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INTRANUCLEAR TRANSPORT OF ANDROSTENEDIONE IN RAT LIVER

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SUMMARY

When castrated male rats were injected intraperitoneally with [1, 2, 6, 7-^{3}H]-testosterone, liver nuclei specifically retained [^{3}H]-androstenedione. Treatment of labelled nuclei with 0.4 M KCl resulted in extraction of an androstenedione–protein complex with a pI of 5.1. An androstenedione–protein complex with identical pI was also found in liver cytosol; this complex had a sedimentation coefficient of 3.3 *s* and was eluted at 0.55 column vol. when chromatographed on a Sephadex G-100 column. The 3.3 *s* protein bound androstenedione with a limited number of binding sites. In reconstitution experiments where labelled liver cytosol was incubated with unlabelled nuclei from castrated male rats it was possible to demonstrate specific nuclear uptake of the androstenedione–protein complex from the cytosol; it could also be shown that this process was saturable and that the uptake increased with time. It is suggested that the androstenedione-binding protein in rat liver cytosol plays a role in the intranuclear transfer of androstenedione.

INTRODUCTION

It was recently demonstrated that the degree of androgen responsiveness of liver enzyme activities in the rat is programmed at birth, leading to higher responsiveness in male than in female rats [1]. The changed androgen responsiveness of liver caused by neonatal imprinting could be due to changed concentration of hepatic receptor proteins for androgens. This prompted us to investigate the possible presence of receptor mechanisms for androgens in rat liver. In a previous publication we reported the occurrence of a high-affinity, low-capacity androstenedione-binding protein in rat liver cytosol [2]. The present communication describes the uptake of the androstenedione–protein complex into rat liver nuclei.

EXPERIMENTAL

Steroids. [1, 2, 6, 7-^{3}H]-Testosterone (specific radioactivity, 84 Ci/mmol) and 4-[1, 2, 6, 7-^{3}H]-androstene-3,17-dione (specific radioactivity, 83 Ci/mmol) were purchased from the Radiomedical Centre, Amersham, England. Unlabelled testosterone and androstenedione were generously supplied by Dr. J. Babcock, Upjohn Co., Kalamazoo, MI, U.S.A.

Animal experiments. Male Sprague–Dawley rats, 8 weeks old, were used in all experiments. In cases where castrated rats were used, the operation was performed under ether anesthesia 14–16 h before the experiment.

In one series of experiments, castrated rats were given an intraperitoneal injection of 250 μCi of [1, 2, 6, 7-^{3}H]-testosterone in 120 μl of acetone. Thirty min later the animals were killed by a blow on the head. The livers were perfused with cold saline, homogenized in TKE-buffer (0.01 M Tris–HCl, pH 7.4—0.01 M KCl—0.001 M EDTA) [3] and cytosol and liver nuclei were prepared as described previously [4]. The nuclear fraction was either taken for identification of retained [^{3}H]-labelled androgen metabolites as described before [5] or extracted for 2 h at 0–2° in 1 ml of TKE-buffer (0.01 M Tris–HCl, pH 7.4—0.01 M KCl—0.001 M EDTA), 0.4 M with respect to KCl. The nuclear suspension was centrifuged at 30,000 *g* for 30 min and the supernatant was analyzed by isoelectric focusing after desalting on Sephadex G-25. The cytosol preparation was chromatographed on a Sephadex G-25 column, equilibrated in TKE-buffer, and the void vol. (containing protein-bound radioactivity) was treated twice with Dextran-coated charcoal [6] and analyzed by isoelectric focusing and sucrose density gradient centrifugation.

Isoelectric focusing. Analysis by isoelectric focusing was carried out essentially as described by Katsumata and Goldman[7]. The columns were fractionated into 30–60 counting vials or test tubes 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, IL, 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.

Density gradient centrifugation. Aliquots (0.2 ml) of the samples to be analyzed were layered on top of linear 5 ml 5–20% (w/v) sucrose gradients in TKE-buffer, 0.01 or 0.4 M with respect to KCl. The tubes were centrifuged at 50,000 rev./min for 20 h at +2° in an SW 50.1 Beckman-Spinco rotor. At the end of the centrifugation, the bottom of the tube was punctured and 3 or 4 drop fractions were collected and

measured for radioactivity. The following markers were used; ovine serum albumin (3.7 *s*), bovine serum albumin (4.6 *s*) and rabbit anti-androstenedione antibody (7 *s*).

