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DRAMATIC PROTECTIVE EFFECT OF LIGAND AGAINST THERMAL DEGRADATION ON MINERALO- AND GLUCOCORTICOID RECEPTORS OF RAT KIDNEY

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SUMMARY

Rat kidney cytosol contains both mineralo- and glucocorticoid receptors. The stability of these receptors was studied by [^{3}H]-aldosterone and [^{3}H]-dexamethasone respectively. Stability experiments at 0°C and 20°C showed more rapid decay of aldosterone binding sites than of dexamethasone receptors. Moreover, a pronounced protective effect of ligand on the stability of binding sites was indicated by a more rapid disappearance rate of aldosterone* and dexamethasone* receptors in the absence of their ligand. The protective effect of ligand on [^{3}H]-aldosterone binding sites was further documented by Scatchard analysis and competition experiments. When aldosterone receptors were protected *during* cytosol preparation by adding [^{3}H]-aldosterone after homogenization: (1) Scatchard plot of [^{3}H]-aldosterone showed two slopes, the steeper slope ($K_{DI} = 3.9 \times 10^{-9}$M) corresponding to the mineralocorticoid receptor; (2) competition experiments showed that aldosterone was the best competitor for [^{3}H]-aldosterone binding sites. When [^{3}H]-aldosterone was added *after* cytosol preparation: (1) only the lower slope ($K_{DII} = 9 \times 10^{-8}$M) corresponding to glucocorticoid receptor was detected; (2) dexamethasone was the best competitor for [^{3}H]-aldosterone binding sites. Various attempts were made to stabilize aldosterone receptors: glycerol and EDTA-containing buffers appeared to be the most effective buffers. No further stability was obtained by the addition of several protease inhibitors.

INTRODUCTION

Edelman *et al.* [1], Sharp *et al.* [2] have demonstrated convincingly that the first step of the mechanism of action of aldosterone in anurian epithelia is its binding to specific proteins located in the cytosol and the nuclei of the target cells. The rat kidney also contains aldosterone binding proteins [3–6] but it has recently been shown that there is in addition a glucocorticoid binding system [7, 8] able to bind aldosterone with less affinity. Rousseau *et al.* [7] and Funder *et al.* [9] have shown that aldosterone binds with maximum affinity to mineralocorticoid receptors and with a lower affinity to glucocorticoid receptors, which show their greatest affinity for dexamethasone [7, 8].

Most of the studies on mineralo- or glucocorticoid cytosolic receptors in the rat have been performed either wholly *in vivo* or in partially *in vitro* systems, such as kidney slices [8–10]. The purpose of this work was to study the binding characteristics of cytosolic mineralo- and glucocorticoid receptors under entirely *in vitro* conditions. (1) In a first series of experiments, the stability of mineralo- and glucocorticoid binding sites has been tested. (2) The differential protective effect of the ligand on mineralo- and glucocorticoid sites has been further documented. (3) Finally, various attempts to stabilize both binding sites for complete *in vitro* studies have been performed.

EXPERIMENTAL

Materials

The following compounds were obtained from the sources indicated: [1, 2-^{3}H]-aldosterone*, 50 Ci/mmole, [1, 2-^{3}H]-dexamethasone, 25 Ci/mmole (Radiochemical Centre, Amersham); aldosterone, dexamethasone D-tryptophan methyl ester, charcoal, norit A (Sigma); Benzamidine (K and K); Dextran T/70 (Pharmacia). Pepstatin was a gift from Dr H. Umezawa and T. Aoyagi. All other products were analytical grade from Merck.

Male Wistar rats (180–200 g) were adrenalectomized 24 h before sacrifice and maintained on normal saline *ad libitum*. On the day of the experiment, the rats were exsanguinated by aortic puncture, the kidneys were removed, decapsulated and rinsed with isotonic saline at 4°C.

