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CHYMOTRYPSINOGEN · INOSITOL PHOSPHATIDE COMPLEXES AND THE TRANSPORT OF DIGESTIVE ENZYME ACROSS MEMBRANES

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Summary

Chymotrypsinogen A was almost quantitatively extracted from aqueous solution in the presence of inositol phosphatides at relatively low concentrations of both ligands. Calcium ion facilitated the interaction at concentrations of 10^{-4}—10^{-5} M. A water-insoluble chymotrypsinogen · Ca^{2+} · inositol phosphatide complex was formed with an apparent stochiometry of 3 mol phospholipid : 3 mol Ca^{2+} : 1 mol protein. Small changes in the structure of the protein prevented complex formation; in particular, the almost identical α-chymotrypsin, did not form complexes under the conditions studied. On the other hand, an homologous, but structurally substantially different, secretory protein, trypsinogen, did form complexes. Water-insoluble complexes were not formed with albumin, carbonic anhydrase or lactic dehydrogenase under the same circumstances. Neither phosphatidylethanolamine nor phosphatidylcholine formed complexes with chymotrypsinogen. Phosphatidylserine formed complexes, but was less effective than inositol phosphatides. Complex formation and stability was dependent upon "critical" concentrations of both Ca^{2+} and H^+. Extraction of the protein from solution increased from neglible to complete when the calcium concentration of the medium was raised slightly from $1.0 \cdot 10^{-4}$ M to $1.5 \cdot 10^{-4}$ M. Conversely, dissociation was complete when H^+ concentration was decreased slightly from pH 6.5 to 7.0. The complex is apparently formed as the result of specific electrostatic interactions between the polar head group of the inositol phosphatide and the protein, with the nonpolar alipathic fatty acid chains of the phospholipid providing a hydrophobic microenvironment for the protein. It is proposed that such complexes could account for the movement of digestive enzyme through membranes.

Introduction

Kinetic evidence suggests that the digestive enzymes produced by the pancreas cross certain biological membranes individually or a small polymers in

response to diffusional forces, i.e., movement is bidirectional and dependent upon concentration gradient [1—11]. The digestive enzymes are water-soluble globular proteins with many polar amino acid side-chains on their surface and their movement across the non-polar lipid bilayer at the membrane's core requires that these charges be masked or otherwise neutralized.

This article describes the formation of complexes between a digestive enzyme, chymotrypsinogen A, and amphipathic phospholipids present in biological membranes, particularly the inositol phosphatides, which results in the essentially complete extraction of dissolved protein from the aqueous phase. Such complexes could account for the movement of charged globular proteins through non-polar regions of biological membranes.

Methods

Chemicals. The following proteins were used: chymotrypsinogen A (bovine, Worthington Biochemical Co. and Calbiochem); α-chymotrypsin (bovine, Worthington Biochemical Co.); bovine serum albumin (Calbiochem); trypsinogen (bovine, Worthington Biochemical Co.); carbonic anhydrase (bovine red blood cell, Worthington Biochemical Co.) and L-lactate dehydrogenase (yeast, Worthington Biochemical Co.).

The following phospholipids were used: inositol phosphatides (from brain cephalin, ICN) [12,13]; phosphatidylcholine (β,-γ-dipalmitoyl-DL-α-lecithin) (ICN); phosphatidylethanolamine (Fraction V, Schwarz-Mann); phosphatidylserine (Schwarz-Mann); L-α-phosphatidylinositol 4-monophosphate (bovine brain, Sigma) [14]; and L-α-phosphatidylinositol 4,5-diphosphate (bovine brain, Sigma) [14].

Quantitative analysis. Protein was estimated with the Folin phenol reagent [15] using standard curves made to each protein. Phospholipid was estimated from its phosphorus content [16]. The calcium content of precipitates was estimated isotopically by adding $^{45}Ca^{2+}$ (0.2 μCi to each test tube) (Amersham-Searle) to a solution of known $CaCl_2$ concentration and applying tracer equilibration kinetics.

Chymotrypsinogen assay. The sample containing 34.5 μg of protein was added to 1.0 ml of 0.05 M Tris (tris(hydroxymethyl)aminomethane) buffer (pH 8.1) containing 10 μg crystalline trypsin (Worthington Biochemical Co.)/ 100 μg bovine serum albumin (fraction V)/0.05 mmol $CaCl_2$. This activation mixture was incubated at 4° C for 2 h [17,18].

