Innervation of Periosteum and Bone by Sympathetic Vasoactive Intestinal Peptide–Containing Nerve Fibers

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Neural control of bone metabolism and growth has been suggested, although the identity of participating neurons and neurotransmitters effecting this control has not been established. Immunohistochemical studies demonstrated a system of vasoactive intestinal peptide (VIP)–immunoreactive nerve fibers that innervate periosteum and bone in several mammalian species. Thoracic sympathetic chain ganglionectomy resulted in an ipsilateral loss of VIP-immunoreactive fibers in the periosteum of ribs, whereas dorsal root ganglionectomy had no effect. Injection of fast blue into rib periosteum labeled a population of VIP-immunoreactive sympathetic postganglionic neurons. Thus, postganglionic sympathetic neurons may provide an important means by which VIP regulates bone mineralization.

REGULATION OF BONE MINERALization and resorption has generally been attributed to blood-borne hormones such as parathyroid hormone, calcitonin, and vitamin D. In vitro studies have demonstrated that vasoactive intestinal peptide (VIP) dramatically stimulates bone resorption (*1*). This action is probably mediated via high-affinity receptors for VIP similar to those recently identified in human osteosarcoma cells (*2*). However, plasma levels of VIP are so low that a hormonal role for VIP in bone resorption seems unlikely. Thus, studies were conducted to assess a cellular source that might deliver sufficient quantities of VIP to osseous cells and thereby regulate bone mineralization.

VIP immunoreactivity was localized (*3*) in varicose nerve fibers in the periosteum of porcine rib, tibia, and vertebral body bone (Fig. 1). Single fibers and large nerve trunks were observed; the latter arborized into what appeared to be terminal networks. Fibers were observed among connective tissue elements and were associated with vascular structures. Similar fibers were observed in the bone of dog, guinea pig, and lamb. VIP fibers were most abundant in the pig. Fiber density was greater in piglets less than 8 weeks old than in mature pigs, hence the choice of 6- to 8-week-old piglets as the primary experimental animal. In sections of porcine vertebral bodies, VIP-immunoreactive fibers were seen extending toward cortical bone and infrequently appeared to terminate at the bone-periosteal junction. Adrenergic fibers have been localized within Haversian canals (*4*); we did not find VIP-immunoreactive fibers in these areas.

Several experiments were performed in 6-week-old piglets to determine the origin of the VIP-immunoreactive fibers. Ribs were chosen as the bone of study because of their easy accessibility and, presumably, most consistently segmental innervation. In one animal, a laminectomy was performed, and three thoracic dorsal root ganglia were removed. No loss of VIP-immunoreactive fibers was seen in adjacent ribs 11 days later, which suggests that the fibers were not sensory. However, VIP immunoreactivity was localized in cell bodies in thoracic sympathetic ganglia (Fig. 2, A and B), and surgical removal of the right thoracic sympathetic chain in a second animal resulted in loss of all VIP-immunoreactive fibers in right ribs 11 days later. No decrease in fibers was noted in the unoperated left side. We obtained additional evidence that VIP-immunoreactive fibers were sympathetic in origin by injecting fast blue (a fluorescent substance carried by retrograde neuronal transport) (*5*) into rib periosteum and localizing this material in VIP-immunoreactive cells in thoracic sympathetic ganglia (Fig. 2, C and D). No VIP-immunoreactive cells were seen in the thoracic spinal cord or in thoracic dorsal root ganglia after ligation of several spinal nerves distal to the ganglia.

In an attempt to characterize further the periosteal nerve fibers, we incubated porcine periosteum with antisera to other neuropeptides. No gastrin or cholecystokinin immunoreactivity was seen. A small number of substance P–immunoreactive fibers was observed (approximately 5 percent of the total number of VIP-immunoreactive fibers); this was in agreement with a recent report of substance P–immunoreactive fibers in human tibial periosteum (*6*).

