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Partial Chemical Characterization of a Natriuretic Substance in Rat Atrial Heart Tissue (41465)

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Abstract. Extracts of atrial and ventricular rat heart tissue were prepared in phosphate-buffered saline and boiled (PBS-boiled) or in acetic acid and fractionated using Sephadex G-75. The extracts were injected intravenously into anesthetized nondiuretic (i.e., not hydrated) rats to assay for natriuretic activity. PBS-boiled atrial extract caused urinary sodium excretion ($U_{Na}V$) to increase significantly ($P < 0.05$) by 672 ± 307% (±SE) during the 10 min after injection; whereas no significant change in $U_{Na}V$ occurred after injection of PBS alone or ventricular extract. U_KV and urine volume increased significantly by 400 ± 236% and 499 ± 105%, respectively, after injection of PBS-boiled atrial extract. Pooled active fractions from Sephadex G-75 of acid atrial extract (lyophilized and reconstituted in PBS) caused $U_{Na}V$ and urine volume to increase significantly by 1015 ± 401% and 807 ± 291%, respectively, and a smaller but significant increase in U_KV of 158 ± 23%. The natriuretic activity of acid atrial extract eluted in the molecular weight range of 3600 to 44,000 daltons, was unaffected by heating in a boiling water bath, treatment with concanavalin A, incubation with plasma, or removing the head of the assay rat, but was abolished by treatment with trypsin. These properties do not generally resemble those of the natriuretic factors in blood and urine previously reported by others.

Since the studies of De Wardener *et al.* (1) suggesting the existence of an unidentified natriuretic hormone, several laboratories have reported on the existence of natriuretic factors in plasma, urine, and renal tissue (2, 3, reviews). Although most of the evidence suggests that the natriuretic factors are small (less than 1000 daltons), heat-stable molecules, it is unclear whether the different laboratories are working with the same substance since they do not all agree on its exact chemical nature. Furthermore, the physiological importance of these natriuretic factors have not been clearly determined, nor has a site of origin been identified. Recently, de Bold *et al.* (4) reported on the presence of a potent fast-acting natriuretic factor in tissue extracts of heart atria in rats. These investigators found that injection of atrial, but not ventricular, extracts into anesthetized rats caused renal sodium chloride excretion to increase 30-fold within a few minutes. Whether the fast-acting natriuretic factor present in atrial tissue is chemically similar to the other natriuretic factors present in body fluids and renal tissue is not known, nor is its physiological relevance known. More information is needed about the physical and chemical nature of the atrial natriuretic factor in order to provide a basis for: (i) comparing it to other natriuretic factors presently studied in other laboratories; and (ii) determining if it is present in blood and urine. Also, it would be of interest to learn more about the chemical structure of this powerful rapidly acting natriuretic substance because its structure might serve as a model for developing new specific natriuretic drugs. The purpose of this study was to partially characterize the chemical and physical properties of the atrial natriuretic factor in order to help provide such a basis for further studies.

Materials and Methods. *Preparation of extracts.* Male Wistar and Sprague–Dawley rats (150–500 g) were used to prepare extracts from atrial and ventricular tissue. Fresh hearts were obtained from animals killed by decapitation preceded by a blow to the head or ether anesthesia. The hearts

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were placed in cold saline (0.9% NaCl) before dissection. Both atria with portions of the inferior and superior vena cava were used for the atrial extracts and a small portion of the apex of the ventricles was used for ventricular extracts. Extracts were also prepared from atria removed from rats that had been used as bioassays (see below). The atria from the bioassay rats were stored at −90° for 2−6 weeks before they were used. The tissue was blotted to remove excess fluid, weighed, and placed in the appropriate solution for homogenization (see below). Extracts were prepared according to two methods using initially 10 ml of solution per gram of tissue (wet weight).

PBS boiled. The tissue was homogenized with a Brinkman Polytron at a setting of 7 (3 × 10 sec) in Dulbecco's phosphate-buffered saline of pH 7.3 (PBS), placed in boiling water for 10 min and centrifuged at 12,000*g* for 10 min. The supernatant was assayed on the same day that the extract was prepared or was lyophilized, stored at −20°, and reconstituted with distilled water before assay.

