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Immunocytochemical localization of atrial natriuretic factor in the heart and salivary glands

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Summary. Antibodies produced in the mouse by repeated intraperitoneal injections of partly purified atrial natriuretic factor (low molecular weight peptide (LMWP) and high molecular weight peptide (HMWP)) have been used to localize these factors by immunohistochemistry (immunofluorescence and immunoperoxidase method) and by immunocytochemistry (protein A-gold technique) in the heart of rats and of a variety of animal species including man and in the rat salivary glands. Immunofluorescence and the immunoperoxidase method gave identical results: in the rat, atrial cardiocytes gave a positive reaction at both nuclear poles while ventricular cardiocytes were consistently negative. The cardiocytes of the right atrial appendage were more intensely reactive than those localized in the left appendage. A decreasing gradient of intensity was observed from the subpericardial to the subendocardial cardiocytes. The cardiocytes of the interatrial septum were only lightly granulated. Sodium deficiency and thirst (deprivation of drinking water for 5 days) produced, as already shown at the ultrastructural level, a marked increase in the reactivity of all cardiocytes from both atria with the same gradient of intensity as in control animals. Cross-reactivity of intragranular peptides with the rat antibodies allowed visualization of specific granules in a variety of animal species (mouse, guinea pig, rabbit, rat, dog) and in human atrial appendages. No reaction could be elicited in the frog atrium and ventricle although, in this species, specific granules have been shown to be present by electron microscopy in all cardiac chambers. With the protein A-gold technique, at the ultrastructural level, single labeling (use of one antibody on one face of a fine section) or double labeling (use of two antibodies on the two faces of a fine section) showed that the two peptides are localized simultaneously in all three types (A, B and D) of specific granules. In the rat salivary glands, immunofluorescence and the immunoperoxidase method showed reactivity exclusively in the acinar cells. The reaction was most intense in the acinar cells of the parotid gland. In the sublingual gland, only the serous cells, sometimes forming abortive "demi-lunes", were reactive. In the submaxillary gland, the reaction was weaker and distributed seemingly haphazardly in the gland. The most constantly reactive cells were localized near the capsule while many cells did not contain visible reaction product.

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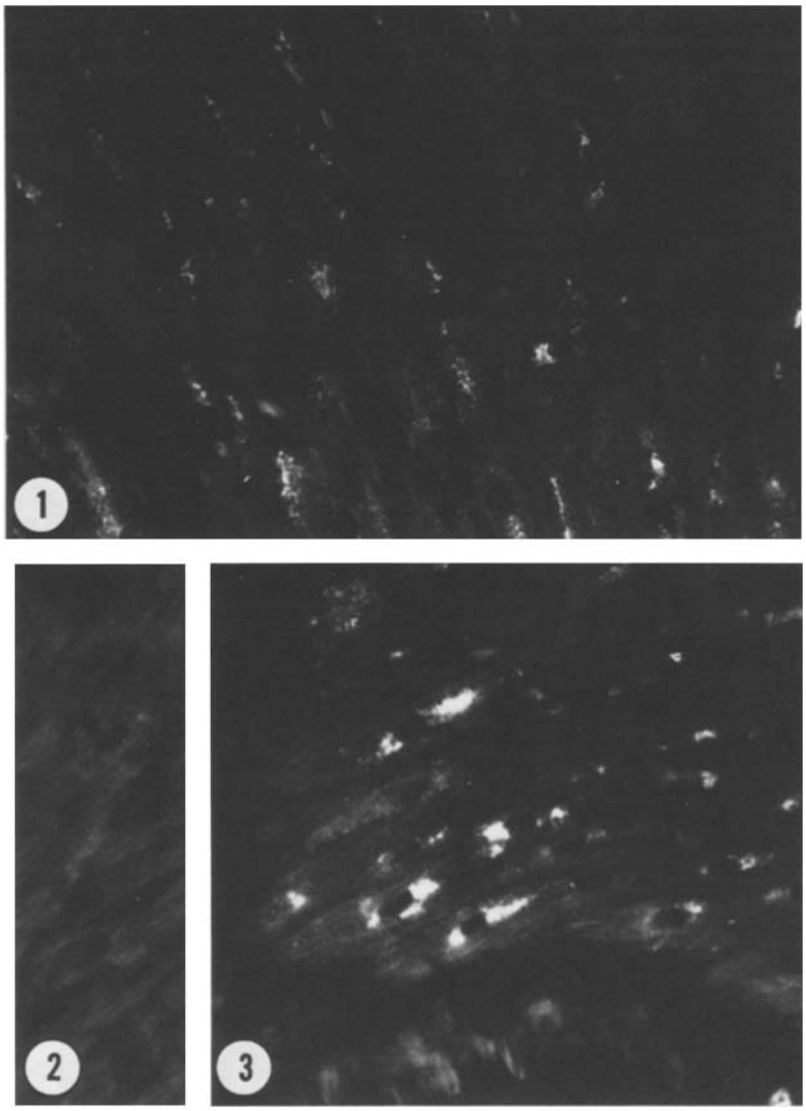
Introduction

The presence of a particular type of granule population in atrial cardiocytes has been discovered by Kisch (1956). This has been confirmed by various investigators during the following years (Poche 1957; Bompiani et al. 1959). Palade (1961) and Jamieson and Palade (1964) described these secretory-like granules in a number of mammalian species and in man and postulated that they were formed in the large Golgi complex characteristic of atrial cardiocytes. These specific granules, found exclusively in the atria of mammals are equally present in the ventricular cardiocytes of a large number of non-mammalian vertebrates such as cyclostomes, amphibians, osteichthyans, reptiles and birds (Bencosme and Berger 1971; Yunge et al. 1980). Based on their morphology, three types of specific granules (A, B and D) are present in mammalian atria and in non-mammalian atrial and ventricular cardiocytes (Bencosme and Berger 1971; Yunge et al. 1980; Cantin et al. 1979). We have already shown that these specific granules are made up of proteins and may be endowed with glycoproteins in rat (Huet and Cantin 1974a; Huet and Cantin 1974b), man (Huet et al. 1974; Cantin et al. 1975) and a variety of animal species (Cantin et al. 1979). We also showed by ultrastructural radioautography that these granules incorporate both ^{3}H-leucine (Yunge et al. 1980) and ^{3}H-fucose (Yunge et al. 1979). The pattern of incorporation, in both cases, was similar to that occurring in the secretory granules of various endocrine cells. In man (Cantin et al. 1975) and in various animal species (Cantin et al. 1979), crinophagy (digestion of secretory granules) occurs in atrial cardiocytes as it does in the prolactin cells of the anterior hypophysis (Farquhar 1971). Following the finding that the number of atrial specific granules seemed to be a function of sodium load and blood volume (Marie et al. 1976; Cantin et al. 1982), it was shown that crude extracts of rat atria had a powerful natriuretic and diuretic effect in the bioassayed rat (Sonnenberg et al. 1980). We have established that these effects are mediated by the specific granules themselves (Garcia et al. 1982).



Figs. 1–3 Illustrate fluorescence microscopy of cardiocytes treated according to Coons' technique

Fig. 1. Section of rat atrium immunostained with low molecular weight peptide (LMWP). Specific granules appear as bright dots near nuclei. (×160)

Fig. 2. Section of rat right ventricular cardiocyte immunostained as in Fig. 1. Note absence of granularity. (×160)

Fig. 3. Section of rat left atrium immunostained with high molecular weight peptide (HMWP). Note bright granular fluorescence near nuclei. (×220)

Table 1. Action of ascites fluid on the diuretic and natriuretic effect of ANF[a] (bioassay)

Treatment	Diuresis[b] [%]	Natriuresis [%]
1) –	564.6 ± 143.8 ($N=10$)	3,628.0 ± 1,464.9 ($N=11$)
2) Ascites (low molecular weight ANF)	93.3 ± 32.2[d] ($N=22$)	269.9 ± 102.2[d] ($N=23$)
3) Ascites (high molecular weight ANF)	49.3 ± 28.7[d] ($N=20$)	192.4 ± 43.0[d] ($N=21$)
4) Unrelated ascites[c]	700.1 ± 411.8 ($N=10$)	1,450.1 ± 1,149.0 ($N=10$)

[a] All rats received ANF (100 µl of the acid extract of pooled atria) either alone (Gr 1) or combined with ascites fluid (Grs 2 to 4)

[b] The diuresis (µl of urine/20 min) and natriuresis (µEq Na^+/20 min) are expressed as the percentage of changes observed during the 20 min period following the injection as compared to the 20 min period preceding the injection

[c] Ascites fluid was induced in mice exactly as for Grs 2 and 3, but with unrelated hybridomas producing monoclonal antibodies against tonin (Ikeda et al. 1981) or apolipoproteins (Milne et al. 1981)

[d] Significant ($p<0.001$) as compared with controls

The aim of the present study was to localize the atrial natriuretic factor in myocardial cardiocytes and in salivary glands by immunohisto- and immunocytochemical methods. Since the number of specific granules is known to increase in water-deprived (de Bold 1979) and in sodium-deficient rats (Cantin et al. 1982), experiments were also conducted to find out if the increase could be visualized by immunohistochemical techniques. Finally attempts were made to find out if the antibodies produced against the rat diuretic and natriuretic factor (ANF) reacted with the atrial specific granules of a number of animal species and of man.

