Journal of Chromatography, 297 (1984) 301–311
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 337

HIGH-PERFORMANCE AND ION-EXCHANGE CHROMATOGRAPHY AND CHROMATOFOCUSING OF THE HUMAN UTERINE PROGESTERONE RECEPTOR: ITS APPLICATION TO THE IDENTIFICATION OF 21-[^{3}H]DEHYDRO ORG 2058-LABELLED RECEPTOR

ARNULF HEUBNER, BERNHARD MANZ*, HANS-JÖRG GRILL and KUNHARD POLLOW
Abteilung für Experimentelle Endokrinologie, Johannes Gutenberg-Universität Mainz, Langenbeckstrasse 1, 6500 Mainz (F.R.G.)

SUMMARY

Two independent lines of evidence were used to identify the human uterine progesterone receptor. First, three differently tritiated progestogens (Org 2058, R 5020, progesterone) were used for reversible labelling of the receptor. Secondly, the highly potent affinity label 21-[^{3}H]dehydro Org 2058 was used to label covalently the steroid-specific binding site of the receptor. The labelled cytosols were chromatographed on a Mono Q high-performance anion-exchange column in the absence or presence of a high molar excess of the respective unlabelled competitor steroids. In the case of 21-[^{3}H]dehydro Org 2058, Org 2058 was used as the unlabelled competitor. After elution with a NaCl gradient, the radioactivity was determined in each fraction and the elution profiles (absorption, A at 280 nm; radioactivity, dpm) were superimposed. Free steroid was eluted with the washing buffer. When the NaCl gradient was performed, two peaks of radioactivity were located. The specifically protein-bound radioactivity was eluted at 0.08 M NaCl. Two non-specific steroid-binding entities were eluted at 0.1 and 0.22 M NaCl, the second of which was identified as albumin. The elution profiles of tritiated progesterone, R 5020, Org 2058 and the affinity label 21-dehydro Org 2058 were identical.

In a second set of experiments, Org 2058- and 21-dehydro Org 2058-labelled cytosols were subjected to high-performance liquid chromatography on a Mono P high-performance chromatofocusing column in the absence or presence of a high molar excess of unlabelled Org 2058. After elution with Polybuffer 74, only one specifically labelled protein (pH 6.4) was detected. When the Mono P-purified receptor was submitted to sodium dodecyl sulphate polyacrylamide gel electrophoresis, two labelled polypeptides with M_r = 45,000 and 27,000 were detectable.

INTRODUCTION

Highly purified steroid receptor proteins are required for studying the complete sequence of events in the interaction of a steroid molecule with the target cell. The reversible nature of the binding of a steroid to its intracellular receptor molecule has

0021-9673/84/$03.00

complicated not only efforts to investigate the fate of a steroid after it enters the cell, but also attempts to purify the steroid receptors. For this reason, affinity-labelled steroids, yielding covalent steroid–receptor complexes, would be of help in the elucidation of the whole sequence of intracellular steroid action. The majority of experiments to date have been performed using electrophilic[1–3] or UV-sensitive steroid analogues[4–7].

In principle, affinity labels may react with numerous other proteins in crude cytosols[8] to yield covalent complexes. Therefore, it is necessary to be able to identify the covalently labelled receptor in the presence of other labelled proteins.

We describe here the first application of high-performance liquid chromatography (HPLC) in the detection and chromatographic resolution of affinity-labelled human uterine progesterone receptors[9,10]. The results of this study suggest that in many experiments HPLC may provide a useful alternative to gel electrophoresis, followed by gel slicing or autoradiography[11].

MATERIALS AND METHODS

Steroids

^{3}H-Org 2058 (specific activity 42 Ci/mmol, 155 TBq/mmol) was a generous gift from Amersham International (Amersham, U.K.). ^{3}H-R 5020 (86 Ci/mmol) and [^{3}H]progesterone (97 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Unlabelled Org 2058 (16α-ethyl-21-hydroxy-19-nor-pregn-4-ene-3,20-dione) was a gift from Organon (Oss, The Netherlands). Unlabelled R 5020 (17α-methyl-20-ethyl-19-nor-4,9-pregnadiene-3,20-dione) was purchased from New England Nuclear. All other chemicals were purchased from Sigma (München, F.R.G.).

