mixtures because of the incorporation of the racemic malonamic acid derivatives; compounds **Ic** and **Id** are single isomers since all the incorporated residues possess a single defined configuration. The in vitro biological activity of these enkephalin analogs is summarized in Table 1. Potency of analogs **Ia** and **Ib** is half that of the closest related analogs with a nonmodified backbone, that is, analogs **g** and **h**, respectively. Analogs **Ic** and **Id** are three times as potent as their corresponding analogs with a nonmodified backbone, that is, analogs **e** and **f**, respectively (see Table 1).

The four analogs were also tested in vivo by injecting them into the cisterna magna of the brains of rats. On a weight basis, **Ic** and **Id** appear to be as potent as β-endorphin in producing catatonia (*7*). The animals remain rigid, displaying general analgesia and a loss of the righting reflex for about 3 hours after an intracisternal injection of 10 μg of **1c** or **1d**. Analogs **Ia** and **Ib** produce a similar syndrome but with effects lasting only 30 to 45 minutes. This state is instantaneously reversed by an injection of naloxone, indicating an opiate receptor mediated effect. In both in vitro and in vivo tests, all the partially modified retro-inverso-isomers tested are considerably longer acting than any of the enkephalin analogs studied previously.

Our findings indicate that this novel approach to structural modification results in analogs with enhanced biological activity. The application of topochemical complementarity can yield spatially equivalent structures when viewed in an extended conformation as shown in Fig. 2. Structure **1** has the same spatial distribution of side chains when the end groups are matched together. Conceptually, the reversal of part of the peptide bonds should not alter biological activity. However, if the modification is carried out properly, compounds with new and enhanced activities result. Thus, the incorporation of *gem*-diamino-, malonyl-, and D-amino acid residues introduces additional possibilities for the synthesis of a new variety of biologically active peptide analogs.

MICHAEL CHOREV
RICK SHAVITZ
MURRAY GOODMAN
Department of Chemistry, University of California, San Diego, La Jolla 92093
SCOTT MINICK
ROGER GUILLEMIN
Salk Institute for Biological Studies, Laboratories for Neuroendocrinology, La Jolla, California 92037

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11. Abbreviations for the amino acid residues are Tyr, tyrosine; Gly, glycine; Met, methionine; Met-ol, methionineol; Met(O)-ol, methionineol sulfoxide; Ala, alanine; Phe, phenylalanine; *N*-MePhe, *N*methyl phenylalanine; Leu, leucine.
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Diffusion-Like Processes Can Account for Protein Secretion by the Pancreas

Abstract. *When fluid secretion by the pancreas was mechanically blocked, amylase secretion into the duct ceased. When flow was reduced in a graded fashion by the application of a back pressure, amylase output was reduced proportionately and amylase concentration in secretion was maintained constant. Thus, the secretion of digestive enzyme from the cell into the duct appears to be dependent upon the concentration of enzyme in the duct system. This behavior is most simply explained by diffusion-like (concentration dependent, bidirectional) fluxes of digestive enzyme across the plasma membrane. A unidirectional process, such as exocytosis, whose rate should be unaffected by fluid flow, cannot readily explain these results.*

Our concept of how proteins are secreted by cells has been greatly influenced by experimental studies on the secretion of digestive enzyme by the exocrine pancreas. During the past 25 years, a rather specific and complex model has been developed which has become the accepted paradigm for this process and for protein secretion in general (*1*). This hypothesis proposes that secretory proteins are "segregated" from the cytoplasm (and other newly synthesized proteins) in special membrane-bound compartments, and subsequently released from one of these intracellular compartments, the secretion granule, into the extracellular environment by a process called exocytosis. In exocytosis, the transfer of secretory product out of the cell is thought to be accomplished by a specialized fusion of the membrane of the secretion granule and the cell membrane, which produces a direct connection between the granule's contents and the extracellular environment.

A variety of experimental evidence from work on the pancreas has made us question this paradigm in general and the final step in the process (exocytosis) in particular (*2*). We have suggested that protein may leave the cell from a free cytoplasmic pool instead of or in addition to exocytosis. In this view, enzyme moves across specialized membranes, particularly granule and cell membranes, by a diffusion-like process in which fluxes are concentration-dependent and bidirectional. We have called this the "equilibrium" hypothesis.