Table 2. Effect of immunohistochemical method on the immunoreactivity of atrial specific granules and salivary glands

Immunohistochemical method	Fixative	Dilution of ascites fluid		Atrial specific granules		Salivary glands		
		LMWP[a] # 3	HMWP # 2	LMWP # 3	HMWP # 2	Parotid	Submandibular	Sublingual "Demi-lune" cells
						Acinar cells		
Immunofluorescence	Bouin	1/20		+++[b]	+++	+++	++	+++
		1/50		+++	+++	+++	++	+++
		1/100		++	++	++	+	++
Immunoperoxidase	Bouin	1/200		+	+	+	+	+
		1/400		+	+	+	0	+
		1/600		+	+	+	0	+
		1/800		+	+	+	0	+
		1/1000		0	0	0	0	0

[a] LMWP = low molecular weight peptide; HMWP = high molecular weight peptide.
[b] +++ = strong reaction; ++ = moderate reaction; + = weak reaction; 0 = no reaction

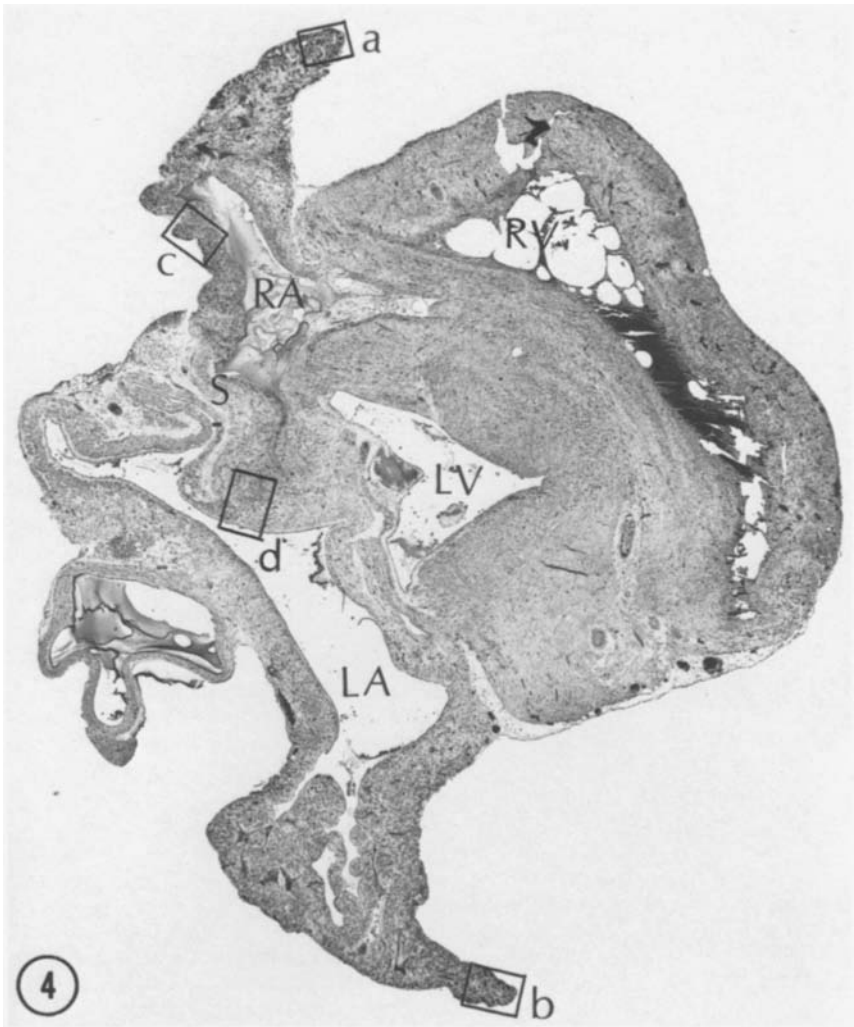


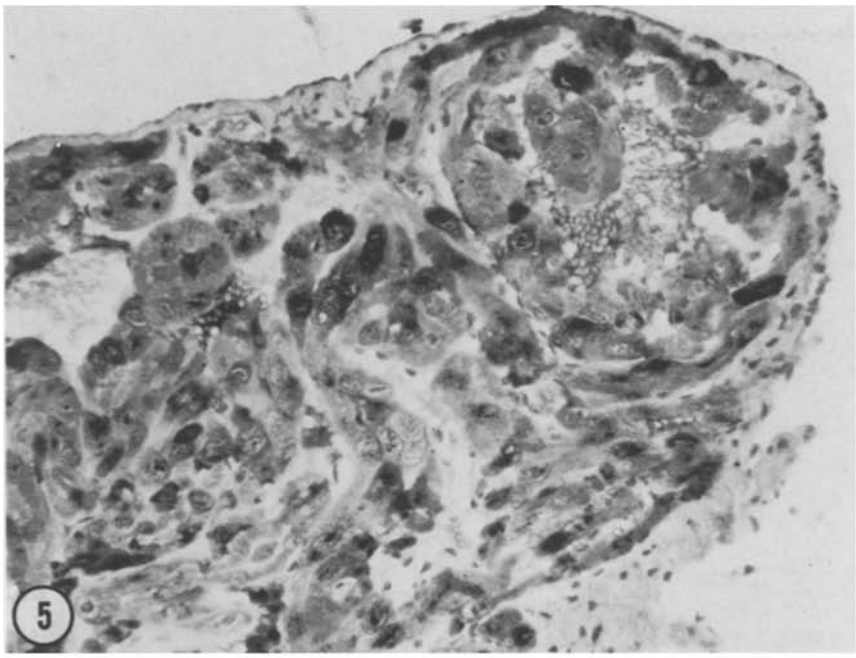
Fig. 4. Survey picture of rat heart cut coronotangentially to conserve atrio-ventricular relationships and immunostained according to the immunoperoxidase technique with low molecular weight peptide antibodies. Right atrium (*RA*); left atrium (*LA*); interatrial septum (*S*); right ventricular cavity (*RV*); left ventricular cavity (*LV*); hematoxylin counterstain (×14)

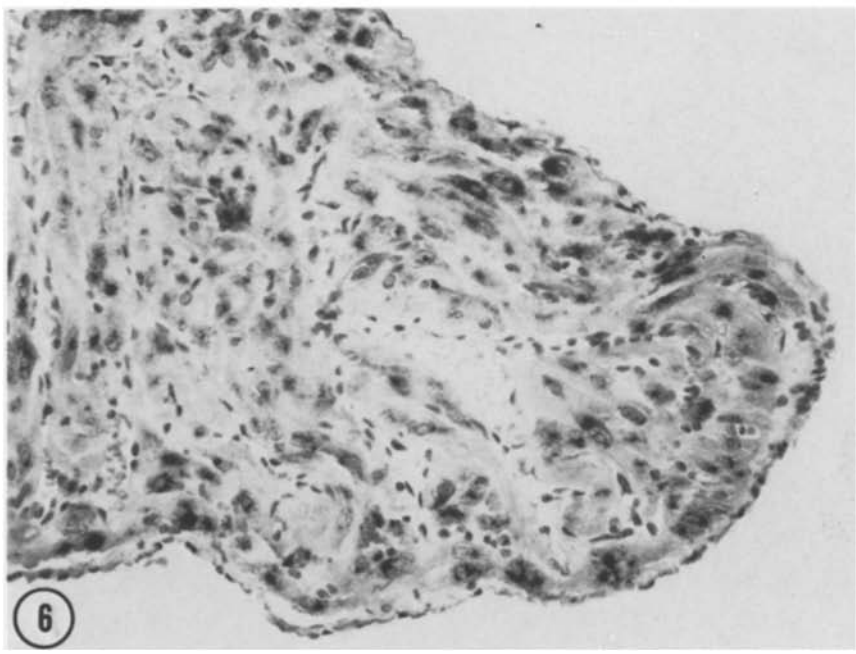
Materials and methods

Purification of ANF

Homogenization of atria in acetic acid and SEP-PAK cartridge extractions were carried out as previously reported (Thibault et al. 1983). The material obtained was then redissolved in 5 ml of 0.1 M acetic acid, deposited onto a 2.5 × 90 cm column (Bio-Gel P10) and eluted with the same acid. Two active regions were found: one (low molecular weight peptide (LMWP)) spreading from 3,000 to 6,000 and the other (high molecular weight peptide (HMWP)) from 6,000 to 15,000 daltons. Each region was pooled and treated separately.

