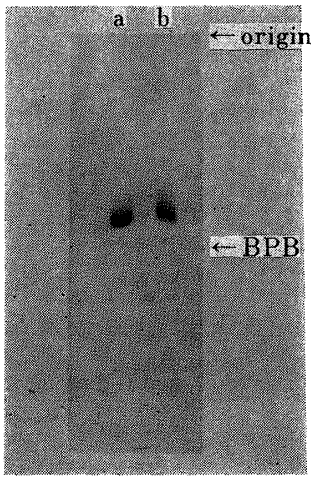

a, untreated tRNA,
b, H_2S–treated tRNA
BPB = Bromphenol blue

Fig. 3. Polyacrylamide Gel Electrophoresis of tRNAs

of the modified tRNA gave a single band, which showed that the sulfhydrolysis of tRNA proceeded without cleavage of the tRNA molecule (Fig. 3).

Sulfhydrolysis of DNA

When heat-denatured DNA was treated at 40° for 72 hr, the UV spectrum of the product showed the presence of 4-thiouracil groups ($A_{330\,nm}/A_{260\,nm}=0.22$). The presence of 2′-deoxy-4-thiouridylate(pdS^{4}U, Fig. 2c) was confirmed by nuclease P_1 digestion followed by two-dimensional thin layer chromatography. Thus, 4-thiouracil groups were also introduced into DNA by the sulfhydrolysis procedure.

Further studies on the sulfhydrolysis of tRNAs for specific amino acids and of certain 5S ribosomal RNAs are in progress in our laboratory.

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Interactions of Sepharose-Bound Neurophysin I and II with Oxytocin and Vasopressin

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The interactions of Sepharose-bound bovine neurophysin I and II with oxytocin and arginine-vasopressin were studied.

The amounts of hormones bound to immobilized neurophysin II were found to depend strongly on temperature, being larger at 4° than at 36°.

It was found that oxytocin and vasopressin were eluted in different fractions from a column of Sepharose-neurophysin I by shallow pH gradients in either direction from pH 5.8, whereas the hormones emerged in the same fractions from a Sepharose-neurophysin II column.

Keywords——(bovine)neurophysin I; (bovine)neurophysin II; oxytocin; vasopressin; immobilized neurophysin; affinity chromatography

The bovine pituitary posterior lobe contains two main hormone-binding proteins, neurophysin I (NP-I) and neurophysin II (NP-II).[2] Several studies have been carried out on the

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2) *a*) M.D. Hollenberg and D.B. Hope, *Biochem. J.*, **106**, 557 (1968); *b*) C.R. Dean and D.B. Hope, *ibid.*, **106**, 565 (1968); *c*) R. Rauch, M.D. Hollenberg, and D.B. Hope, *ibid.*, **115**, 473 (1969).

molecular features of the hormone-neurophysin (NP) interaction[3] and it has been shown that purified NP-I and NP-II can bind with both oxytocin and vasopressin with essentially identical affinity in solution,[3c] though they have somewhat different pH-profiles of binding to each hormone.[3e,h,i] Although these studies in solution suggest that the NP-I and NP-II molecules both partially change their conformations for complete binding with hormones,[3d,f-i] it has been found that immobilized NPs attached to insoluble supports such as agarose or cellulose still retain their ability to bind hormones.[4] This indicates that the region of NP involved in the hormone-binding is not significantly affected by the immobilization. Therefore it may be possible to use immobilized NP in studies of the basic mechanism involved in hormone-protein interactions, as well as in analytical methods for the hormones. However, there has been no comparative study of the interactions between the hormones and immobilized NP-I and NP-II.

Recently, Robinson and Walker unsuccessfully attempted to separate oxytocin and vasopressin on a column of agarose-NP.[4c] This failure might have been due to the NP used, which was not separated into I and II.

In the present work we have prepared Sepharose-bound NP-I and NP-II separately, and studied the hormone-immobilized NP interactions as a function of pH.

