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 An arterial reference blood sample used to calculate tissue flow and cardiac output was withdrawn from the aorta at a rate of 7.5 ml/min for 2 minutes.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.

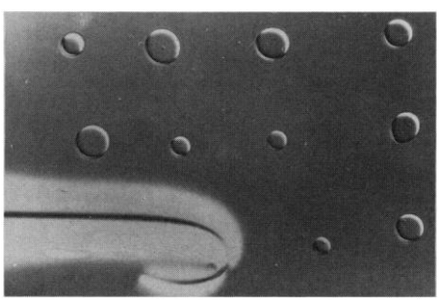

Fig. 1. Dried droplets of a suspension of zymogen granules. Pancreatic zymogen granules were prepared from rat pancreas by the method of Harper *et al.* (*7*), but with a homogenizing fluid of 0.65*M* sucrose, 5 m*M* 2-(*N*-morpholino) ethane sulfonate, *p*H 5.5. The pellet from one-half the homogenate 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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amount of lysate assayed and the ratio of amylase to chymotrypsin content was equivalent to their ratio measured with conventional, milliliter-scale assays. Second, the ratio of chymotrypsin to amylase found in the lysates of granules, 0.69, was equivalent to the mean ratio calculated from individual granule measurements. These control studies were conducted over the range of activities observed in samples of individual granules (Fig. 2b) and indicate that variation measured between granules reflects variation in secretory protein content of the granules.

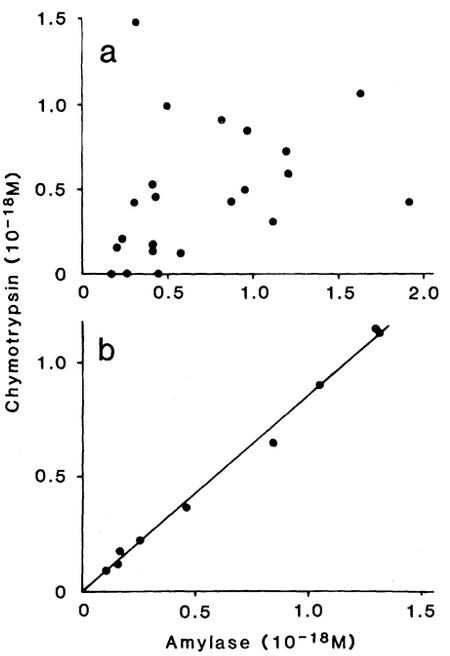
The 14% standard deviation reported for granule diameters (*9*) would mean a 48% standard deviation in granule volumes, and presumably thus in enzyme contents as well. Such variation in overall content is consistent with our activity data. The magnitudes of the activities found are consistent with those expected on the basis of reported values for zymogen granule size and protein content (*9, 10*) and for overall ratios and activities of these enzymes from rat pancreas (*11*).

The distributions of chymotrypsin and of amylase contents among the granules appeared to be quantized (Fig. 3). The chymotrypsin distribution is consistent with a Poisson distribution having a "quantum size" (amount of enzyme per subunit) of 0.24 attomoles (amol) and an average of 2.0 chymotrypsin quanta per granule ($P > 0.25$ by χ^2) (*12*). The amylase distribution is consistent with a Poisson distribution having a quantum size of 0.35 amol, and an average of 1.30 amylase quanta per zymogen granule, above a basal level of 0.25 amol per granule ($P > 0.25$ by χ^2) (*13*). Such distributions might be expected if zymogen granules were constructed from a number of subunits, each subunit containing a fixed amount of one secretory protein. The existence of subunits in the construction of zymogen granules has been demonstrated (*14*). Alternatively, such quantized distributions of secretory protein contents among granules could arise from differences among cells in the number of subunits of zymogen that are packaged into the zymogen granules in each cell. If differences in protein processing among cells were to be combined with differential sensitivity to secretagogues among cells, it would be possible for exocytosis to lead to nonparallel secretion.

Quantized distributions of amylase and chymotrypsin were also observed when granules were prepared from one animal that had been maintained on a high-protein diet. In that animal, although almost all of 30 granules analyzed still showed both amylase and chymotrypsin activity, the mean amylase content per granule was an order of magnitude lower than the mean chymotrypsin content (0.072 ± 0.008 SEM amol of amylase per granule relative to 1.09 ± 0.12 SEM amol of chymotrypsin per granule). The basal level of amylase, the amylase quantum size, and the mean number of amylase quanta per granule were all lower than seen for a normal diet.