To confirm that VIP immunoreactivity found in bone was actually VIP and not a cross-reacting peptide, we used radioimmunoassay and reversed-phase high-performance liquid chromatography (HPLC) techniques to compare endogenous, extractable VIP with a synthetic standard (*7*). VIP immunoreactivity was found to elute with the same retention time as synthetic VIP (Fig. 3). Dog and pig periosteum contained 15 and 18 pg of VIP per milligram (wet weight) of tissue, respectively. Bone from these animals contained 1 to 5 percent VIP per milligram of tissue (wet weight).

Because vasodilation is a proposed physiological role for VIP in other tissues, microspheres labeled with gamma emitters were used to investigate the effect of VIP on bone and periosteal vessels. Regional blood flows obtained by the microsphere technique are believed to reflect true blood flows in most tissues (*8*), and this method has been used to measure blood flow changes in

Table 1. Effects of short-term infusion of VIP in piglets. Radiolabeled microsphere studies were performed in unanesthetized, unsedated piglets, 6 to 8 weeks old, weighing 10 to 14 kg (*18*). Microspheres of the size used are believed to be trapped completely in small arterioles on the first pass through the microcirculation (*8*). Hemodynamic parameters were measured by Statham transducers attached to fluid-filled catheters and recorded on an Electronics for Medicine DR 12 recorder (Honeywell). Plasma VIP was measured by radioimmunoassay. Resistances are expressed in resistance units where one unit = 1 mmHg/[ml(min · g)$^{-1}$]. Control values for arterial *p*H, PO_2, PCO_2, and hematocrit were 7.53 ± 0.02, 82.3 ± 2.5 mmHg, 28.6 ± 2.0 mmHg, and 30 ± 2%, respectively. Values are means ± SEM.

Hemodynamic parameters	Vehicle infusion	VIP infusion
Plasma VIP (ng/ml)	0.059 ± 0.02	8.98 ± 1.95**
Heart rate (beat per minute)	138 ± 7	214 ± 17**
Mean aortic pressure (mmHg)	92 ± 3	64 ± 7***
Mean left atrial pressure (mmHg)	7 ± 1	2 ± 1*
Mean pulmonary artery pressure (mmHg)	18 ± 2	16 ± 2
Cardiac index [ml/(min · kg)]	261 ± 20	276 ± 87
Systemic vascular resistance (resistance units)	364 ± 26	244 ± 37*
Pulmonary vascular resistance (resistance units)	71 ± 10	62 ± 10

*$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. Student's two-tailed paired *t* test was used.

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bone (*9*). Infusion of 1 μg/(kg · min) of VIP in piglets decreased mean aortic and left atrial pressure, decreased systemic vascular resistance, and increased heart rate in a reflex manner (Table 1). These results are consistent with the known systemic vasodilatory effect of VIP (*10*). A flushing response was seen in all lightly pigmented piglets within 3 minutes of VIP infusion, and the vascular resistance of the skin dropped significantly. This is in agreement with the results of studies performed in humans (*11, 12*).

We found no increase in blood flow or decrease in vascular resistance in bone at periosteal beds in any bone studied. Bone blood flow, similar to renal, intestinal, and cerebral cortical flow, actually decreased during VIP infusion, possibly as a result of shunting of blood to other areas or of venous pooling, thereby limiting cardiac output (Table 2). Control values for blood flow to bone varied considerably and correlated with the gross appearance of the bone, skull being the least vascular and vertebral body the most vascular. The calculated vascular resistances in all bones increased, reaching statistical significance in the skull only (Table 2). We conclude that, unlike some other vascular beds in piglets, bone and periosteal beds are not responsive to the vasodilatory effects of VIP alone. These studies suggest that vasodilation is probably not the primary action of VIP in bone.