Acid, partially purified. The tissue was homogenized with a Polytron (3 × 3 sec) in 10 vol of 1.0 *M* acetic acid and centrifuged as above. The pellet was reextracted in one-seventh the original volume of acetic acid, and the combined supernatants were lyophilized and stored at −20°. Lyophilizates of the acid extract having an original volume of 6−8 ml were reconstituted in 1.5−2.0 ml of 0.5 *M* acetic acid, homogenized, and centrifuged at 12,000*g* for 10 min. The supernatant was placed on a column (1.5 × 85 cm) of Sephadex G-75 equilibrated in 0.5 *M* acetic acid. The eluate was monitored at 280 nm and 6-ml fractions were collected at 4° at a flow rate of 0.6 ml/min. The fractions were lyophilized, stored at −20°, and dissolved in PBS for assay of natriuretic activity as described below. Natriuretic activity was found mostly in fractions 11−18, which represented the elution volume between 57 and 105 ml (correcting for a 3-ml dead space in the collection line). For subsequent experiments requiring partially purified acid extract, fractions 11−18 were pooled, lyophilized, and dissolved in PBS for treatment and/or bioassay.

The column of Sephadex was calibrated with the following proteins: ovalbumin (43,000 daltons), chymotrypsinogen A (25,000 daltons), and ribonuclease A (13,700 daltons). As reported by others (5) the calibration data from the marker proteins were analyzed by linear regression using the method of least squares.

Bioassay. Male Wistar and Sprague−Dawley rats maintained on laboratory chow (containing 0.4% sodium and 1% potassium) and water *ad lib* were used to assay for natriuretic activity. The rats were anesthetized with ether and catheters were placed in the trachea and in the left femoral vein and artery. Silastic tubing (1-mm i.d., 2-mm o.d., 15 mm in length) with windows cut through the wall at one end was inserted into the urinary bladder (windows inside bladder) through an abdominal incision and secured with suture. The rat was allowed to stabilize for 30 min after surgery before collecting urine for control measurements. Mean arterial pressure was monitored continuously with a Statham transducer and a Grass polygraph. Control urine output was determined during three 10-min periods. Rats that had a control urine output less than 0.01 or greater than 0.1 ml/10 min were discarded. To collect urine, a catheter constructed of polyethylene (PE) 90 tubing (Intramedic), 7 mm in length connected to PE 190 tubing, 40 mm in length, was inserted into the 4-mm portion of the silastic tubing protruding from the bladder. The PE 90-190 tubing together with a small plastic vial were weighed on an analytical balance before and after collecting urine. New PE 90-190 tubing and vials were used for each 10-min collection period. Any urine remaining in the tubing after the second weighing was flushed into the vial by forcing air through it with a syringe.

After determining control urine output by weight, the test substance (extract, PBS or saline) was injected intravenously (4 ml/kg) over a 3-min period. Urine was collected for two or three 10-min periods after the start of injection. The urine samples were stored at −20° until analyzed for sodium

and potassium concentration by flame photometry. All samples were diluted with 0.5 ml of distilled water before analysis. Natriuretic activity was taken as the percentage change in sodium excretion (from the average 10-min control level) during the first 10-min period after injection ($\%\Delta U_{Na}V$). Protein concentration of the reconstituted fractions of partially purified acid extract was determined by the method of Lowry *et al.* (6).

Treatments. To test for the presence of inhibitors or activators of atrial natriuretic activity in plasma, PBS-boiled atrial extract was incubated with fresh heparinized plasma from donor rats. For each assay 1 ml of extract was incubated with 1 ml of rat plasma for 1 hr at 37°, and 8 ml/kg of this mixture was injected into the assay rats. For control, 4 ml/kg of PBS-boiled atrial extract kept at 37° for 1 hr was injected into assay rats and then immediately 4 ml/kg of plasma was injected so that the control rats received the same volume of extract and plasma as the test rats, but without the extract and plasma coming into contact before injection.