In selected cases, aliquots of the focused radioactive peaks and the radioactive peaks from the sucrose gradient centrifugations were taken for identification of radioactive metabolites. Twenty vol. of acetone–ethanol, 1:1 (v/v), were added to the samples. After filtration, the extracts were evaporated to dryness and the residues were dissolved in double-distilled water and passed through an Amberlite XAD-column. The steroids were eluted with methanol. Identification of the radioactive metabolites was performed by t.l.c. and radio–gas chromatography [4]. A steroid was considered identified if it had the same thin-layer chromatographic mobility and the same retention time on SE-30 and on OV-17 as the reference steroid.

Nuclear uptake in vitro *of protein-bound androgen.* Unlabelled nuclei were prepared from liver or kidney from male animals castrated 14–16 h prior to killing. The nuclear fractions were resuspended in [^{3}H]-labelled cytosol ($2 \cdot 10^7$ nuclei/ml of cytosol) prepared from liver of castrated male rats given 250 μCi of [1, 2, 6, 7-^{3}H]-testosterone 30 min before death. The nuclear–cytosol mixtures were generally incubated for 60 min at 37°. Control incubations of [^{3}H]-labelled cytosol without added nuclei were also carried out. After incubation, the mixtures were cooled and centrifuged at 30,000 *g* for 20 min. The supernatants were passed through Sephadex G-25 columns and the void vols were treated with Dextran-coated charcoal before analysis by isoelectric focusing.

The nuclear sediments were resuspended in medium A (0.88 M sucrose–1.5 mM $CaCl_2$–1 mM $MgSO_4$–0.01 M Tris–HCl, pH 7.4) [8], layered above 2.2 M sucrose and centrifuged for 30 min at 58,000 *g* [4]. The nuclear pellets were resuspended in TKE-buffer and aliquots were taken off for determination of DNA according to Burton[9]. Further analyses of the nuclear fractions were carried out as described above for liver cell nuclei isolated from animals given [1, 2, 6, 7-^{3}H]-testosterone.

RESULTS AND DISCUSSION

In vivo *experiments*

The radioactivity present in liver nuclei 30 min after intraperitoneal administration of [1, 2, 6, 7-^{3}H]-testosterone to castrated male or female rats was identified as [^{3}H]-androstenedione (Fig. 1). Isoelectric focusing of the hepatic nuclear protein extract from male rats given [1, 2, 6, 7-^{3}H]-testosterone showed one single radioactive peak with a pI of 5.1 (Fig. 2).

When the [^{3}H]-labelled cytosol obtained from castrated male rats given [1, 2, 6, 7-^{3}H]-testosterone was fractionated on a Sephadex G-25 column equilibrated with TKE-buffer usually about 5–10% of the radioactivity was recovered in the void vol. When an aliquot of this fraction was analyzed by sucrose density gradient centrifugation a radioactive peak was observed at 3.3 *s* (Fig. 3). In contrast to other regions of the gradient that contained mainly water-soluble labelled metabolites, the 3.3 *s* region was characterized by the presence of ethyl acetate extractable radioactive material. With material pooled from several gradients it could be shown that one single metabolite was present in the free steroid fraction of the 3.3 *s* region of the gradient, namely [^{3}H]-androstenedione.

In order to investigate whether the 3.3 *s* androstenedione-binding protein represented a high- or a low-capacity binding protein, the following experiments were carried out. Thirty min prior to administration of [1, 2, 6, 7-^{3}H]-testosterone, the castrated rat was given 10 mg of unlabelled testosterone. Sucrose density gradient centrifugation showed that this procedure resulted in a reduction of the radioactive 3.3 *s* peak to 20% of the control value. Furthermore, unlabelled liver cytosol prepared from castrated male rats was incubated at 37° for 30 min with 10^{-8} M [1, 2, 6, 7-^{3}H]-testosterone in the absence or presence of 10^{-6} M unlabelled testosterone and subsequently analyzed by sucrose density gradient centrifugation (Fig. 4). Also under these conditions, a complex appeared with a sedimentation coefficient of 3.3 *s* that was partially displaceable with an excess of unlabelled testosterone (Fig. 4). Both of these experiments indi-