Each pool was placed on a cationic exchanger, a CM Bio-Gel





Figs. 5–8 Consist of higher magnifications of areas (*a, b, c, d*) framed in Fig. 4

Fig. 5. Right atrial appendage (*a*). Most cardiocytes exhibit an intense reaction with the most granulated cells located near the pericardium. Hematoxylin counterstain. (× 160)

Fig. 6. Left atrial appendage (*b*). Most cardiocytes are markedly granulated but less so than in the right atrial appendage. Hematoxylin counterstain. (× 160)

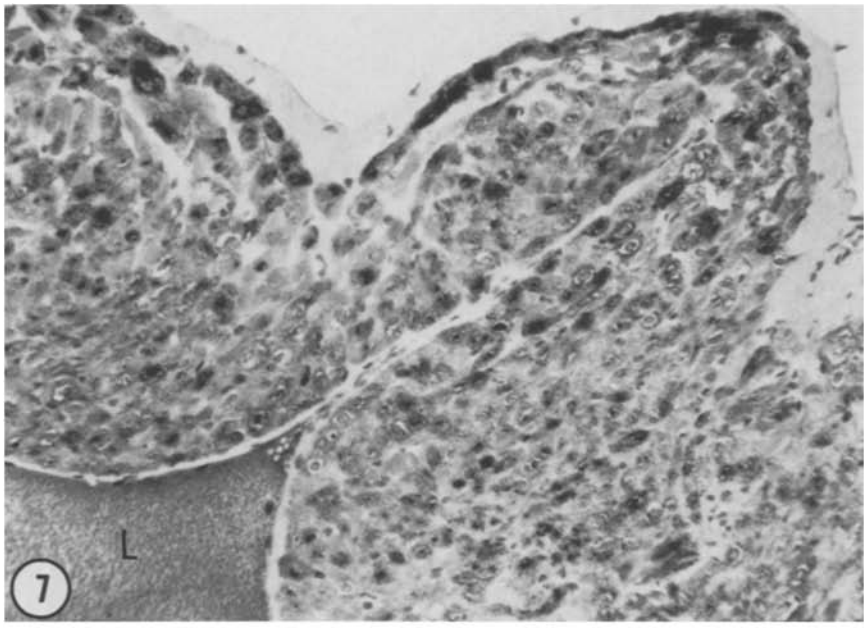
A column (1 × 20 cm) equilibrated with 0.01 M ammonium acetate (pH 5.0) and eluted with a linear gradient of 450 ml of 0.01 to 1.0 M ammonium acetate (pH 5.0). Both LMWP and HMWP behaved in exactly the same way and eluted between 0.1 and 0.25 M ammonium acetate.

Each pool was further purified on another cationic exchanger, a Mono S HRS/5 column coupled to a Varian 5060 liquid chromatograph. Elution was performed with a gradient of 0.02 to 1.0 M triethylamine acetate pH 6.5 at a flow rate of 1 ml with variable gradient slopes: 0.1 M/min between 0.02 to 0.4 M and 0.016 M/min for 0.4 to 1.0 M. LMWP eluted at 0.7 M and the HMWP at 0.5 M. When bioassayed as already reported (Thibault et al. 1983) LMWP was found to have a specific activity of 46,370 μEq/Na^+/20 min/mg protein and HMWP, a specific activity of 13,450 μEq/Na^+/20 min/mg protein.

Production of antibodies

BALB/C female mice, 12 to 15 week old, were injected intraperitoneally with 20 μg of either LMWP or HMWP in complete Freund's adjuvant (20 μl of the peptide with 180 μl of Freund's adjuvant), according to the method of Tung et al. (1976) as pioneered by Munoz (1957). They were injected on day 1, 14 and 21 of the experiment. Ascites fluid was first collected 5 weeks after the first injection and at weekly intervals thereafter. Eighteen samples of ascites fluid (either from LMWP- or HMWP-injected mice) were thus collected.

For control purposes, hybridomas producing monoclonal antibodies against tonin (Ikeda et al. 1981) or apolipoprotein (Milne et al. 1981) were implanted into the peritoneal cavity of BALB/C female mice of the same age and ascites fluid collected after appro-



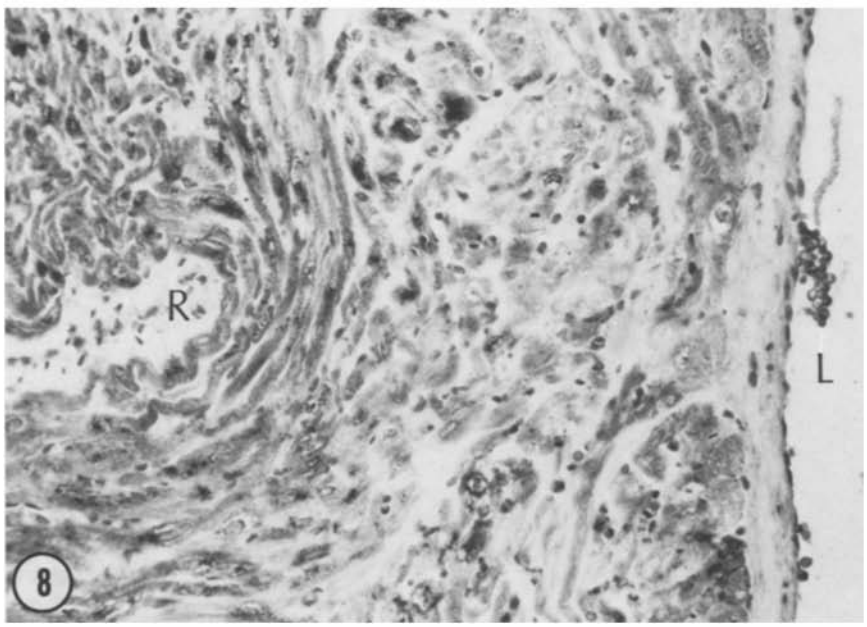


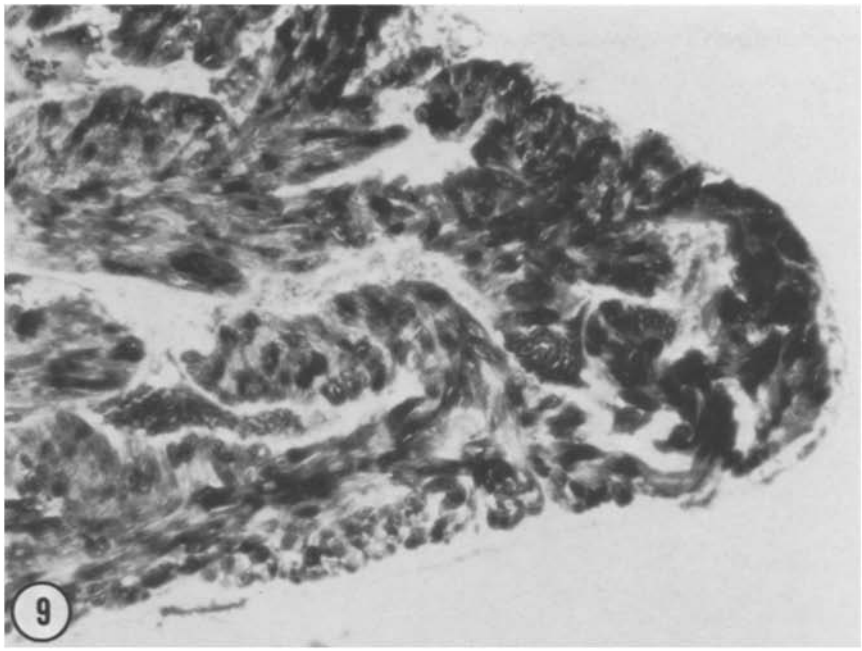
Fig. 7. Part of the right atrial free wall (*c*). *L*, lumen. The most intensely reactive cardiocytes are located near the pericardium. Hematoxylin counterstain. (× 160)

Fig. 8. Septum between right atrial (*R*) and left atrial lumina (*L*) (*d*). Several cardiocytes are lightly granulated while most are not. Hematoxylin counterstain. (× 250)

priate intervals (McKean 1980). In all cases sodium azide (0.02%) was added to ascites fluid immediately upon withdrawal from the peritonium. It was then centrifuged and the supernatant kept at −70° C.