Pancreatic zymogen granules from a single animal thus can differ quantitatively in composition. Short-term changes in the composition of pancreatic juice, as seen in the study by Adelson and Miller (*2*), could result from selective secretion of granules with different compositions. Further studies are required to determine if differences among granules reflect differences among or within cells, and to determine the functional significance of this quantitative heterogeneity.

Fig. 2. Activities of amylase and chymotrypsin in single zymogen granules (a) and in samples of a lysed suspension of granules (b) from the same animal. Droplets with individual granules under oil were obtained as described (Fig. 1), but with melibiose instead of sucrose in the homogenizing fluid (to remove interference with the amylase assay). Micropipetting and microfluorescence procedures were as described (*4–6*). Droplets with single visible granules were dissolved in 140 pl of a lysing solution [100 m*M* NaCl, 100 m*M* KCl, 100 m*M* piperazine-*N*,*N*′-bis(2-hydroxypropane sulfonate), bovine albumin (2 mg/ml), *p*H 8.0]; the granule was usually seen to lyse during application of this solution. The lysate was then taken back up into the pipette, and transferred to a glass slide for assay. Thirty-five picoliters of lysate was activated with 90 pl of trypsin (5 μg/ml) for 60 minutes, and then assayed at 27°C for chymotrypsin (*5*). Another 35 pl was analyzed for amylase by micromodification of the method of Guilbault and Rietz (*15*). Fluorescence increases were related to NADH production with standard NADH, and activities of the 35-pl samples were corrected back to the original lysate, with correction for evaporation of the 140-pl lysate between the time of dissolving the droplet and that of taking the 35-pl samples. Results are presented as the number of attomoles of standard enzyme (porcine amylase, M_r 45,000; bovine chymotrypsin A, M_r 22,000) that would have produced the observed enzyme activities. In (b), granules from this animal were lysed and several dilutions were made at the milliliter scale. Microsamples of 135 pl were put under oil and assayed for amylase and chymotrypsin in a manner identical with that used for lysates of individual granules. The line is the linear least-square regression line of chymotrypsin on amylase.



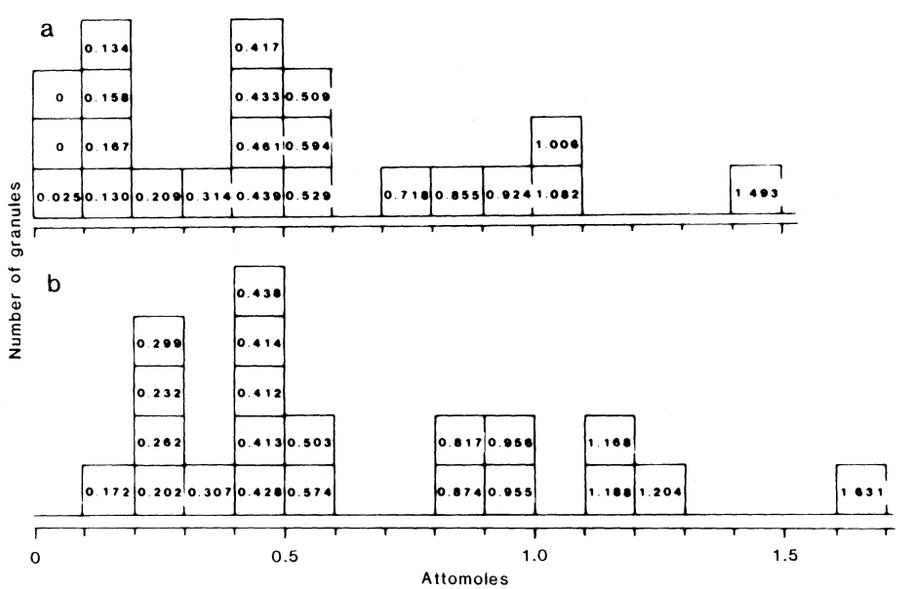


Fig. 3. Histograms of chymotrypsin (a) and amylase (b) activities among granules shown in Fig. 2a. The number in each box is the number of attomoles of enzyme estimated for a single granule. The highest value of amylase, 1.91 amol, is off the scale and not shown.

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Cloned Fragment of the Hepatitis Delta Virus RNA Genome: Sequence and Diagnostic Application

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Hepatitis delta virus (HDV) is a replication-defective etiological agent of hepatitis that requires hepatitis B virus (HBV) as a helper. A complementary DNA (cDNA) fragment of the RNA genome of HDV was cloned into the plasmid vector pBR322, and the primary nucleotide sequence and predicted protein products of the cDNA fragment were determined. This cloned cDNA fragment has been used as a sensitive radioactive probe for the detection of HDV RNA in the serum of patients with either acute or chronic HDV infections.

