuclei with a phosphate buffer, and in the nuclear extract prepared by direct extraction of the nuclei by NaCl. The results are given in Table 2. It seems that the major part of the bound radioactivity is made up of (^{3}H)testosterone. The levels of dihydrotestosterone are not very high, and are in keeping with other findings on the cytoplasmic receptors of the hypophysis [3, 4]. The presence of a non polar steroid in the bound fractions must be stressed again. We have already reported its association with the proteins of the cytoplasm of the hypophysis [3, 4]. In the cytosol, as in the nuclei, this non polar derivate is present exclusively in the bound fractions.

We have also examined the nature of the free steroids, these are tentatively identified as testosterone (85%) DHT (5%) and androstane dione (3%). The direct extraction of the steroids, present in the nuclei by chloroform: methanol

Table 2. Nature of the steroids bound to the nuclear "receptors" of the hypophysis after 30 min incubation of the slices in the presence of (^{3}H)testosterone.

	Extracts		
	0·05 M Phosphate buffer	M NaCl (1)	M NaCl alone (2)
Androstane diol	7		
Testosterone	62	70	66
Dihydrotestosterone	2	8·5	7
Steroid X	15	8	7

(1) M NaCl extract obtained after extraction by 0·05 mol/l phosphate buffer.

(2) Extract obtained after homogenization of the purified nuclei in M NaCl. The levels of steroids are given in % of the total radioactivity of the chromatograms.

mixture (2·1 v/v) and their analysis by chromatography confirmed the results obtained starting from bound and free fractions.

DISCUSSION AND CONCLUSIONS

The study of hypophyseal nuclear "receptors" was made after incubation of hypophysis and preparation of purified nuclei. Is seems indispensable to use pure nuclear preparations, free from cytoplasmic contamination, in order to avoid the undesirable presence of cytoplasmic "receptors". Examination by optical and electron microscopy indicated the purity of the nuclei thus obtained. The purity of the nuclei was further checked by measuring RNA and DNA and establishing the RNA/DNA ratio. The level of recuperation calculated from the DNA dosage is relatively satisfactory because it is between 30 and 40%. The electron microscope examination showed, among other things, that the structure of the nuclei was well preserved.

The study of the penetration *in vitro* of [^{3}H]-testosterone into the nuclei as a function of the length of the incubation and the specific activity of the nuclear "receptors" seems to indicate that the saturation of the latter is reached between 30 and 60 min. Our results are in agreement with those of Mainwaring and Peterken who obtained a maximal concentration of radioactivity in prostate nuclei 30 min after an injection s/c of [^{3}H]-testosterone [13]. Similarly, Unhjem noted optimum labelling of nuclei 30 min after incubation of prostate slices [14]. It seems that, for the prostate, this period of incubation is sufficient for the transformation of the testosterone into dihydrotestosterone, and for the formation of macromolecular cytoplasmic associations and their passage into the nuclei.

The treatment of the nuclei by 0·05 M phosphate buffer at pH 7·4, extracts 11% of the nuclear proteins and 66% of the total radioactivity (average of 8 experiments). Nevertheless the quantity of bound tritium, evaluated by passage on column of Sephadex G 25, is slight, because it represents only 6·5% of the extracted radioactivity. The concentration of the bound radioactive material is $0{\cdot}5 \times 10^{-13}$ mol per mg of proteins. These experiments showed that after being incubated at 37°C for 30 min, the slices contained a large quantity of free radioactivity. The same holds true for the nuclei of the ventral prostate after incubation *in vitro* [14–16]. It is possible that the free radioactive steroid penetrates into the nuclei by a simple passive diffusion phenomenon and that it has no biological significance.

The phosphate extract, examined by chromatography on Sephadex gel G 200 equilibrated in phosphate buffer, contains only a fraction of protein excluded from the gel and coincides with the passage of the bound radioactivity (Fig. 3). It is probable, considering the low ionic force of the phosphage buffer, that the macromolecular association obtained corresponds to the soluble nucleoplasmic proteins. It is however possible that the protein and radioactivity have been bound to the outer surface of the nuclei.

After treating the nuclei by phosphate buffer the nuclear residue was extracted by M NaCl. This yields 27% of the total proteins and 51% of the residual radioactivity. By gel filtration on Sephadex G 25 equilibrated with M NaCl, the radioactivity was separated into two fractions, bound and free. The former represents 15% of the total radioactivity extracted by M NaCl. By gel filtration on Sephadex G 200 prepared in M NaCl, the nuclear proteins, dissolved in sodium chloride, were separated into two principal fractions. The first is excluded from

the gel, the second is retained. This latter is eluted between 60 and 120 ml and corresponds to the histones. The bound radioactivity is eluted at the same time as the excluded protein fraction. The androgen concentration of this fraction is identical to the precedent. It is $0{\cdot}5 \times 10^{-13}$ mol per mg of proteins. We do not know for the moment, if there is an identity between the macromolecular association obtained from the phosphate buffer and that extracted by NaCl.

