Antivasopressin Antibody. Characterization of High-Affinity Rabbit Antibody with Limited Association Constant Heterogeneity*

Wen-Hsien Wu† and John H. Rockey

ABSTRACT: High-affinity antivasopressin antibody with limited association constant heterogeneity has been induced in rabbits by immunization with vasopressin coupled by carbodiimide-initiated amide linkage to equine γGab-globulins, and also by immunization with unmodified vasopressin. The antivasopressin antibodies of hyperimmune antisera have been shown to be 7S γG-globulins by radioimmunoelectrophoresis and sucrose density gradient ultracentrifugation. Radioimmunoprecipitation vasopressin binding curves have been constructed with radioiodinated vasopressin, employing sheep antirabbit γG-globulin serum to precipitate the complexes of rabbit antivasopressin antibody and radioiodinated vasopressin. The average intrinsic association constants, K_0, of the antivasopressin antibodies did not vary substantially during the immunization schedules, or between animals immunized either with unmodified vasopressin or with vasopressin covalently linked to equine γGab-globulins (K_0 = 1.6–4.5 × 10^9 M^{-1}). The r/c *vs.* r plots (r = moles of hapten bound per mole of antibody; c = free hapten concentration) were linear over a wide range of values for r (r = 0.1–1.9). Analysis of the binding data by use of the Sips distribution function ($\log (r/(n - r)) = a \log c + a \log K_0$) furnished values for the index of heterogeneity, a, which were near unity, again indicating that there was no substantial heterogeneity of the association constants of the antibodies of individual sera. The ability of analogs of vasopressin and chemically modified vasopressin to displace radioiodinated vasopressin from antivasopressin antibody has been evaluated by constructing radioimmunoassay inhibition curves. 8-Lys-vasopressin and 8-Arg-vasopressin displayed equal capacities to displace radioiodinated vasopressin from rabbit antivasopressin antibody. Oxytocin (3-Ile-8-Leu-vasopressin) required a concentration ten times greater than 8-Lys-vasopressin or 8-Arg-vasopressin to effect a similar result. Performic acid oxidized vasopressin required a concentration 10^4 times greater than native vasopressin to displace an equivalent amount of radioiodinated vasopressin from antibody, indicating that disruption of the disulfide bond of the nonapeptide and conversion of the half-cystinyl residues at positions 1 and 6 into cysteic acid residues resulted in a profound alteration in the antigenic structure of the hormone. Conversion of the half-cystinyl residues into neutral *S*-carbamidomethylcysteinyl residues by reduction with 2-mercaptoethanol and alkylation with iodoacetamide also led to a marked alteration in the antigenic structure of the nonapeptide. Space-filling molecular models of vasopressin and of chemically modified vasopressin have been constructed and used in the analysis of the experimental findings.

Experimental evidence has indicated that antibody specificity is a function of the unique amino acid sequences of the constitutive polypeptide chain subunits of the immunoglobulin molecule (Haber, 1964; Whitney and Tanford, 1965; Freedman and Sela, 1966; Koshland *et al.*, 1966). A number of mechanisms for generating the required antibody amino acid sequence variations have been proposed, largely upon the basis of observed differences in primary structure of Bence Jones proteins (Dreyer and Bennett, 1965; Brenner and Milstein, 1966; Smithies, 1967; Hood and Ein, 1968). However, a fully adequate delineation of the biosynthetic mechanisms which lead to the translation of the three-dimensional stereoconfigurational information of an antigenic determinant (Kabat, 1966) into the required antibody subunit amino acid sequence alteraltions has yet to be obtained. An additional relevant problem is posed by the observed diversity in structure of the classes and subclasses of immunoglobulins of a single species (Cohen and Porter, 1964; Rockey, 1967). A single animal may produce a number of molecular forms of antibody which vary in primary structure in ways that are not directly relevant to the structure of the antibody combining site (Rockey, 1967). The immunoglobulins of the individual members of a given species also may show allotypic variations which are not directly related to antibody specificity (Dubiski *et al.*, 1961; Allen *et al.*, 1964; Koshland, 1967; Inman and Reisfeld, 1968). These diversities in immunoglobulin structure pose the question of whether or not each class, subclass, and allotypic variation of protein, with a stable amino acid sequence alteration not directly related to antibody specificity, requires a separate mutational event to generate a given antibody specificity, or, alternately, whether or not there exists a common mechanism for generating similar antibody specificities in the diverse proteins. A definition of the amino acid sequence variations that determine antibody specificity, and a delineation of the biosynthetic mechanisms responsible for the generation of antibody specificity may require the study of a number of homogeneous antibodies which differ in specificity,

* From the Departments of Anesthesiology and Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received February 4, 1969.* This work was supported by U.S. Public Health Service Grants 5-P01-GM-09070, 1-P 01-GM-15430, 5-T 01-GM-215, and AI-05305.

† Present address: Department of Anesthesiology, Episcopal Hospital, School of Medicine, Temple University, Philadelphia, Pa. 19125.

and of antibodies with similar specificity but which are members of different classes, subclasses, and allotypes of proteins.

Antibodies produced by individual animals against small haptenic groupings in general have displayed a substantial heterogeneity in intrinsic association constants (Eisen and Siskind, 1964; Kabat, 1966; Fujio and Karush, 1966; Rockey, 1967). Although the observed heterogeneity of antibody affinities may in part be due to the fact that the antibody population is composed of a number of molecular forms of immunoglobulins (Rockey, 1967), the heterogeneity principally has been ascribed to variations in the structure of the antigenic determinant contributed by the topology of the molecular surfaces adjacent to the small haptenic grouping (Kabat, 1966; Richards *et al.*, 1967; Schlossman *et al.*, 1968). Recent efforts in several laboratories have been directed toward generating antibodies with more homogeneous binding characteristics by employing larger and more uniform antigenic determinants (Richards *et al.*, 1967; Haber *et al.*, 1967). We have employed the biologically interesting nonapeptide vasopressin as an antigenic determinant and have studied high-affinity rabbit antibody produced against the unmodified peptide and against the peptide coupled to a carrier protein. Each method of immunization has led to the production of antibodies with restricted association constant heterogeneity. The role of the disulfide bond of vasopressin in determining the antigenic structure of the nonapeptide also has been investigated.

Materials and Methods

Preparation of Antigens. The γG-globulins were precipitated from equine serum with 33% saturated ammonium sulfate, taken up in distilled water, transferred to 0.01 M sodium phosphate buffer (pH 8) by dialysis, and freed of other serum proteins by passage over a column of DEAE-cellulose (Whatman DE 52, 1.0 mequiv/g) equilibrated with the same solvent. The proteins were examined by immunoelectrophoresis and found to be γGab-globulins (Rockey, 1967) free of other serum proteins. Vasopressin was coupled to equine γG-globulins by employing either of two carbodiimides, ECDI[1] (Ott Chemical Co., Muskegan, Mich.) or MCDI (Aldrich Chemical Co., Milwaukee, Wis.) (Goodfriend *et al.*, 1964; Permutt *et al.*, 1966); 21 mg of equine γG and 500 PU (pressor units, USP) of Pitressin powder, a mixture of 8-Lys-vasopressin and 8-Arg-vasopressin (Parke Davis and Co., Detroit, Mich.), was dissolved in 1 ml of distilled water and the pH of the solution was adjusted to 7.45 with 0.1 N sodium hydroxide; 15 mg of either ECDI or MCDI was added and the mixture was incubated at room temperature for 18 hr. The extent of coupling of vasopressin to equine γG was evaluated by adding radioiodinated vasopressin (*vide infra*) to the mixture prior to the carbodiimide coupling and determining the percentage of the radioactivity of the reaction product which was specifically precipitable with an excess of rabbit antiequine γG serum. In control experiments in which the carbodiimide had been omitted, less than 5% of the added radioiodinated vasopressin was precipitated with the equine γG. After carbodiimide linkage, 75–85% of the radioiodinated vasopressin was precipitated with the equine γG by rabbit antiequine γG serum. The linkage of radioiodinated vasopressin to equine γG also was demonstrated by radioimmunoelectrophoresis (Morse and Heremans, 1962). The ratio (moles of vasopressin/mole of equine γG) was calculated to be 7–8 to 1 on the basis of the extent of linkage determined with radioiodinated vasopressin.