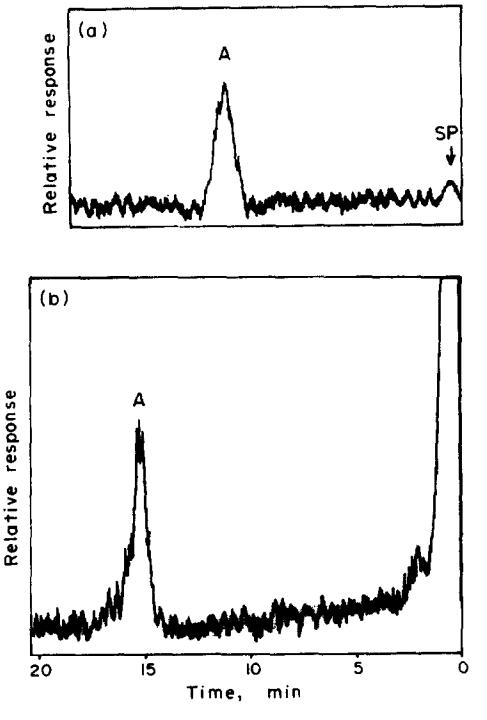

Fig. 1. Thin-layer chromatogram scan (Fig. 1a) of radioactivity extracted from liver nuclei from castrated male rats after intraperitoneal injection of [1, 2, 6, 7-^{3}H]-testosterone. Radio–gas chromatographic analysis (1% OV-17) (Fig. 1b) of the radioactive peak eluted from the thin-layer plate scanned in Fig. 1a. Abbreviations: SP = Starting point; A = [^{3}H]-androstenedione.

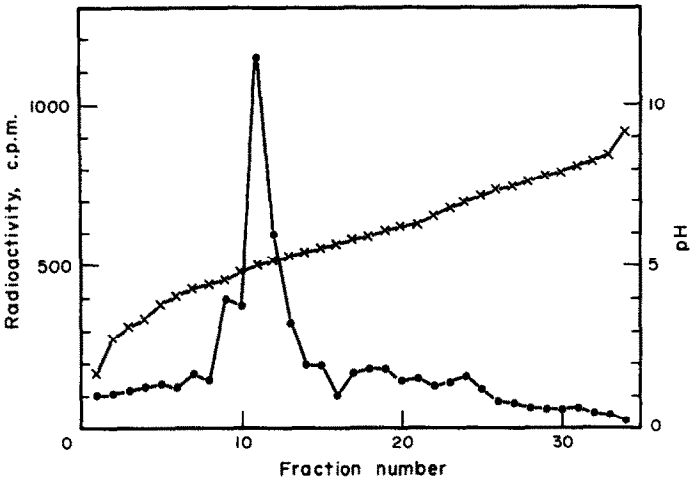

Fig. 2. Isoelectric focusing of TKE-0.4 M KCl extract of liver nuclei from a castrated male rat given 250 μCi of [1, 2, 6, 7-^{3}H]-testosterone 30 min before death. Each fraction had a vol. of 0.3 ml. Explanations: —●—, radioactivity; — x —, pH.

cate that the protein sedimenting at 3.3 *s* binds androstenedione with a limited number of binding sites.

When the void vol. from the Sephadex G-25-chromatography of [^{3}H]-labelled cytosol was treated with Dextran-coated charcoal and analyzed by isoelectric focusing, a major radioactive peak appeared at pH 5.1 (Fig. 5). Further analysis of this peak showed that it contained about equal amounts of [^{3}H]-androstenedione and [^{3}H]-testosterone. Sucrose gradient analysis of the radioactive material focusing between pH 5.0 and 5.2 revealed a homogeneous peak with