The output or amount of protein that would be secreted (the net secretory flux) in a given state by the two mechanisms that have been proposed—exocytosis and equilibrium-dependent mem-

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brane transport—would depend upon different variables. For exocytosis, a unidirectional release mechanism, the rate of protein secretion would be determined intracellularly by the rate at which secretion granules fuse with plasma membrane; it would be essentially independent of the concentration of secretory product in the extracellular environment. On the other hand, secretion in an equilibrium-based system would be crucially dependent on the concentration of product in the extracellular compartment (the duct system for glands such as the pancreas).

In the present experiments, employing a preparation of whole pancreas in vitro (*3*), we used this difference to try to distinguish between these two mechanisms. When fluid secretion is artificially stopped, preventing outflow from the duct system by closing an indwelling catheter, or reduced by applying a back pressure to the column of fluid in the duct system, enzyme secretion should continue unabated if secretion occurs as proposed by the exocytosis model. The concentration of enzyme in the duct would simply increase with time as fusion continues. It should be able to increase greatly, certainly to the magnitudinally higher concentrations seen when secretion is driven by stimulants and perhaps as high or higher than the solubility of the enzymes themselves in water. In contrast, the membrane transport model predicts that the concentration of enzyme at the site of secretion and throughout the duct system would be limited to the value at which duct-to-cell and cell-to-duct fluxes become equal, that is, the concentration at which a net flux no longer occurs. This limiting value would either be the concentration of enzyme in the cytoplasm itself, for a simple equilibrium, or, if an active step occurred between cytoplasm and duct, then this limiting concentration would be that necessary to bring the net flux to zero. In either case, secretion should be greatly inhibited if enzyme is not removed from the site of secretion by the flow of fluid.

New Zealand White rabbits were anesthetized with a mixture of allobarbital (0.34 mmole per kilogram of body weight), urethan (3.7 mmole per kilogram of body weight), and monoethylurea (3.2 mmole per kilogram of body weight) administered intraperitoneally after a 17- to 20-hour fast. After the animals were laparotomized, the pancreatic duct was cannulated through the intestinal papilla with polyethylene tubing. The pancreas was then excised from the animal and mounted whole in a Plexiglas chamber by techniques described elsewhere (*3*). The gland was bathed in 400 ml of a bicarbonate-buffered Krebs-Henseleit solution (*4*), enriched with a defined mixture of amino acids (*5*), and 5.5 m*M* glucose. The fluid was gassed with 95 percent O_2 and 5 percent CO_2 and the temperature maintained at 31°C.

In some experiments, ductal secretion was collected for three 1-hour periods, followed by a 10-minute period, after which the end of the cannula was fused closed to prevent fluid flow. Blockage was maintained for 90 minutes, at which time the tube was reopened. Immediately following the resumption of flow (postblockage), the secretion products were collected for 15 minutes at either 2-, 3-, and two 5-minute intervals, or three 5-minute intervals, depending on the volume of secretion. Two additional 15-minute collection periods followed.

In other preparations, after a 1-hour equilibration period, a back pressure was applied to the duct system by raising the end of the cannula by increments of approximately 10 mm (0.74 mm-Hg back pressure), starting at the level of the papilla and continuing until secretion was reduced to almost zero (the organ was mounted vertically with the papilla in the most dependent position). The height of the column of fluid was then lowered back to the level of the papilla in steps of approximately 20 to 50 mm. At each level, secretion was collected for 5 to 10 minutes, depending upon flow rate. These sampling periods reflected steady-state flow for all levels at which the cannula was set; that is, for each setting an additional 5- to 10-minute collection period produced the identical flow.