NMR spectra were obtained with a field strength of 90 MHz on samples in C^2HCl_3 on a Bruker MX 90, using tetramethylsilane as an internal standard. Mass spectra were obtained with a Varian Mat 711 (100 eV). Analytical and preparative thin-layer chromatography (TLC) were carried out using precoated silica gel F_{254} plates (Merck, Darmstadt, F.R.G.). Thin-layer plates were developed in the following solvent systems: A, chloroform–methanol (9:1, v/v); B, *n*-butanol–glacial acetic acid–water (12:3:5, v/v/v).

Synthesis of 16α-ethyl-19-nor-4-pregnene-3,20-dione-21-al (21-dehydro Org 2058; Fig. 1)

To Org 2058 (0.1 g), dissolved in 150 ml methanol, 12.5 mg of cupric acetate, dissolved in 100 ml methanol, were added and air was bubbled through the well stirred solution. The reaction was stopped after 30 min by adding 200 ml water. The solvents were evaporated under reduced pressure and the remaining yellow oil was dissolved in ethyl acetate. The organic phase was washed twice with sodium hydrogencarbonate (1%) and water. The solvent was evaporated and the remaining oil further purified by preparative TLC in solvent system B. Mass spectrum (high resolution): *m/e* 342.2592, $C_{22}H_{30}O_3$. NMR: δ 9.15 (aldehyde). R_F: in solvent system A, 0.97; in solvent system B, 0.8.

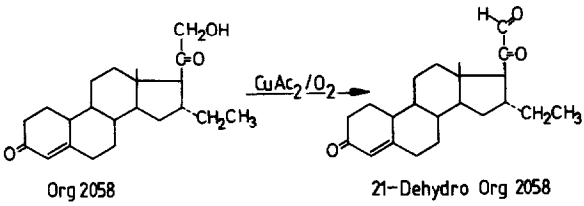

Fig. 1. Synthesis of 21-dehydro Org 2058. Ac = Acetate.

Synthesis of 16α-ethyl-19-nor-4-pregnene-3,20-dione-21-[^{3}H]al (21-[^{3}H]dehydro Org 2058)

^{3}H-Org 2058 (48 nmol; 2 mCi, specific activity 42 Ci/mmol) in 2 ml of toluene–ethanol (9:1, v/v) was evaporated to dryness in a round-bottomed flask and redissolved in 2 ml methanol. The flask was equipped with a magnetic stirrer and air was bubbled into the solution through a capillary tube. Cupric acetate (300 μl, 0.37 μmol) in methanol was added to the well stirred solution and the oxidation was complete within 30 min. During the reaction it was necessary to wash the glass walls of the reaction flask with an additional 1 ml methanol to minimize unreacted starting material. The reaction was stopped by adding the same volume of distilled water. The organic solvent was partly removed and the steroid extracted three times with 15 ml ethyl acetate. The combined organic extracts were evaporated to dryness, the residue was again dissolved in 200 μl ethyl acetate and further purified on thin-layer plates in solvent system B. The identity of the purified steroid was confirmed by co-elution with unlabelled 21-dehydro Org 2058 on thin-layer plates in solvent systems A and B. No attempts were made to determine the specific activity of 21-[^{3}H] dehydro Org 2058. Radiochemical yield: about 70–80%.

Preparation of cytosols

Human tissues were quick frozen in liquid nitrogen and stored at −80°C until use. Tissue homogenization was performed in ice-cold TE buffer (10 m*M* Tris–HCl, 1.5 m*M* EDTA, pH 7.5) or b-TI buffer (25 m*M* BisTris, iminodiacetic acid, pH 7.1) using an Ultra Turrax. The homogenates were centrifuged for 1 h at 105,000 *g* and the supernatants taken as cytosol.

Competition assay

The tubes were prepared as follows: into each tube 0.1 ml of ^{3}H-Org 2058 (in TE buffer) was pipetted, to give a final concentration of 32 n*M*. Then aliquots of 0.1 ml containing the various competitors (in TE buffer) at ten different concentrations (3.2–640 nmol/l) were added. Finally, 0.2 ml of cytosol were added to each tube and the tubes were incubated for 4 h at 0°C. Incubation was terminated by addition of 0.5 ml dextran-coated charcoal (DCC) suspension. The final concentrations were 5 g/l of charcoal and 0.5 g/l of dextran T500. After 10 min of incubation the tubes were centrifuged for 10 min at 5000 *g*. One ml of the supernatant was withdrawn and counted for radioactivity. All determinations were carried out in triplicate.

