such as biomaterials or devitalized bone remains to be investigated. It seems logical to assume that, all other factors being equal, random contact between bacterial cells and substrate increases as substrate area increases.

The matrix formed by the exopolysaccharide polymers serves not only as an adhesive mechanism but also as a nutrient-trapping ion-exchange material (*11*), and it allows the bacteria to resist engulfment by phagocytic cells. It appears to be virulence-related (*1, 4–7*), and it has been shown to confer resistance to host defense mechanisms such as surfactants (*26*) and antibodies (*27*) and to the effective penetration of antibiotics (*27, 28*). The stability of this biofilm appears to be a major factor in the persistence of many chronic orthopedic infections (*4–7*).

This study suggests that compromised, dead, or sequestrated bone is a suitable substrate for bacterial colonization and biofilm formation. There are indications that the adherent state mediated by the exopolysaccharide polymers enhances the virulence of the bacteria in the biofilm. In such states progressive microcolonial habitation occurs with development of the complex consortia seen in chronic osteomyelitis. We believe that the adherent form of infection is natural in acute and chronic osteomyelitis and that adherence explains in part the resistance of this disease to antibiotic therapy and its persistence until all dead bone and compromised tissues have been removed.

The infections in the cases presented here share some qualities of the adherent state in nature and in certain diseases and biomaterial-related infections. There appears to be no contradiction in these shared qualities, but rather a compelling unity that suggests common causal mechanisms for all adherent bacterial infections.

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Pancreatic Secretion by Nonparallel Exocytosis: Potential Resolution of a Long Controversy

Abstract. *The idea that pancreatic digestive enzyme secretion can occur in a nonparallel manner has been controversial because of its presumed incompatibility with the exocytosis secretory mechanism. Correlation and regression analysis of enzyme output by the rabbit pancreas after it is stimulated with cholecystokinin and chymodenin revealed that digestive enzymes are secreted in a highly linked fashion, compatible with exocytosis and with nonparallel secretion. Thus, exocytosis and nonparallel secretion are not contradictory processes, but rather nonparallel secretion is due to exocytosis from heterogeneous sources within the pancreas.*

The physiological and cell biological phenomena underlying the secretion of digestive enzymes by the mammalian pancreas have been the subject of an intense ongoing controversy for over a decade. Palade, in his 1975 Nobel Prize address (*1*), summarized the process of synthesis, segregation, storage, and final exocytotic expulsion of the several digestive enzyme species from the pancreatic acinar cell. Rothman (*2*) criticized the exocytosis hypothesis of enzyme secretion as being based on incomplete and insufficient evidence and contradictory to the observed phenomena of nonparallel or enzyme-specific secretion of digestive enzymes. In place of the exocytosis hypothesis, Rothman (*2, 3*) offered as an alternative model of cellular secretory protein routing the "equilibrium hypothesis." In this model, secreted proteins cross directly through the membranes of the cell in a regulated fashion, rather than being totally segregated from the cytosol and stored within the membranes. The controversy has continued to the present, with laboratories in several countries contributing evidence favoring one side of the argument or the other.

Observations from various laboratories have shown rapid nonparallel or enzyme-specific secretion of digestive enzymes after administration of diverse hormones, neurotransmitters, metabolites, or digestive stimuli to various whole-pancreas preparations both in vitro and in vivo (*4*). According to the exocytosis hypothesis, distinct species of digestive enzymes are synthesized, processed, segregated from the cytosol, and stored in the zymogen granules, in parallel with each other, within 45 minutes to 1 hour (*1, 5*). Further, the zymogen granules appear in electron micrographs to be homogeneous, even when their contents are assessed by immunohistochemical methods (*6*). Thus, either the observations of nonparallel secretion are incorrect [which has been the claim of some cell biologists (*7*)] or exocytosis is not a satisfactory mechanism to explain the secretory phenomena (*2*).