Methods

Stability experiments. The stability at 0°C of [^{3}H]-aldosterone and [^{3}H]-dexamethasone *filled* binding sites was studied as follows. Kidneys from

* *Systematic names*: aldosterone, 11β, 21-dihydroxy-3, 20 dioxo-4-pregnen-18 al (11→18)-lactol; dexamethasone, 9α-fluoro-11β, 17α, 21-trihydroxy-16α methyl-1, 4-pregnadiene-3, 20-dione. *Enzymes*: chymotrypsin (EC 3.4.4.5); trypsin (EC 3.4.4.4).

adrenalectomized rats were minced and homogenized in 0.01 M Tris–HCl, 1 mM EDTA, pH 7.4, containing 10% glycerol. The homogenate was centrifuged at 700 *g* for 10 min. The resulting supernatant was saturated with 10^{-7}M [^{3}H]-aldosterone or [^{3}H]-dexamethasone and immediately centrifuged at 105,000 *g* for 1 h. The specific radioactivity bound was determined each h by separating bound from free hormone using the charcoal technique [11]. The aqueous samples were counted after addition of 10 ml of Unisolve (Koch–Light) in a Packard Tricarb 3380 scintillation spectrometer with a 34% efficiency. Correction for quenching was made.

Similar experiments were performed for determining the stability at 20°C of the filled sites. For each determination the cytosol was chilled for 15 min at 4°C before the charcoal dextranassay.

For the stability of the unoccupied binding sites the cytosol was prepared as described except that addition of labelled hormone was omitted after the 700 *g* centrifugation. The amount of radioactivity bound was determined, after various times, by adding a saturating solution of 10^{-7} M tritiated hormone to the cytosol samples for 1 h.

All incubations were done in parallel with controls containing a 1000-fold excess of unlabelled steroid in order to determine non specific binding which was then substracted from the total bound.

In order to verify that [^{3}H]-aldosterone and [^{3}H]-dexamethasone were not metabolized in the cytosol, even after 10 h of incubation the tritiated hormone was extracted with ether and chromatographed on silica gel with chloroform–acetone (1:1 v/v) for [^{3}H]-aldosterone and chloroform–ethanol (10:3 v/v) for [^{3}H]-dexamethasone. A single peak of radioactivity coincident with the standard was found.

Scatchard analysis and competition experiments

Kidney slice experiments: Kidney slices (250 μm) from adrenalectomized rats were prepared and incubated for 20 min at 20°C, as described by Funder *et al.* [9]. At the end of the incubation, slices were drained under suction, rinsed with chilled incubation buffer and homogenized at 4°C with 0.25 M sucrose, 1 mM $MgCl_2$. The cytosol (105,000 *g* supernatant) was then prepared and bound from free radioactivity separated by charcoal adsorption.

Direct in vitro *incubation.* The 700 *g* supernatant from kidney homogenate was prepared as described in the stability experiments. Labelled and unlabelled hormones were added to the 700 *g* supernatant which was centrifuged for 1 h at 105,000 *g*. The total incubation time was 2 h 30 min for [^{3}H]-aldosterone and 3 h 30 min for [^{3}H]-dexamethasone. These incubation periods were selected according to the results of the stability studies.

Delayed in vitro *incubation.* The cytosol was prepared without any addition of steroid to the 700 *g* supernatant. Radioactive and non-radioactive steroids were added to the cytosol with the same incubation times as in previous series.

Protein content was determined by the method of Lowry [12].

Attempts to increase [^{3}H]-aldosterone and [^{3}H]-dexamethasone binding sites. Several experiments were performed to try to stabilize and/or to increase the apparent number of [^{3}H]-aldosterone and [^{3}H]-dexamethasone binding sites. Kidneys were homogenized in the buffer tested and the cytosol prepared as described before in stability experiments of filled binding sites. Measurements of [^{3}H]-aldosterone and [^{3}H]-dexamethasone concentration of sites were performed at equilibrium and every h for five consecutive h.

RESULTS

Stability of aldosterone and dexamethasone binding sites

Figures 1a and 1b show the stability of filled and unfilled aldosterone and dexamethasone receptors at 0°C. When saturating concentrations of tritiated hormone were added to the 700 *g* supernatant the maximum binding was obtained after an incubation period of 2 h 30 min. This time point was thus chosen to represent 0 h for the disappearance studies. As shown