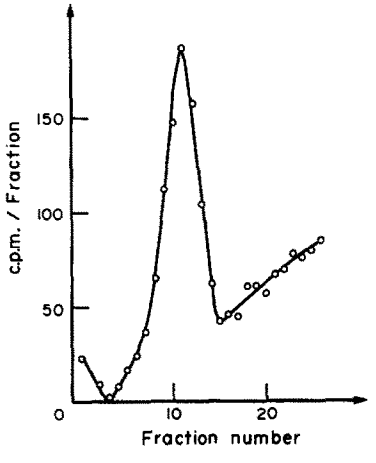

Fig. 3. Sucrose density gradient centrifugation of protein-bound fraction recovered after chromatography on Sephadex G-25 of liver cytosol from a castrated male rat given 250 μCi of [1, 2, 6, 7-^{3}H]-testosterone 30 min before death. Centrifugation was performed in a linear 5 ml 5–20% (w/v) sucrose gradient in 0.01 M KCl–TKE-buffer at 50,000 rev./min for 20 h at +2° in an SW 50.1 rotor. After centrifugation the bottom of the tube was punctured and fractions were collected. The radioactive peak corresponds to a protein with a sedimentation coefficient of 3.3 *s*.

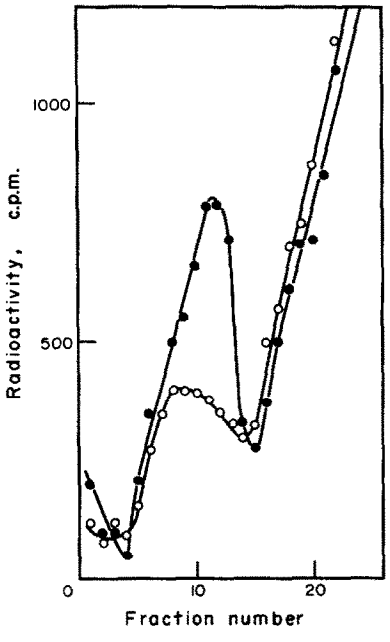

Fig. 4. Sucrose density gradient centrifugation of protein-bound fraction recovered after chromatography on Sephadex G-25 of liver cytosol from a castrated male rat incubated with 10^{-8} M [1, 2, 6, 7-^{3}H]-testosterone in the absence (—●—) or presence (—○—) of 10^{-6} M unlabelled testosterone. Cytosol was prepared in TKE-buffer and incubation was performed in the same medium for 30 min at 37°. Subsequently, the incubation mixture was chromatographed on a Sephadex G-25 column equilibrated with TKE-buffer and the void volume recovered from the column was treated with Dextran-coated charcoal and analyzed by sucrose density gradient centrifugation. Centrifugation was performed in a linear 5 ml 5 to 20% (w/v) sucrose gradient in 0.01 M KCl—TKE-buffer at 50,000 rev./min for 20 h at +2° in an SW 50.1 rotor. After centrifugation the bottom of the tube was punctured and fractions were collected. The radioactive peak corresponds to a protein with a sedimentation coefficient of 3.3 *s*.

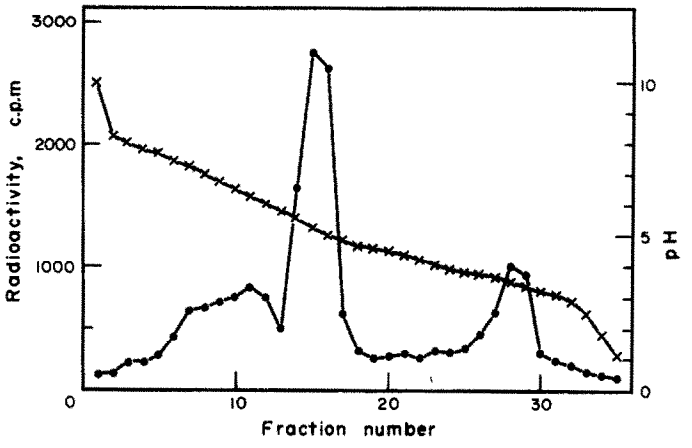

Fig. 5. Isoelectric focusing of Dextran-coated charcoal-treated cytosol from liver of a castrated male rat given 250 μCi of [1, 2, 6, 7-^{3}H]-testosterone 30 min before death. Each fraction had a vol. of 0.3 ml. Explanations: —●—, radioactivity; —×—, pH.

a sedimentation coefficient of 3.3 *s*, both in high and low ionic strength. When the pI 5.0–5.2-peak was chromatographed on Sephadex G-100 equilibrated in TKE-buffer, it was eluted at 0.55 column vols (corresponding to a distribution coefficient of 0.30 (*cf.* ref. 10)).