Affinity labelling

Cytosol (approximately 5 mg protein per ml) was incubated with 32 nmol/l

21-[^{3}H]dehydro Org 2058 for 4 h at 0–4°C. Sodium tetrahydroborate (10 mmol/l) was added and the reactants kept for 16 h at 0–4°C. After gel filtration on Sephadex G-50 fine (Pharmacia, Uppsala, Sweden), equilibrated with TE or b-TI buffer, samples were stored at −20°C until used.

High-performance liquid chromatography

Chromatography was carried out on the Pharmacia FPLC system, fitted with either I or II. I was a pre-packed Mono Q column (high efficiency anion exchanger based on monodisperse 10-μm spheres, Pharmacia). The column was equilibrated in TE buffer at 25°C and eluted at different flow-rates (0.75–2 ml/min) with a NaCl gradient to 1 mol/l.

II was a pre-packed Mono P column (polybuffer exchanger, chromatofocusing, Pharmacia). The column was equilibrated in b-TI buffer at 25°C and eluted at a flow-rate of 1 ml/min with 100 ml polybuffer 74, pH 4.0, iminodiacetic acid (1:10 dilution of polybuffer 74 stock solution; Pharmacia).

Protein was monitored at 280 nm. 500-μl Fractions were collected and radioactivity was monitored by liquid scintillation counting (Ready-Solv HP; Beckman Instruments, München, F.R.G.).

RESULTS

Binding of 21-dehydro Org 2058 to human uterine cytosol receptors

Cupric acetate-catalyzed oxidation of Org 2058 by oxygen led to the 21-de-

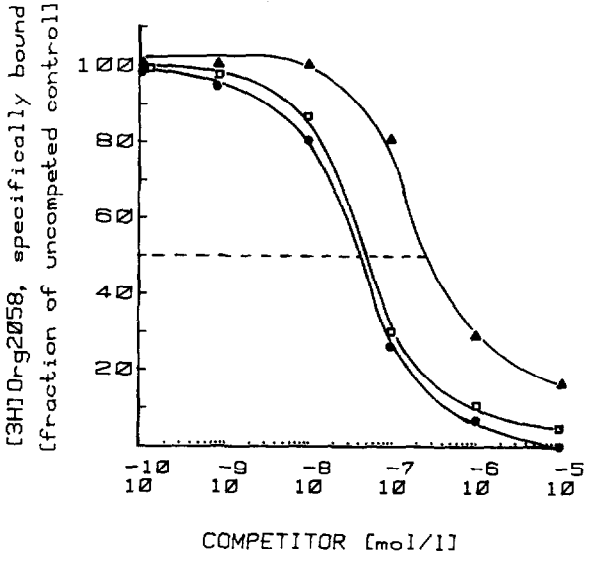

Fig. 2. Competitive binding of progesterone (▲), Org 2058 (●) and 21-dehydro Org 2058 (□) to uterine cytosol progesterone receptors. Cytosol was incubated for 4 h at 4°C with 32 n*M* ^{3}H-Org 2058 in the presence of various concentrations of the above reagents as described previously[13]. Each point represents the mean of triplicate DCC assays. Bound ^{3}H-Org 2058 is expressed as a percentage of that bound in the absence of competitors. Points of intersection with the dashed line (50%-binding) represent the relative binding affinity (RBA-value).

hydro derivative in almost quantitative yield (Fig. 1). In order to assess the interaction of this derivative with the uterine progesterone receptor, we first examined its ability to compete with ^{3}H-Org 2058, a well characterized progesterone receptor ligand[12,13], for binding to the receptor in crude cytosol. Fig. 2 shows that the loss of the 21-hydroxy group of Org 2058 is accompanied by a significant reduction of binding ability. In the hierarchy of relative binding affinities (referred to Org 2058), however, 21-dehydro Org 2058 is still superior to progesterone.

