ISOLATION OF ANTIBODIES TO [8-LYSINE]-VASOPRESSIN BY AFFINITY CHROMATOGRAPHY

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[8-Lysine]-vasopressin was coupled *via* its NH_2-groups to Sepharose 4 B and Spheron P-300 which had been activated by CNBr. These modified supports were used for the isolation of antibodies to [8-lysine]-vasopressin from rabbit antiserum. The production of antibodies in rabbits was induced by administration of a conjugate of bovine serum albumin with [8-lysine]-vasopressin which had been coupled to the albumin also *via* its NH_2-groups. Unlike the unmodified supports, both immunosorbents effectively adsorbed antibodies to [8-lysine]-vasopressin. Between 20 and 30% of serum antibodies applied were desorbed by elution of the immunosorbents by a citrate buffer at pH 3·2 or by 6M urea or 6M guanidine.

The neurohypophysial hormones oxytocin and vasopressin are regarded as very little effective antigens. The most usual method of induction of formation of antibodies to these hormones is their application in the form of protein conjugates or in the form of products obtained by adsorption of the hormones to carbon microparticles. The size of the neurohypophysial hormones, which is comparable with that of the antibody binding site, should give rise to a high homogeneity of the antibodies produced[1]. If, however, the antigenicity of the neurohypophysial hormones is enhanced by their coupling to a high molecular weight support (together with the use of adjuvants), the heterogeneity of the antibodies produced will increase[2-4] since the parts of the support which are close to the hormone attached also contribute to the antibody specificity.

This study was aimed at the isolation of specific antibodies to a neurohypophysial hormone, [8-lysine]-vasopressin, by affinity chromatography. We used for this purpose specific adsorbents with neurohypophysial hormones attached in a manner similar to that used with protein conjugates for immunization.

EXPERIMENTAL

Materials

Bovine serum albumin was purchased from the Institute for Sera and Vaccines, Prague. [8-Lysine]-vasopressin was a commercial product of Léčiva, Modřany; it was purified by ion-exchange

chromatography and carrier-free electrophoresis. Its purity was checked by paper electrophoresis, amino-acid analysis, and by the biological test according to Coon[5], as modified by Krejčí and coworkers[6]. 1-Ethyl-3-(3-dimethylamino)propylcarbodiimide methiodide was from Fluka AG Buchs, Switzerland, Al-Span-Oil was from Velaz, Prague. ^{125}I (NaI) was purchased from the Institute of Isotopes, Hungary, Sepharose 4 B from Pharmacia, Uppsala, Sweden, Spheron P-300 from Lachema, Brno. We are indebted to Dr J. Čoupek, Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, for a standard sample of Spheron P-300. Cyanogen bromide was kindly provided by Dr J. Turková, Department of Protein Chemistry, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences. The remaining chemicals used were of analytical purity.

Methods

Preparation of antiserum: The antisera were prepared by immunization of rabbits with a conjugate of bovine serum albumin and [8-lysine]-vasopressin[7,8]. Twenty mg of bovine serum albumin was dissolved in physiological saline, 10 mg of [8-lysine]-vasopressin in 0·5 ml of H_2O and 10 mg of soluble carbodiimide was added. The mixture was stirred 2 h at room temperature and then treated with additional 10 mg of carbodiimide. Subsequently, the mixture was stirred for 3 more hours and then dialyzed 24 h at 4°C against physiological saline (repeatedly replaced). From earlier experiments (J. Vaněčková, unpublished observations) with [^{3}H] [8-lysine] vasopressin, the conjugate contained approximately 2 mol of [8-lysine]-vasopressin per mol of bovine serum albumin. The mixture of the antigen with Al-Span-Oil[9] adjuvant was administered to the rabbits by subcutaneous injection to paws and dorsal areas of the body, 3-times at intervals of 10—14 days. Blood was withdrawn by cardiac puncture 10 days after the last injection; the serum was isolated and stored at —20°C.

Immunologic test. The iodination of [8-lysine]-vasopressin was carried out according to Hunter and Greenwood[10]. Iodinated [8-lysine]-vasopressin, purified by ion-exchange chromatography on Dowex 1X8 in Cl-form[3] and by gel filtration on Sephadex G-25, was stored at —20°C. Rabbit anti serum with an antibody titer 1 : 200 (the titer corresponds to a dilution of antiserum at which 50% of labelled hormone is fixed in the reaction) was used for the reaction. The antiserum (0·4 ml, appropriately diluted by physiological saline containing 0·1% of bovine serum albumin and adjusted to pH 7·3 by 0·05M sodium phosphate buffer), 0·1 ml of a solution of [8-lysine]-vasopressin (10^{-6} — 10^{-3} mg), also adjusted to pH 7·3, or 0·1 ml of buffer, and 0·1 ml of a solution of ^{125}I [8-lysine]-vasopressin (approximately 5000—6000 c.p.m.) were incubated 20 h at 4°C. The hormone bound in the hormone-antibody complex was separated by the addition of 1 ml of a suspension of dextran-coated charcoal to which the free hormone is bound. The suspension contained 2·5% of charcoal and 0·25% of dextran in buffered physiological saline[11]. After the reacting components had been mixed, the suspension was centrifuged, a part of the supernatant was removed and its radioactivity measured after the addition of 10 ml of scintillation solution prepared according to Bray[12].