The activation mixture was then added to 1.0 ml of 0.1 M Tris buffer (pH 7.4) and 3.0 ml of the substrate (80 mM suspension of acetyl tyrosine ethyl ester in 30% methanol) and H^+ liberation followed at 25° C at pH 7.4 by pH-stat techniques. The initial rate of substrate hydrolysis was followed.

Experimental procedure. The formation of phospholipid · protein complexes was tested for by mixing equal volumes (either 0.5 or 1.0 ml) of varying concentrations of different proteins, phospholipids, and calcium salt. The turbidity of the resultant mixture was measured at 540 nm to estimate the extent of precipitation. Very small precipitates or light scattering changes that may occur when complexes are formed in the absence of precipitation are not detectable by this method. Quantitative estimates of precipitate formation were made

chemically after separation of the precipitate from the solution by centrifugation (10 000 × *g* for 10 min). The order in which reagents were added did not alter the extent of precipitation in any of the cases tested. In some situations, after the precipitate was separated from the supernatant an additional precipitate formed. The date presented below only include the material present in the first sediment. For chymotrypsinogen · inositol phosphatide complexes it contained approximately 90% of the total precipitable material.

Results

The chymotrypsinogen · inositol phosphatide complex

Phosphatides. Over 90% of the chymotrypsinogen A could be extracted from solution in a precipitate when inositol phosphatides were added to the medium (Fig. 1 and Table I). Visible precipitates were formed at inositol phosphatide concentrations as low as 17 μM in the presence of calcium ion *. When pure inositol phosphatides [14] (L-α-phosphatidylinositol 4-monophosphate or L-α-phosphatidylinositol 4,5-diphosphate) were tested instead of the Folch-Pi mixture of mono-, di- and tri-phosphatides [12,13], equivalently effective extraction was observed (Table II). Another polar phospholipid, phosphatidylserine, was somewhat less effective. It only extracted about 50—60% of the chymotrypsinogen from solution (Table II). For the same conditions, protein-containing precipitates were not formed with two Zwitterionic phosphatides, phosphatidylcholine and phosphatidylethanolamine.

When the concentration of inositol phosphatides in the mixture was increased, the turbidity of the suspension also increased reaching an apparent plateau between 0.33 and 0.83 mM phosphatide for the concentrations of ligands shown in Fig. 1. An additional increase in turbidity was seen when the phosphatide concentration was increased further to another plateau between 1.0 and 1.25 mM phosphatide (Fig. 1). Above 1.25 mM phosphatide concentration there was a precipitous decrease in turbidity, about 85% reduction between 1.25 and 1.33 mM (Fig. 1). This sharp decrease in the extent of precipitation as the result of a small increase in phosphatide concentration suggests the attainment of a critical micellar concentration at which "pure" phospholipid micelles form which exclude chymotrypsinogen; i.e., the phospholipid activity in solution is dramatically reduced by increasing its concentration.

Proteins. Chymotrypsinogen was fully extracted from solution at concentrations ranging from 20 to 100 μM. The percentage of enzyme precipitated (about 90%) remained relatively constant over this range as long as calcium and phosphatide concentrations were changed roughly in proportion to chymotrypsinogen (Table I). When the concentration of chymotrypsinogen was increased in the absence of concomitant increases in the concentration of the other two ligands, an apparent maximum capacity for extraction was approached (Fig. 2, +calcium). Trypsinogen, a digestive enzyme with considerable structural homology to chymotrypsinogen, also formed precipitates with inositol phos-

* Inositol phosphatide molarity is an estimate based on an assumed average of 2 gram atoms of phosphorus per mole of phospholipid; assuming a molecular weight of 900 and determining molarity by weight gives a roughly comparable value (+11%).