The results obtained with M NaCl encouraged us to directly extract the purified nuclei by this solution. This method offers the advantage of dissolving the nucleoproteins and dissociating a part of the histones[17, 18]. It was used by Mainwaring[15] and by Unhjem[14] to extract the nuclear receptors of the prostate. Bruchovsky and Wilson also demonstrated the efficiency of 0·6 M NaCl and 1 M NaCl[16], as well as King *et al.* and Alberga *et al.* for the isolation of nuclear receptors of estradiol[19, 20]. In fact, M NaCl extracts 93% of DNA, 61% of the proteins and 73% of the total radioactivity of the hypophysis nuclei. This is made up of 10% of the bound radioactivity.

The fractionation of the proteins on Sephadex gel G 200 equilibrated in M NaCl shows two protein peaks. The excluded one would seem to correspond to the acid proteins of the chromatin, the reatined one seems to be made up of histones (Fig. 4). The DNA extracted by M NaCl is eluted between 40 and 60 ml (Fig. 4). The bound radioactivity is dissociated into two peaks, the first of which is collected between 40 and 56 ml, the second between 56 and 82 ml (Fig. 4). The androgen concentration of these macromolecular associations is respectively $0{\cdot}6 \times 10^{-13}$ mol and $2{\cdot}1 \times 10^{-13}$ mol per mg of proteins.

The demonstration of the existence of two types of associations of radioactivity in the hypophysis nuclei is not surprising. In 1968, Bruchovsky and Wilson had already identified two radioactive fractions in the prostate nuclei by chromatography on Sephadex G 200. One fraction was excluded and eluted with the proteins and the DNA, the other was retained slightly and associated with other proteins[16]. In the same way, O'Malley *et al.* have, using chromatography on DEAE cellulose, dissociated into two fractions, A and B, the progesterone receptor of the chicken oviduct[21]. King *et al.* for their part, have obtained several forms of estradiol association in the nuclei of a mammillary tumor, after chromatrography on a hydroxyapatite column[19]. Giannopoulos and Gorski have separated two forms of nuclear "receptors" of estradiol in the uterus by gel filtration on Sephadex G 200[22]. These results, added to ours, tend to prove that in the nuclei, there can exist several types of macromolecular associations of the hormone, some specific, others not so, especially when they were obtained *in vitro* after incubation of slices, of nuclei, or of reconstituted acellular systems. It is not impossible that in certain cases, we are dealing with the same entity, but in different forms. It seems in fact, that in our experiments, two types of macromolecular associations of testosterone are present in the hypophysis nuclei, one of which is easily extracted with 0·05 mol/l phosphate buffer, the other by M NaCl. Because of its weak ionic force, the phosphate buffer extracts the soluble proteins, probably acid, of the nucleoplasm. This protein-radioactivity association is excluded from the Sephadex gel G 200, like the cytoplasmic "receptor" of the hypophysis, and like it, is soluble in phosphate buffer[1, 2, 4].

The direct extraction of purified nuclei by M NaCl is characterized by its efficacity from the stand point of the recuperation of radioactivity and of desoxyribonucleoproteins. Such an extract is divided into two parts by gel filtration

on Sephadex G 200. The DNA and the acid proteins are collected with the void volume, the histones are retained. The bound radioactivity is itself separated into two fractions, one excluded, the other retained slightly. It does not seem that there are any bindings between the radioactivity and the histones of the hypophysis, which agrees with the observations which were made on rat prostate and chicken oviduct[13, 16, 21, 23] and on the nuclear receptors of estradiol[19, 20]. The nature of the two types of macromolecular associations identified by chromatography on Sephadex G 200, remains to be seen. Does the association excluded from the gel and eluted at the same time as the DNA and some proteins represent an association DNA-acid protein-testosterone? Is the association eluted later but before the histones, an acid protein testosterone combination? For the moment, it is difficult to decide, but our research should be directed towards the identification of the nature of these nuclear associations and their specificity towards the hormone.

The study of the nature of the androgens bound to the nuclear macromolecules, after incubation in the presence of (^{3}H)testosterone shows their diversity. It confirms our previous results concerning the cytoplasmic "receptor" of the anterior hypophysis[3, 4]. The bound radioactivity is constituted mainly of testosterone. There is therefore a fundamental difference between the hypophysis and the prostate. It is in fact now well established that in this organ dihydrotestosterone represents the most important ligand of the nuclear "receptors" [13, 14, 16, 23–25].

The small quantity of DHT detected in the cytoplasmic "receptors", as well as in the nuclear "receptors",[3, 4] proves the existence of 5 α-reductase in the hypophysis, but also proves its weak ratio. This is in agreement with the work done by Perez-Palacios *et al.*[28] and with the work of Rommerts and Van der Molen [27] on the metabolism of androgens in the hypophysis and in the superior nervous centers of dogs and rats.

We must mention again the presence in the fraction bound to the nuclear macromolecules, of a non identified non polar steriod, we have already reported its binding to the cytoplasmic "receptors" of the hypophysis[3, 4]. A similar apolar compound has been likewise detected by us in the cytoplasmic "receptors" of the prostate[3, 4], by Fang *et al.* (24), and by Perez-Palacios *et al.* in the hypophysis and the brain[26]. The determination of its nature is in progress.

After this preliminary research on the existence of a macromolecular binding of androgens in the anterior hypophysis of rats, we are continuing our work in order to discover the specificity and the nature of the concerned protein.

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