When plasma from rats given [1, 2, 6, 7-^{3}H]-testosterone was chromatographed on Sephadex G-25, treated with Dextran-coated charcoal and analyzed by isoelectric focusing, no radioactive peak was found to focus. Hence it is concluded that the androgen-binding protein with a pI of 5.1 was formed in the liver itself and was not transported into the hepatocytes from blood.

In vitro *experiments*

As can be seen from Table 1, nuclear uptake of radioactivity was 5–10 times higher when unlabelled liver nuclei were incubated with [1, 2, 6, 7-^{3}H]-testosterone in the presence of unlabelled liver cytosol from castrated male rats than in the presence of buffer only. These experiments indicated the importance of cytosol components in nuclear uptake of androgen. The uptake of radioactive steroid into nuclei was not temperature-dependent (Table 2). In reconstitution experiments with labelled cytosol and unlabelled nuclei it was shown that the pI 5.1-complex was specifically decreased in cytosol during incubation (Fig. 6). Furthermore, the pI 5.1-complex was extractable from the repurified nuclei.

Table 1. Dependence of nuclear uptake of androgen on the presence of cytosol

Incubation medium	Radioactivity in the nuclear fraction (pmol/mg DNA)	
	Experiment I	Experiment II
Cytosol	0.18	0.15
TKE-buffer	0.018	0.028

Liver nuclei, $2 \cdot 10^7$, prepared from castrated male rats were incubated for 30 min at 37° with $2 \cdot 10^5$ d.p.m. of [1, 2, 6, 7-^{3}H]-testosterone in 1 ml of TKE-buffer or in 1 ml of liver cytosol prepared from castrated male rats. After the incubation the nuclei were repurified and aliquots were taken for calculation of amount of DNA and for calculation of radioactivity

Table 2. Dependence of nuclear uptake of androgen on temperature

Temperature	Radioactivity in the nuclear fraction (pmol/mg DNA)
0°C	0.13
20°C	0.21
37°C	0.19

Aliquots, 1 ml, of cytosol prepared from castrated male rats killed 30 min after intraperitoneal administration of 250 μCi of [1, 2, 6, 7-^{3}H]-testosterone, were incubated for 60 min with $2 \cdot 10^7$ nuclei prepared from castrated male rats. After incubation the nuclei were repurified and aliquots of the nuclear suspension were taken for calculations of radioactivity and for calculation of amount of DNA.

In a series of reconstitution experiments with [^{3}H]-labelled cytosol and unlabelled liver nuclei from castrated male rats, the amount of androstenedione–protein complex in cytosol was measured as the peak sedimenting at 3.3 *s* on sucrose gradients. In control experiments, liver cytosol isolated from castrated male rats 30 min after injection of [1, 2, 6, 7-^{3}H]-testosterone showed no significant decrease in the amount of androstenedione–protein complex when kept at 37° for 300 min. When unlabelled liver nuclei were incubated with the labelled cytosol preparation (standard incubation conditions: 2×10^7 nuclei, corresponding to about 1/50 of a total liver homogenate, per ml cytosol at 37° for 60 min) they took up radioactivity that was retained even after extensive repurification of the nuclei. Extraction of the nuclear radioactivity with organic solvents followed by thin-layer and radio–gas chromatographic analysis of the extract

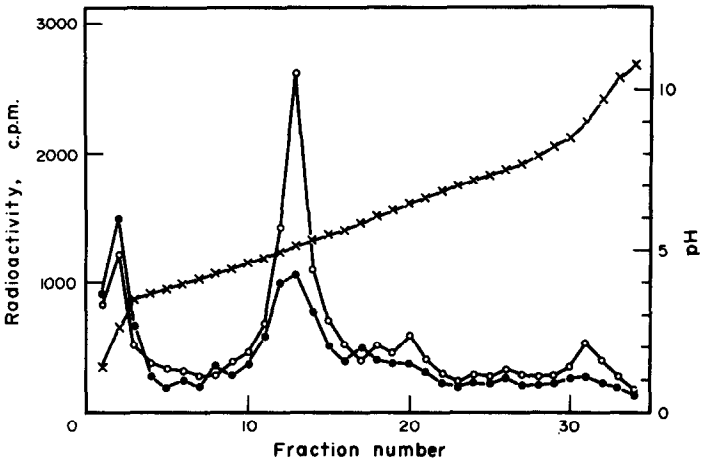

Fig. 6. Reconstitution experiment with labelled cytosol from liver of castrated male rats given [1, 2, 6, 7-^{3}H]-testosterone and unlabelled liver nuclei from castrated male rats. Isoelectric focusing of (a) Dextran-coated charcoal-treated labelled cytosol heated at 37° for 30 min (control experiment;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