Attempts to determine the constants for binding of 21-[^{3}H]dehydro Org 2058 to the progesterone receptor by Scatchard plot analysis[14] failed. Following an overnight incubation of 32 n*M* of the tritiated derivative with or without a 200-fold excess of unlabelled Org 2058, only small differences between total and non-specific binding could be detected. In addition, the total radioactivity after charcoal treatment was significantly higher than that found when ^{3}H-Org 2058 was used as ligand. As our competition experiments (Fig. 2) with unlabelled 21-dehydro Org 2058 and ^{3}H-Org 2058 as ligands gave no clue to the reasons for these discrepancies, the reversibility of the steroid receptor interaction was studied. To control the exchange conditions, 3200 n*M* of unlabelled Org 2058 were incubated with the same uterine cytosol for 2 h and processed as described in the legend to Fig. 2. The exchange was performed

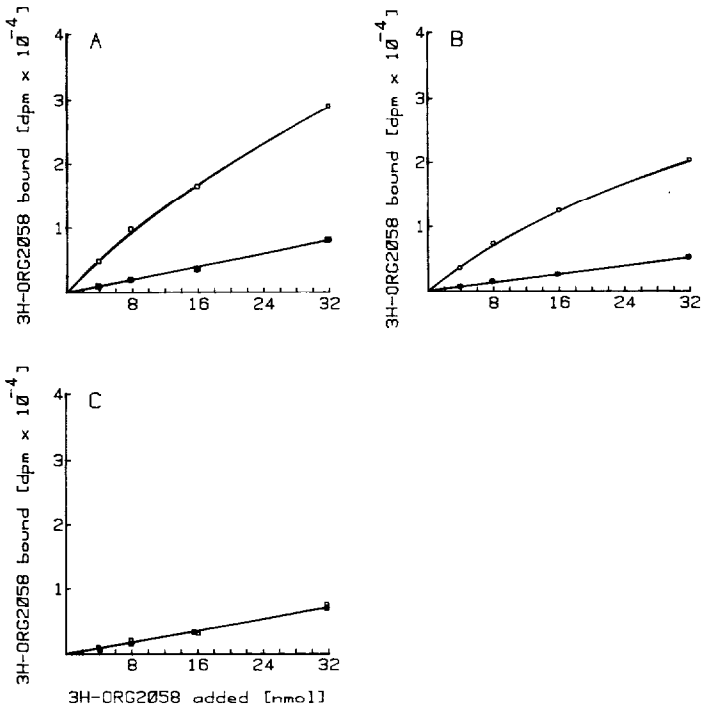

Fig. 3. Exchange of receptor-bound unlabelled Org 2058 and 21-dehydro Org 2058 against ^{3}H-Org 2058. Uterine cytosol was divided into three equal batches. One batch was kept at 4°C until use (A). The remaining batches were incubated with either Org 2058 (B) or 21-dehydro Org 2058 (C), final concentration 3200 n*M*. After 2 h the unbound steroids were removed with DCC and both cytosols, together with batch A, were incubated with increasing concentrations (1–32 n*M*) of ^{3}H-Org 2058 (open symbols) with or without a 200-fold excess of Org 2058 (closed symbols) for 2 h at 25°C. The samples were then cooled for 10 min in an ice-bath and assayed for specifically bound ^{3}H-Org 2058 as described previously[13].

