Chou protein just as well. More work such as tryptic peptide composition and sequence analysis of the Einstein-Chou fragment and the larger protein is necessary to identify the relative order of these three active fragments in the larger encephalitogen. In any event, there is no overlap between our protein and the Eylar-Hashim peptide, thus indicating the presence of at least two separate encephalitogenic sites. Together these two fragments represent approximately half of the larger protein.

Since our active fragment has been tested only in rabbits, and the other two only in guinea pigs, it will be important to determine activities in other species. In rabbits, the critical amount of our protein for encephalitogenic activity seems to be 50 μg. Injections of 100 or 200 μg produced experimental allergic encephalomyelitis (EAE) in over 90 percent of injected rabbits with the average day of onset being around 14 days (*3*). In most experiments, 50 μg exhibited the same degree of activity, while in other experiments the incidence was lower and the day of onset was prolonged. Below 50 μg activity fell off; that is, 25 μg produced EAE in three-fourths of the rabbits with an average onset of 17 days, and 15 μg produced this disease in five-eighths of the rabbits with an average onset of 25 days. A tryptic digest of our protein was inactive but peptide 16 to 45, obtained by dilute acid hydrolysis, produced EAE in two out of two rabbits on days 14 and 21, respectively, in a dose of approximately 25 μg.

The amino acid sequence of the encephalitogenic protein from bovine cord and rabbit brain reported here is also of importance since we are dealing with a membrane protein. One might predict that such proteins must have unique properties that result primarily from their amino acid sequence. It is interesting to note (Fig. 1) that portions of our protein can be fitted to a repeating sequence of three apolar amino acids followed by two polar ones (residues 1 to 15 and 37 to 44 in the bovine preparation, and residues through the NH_2-terminal aspartic acid of peptide T_6 and 37 to 44 in the rabbit preparation). In a discussion of interaction of membrane protein and lipid, Wallach (*13*) "conjectured that the distribution of polar amino acids is such that their side chains lie at the membrane surfaces, or cluster round the central axis of each subunit, or both, possibly producing aqueous channels which penetrate the membrane." This requires that the protein segments have a polar face opposite the apolar one. Such an arrangement could be made with a repeating sequence of polar and apolar residues.

The foregoing evidence suggests that only one basic protein exists in bovine myelin and that this protein contains more than one encephalitogenic site, as demonstrated by sequence analysis on encephalitogenic fragments which have been shown to be part of the larger protein. It is conceivable that other portions of the protein are also active.

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Plasma Saluretic Activity: Its Nature and Relation to Oxytocin Analogs

Abstract. *Plasma natriuretic activity was evoked in cows and dogs by infusion of saline with or without dextran. Deproteinized samples were fractionated on both Sephadex and Bio-Gel columns; the activity was separated, the approximate molecular weight being in the region of 1000. Incubation with chymotrypsin destroyed the activity, suggesting that it might be a polypeptide. A similar activity in blood resulted from intracarotid injection of either oxytocin or either of two synthetic analogs. Possibly the latter are saluretic by virtue of a releasing action on some intracranial structure for another natriuretic peptide.*

Knowledge of the chemical nature of "natriuretic hormone" has thus far been acquired from an active factor in the plasma of dogs, cats, and cows sampled after a variety of stimuli (infusion of saline with and without colloid, carotid-artery occlusion) as well as from uremic man (*1–3*). This evidence suggests that the molecule concerned is not precipitated with plasma proteins, is heat-stable after protein is removed, is polypeptide in nature, contains at least one basic amino acid and at least one free amino group, and has a molecular weight of less than 20,000 (*2*) or less than 2000 (*3*). Until the exact chemical identity has been established, there is no way of determining whether the various experimental results deal with the same molecular entity. The cow is an adequate experimental animal for the production of saluretic responses to such "volume" stimuli as the infusion of isosmotic, isooncotic dextran, accompanied by the appearance of natriuretic activity in plasma (*4*). There is the particular advantage that fairly large blood samples can be withdrawn.

Six cows were investigated by described methods (*4*). A cardiac catheter was introduced through the external jugular vein into the innominate vein in order to collect blood mixed with the

venous return from the brain. Samples (500 ml) were collected before the onset of dextran infusion; 15 liters of 6 percent (weight to volume) dextran in physiological saline were then infused rapidly. At the height of the diuretic response a further blood sample (1500 ml) was withdrawn through the same catheter. The second sample was collected, on the average, 1.3 hours after the first. All blood samples were immediately deproteinized by addition to cold 30 percent (weight to volume) trichloroacetic acid to a final concentration of 5 percent (weight to volume). After centrifugation in the cold, the supernatant was extracted three times with five volumes of diethyl ether to a final *p*H in the aqueous phase of 5.5, in order to remove free trichloroacetic acid. The ether was then removed under reduced pressure, and the natriuretic activity of the residue, adjusted to *p*H 7.5 (0.2-ml samples), was determined in hydrated rats anesthetized with ethanol (*2*). No natriuretic activity was found in control samples taken before infusion; an average activity of +86.3 percent change (percent change in the tubular rejection fraction of Na) was found in the samples taken after infusion.