Experimental

Neurophysins——Bovine NP-I and NP-II were prepared as described previously from acetone-dried powder of bovine pituitary posterior lobes, and the purity of the preparations was confirmed by means of electrophoresis and amino acid analysis.[3h]

Hormones——A solution of NP-free hormones, oxytocin and arginine-vasopressin (about 60 units each/ml) was prepared from the NP-hormone complexes by gel filtration on Sephadex G-25 at pH 2.0, as described previously.[3h]

Preparation of Sepharose-NP——NP-I or NP-II (20 mg) was allowed to react with 1.5 g of cyanogen bromide-activated Sepharose (CNBr-activated Sepharose 4B, Pharmacia Fine Chemicals) in 10 ml of 0.1 M borate buffer, pH 8.05, containing 0.5 M sodium chloride for 20 hr at 4°. The derivatives were treated with 0.1 M ethanolamine for 2 hr at room temperature. The Sepharose derivatives were washed extensively with 0.1 M sodium acetate, pH 4.0, containing 1 M sodium chloride, on a sintered glass filter, then with 0.1 M borate buffer, pH 8.05, containing 1 M sodium chloride, and transferred to a column for chromatography (1.0 × 15 cm). Each column was equilibrated with buffer, as described in "Results and Discussion," before use.

Amounts of NPs coupled to the Gel——A portion of swollen NP-coupled gel was washed with acetone in order to dry it as completely as possible, and the dried product was hydrolyzed with 6 N HCl at 110° for 22 hr *in vacuo*. The hydrolysate was evaporated to dryness under reduced pressure, dissolved in sodium citrate buffer (Na concentration = 0.2 N, pH 2.2), and analyzed for amino acids. The amounts of NP-I and NP-II coupled to the gel were estimated as 6.5 mg and 11.3 mg per 1.5 g of CNBr-activated Sepharose 4B, respectively, based on the amounts of serine and glutamic acid. The recovery of these amino acids, serine and glutamic acid, of NPs was confirmed to be constant under these amino acid analysis conditions in preliminary experiments.[5]

Hormone Activities——Oxytocin and vasopressin were assayed by the fowl blood pressure method and the spinal rat blood pressure method, respectively, as detailed in the Pharmacopoeia of Japan.

3) *a*) E. Breslow and L. Abrash, *Proc. Nat. Acad. Sci. U.S.A.*, **56**, 640 (1966); *b*) E. Breslow, H.L. Aanning, L. Abrash, and M. Schmir, *J. Biol. Chem.*, **246**, 5179 (1971); *c*) E. Breslow and R. Walter, *Mol. Pharmacol.*, **8**, 75 (1972); *d*) E. Breslow and J. Weis, *Biochemistry*, **11**, 3474 (1972); *e*) M. Camier, R. Alazard, P. Cohen, P. Pradelles, J.L. Morgat, and P. Fromageot, *Eur. J. Biochem.*, **32**, 207 (1973); *f*) J.H. Griffin, R. Alazard, and P. Cohen, *J. Biol. Chem.*, **248**, 7975 (1975); *g*) J. Wolff, R. Alazard, M. Camier, J.H. Griffin, and P. Cohen, *ibid.*, **250**, 5215 (1975); *h*) H. Fukuda, T. Hayakawa, J. Kawamura, and Y. Aizawa, *Chem. Pharm. Bull.*, **24**, 36 (1976); *i*) H. Fukuda, T. Hayakawa, J. Kawamura, and Y. Aizawa, *ibid.*, **24**, 2043 (1976); *j*) M. Blumenstein and V.J. Hruby, *Biochemistry*, **16**, 5169 (1977).

4) *a*) R. Portanova and H. Sachs, *Endocrinology*, **80**, 527 (1967); *b*) V. Pliska and H. Sachs, *Eur. J. Biochem.*, **41**, 229 (1974); *c*) I.C.A.F. Robinson and J.M. Walker, *J. Endocrinol.*, **80**, 191 (1979).

5) Unpublished.

Results and Discussion

Effect of Temperature on Hormone-Binding Capacity

To investigate the effect of temperature on the maximum hormone-binding to Sepharose-NP, a solution containing a large excess of oxytocin or vasopressin (about 100 units) was charged onto a column of Sepharose-NP-II (1.0×1.0 cm), which had been equilibrated with 0.1 M pyridine–acetic acid buffer (pH 5.7), at 4° or 36°. After the column had been thoroughly washed with the same buffer, the hormone was eluted with 10 ml of 0.01 N HCl. The amounts of hormones bound to the column at 4° and 36° are shown in Table I. The amounts of hormones bound to immobilized NP-II were found to depend strongly on temperature, being larger at 4° than at 36°.

A hydrophobic interaction should, in contrast to an electrostatic one, decrease when the temperature is decreased.[6] Thus, the results obtained here suggest that electrostatic forces may be more signifcant in the binding of immobilized NP and hormone than hydrophobic interactions.

It seems likely that affinity chromatography using Sepharose-NP may be performed effectively at low temperature. Thus, the following experiments were all carried out at 4°.