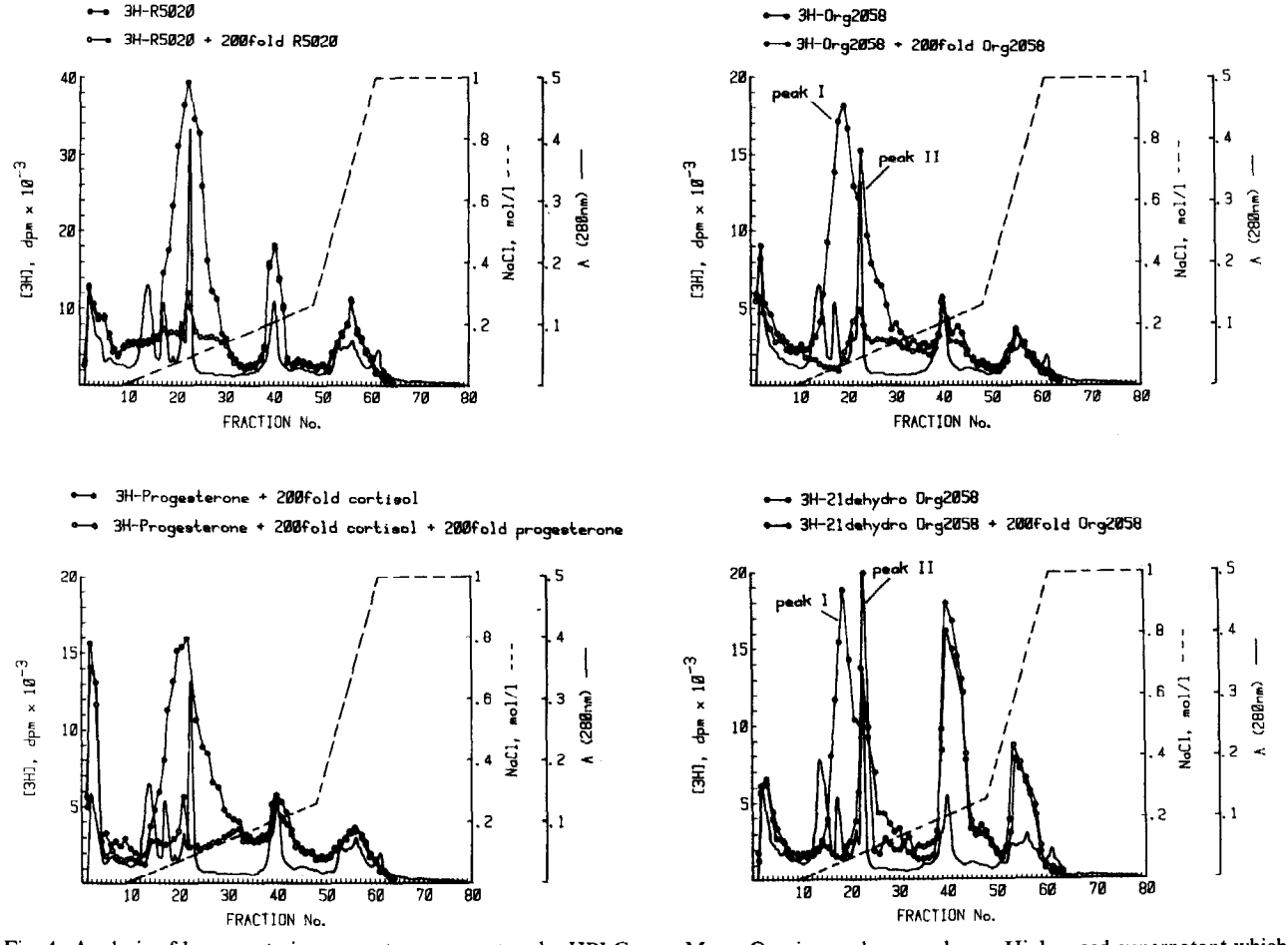

Fig. 4. Analysis of human uterine progesterone receptors by HPLC on a Mono Q anion-exchange column. High speed supernatant which had been exposed to [^{3}H]steroid (32 n*M*) with (○) or without (●) non-radioactive competitor (6.4 μM), (500 μl) was applied to the column. The elution was performed as described under Materials and Methods. Absorbance determinations at 280 nm are shown by the full line, NaCl gradients by the dashed lines.

at different temperatures (data not shown) and an exchange lasting for 2 h at 25°C was found to be optimal, although a 100% exchange was never obtained. Fig. 3 summarizes the results. The steady-state binding of ^{3}H-Org 2058 to the untreated (Fig. 3A) or Org 2058-labelled (Fig. 3B) cytosols was, except for the total amount of available progesterone receptors, very similar. 21-Dehydro Org 2058 (Fig. 3C, however, irreversibly inactivated the progesterone receptor and only non-specific binding was detectable.

HPLC of human uterine progesterone receptor

The results in Fig. 4 illustrate the HPLC of differently labelled uterine progesterone receptors on a Mono Q anion-exchange column. Independent of the