Bone-resorbing hormones may act by mediating biochemical or morphological changes in osteoblasts, which in turn allow osteoclasts access to resorption surfaces (*13*). In vitro studies (*1, 2*) show a dramatic response of osseous tissues to VIP: osteoblastic osteosarcoma cells respond to nanomolar concentrations of VIP with increases in adenosine 3′,5′-monophosphate, and whole mouse calvaria are resorbed under the influence of VIP. Our morphological studies demonstrate VIP-immunoreactive nerves at sites where released VIP could reach osteoblasts, effecting resorption; in our physiological studies we found evidence against a primary role for VIP in vasodilation of bone vessels. Neural control of bone metabolism has been proposed, but no specific neurotransmitters have been found to be involved. For example, surgically and chemically sympathectomized rats have decreased osteoblastic activity, as measured by uptake of tritiated proline (*14*). Surgical sympathectomy was formerly used thera-

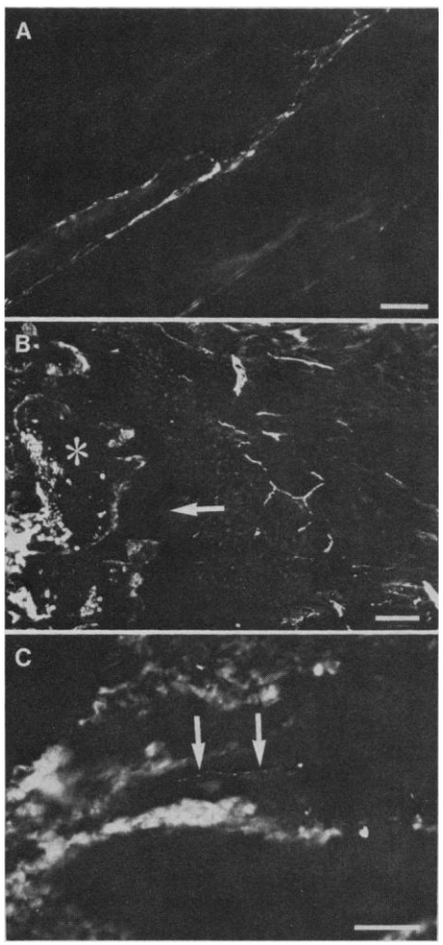

Fig. 1. Immunofluorescent photomicrographs of porcine tissues after staining for VIP. (A) VIP immunoreactivity in varicose fibers in longitudinally sectioned rib periosteum. Scale bar, 50 μm. (B) VIP immunoreactivity in periosteum of vertebral body. Arrow denotes bone; asterisk denotes marrow cavity. Scale bar, 100 μm. (C) Arrows mark VIP-immunoreactive fiber between trabeculae of subcortical tibial bone. Scale bar, 50 μm. No fibers were seen in absorption controls of serial sections incubated with antisera to VIP and excess synthetic VIP.