We explored the possibility that the rabbit pancreas in vitro and in situ might secrete enzymes in a nonparallel fashion, and we simultaneously determined whether the enzyme species were secreted in groups, as expected of exocytosis, or independently, as expected of an equilibrium-type mechanism. The existence of nonparallel secretion was explored by regression analysis of the secretion of paired digestive enzymes under several different conditions of stimulation. Sig-

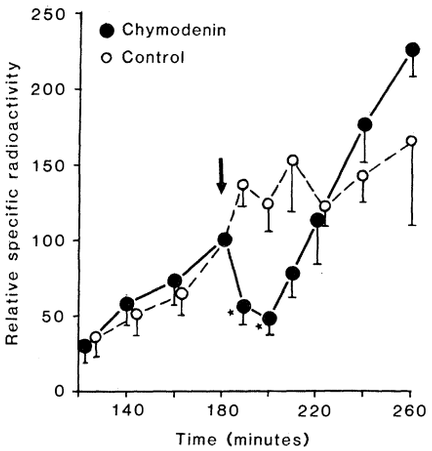

Fig. 1. [^{14}C]Leucine (5 μCi) (Amersham) was added to the medium bathing the in vitro preparation (Table 1) for a 3-hour basal period. The bath was renewed hourly with fresh medium containing 5 μCi of [^{14}C]leucine before the addition of chymodenin (1.6×10^{-8} *M*) or diluent alone (controls). Radioactive labeled protein was measured by the method of Rothman and Isenman (*14*), with 10 percent trichloroacetic acid (TCA) precipitation at 4°C for 30 minutes and filtration onto 0.3-μm nitrocellulose and cellulose-acetate filters (Millipore) under reduced pressure. The filters were washed with 10 ml of 20 percent TCA containing 10 m*M* "cold" leucine and counted in vials containing PCS II (Amersham). The specific radioactivity (disintegrations per minute per microgram of protein) was normalized relative to the period preceding the addition of chymodenin or diluent alone (arrow). There were nine observations for controls and seven for chymodenin-treatment for each period. Stars indicate a significant difference from the time-paired controls in an unpaired *t*-test at $P < 0.01$.

nificant differences in the slopes of the curves for the secretion of pairs of enzymes, as revealed by regression analysis, would be evidence of nonparallel secretion, whereas identical slopes would indicate parallel secretion. To explore possible linkage or grouping of enzymes during pancreatic secretion, we compared enzymes in pairwise fashion by correlation analysis of the enzyme output data on a point-by-point basis. A high degree of correlation would be expected for a simple exocytosis mechanism in which the various enzyme species are stored together in zymogen granules and expelled in groups during secretion. In contrast, a simple equilibrium mechanism in which individual enzyme species can permeate the intracellular and plasma membranes bidirectionally (enzyme-specific secretion) should produce a secretory pattern in which the various enzymes species are unlinked or independent. Taken together, the results of the regression and correlation analyses should produce one of the following patterns consistent with a specific secretory mechanism: (i) parallel secretion and high correlation (exocytosis); (ii) nonparallel secretion and low correlation (equilibrium); or (iii) nonparallel secretion and high correlation (exocytosis from heterogeneous sources within the pancreas). We report here the unambiguous finding of the third pattern in both in vitro and in situ studies.

Treatment of the rabbit pancreas in vitro by addition of pure chymodenin (1.6×10^{-8} *M*)—a candidate gastrointestinal hormone (*8*)—to the medium bathing the gland produced an immediate and significant increase in the correlation coefficients observed in the output of all pairs of enzymes, or of enzyme and total protein, as compared with the control-treated gland (*9*) (Table 1). The degree to which the variance in one enzyme output can be attributed to the variance of a second enzyme output is measured by the square of the correlation coefficient (r^2) (*9*). For the control group, r^2 was 23 percent whereas in the experimental group the linkage was 66 percent by this criterion. The mean r^2 for the chymodenin-treated enzyme pairs (as distinguished from enzymes paired with total protein) was 85 percent and for the control group was 53 percent (*10*).

As the correlation coefficient increased for secretion by enzyme pairs in the group treated with chymodenin in vitro, the source of the secreted enzymes within the pancreas, which under basal conditions had been a newly synthesized pool, changed to an older, prestored pool. This was shown by an immediate drop in specific radioactivity of newly secreted protein after chymodenin was added. When [^{14}C]leucine was continually incubated with the pancreas under basal (that is, unstimulated) conditions, radioactively labeled protein was secreted, beginning approximately 1 hour after the ^{14}C was added, and the specific radioactivity increased with time (Fig. 1). The addition of chymodenin (1.6×10^{-8} *M*) resulted in a minor (20 percent; not significant) increase in total protein output which, accompanied by a net 40 percent decrease ($P < 0.005$) in the output of radioactive protein, gave a 60 percent decrease ($P < 0.01$) in the specific radioactivity of the secreted protein.