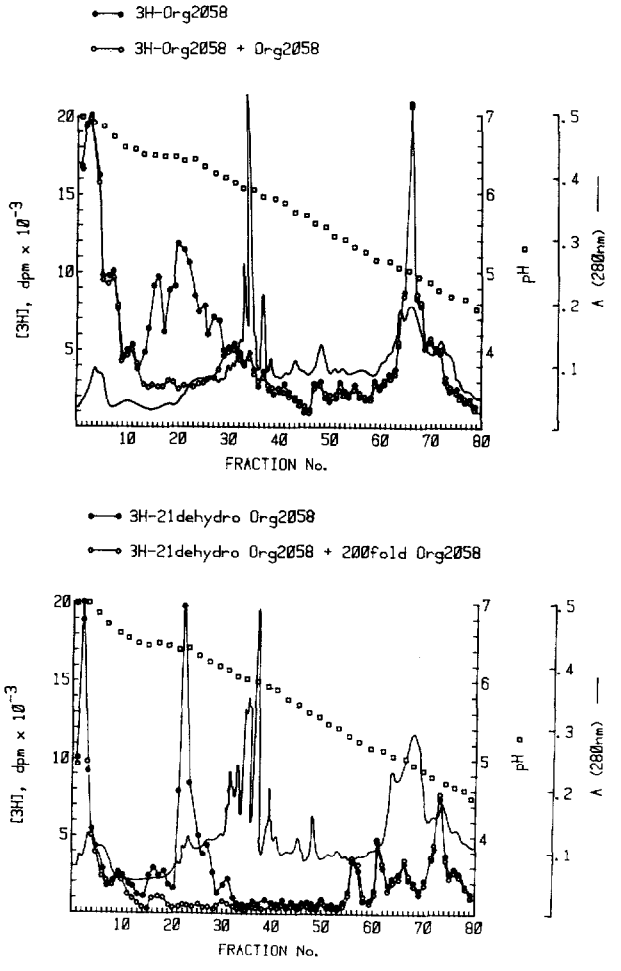

Fig. 5. Chromatofocusing of human uterine progesterone receptors by HPLC on a Mono P polybuffer exchanger. High speed supernatant which had been exposed to [^{3}H]steroid (32 nM) with (○) or without (●) non-radioactive competitor (6.4 μM, 500 μl) was applied to the column. The elution was performed as described under Materials and Methods. Absorbance determinations at 280 nm are shown by the continuous line, pH gradients by the squares.

[^{3}H]progestine used, the specifically labelled proteins were eluted as single peaks (peak I) at approximately 0.075 *M* NaCl. The non-specifically labelled protein eluted at 0.22 *M* NaCl was generally found in affinity-labelled preparations (Fig. 4) and was identified as serum albumin contamination (LC-Partigen Albumin, immunodiffusion plates; Behring-Werke, Marburg, F.R.G.; data not shown). Due to the higher dissociation rates of ^{3}H-R 5020- and ^{3}H-progesterone–receptor complexes[15,16], no significant resolution of peaks I and II on the Mono Q column was obtained. Varying the flow-rate had no impact on the resolution of the peaks. Yet, the slower the flow-rate, the smaller were the radioactivity peaks and the more radioactivity could be found in the void volume.

The comparison of ^{3}H-Org 2058- and 21-[^{3}H]dehydro Org 2058-labelled uterine cytosols is shown in Fig. 4. In the case of affinity-labelled cytosols, very sharp resolution of peaks I and II was possible.

The results in Fig. 5 illustrate chromatofocusing of ^{3}H-Org 2058- and 21-[^{3}H] dehydro Org 2058-labelled uterine cytosols. In case of Org 2058, a heterogeneous pattern of specifically labelled proteins was obtained.

As chromatofocusing is much more time-consuming than anion-exchange chromatography, lower levels of labelled receptor were obtained. On the contrary, when affinity-labelled cytosol was applied onto the column, one sharp peak of specifically labelled protein was detectable (Fig. 5). The non-specifically labelled proteins showed an heterogeneous pattern. Nevertheless it should be emphasized that a two-fold higher yield of specifically and non-specifically labelled proteins was obtained when affinity-labelled cytosols were used and that no precipitates were caused by the pH gradient.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

To determine an approximate value of the molecular weight of the affinity-

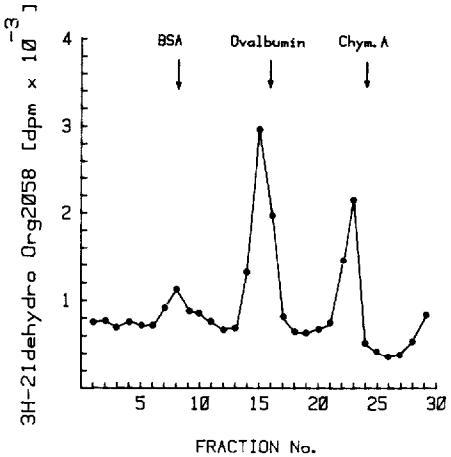

Fig. 6. SDS-PAGE of HPLC-purified 21-[^{3}H]dehydro Org 2058-labelled receptor. The labelled receptor was partly purified by chromatofocusing on a Mono P polybuffer exchanger (Fig. 5). The receptor fractions were pooled and precipitated with 10% trichloroacetic acid. SDS-PAGE was performed according to Laemmli[21], the gel sliced (3-mm slices) and digested overnight in 0.5 ml of 30% H_2O_2 and then the radioactivities were measured. BSA = Bovine serum albumin.

