Résumé. L'injection intraventriculaire d'acetylcholine provoque chez le rat mâle une sécrétion accrue de LH. Cet effet de l'acetylcholine est inhibé par l'administration intraventriculaire d'atropine, et est augmenté par l'injection intraventriculaire de prostigmine.

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Purification of Rabbit Angiotensin II-Antibodies by Affinity Chromatography

Antibodies against angiotensin II have been used repeatedly for in vivo experiments, in which the participation of the renin-angiotensin system in various forms of experimental hypertension was studied (for review see CHRISTLIEB and HICKLER[1]). In most of these studies crude antisera have been injected into the test animal with the obvious disadvantage that not only the antibody, but also considerable amounts of heterologous protein, renin, renin substrate and angiotensin II (up to 300 ng/ml) were injected. In order to provide a more defined basis for in vivo studies, the conditions for purification of angiotensin-antibodies by affinity chromatography were studied.

Materials and methods. Anti-sera were raised in male white New Zealand rabbits, weighing 2000–2500 g, by immunization with asp[1]-ileu[5]-angiotensin II (Schwarz/Mann, Orangeburg, USA) coupled to porcine γ-globulin by the carbodiimide method, as described by GOODFRIEND et al.[3]. After 3 to 5 months all animals developed antibody titers ranging between 1:13000 and 1:180000.

Affinity columns for most of the separation experiments were prepared by coupling angiotensin II to the sepharose matrix via albumin as a spacer, a principle first proposed by CUATRECASAS et al.[4]. In a typical experiment 3 g of cyanogenbromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) were washed in 1 m*M tris*-HCl buffer pH 6.0, and adjusted to pH 8.0 with $NaHCO_3$ buffer. Bovine serum albumin (Behring Werke, Marburg), 20 mg in 0.1 *M* $NaHCO_3$ buffer pH 8.0, containing 0.5 *M* NaCl, was added. After 2 h of incubation, about 65% of the albumin had been bound to the sepharose as judged by the decrease in absorbancy at 280 nm and protein content in the supernatant[5]. Following treatment with 1 *M* ethanolamine at pH 8 for 2 h, the product was washed 4 times alternately with 0.1 *M* borate buffer pH 8.0, and 0.1 *M* acetate buffer pH 4.0, and finally adjusted to pH 6.0. subsequently 2 mg angiotensin II were coupled to the sepharose bound albumin by the addition of 1.2 g of N-ethyl-N-dimethyl-aminopropyl-carbodiimide at pH 6.0. A small amount of I^{125}-labelled angiotensin II was added to the reaction mixture, and the radioactivity of the sepharose-free supernatant was determined at various time intervals. Furthermore, the amount of angiotensin II remaining in the supernatant was estimated by rat blood pressure bioassay[6]. The coupling product was again washed alternately with acidic and alkaline buffer was described above, and packed in a 1.1×21 cm column and equilibrated with 0.1 *M* tris-HCl pH 7.4. Angiotensin II-antiserum (9 ml) was diluted 1:3 with the same buffer and poured through the column at a flow rate of about 0.5 ml/min. The column was washed with 50 ml 0.1 *M* tris-HCl pH 7.4 and eluted in batches with 50 ml each of 0.1 *M* Na-citrate-HCl buffer of pH 5, 4, 3, 2, 1.2 and finally pH 1.2 containing 0.5 *M* NaCl. The eluate was collected in 10 ml fractions into tubes kept at 0° and containing 2 ml 3 *M tris*-HCl pH 7.4, to immediately neutralize the acidic eluate.

In a separate experiment angiotensin II was coupled directly to CNBr-activated Sepharose without using albumin as a spacer.

Antibody concentrations were estimated by titer determination. The titer is arbitrarily defined as the dilution of an antibody preparation, at which exactly half of 10 pg of I^{125}-angiotensin II is bound to the antibody in a standard radioimmunoassay system[7] (250 µl of 0.1 *M tris*-acetate buffer pH 7.4 with 0.1% human serum albumin, 20 h equilibration at 0°). Assuming that under these conditions of extreme dilution (e.g. antiserum is diluted at least 15000-fold), there is no interference by substances present in the original material, the amount of antibody which binds 5 pg of angiotensin II can be taken as an arbitrary antibody unit, provided that no significant differences in affinity exist. Thus, the antibody concentration of a given preparation can be expressed as the reciprocal of the titer. The radioimmunoassay system for angiotensin II and the estimation of cross reactivity with analogous peptides has been described previously[7]. The apparent affinity constants of the antibody preparations were estimated from double-reciprocal plots according to NISONOFF and PRESSMAN[8].