Inhibition of the diuretic and natriuretic effects of ANF by antibodies

In order to assess if antibodies were produced against LMWP or HMWP, we used a modification of the standard bioassay of atrial natriuretic factor (ANF) (Garcia et al. 1982; Thibault et al. 1983). Atria from 20 female Sprague-Dawley rats (190–220 g) were homogenized in 1.0 M acetic acid (2 ml for atria from one rat) and centrifuged at 40,000 *g* for 20 min. The supernatants were lyophilized overnight and the powder redissolved in sodium phosphate buffer (pH 6.0) (500 µl for 2 atria). The precipitates were separated by centrifugation at 40,000 *g* for 10 min and the supernatants were pooled. Protein concentrations determined by the method of Bradford (1976) amounted to 0.15 mg/ml of the pool of supernatants. One hundred microliters of the pooled supernatant was mixed with 200 µl of ascites fluid and incubated at 4° C for 24 h with constant agitation. This was then centrifuged and the supernatants completed to 1.0 ml with Krebs fluid (pH 7.2) just before injection into the bioassay rats. Female Sprague-Dawley rats (200–225 g body weight) were used as bioassay animals. They were anesthetized with pentobarbital (60 mg/kg i.p.) and a bladder catheter and intrajugular catheter were installed. The animals received an infusion of 5% dextrose (3 ml/h) for 10 min before the assay and during the evaluation period. Urine was collected in preweighed vials for 20 min intervals. After a basal collection period, samples of ANF or of the ANF-antibody mixtures were injected to test their diuretic and natriuretic activity. Sodium concentration was measured in a flame photometer. Results are expressed as the percentage of change in diuresis and natriuresis between the 20 min basal period and the 20 min period immediately following the injection.



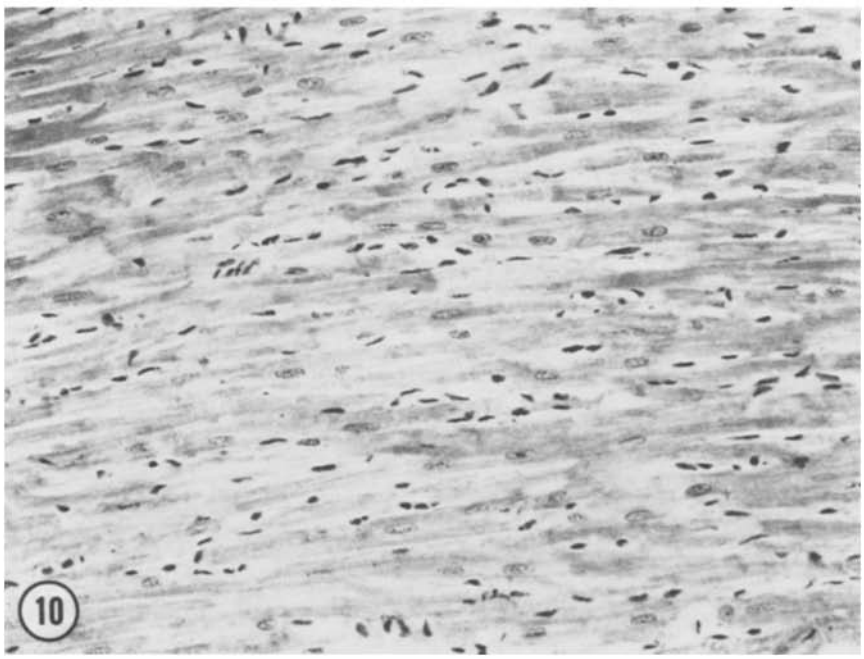


Fig. 9. Left atrial appendage of sodium-deficient rat treated as in Fig. 4. All cardiocytes exhibit an intense reaction particularly near the pericardium. (×160)

Fig. 10. Left ventricular myocardium of sodium-deficient rat treated as in Fig. 4. Note total absence of reactivity in all cardiocytes. Hematoxylin counterstain. (×250)

Effects of sodium deficiency and water deprivation

Sodium deficiency. Previous studies done at the ultrastructural level have shown that the number of specific granules increases in atria after a period of sodium deficiency (Marie et al. 1976; Cantin et al. 1982). In order to find out if this increase in granularity could be visualized by immunohistochemical methods, 15 female Sprague-Dawley rats (average body weight 100 g; range 90–110 g) were fed a sodium-deficient diet (ICN Biochemicals) for 30 days and allowed distilled water ad libitum. Fifteen control rats of the same sex, breed and body weight were fed Purina Laboratory chow and allowed tap water for an identical time period. Eight animals in each group were sacrified under ether anesthesia and their heart fixed for 24 h in Bouin fluid. The right atrium of the remaining animals in each group was perfused with 2% glutaraldehyde through the right ventricle and prepared for ultrastructural morphometry as already described (Cantin et al. 1979).

Water deprivation. One group of 15 Sprague-Dawley rats was completely deprived of water for 5 days with access to food, while a control group, also of 15 rats, was allowed free access to water and food. At the end of the experiments, the hearts of 8 rats in each group were treated as above. The remaining animals in each group were perfused through the right ventricle with 2% glutaraldehyde and the right atria prepared for ultrastructural morphometry.

Immunohistochemistry

For both immunofluorescence and immunoperoxidase techniques the hearts from control female Sprague-Dawley rats and sodium-deficient or water-deprived rats were fixed in Bouin fluid, dehydrated, embedded in paraffin and sectioned serially at 5 μm either longitudinally, i.e. parallel to the long axis of the heart or corono-

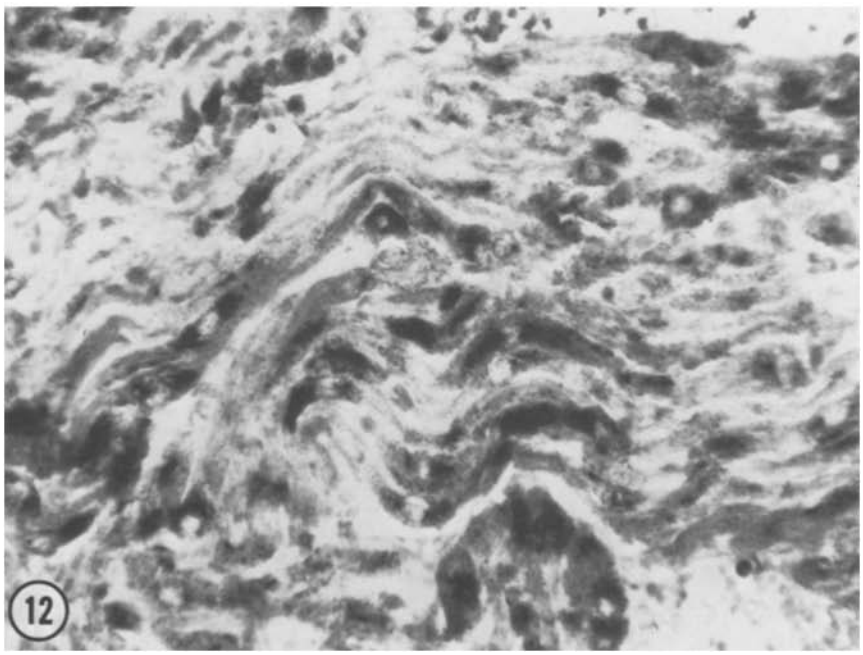


Fig. 11. High power view of right atrial myocardium of control rat treated as in Fig. 4. Note paranuclear localization of reaction sites. One cardiocyte (arrow) is binucleated. Hematoxylin counterstain. (× 630)

Fig. 12. High power view of right atrial myocardium of water-deprived rat treated as in Fig. 4. Most cardiocytes exhibit an intense paranuclear reaction. (× 250)

tangentially. The parotid gland and the submaxillary and sublingual glands of control rats were treated similarly and cut longitudinally along their long axis.

Crossreactivity of LMWP or HMWP antibodies with the atrial specific granules of various species

The heart of 5 frogs (*Rana catesbeiana*) and the atria of 5 adult female mice, guinea pigs, rabbits, cats and dogs and the atrial appendages of 5 patients were fixed in Bouin fluid for 24 h, dehydrated and embedded in paraffin. They were cut at 5 μm and immunostained as described below. The patients (3 males and 2 females, 43 to 63 year old) had died within 24 h of an acute myocardial infarction. At autopsy the atria were not found to be involved and their morphology did not present any evidence of other forms of damage. The atrial appendages were fixed within 10 h of death.

Immunofluorescence

Fluorescence staining by the indirect method was based on Coons' sandwich technique (1958). The sections were incubated at 37° C for 30 min with ascites fluid diluted 1:20, washed for three 10 min periods in PBS and then incubated again at 37° C for 30 min with FITC (fluorescein isothiocyanate-conjugated rabbit immunoglobulins to mouse immunoglobulins (DAKO, Denmark) diluted 1:20 with PBS. They were then washed for three 10 min periods in PBS and mounted in 90% glycerol in PBS. Adjacent sections stained according to the periodic acid Schiff (PAS) technique were used for orientation. Controls included, omission of ascites fluid, use of unrelated ascites fluid and omission of FITC. They gave negative results.