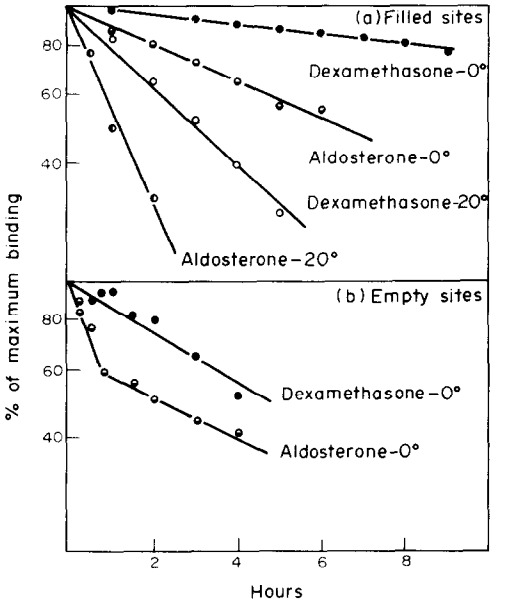

Fig. 1. Stability of aldosterone and dexamethasone binding sites at 0° and 20°. (a) Stability of filled binding sites. Aldosterone and dexamethasone receptors were saturated at 0°C with 10^{-7}M tritiated hormone in the 700 *g* supernatant. After 1 h centrifugation at 30 000 *g* the cytosol was kept for various time at 0°C ([^{3}H]-aldosterone ⊖——⊖, ^{3}H-dexamethasone ●——●) or at 20°C ([^{3}H]-aldosterone ◐——◐, [^{3}H]-dexamethasone ○——○) before the charcoal assay. Results are expressed as specific binding. (b) Stability of empty binding sites. Aldosterone and dexamethasone binding sites were saturated with [^{3}H]-aldosterone (⊖——⊖) or [^{3}H]-dexamethasone (●——●) after keeping the cytosol, free of steroid, for various times. Data are corrected for non specific binding.

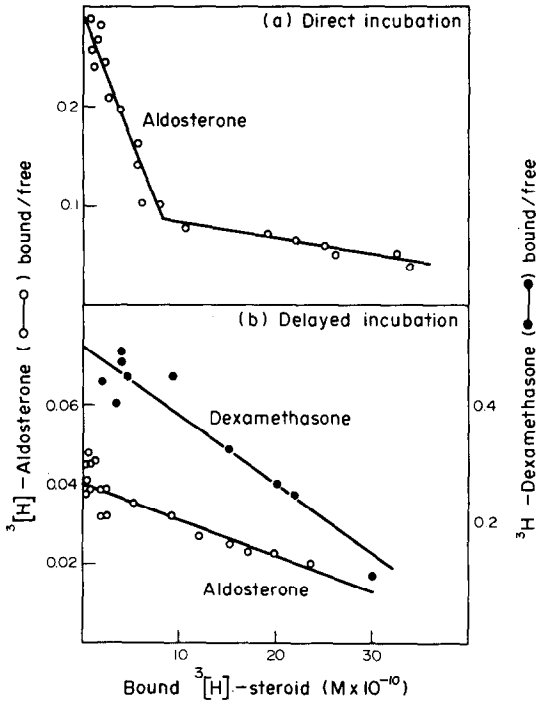

Fig. 2. Scatchard analysis of aldosterone and dexamethasome binding in rat kidney cytosol. (a) Direct incubation. Increasing concentrations of [^{3}H]-aldosterone were added to the 700 *g* supernatant and the cytosol was prepared. After a total incubation time of 2 h 30 min bound and free hormone were separated using the charcoal technique. (b) Delayed incubation. The cytosol was prepared without ligand and subsequently incubated with varying concentrations of [^{3}H]-aldosterone (o——o) for 2 h 30 min or with [^{3}H]-dexamethasone (●——●) for 3 h 30 min. All data are expressed as specific binding.

in Fig. 1a there is a linear decrease with time in the number of binding sites for both steroids. However the loss of aldosterone receptors is faster than that of dexamethasone receptors since after 8 h more than 50% of aldosterone binding sites are lost whereas 80% of the dexamethasone receptors are still present. The maximum binding of unfilled sites was obtained just after the 105,000 *g* centrifugation. This was thus taken as the initial time point for the kinetic studies. Figure 1b shows that the decrease in the binding capacity, of both aldosterone and dexamethasone receptors is greater when the sites are unfilled. The disappearance curve of dexamethasone binding sites was clearly linear throughout the period of incubation, whereas that of aldosterone sites showed two different regression lines. The first regression line was very steep and corresponded to a loss of 40% of specific [^{3}H]-aldosterone binding within approximately 1 h. This suggested that there were two classes of aldosterone binding sites, one highly unstable that could be lost within 1 h, when unprotected by their ligand, and a second group which decayed more slowly.