Preparation of immunosorbents. One ml of Sepharose 4 B or 500 mg of Spheron P-300 respectively was mixed with 1 ml of H_2O. The suspension was stirred and treated stepwise with 1 ml of 10% solution of CNBr. The pH was kept 12 min at 11 by the additon of 2M-NaOH. The suspension was quickly washed on a Büchner funnel with 50 ml of cold 0·1M-$NaHCO_3$, pH 9, and mixed with 2 μmol (2 mg) of [8-lysine]-vasopressin. The mixture was stirred 16 h at 2—3°C, then filtered on a Büchner funnel, washed twice with 5 ml of 1M-NaCl in 0·05M sodium phosphate buffer at pH 7·5 and with 25 ml of 0·05M sodium phosphate buffer, pH 7·5. Lastly, the suspension was mixed with 5 ml of the same buffer and stored at 2°C.

TABLE I

Adsorption and Elution of Antibodies to [8-Lysine]-vasopressin Using Specific Immunosorbents

The values are expressed in per cent of binding of the original serum whose binding ability for [8-lysine]-vasopressin labelled with ^{125}I has been taken equal 100%.

Fraction	Modified Sepharose 4 B				Modified Spheron P-300				Unmodified Sepharose 4 B Spheron P-300	
	citrate buffer pH 3·2	1M-NaCl or 0·1M-HCl	urea 6M	guanidine 6M	citrate buffer pH 3·2	1M-NaCl or 0·1M-HCl	urea 6M	guanidine 6M	guanidine 6M	
Antibodies not adsorbed	9·0	1·5	1·8	2·0	0·5	3·0	0·5	1·0	82·0	90·0
Eluting buffer total volume 12 ml	0·5	0	0	0	0	0·4	1·0	0	8·0	13·0
1st effluent fraction, total volume 6 ml	8·5	0	21·0	19·5	0	0	15·0	18·4	0	0
2nd effluent fraction, total volume 6 ml	10·0	0	10·2	9·4	4·3	0	6·6	8·7	0	0
Elution buffer, total volume 6 ml	0	0	0	0	0	0	0	0	0	—

Isolation of antibodies by affinity chromatography. The immunosorbent (2 ml), washed before use with 0·02M sodium phosphate buffer, pH 7·5, was poured into a column. The antiserum (0·6—1·0 ml) was diluted 10-times and passed over the column at a rate of 1 ml/5 min. The column was washed with sodium phosphate buffer, pH 7·5. The following solutions were used for the elution of antibodies (carried out twice, in subsequent runs with the same volume of eluant at a rate of 1 ml/5 min): *a*) 1M-NaCl, *b*) citrate buffer, pH 3·2 in 0·1% solution of bovine serum albumin, *c*) 6M urea, *d*) 6M guanidine hydrochloride, and *e*) 0·1M-HCl (the eluate was immediately neutralized). Effluent fractions were dialyzed 24 h at 4°C against physiological saline. The volume of the fractions was made up to the original volume of the serum sample applied with sodium phosphate buffer, pH 7·5; the binding capacity of [^{125}I] [8-lysine]-vasopressin of these samples was determined.

RESULTS AND DISCUSSION

The results of the individual experiments are summarized in Table I. The binding of the labeled hormone by the original serum is taken equal 100% (at the dilution of serum chosen, a 25—35% binding of the quantity of labeled hormone applied was achieved). The quantity of the labeled hormone bound in the individual fractions from the column is expressed in per cent of binding of the original serum. As follows from the Table, unmodified supports, *i.e.* Spheron P-300 and Sepharose 4 B without [8-lysine]-vasopressin attached, do not pick up antibodies to [8-lysine]-vasopressin from the serum. By contrast, both modified Sepharose and Spheron bind antibodies with a high efficiency. Antibodies are adsorbed to 91—98% by modified Sepharose and to 97—99·5% by Spheron. We have tested several systems as eluants of antibodies bound to the column. The antibodies are not eluted by 1M-NaCl and 0·1M-HCl and only a small quantity of antibodies is eluted from modified Sepharose by the citrate buffer. Both 6M urea and 6M guanidine were used with a relatively good result. The yields obtained by us, *i.e.* approximately 20—30% are relatively low when we compare them to the results of elution of antibodies to insulin[13], serum albumin[14], or trinitrophenyl-DNA or-protein *via* lysine[15] from immunosorbents under conditions which were inefficient in our study. We did not try to elute the antibodies by the hapten, *i.e.* by [8-lysine]-vasopressin, in view of the complications involved in the necessity of perfect separation of [8-lysine]-vasopressin from the eluted antibodies, which is a condition of determination of the yield of elution by the radioimmunologic method. We ascribe so far the lower yields of antibodies to [8-lysine]-vasopressin to their instability during their dialysis. When 6M guanidine is used for elution, the antibodies can even be impaired irreversibly[16,17].

The possibility of attachment of the hormone to the functional group of the support by a covalent bond enables us also in the case of [8-lysine]-vasopressin the preparation of defined antigens as well as of insoluble supports specifically adsorbing antibodies prepared by the application of antigens obtained earlier.

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Note added in proof: During the preparation of our manuscript a paper appeared by J. P. Frénoy and coworkers (Eur. J. Biochem. *43*, 371 (1974)) dealing with the isolation of [8-lysine]-vasopressin antibodies by means of affinity chromatography.

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