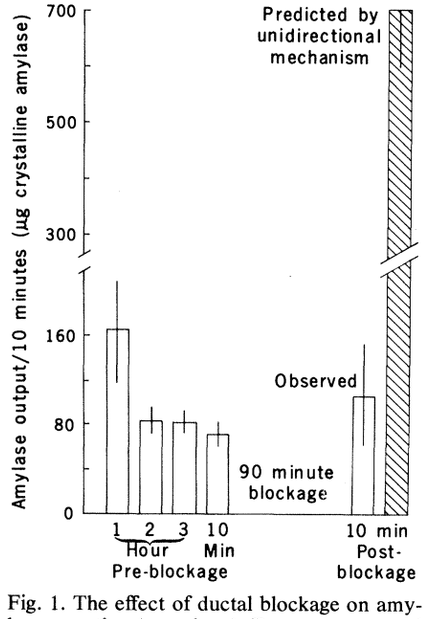


Fig. 1. The effect of ductal blockage on amylase secretion (open bars). Data are presented as mean amylase output per 10 minutes ± standard error (N = 4). Samples were collected at hourly intervals for 3 hours and for a 10-minute period immediately before blockade. Postblockage data are given for the first 10-minute period and compared to the value predicted by a unidirectional transfer mechanism (hatched bar). Secretion during the first period after blockage (10-minute postblockage) was close to that observed for a similar time period just prior to blockage, indicating that secretion did not accumulate to any degree in the duct system during blockade, as would have been expected if a vectorial or unidirectional secretory process accounted for enzyme secretion.

Secretion was assayed for amylase activity by a modification of the method of Rinderknecht *et al.* (*6*). Porcine α-amylase (2× crystallized) with a specific activity of 506 international units per milligram (Worthington) was used as a standard. Little change was seen in either amylase output (Fig. 1) or concentration in secretion collected immediately after a 90-minute duct blockade. The amylase concentration in the 10 minutes before blockage was 0.89 μg standard ± 0.18 μl (mean ± standard error) compared to 0.93 ± 0.27 μg/μl in the 2- to 5-minute postblockage (N = 5). Furthermore, amylase concentration in samples collected as long as 45 minutes postblockage remained relatively unchanged (5- to 15-minute interval, 0.72 ± 0.17 μg/μl; 15- to 30-minute interval, 0.64 ± 0.22; 30- to 45-minute interval, 0.80 ± 0.23). Similar results have been obtained in situ in anesthetized rabbits (data not shown). Thus, enzyme secretion virtually ceased in the absence of fluid secretion, with the minor exception of the filling of an apparent intraductal volume capacitance (*7*). If enzyme secretion had continued unabated during the 90-minute period, as the exocytosis model would predict, then the output and concentration of amylase should have been increased about an order of magnitude in samples collected immediately after blockage (Fig. 1).

When a graded back pressure was applied to the column of fluid in the duct, to reduce flow progressively, the results were similar. That is, the concentration of amylase remained relatively constant over the range of flows produced in this manner (Figs. 2 and 3). Output decreased in proportion to decreased flow, and amylase concentration did not increase as would have been expected if enzyme secretion had continued without decrement as flow was decreased (Figs. 2 and 3). For the lowest flows (highest back pressure), an increase in amylase

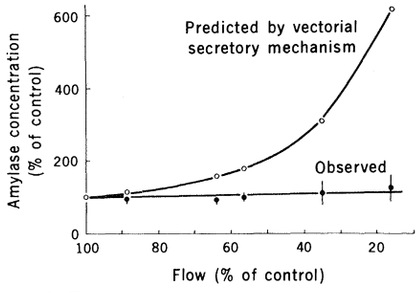


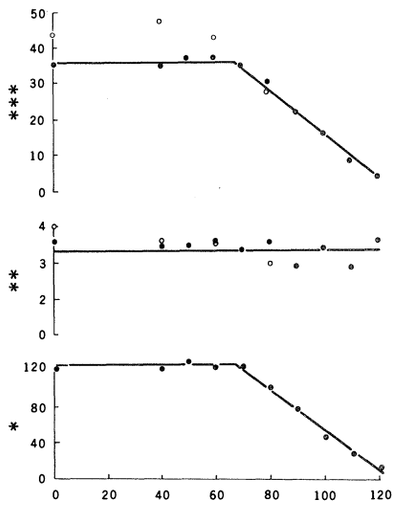
Fig. 2. The concentration of amylase in secretion as flow is progressively decreased by the application of a graded back pressure. The amylase concentration observed in secretion (closed circles) remained essentially constant over the wide range of flows studied. Data represent the mean ± standard error for four experiments. Open circles represent the increase in concentration that would be predicted if enzyme secretion continued at a constant rate independent of fluid flow, as in a unidirectional (vectorial) secretory process.