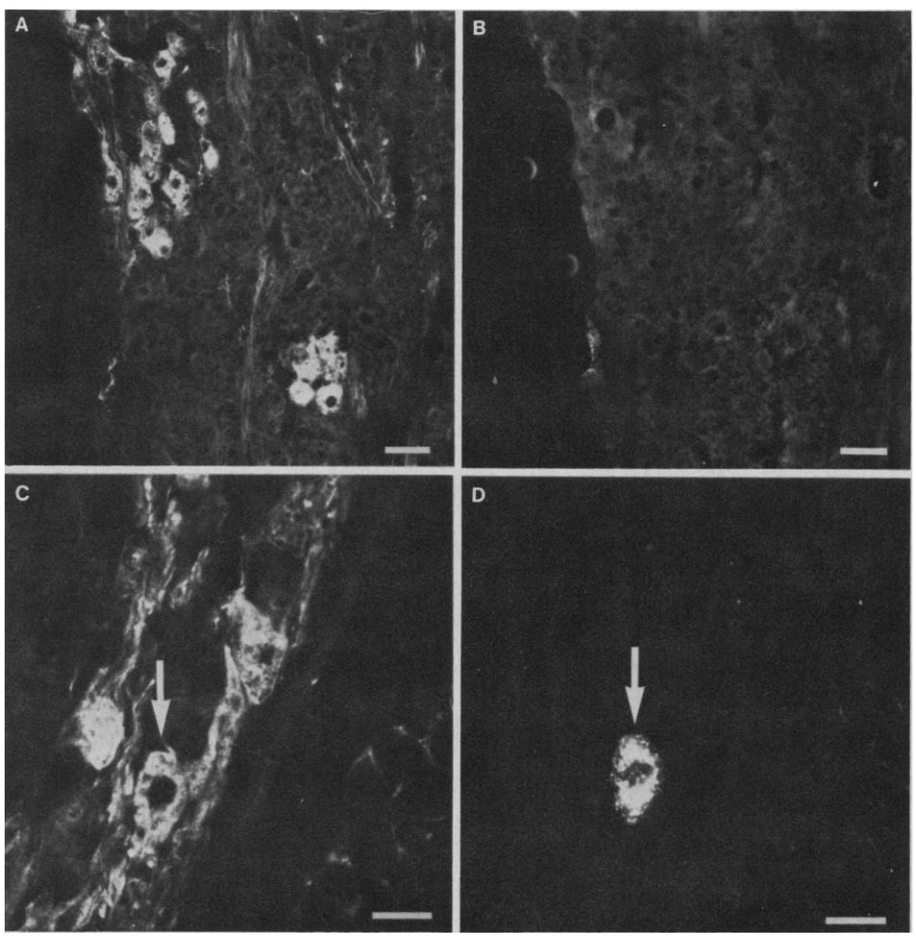

Fig. 2. Immunofluorescent photomicrographs of porcine thoracic sympathetic chain ganglia. (A) Clusters of VIP-immunoreactive cell bodies with characteristic perinuclear staining and bundles of VIP-immunoreactive fibers (seen in all ganglia examined). (B) In serial sections incubated with antisera to VIP pretreated with synthetic VIP, no immunoreactive cells or fibers were seen. (C) Subperiosteal injection of fast blue (*7*) resulted in visualization of fast blue–containing cells in thoracic sympathetic chain ganglia 3 days later (transmitted illumination, UG 1 excitation filter, No. 41 barrier filter). Tracer was contained in vesicles characteristic of material moved via retrograde axonal transport. (D) A photomicrograph of the same section as (C) when stained for VIP immunoreactivity (epifluorescence) shows a fast blue–containing cell, which also contains VIP. The arrows indicate same cell in (C) and (D). Cells containing only fast blue were also observed, presumably the source of numerous adrenergic fibers to bone and periosteum described by others (*17*). Scale bars, 50 μm (A and B); 20 μm (C and D).

Table 2. Shortly after microsphere injection piglets were killed, and two or more samples of each tissue were removed for gamma counting (Packard Autogamma model 5912/9771). Blood flow values shown are means ± SEM of the weighted averages of individual tissue samples. Resistances are expressed in resistance units where one unit = 1 mmHg/[ml(min · g)$^{-1}$]; values are means ± SEM. We calculated regional vascular resistances by dividing mean aortic or pulmonary artery pressures by cardiac index (index expressed in milliliters per minute per gram). Rib, spinous process, and vertebral body samples were taken from the thoracic region (T_5–T_7). Skull samples included both frontal and parietal bone. All bone samples included intact periosteum, and therefore all bone flows represent combined bone and periosteal flows. Submandibular salivary glands were used.

Tissues	Blood flow [ml/(min · g)]		Regional vascular resistance (resistance units)	
	Vehicle	VIP	Vehicle	VIP
Ribs	0.26 ± 0.02	0.10 ± 0.02**	365 ± 37	887 ± 210
Vertebral bodies†	0.41 ± 0.04	0.18 ± 0.14***	227 ± 18	387 ± 109
Spinous processes†	0.30 ± 0.02	0.13 ± 0.02***	309 ± 27	503 ± 102
Tibial diaphysis	0.17 ± 0.03	0.07 ± 0.02**	648 ± 116	2180 ± 979
Skull	0.15 ± 0.02	0.02 ± 0.01**	754 ± 163	1334 ± 261***
Skin†	0.06 ± 0.01	0.16 ± 0.04	1369 ± 322	480 ± 168***
Salivary gland†	0.73 ± 0.14	1.01 ± 0.24	143 ± 30	77 ± 26**
Adrenal gland	1.85 ± 0.11	2.80 ± 0.28*	51 ± 3	25 ± 4***
Masseter muscle	0.07 ± 0.01	0.12 ± 0.03	1640 ± 320	603 ± 89**
Triceps muscle	0.13 ± 0.03	0.17 ± 0.04	961 ± 234	739 ± 207
Cerebral cortex	0.96 ± 0.07	0.76 ± 0.03*	99 ± 10	86 ± 9
Dura†	0.41 ± 0.06	0.35 ± 0.08	219 ± 30	254 ± 113
Renal cortex	5.53 ± 0.50	2.60 ± 0.53**	18 ± 2	31 ± 8
Small intestine	1.03 ± 0.09	0.57 ± 0.08**	94 ± 8	134 ± 28

*$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. Student's two-tailed paired t test was used. †$n = 5$; for other tissues, $n = 8$.