Figure 1a shows the influence of temperature (20°C) on saturated binding sites. Again, dexamethasone binding sites were lost more slowly than aldosterone sites, since 10% of the initial aldosterone binding sites were found after 2 h, at which time about 70% of dexamethasone sites remained. Without saturation at 20°C both gluco- and mineralocorticoid receptors were totally degraded in less than 1 h (not shown).

From these experiments, aldosterone and dexamethasone binding sites appeared to be dramatically stabilized by their ligand. It was therefore of interest to document further these relative instabilities and to see if the loss of these binding sites could be prevented.

Scatchard analysis of aldosterone and dexamethasone binding sites

In a first series of experiments, kidney slices were incubated at 20°C with increasing concentrations of [^{3}H]-aldosterone. Specific binding plotted by the method of Scatchard [13] (not shown) indicated that aldosterone was bound to two different classes of binding sites, one with a high affinity ($K_{DI} = 3.5 \times 10^{-9}$ M) and the second with a lower affinity ($K_{DII} = 4.7 \times 10^{-8}$ M).

The same binding experiments were performed directly *in vitro*. When [^{3}H]-aldosterone was added to the 700 *g* supernatant (direct incubation), the Scatchard plot showed two slopes (Fig. 2a), as in kidney slice experiments. The dissociation constants of the two sites have been estimated, they are for the first and the second set of sites respectively: $K_{DI} = 3.9 \times 10^{-9}$ M and $K_{DII} = 5.3 \times 10^{-8}$ M. The apparent numbers of binding sites were respectively $N_I = 9 \times 10^{-14}$ and $N_{II} = 39 \times 10^{-14}$ mol/mg of proteins. In contrast, when cytosol was first prepared and then incubated with [^{3}H]-aldosterone (delayed incubation), aldosterone became bound to only a single class of sites (Fig. 2b). The apparent K_D was 9×10^{-8} M and the concentration of binding sites was 2.3×10^{-13} mol/mg of proteins. Since the high affinity and low capacity class of sites has previously been shown to be mineralocorticoid sites [7, 9] these experiments suggested that mineralocorticoid sites were lost during the preparation of cytosol when they were not protected by their ligand as in direct incubation.

The Scatchard analysis of dexamethasone binding sites was not dependent on the preparation of cytosol. Direct and delayed incubation experiments showed that, in both cases, dexamethasone bound to a single set of binding sites with a K_D of 8.4×10^{-9} M and a number of binding sites of 3.1×10^{-13} mol/mg of proteins (Fig. 2b).

Competition experiments

The lability of unfilled aldosterone sites was further documented by competition experiments. In *in vitro* experiments, (Table 1), unlabelled aldosterone was the best competitor for [^{3}H]-aldosterone binding sites

Table 1. Concentration of competing steroid necessary to displace 50% of labelled aldosterone or dexamethasone in direct or delayed *in vitro* incubation

	Aldosterone ($\times 10^{-8}$ M)	Dexamethasone ($\times 10^{-8}$ M)
Direct incubation ([^{3}H]-aldosterone: 5×10^{-10} M)	0.71	1.4
Delayed incubation ([^{3}H]-aldosterone: 5×10^{-10} M)	2.0	1.2
Direct incubation Delayed incubation ([^{3}H]-dexamethasone: 5×10^{-9} M)	7.5	1.9

when incubations were performed on the 700 *g* supernatant. However, in delayed incubations, dexamethasone competed more than aldosterone for displacing [^{3}H]-aldosterone, suggesting again that mineralocorticoid sites specificity was lost in this preparation. In contrast, the concentrations of competitor (aldosterone or dexamethasone) used to obtain 50% of [^{3}H]-dexamethasone binding were the same with direct and with direct and with delayed incubation (Table 1).