Correlation and regression data are

Table 1. The pancreas from the male albino New Zealand rabbit (2.0 to 2.5 kg) was used (*15, 16*). For the in vitro experiment, seven glands were treated with 25 μg of pure chymodenin (1.6×10^{-8} *M*) (*8*) after a 3-hour basal period of 20-minute sequential collections. Nine control glands were treated with diluent alone. Secretion was then collected for four 10-minute periods, after which the 20-minute collections were resumed. For the in situ experiment, after 1 hour of unstimulated secretion, cholecystokinin (1.6 IDU/kg per hour) (Boots) was infused via the femoral vein for 2 hours. Four rabbits received 25 μg of pure chymodenin as an intravenously injected bolus after the first hour; four controls received a bolus of diluent only. Twenty-minute aliquots were collected during cholecystokinin infusion; ten-minute collections were made after chymodenin or control injections. For the in situ regression data, see Fig. 2. Chymotrypsinogen and trypsinogen were activated by the technique of Glazer and Steer (*16*) within the protein concentration limits specified by them. Maximal activation of zymogens and the stable maintenance of this activity for several hours was obtained as described by Glazer and Steer. All enzymes and total protein were measured by standard methods (*17*). Enzyme output is expressed as total enzyme secreted per minute. Correlations (*r*) were significant for all values at $P < 0.004$ except where indicated as not significant (N.S.). There were 20 observations for each value in the in vitro series and 24 observations in the in situ series. All statistical analyses were performed with SAS programs (*18*) on an IBM computer.

Pair	Correlation coefficient (*r*)	
In vitro	Control	Chymodenin
Chymotrypsinogen-amylase	0.66	0.90
Chymotrypsinogen-lipase	0.74	0.93
Amylase-lipase	0.78	0.93
Chymotrypsinogen–total protein	0.47	0.69
Amylase–total protein	0.16 (N.S.)	0.70
Lipase–total protein	0.08 (N.S.)	0.76

In situ	Unstimulated	Cholecystokinin	Cholecystokinin plus Chymodenin
Chymotrypsinogen-amylase	0.66	0.72	0.82
Chymotrypsinogen-lipase	0.81	0.70	0.96
Chymotrypsinogen-trypsinogen	0.81	0.74	0.92
Chymotrypsinogen–total protein	0.80	0.83	0.96
Amylase-lipase	0.82	0.95	0.91
Amylase-trypsinogen	0.93	0.92	0.85
Amylase–total protein	0.91	0.97	0.90
Lipase–trypsinogen	0.86	0.96	0.92
Lipase–total protein	0.93	0.95	0.98
Trypsinogen–total protein	0.91	0.95	0.94

presentedin Table 1 and Fig. 2 for enzyme secetion in the in situ pancreas under thre sets of conditions: basal, cholecystokinin infusion at a rate sufficient to produce a modest increase in protein ouput, and continuous cholecystokinin infision with a superimposed single bolus of chymodenin. These studies revealed ahigh degree of linkage among enzymes during their secretion (Table 1), and simultaneous nonparallel changes in their ratios (Fig. 2). Under the in situ conditions, the correlations were extraordinarly high for a biological system, and correlation values approaching 1 were observed for specific enzyme pairs. The unstimulated gland in situ (which secretes protein at a rate 2 to 5 times that of the gland in vitro) gave a mean correlation of $r = 0.84$ ($r^2 = 0.71$) for all of the enzyme pairs; cholecystokinin infusion produced a correlation of $r = 0.89$ ($r^2 = 0.79$); and a chymodenin bolus superimposed on cholecystokinin infusion gave $r = 0.92$ ($r^2 = 0.84$).