labelled receptor, the Mono P purified progesterone-receptor preparation (Fig. 5) was precipitated with trichloroacetic acid and submitted to SDS-PAGE (Fig. 6). The Mono P fraction was preferred to the Mono Q purified fraction because of its higher purity. As the total amount of affinity-labelled receptor (computed on the basis of an specific activity of 21-[^{3}H]dehydro Org 2058 of 42 Ci/mmol and a molecular weight of 45,000[6,13]) is less than 10 pg of protein per chromatogram, no attempts were made to identify the receptor by protein-staining procedures. Although gel slicing does not allow the exact determination of the molecular weights of labelled proteins, two radioactive peaks corresponding to $M_r \approx 45{,}000$ and $\approx 27{,}000$ were always detectable.

DISCUSSION

HPLC has been the method of choice for the detection and purification of a host of biopolymers[17]. The method is rapid, sensitive and ideally suited for the detection of labile, radiolabelled proteins such as steroid hormone–receptor complexes (Figs. 4 and 5) which comprise only a very small portion of the cell extract[18]. The most impressive results, however, were obtained when affinity-labelled cytosols were investigated. Despite the lability of hormone–receptor complexes at elevated temperatures (25°C), [^{3}H]progesterone- and 21-[^{3}H]dehydro Org 2058-labelled receptors are eluted at superimposable positions (Figs. 4 and 5) and only one specifically labelled protein is detectable. In addition, SDS-PAGE of partly purified (Fig. 6) labelled receptor reveals two polypeptides ($M_r \approx 45{,}000$ and $\approx 27{,}000$) whose molecular weights are in excellent accord with previously published data[6,10,19,20].

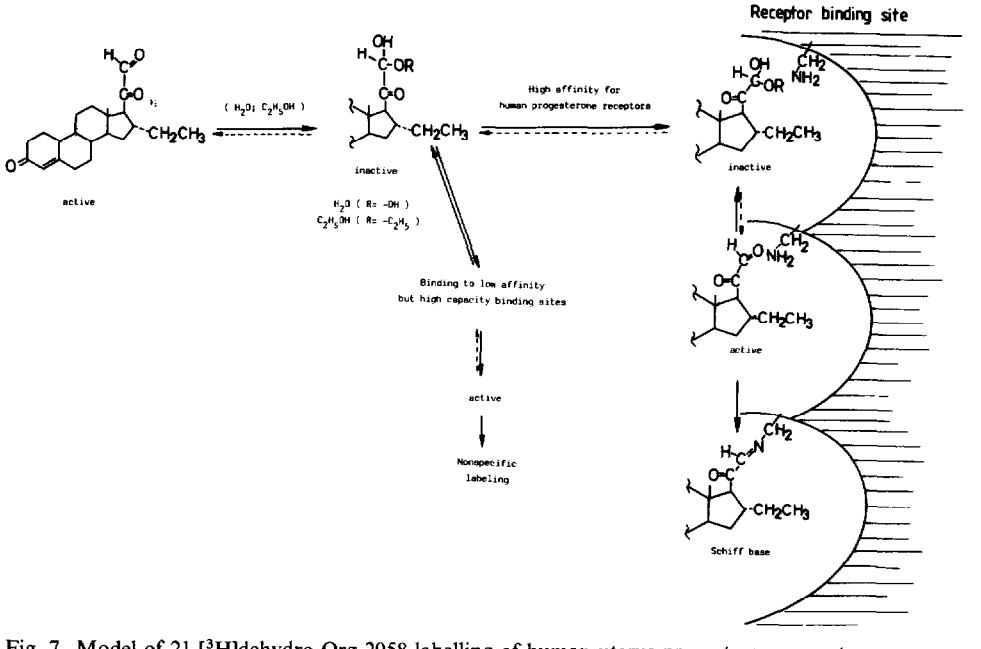

Fig. 7. Model of 21-[^{3}H]dehydro Org 2058 labelling of human uterus progesterone receptor.

Affinity labelling with 21-dehydro Org 2058 through an azomethine intermediate requires the presence of an appropriate primary amine at the protein binding site for the ligand. As the amount of covalent labelling that takes place in a complex mixture of proteins is a function of the availability of the amine and the affinity of the label for the site, low-affinity binding sites occurring in great abundance may yield as many or even more covalent adducts (azomethines) as arise from high-affinity binding sites. Thus, a significant quantity of 21-[^{3}H]dehydro Org 2058 binds to albumin (Fig. 4), as could be shown by immunochemical methods (data not shown).