To determine if the atrial natriuretic factor was active without the presence of the central nervous system in the assay rats, PBS-boiled atrial extract was injected into headless rats maintained at the level of arterial blood pressure similar to that of intact assay rats by infusing epinephrine (0.5 μg/kg/min) and vasopressin (10 ng/kg per min). Vice-lock pliers and two steel bars were used to crush and clamp tissue in the cervical area similar to that described by Cowley and Guyton in dogs (7), so that the head could be removed without blood loss. Saline (4 ml/kg) was injected into five headless rats to determine the effects of volume alone.

To determine whether or not the activity of the atrial natriuretic factor was susceptible to proteolysis, partially purified acid extracts were treated with a suspension of insoluble bovine pancreatic trypsin–agarose (Sigma). One milliliter of the suspension yielded approximately 0.5 ml of packed gel and there were 86 units/ml packed gel. One unit hydrolyzed 1.0 μmol of BAEE per minute at pH 8.0 at 30°. One milliliter of extract was mixed and incubated with 0.1 ml of a suspension of PBS-washed insoluble trypsin–agarose (43 units/ml of suspension) for 1 hr at 37°. The extract and trypsin–agarose were centrifuged causing the trypsin–agarose to form a pellet at the bottom of the tube. The supernatant (trypsin-free extract) was injected into the assay rats.

To determine whether or not the atrial natriuretic factor contains a carbohydrate moity, partially purified acid extract reconstituted in PBS was treated with concanavalin A–Sepharose 4B (Sigma) since this substance is known to form insoluble complexes with polysaccharides and glycoproteins (8, 9). One milliliter of extract was mixed and incubated with 0.1 ml of a suspension of PBS-washed insoluble concanavalin A (8 mg/ml) for 1 hr at 37°. The insoluble concanavalin A was removed from the extract by centrifugation before the extract was injected into the assay rats.

To determine whether or not the natriuretic activity of partially purified acid atrial extract was resistant to heat, 1 ml from five different batches of this type of extract was placed in boiling water for 10 min, cooled in ice, and then assayed.

To determine the effect of pH adjustment, acid extracts were prepared from Sprague–Dawley rats as described above using 1.0 *M* acetic acid. Ammonium hydroxide (14.8 *M*) was added to the supernatants of the homogenates in order to adjust the pH to 8.2–8.6. This process caused the formation of a precipitate which was removed by centrifugation. The supernatants of the pH-adjusted (alkaline) extracts were lyophilized, reconstituted in 0.5 *M* acetic acid, and fractionated using Sephadex G-75 equilibrated with 0.5 *M* acetic acid as described above.

All injections, unless stated otherwise, were 4 ml in volume per kilogram body weight of the assay rat and were infused intravenously over a 1- to 3-min period. Lyophilizates originally containing PBS, the salts of which were not removed by lyophilization, were reconstituted with distilled water. Lyophilizates originally con-

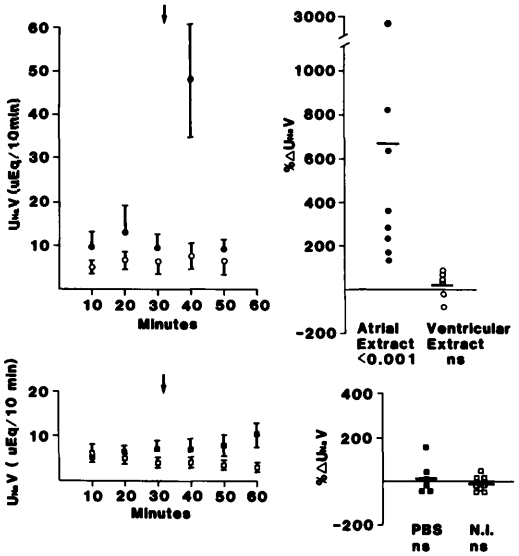

FIG. 1. The graphs on the left show the urinary sodium excretion ($U_{Na}V$) of anesthetized rats during sequential 10-min periods. Each point in the left-hand graphs represents the mean ±SE of six to eight observations. The arrows indicate the time of injection of test solutions (4 ml/kg). The $U_{Na}V$ for each rat was averaged for the first three 10-min periods and the percentage change from this average during the next 10-min period (an indication of the natriuretic activity of the test solutions) is shown in the graphs at the right. Each point in the right-hand graphs represents a single observation, and the horizontal lines represent the averages for the groups. The level of significance in each group for the difference between the average of the first three 10-min $U_{Na}V$ and the fourth 10-min $U_{Na}V$ are given in the right-hand graphs (ns, not significant). ●, Wistar rats receiving PBS-boiled atrial extract; body weight 226 ± 17 g. ○, Wistar rats receiving PBS-boiled ventricular extract; body weight 216 ± 16 g. ■, Wistar rats receiving phosphate-buffered saline (PBS); body weight 209 ± 5 g. □, Sprague–Dawley rats receiving no injection (NI); body weight 255 ± 6 g.