concentration may not have been seen for a particular period because of a possible time delay in traversing the potential dead space of the duct system. We checked for this by continuing to measure amylase concentration in secretion when the procedure was reversed, and the height of the column sequentially lowered back to the level of the papilla. No increase in enzyme concentration was observed under these conditions (Fig. 3).

The hydrodynamic pressure of secretion (the secretory pressure) is thought to be the consequence of the osmotic movement of water into the duct lumen, and fluid secretion should cease when this pressure is counterbalanced by an equal and opposite pressure such as the applied end pressure in our experiments. Thus, the pressure gradient which determines the rate of fluid flow (intraductal pressure minus back pressure) is altered with changing end pressure. Increases in column height, however, did not reduce flow at the steady state until approximately 90 ± 16 mm-H_2O (mean ± standard error) (6.7 mm-Hg) above the papilla (Figs. 3 and 4). Therefore, up to this point, the system compensated in some manner for the diminished driving force. Above 90 mm-H_2O, fluid flow decreased in a linear fashion as the applied back pressure was increased (Figs. 3 and 4).

We should consider the possibility that the inhibition of enzyme secretion was a direct effect of the applied hydrostatic pressure, rather than an indirect effect caused by the reduction in fluid flow. If this were so, then hydrostatic pressure would have had to have altered both fluid flow and enzyme secretion identically; that is, both the pressure at which inhibition begins as well as the rate of decline after its onset would have to have been exactly the same for the two supposedly independent variables, and this seems unlikely. Furthermore, a similar coupling of fluid flow and enzyme secretion has been observed when flow was reduced by means other than an applied back pressure (see note added in proof).

These experiments demonstrate that the outflow of water from the duct system is a critical determinant of the net flux of digestive enzyme across the apical or duct-facing plasma membrane of the secretory (acinar) cell. That is, the net transfer of enzyme across this membrane ceases when the "excretion" of water ceases, and is reduced proportionately when flow is reduced in a graded fashion. Such behavior suggests and is consistent with the hypothesis that the net flux of enzyme across the membrane, its "secretion," is dependent upon the transmembrane enzyme concentration gradient, as proposed by the equilibrium theory. The prediction of the unidirectional or "vectorial" exocytosis hypothesis that the concentration of enzyme would greatly increase in ductal fluid during blockage was not borne out by the evidence. Of course, we cannot exclude the possibility that a vesicular mechanism of much greater complexity than has been envisioned in the past might exist that could account for the present results (*8*).



*Amylase output/5 minutes (μg crystalline amylase)
**Amylase concentration (μg crystalline amylase/μl)
***Flow (μl/5 minutes)

Fig. 3. Fluid secretion (flow), amylase concentration, and amylase output as a function of back pressure applied to the column of fluid in the pancreatic duct for a single experiment (closed circles). As back pressure was increased, both flow and output decreased in tandem leaving concentration essentially unchanged. Pressure is given in millimeters of water above the level of the pancreatic papilla (see text). Open circles represent values for flow and concentration when the back pressure was reduced back toward zero.

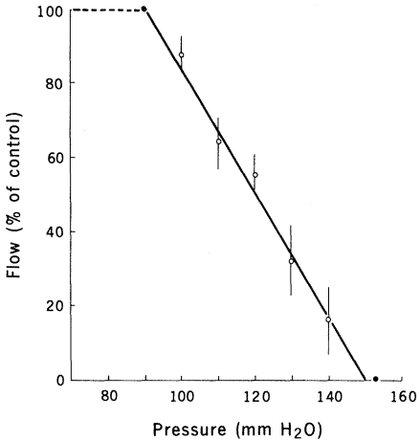


Fig. 4. Flow as a function of the applied back pressure. Data are given as the mean ± standard error for four experiments. The mean fall in flow, relative to the period prior to the decline, is shown for each 10-mm-H_2O increment in pressure (open circles) and is set to the average value for the beginning of the decline (90 mm-H_2O; closed circles). The average zero flow intercept was 154 mm-H_2O (closed circle), which is in good agreement with the intercept of 150 calculated from the regression line.