HEPATITIS DELTA VIRUS (HDV), a naturally occurring defective virus of man, depends on hepatitis B virus (HBV) for its replication and expression (*1, 2*). For the large population of chronic HBV carriers worldwide, HDV represents a serious medical problem because HDV infection frequently results in severe and progressive liver disease, delta hepatitis. Epidemics of delta hepatitis have occurred in the general population of underdeveloped countries where HBV is endemic. In developed countries the disease appears to be limited to certain high-risk groups, including intravenous drug addicts and hemophiliacs (*2*).

The viral nature of the HDV was established in chronically HBV-infected chimpanzees, an experimental model of acute HDV infection and disease (*3*). In acute phase serum, HDV circulates as a 36-nanometer particle having the HBV surface antigen (HBs) on its surface and the delta antigen (HDAg) and HDV RNA within the particle (*4*). The 1.75-kilobase (kb) HDV RNA is not polyadenylated, has considerable secondary structure, and appears to be single-stranded (*5*). The physical association of HDV RNA with HDAg (an established marker of HDV infection) within the viral particle (*1, 4, 5*), the lack of hybridization between complementary DNA (cDNA) transcripts of HDV RNA and HBV or host DNA's (*5*), and, most important, the near equivalence between the HDV RNA concentration and the HDV infectivity titer of an acute phase chimpanzee serum (*2, 6*) indicate that HDV RNA is the genome of this unusual viral agent. No information has been reported on the pri-

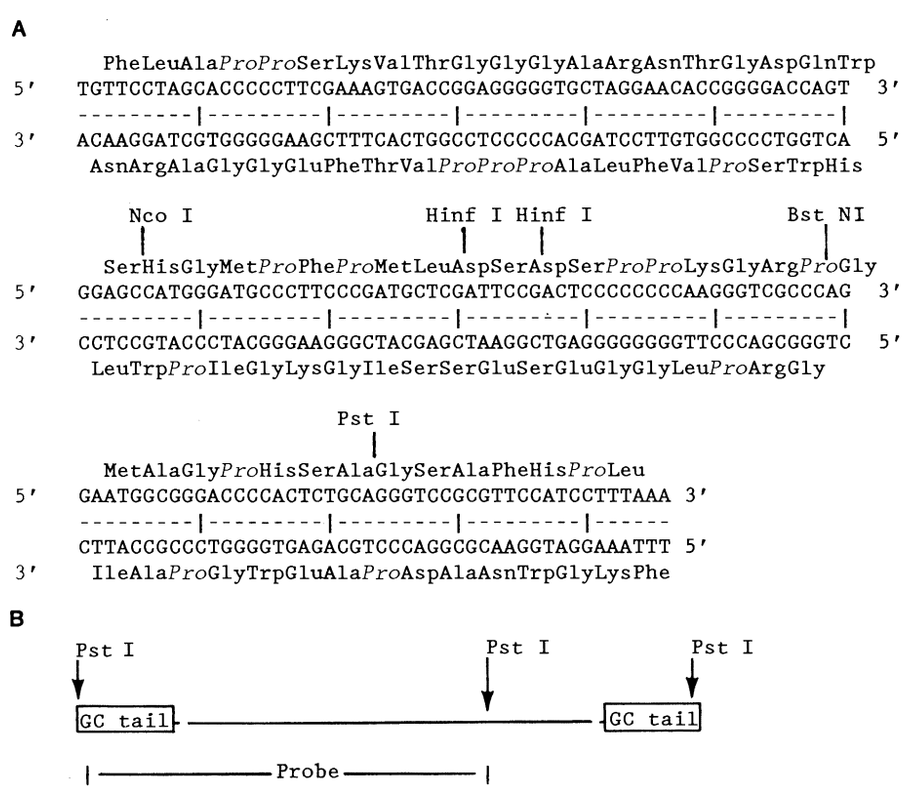

Fig. 1. (A) The primary nucleotide sequence of the 166-bp HDV RNA cDNA clone, pKD3. Above and below the nucleotide sequence are the predicted amino acid sequences of the single open reading frames in each strand. The sequence was obtained by the method of Maxam and Gilbert (*14*). Nucleotide sequence determinations were made from 3′-end-labeled Pst I termini and 5′-end-labeled Nco I, Hinf I, and Bst NI sites. These sites are indicated above the sequence. (B) The Pst I subfragment (139 bp + 14 bp GC tail) was used to make the hybridization probe.

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