These high correlations were accompanied by clear evidence of nonparallel secretion (Fig. 2). Infusion of cholecytokinin alone did not change the proportional relation in the secretion of paired enzymes from the values obtained under basal conditions, despite the 2.5-fold increase in overall protein output. However, superimposition of a chymodenin bolus on continual infusion of cholecystokinin resulted in a significant change in the regression relation for five enzyme pairs, without altering the rate of the ongoing protein output (*11*). The chymotrypsinogen-lipase pair, for example, shifted from secretion from one pool under the basal and cholecystokinin-stimulated conditions, to secretion from a second pool that was richer in chymotrypsinogen after chymodenin was added. Data obtained nearly a decade ago (Fig. 2, inset) showed a similar rapid, nonparallel shift, with chymotrypsinogen increased relative to lipase (*12*). The activity of chymodenin in selecting for secretion from a specific pool without altering the overall secretory rate is unique for a gastrointestinal hormone and is to be contrasted with the stimulation of overall digestive enzyme secretion and with stimulation of secretion of a single enzyme type. The regulation of the composition of the digestive enzyme mixture appears to be independent of the regulation of the rate of digestive enzyme secretion.

Thus, confrontation of the apparent paradox between nonparallel secretion and exocytosis produced clear evidence consistent with both processes in the same system. The pancreas appears to be a heterogeneous organ, secreting digestive enzymes from several distinct sources within the gland. Heterogeneity based on a morphological distinction between the total enzyme content of "peri-insular" and "tele-insular" acinar cells (*13*) has been reported but has never been connected to functional secretory data. Rothman and Isenman (*14*) showed