Table I. Effect of Temperature on the binding of Hormones to the Sepharose-Neurophysin II Column (1×1 cm)

Bound hormone units			
at 4°		at 36°	
Oxyto.	Vasop.	Oxyto.	Vasop.
21.4	22.6	9.4	10.4

Elution Profiles of Hormones from Sepharose-NP-I and -II Columns

Figure 1 shows the results of a typical experiment in which oxytocin and vasopressin were loaded onto a column of Sepharose–NP-I. In the case of Fig. 1A, a solution of oxytocin and vasopressin (pH 5.8) was applied to a column (1.0×6.0 cm) saturated with 0.1 M Na_2HPO_4–citric acid buffer (pH 5.8) and eluted with the same buffer system, gradually decreasing the pH. Vasopressin emerged from the column faster than oxytocin. A part of the oxytocic activity appeared in the fractions in which vasopressin appeared. Although we have no direct evidence, this oxytocic activity may not be attributable to oxytocin because it is known that arginine-vasopressin possesses chicken blood depressor activity amounting to about 15% of its rat blood pressor activity.[7] In the case of Fig. 1B, the hormones were applied to the above column saturated with 0.1 M phosphate buffer (pH 5.8) and eluted with the same buffer system with a gradual increase of the pH to 7.4, followed by 0.1 M borate buffer with a pH gradient from 7.4 to 9.0. On the basic side, oxytocin and vasopressin also emerged in different fractions and in this case the former emerged faster than the latter.

Figure 2 shows the results of similar experiments with a column of Sepharose–NP-II. As shown in Fig. 2A and 2B, the two hormones emerged from the column at the same or almost the same pH upon gradient elution in either direction from pH 5.8.

The pHs of the peaks in the elution profiles of the hormones may be assumed to be critical for the binding of each hormone to each column. A comparison of these values with the ap-

6) *a*) S. Hjerten, *J. Chromatogr.*, **87**, 325 (1973); *b*) S. Hjerten, J. Rosengren, and S. Påhlman, *J. Chromatogr.*, **101**, 281 (1974).

7) "Handbook of Biochemistry," ed. by H.A. Sober, The Chemical Rubber Co., Cleveland, Ohio, 1968, p. C-95.

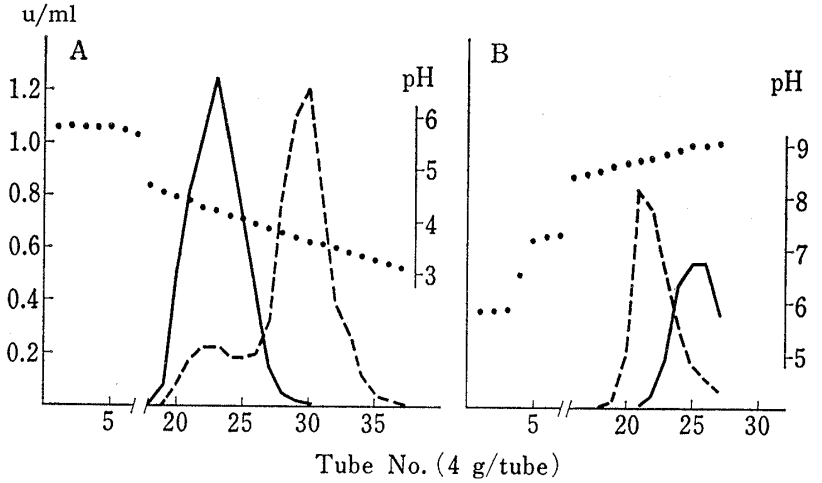

Fig. 1. Elution Profiles of Oxytocin and Vasopressin from a Column of Sepharose-Neurophysin I (1×6 cm) obtained by pH Gradient Elution from pH 5.8 to pH 3.0 (A) and from pH 5.8 to pH 9.1 (B)

--------; oxytocin, ———; vasopressin.