Samples (100 ml) of both active and control deproteinized plasma were applied to a G-25 Sephadex column (total volume 1200 ml) charged with 0.1*M* acetic acid, and 15-ml fractions were collected at 15-minute intervals. These fractions were analyzed for conductivity, Na concentration, absorbancy at 280 nm, natriuretic activity, and dextran content (anthrone reaction). The fractions were lyophilized, and natriuretic activity was determined again after the material was redissolved in 4 ml of distilled water. Activity was found in only one fraction from the G-25 separation, and this was stable after lyophilization. The active fractions were then applied to a Bio-Gel P-2 column (total volume 148 ml) charged with 0.1*M* acetic acid for further separation; 2.5-ml fractions were collected at 20-minute intervals, lyophilized, redissolved in 4 ml of distilled water, and subjected to the same analyses as before.

C^{14}-Vasopressin (5 μc) was dissolved in an active G-25 fraction, and the mixture was applied to the same P-2 column. In this experiment radioactivity of the fractions was measured in addition to the other characteristics.

Finally, P-2 fractions with natriuretic activity were incubated for 2 hours at 37°C in 0.2*M* phosphate buffer (*p*H 7.6) in a Dubnoff shaker. To blank samples, 5 ml of distilled water was added; to the remainder, 25 mg of chymotrypsin contained in the same volume was added. The reaction was terminated by the addition of glacial acetic acid to a *p*H of 2.0 and exposure to 100°C for 3 minutes. The *p*H of both control and experimental samples was then corrected to 7.5, and natriuretic activity was determined.

Figure 1 shows the fractional results of the P-2 separation of active samples from the G-25 column. There were typical and repeatable absorbancy (280 nm) and conductivity curves, and natriuretic activity appeared in only one pooled fraction associated with the middle absorbancy (280 nm) peak. Separation had resulted in concentration of the activity to an average value of + 310 percent change in tubular rejection fraction of Na. Continuous recording of both ultraviolet absorption and

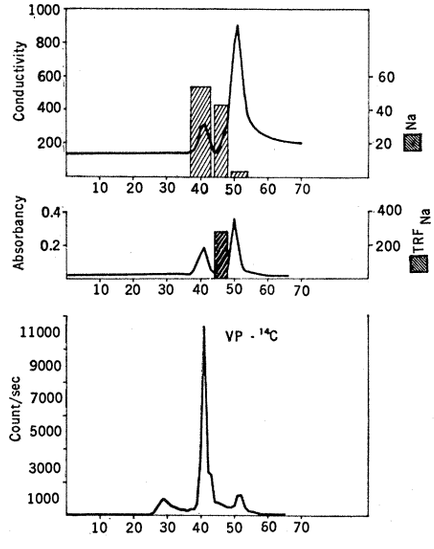

Fig. 1. Gel separation of deproteinized bovine plasma with natriuretic activity on Bio-Gel P-2, after preliminary separation on Sephadex G-25. Abscissa for all three graphs gives the fraction number. Ordinates: The top curve is conductivity in reciprocal ohms. The columns in the top graph represent Na concentration in milliequivalents per liter in pooled fractions (from left to right, the three columns represent 8, 4, and 4 pooled fractions, respectively). Absorbancy at 280 nm is shown in the middle graph. TRF_{Na} (column, middle graph) represents the tubular rejection fraction of sodium (in assay rats) in percentage change (natriuretic activity) in four pooled fractions. The curve in the bottom graph shows the number of counts per second of C^{14}-vasopressin (VP-^{14}C).

conductivity was of advantage in more rapid processing of the gel separation. No dextrans were contained in the active samples, and the concentration of Na was 45 meq/liter; that is, the tested samples were hypotonic.

After incubation of the mixtures with chymotrypsin, activity remained in the control mixtures without enzyme, but was completely absent from the mixtures containing chymotrypsin. The radioactivity (count/sec) in the P-2 fractions showed that C^{14}-vasopressin separated out from the column in fractions just preceding the natriuretic activity.

In control samples, a small amount of natriuretic activity separated out from the G-25 column in the same fractions as from the active samples taken after infusion, despite the lack of measurable activity in the initial deproteinized plasma. The difference in activity between samples taken before and after infusion was highly significant ($P < .001$) after G-25 separation.

This same experiment was repeated with eight dogs infused with physiological saline. The animals were anesthetized with chloralose (80 mg/kg) given intravenously; 50 ml of blood was sampled before infusion, and 200 ml was taken at the height of the diuretic-saluretic response. This latter balanced state was reached after 30 to 90 minutes and after infusion of 1.5 to 5.0 liters of saline. Samples were processed in the same manner, with results similar to those reported above for bovine plasma.