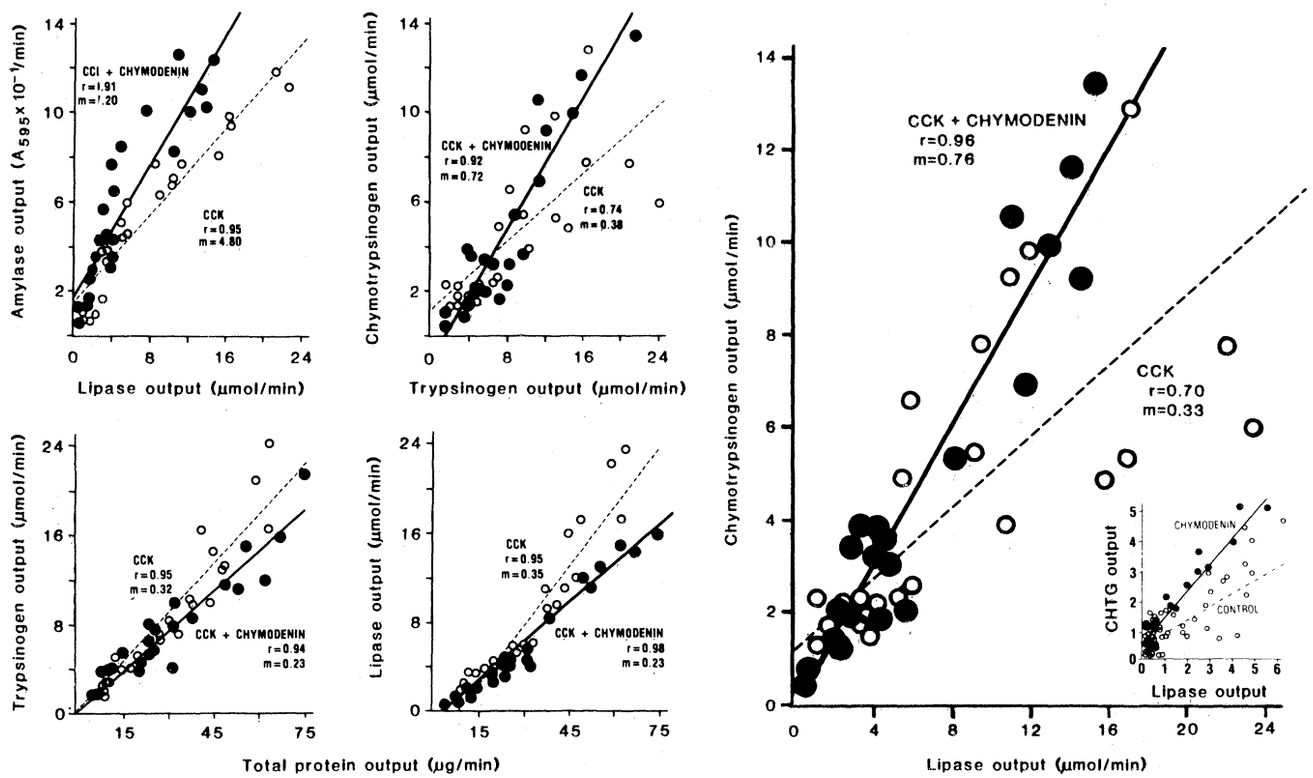

Fig. 2. Regression changes in slope of enzyme outputs by pairs after cholecystokinin (CCK) and chymodenin treatment of the rabbit pancreas in situ. Individual data points are shown for the hour after bolus injection of chymodenin (25 μg) superimposed on cholecystokinin infusion (1.6 IDU/per kilogram per hour) (●) and for controls that received diluent alone superimposed on cholecystokinin infusion (○). The computed regression lines, the correlation coefficients (r) from Table 1, and the slopes (m) are also shown. The slopes ± standard errors of the mean for cholecystokinin and for cholecystokinin plus chymodenin, respectively, were: amylase-lipase, 4.8 ± 0.03 and 7.20 ± 0.7; chymotrypsinogen-trypsinogen, 0.38 ± 0.07 and 0.72 ± 0.07; trypsinogen–total protein, 0.32 ± 0.02 and 0.23 ± 0.02; lipase–total protein, 0.35 ± 0.02 and 0.23 ± 0.01; and chymotrypsinogen-lipase, 0.33 ± 0.07 and 0.76 ± 0.05. There were four animals and 24 observations for each group. Correlations were significant at $P < 0.0001$. The slopes differed significantly for each enzyme pair at $F < 0.002$ or less as determined by a test for heterogeneity of slopes based on covariance of the regression lines (*9*). With the Bonferroni transformation, the probabilities are each significant at $F < 0.02$ or less (*9*). For comparison, the effects of chymodenin on the chymotrypsinogen-lipase pair in two different series of experiments conducted nearly a decade apart, are shown in the large figure (present series) and inset [1975 series (*12*)]. (In the inset, CHTG represents chymotrypsinogen.) In 1975, chymodenin was given as a bolus alone and compared with a saline control in vitro (for the control, $r = 0.53$ and $m = 0.395$ and for chymodenin treatment $r = 0.96$ and $m = 0.898$, the slopes being significantly different at $P < 0.001$).

functional heterogeneity in protein sources within the gland after a cholecystokinin- or methacholine-stimulated switch from a basal pool to a prestored pool of enzyme; their interpretation of the data, in terms of the equilibrium hypothesis, was that newly synthesized and old enzyme were mixed in the cytosol. In contrast, our data directly confirm that enzymes are secreted in groups, in a linked fashion from prepackaged organelles, exactly as expected of exocytosis. Instead of a unique secretory pathway as postulated in the original exocytosis model, our observations of nonparallel secretion under exocytotic conditions suggest the existence of multiple inter- or intracellular exocytotic pathways.