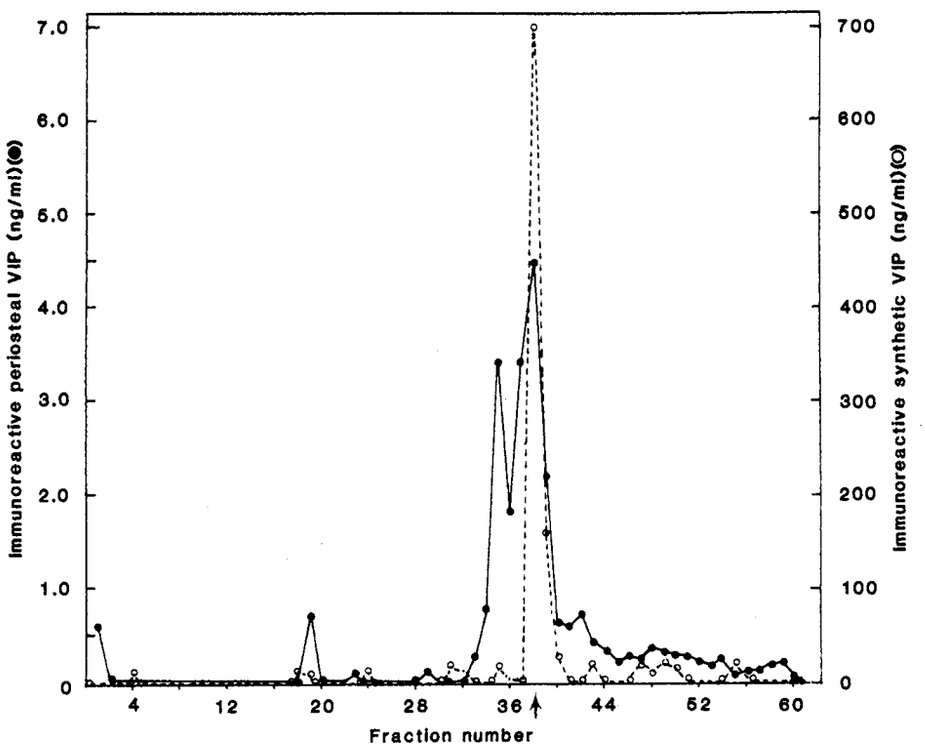

Fig. 3. Elution profiles of porcine periosteal extract and synthetic VIP after reversed-phase HPLC and radioimmunoassay for VIP. VIP was partially purified from pig rib periosteum and subsequently chromatographed (*7*). One-milliliter fractions were collected, and small aliquots were assayed by radioimmunoassay for VIP (ImmunoNuclear, Stillwater, MN), as shown by the solid line. Synthetic VIP (Sigma, St. Louis, MO) was run in parallel, as shown by the dashed line. The arrow denotes the elution time of 37.9 minutes for the synthetic VIP as measured by the appearance of a single peak in ultraviolet absorbance at 256 nm. The nature of the smaller immunoreactive peak in the extract is not clear but probably represents a fragment or degradation product of VIP, as a similar doublet was occasionally seen in chromatographs of synthetic VIP. When an extract of dog periosteum was chromatographed, a single immunoreactive peak was obtained. In experiments with synthetic and ^{125}I-labeled synthetic VIP, approximately 75% of added VIP could be recovered from samples by this technique.

peutically to decrease discrepancies in limb length in children affected by poliomyelitis (*15*), and severing large nerve trunks results in greatly increased callus formation in rats (*16*). Because of its anatomical location and in vitro effect, we hypothesize that VIP may modulate bone resorption in vivo. Neural control of bone resorption may be important in other conditions in which calcium mobilization occurs, such as osteoporosis, lactation, and loss of mineral associated with zero gravity. Further neurophysiological studies may clarify the role of VIP and other transmitters in such states.