Rabbit γG-globulins were prepared by DEAE-cellulose column chromatography (0.015 M sodium phosphate buffer, pH 8) and Pevikon block zone electrophoresis (Müller-Eberhard, 1960).

Preparation of Antisera. Rabbit antivasopressin sera were induced by immunizing randomly bred New Zealand red and white rabbits with either vasopressin–equine γG or uncoupled vasopressin. Two rabbits were immunized with vasopressin–equine γG. The initial injection contained an equivalent of 250–500 PU of vasopressin–equine γG emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) and was administered into the four footpads. Each rabbit then received one-half the initial quantity of vasopressin–equine γG mixed with complete adjuvant, repeatedly injected subcutaneously at 3-week intervals. Seven rabbits were immunized with Pitressin which had not been coupled to a protein carrier. Four rabbits received repeated injections of 100–250 PU of Pitressin mixed with complete Freund's adjuvant. Three rabbits received injections of 250 PU of Pitressin mixed with complete Freund's adjuvant and Pertussis vaccine (USP, Eli Lilly and Co., Indianapolis, Ind.) (Wright and Norman, 1966). Antigens were injected initially into the footpads and thereafter at multiple subcutaneous sites at monthly intervals. The rabbits were bled by cardiac puncture 7–10 days after each antigen administration and the sera were stored at −20° until used.

Sheep antirabbit γG-globulin serum was produced by repeatedly immunizing a single animal with rabbit γG mixed with complete Freund's adjuvant.

Purification of Vasopressin. Vasopressin was purified from bovine protopituitrin (8-Arg-vasopressin) and from a mixture of bovine and porcine protopituitrin (8-Arg-vasopressin and 8-Lys-vasopressin) (Parke Davis and Co.) by the method of Schally *et al.* (1964) with minor modifications; 1 g of protopituitrin powder was extracted three times with 1 ml of 0.02 M ammonium acetate buffer (pH 4.5). Insoluble materials were removed by centrifugation, and the pH of the supernatant was adjusted to 6.0 with 0.4 M ammonium hydroxide. The material was applied to a 1 × 50 cm column of CM-cellulose (Whatman CM 52, 1.0 mequiv/g) which had been equilibrated with 0.02 M ammonium acetate buffer (pH 6.0) and the column was washed with the same solvent. The column effluent (flow rate 0.5–1.0 ml/min, collected in 1–2-ml fractions) was monitored at 254 mμ with an ultraviolet spectrometer (LKB Uvicord type 4701 A) by the modified method of Folin–Ciocalteu (Lowry and Bessey, 1964) and by bioassay for pressor activity in the rat (Dekanski, 1952). Vasopressin was eluted from the washed column with a linear positive gradient with a final buffer concentration of 0.2 M ammonium acetate (pH 7.0). The fractions containing the maximum rat pressor activity were pooled, lyophilized, taken up in 0.25% acetic acid, and filtered through a 1 × 175 cm column of Sephadex G-25 (Pharmacia) in 0.25% acetic acid at a flow rate of 3–4 ml/hr; 1-ml fractions were collected and assayed optically by

[1] Abbreviations used: ECDI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; MCDI, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; PU, USP pressor units; vasopressin–equine γG, vasopressin covalently linked to equine γG-globulins.

the rat pressor bioassay and by ascending paper chromatography (Whatman No. 3MM paper) in butanol–acetic acid–water (4:2:5, v/v), employing synthetic 8-Lys-vasopressin and 8-Arg-vasopressin as reference markers. The fractions containing the maximum pressor activity were pooled and lyophilized. In some instances, the preparation of vasopressin was subjected to preparative paper chromatography or preparative high-voltage paper electrophoresis (*vide infra*) as an additional purification procedure. The absorption spectrum of the purified vasopressin in 0.1 N sodium hydroxide was examined with a Zeiss PMQII spectrophotometer. Vasopressin concentrations were determined from the optical density at 294 mμ in 0.1 N sodium hydroxide, employing a molar extinction coefficient, $\epsilon^{1\text{ cm}}_{\lambda 294\text{m}\mu}$, for tyrosine of 2.3×10^3. Purified vasopressin was subjected to amino acid analysis and these data were also used to calculate hormone concentrations. Vasopressin was stored in 0.25% acetic acid at −20° until used.

Synthetic 8-Lys-vasopressin (270 PU/mg), 8-Arg-vasopressin (385 PU/mg), and oxytocin (450 USP units/mg) were generous gifts from Dr. P. S. Arcese, Sandoz Pharmaceuticals (Hanover, N. J.).

Rat Pressor Bioassay. Vasopressin preparations were assayed for pressor activity in the rat by the method of Dekanski (1952). Carotid arterial pressures were monitored with a Statham pressure transducer and recorded with a Grass Model 5 D polygraph. USP Posterior Pituitary Reference Standard (USP Reference Standards, New York, N. Y.) was used to define the pressor activity of unknowns.

Radioiodination of Vasopressin. Vasopressin was labeled with ^{131}I (specific activity 20–25 mCi/μg, Cambridge Nuclear Corp., Cambridge, Mass.) by the method of Hunter and Greenwood (1962) with the modification that sodium metabisulfite was omitted to avoid reduction of the disulfide bond (Roth *et al.*, 1966). Radioactivity was measured with a Packard Model 5212 automatic gamma counter at a counting efficiency of 32.3%. Three procedures were employed to purify the radioiodinated vasopressin. In the first procedure, the reaction mixture (vasopressin radioiodinated in 0.5 M sodium phosphate buffer, pH 7.5) was filtered through a lead-shielded 1 × 25 cm column of Sephadex G-25 in 0.25% acetic acid. Free ^{131}I and damaged materials were retarded by the column. In the second procedure, the molarity and pH of the reaction mixture (vasopressin radioiodinated in 0.3 M ammonium acetate buffer, pH 7.5) were adjusted to 0.02 M and pH 5.5, respectively, by the addition of predetermined amounts of 0.3 M ammonium acetate buffer (pH 4.0) and distilled water. The material was applied to a 1 × 4 cm column of CM-cellulose equilibrated with 0.02 M ammonium acetate buffer (pH 5.5) and free ^{131}I and damaged materials were eluted under positive air pressure with the same solvent. Radioiodinated vasopressin was eluted from the washed column with 0.2 M ammonium acetate buffer (pH 7.0). In the third procedure, the reaction mixture in 0.02 M ammonium acetate buffer (pH 5.5) was passed over a 1 × 4 cm column of DEAE-cellulose equilibrated with the same solvent. Radioiodinated vasopressin was eluted under positive air pressure with the initial solvent. Free ^{131}I and damaged materials were retained by the column.

The purified radioiodinated vasopressin was examined by paper chromatoelectrophoresis (Berson *et al.*, 1956) at 4° in a barbital buffered solvent of ionic strength 0.05, pH 8.6 (500 V applied for 1 hr), and by ascending paper chromatography. The paper strips were scanned with a Packard Model 7200 radiochromatogram scanner and stained with ninhydrin. The percentage of the radioactivity of purified radioiodinated vasopressin specifically precipitable with rabbit antivasopressin serum and an excess of sheep antirabbit γG-globulin serum was determined by radioimmunoprecipitation (*vide infra*). Radioiodinated vasopressin prepared by Sephadex G-25 gel filtration frequently contained small quantities of radioactive contaminants. These were removed by the addition of Dowex 1-X10 resin (Roth *et al.*, 1966). Radioiodinated vasopressin prepared by CM-cellulose or DEAE-cellulose chromatography was free of radioactive contaminants.