Results and discussion. The coupling of angiotensin II to the sepharose-albumin complex appeared to be complete after 2 h of incubation, since at that time there was no detectable biological activity in the supernatant, as judged by rat blood pressure assay. However, at the same time, half of the radioactivity of I^{125}-labelled angiotensin II, which had been added at the beginning of the coupling reaction, was still in the soluble form. This discrepancy between biological activity and radioactivity may find its explanation in a partial polymerization of angiotensin, i.e. the formation of dimers or trimers of angiotensin II by the carbodiimid reaction, since the immobilized albumin is less readily available as a reaction partner. This interpretation is supported by the following experiment. The product of a reaction mixture identical

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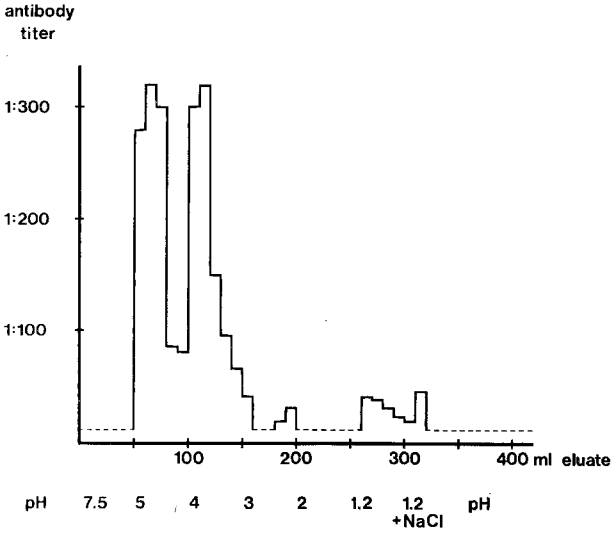
to that described above, except for omission of sepharose-albumin, was chromatographed on a sephadex G 15 column. Most of the radioactivity was eluted with the void volume, indicating a molecular weight of more than 1500, whereas radioactivity due to unreacted angiotensin II eluted later in the fractionation range–clearly separate from the void volume. It is therefore conceivable that the final sepharose coupling-product contains not only angiotensin monomers coupled to albumin, but also dimers and, to a lesser extent, other polymerized conjugates of angiotensin linked to the matrix.

Chromatography of angiotensin II-antisera by a discontinuous pH-gradient resulted in the elution of several distinct fractions. A typical elution profile is shown in the Figure. Main peaks appear at an elution pH of 5, at pH 4 and 1.2. These peaks were seen in all experiments, the quantitative distribution of antibodies among these peaks, however, varied among different experiments.

Total recovery of antibody from the column was about 60%. The purification was more than 100-fold in terms of antibody content per mg protein. The purified antibody preparation was free of renin substrate, but contained still some angiotensin II, which was removed by extraction with a cation exchange resin (Biorad AG 50 WX2 H^+-form)[7]. In contrast, the recovery from sepharose-angiotensin II-columns (without albumin) was only 20%, most of which eluted at pH 1.2. It was not possible to increase the yield from these columns by using more drastic elution conditions such as alkaline elution media, stepwise increase of acidity to 3 *N* HCl – a procedure used succesfully by CUATRECASAS[9] with insulin antibodies – or with lauryl-sulfate.

The elution of angiotensin antibodies in several distinct fractions may reflect antibody heterogeneity, i.e. separation of different antibody populations by differences in affinity for angiotensin II. However, besides a moderate increase in apparent affinity by the purification from 2.5×10^{10} l/mole to 5×10^{10} l/mole, there was no difference in affinity between antibody fractions eluted at pH 4 and 5 (combined to pool 1) and pH 1.2 (pool 2), which is also reflected in comparable sensitivity in the standard radioimmunoassay system.

In addition, only minor differences in specificity were found (Table) when the cross-reactions with several angiotensin analogues were estimated. These minor differences in the properties of pool 1 and 2 antibodies do not satisfactorily explain the drastic difference in the elution behaviour, i.e. elution at pH 5 versus pH 1.2. It has to be considered that the fractionation of antibody, as shown in the Figure, is partly due to heterogeneity of the affinity ligands on the column, as has been pointed out above. Since the aim of the present study was primarily to obtain purified antibody for in vivo studies, this problem was not further investigated[10].



Affinity chromatography of angiotensin II-antibody on sepharose-albumin-angiotensin II-columns. Elution by discontinuous pH-gradient. Antibody concentration was estimated by titer determination.

Zusammenfassung. Antisera gegen Angiotensin II wurden durch Immunisierung von Kaninchen mit Angiotensin II-Schweineglobulin-Konjugaten erhalten. Aus diesen Antiseren konnten Angiotensin II Antikörper durch Affinitätschromatographie auf Sepharose-Albumin-Angiotensin II-Säulen mehr als 100-fach angereichert werden. Durch diskontinuierliche pH-Gradienten-Elution wurden verschiedene Antikörper-Fraktionen erhalten, die sich jedoch nur geringfügig in Affinität und Spezifität unterschieden. Die mögliche Beteiligung unterschiedlicher Liganden der Affinitätsmatrix an der Fraktionierung wird diskutiert.

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Crossreactivity of angiotensin II-antiserum and purified antibody fractions with some angiotensin analogues

Angiotensin II analogue	Crossreaction of antibody of ileu^5-angiotensin II (%)		
	Untreated antiserum	Pool 1	Pool 2
Ileu^5-angiotensin II	100	100	100
Val^5-angiotensin II	67	69	41
Ileu^5-angiotensin I	0.6	0.5	0.2
Arg^1-heptapeptide	40	48	28
Val^1-hexapeptide	35	44	35

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