showed in several repeated experiments that the retained radioactivity exclusively consisted of [^{3}H]-androstenedione. When the repurified nuclei were extracted with TKE-buffer, 0.4 M with respect to KCl, and the extract was desalted and analyzed by isoelectric focusing, the nuclear radioactivity focused at pH 5.1. Incubation of a fixed amount of unlabelled liver nuclei with increasing amounts of labelled cytosol showed that the nuclear uptake of androstenedione–protein complex was a saturable process (Fig. 7a). A somewhat larger amount of radioactivity was taken up by the nuclei than the decrease in amount of androstenedione–protein complex observed in the cytosol. This tendency became more pronounced with increasing amounts of cytosol. The same phenomenon was observed in another series of reconstitution experiments where the dependency of the nuclear uptake of androstenedione–protein complex on time was investigated (Fig. 7b). The nuclear uptake increased in an almost linear fashion during at least 300 min and it could be shown that this radioactivity exclusively consisted of [^{3}H]-androstenedione. However, the decrease in amount of androstenedione–protein complex in the cytosol during the same time was less than the increase in nuclear radioactivity. The reason for this discrepancy is not known but it could possibly be reformation of androstenedione–protein complex in the cytosol during the course of the reconstitution experiment.

The reconstitution experiments described above indicate the occurrence of a specific mechanism for the transfer of androstenedione from liver cytosol to nuclei. In another type of reconstitution experiments, 5 ml aliquots of [^{3}H]-labelled cytosol were also incubated with equal amounts of unlabelled liver or kidney nuclei ($2 \cdot 10^7$/ml of cytosol) respectively. After repurification of the nuclei, liver nuclei were found to contain twice the amount of radioactivity found in kidney nuclei, calculated per μg of DNA. These results indicate a certain degree of tissue specificity in nuclear uptake of the androstenedione–protein complex.

In conclusion, the present investigation has demonstrated the binding of androstenedione to a specific protein in rat liver cytosol and nuclei with a high affinity for androstenedione. A role is suggested for the androstenedione–protein complex in intranuclear transfer of androstenedione. The exact relationship between this 3.3 *s* androstenedione–protein complex and the 10 *s* androstenedione–protein complex described in rat liver cytosol in a previous publication [2] is not clear. It is possible that the androstenedione–protein complex can appear in more or less aggregated forms depending on the experimental conditions; a tendency to aggregate seems to be characteristic of certain androgen receptor proteins [11, 12].

The physiological importance of the androstenedione-binding protein in rat liver cytosol and nuclei is unclear at the present time. Androgens are known to be involved in certain hepatic functions, *e.g.* the formation of the male-specific α_2 urinary globulin in rats [13] and the differentiation of the steroid metabolizing enzymes in rat liver [14]. However, some of these effects of androgens have recently been shown to be mediated *via* the pituitary [15] and it is uncertain to what extent androgen receptor proteins in the liver are involved in these effects. Androstenedione has been found to have a growth-stimulatory and an anabolic effect similar to that of testosterone [16] and it is possible that the hepatic androstenedione-binding protein plays a role in these actions of the hormone. Relatively little is known about the occurrence of receptor proteins for androstenedione in mammalian cells and the present investigation calls for a more