Radioimmunoprecipitation. Rabbit sera were screened for antivasopressin antibody by mixing 0.1 ml of serum with radioiodinated vasopressin (2000–3000 cpm) and precipitating the radioiodinated vasopressin–rabbit antivasopressin antibody complexes with an excess of sheep antirabbit γG-globulin serum. The mixtures of rabbit serum, radioiodinated vasopressin, and sheep antirabbit γG-globulin serum were diluted to a final volume of 1 ml with 0.15 M sodium chloride–0.01 M sodium phosphate buffer (pH 7.4) containing 0.5% human serum albumin (w/v) and incubated at 4° for 24 hr. The total radioactivity of each tube was determined, and the tube was centrifuged at 950*g* for 20 min at 4°. The supernatant was removed, the precipitate was washed with the above diluent, and the radioactivity of the precipitate (and in some instances of the pooled supernatant and washings) was determined. The amount of sheep antirabbit γG-globulin serum that was required to precipitate all of the rabbit γG had been determined in prior experiments by constructing quantitative precipitin curves (Kabat, 1961) with rabbit γG or rabbit serum. Radioprecipitin curves also were constructed to determine the optimum concentration of sheep antirabbit γG-globulin serum required to precipitate the radioiodinated vasopressin–rabbit antivasopressin antibody complexes.

Radioimmunoprecipitation vasopressin binding curves were constructed at 4° by adding increasing quantities (0–15 mμg) of radioiodinated vasopressin (delivered with a microburet syringe) to an optimal dilution of rabbit antivasopressin serum and then precipitating all of the rabbit γG with sheep antirabbit γG-globulin serum; 0.1 ml of a dilution ($^1/_{50}$ to $^1/_{400}$) of rabbit antivasopressin serum was mixed with the radioiodinated vasopressin. Each tube was prepared in duplicate; 0.1 ml of a $^1/_{100}$ dilution of nonimmune rabbit serum was included whenever the dilution of rabbit antivasopressin serum was $^1/_{200}$ or greater, to increase the quantity of precipitate. A predetermined amount of sheep antirabbit γG-globulin serum was added and the mixture was diluted to a final volume of 1 ml. The mixture was incubated for 24 hr at 4° and the radioactivity in the precipitate and in the supernatant was determined after centrifugation at 4°. The data were corrected for radioactivity included nonspecifically in the precipitates by the use of standard curves constructed with nonimmune rabbit serum, where the per cent of the total radioactivity included in the precipitate was expressed as a function of the concentration of free radioiodinated vasopressin. The radioactivity included nonspecifically in the precipitate never exceeded 2% of the total radioactivity added. The radioiodinated vasopressin binding data were expressed in terms of r and c, where r is the moles of radioiodinated vasopressin bound per mole of antivasopressin antibody in the precipitate and c is the concentration of free radioiodinated vasopressin

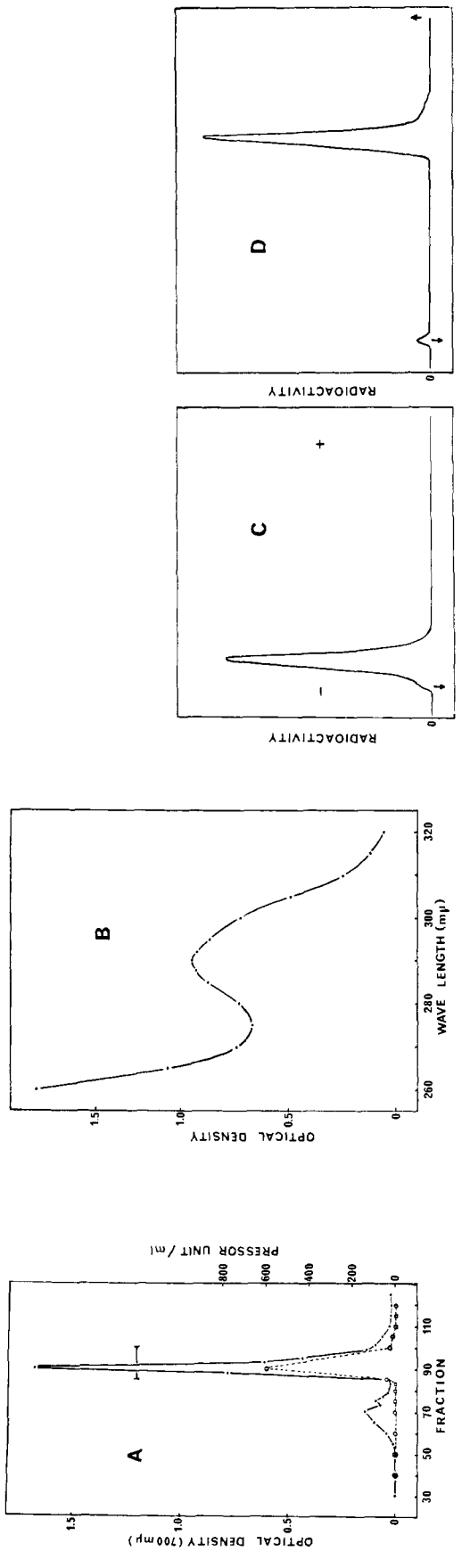

FIGURE 1: Vasopressin studies. (A) Gel filtration of vasopressin (Lys-vasopressin and Arg-vasopressin), purified by CM-cellulose chromatography, through a 1 × 175 cm column of Sephadex G-25 in 0.25% acetic acid. Optical density (●—●) and rat bioassay pressor activity (○—○). (B) Absorption spectrum of purified vasopressin in 0.1 N sodium hydroxide. (C) Examination of radioiodinated vasopressin purified by DEAE-cellulose chromatography, by chromatoelectrophoresis at pH 8.6. Origin, ↓. Anode to the right. (D) Examination of DEAE-cellulose purified radioiodinated vasopressin by ascending paper chromatography. Solvent, butanol–acetic acid–water (4:2:5, v/v). Origin, ↓. Solvent front, ↑.

in the supernatant. The ratio r/c was plotted as a function of r, and the curves were extrapolated to infinite free radioiodinated vasopressin concentration to obtain the molar concentration of antibody combining sites. The valence of the antibody, n, was taken as 2. Average intrinsic association constants, K_0, were obtained as the reciprocal of the molar concentration of free radioiodinated vasopressin in the supernatant at that point on the binding curve where one-half of the antivasopressin antibody combining sites had been occupied by radioiodinated vasopressin (Karush, 1962; Eisen and Siskind, 1964; Rockey, 1967). The data also were analyzed according to the Sips distribution function (Sips, 1948; Karush, 1962)

$$\log\left(\frac{r}{n-r}\right) = a \log c + a \log K_0$$

Log $(r/(n-r))$ was plotted as a function of log c, and the data were treated by the method of least squares to obtain a, the index of heterogeneity.

Radioimmunoassay Inhibition Curves. Inhibition curves were constructed at 4° by adding increasing quantities of unlabeled vasopressin or vasopressin analog (0–10 μg) to a set of tubes containing a constant amount of rabbit antivasopressin serum and radioiodinated vasopressin. Sufficient sheep anti-rabbit γG-globulin serum then was added to precipitate all of the rabbit γG. The dilution of rabbit antivasopressin serum and the quantity of radioiodinated vasopressin were selected on the basis of preliminary experiments so that 30–50% of the radioiodinated vasopressin was precipitated in the absence of inhibitor. The percentage of the total radioactivity included in the precipitate was plotted as a function of the logarithm of the concentration of added unlabeled vasopressin or vasopressin analog.

Performic Acid Oxidation. Vasopressin was oxidized with performic acid by the method of Hirs (1956). Precooled (0°) performic acid was added to a sample of vasopressin and the reaction mixture was maintained at 0° for 2.5 hr, diluted with distilled water, and lyophilized. Control samples of vasopressin were treated in an identical manner with the exception that the performic acid was replaced by distilled water.