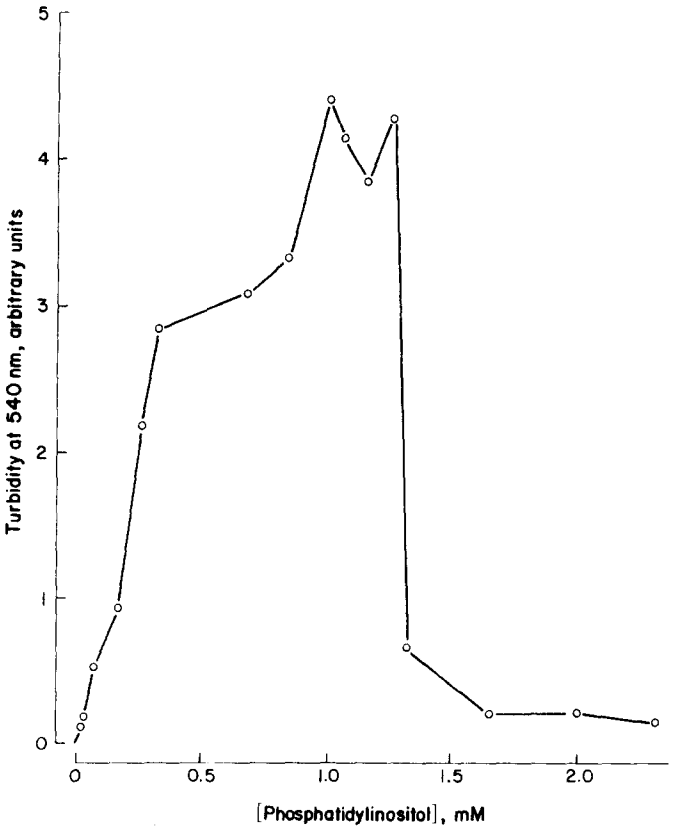

Fig. 1. The effect of inositol phosphatide concentration on the formation of inositol phosphatide · Ca^{2+} · chymotrypsinogen complexes as estimated from the turbidity of suspensions. Chymotrypsinogen and $CaCl_2$ were present at 41 μM and 0.3 mM concentrations, respectively. Values are the mean of a minimum of 3 observations at each point.

phatides. On the other hand, α-chymotrypsin, an active form of chymotrypsinogen A, did not form precipitates under the same conditions (Table I). Serum albumin, which also complexes with inositol phosphatides [19], was not extracted from solution in any quantity under these conditions (Table I and Fig. 3). We were unable to demonstrate the precipitation of two cytoplasmic enzymes, carbonic anhydrase and lactate dehydrogenase, in the presence of inositol phosphatides (Table I).

Calcium. Calcium ion greatly enhanced the propensity for precipitate formation, and while chymotrypsinogen · inositol phosphatide complexes were formed in its absence, higher concentrations of protein were required (Fig. 2). (From the turbidity data in Fig. 2 the apparent K_a was decreased by about 60%.) Under circumstances in which calcium enhances precipitation (i.e., at relatively low concentrations of chymotrypsinogen), its presence is crucial, and precipitation was not observed in its absence (Figs. 2 and 3).

Only relatively low concentrations of calcium ion were required for precipitation and essentially complete extraction of the protein was obtained at calcium concentrations as low as $6 \cdot 10^{-5}$ M, the lowest concentration tested

TABLE I

PROTEIN-PHOSPHOLIPID INTERACTIONS: VARYING PROTEIN

All values are for a minimum of three separate measurements done in duplicate or triplicate. For the two conditions for which S.E. is shown, $n = 5$ for α-chymotrypsin and 12 for trypsinogen.

Protein	% Protein sedimented	Other reagents
Chymotrypsinogen A (bovine) (20.5 μM)	95.0	0.17 mM PI * 0.06 mM $CaCl_2$ (A)
Chymotrypsinogen A (41 μM)	87.5	0.34 mM PI 0.12 mM $CaCl_2$ (B)
Chymotrypsinogen A (75 μM)	90.0	0.68 mM PI 0.21 mM $CaCl_2$ (C)
Chymotrypsinogen A (102.5 μM)	93.0	1.0 mM PI 0.30 mM $CaCl_2$ (D)
α-Chymotrypsin (bovine) (20.5 μM)	8.4	Same as A
α-Chymotrypsin (41 μM)	11.3	Same as B
α-Chymotrypsin (75 μM)	3.1	Same as C
α-Chymotrypsin (102.5 μM)	6.7 ± 1.1	Same as D
Trypsinogen (102.5 μM)	55.7 ± 3.4	Same as D
Bovine serum albumin	3.4	Same as D
Serum albumin (14.9 μM)	11.4	1.0 mM PI 5.0 mM $CaCl_2$
Carbonic anhydrase (bovine erythrocyte) (13.3 and 80.7 μM)	no visible precipitation	Same as D
L-Lactate dehydrogenase (yeast) (10 μM)	no visible precipitation	1.0 mM PI 5.0 mM $CaCl_2$

* PI, inositol phosphatide.