Whereas oxytocin is moderately saluretic when administered intravenously, a far greater natriuresis can be produced by administration of the same dose into the carotid arteries (*5*). The 4-leucyl analog of oxytocin has only natriuretic and diuretic properties (*6*); that is, the antidiuretic and vasomotor activities of the parent hormone are suppressed by the alteration in sequence position 4. In such an analog, polypeptide-induced saluresis can be studied as a more isolated phenomenon, without the usual spectrum of other activities. We administered synthetic oxytocin and two analogs, 4-leucyl-8-isoleucyl- and 4-prolyl-8-isoleucyl-ocytocin, to dogs and cats. For each of the three peptides (Table 1), the selected dose administered intravenously or into the renal arteries produced a small to moderate degree of saluresis. The same dose was then given into a carotid artery, and arterial blood

Table 1. Cat assay of natriuretic activity of oxytocin (OT), synthetic analogs of OT, deproteinized canine plasma before and after saline loading, and gel fractions of the latter. Samples were administered to cats by one of three routes: intravenously, into the renal arteries, into a carotid artery. TRF, tubular rejection fraction.

Substance injected	Amount injected per kilogram	Average percentage of change (± SE) in TRF of Na		
		Femoral vein	Renal artery	Carotid artery
OT	1 μg	+ 18 ± 2	+ 66 ± 7	+ 347 ± 38
4-leucyl-8-isoleucyl-OT	3 μg	10 ± 2	74 ± 6	487 ± 41
4-prolyl-8-isoleucyl-OT	8 μg	0	53 ± 5	241 ± 26
Control deprot. canine plasma	0.3 ml	0	0	0
P-2 fractions 44-48, control canine plasma	0.3 ml	0	16 ± 4	0
Experimental deprot. canine plasma	0.3 ml	13 ± 3	76 ± 7	0
P-2 fractions 44-48, exp. canine plasma	0.3 ml	48 ± 6	280 ± 23	0

Table 2. Assay of deproteinized arterial cat plasma before and 10 minutes after intracarotid injection of 4-Leu-8-Ileu-OT (3 μg/kg) and before and after incubation with chymotrypsin.

Substance injected	Amount injected	Average percentage of change (± SE) in TRF of Na		
		Femoral vein	Renal artery	Carotid artery
Control deproteinized plasma	0.3 ml/kg	No test	+ 10 ± 3	0
Control plasma after chymotrypsin	Same original volume	No test	12 ± 4	0
Experimental deproteinized plasma	0.3 ml/kg	No test	462 ± 37	0
Experimental plasma after chymotrypsin	Same original volume	No test	18 ± 4	0

was collected 10 minutes later (30 ml from cats, 150 ml from dogs). There were ten animals in each series. This blood was immediately deproteinized in the cold as above, and natriuretic activity was assayed on cats before and after the sample was incubated with chymotrypsin [for assay details, anesthesia, and surgical preparation of the animals, see (*2*)]. Included in the same assay were samples of canine plasma, before and after fractionation, from saline-loading experiments.

All the synthetic peptides were most saluretically active after carotid artery injection (Table 1). All the natriuretically active plasma samples—whether or not they were fractionated and without regard to the provoking stimulus for the activity (infusion or intracarotid peptide injection)—increased Na rejection in the assay animals after injection into the renal arteries but not after injection into the carotid artery. Incubation with chymotrypsin destroyed the natriuretic activity which followed upon intracarotid peptide injection.

These results indicate that, like activity induced by carotid-artery occlusion in cats, activities produced by dextran infusion in cows and by saline infusion in dogs are also represented by a polypeptide of small molecular weight. From the sharpness of the separation, "natriuretic hormone" in this case appears to be a single molecular species, and the separation and comparison with C^{14}-vasopressin behavior indicate that the active moieties in dogs and cats are similar in molecular size to vasopressin. This comparison is based on the assumption of a structural similarity between the two polypeptides, as suggested by the proposed site of origin in the diencephalon (*7*), the ability of synthetic oxytocin antagonists to inhibit reversibly the saluretic component of the response to carotid-artery occlusion (*8*), and our results. The isolation procedures possibly result in splitting of an originally larger molecule, with fragments of the latter retaining natriuretic activity.

Explanation of how peptide hormones, such as oxytocin, increase sodium transport in isolated systems such as the frog skin, and yet at the same time produce natriuresis—rather than the reverse from increased tubular transport—in intact animals is difficult. Our data suggest that at least part of the saluresis may result from a secondary, rather than primary, hormone-kidney interaction. The three peptides we used caused the appearance of significant natriuretic activity in arterial blood after injection into a carotid artery. Since the latter activity disappeared after incubation with chymotrypsin, it was also due to a peptide of small molecular weight. It might be objected that a natriuretic peptide was injected into the brain and collected again from arteries, and that we are assaying what we injected. Quantitative estimates eliminate this possibility, however. A standard dose of provoking peptide was six times as active when given into a carotid artery as when given by any other route—after dilution in that volume of blood contained between the carotid and renal arteries. Ten minutes after intracarotid injection, 1 ml of deproteinized plasma was significantly more natriuretic in the cat assay than the total original dose of the provoking peptide when both samples were given into the renal artery circulation. Moreover, experiments with the isolated, perfused dog kidney have failed to show any natriuresis whatsoever after administration of even very large doses of the three synthetic peptides used in our work (Nizet and Cort, in preparation). In other words, it would appear that oxytocin and the two analogs cause liberation of another peptide of small molecular weight from some intracranial structure, presumably nervous, and that the latter directly increases tubular Na rejection.

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