Reduction and Alkylation. Vasopressin was reduced at pH 8 for 24 hr at room temperature in a closed reaction vessel under nitrogen with 0.6 M 2-mercaptoethanol, and alkylated with a 20% molar excess of iodoacetamide (Mann Research Laboratories, recrystallized twice from ethanol and then twice from water). The pH was maintained at 8 by the addition of solid Tris.

High-Voltage Electrophoresis. High-voltage paper electrophoresis was accomplished in a tap-water-cooled Gilson Model D electrophorator (Gilson Medical Electronics, Middleton, Wis.) on Whatman No. 3MM paper in a pyridine–acetic acid–water (1:10:289, v/v) solvent of pH 3.7 (Katz *et al.*, 1959). Vasopressin was localized with ninhydrin and eluted from unstained preparative electrophoresis strips with 0.25% acetic acid.

Amino Acid Analysis. Lyophilized peptides were dissolved in glass-redistilled constant-boiling (approximately 5.7 N) hydrochloric acid in heavy-walled Pyrex combustion tubes and frozen in a bath of solid carbon dioxide and methyl Cellosolve. The tubes were evacuated and the samples were thawed and degassed. The tubes then were sealed under vac-

TABLE I: Amino Acid Composition of Preparations of Vasopressin and Chemically Modified Vasopressin Used to Construct Radioimmunoassay Inhibition Curves.

	Lys-vasopressin and Arg-vasopressin			Synthetic Lys-vasopressin	
Amino Acid	Native Hormone	Performic Acid Oxidized[a]	Reduced and Alkylated[b]	Native Hormone	Performic Acid Oxidized[c]
Half-Cys	1.5[d]			1.4[d]	
Cysteic acid		1.8			1.9
S-Carboxymethyl-cysteine			1.6		
Tyr	0.7[d]	0.8[d]	ND[e]	0.7[d]	0.2[d]
Phe	1.0	1.0	ND	0.9	0.9
Glutamic acid	0.9	0.9	0.9	1.0	1.0
Aspartic acid	1.0	1.0	1.0	1.0	1.0
Pro	0.9	1.1	1.1	1.0	1.0
Lys	0.7	0.7	0.8	1.0	1.0
Arg	0.4	0.4	0.3		
Gly	1.0	1.0	ND	1.0	1.0

[a] Mixture of Lys-vasopressin and Arg-vasopressin oxidized with performic acid in the absence of chloride ion. [b] Lys-vasopressin and Arg-vasopressin reduced with 2-mercaptoethanol and alkylated with iodoacetamide. [c] Synthetic Lys-vasopressin oxidized in the presence of chloride ion and purified by high-voltage paper electrophoresis. [d] 24-hr data uncorrected for destruction during acid hydrolysis. [e] Not determined because of the presence of interfering substances contributed by the solvent used during the reduction and alkylation.

uum (less than 50-μ residual pressure) and the samples were hydrolyzed at 110 ± 1° for 24 hr (Moore and Stein, 1963). Amino acid analysis was performed with a Technicon single-column amino acid analyzed equipped with an Infotronics Model CRS-10ABT digital read-out system. Norleucine was incorporated into each analysis as an internal standard.

Radioimmunoelectrophoresis. Radioimmunoelectrophoresis was performed by the method of Morse and Heremans (1962).

Density Gradient Ultracentrifugation. Sucrose density gradient ultracentrifugation was accomplished as previously described (Rockey and Kunkel, 1962). The hemolytic activities of rabbit 7S and 19S antisheep red blood cell antibodies were used as internal density gradient sedimentation standards (Rockey and Kunkel, 1961).

Molecular Models. Space-filling molecular models of vasopressin and vasopressin analogs were constructed with an Ealing CPK (Corey–Pauling–Koltun) atomic model system (Ealing Corp., Cambridge, Mass.).

Experimental Results

Characterization of Vasopressin. Protopituitrin powder (1 g) yielded 12–15 mg of purified vasopressin. Figure 1 illustrates the elution profile obtained upon Sephadex G-25 gel filtration of a mixture of 8-Lys-vasopressin and 8-Arg-vasopressin (Figure 1A) and the absorption spectrum of the purified hormone in 0.1 N sodium hydroxide (Figure 1B). The amino acid composition of the product is presented in Table I. The purified vasopressin moved on ascending paper chromatography as a single ninhydrin-positive component with an R_F of 0.7–0.8. The rat pressor activity of purified vasopressin was 300–350 PU/mg. Figure 1 also presents the results of examination of chromatographically purified (DEAE-cellulose) radioiodinated vasopressin by chromatoelectrophoresis (Figure 1C), and by ascending paper chromatography (Figure 1D). The specific activity of the radioiodinated vasopressin ranged from 100 to 180 μCi per μg. This corresponded to an average of 1 ^{131}I atom/15–27 vasopressin molecules. When the radioiodinated vasopressin was examined for pressor activity in the rat, it was found that 70–80% of the initial vasopressin pressor activity was recovered in the chromatographically purified radioiodinated product. Under optimal condition for maximum radioimmunoprecipitation of radioiodinated vasopressin, 92–97% of the radioactivity of the preparations of radioiodinated vasopression employed in the present studies was precipitated by rabbit antivasopressin antibody and an excess of sheep antirabbit γG-globulin serum. Variation of the time of incubation of the reaction mixture from 18 to 96 hr did not alter the percentage of the radioiodinated vasopressin precipitated with specific antibody. In control experiments where nonimmune rabbit serum replaced the rabbit antivasopressin serum, less than 2% of the radioactivity of the radioiodinated vasopressin was precipitated.

Radioimmunoelectrophoresis and Density Gradient Ultracentrifugation. The antivasopressin antibody of hyperimmune rabbit antiserum was shown to be a 7S γG-globulin by radioimmunoelectrophoresis and sucrose density gradient ultracentrifugation (Figure 2). The γG-globulins of nonimmune rabbit sera failed to bind radioiodinated vasopressin when examined under similar conditions.

Radioimmunoprecipitation Vasopressin Binding Curves. The molar concentration of antibody combining sites in a diluted sample of rabbit antivasopressin serum was determined from the radioimmunoprecipitation vasopressin binding data by extrapolating the *r/c vs. r* curve to infinite free vasopressin

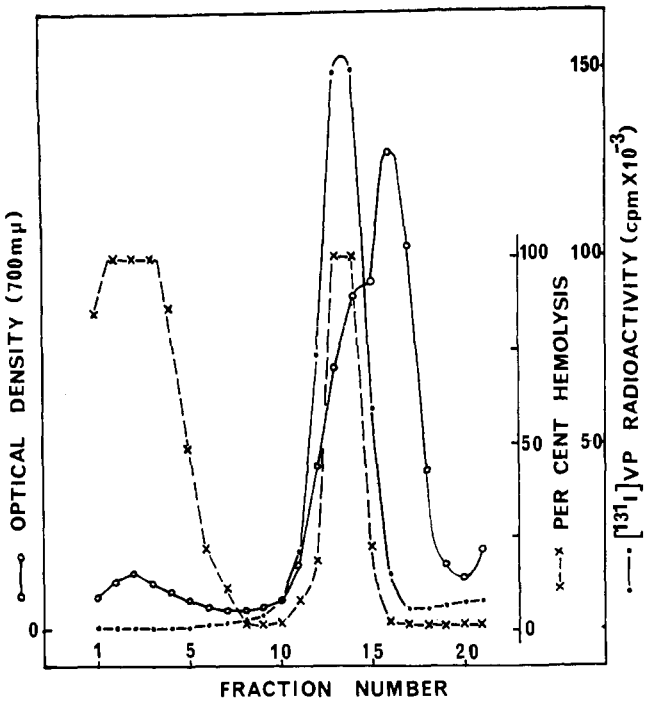

FIGURE 2: Sucrose density gradient ultracentrifugation of a mixture of a hyperimmune rabbit antivasopressin serum equilibrated with radioiodinated vasopressin, and a rabbit antisheep red blood cell serum. (O—O) Protein distribution (optical density at 700 mμ), (●—●) radioiodinated vasopressin radioactivity, and hemolytic activity of antisheep red blood cell antibodies, (×---×). The hemolytic activity was associated with both 7S and 19S antibodies. The antivasopressin antibody activity was restricted to the 7S immunoglobulins.