(Tables I and II). Moreover, the calcium concentration necessary for protein precipitation had a critical range. For the conditions shown in Fig. 3, precipitation was minimal at 0.00010 M calcium and became virtually maximal when its

TABLE II

PROTEIN-PHOSPHOLIPID INTERACTIONS: VARYING PHOSPHOLIPID

All values are for a minimum of three separate measurements, done in duplicate or triplicate.

Phospholipid	% Protein sedimented	Other reagents
Phosphatidylcholine (0.17 mM)	3.8	20.5 μM chymotrypsinogen 0.06 mM $CaCl_2$ (A)
Phosphatidylethanolamine (0.17 mM)	5.7	Same as A
Phosphatidylserine (0.17 mM)	52.8	Same as A
Phosphatidylinositol (0.17 mM)	95.0	Same as A
L-α-Phosphatidylinositol 4-monophosphate (bovine brain) (0.34 mM)	98.9	41 μM chymotrypsinogen 0.12 $CaCl_2$
L-α-Phosphatidylinositol 4,5-di phosphate (bovine brain) (0.34 mM)	95.4	41 μM chymotrypsinogen 0.12 mM $CaCl_2$

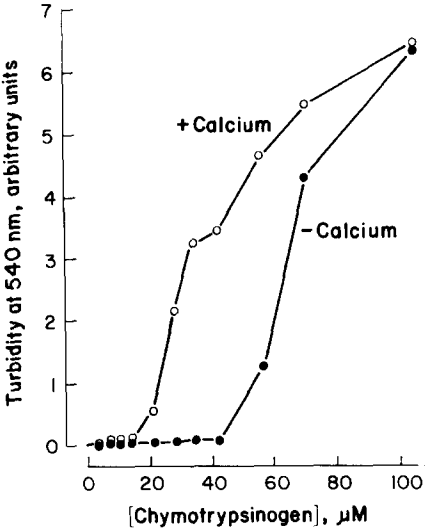

Fig. 2. The effect of chymotrypsinogen concentration on the formation of inositol phosphatide · chymotrypsinogen complexes as estimated from the turbidity of suspensions in the presence (open circles) or absence (closed circles) of calcium ion. Inositol phosphatide concentration was 1.0 mM and $CaCl_2$ concentration 0.3 mM when present. Values are the mean of a minimum of 4 observations at each point for "+calcium" and 3 for "—calcium".

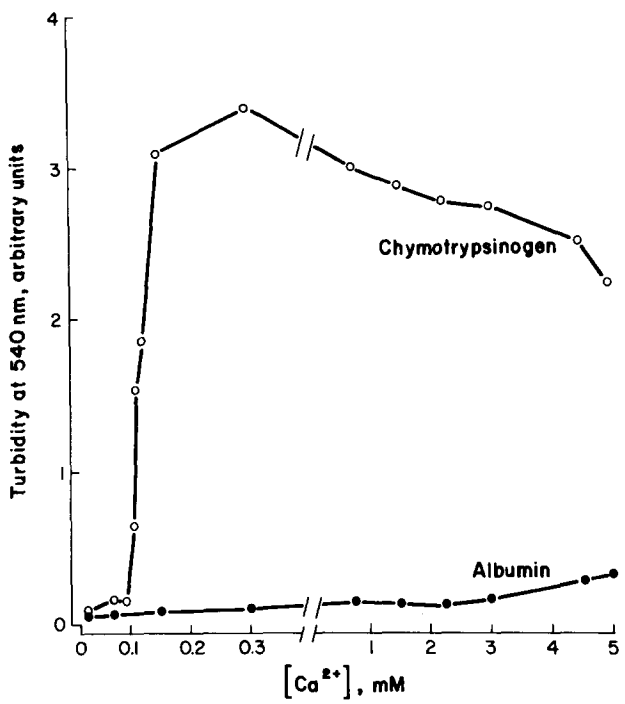

Fig. 3. The effect of calcium concentration on the formation of inositol phosphatide · chymotrypsinogen and inositol phosphatide · albumin complexes as estimated from the turbidity of suspensions. Chymotrypsinogen (open circles) concentration was 41 μM and albumin (closed circles) concentration was 15 μM. Inositol phosphatide concentration was 1.0 mM for both albumin and chymotrypsinogen cases. Values are the mean of a minimum of 3 observations at each point for both chymotrypsinogen and albumin.