Studies on the stabilization of the [^{3}H]-aldosterone and [^{3}H]-dexamethasone binding sites

Several trials were performed in order to stabilize [^{3}H]-aldosterone and [^{3}H]-dexamethasone binding sites. First, the composition of various buffers was tested. Table 2 shows the results obtained at equilibrium for [^{3}H]-aldosterone and [^{3}H]-dexamethasone binding sites. Sucrose and glycerol appeared clearly to increase the apparent number of [^{3}H]-aldosterone binding sites. 1 mM EDTA also increased the binding of [^{3}H]-aldosterone and [^{3}H]-dexamethasone binding sites. However, [^{3}H]-aldosterone and [^{3}H]-dexamethasone binding sites were lost at the same rate for 5 h whatever the buffers used, suggesting that no stabilization could be obtained in the conditions used. The use of different protease inhibitors such as: D-tryptophan methyl ester (1 mM), a chymotrypsin inhibitor [14]; benzamidine (1 mM), a trypsin inhibitor [15]; sodium tetrathionate (1 mM), phenylmethanesulfonylfluoride (1 mM), diisopropylphosphofluoridate (1 mM), pepstatin (0.01 mM), protease inhibitors [16] failed to prevent the loss of mineralo- and glucocorticoid sites.

DISCUSSION

Since the very first studies on aldosterone binding proteins, in rat kidney, a great difficulty of working on these receptors in complete *in vitro* experiments has been reported [17, 18]. It is difficult to compare these experiments to the more recent studies [7–9] since aldosterone binding to mineralo- and glucocorticoid receptors was not yet distinguished. However, Robinson and Fanestil [17] showed that 25% of unfilled aldosterone binding sites were lost within 2 h at 4°C and maximum 'binding aldosterone capacity' could only be seen in 10,000 *g* supernatant after 15 min of centrifugation. Ludens and Fanestil [18] also pointed out that *in vitro* aldosterone bound to a high affinity, low capacity set of sites which probably represented the mineralocorticoid sites, but the data points showed a high degree of scatter, probably due to the method of preparation of the supernatant fraction. These results could be explained by the unstability of aldosterone and dexamethasone receptors.

In the present study a series of experiments were conducted in order to define the optimal conditions

Table 2. Influence of buffer composition on [^{3}H]-aldosterone and [^{3}H]-dexamethasone binding

Buffer	[^{3}H]-Aldosterone binding	[^{3}H]-Dexamethasone binding
A(10 mM Tris–HCl, pH 7.4)	100	100
A + 0.1 M Sucrose	102	91
A + 0.25 M Sucrose	153	111
A + 10% glycerol	167	117
B (10 mM Tris–HCl, pH 7.4 10% glycerol)	100	100
B + 3 mM $CaCl_2$	78	79
B + 3 mM $MgCl_2$	72	79
B + 1 mM EDTA	120	116

Specific binding is determined after direct incubation with 10^{-7} M [^{3}H]-aldosterone for 2 h 30 min and with 10^{-7} M [^{3}H]-dexamethasone for 3 h 30 min. Results are expressed as % of binding with reference made for Buffer A or B (100% binding).

for studying *in vitro* renal mineralo- and glucocorticoid sites. The ability of ligand to protect against denaturation or inactivation mineralo- and glucocorticoid sites has been documented by stability studies, Scatchard plot analysis and specificity of mineralocorticoid sites as tested by competition experiments.

The stability studies showed that mineralocorticoid sites are far more unstable than glucocorticoid binding sites. When unfilled, the aldosterone binding sites were rapidly lost whereas, under the same conditions, glucocorticoid sites were still recovered. The addition of ligand after homogenization is an absolute requirement in order to preserve mineralocorticoid sites, but this precaution is not mandatory for the study of glucocorticoid binding sites since unprotected dexamethasone binding sites kept their binding characteristics. Therefore the studies on aldosterone binding sites must be done with precautions to preserve the stability of the aldosterone receptor. The binding proteins must be protected by the presence of ligand, low temperature and speed of manipulation, otherwise most of the binding observed may be primarily to the dexamethasone receptor. Moreover, besides their binding affinity and specificity, the gluco- and mineralocorticoid binding sites can be distinguished according to their stability. A protective effect of ligand against steroid receptor denaturation has already been reported for the estradiol receptor [19–21] and for glucocorticoid receptors [22–23] although in no case complete loss of steroid binding proteins activity was observed.