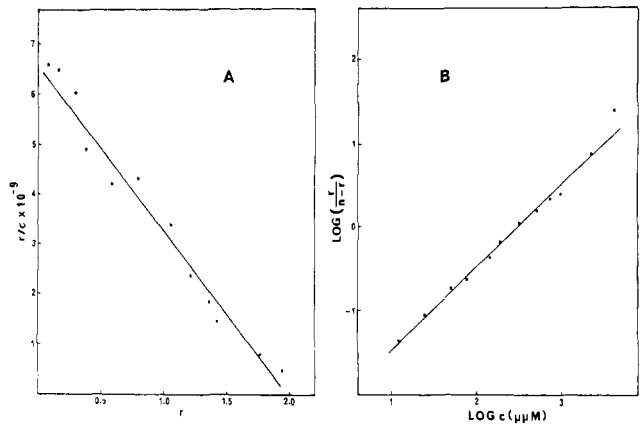

FIGURE 3: Radioimmunoprecipitation vasopressin binding curves constructed with radioiodinated vasopressin and rabbit 17 antivasopressin serum. (A) Binding data presented in terms of r and c, where r = moles of hapten bound/mole of antibody, and c = free hapten concentration ($r/c = (n - r)K_0$, $n = 2$). Average intrinsic association constant, $K_0 = 3.2 \times 10^9$ M^{-1}. (B) Binding data expressed in terms of the Sips distribution function, $\log (r/(n - r)) = a \log c + a \log K_0$. Data fitted to a linear curve by the method of least squares. Index of heterogeneity, a, is 1.04.

concentration (*i.e.*, r/c approached 0, and r extrapolated to n) (Figure 3). The valence, n, of the rabbit γG antibody was taken as 2 (Eisen and Siskind, 1964). A representative binding curve is presented in Figure 3. Reproducible binding curves could be constructed with high-affinity rabbit antivasopressin serum even when the antibody concentration had been reduced so that only one-tenth of the total radioiodinated vasopressin was specifically precipitated at $r = 1$. Radioimmunoprecipitation therefore was capable of detecting high-affinity antibody at concentrations as low as 10^{-10}–10^{-11} M. Both of the rabbits immunized with vasopressin–equine γG, and five of the seven rabbits immunized with uncoupled vasopressin produced measurable antivasopressin antibody. The sera from two rabbits, one immunized with vasopressin not specifically coupled to a protein carrier, the other immunized with vasopressin–equine γG, were selected for more detailed study. The concentrations of antibody in the sera at different intervals in the immunization schedule are summarized in Table II. Antibody concentrations did not vary greatly during the immunization schedule. Average intrinsic association constants, K_0, were obtained from the radioimmunoprecipitation vasopressin binding curves as the reciprocal of the free hapten concentration, c, at that point on the r/c *vs.* r plot where one-half of the antibody combining sites had been occupied by the vasopressin hapten (*i.e.*, $r = 1$). The average association constants determined for antibodies in the sera of the two rabbits at different intervals during the immunization schedule are summarized in Table II. Within the limits of experimental error, a variation in the concentration of rabbit antivasopressin antibody over a 20-fold range did not alter the value of the average association constant obtained for a given rabbit antivasopressin serum. High-affinity ($K_0 = 10^9$ M^{-1} or greater) antivasopressin antibodies were produced by rabbits immunized with vasopressin–equine γG and also by rabbits immunized with vasopressin not specifically coupled to a protein carrier (Table II). There was no substantial variation in the average association constants over the immunization schedule (Table II).

Analysis of the r/c *vs.* r data over a wide range of values for r (r = 0.1–1.9) failed to demonstrate any substantial heterogeneity in the association constants of the antivasopressin antibodies in an individual serum. A representative binding curve is presented in Figure 3A. The r/c *vs.* r data fitted, within experimental error, on a linear curve. Analysis of the vasopressin binding data by the Sips relationship also failed to demonstrate any substantial heterogeneity of the association constants. Treatment of the log $(r/(n - r))$ *vs.* log c data of Figure 3B by the method of least squares gave a value for the index of heterogeneity, a, of 1.04.

Performic Acid Oxidation of Vasopressin. Synthetic 8-Lys-vasopressin (1.75 mg) was oxidized at 0° in 75 ml of performic acid. Oxidized 8-Lys-vasopressin and control 8-Lys-vasopressin were examined by high-voltage paper electrophoresis and by amino acid analysis. The oxidized 8-Lys-vasopressin migrated as a single ninhydrin-positive component, and at pH 3.7 had a substantially lower electrophoretic mobility toward the cathode than did the positively charged control 8-Lys-vasopressin. Samples of the oxidized 8-Lys-vasopressin and the control 8-Lys-vasopressin were isolated by preparative high-voltage paper electrophoresis and portions of the electrophoretically purified peptides were subjected to amino acid analysis (Table I). Performic acid oxidized 8-Lys-vasopressin contained two residues of cysteic acid but only one-third the tyrosine content of the control (Table I). The 8-Lys-vasopressin solvent contained chloride ion, and the substantial loss of tyrosine was

TABLE II: Hapten Binding Characteristics of Rabbit Antivasopressin Antibodies.

Rabbit	Antigen	Immunization Interval (Days)	Reciprocal of Serum Dilution	$K_0 \times 10^{-9}$, 4° (l./mole)	ΔF_u[a] (kcal/mole)	a[b]	Serum Antibody Concn (μM)
12[c,d]	Unmodified vasopressin	92	1000	1.8	−13.9	0.95	0.11
		247	1000	1.6	−13.9		0.89
		377	1000	2.0	−14.0	0.84	0.50
17[e]	Vasopressin–equine γG	131	500	2.4	−14.1	1.06	0.28
			1000	2.0	−14.0		0.15
			2000	4.5	−14.4	0.93	0.14
			4000	1.7	−13.9		0.32
		168	1000	3.7	−14.3		0.16
		197	1000	3.2	−14.2	1.04	0.28
		227	1000	4.0	−14.4		0.20

[a] Unitary free-energy change, $\Delta F_u = \Delta F° - 7.98T = -RT \ln K_0 - 7.98T$ (Karush, 1962). [b] Index of heterogeneity, a, obtained from the Sips distribution function, $\log (r/(n - r)) = a \log c + a \log K_0$. [c] Rabbit 12 developed pronounced polydipsia and polyuria during the course of immunization. [d] Allotypic specificities of rabbit 12 γG-globulins, A (1+, 2−, 3+, 4+, 5−, 6−, 9−). Agar diffusion studies with specific antiallotype sera and radioiodinated vasopressin have demonstrated both heavy-chain Fd fragment allotypic variants (A1, A3) of antivasopressin antibody in serum 12 (J. H. Rockey, W.-H. Wu, and S. Dubiski, unpublished observation). [e] Allotypic specificity of rabbit 17 γG-globulins, A(1+, 2−, 3−, 4+, 5−, 6−, 9−).

considered to be due to the formation of chlorotyrosine (Hirs, 1956). Samples of oxidized 8-Lys-vasopressin and control 8-Lys-vasopressin, taken both before and after preparative high-voltage paper electrophoresis, were used to construct radioimmunoassay inhibition curves (*vide infra*).