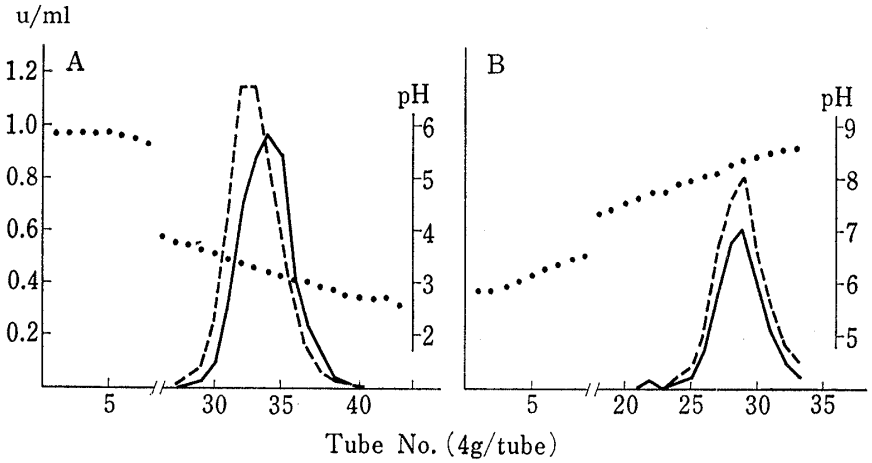

Fig. 2. Elution Profiles of Oxytocin and Vasopressin from a Column of Sepharose-Neurophysin II (1×6 cm) obtained by pH Gradient Elution from pH 5.8 to pH 2.5 (A) and from pH 5.8 to pH 8.6 (B)

--------; oxytocin, ———; vasopressin.

parent pK values for free NPs[3e] is shown in Table II. It was found that the values for the immobilized NPs were all shifted outside the corresponding acidic and basic values for free NPs. It seems likely that the electronic environment of the functional group(s) of the immobilized protein involved in the interaction may differ from that of free NPs. This might be a result of the partial loss of flexibility of immobilized NPs in relation to the conformational change which is thought to be required for complete hormone binding, since NP molecules must be immobilized on Sepharose at more than one point because they possess several free amino groups. The significant shift of the critical pH for hormone-protein binding outside the apparent pK values for free NPs suggests that the functional group(s) in NPs may become more exposed upon immobilization. At any rate, it should be noted that elutions of these hormones from Sepharose–NP-I and –NP-II are greatly affected by the pH of the eluent, and the elution profiles coincide well with the pH profiles for hormone-NP binding. This strongly suggests that the binding process is dependent on at least two distinct ionizable groups in the case of the immobilized NPs. These findings, together with the results obtained on the influence of temperature on the hormone-binding capacity, indicate that electrostatic binding

Table II. Comparison of Elution pHs of Oxytocin and Vasopressin from immobilized NPs Columns and pK Values for the Hormone-binding to Free NPs

		For oxytocin		For vasopressin	
		Acidic side	Basic side	Acidic side	Basic side
NP-I	Free[a]	4.65	7.15	4.6	6.4
	Immobilized[b]	3.6	8.7	4.2	9.0
NP-II	Free[a]	4.05	6.75	4.75	6.4
	Immobilized[b]	3.3	8.35	3.2	8.35

a) apparent pK values given by Camier *et al.*[3e]
b) pHs of the peaks in the elution profiles.

is an essential element in hormone recognition by NPs, and this is the reason why Sepharose–NPs retain the ability to bind the hormones. [1-Desamino]-oxytocin, which lacks the α-amino group in the cysteine residue, does not bind to agarose-NP.[4c] This result also supports the existence of electrostatic interaction between NPs and hormones.

Another interesting finding can be seen in Fig. 1, *i.e.*, that oxytocin and vasopressin can be separated on a Sepharose–NP-I column. The attempts of Robinson and Walker to elute oxytocin and vasopressin separately from a column of agarose–NP by the use of shallow pH gradients in either direction from pH 5.8 were unsuccessful.[4c] They used a mixture of NP-I and NP-II to prepare their immobilized protein column. Since oxytocin and vasopressin are hardly separated on a Sepharose–NP-II column (Fig. 2), their use of the mixture may account for their lack of success. On the other hand, Sepharose bound NP-I does have the ability to separate the two hormones (Fig. 1) and thus may represent a useful analytical tool for studies on these hormones.

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A Simple Purification Procedure for Rat Pancreatic Elastase and Radioimmunoassay of the Enzyme

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Rat pancreatic elastase was purified on a column of lima bean trypsin inhibitor-Sepharose after inactivating trypsin and chymotrypsin in the pancreatic homogenate by treatment with N$^{\alpha}$-*p*-tosyl-L-lysylchloromethyl ketone and N-tosyl-L-phenylalanylchloromethyl ketone. The molecular weight of the enzyme was 25000, as determined by polyacrylamide gel electrophoresis. A radioimmunoassay method was established for rat elastase; the enzyme level could be determined over the range of 2.5—300 ng of the enzyme per ml.

Keywords——rat pancreatic elastase; purification; characterization; radioimmunoassay; tissue level of immunoreactive elastase

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