Table 3. Effect of water deprivation and sodium deficiency on the specific granule number in the right atrial appendage of the rat (ultrastructural study)

Treatment	Total number of specific granules[a] in the right atrial appendage
–	105.8 ± 7.5
Water deprivation (5 days)	148.5 ± 9.2[b]
Sodium deficiency (30 days)	150.0 ± 8.9[b]

[a] The granules (A, B and D) were counted in twenty-five 8 × 10 inch prints (final magnification of × 13,380) corresponding to an area of 386.8 μm², for each animal. Each result is the mean ± SE obtained from five animals

[b] Significant ($p < 0.001$) as compared with controls

Table 4. Cross-reactivity of atrial natriuretic factor antibodies with the specific granules of the atria in various species

Species	Intensity of immunoperoxidase reaction	
	LMWP[a] antibodies	HMWP antibodies
Frog (*Rana catesbeiana*)	0[b]	0
Mouse	+ +	+ +
Guinea pig	+ + +	+ + +
Rabbit	+ +	+ +
Cat	+ +	+ +
Dog	+ +	+ +
Man (atrial appendage)	+ + +	+ + +

[a] LMWP (low molecular weight factor antibodies) and HMWP (high molecular weight factor antibodies) were diluted 1:50

[b] 0 = no reaction; + = weak reaction; + + = moderate reaction; + + + = strong reaction

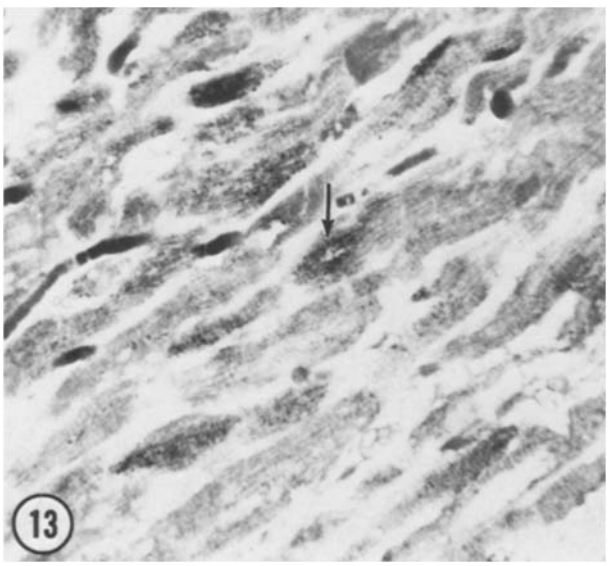


Fig. 13. Sections of human right atrial appendage treated as in Fig. 4. Note granularity (arrow) in a paranuclear position in several cardiocytes. (× 160)

Immunoperoxidase technique

Following deparaffinization and hydration, the sections were exposed: 1) for 46 h at 4° C plus 2 h at room temperature to ascites fluid diluted 1:50 to 1:1,000; 2) for 30 min at 22° C to peroxidase-conjugated rabbit immunoglobulins to mouse immunoglobulins (DAKO, Denmark) diluted 1:50. The peroxidase was visualized by incubation in a medium containing 3 mg of 3-3′diaminobenzidine per 10 ml of 0.05 M Tris-HCl, pH 7.6 and 0.1% H_2O_2 for 10 min in the dark at 22° C (Cantin et al. 1983). Sections were examined as such or counterstained with hematoxylin or according to the periodic acid Schiff (PAS) technique. With both techniques, the specificity of the ascites fluid was assessed by solid phase immunoadsorption (Swaab and Pool 1975) as already described (Cantin et al. 1983) using concentrations of LMWP or HMWP ranging from 1 to 20 μg depending on the dilution of ascites fluid used. Unrelated mouse ascites fluids were used as controls instead of the primary ascites fluid. Controls also included: omission of the ascites fluid or of rabbit anti-mouse immunoglobulins. All these controls gave negative results.

Ultrastructural morphometry

Ultrastructural morphometry of the cardiocytes located in the right atrial appendage was performed as already described (Cantin et al. 1979). After perfusion with 2% glutaraldehyde buffered with cacodylate-HCl (0.1 M pH 7.6), the right atrial appendage was minced and fixed for a further 2 h. The specimens were rinsed with cacodylate buffer, post-fixed with osmium tetroxide in veronal buffer, dehydrated and embedded in Araldite. Semi-fine sections of blocks of tissues were cut in a Reichert ultramicrotome (OMU_2) to find longitudinally oriented cardiocytes. The tissues containing such oriented muscle fibers were cut to make fine sections stained with uranyl acetate and lead citrate and viewed in a Jeol 100CX electron microscope at a magnification of 4773 (high enough magnification to differentiate between A and B granules and low enough to identify D granules located between sarcomeres). Only longitudinal sections framed on two sides by myofilaments and containing in their center part of a nucleus and at least one Golgi complex were photographed without taking into account the number of specific granules present. For each animal, 25 photographs corresponding to those criteria were chosen. The specific granule count was done on 8 inch × 10 inch prints at a final average magnification of 13,800 corresponding to an area of 386.8 μm². The prints were covered by a transparent overlay containing a 1 cm square lattice to facilitate granule count. The results (Table 1) are the means (± SE) of counts done in 5 animals.

Immunocytochemistry: Protein A-gold technique

Preparation for electron microscopy. The right and left atria of control female Sprague-Dawley rats (190–210 g) were fixed with 1% glutaraldehyde buffered with cacodylate HCl (0.1 M, pH 7.1) perfused either through the right or left cardiac ventricle respectively for 10 min. The specimens were then minced, fixed for a further hour in the same manner and placed in cacodylate buffer containing 2% sucrose at 4° C for 24 h. The specimens were embedded in Lowicryl K_4M at −45° C (Carlemalm et al. 1982; Roth et al. 1981) or in glycol methacrylate (GMA) (Cantin and Benchimol 1975) at 4° C.

Preparation of the protein A-gold complexes. Colloidal gold was prepared according to the method of Frens (1973) as already described (Cantin et al. 1983). Small gold particles (pAgS; 12.69 ± 0.16 nm: $N = 100$) were obtained by adding 7.5 ml of sodium citrate to 100 ml of the tetraaurochloric acid solution. Large particles (pAgL; 22.29 ± 0.21 nm: $N = 100$) were prepared in the same manner but only 2 ml of sodium citrate were added. The gold suspensions were then boiled for an additonal 15 min, adjusted to pH 6.9 with 0.2 M K_2CO_3 and then used for labeling protein A as described (Roth et al. 1978).