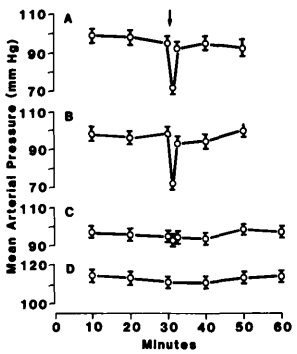

FIG. 2. Mean arterial pressure in rat assays shown in Fig. 1. The arrow indicates the time of injection. Assay rats received PBS-boiled atrial extract (A), PBS-boiled ventricular extract (B), PBS alone (C), or no injection (D). Means ± SE are indicated.

taining acetic acid, which was completely removed by lyophilization, were reconstituted with PBS. Extracts prepared from Wistar rats were assayed in Wistar rats and extracts prepared from Sprague–Dawley rats were assayed in Sprague–Dawley rats.

Statistics. Student's *t* test with paired analysis was used to determine the significance of difference between the average 10-min sodium excretion during the control period and the sodium excretion during the 10 min after injection of the test substance. Where appropriate, unpaired analysis was used for analyzing the difference in percentage $\Delta U_{Na}V$ between groups. $P < 0.05$ was considered significant. Values are expressed as means ±SE.

Results. PBS-boiled atrial extract caused $U_{Na}V$ to increase significantly by 672 ± 307% during the first 10 min after injection into the assay rats, whereas no significant change in $U_{Na}V$ occurred after injection of PBS alone or ventricular extract (Fig. 1). The changes that occurred were essentially complete by the end of the second 10-min period after injection. U_KV increased significantly by 400 ± 236% and urine volume increased significantly by 499 ± 105% after injection of PBS-boiled atrial extract, while neither changed significantly after PBS alone or ventricular extract was injected. In the group of rats not receiving an injection, no significant change occurred in $U_{Na}V$ (Fig. 1), U_KV or urine volume during the 10-min period following control. Mean arterial pressure decreased transiently after injection of PBS-boiled atrial and ventricular extracts, but changed little after injection of PBS alone or in rats not receiving an injection (Fig. 2).

In the assay rats receiving PBS-boiled atrial extract pretreated with plasma, $U_{Na}V$ increased by 765 ± 226%, which was not significantly different from the 748 ± 188%

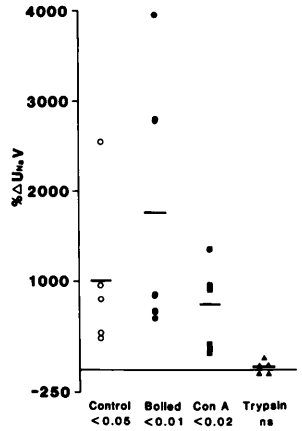

FIG. 3. Natriuretic activity ($\%\Delta U_{Na}V$) of partially purified acid atrial extracts in Sprague–Dawley rats. Each point represents a single observation and the horizontal lines represent the averages of the groups. Five different batches of extract were divided into four 1-ml aliquots, which were subjected to one of the following treatments before assaying: control (no treatment); boiled (placed in a boiling water bath for 10 min); con A (treated with insoluble concanavalin A); trypsin (treated with insoluble trypsin). The *P* value in each group for the difference between the average of the first three 10-min $U_{Na}V$ and the 10-min $U_{Na}V$ after injection are given (ns, not significant). Body weights of the rat assays were 191 ± 7, 188 ± 8, 183 ± 12, and 188 ± 10 g for the control, boiled, Con A, and trypsin groups, respectively.