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8. This was the dose that was originally found to cause enhanced secretion of chymotrypsinogen in the pancreas in vitro (*12*). For a review of the chemistry and purification of chymodenin, see J. W. Adelson *et al.*, in *Gastrointestinal Hormones*, G. B. Jerzy-Glass, Ed. (Raven, New York, 1980), pp. 387–396.
9. The correlation coefficient for the output of each pair of enzymes in vitro was tested for an increase in the chymodenin treatment values over the control value by Fisher's conversion of the r values to a normal z distribution by the formula $z(r) = 1/2\ln[(1 + r)/(1 - r)]$ followed by a t-test of the differences of the z values [G. W. Snedecor and W. G. Cochran, *Statistical Methods* (Iowa State Univ. Press, Ames, 1980)]. The results were significant for chymotrypsinogen-amylase, chymotrypsinogen-lipase, amylase-lipase, amylase–total protein, and lipase–total protein. To further ensure that the high correlation coefficients obtained in vitro after chymodenin treatment were not an artifact, the data were challenged by testing the possibility that the variance of the numerical ratios of the enzyme outputs would decrease, as would be expected. The results were: chymotrypsinogen-lipase, $F = 0.002$; chymotrypsinogen–total protein, $F = 0.02$; amylase-lipase, $F = 0.0001$; amylase–total protein, $F = 0.002$; and lipase–total protein, $F = 0.02$. For chymotrypsinogen-amylase, F was 0.09. The chymodenin-treated population was not different from the control population before treatment: the mean pretreatment correlation for the chymodenin-treated population was $r = 0.73$ and for the control treated population was $r = 0.70$. When the data were divided into two groups, one above and one below the mean output of each enzyme, correlation analysis revealed that the coefficients were significant and comparable in magnitude to those of the entire population, indicating the lack of an anomalous effect of output range on the correlations obtained.
10. A statistical comparison of the mean correlation coefficients for enzyme output in the chymodenin-treated group with the mean correlation coefficients in control group would not be valid because the coefficients are not independent observations; thus the standard error cannot be derived. The increased correlations in the chymodenin-treated group were observed in six of six cases, were of a large magnitude, and were consistent with chymodenin causing the release of enzymes in more tightly linked groups than in the controls.
11. The enzyme output data were not distributed in isolated data clusters on an animal-by-animal basis; the data among individual animals overlapped with each other to a large extent. Further, Adelson and Rothman (*12*) earlier showed that the inter-animal and interperiod variances in enzyme output did not differ significantly. The differences of proportionality in situ were not significantly dependent on an initial pretreatment difference between the populations treated with cholecystokinin only and cholecystokinin plus chymodenin; when the two populations were tested separately for changes in the regression before and after chymodenin treatment, the changes in slope for the chymodenin-treated population were similar to those for time-paired controls.
12. One of us (J.W.A.) and Rothman reported earlier that chymodenin selectively stimulated pancreatic secretion of chymotrypsinogen; this was based on evidence that chymotrypsinogen secretion increased two- to threefold on stimulation by chymodenin whereas lipase secretion remained unchanged in experiments with the rabbit pancreas in vitro and in situ [J. W. Adelson and S. S. Rothman, *Science* **183**, 1087 (1974); *Am. J. Physiol.* **229**, 160 (1975)]. Although it appeared that chymotrypsinogen was secreted independently of other enzymes because its output increased compared with the output of lipase, which did not change, we believe that the original interpretation of the data was incorrect because this conclusion was arrived at without consideration of the link between the secretion of chymotrypsinogen and the secretion of lipase ($r = 0.90$) in both the earlier study and the present ones. Thus the two enzymes were secreted together in a fixed ratio of chymotrypsinogen to lipase, which was greater than that in the basal state.
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Immunogenicity of Synthetic Peptides from Circumsporozoite Protein of *Plasmodium falciparum*

Abstract. *In a study of recombinant proteins that might be useful in developing a vaccine against malaria, synthetic peptides from the circumsporozoite (CS) protein of* Plasmodium falciparum *were found to be immunogenic for mice and rabbits. Antibody to peptides from the repeating region of the CS protein recognized native CS protein and blocked sporozoite invasion of human hepatoma cells in vitro. Antibodies to peptides from regions I and II had no biologic activity, although antibody to region I recognized processed CS protein by Western blot analysis. These data support the feasibility of developing a vaccine against the sporozoite stage of the malaria parasite by using synthetic peptides of the repeating region of the CS protein conjugated to a carrier protein.*

When injected into humans and other animals, irradiated sporozoites of the malaria parasite, *Plasmodium*, provide protection against further challenge with viable sporozoites (*1–3*). This protection is mediated, at least in part, by antibodies to the circumsporozoite (CS) protein present on the sporozoite's surface (*4*). Recently, the CS genes encoding the CS proteins of *Plasmodium knowlesi* and *P. falciparum* were cloned (*5*) and sequenced (*6*). The CS gene for *P. falciparum* encodes for 41 tetrapeptide repeating units flanked by two regions showing homology between *P. falciparum* and *P. knowlesi*. It was suggested by Dame *et al.* (*6*) that these two small conserved sequences might have an important biological function and, along with the repeat region, be useful targets for vaccine development.

We synthesized peptides from the repeating and conserved regions of the CS protein of *P. falciparum* (Table 1) (*7*). Repeating sequences and peptides from region I were conjugated to bovine serum albumin (BSA) with the use of succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), or to thyroglobulin with the use of *m*-maleimi-