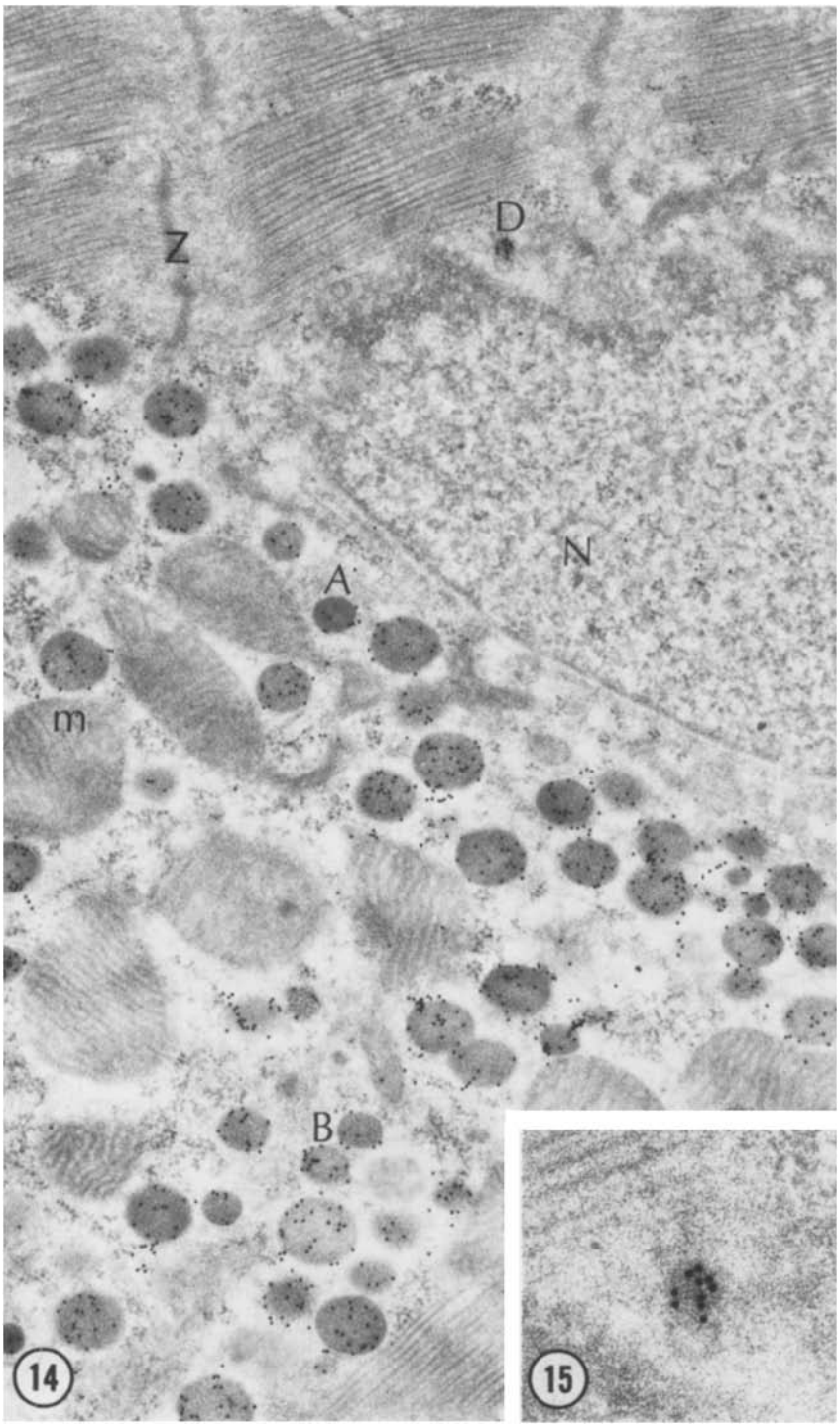


Fig. 14. Fine sections of control rat right atrial cardiocyte embedded in Lowicryl K_4M and treated according to the protein A-gold technique with low molecular weight peptide antibodies. Gold particles are present over all types of specific granules: type A (*A*), type B (*B*) and type D (*D*). Mitochondrion (*m*), nucleus (*N*), myofilaments with Z bands (*Z*). (×31,600)

Fig. 15. Higher magnification of type D specific granule illustrated in Fig. 14 and overlain with gold particles (×85,600)

Immunocytochemical labeling

Single labeling. As already described (Cantin et al. 1983), the nickel grids with mounted fine sections were incubated on a drop of phosphate-buffered saline (PBS; 0.01 M Na-phosphate, 0.14 M NaCl pH 7.4) containing 1% ovalbumin for 5 min. The grids were transferred on a drop of ascites fluid (either LMWP or HMWP) diluted 1:20 to 1:700 in PBS and incubated for 60 min at room temperature. After a rapid rinse in PBS, the grids were then incubated with the pAg complexes for 30 min at room temperature. The grids were thoroughly washed in PBS, rinsed in distilled water and dried. The sections were then stained with uranyl acetate and lead citrate and examined in a Jeol 1200EX electron microscope.

Double labeling (Cantin et al. 1983). First the A face of the section was exposed to 1% ovalbumin in PBS as above. The grids were then transferred on a drop of the first ascites fluid diluted 1:700 in PBS (either LMWP or HMWP) and incubated for 30 min at room temperature. The incubation was performed with the grids floating face A down. After the incubation, the grids were rapidly rinsed by floating them sucessively on several drops of PBS and then incubated face A down on a drop of pAgS solution for 30 min at room temperature. The sections were then thoroughly washed

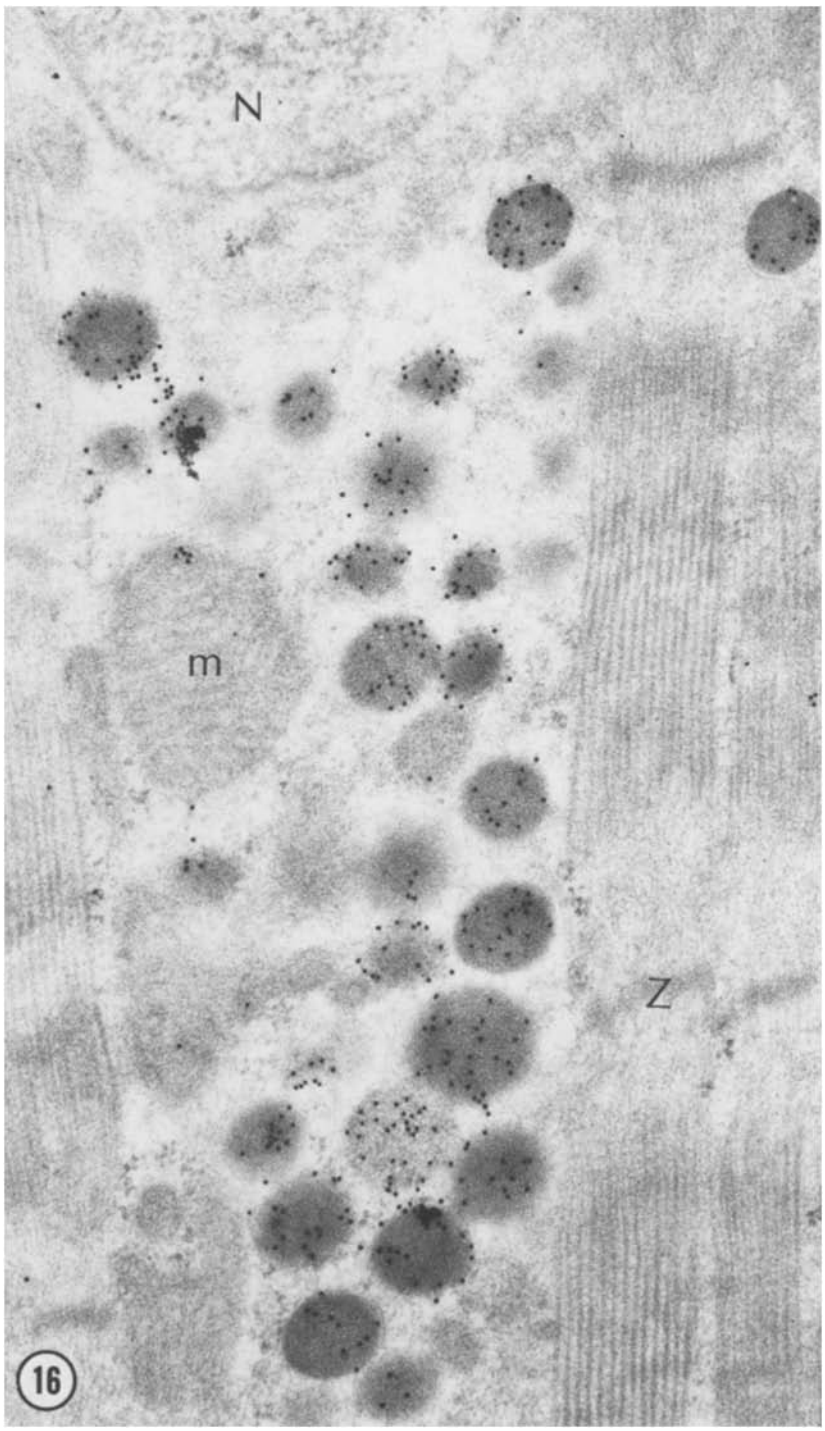


Fig. 16. Fine sections of control left atrial cardiocyte embedded in Lowicryl K_4M and treated according to the protein A-gold technique with high molecular weight peptide antibodies. Nucleus (*N*), Mitochondrion (*m*), myofilament with Z band (*Z*), all specific granules are overlain with gold particles. (×48,960)

in PBS, rinsed in distilled water and dried. The procedure was exactly repeated using the B face of the section, the other ascites fluid and pAgL. Care was taken in all these procedures not to wet the opposite face during the incubation. The reverse procedure was used on alternate sections, using first pAgL and then pAgS. Staining with uranyl acetate and lead citrate was performed on face A of the sections.

In some instances, staining was performed after the labeling of face A, following by examination with the microscope. The grids were then processed for the labeling of face B and the sections reexamined.

The specificity of immunostaining was assessed with the following controls (Cantin et al. 1983); 1) Incubation of the thin sections with ascites fluid previously adsorbed with either LMWP or HMWP (solid phase immunoadsorption as above); for each 1 ml of ascites fluid diluted 1:700, 1.0 μg of either LMWP or HMWP were used. 2) Incubation of the thin sections with ascites fluid followed by 1 h of incubation with unlabeled protein A (0.2 mg/ml) and then with the pAg solution. 3) Omission of the antiserum step and application of the pAg solution alone. 4) Immunostaining of the thin sections with unrelated ascites fluid (tonin or apolipoprotein) diluted 1:10 to 1:1,000 and then with protein A-gold as above. All these controls gave negative results, i.e. insignificant random deposits of gold particles on the sections.