To eliminate the formation of chlorotyrosine as a possible consideration in evaluating alterations in antigenic structure of vasopressin resulting from performic acid oxidation, 3.3 mg of a chromatographically purified chloride-free mixture of 8-Lys-vasopressin and 8-Arg-vasopressin, isolated from protopituitrin powder, was oxidized at 0° in 3 ml of performic acid. Amino acid analysis of the reaction product indicated that, under these conditions, performic acid oxidation converted the cystine of vasopressin into two cysteic acid residues without a loss of tyrosine (Table I). This material also was used to construct radioimmunoassay inhibition curves.

Reduction and Alkylation of Vasopressin. Vasopressin (1.1 mg of 8-Lys-vasopressin and 8-Arg-vasopressin, purified by paper chromatography) was reduced with 0.6 M 2-mercaptoethanol and alkylated with iodoacetamide in 0.002 M EDTA–0.5 M Tris-HCl buffer, pH 8 (total volume 1.1 ml). A sample of the reaction product was subjected to amino acid analysis in the present of the solvent components. Solvent controls for the amino acid analysis were prepared in an identical manner with the exception that vasopressin was either eliminated or replaced with a standard mixture of amino acids. The presence of the solvent did not interfere with the determination of *S*-carboxymethylcysteine, aspartic acid, glutamic acid, Pro, Lys, or Arg. Reaction products contributed by the solvent interferred with the determination of the remaining amino acids of vasopressin. The amino acid compositions of control vasopressin (8-Lys-vasopressin and 8-Arg-vasopressin) and of the reduced and alkylated vasopressin are presented in Table I. The reduced and alkylated vasopressin was used to construct radioimmunoassay inhibition curves.

Radioimmunoassay Inhibition Curves. The ability of the vasopressin analogs (8-Lys-vasopressin, 8-Arg-vasopressin, and oxytocin) and of chemically modified vasopressin to compete with radioiodinated vasopressin for rabbit antivasopressin antibody was assayed by constructing radioimmunoassay inhibition curves with a high-affinity antiserum from rabbit 17 (Table II). Representative inhibition curves are presented in Figure 4. 8-Lys-vasopressin and 8-Arg-vasopressin each had an equal capacities to displace radioiodinated vasopressin from the rabbit antivasopressin antibody. Oxytocin required a concentration ten times greater than either 8-Lys-vasopressin or 8-Arg-vasopressin to effect an equivalent displacement (Figure 4). 8-Lys-vasopressin, 8-Arg-vasopressin, and oxytocin each, at high peptide concentrations, were capable of displacing more than 98% of the radioiodinated vasopressin from the rabbit antivasopressin antibody (Figure 4), indicating that each vasopressin analog was competing with the radioiodinated vasopressin for the same antibody population. Disruption of the disulfide bond of vasopressin either by performic acid oxidation or by reduction and alkylation produced a marked alteration in the antigenic structure of the peptide. Synthetic 8-Lys-vasopressin, oxidized with performic acid in the presence of chloride ion, required a concentration 10^4 times greater than control 8-Lys-vasopressin to compete effectively with radioiodinated vasopressin for rabbit antivasopressin antibody. Performic acid oxidized 8-Lys-vasopressin, isolated by preparative high-voltage paper electrophoresis (Table I), retained approximately the same capacity to displace radioiodinated vasopressin as did the oxidized 8-Lys-vasopressin prior to electrophoretic purification (Figure 4), indicating that the inhibition obtained at high peptide concentrations was not due to contaminating nonoxidized hormone, and that the oxidized peptide retained a part of the antigenic structure of the native vasopressin. Vasopressin which had been oxidized with performic acid in

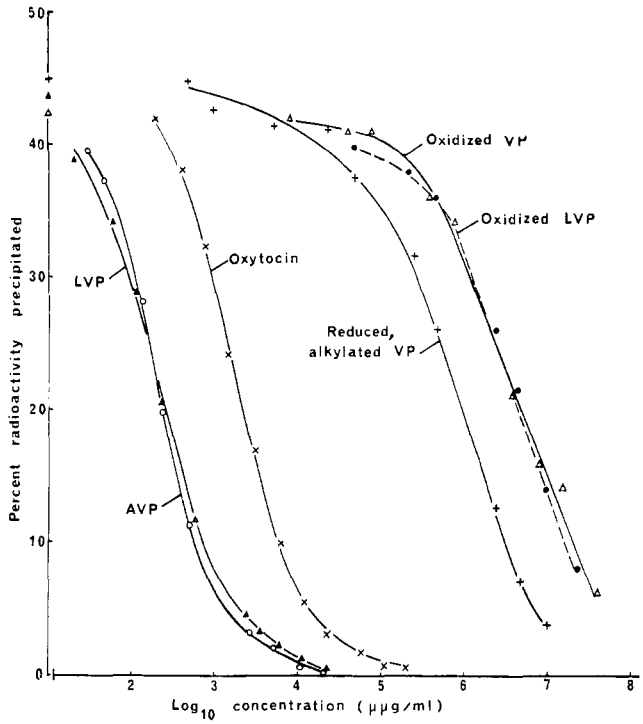

FIGURE 4: Radioimmunoassay inhibition curves constructed with the antivasopressin serum from rabbit 17. (▲—▲) Synthetic Lys-vasopressin, (●---●) performic acid oxidized Lys-vasopressin isolated by high-voltage paper electrophoresis, (○—○) synthetic Arg-vasopressin, (△—△) chloride-free mixture of Lys-vasopressin and Arg-vasopressin oxidized with performic acid, (+—+) Lys-vasopressin and Arg-vasopressin mixture reduced with 2-mercaptoethanol and alkylated with iodoacetamide, and (×—×) oxytocin. The percentage of the radioiodinated vasopressin radioactivity precipitated has been plotted as a function of the logarithm of the concentration of added unlabeled inhibitor. The points on the ordinate are the percentages of the radioiodinated vasopressin radioactivity precipitated in the absence of added unlabeled inhibitor.

the absence of chloride ion (Table I) also required a concentration approximately 10^4 times greater than control to displace radioiodinated vasopressin from rabbit antivasopressin antibody (Figure 4). Vasopressin which had been reduced with 2-mercaptoethanol and alkylated with iodoacetamide required a concentration more than 10^3 times that of control to effect an equivalent displacement of radioiodinated vasopressin from antibody (Figure 4). The greater residual inhibiting capacity of the reduced and alkylated vasopressin in comparison with the performic acid oxidized vasopressin (Figure 4) may reflect incomplete reduction and alkylation.

Molecular Models. Stereochemically feasible space-filling molecular models of vasopressin, vasopressin analogs, and chemically modified vasopressin were constructed with the following restrictions. All amino acid residues were in the L configuration. Peptide units were planar and in the *trans* configuration. The N–C^α–C′ (τ) bond angles were maintained near 110°. The dihedral angle pairs ϕ(N–C^α) and ψ(C^α–C′) were restricted by the van der Waals contacts between nonbonded neighboring atoms (Ramachandran *et al.*, 1966). The greatest restriction on the possible conformations of vasopressin was imposed by the necessity of forming a disulfide linkage between the half-cystinyl residues at positions 1 and 6. A preferred, but not necessarily unique (Gibson and Scheraga, 1967), structure for iodinated 8-Lys-vasopressin is presented in Figure 5A. The disulfide bond has a right-handed helical configuration (*cf.* Urry *et al.*, 1968). The first five amino acid residues form the initial turn of a right-handed α helix, with a hydrogen bond linking the carbonyl oxygen of half-cystinyl residue 1 and the α-amino nitrogen of asparaginyl residue 5. The next amide hydrogen bond of the α helix cannot be formed because of the rotation required about the asparaginyl ϕ(N–C^α) dihedral angle for the formation of the disulfide bridge. The tyrosinyl and phenylalanyl side groups are held in near-planar aposition by π–π orbital interactions. A value near 150° has been selected for the ψ(C^α–C′) dihedral angle of proline (*cf.* Perutz *et al.*, 1968). Amide carbonyl oxygens are concentrated on the side of the model opposite to the disulfide bond. The iodotyrosinyl residue and the two free amino groups (N-terminal α-amino group of half-cystinyl residue 1 and ϵ-amino group of lysinyl residue 8) are in near proximity to the disulfide bond.