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7. Several ribs were removed from a 6-month-old pig immediately after death. Periosteum (3 g) was dissected free from bone and surrounding fat and muscle. The tissue was homogenized in 3*N* acetic acid, heated for 5 minutes to 100°C, and allowed to stand overnight at 4°C. The homogenate was centrifuged, and lipid was extracted from the supernatant with four volumes of HPLC-grade hexanes. VIP was partially purified from the extract by adsorption to XAD-2 resin by the method of Chang and coworkers [T. Chang, F. L. Roth, H. Tai, W. J. Chey, *Anal. Biochem.* **97**, 286 (1979)]. Extract was applied to a C-18 column and eluted with a 0 to 60% gradient of acetonitrile in water, 1% per minute, both solvents containing 0.1% trifluoroacetic acid.
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12. In contrast to the results of studies in neonatal lambs in which a role for VIP in pulmonary vasodilation was proposed [T. Kulik, D. E. Johnson, R. P. Elde, J. E. Lock, *Am. J. Physiol.* **246**, H716 (1981)], we found no significant reduction in pulmonary artery pressure in this animal model. Analysis of regional blood flows and peripheral vascular resistances showed differential sensitivity of various vascular beds to VIP, a phenomenon seen with other vasodilators [S. Einzig, G. H. Rao, J. G. White, *Can. J. Physiol. Pharmacol.* **14**, 396 (1981)]. In agreement with other studies, vascular resistance decreased significantly in salivary glands [J. M. Lundberg, A. Anggard, J. Fahrenkrug, *Acta Physiol. Scand.* **114**, 329 (1982)] and cephalic muscle [D. A. Wilson, J. T. O'Neill, S. I. Said, R. J. Traystman, *Circ. Res.* **48**, 138 (1983)]. A significant increase in adrenal blood flow with a corresponding drop in resistance was also observed. Blood flow decrease did not always

reach statistical significance because of the small number of animals used. Despite unchanged cardiac output, decreased resistance, possibly with increased flow, indicated that VIP was acting as a vasodilator in these tissues.
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18. Animals were fitted with left atrial, aortic, and pulmonary artery catheters 1 week before experimentation. Microsphere studies were carried out as described (*12*). Briefly, radiolabeled spheres 15 μm in diameter (^{141}Ce, ^{85}Sr, ^{46}Sc, or ^{95}Nb; 3M Company, St. Paul, MN) were injected into the left atrium after 10 minutes of intravenous infusion of vehicle alone (0.1*M* phosphate-buffered saline containing 5% pig plasma and 500 kallikrein inactivated units of aprotinin, 0.38 ml/min) and after 10 minutes of infusion of VIP at a rate of 1 μg/(kg · min). An arterial reference blood sample used to calculate tissue flow and cardiac output was withdrawn from the aorta at a rate of 7.5 ml/min for 2 minutes.
19. We thank D. C. Seldin and W. Mahoney for assistance with HPLC and E. Skjelstad-Lorenz for assistance with blood flow studies. This work was supported by grants from the Minnesota Medical Foundation, Immunonuclear Corporation, and the Veterans Administration Research Service.

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Pancreatic Zymogen Granules Differ Markedly in Protein Composition

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The activities of both chymotrypsin and amylase in individual zymogen granules of rat pancreas were measured by means of micromanipulation and microfluorometric methods. The enzyme content and the ratio of amylase to chymotrypsin varied widely among granules taken from the same animal. These results are compatible with short-term nonparallel bulk secretion of the two enzymes through exocytosis. The distribution of each enzyme activity in a population of granules suggests quantal packaging of amylase and chymotrypsinogen into the granules.