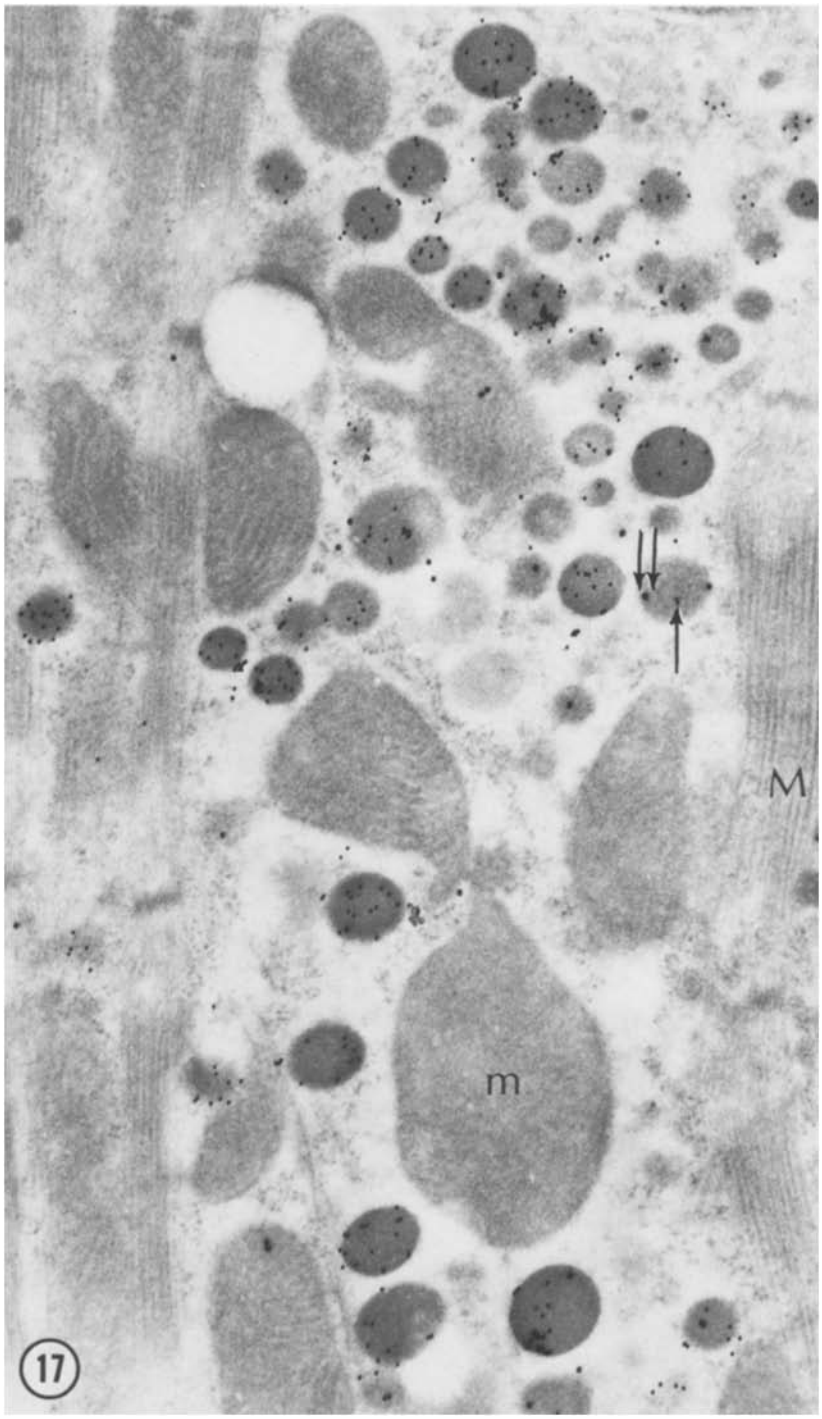


Fig. 17. Fine sections of right atrial cardiocyte from control rat embedded in Lowicryl K_4M and immunostained according to the protein A-gold technique: double labeling with low molecular and high molecular weight peptides. Note the presence of small (pAgS) (arrow) and large (pAgL) (double arrow) gold particles on most specific granules. Mitochondrion (*m*), myofilament (*M*). (×36,450)

Results

Inhibition of the diuretic and natriuretic effect of LMWP or HMWP by antibodies

As can be seen in Table 1, immunoadsorption of atrial homogenates with either LMWP or HMWP antibodies produced a significant inhibition of the diuretic and natriuretic effect whereas immunoadsorption with unrelated antibodies had no significant inhibitory effect.

Immunohistochemistry

Rat heart

Control animals. Since the results obtained by immunofluorescence (Figs. 1–3) and by the immunoperoxidase technique (Figs. 4–13) were identical (Table 2), they will be described together. Ascites fluid from either LMWP or HMWP produced identical staining patterns. There were only slight variations in intensity of staining between the ascites fluid collected at various time intervals after the first intraperi-

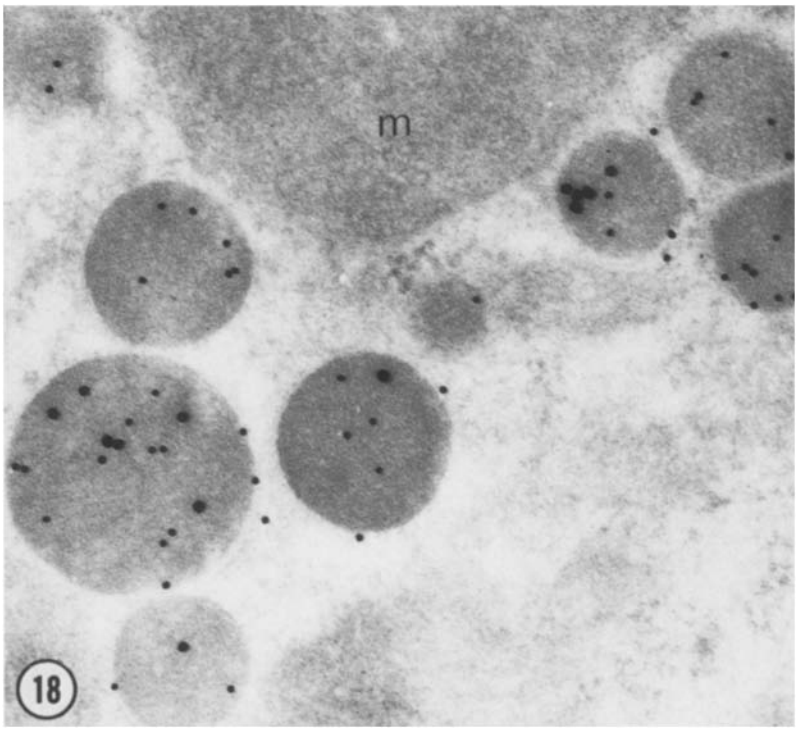


Fig. 18. Fine sections of left atrial cardiocyte from control rat embedded in Lowicryl K_4M and immunostained according to the protein A-gold technique: double labeling with low molecular weight and high molecular weight peptides. Note the presence of small (pAgS) and large (pAgL) gold particles on most specific granules. Mitochondrion (*m*). (×81,600)

toneal injection of antigen. There was no reaction in ventricular cardiocytes (Figs. 2 and 10). In the atria the intensity of the reaction varied depending on the anatomic localization of cardiocytes (Figs. 4–8). The most intensely granulated cardiocytes were localized in atrial appendages and the number of densely granulated cells was greater in the right (Fig. 5) than in the left atrium (Fig. 6). In many sections, the subpericardial cardiocytes from all regions of the atria were more granulated (Fig. 7) than those located in the center of the atrial wall or in the subendocardial area. In the septum many cardiocytes appeared deprived of granularity (Fig. 8). The granularity in all cases was most intense in the paranuclear areas (Fig. 11).

Sodium-deficient rats. There was a marked increase in the degree of granulation (Fig. 9) of both atrial chambers with here again, a preponderance in the right atrium. All atrial cardiocytes even those located in the mid region of the septum exhibited granulation. The gradient of granule density from the subpericardial to the subendocardial area of control atria was also present in these animals.

Water-deprived rats. Water deprivation produced a marked increase in atrial granularity (Fig. 12) in both atria with a preponderance on the right. Here again subpericardial cardiocytes were more granulated than subendocardial ones.

Ultrastructural morphometry. As already described (Cantin et al. 1975, 1982; de Bold 1979), cardiocytes in the atrial appendages of water-deprived or sodium-deficient rats were significantly more granulated than in controls (Table 3).