A significantly different structure is easily obtained after disruption of the disulfide bond. The model of performic acid oxidized 8-Lys-vasopressin presented in Figure 5B was generated by simply rotating the two halves of the nonapeptide about the N–C^α bond (ϕ dihedral angle) of asparaginyl residue 5, and then forming the next amide hydrogen bond of the right-handed α helix between the carbonyl oxygen of residue 2 and the α-amino nitrogen of residue 6. The negatively charged cysteic acid residues were thereby widely separated. A similar structure may be favored for reduced and *S*-carbamidomethylated vasopressin by like-charge repulsion between the positively charged terminal α-amino group of the half-cystinyl residue 1 and either the ϵ-amino group of the lysinyl residue (8-Lys-vasopressin) or the guanidino group of the arginyl residue (8-Arg-vasopressin) at position 8.

Discussion

Vasopressin presents a number of molecular features as an antigenic determinant which may contribute to its usefulness in eliciting antibodies with restricted association constant heterogeneity. Studies of the quantitative inhibition of antigen–antibody reactions with polysaccharide (Kabat, 1961), polypeptide (Maurer, 1964; Arnon *et al.*, 1965; Schlossman *et al.*, 1968) and polynucleotide (Stollar *et al.*, 1962) segments of the larger immunizing antigens have indicated that the upper limits for the dimensions of the most extended form of an antigenic determinant complementary in size to the antibody combining site are of the order of 25–36 × 10–17 × 6–7 Å (Kabat, 1966). The molecular model of native vasopressin has the dimensions 19 × 14 × 11 Å. Vasopressin, therefore, may be of sufficient size to completely occupy an antibody combining site as a single determinant. The disulfide linkage between the half-cystinyl residues at positions 1 and 6 may severely restrict the possible conformations of the nonapeptide (Gibson and Scheraga, 1967), and vasopressin may present a more rigid antigenic determinant than do other peptides of similar size which lack a restrictive and stabilizing cyclic structure. Disruption of the disulfide bond of vasopressin by performic acid oxidation and conversion of the two half-cystinyl residues into negatively charged cysteic acid residues resulted in a profound alteration in the antigenic structure of the nonapeptide. Conversion of the half-cystinyl residues

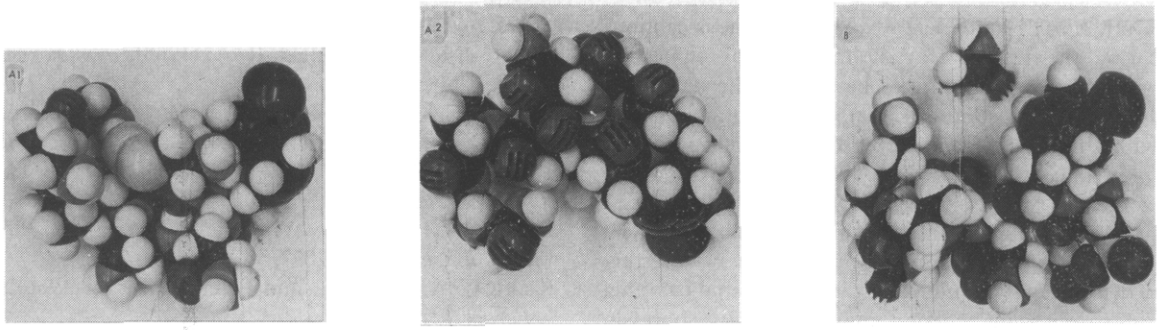

FIGURE 5: Space-filling three-dimensional Corey–Pauling–Koltun molecular models of radioiodinated Lys-vasopressin (A) and performic acid oxidized Lys-vasopressin (B). Two views of the native hormone are presented in A1 and A2. The dimensions of the native molecule are 19 × 14 × 11 Å. A model of the *S*-carbamidomethyl group of reduced and alkylated vasopressin also is shown in B.

into neutral *S*-carbamidomethylcysteinyl residues also led to a marked change in the antigenic structure of the hormone.

High-affinity antivasopressin antibody has been induced in rabbits by immunization either with vasopressin covalently coupled by carbodiimide-initiated amide cross-linkage (Goodfriend *et al.*, 1964; Permutt *et al.*, 1966) to equine γ-Gab-globulins or with vasopressin not covalently linked to a larger carrier molecule. The observation that the nonapeptide is capable of eliciting antibodies without prior covalent linkage to a larger carrier protein confirms the findings previously reported by Roth *et al.* (1966). It is of special interest that an antigenic determinant complementary in size to the antibody combining site is antigenic without further modification. Other small antigenic determinants which have been shown to be antigenic without covalent linkage to a large carrier molecule are glucagon (Unger *et al.*, 1961; Rockey and Kunkel, 1962), oxytocin (Gilliland and Prout, 1965), angiotensin (Dietrich, 1966; Boyd and Peart, 1968), gastrin (Schneider *et al.*, 1967), and α-DNP-hepta-L-lysine (Schlossman *et al.*, 1965). Covalent linkage of vasopressin to a larger protein molecule by amide linkage *via* the α-amino group of the terminal half-cystinyl residue at position 1 (8-Arg-vasopressin and 8-Lys-vasopressin) or *via* the ε-amino group of a lysinyl residue at position 8 (8-Lys-vasopressin) should restrict the orientation of the nonapeptide as an antigenic determinant. In this regard it is of interest to note that in the space-filling molecular model of 8-Lys-vasopressin, the two available amino groups are in near proximity. The average intrinsic association constants, K_0, of the antibodies produced against the covalently linked vasopressin did not differ, within the limits of experimental error, from those of the antibodies produced against the unmodified nonapeptide. These experiments therefore failed to demonstrate any effect of a restrictive orientation of vasopressin by covalent linkaged on the specificity of the induced antibodies. The linearity of the *r/c vs. r* data, and the near unity of the index of heterogeneity, *a*, of the Sips distribution function indicated that there was no substantial heterogeneity in the association constants of the antibodies in the selected sera examined. Therefore, there was no evidence that the variable topology of the adjacent surfaces of the γGab-globulin carrier proteins (Rockey, 1967) contributed to the antigenic structure of the covalently linked vasopressin determinant. Examination of the antibody contained in sera obtained at different intervals during the immunization schedule also failed to demonstrate a variation in average intrinsic association constants indicative of an alteration of the specificity of the antibody with prolonged immunization.

The fact that antibodies of a serum do not display association constant heterogeneity in no way precludes the presence of multiple molecular forms of antibodies. The antivasopressin antibodies of rabbit 12 (Table II), which showed limited association constant heterogeneity, were composed of two heavy-chain allotypic variants (A1 and A3). The Fd fragments (N-terminal halves) of the heavy chains from the two γG-globulin allotypes have been reported to differ significantly in amino acid composition (Inman and Reisfeld, 1968).