The mechanism of destruction or inactivation of steroid receptors in rat kidney is not clear. Glycerol- or sucrose-containing buffers increased the apparent number of binding sites but did not stabilize these proteins. This improvement in the apparent number of cytosolic or chromatin binding sites by addition of sugar solutions had already been noted for aldosterone binding proteins [9, 5, 17] and is shared by other steroid receptors [24]; it could be due to a prevention of the aggregation of steroid-binding proteins. The addition of Ca^{2+} to the preparation decreased the number of sites; this could be due to a calcium-activated proteolysis since some proteolytic enzymes are activated by Ca^{2+} [25]. Moreover, it has been shown that estradiol receptor undergoes a transformation in the presence of Ca^{2+} ions and it has been suggested that this is a Ca^{2+} activated proteolysis [26]. However, attempts to decrease proteolysis by various protease inhibitors were unsuccessful.

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REFERENCES

1. Edelman I. S., Bogoroch R. and Porter G. A.: *Proc. natn. Acad. Sci. U.S.A.* **50** (1963) 1169–1177.
2. Sharp G. W. G., Komack C. L. and Leaf A.: *J. clin. Invest.* **45** (1966) 450–459.
3. Fanestil D. D. and Edelman I. S.: *Proc. natn. Acad. Sci. U.S.A.* **56** (1966) 872–879.
4. Herman T. S., Fimognari G. M., and Edelman I. S.: *J. biol. Chem.* **243** (1968) 3849–3856.
5. Swaneck G. E., Chu L. L. H. and Edelman I. S.: *J. biol. Chem.* **245** (1970) 5382–5389.
6. Marver D., Goodman D. and Edelman I. S.: *Kidney International* **1** (1972) 210–223.
7. Rousseau G., Baxter J. D., Funder J. W., Edelman I. S. and Tomkins G. M.: *J. steriod Biochem.* **3** (1972) 219–227.
8. Funder J. W., Feldman D. and Edelman I. S.: *Endocrinology* **92** (1973) 1005–1013.
9. Funder J. W., Feldman D. and Edelman I. S.: *Endocrinology* **92** (1973) 994–1004.
10. Funder J. W., Feldman D., Highland E. and Edelman I. S.: *Biochem. Pharmacol.* **23** (1974) 1493–1501.
11. Ulmann A., Menard J. and Corvol P.: *Endocrinology* **97** (1975) 46–51.
12. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: *J. biol. Chem.* **193** (1951) 265–275.
13. Scatchard G.: *Ann. N.Y. Acad. Sci.* **51** (1949) 660–672.
14. Huang H. T. and Niemann C.: *J. Am. chem. Soc.* **73** (1951) 1541–1548.
15. Tanizawa K., Ishii S., Hamaguchi K. and Kanaoka Y.: *J. Biochem. (Japan)* **69** (1971) 893–899.
16. Aoyagi T., Kunimoto S., Morishima H., Takeuchi T. and Umezawa H.: *J. Antibiotics* **24** (1971) 687–694.
17. Robinson R. G. and Fanestil D. D.: *Acta endocr.* Supplt. **147** (1970) 275–288.
18. Ludens J. H. and Fanestil D. D.: *Biochim. biophys. Acta* **244** (1971) 360–371.
19. Baulieu E. E., Alberga A., Jung I., Lebeau M. C., Mercier-Bodard C., Milgrom E., Raynaud J. P., Raynaud-Jammet C., Rochefort H., Truong H. and Robel P.: *Recent Prog. Horm. Res.* **27** (1971) 351–419.
20. Puca G. A., Nola E., Sica V. and Bresciani F.: *Biochemistry* **10** (1971) 3769–3779.
21. Katzenellenbogen J. A., Johnson H. J. Jr. and Carlson K. E.: *Biochemistry* **12** (1973) 4092–4099.
22. Bell P. A. and Munck A.: *Biochem. J.* **136** (1973) 97–107.
23. Watanabe H.: *J. steroid Biochem.* **6** (1975) 27–33.
24. Feil P. D., Glasser S. R., Toft D. O. and O'Malley B. W.: *Endocrinology* **91** (1972) 738–746.
25. Schulze I. T. and Colowick S. P.: *J. biol. Chem.* **244** (1969) 2306–2315.
26. Puca G. A., Nola E., Sica V. and Bresciani F.: *Endocrinology* **11** (1971) 4157–4167.