Since α-keto aldehydes react readily and almost exclusively with amines, the high selectivity of labelling of receptor by 21-[^{3}H]dehydro Org 2058 needs to be discussed (Fig. 7). It is unlikely that the label exists in the α-keto aldehyde form during storage in the ethanolic stock solution or when diluted in buffer. Thus, the high receptor labelling efficiency (more than 50% of the label is specifically bound) might be the consequence of a preceding addition of ethanol or water to the aldehyde. The label would then be inactivated until bound to the receptor.

The small portion of free α-keto aldehyde, always present under equilibrium conditions, could be fixed at an optimum distance to a free amino group in the hormone binding site. According to LeChatelier's principle, the formation of azomethines (Schiff bases) would change the equilibrium, new free α-keto aldehyde being formed and allowing the labelling reaction to proceed. This hypothesis is confirmed by the observation that prolonged incubation times raise the amount of irreversibly labelled receptor (data not shown). The non-specific labelling of cytosolic proteins should be low (because of the very low concentrations of free α-keto aldehyde) whereas low-affinity binders such as serum albumin should become labelled. These predictions are in excellent accord with our HPLC data.

ACKNOWLEDGEMENT

This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

REFERENCES

1 J. C. Warren, F. Arias and F. Sweet, *Methods Enzymol.*, 36 (1975) 374 410.
2 F. Sweet, R. C. Strickler and J. C. Warren, *J. Biol. Chem.*, 253 (1978) 1385–1392.
3 S. S. Simons, Jr. and E. B. Thompson, *Proc. Nat. Acad. Sci. U.S.A.*, 78 (1981) 3541–3545.
4 J. A. Katzenellenbogen, H. J. Johnson, K. E. Carlson and H. N. Myers, *Biochemistry*, 13 (1974) 2986–2994.
5 H. M. Westphal, G. Fleischmann and M. Beato, *Eur. J. Biochem.*, 119 (1981) 101–106.
6 R. G. Smith, M. d'Jstria and N. M. Van, *Biochemistry*, 20 (1981) 5557–5565.
7 B. A. Lessey, P. S. Alexander and K. B. Horwitz, *Endocrinology*, 112 (1983) 1267–1274.
8 H. J. Eisen, R. E. Schleenbaker and S. S. Simons, Jr., *J. Biol. Chem.*, 256 (1981) 12920–12929.
9 B. Manz, A. Heubner and K. Pollow, *Eur. J. Cell Biol. Suppl.*, 2 (1983) 26.
10 B. Manz, A. Heubner, H-J. Grill, H-J. Thimm, I. Köhler and K. Pollow, *Eur. J. Biochem.*, (1983) submitted for publication.
11 V. Chowdhry and F. H. Westheimer, *Annu. Rev. Biochem.*, 48 (1979) 293–325.
12 H. M. Westphal, G. Fleischmann and M. Beato, *Eur. J. Biochem.*, 119 (1981) 101–106.
13 B. Manz, H-J. Grill, I. Köhler, A. Heubner and K. Pollow, *Eur. J. Biochem.*, 131 (1983) 333–338.
14 G. Scatchard, *Ann. N.Y. Acad. Sci.*, 51 (1949) 660.
15 O. Jänne, *Med. Biol.*, 56 (1978) 225–248.

16 J. P. Raynaud, in F. B. Thompson and M. E. Lippmann (Editors), *Steroid Receptors and the Management of Cancer*, Vol. 1, CRC Press, Boca Raton, 1979, Ch. 14.
17 J. Richey, *Technical Reviews Series*, Pharmacia Fine Chemicals, Uppsala, 1983.
18 G. C. Chamness and W. L. McGuire, in E. B. Thompson and M. E. Lippman (Editors), *Steroid Receptors and the Management of Cancer*, Vol. 1, CRC Press, Boca Raton, 1979, Ch. 1.
19 S. D. Holmes, N. T. Van, S. Stevens and R. G. Smith, *Endocrinology*, 109 (1981) 670–672.
20 S. D. Holmes and R. G. Smith, *Biochemistry*, 22 (1983) 1729–1734.
21 U. K. Laemmli, *Nature (London)*, 227 (1970) 680–682.