Cross-reactivity of LMWP or HMWP antibodies with the atrial specific granules of various species

As can be seen in Table 4, the atria of various animal species and human atrial appendages (Fig. 13) contained granules which were immunostained with either LMWP or HMWP antibodies. No reaction could be elicited in either the atria or the ventricle of frogs although the cardiocytes from all chambers of the frog heart are known, from ultrastructural studies (Yunge et al. 1980) to contain all three types of specific granules.

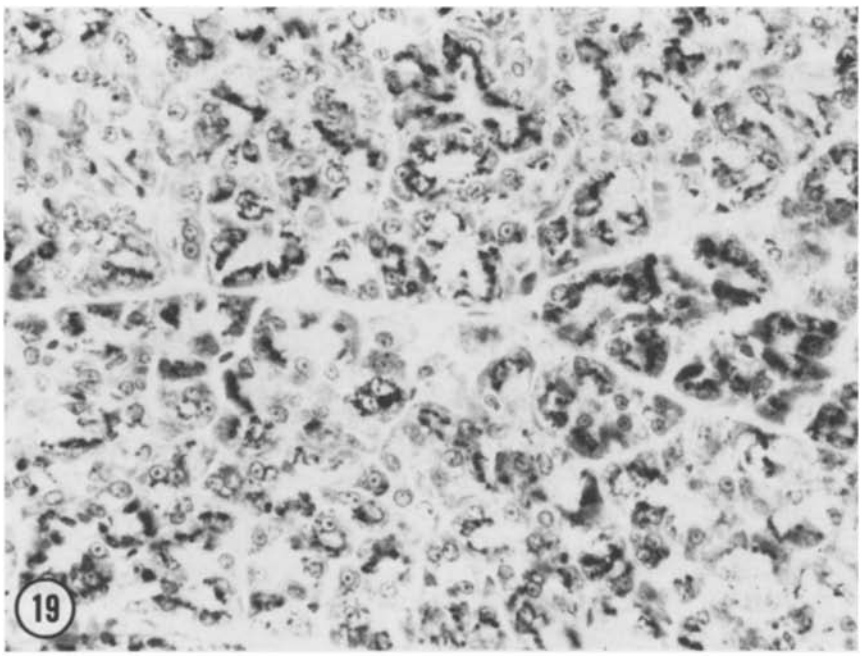
Immunocytochemistry

Single labeling. With ascites fluid from either LMWP- or HMWP-injected mice, all specific granules of both atria whether of the A, B or D types were reactive (Figs. 14–16). Gold particles were not present on any other structure in any significant number. Identical results were obtained in Lowicryl K_4M-or in GMA-embedded atria.

Double labeling. In Lowicryl K_4M-embedded material both types of gold particles, i.e. pAgS and pAgL were present simultaneously on all three types of specific granules of both atria (Figs. 17 and 18).

Salivary gland immunohistochemistry

Here again the results obtained by immunofluorescence and immunoperoxidase techniques were identical and will be described together. The various types of ductal cells in all three salivary glands constantly remained unreactive (Figs. 19 and 20). The reaction was most intense in the acinar cells of the parotid gland (Fig. 19). All acinar cells were reactive. Reaction product was localized near the base



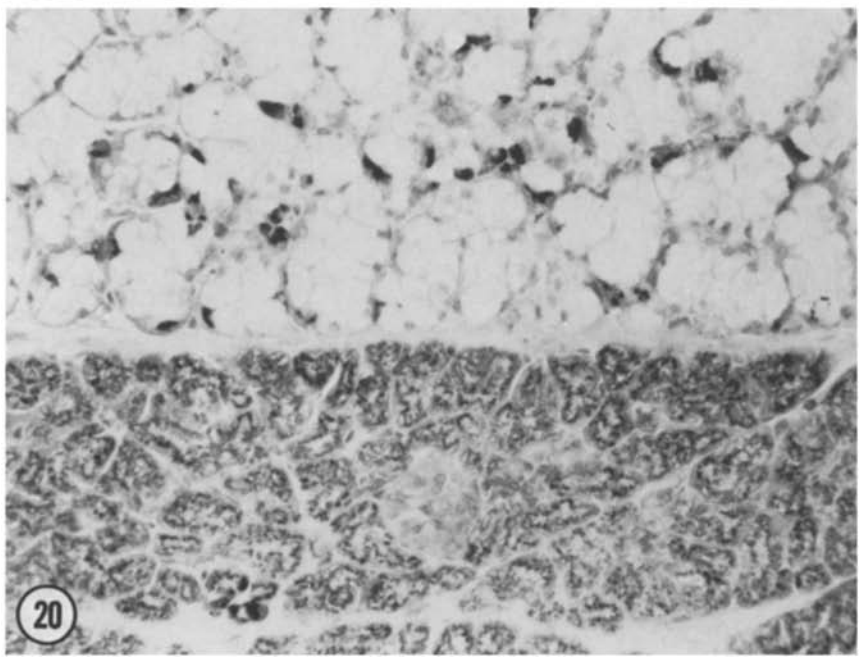


Fig. 19. Section of parotid gland of control rat treated according to the immunoperoxidase technique (low molecular weight peptide antibodies) and counterstained with hematoxylin. Note reaction sites in all acinar cells in a basilar, paranuclear position. (× 250)

Fig. 20. Section at the junction of sublingual (above) and submandibular gland (below) of control rat treated according to the immunoperoxidase technique (high molecular weight peptide antibodies) and counterstained with hematoxylin. Note reaction product filling the cytoplasm of paraacinar cells either single or forming "demi-lunes" (arrow) in the sublingual gland. The acinar cells are unreactive. In the submandibular gland, the reaction is present in the cytoplasm of acinar cells but is less marked than in parotid gland acinar cells. (× 250)

of the cells in a paranuclear position and appeared granular. In the sublingual gland (Fig. 20), only the serous cells, sometimes forming abortive "demi-lunes" were reactive. These cells, in the rat, have scanty cytoplasm which was filled with reaction product. In the submaxillary gland (Fig. 20), the reaction was weaker and distributed seemingly haphazardly in the gland. The most constantly reactive cells were localized near the capsule. Many cells did not contain visible reaction product.

Discussion

The present results indicate that mice injected with LMWP or HMWP produce antibodies against the factor(s) responsible for the powerful diuretic and natriuretic effect of atrial extracts. These antibodies are also specific since immunostaining at both the light and electron microscopic levels is inhibited following immunoadsorption. A further proof of their specificity is the absence of significant staining of any other myocardial antigenic site. While LMWP may be the active factor, HMWP may represent a precursor product. Our immunocytochemical results indicate that both factors are contained in all three types of specific granules in both atria. Thus, despite their morphological heterogeneity, all these granules store the same product. Our results also indicate that most atrial cardiocytes are granulated while all ventricular cardiocytes are not. Water deprivation and sodium deficiency produce a tremendous increase in the granularity of practically all atrial cardiocytes thus confirming and extending our ultrastructural studies.

The morphologic pattern of reactivity we observed in control and experimental animals may be explained by Laplace's law concerning the mathematical analysis of stress distribution within distensible membranes of spherical or cylindrical shape (Badeer 1963). This may be expressed as follows:

$$T = Pr/2h,$$

where T is the mean force per unit cross-sectional area, P the mean transmural pressure across the membrane, r the radius of the structure and h its thickness. This equation has been used to show that in the left cardiac ventricle, radial pressure in control conditions decreases in a non-linear fashion from the inner to the outer surface (Wong and Rautaharju 1968). Thus, the high density distribution of specific granules in the subpericardial area of the atrial free wall could be the result of the lower tension or local stress at these sites. Likewise, the increase of the specific granule number in the atrial wall of sodium-deficient or water-deprived animals can be explained by decrease in atrial pressure and radius and a resultant increase in the thickness leading to a decrease in wall tension. Likewise, the higher level of granulation in the right atrial appendage may be due to the well known pressure differential between the two atria (Marie et al. 1976). If this is so, the degree of stretch of atrial cardiocytes would control their granularity and possibly the secretion of the diuretic and natriuretic factor. The presence of substances immunologically identical to LMWP and HMWP of atrial origin in salivary gland secretory cells may indicate that they are involved in the handling of sodium and water by the ductal cells of these glands. This is not surprising since several laboratory data indicate that ductal cells of salivary glands and renal tubular cells share several functions (Knauf et al. 1982).

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Note added in proof: The immunohisto- and cytochemical results presently described have been confirmed with antibodies raised in the mouse by the technique of Tung against the 1–16 amino acids synthetic fragment of the 1–33 amino acids molecule which has now been isolated, sequenced and synthetized (1). These antibodies, however, do not cross-react significantly with human atrial specific granules which indicates that the structure of human ANF might be slightly different. The synthesis of the 1–16 fragment was done by Dr. P. Schiller, Director, Polypeptides and Chemical Biology Laboratory, Clinical Research Institute of Montreal.

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