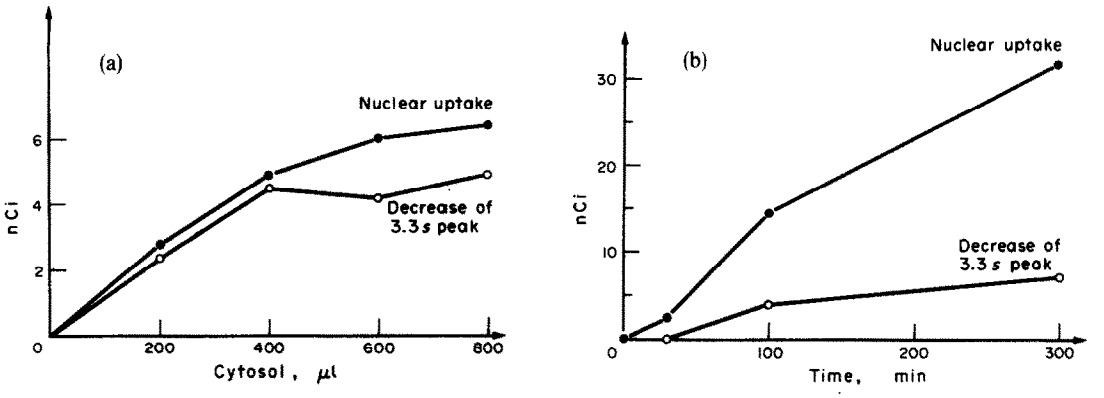

Fig. 7. Reconstitution experiments with labelled cytosol from liver of castrated male rats given [1, 2, 6, 7-^{3}H]-testosterone and unlabelled liver nuclei from castrated male rats. (a) Influence of increasing amounts of cytosol. The nuclear fraction ($2 \cdot 10^7$ nuclei) was resuspended in 200, 400, 600 or 800 μl of *in vivo*-labelled cytosol (see text). The nuclear–cytosol mixtures were incubated for 60 min at 37°. The decrease of the 3.3 *s* complex was calculated as the difference between the amount of 3.3 *s* complex in control incubations without nuclei and in nuclear–cytosol incubation mixtures. (b) Influence of time. The nuclear fraction ($2 \cdot 10^7$ nuclei) was resuspended in 1 ml of *in vivo*-labelled cytosol and the mixtures were incubated for 30, 100 and 300 min. The decrease of the 3.3 *s* complex was calculated as indicated above. After the incubations, the mixtures were cooled and centrifuged at 30,000 *g* for 20 min. The supernatants were passed through Sephadex G-25 columns and aliquots of the void volumes were analyzed by sucrose density gradient centrifugation. The nuclear fraction was purified as described in the test and aliquots were taken for measurement of radioactivity.

thorough search for androstenedione-binding proteins in rat and human tissues.

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REFERENCES

1. Gustafsson J.-Å. and Stenberg Å.: *J. biol. Chem.* **249** (1974) 719–723.
2. Gustafsson J.-Å., Pousette Å., Stenberg Å. and Wrange Ö.: *Biochemistry* **14** (1975) 3942–3948.
3. Puca G. A. and Bresciani F.: *Nature, Lond.* **218** (1968) 967–969.
4. Gustafsson J.-Å. and Pousette Å.: *Biochemistry* **13** (1974) 875–881.
5. Gustafsson J.-Å. and Pousette Å.: *Biochemistry* **14** (1975) 3094–3101.
6. Beato M. and Feigelson P.: *J. biol. Chem.* **247** (1972) 7890–7896.
7. Katsumata M. and Goldman A. S.: *Biochim. biophys. Acta* **359** (1974) 112–129.
8. Verhoeven G. and DeMoor P.: *Endocrinology* **91** (1972) 54–64.
9. Burton K.: *Biochem. J.* **62** (1956) 315–323.
10. Gelotte B.: *J. Chromatogr.* **3** (1969) 330–342.
11. Mainwaring W. I. P. and Irving R.: *Biochem. J.* **134** (1973) 113–127.
12. Liao, S. and Fang, S.: *Vitams Horm.* **27** (1969) 17–90.
13. Roy A. K. and Neuhaus O. W.: *Biochim. biophys. Acta* **127** (1966) 82–87.
14. Gustafsson J.-Å., Ingelman-Sundberg M. and Stenberg Å.: *J. steroid Biochem.* **6** (1975) 643–649.
15. Gustafsson J.-Å. and Stenberg Å.: *Endocrinology* **95** (1974) 891–896.
16. Kochakian C. D.: *Ala. J. Med. Sci.* **1** (1964) 24.