Our observations are explained most simply by a diffusion-like process. In this context, the fluxes across the membrane in the free-flow situation must have maintained the enzyme concentration in the duct system at, or close to its maximum value for the gland's unstimulated state. This is so because the enzyme concentration in the duct remained constant instead of increasing during both total blockage and reduced outflow conditions. Thus, either the proteins are in equilibrium across the apical membrane (that is, the concentration of free enzyme in the cytoplasm and the duct lumen are roughly the same), or a steady-state concentration gradient is maintained between cell and duct (presumably by an energy-dependent efflux process). In either case, the net secretory flux would be produced by the removal of fluid from the secretory site (*9*).

Note added in proof: Ho and one of us (S.S.R.), using the same biological preparation, have shown a similar constancy in the enzyme concentration of secretion in the stimulated state in the face of decreasing fluid secretion produced by either ouabain or a low Na^+ medium,

rather than by mechanically reducing outflow. Therefore, a reduction in water and salt secretion by whatever means can apparently produce proportional decreases in enzyme secretion.

LOIS D. ISENMAN
S. S. ROTHMAN

Department of Physiology,
School of Medicine,
University of California,
San Francisco 94143

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7. Flow during the first collection period of 2 to 5 minutes after 90 minutes of blockage was substantially augmented relative to prior control flow rates (2.7 times on the average). The total cumulative increase in flow over controls amounted to a mean increase of 95 μl. This suggests that the duct system has a substantial capacitance, most probably in the larger collecting vessels. The capacitance would be of about the same volume as that of the duct itself, as estimated by extracellular space markers in the free-flow situation [M. Rossier and S. S. Rothman, *Am. J. Physiol.* **228**, 1199 (1975)]. The presence of a capacitance can also be inferred in another manner. When an open cannula was raised to the column height at which flow just ceased, flow began again in a matter of seconds. The column was continuously raised in this manner, until flow stopped permanently. This is consistent with the gradual filling of a capacitance.
8. The more complex model of bidirectional exocytosis or exocytosis linked to endocytosis cannot explain the present results in a simple fashion. For example, in a bidirectional model, the reduced output would presumably result from increased reuptake of enzyme. However, increased reuptake would have to be regulated relative to flow in order to maintain the concentration of enzyme in the duct system constant over a wide range of flow and during total blockage; that is, it would have to increase in exact proportion to the reduction in flow. Furthermore, in order to explain the fact that the concentration of enzyme in the duct increases and decreases independently of flow under stimulated conditions, we would have to further hypothesize that the regulated reuptake could itself be regulated; namely, the relationship between protein efflux from the cell and its reuptake could be varied independently of flow.
9. These experiments also bear upon another aspect of pancreatic secretion. It has been proposed that cells lining the ducts, as opposed to or in addition to cells in or near the acinus, are responsible for the addition of a substantial proportion of the secreted fluid [I. Schulz, A. Yamagata, M. Weske, *Pfluegers Arch. Gesamte Physiol. Menschen Tiere* **308**, 277 (1969); S. A. Mangos and N. R. McSherry, *Am. J. Physiol.* **221**, 496 (1977)]. If this is true, then the acinar secretion of digestive enzyme would be diluted by fluid as it traveled down the ducts. In this case, during a flow reduction produced by blockage or back pressure, the concentration of enzyme throughout the duct system would increase over time toward that at the site of enzyme secretion. Since the concentration of enzyme did not increase during 90 minutes of blockage, it can reasonably be concluded that an admixture of a significant amount of fluid by cells lining the ducts during spontaneous flow does not occur.
10. We thank Dr. J. Ho and R. Fielding for their valuable discussions. Supported by NIH research grant AM16990 and training grant GM00927.