A rigid asymmetric antigenic determinant, capable of completely occupying an antibody combining site, may, nevertheless, be able to generate antibodies which differ in the structure of their combining sites. The space-filling molecular model of vasopressin in asymmetric. More than a single orientation of the nonapeptide in the antibody combining site may be envisioned. For example, if both the heavy and the light chains of the antibody contribute to the topology of the combining site (Utsumi and Karush, 1964; Rockey, 1967; Yoo *et al.*, 1967), a distinct region of the vasopressin molecule could be in contact with heavy-chain residues in one configuration and with light-chain residues in a second configuration. The construction of comparative radioimmunoassay inhibition curves with analogs of vasopressin furnishes an additional parameter for evaluating the specificity of the antibodies produced after different methods of immunization, by individual animals, and in different species. More than 120 analogs of vasopressin have been described (Schwartz and Livingston, 1964). Oxytocin, which differs from vasopressin by but two amino acid residues (Ile at position 3, and Leu at position 8), required a concentration ten times greater than either 8-Lys-vasopressin or 8-Arg-vasopressin to effect an equivalent displacement of radioiodinated vasopressin from the antivasopressin antibody of rabbit 17. Permutt *et al.* have reported that a concentration of oxytocin 4×10^3 times greater than 8-Lys-vasopressin was required to effect an equivalent displacement of radioiodinated 8-Lys-vasopressin from a rabbit anti-8-Lys-vasopressin antibody (Permutt *et al.*, 1966). Roth *et al.* have reported that a concentration of oxytocin 10^2–10^3 times that of vasopressin was needed to effect a comparable result on radioimmunoassay with rabbit antivasopressin antibody, and also have described other antisera capable of distinguishing between 8-Lys-vasopressin and 8-Arg-vaso-

pressin (Roth *et al.*, 1966; Klein *et al.*, 1966). One is cautious in comparing the results of radioimmunoassay inhibition studies from different laboratories because of the possibility of variations in the vasopressin analog preparations. Initial studies with the serum from rabbit 12 have indicated that here again a concentration of oxytocin ten times greater than 8-Lys-vasopressin was required to effect an equivalent displacement of radioiodinated vasopressin from antibody (W.-H. Wu and J. H. Rockey, unpublished observations). Nevertheless, more detailed radioimmunoassay inhibition studies with a number of vasopressin analogs, employing antivasopressin antibodies from different animals and different species and antisera specific for distinct classes, subclasses, and allotypes of immunoglobulins, may prove the most useful method for defining similarities and differences between distinct antivasopressin antibodies. The system also offers a useful method for comparing the topology of vasopressin analogs which vary in hormonal activity.

Acknowledgment

We thank Dr. Jesse Roth and Dr. Allen W. Root for helpful discussions during the initial phase of these studies. The allotypic specificities of the rabbit immunoglobulins kindly were determined by Dr. Stanislaw R. G. Dubiski. We are especially indebted to Miss Ada C. Bello for excellent technical assistance.

References

Allen, J. C., Kunkel, H. G., and Kabat, E. A. (1964), *J. Exptl. Med. 119*, 453.

Arnon, R., Sela, M., Yaron, A., and Sober, H. A. (1965), *Biochemistry 4*, 948.

Berson, S. A., Yalow, R. S., Bauman, A., Rothschild, M. A., and Newerly, K. (1956), *J. Clin. Invest. 35*, 170.

Boyd, G. W., and Peart, W. S. (1968), *Lancet 2*, 129.

Brenner, S., and Milstein, C. (1966), *Nature 211*, 242.

Cohen, S., and Porter, R. R. (1964), *Advan. Immunol. 4*, 287.

Dekanski, J. (1952), *Brit. J. Pharmacol. 7*, 567.

Dietrich, F. M. (1966), *Intern. Arch. Allergy Applied Immunol. 30*, 497.

Dreyer, W. J., and Bennett, J. C. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 864.

Dubiski, S., Duziak, Z., Skalba, D., and Kelus, A. (1961), *Immunology 4*, 236.

Eisen, H. N., and Siskind, G. W. (1964), *Biochemistry 3*, 996.

Freedman, M. H., and Sela, M. (1966), *J. Biol. Chem. 241*, 5225.

Fujio, H., and Karush, F. (1966), *Biochemistry 5*, 1856.

Gibson, K. D., and Scheraga, H. A. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 1317.

Gilliland, P. F., and Prout, T. E. (1965), *Metabolism 14*, 918.

Goodfriend, T. L., Levine, L., and Fasman, G. D. (1964), *Science 144*, 1344.

Haber, E. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 1099.

Haber, E., Richards, F. F., Spragg, J., Austen, K. F., Vallotton, M., and Page, L. B. (1967), *Cold Spring Harbor Symp. Quant. Biol. 32*, 299.

Hirs, C. W. H. (1956), *J. Biol. Chem. 219*, 611.

Hood, L., and Ein, D. (1968), *Nature 220*, 764.

Hunter, W. M., and Greenwood, F. C. (1962), *Nature 194*, 495.

Inman, J. K., and Reisfeld, R. A. (1968), *Immunochemistry 5*, 415.

Kabat, E. A. (1961), Kabat and Mayers Experimental Immunochemistry, 2nd ed, Springfield, Ill., C. C Thomas.

Kabat, E. A. (1966), *J. Immunol. 97*, 1.

Karush, F. (1962), *Advan. Immunol. 2*, 1.

Katz, A. M., Dreyer, W. J., and Anfinsen, C. B. (1959), *J. Biol. Chem. 234*, 2897.

Klein, L. A., Roth, J., and Petersen, M. J. (1966), *Surgical Forum 17*, 240.

Koshland, M. E. (1967), *Cold Spring Harbor Symp. Quant. Biol. 32*, 119.

Koshland, M. E., Englberger, F. M., and Shapanka, R. (1966), *Biochemistry 5*, 641.

Lowry, O. H., and Bessey, O. A. (1946), *J. Biol. Chem. 163*, 633.

Maurer, P. H. (1964), *Progr. Allergy 8*, 1.

Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6*, 819.

Morse, J. H., and Heremans, J. F. (1962), *J. Lab. Clin. Med. 59*, 891.

Müller-Eberhard, H. J. (1960), *Scand. J. Clin. Lab. Invest. 12*, 33.

Permutt, M. A., Parker, C. W., and Utiger, R. D. (1966), *Endocrinology 78*, 809.

Perutz, M. F., Muirhead, H., Cox, J. M., and Goaman, L. C. G. (1968), *Nature 219*, 131.

Ramachandran, G. N., Venkatachalam, C. M., and Krimm, S. (1966), *Biophys. J. 6*, 849.

Richards, F. F., Sloane, R. W., Jr., and Haber, E. (1967), *Biochemistry 6*, 476.

Rockey, J. H. (1967), *J. Exptl. Med. 125*, 249.

Rockey, J. H., and Kunkel, H. G. (1961), *Arthritis Rheumat. 4*, 449.

Rockey, J. H., and Kunkel, H. G. (1962), *Proc. Soc. Exptl. Biol. Med. 110*, 101.

Roth, J., Glick, S. M., Klein, L. A., and Peterson, M. J. (1966), *J. Clin. Endocrinol. Metab. 26*, 671.

Schally, A. V., Bowers, C. Y., Carter, W. H., and Hearn, I. C. P. (1964), *Arch. Biochem. Biophys. 107*, 332.

Schlossman, S. F., Levine, H., and Yaron, A. (1968), *Biochemistry 7*, 1.

Schlossman, S. F., Yaron, A., Ben-Efraim, S., and Sober, H. A. (1965), *Biochemistry 4*, 1638.

Schneider, D. R., Endahl, G. L. Dodd, M. C., Jesseph, J. E., Bigley, N. J., and Zollinger, R. M. (1967), *Science 156*, 391.

Schwartz, I. L., and Livingston, L. M. (1964), *Vitamins Hormones 22*, 261.

Sips, R. (1948), *J. Chem. Phys. 16*, 490.

Smithies, O. (1967), *Science 157*, 267.

Stollar, D., Levine, L., Lehrer, H. I., and Van Vunakis, H. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 874.

Unger, R. H., Eisentraut, A. M., McCall, M. S., and Madison, L. L. (1961), *J. Clin. Invest. 40*, 1280.

Urry, D. W., Quadrifoglio, F., Walter, R., and Schwartz, I. L. (1968), *Proc. Natl. Acad. Sci. U. S. 60*, 967.

Utsumi, S., and Karush, F. (1964), *Biochemistry 3*, 1329.

Whitney, P. L., and Tanford, C. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 524.

Wright, P. H., and Norman, L. L. (1966), *Diabetes 15*, 668.

Yoo, T. Y., Roholt, O. A., and Pressman, D. (1967), *Science 157*, 707.