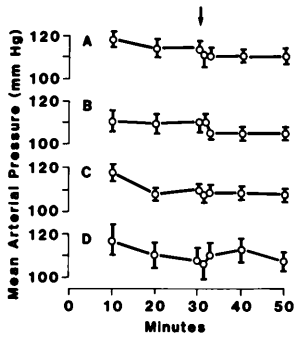

FIG. 4. Mean arterial pressure in rat assays shown in Fig. 2. The arrow indicates time of injection. Assay rats received partially purified acid atrial extract untreated (A) or treated by boiling water (B), concanavalin (C), and trypsin (D). Means ± SE are indicated.

increase in the rats receiving plasma and extract that was not incubated together before injection. In the headless rats, PBS-boiled atrial extract caused $U_{Na}V$ to increase by 998 ± 352%, which was significantly different from the −4 ± 15% change in the headless rats receiving saline.

Untreated partially purified atrial extract caused $U_{Na}V$ to increase significantly by 1015 ± 401% during the first 10 min after injection (control, Fig. 3) and U_KV and urine volume to increase significantly by 158 ± 23% and 807 ± 291%, respectively. Partially purified acid atrial extract boiled for 10 min or treated with concanavalin A caused $U_{Na}V$ to increase significantly by 1765 ± 679% and 734 ± 484%, respectively, but after treatment with trypsin it caused $U_{Na}V$ to change insignificantly by 52 ± 33% (Fig. 3).

Partially purified atrial extract, whether untreated, boiled, treated with concanavalin A, or treated with trypsin had only a small effect on mean arterial pressure (Fig. 4).

Calibration data for the column of Sephadex G-75 fit the equation log MW = 5.9−0.9 (V_e/V_0), where MW, V_e, and V_0 are molecular weight, elution volume, and void volume, respectively. The greatest natriuretic activity of the atrial extracts that remained acid eluted at 1.5−1.4 V_e/V_0 (36,000−44,000 daltons), with moderate natriuretic activity trailing in the smaller MW range (Fig. 5). The greatest natriuretic activity of atrial extracts that were adjusted to an alkaline pH eluted at 2.6−2.4 V_e/V_0 (3600−5500 daltons) with less natriuretic activity spreading in the larger MW range (Fig. 6). In two experiments the precipitate of the pH-adjusted extracts were reextracted in 1.0 *M* acetic acid and concentrated hydrochloric acid such that the pH was less than 3.0. They were lyophilized and tested for natriuretic activity. In both cases $U_{Na}V$ increased by greater than 2000% in the rat assays.

Discussion. The results of this study confirm the findings of de Bold *et al.* (4) showing that there resides in atrial, but not in ventricular, rat heart tissue a substance that causes marked, short-lived natriuresis in anesthetized rats. As demonstrated by de Bold (10), this tissue specificity is due to the presence of specific atrial granules, found in abundance in atrial but not in ventricular mammalian cardiocytes.

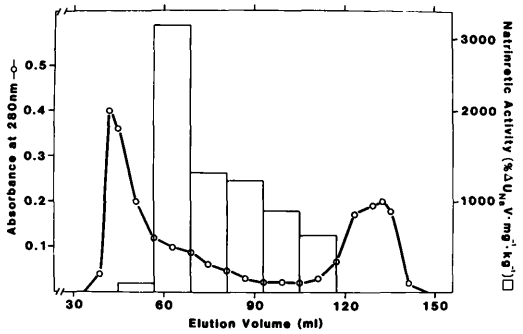

FIG. 5. The average gel filtration (Sephadex G-75) results from four batches of acid atrial extract (Sprague–Dawley) with no pH adjustment. Natriuretic activity is expressed as $\%\Delta U_{Na}V$ per mg protein injected per kg weight of the assay rat. No natriuretic activity was detected at elution volumes less than 45 ml or greater than 117 ml.

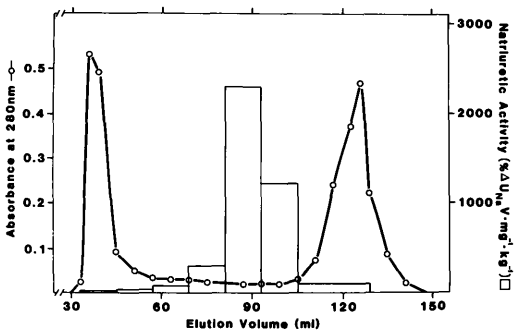

FIG. 6. The average gel filtration (Sephadex G-75) results from three batches of acid atrial extract (Sprague–Dawley) with pH adjusted to alkalinity before processing for chromatography.