SECRETORY CELLS MAY SYNTHESIZE and export more than one substance. The relative amounts of these substances secreted at the organ level may change with physiological needs. To understand the control of secretion by a cell that exports more than one substance it is necessary to determine whether or not the substances are packaged in a fixed ratio within and among cells. The exocrine pancreas, which exports at least 19 secretory proteins, is a useful model for studying this problem. Export is via exocytosis of proteins packaged within zymogen granules (*1*). For exocytosis to be compatible with the observed short-term variations of the relative amounts of secretory proteins in the pancreatic juice (*2*), the ratio of secretory proteins has to be different among granules. Immunohistochemical studies have suggested that all secretory proteins are present in all zymogen granules (*3*), but these studies could not have distinguished quantitative differences in the ratios of the secretory proteins among granules.

We have directly addressed the problem of relative packaging of secretory proteins among granules by measuring the content of both amylase and chymotrypsinogen in individual zymogen granules. We have found that the composition of individual pancreatic zymogen granules isolated from a single animal can markedly differ. This observation may reconcile the theory of exocytosis with the finding of short-term changes in the composition of pancreatic exocrine secretion.

Analysis of amylase and chymotrypsinogen in individual zymogen granules was performed by means of a general microfluorescence method (*4*) as adapted to enzyme analysis (*5*) and to the quantitation of fluorescence in liquid droplets (*6*). Zymogen granules were prepared from individual adult female Wistar rats by a modification of the method of Harper *et al.* (*7*). Individual granules were obtained by spraying a suspension of granules onto a cover slip, examining the air-dried droplets of suspension via differential interference-contrast optics (Fig. 1), and selecting droplets with single zymogen granules. Each droplet thus selected was dissolved and its granule lysed in 140 picoliters (pl) of a buffered salt solution. Portions of the lysate of a single granule were then analyzed by microfluorometric methods for their amylase and chymotrypsin activities, the latter after activation with trypsin.

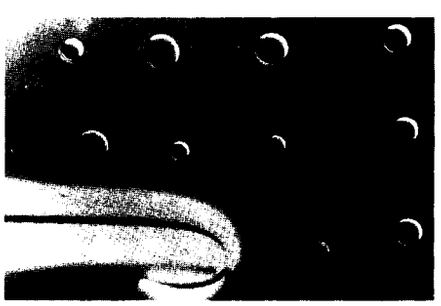

Fig. 1. Dried droplets of a suspension of zymogen granules. Pancreatic zymogen granules were prepared from rat pancreas by the method of Harper *et al.* (*7*), but with a homogenizing fluid of 0.65*M* sucrose, 5 m*M* 2-(*N*-morpholino) ethane sulfonate, *p*H 5.5. The pellet from one-half the homogenate of a single pancreas was gently resuspended in 1 ml of homogenizing fluid and sprayed via an Effa Spray Mounter (E. F. Fullam, Schenectady, NY) onto a siliconized glass cover slip. The cover slip was covered with paraffin oil, and observed with Nomarksi optics. For this photo, individual droplets were dislodged from the cover slip with the small glass hook shown and moved into an array. Final magnification, ×330.

In Fig. 2a, the chymotrypsin content is plotted against the amylase content for 22 individual zymogen granules from a single animal. The mean amount of chymotrypsin per granule was 0.482×10^{-18} mol (±0.084 SEM) and the mean amount of amylase per granule was 0.698×10^{-18} mol (±0.103 SEM). The ratio of mean chymotrypsin to amylase was 0.69. Absolute amounts per granule of both enzyme activities, however, varied over wide ranges. The median content for chymotrypsin and amylase was 0.4×10^{-18} mol per granule. There was no significant correlation between amylase and chymotrypsin content among granules. These results support the idea that amylase and chymotrypsinogen are not packaged into individual granules in a fixed ratio. Differential packaging of secretory products among and within cells has also been observed in the bovine pituitary (*8*). To determine if our results could be attributed to imprecision of the measurements, we lysed a suspension of granules to produce a solution of homogeneous composition, and handled and assayed picoliter volumes of lysate as if they were lysates of individual granules. These results validated the microassay of individual granules in two ways. First, the activities of amylase and chymotrypsin were each proportional to the

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