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Tissue Perfusion Rate Determined from the Decay of Oxygen-15 Activity After Photon Activation in situ

Abstract. *Rates of cerebral perfusion were obtained from measurement of the disappearance (wash-out) of oxygen-15 after in situ tissue activation with 45-million-volt x-rays. In an anesthetized cat, typical values were 90 milliliters per minute per 100 grams of tissue, with 55 percent wash-out. In a specific radiotherapy patient, the value was 65 milliliters per minute per 100 grams of tissue, with 63 percent wash-out of oxygen-15 through incorporation into tissue water.*

Continuous perfusion of all body tissues by blood is required for their vitality. Measurement of the distribution of cerebral perfusion in patients with occlusive cerebral disorders can reveal the location of infarcted tissue and the degree to which blood flow to this tissue can be restored by surgical, radiotherapeutic, or pharmacological intervention. In cancer patients, the rate of tumor perfusion influences significantly the transport of both oxygen and drugs to malignant tissue volumes. Measurement of tumor perfusion and its manipulation by the introduction of heat, anesthesia, and vasoactive drugs can lead to improved radiotherapy and chemotherapy. The radioisotope clearance technique, which utilizes gamma and positron cameras to image the accumulation (wash-in) or removal (wash-out) of radioactivity in the region of interest after the intravascular injection or inhalation of radiopharmaceuticals, has been employed to study regional tissue perfusion in animal and human subjects (*1*, *2*). However, the technique has often been limited in its ability to quantitate blood flow in poorly perfused tissue. The signal obtained from the area of low blood flow can be substantially contaminated by detected emissions from adjacent, well-perfused tissue, with its correspondingly greater uptake of radioactive indicator (*3*). Where clinically feasible, injection of the indicator directly into the poorly perfused area can, in principle, overcome the above limitations, and has indeed been employed to study tumor perfusion by radioisotope wash-out (*4*). However, the results obtained by direct injection can be influenced significantly by the degree to which the microcirculation is perturbed by tissue pressure gradients, and by the failure of the injected indicator to adequately diffuse throughout the relevant volume prior to the initiation of radioisotope imaging.

Radioactive isotopes of carbon, nitrogen, and oxygen can be introduced directly, uniformly, and noninvasively into the tissue region of interest through activation of the tissue by high-energy radiation. Reports of activation of tissue elements in vivo by 33- and 45-MV x-rays (*5*, *6*) and 200-MeV protons (*7*) may be found in the literature. We report here the results of quantitative studies of regional tissue perfusion employing in situ activation by photons in a 45-MV clinical betatron x-ray beam. Interaction of beam photons with tissue element nuclei produces ^{11}C, ^{13}N, and ^{15}O, all of which decay by positron emission, with half-lives of 20.5, 10.0, and 2.0 minutes, respectively.

An emitted positron interacts with tissue atoms and, with an atomic electron, is quickly annihilated, producing two gamma-ray photons of energy 0.51 MeV, approximately 180° apart. After activation, the positron activity from a given volume of irradiated tissue is monitored as a function of time, employing standard coincidence counting apparatus and techniques to detect pairs of annihilation gamma rays. Analysis of the time dependence of positron emission from a static, tissue-like gelatin cylinder revealed that the separate contributions to the total activity from ^{15}O and ^{11}C could be identified, and that the contribution from ^{13}N was negligible. The analysis also established that, during the first few minutes after the completion of activation, nearly all of the coincidence signal was due to the decay of ^{15}O in the activated volume. Therefore, in our studies of tissue perfusion, the radioactive indicator must be ^{15}O, and the rate of tissue perfusion must ultimately be determined from the measured half time for wash-out of this radioisotope from the imaged volume of interest.

The time dependence of the measured decay of ^{15}O activity from a volume of living tissue is governed by the fate of this radioisotope after it is produced. The ^{15}O nuclei emerge from photonuclear reactions as recoil ions moving through the tissue medium. A certain fraction of these "hot" ions will attach to cell proteins (or protein fragments) and, as the labeled macromolecules cannot diffuse through capillary walls, the decay of the signal from such a "fixed fraction" will be purely physical, with a half-life of 2.0 minutes. The measure of

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