In a preliminary study using an acetic acid extract of rat atrial tissue and adjusting the pH to 7.6 before gel filtration in Sephadex G-75, de Bold found that natriuretic activity eluted mostly in the fractionation range below 6000 daltons (11). In the present study using the same acid extraction and gel filtration, but without adjusting the pH of the atrial extracts we found the greatest natriuretic activity in the 36,000- to 44,000-dalton range with moderate amounts of activity trailing in the lower molecular weight range (Fig. 5). To determine if these disparate results were related to the difference in pH adjustment acid atrial extracts were prepared as before, but in addition the pH of the extracts was adjusted to 8.2–8.6 before processing for gel filtration. This pH range was chosen because it was found that the titration curve was very steep at pH 7.0–8.0, causing pH adjustment in that range to be difficult. The greatest natriuretic activity of the extracts adjusted to an alkaline pH was found in the 3600- to 5500-dalton range with much less activity spreading in the larger molecular weight range (Fig. 6). These findings were similar to those of de Bold (11). Furthermore natriuretic activity was also recovered from the precipitate of the pH-adjusted extracts.

At least three possible interpretations can be applied to these findings. (a) The atrial natriuretic factor has a molecular weight between 36,000 and 44,000 daltons and the smaller active particles found in the pH-adjusted extracts represent active split products. (b) The molecular weight of the atrial natriuretic factor is between 3600 and 5500 daltons and the larger active particles represent aggregates of this smaller molecule or the smaller active molecule bound to a larger inactive molecule. (c) There are two or more active molecules with molecular weights ranging between 3600 and 44,000 daltons. Thus, the process of adjusting the pH of the acid extracts to alkalinity might lead to a greater precipitation of the larger active particles and/or cause disaggregation of smaller active particles. Finally, it must be stated that a formal determination of the molecular weight of the atrial natriuretic factor(s) can be conducted only after greater purification has been achieved.

The natriuretic activity of the atrial extracts was abolished by treatment with trypsin but was resistant to heat or treatment with concanavalin A. These results suggest, but do not establish, that the active molecule contains peptide bonds and is free of carbohydrates. Incubation of the PBS-boiled atrial extract with plasma before injection into the rat assay, did not alter its natriuretic activity, suggesting that the rapid abatement of its activity may be due to a metabolic process involving more than circulating factors. These results also suggest that plasma does not contain elements that potentiate the activity of the

atrial natriuretic factor. The observation that atrial extract increased natriuresis in headless rats suggests that its action does not require the presence of the central nervous system or the release of any factors from the brain.

Thus, the atrial natriuretic factor was found to be extractable in acid, heat stable, of molecular weight between 3600 and 44,000 daltons, destroyed by trypsin, and uninfluenced by treatment with insoluble concanavalin, plasma incubation, or the presence of the central nervous system in the rat assay. These properties do not generally resemble those of the natriuretic factors presently studied in several other laboratories, since most of those substances appear to be of molecular weight less than 1000 daltons, and/or have a time of onset and duration of action greater than that of the atrial natriuretic factor examined in the present study (1, 2, 12, 13).

The physiological relevance (if any) of the atrial natriuretic factor has not been determined, although a recent study by Sonnenberg *et al.* (14) showed that inhibiting proximal tubular organic acid secretion by probenecid attenuated the natriuretic response in rats to both atrial natriuretic factor and acute volume expansion. These data provide circumstantial evidence that the atrial natriuretic factor plays a role in the natriuretic response to acute volume expansion and also suggests that it may act through a mechanism similar to that of furosemide, which is also blocked by probenecid.

It is not known whether the atrial natriuretic factor is released into the blood stream, which is requisite for it to have any direct physiologic effects on the kidney. Although this study gives no evidence in this regard, it provides information about the chemical and physical properties of the atrial natriuretic factor which can be used in future studies to determine whether or not this substance is present in blood and urine.

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