TABLE III

STOCHIOMETRY OF THE INOSITOL PHOSPHATIDE · CALCIUM · CHYMOTRYPSINOGEN COMPLEX (mol/mol)

The number of measurements included in the calculation is shown in parentheses.

Phosphorus · protein *	7.02 ± 0.05 S.E. (34)
Inositol phosphatide · protein (assuming 2 mol P/mol phospholipid)	3.51
Inositol phosphatide · protein (assuming molecular weight 900)	3.15
Calcium · protein **	2.99 ± 0.25 (12)
Phospholipid · calcium · protein	≈3 : 3 : 1

* For A, B, C, and D in Table I.
** For A and D in Table I.

concentration was increased by 0.00005 M; an extremely sharp concentration dependence at a relatively low calcium concentration. As the concentration of calcium was increased above 1 mM, its presence interfered with precipitate formation and the turbidity of the suspension was gradually reduced by increasing the calcium concentration (Fig. 3). Even though albumin · inositol phosphatide complexes are also calcium requiring [19], the low concentration of calcium that was effective in enhancing chymotrypsinogen precipitation did not produce albumin-containing precipitates (Fig. 3 and Table I).

The stochiometry of the chymotrypsinogen · inositol phosphatide complex. Relatively few moles of phospholipid were able to extract a mole of chymotrypsinogen from solution. The ratio of phosphorus to protein in the precipitate was 7.02 ± 0.05 S.E. (calculated from the slope ± the error of the regression for a plot of 34 observations made with various concentrations of the ligands (A, B, C and D as given in Table I)) (Table III). An assumed average of 2 moles of phosphorus/mole of phospholipid gives a phospholipid to protein ratio of 3.5 : 1; while an average molecular weight of 900 for the phospholipid gives a ratio of 3.15 : 1.

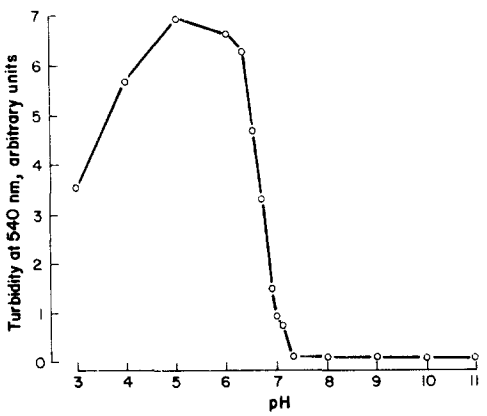

Fig. 4. The effect of pH on the formation of inositol phosphatide · Ca^{2+} · chymotrypsinogen complexes as estimated from the turbidity of suspensions. Inositol phosphatide, $CaCl_2$, and chymotrypsinogen were present at 1.0 mM, 0.3 mM, and 102.5 μM concentrations, respectively. Values are the mean of 3—4 observations at each point.

TABLE IV

EFFECT OF IONIC STRENGTH ON THE STABILITY OF THE INOSITOL PHOSPHATIDE · CHYMOTRYPSINOGEN COMPLEX

Salt solution	% Protein in sediment
1 M NaCl *	2.1, 1.3
1 M KCl	13.0, 9.4
0.15 M NaCl	2.8, 1.9
0.15 M KCl	1.6, 1.7
H_2O alone	89.7, 94.4

* 1 ml each of the same reagents as D in Table I plus 1 ml of salt or H_2O as indicated.

Similarly, the molar ratio of calcium to chymotrypsinogen was 2.99 ± 0.25 S.E. for precipitates formed with the same inositol phosphatide mixtures (A and D in Table I) (Table III). Thus, the overall average molar ratio for phospholipid : calcium : protein was approximately 3 : 3 : 1.

The effect of pH. The stability of the complex was dependent upon the pH of the medium. The complex was most stable at slightly acidic pH (between 5 and 6.5) and was completely dissociated at slightly alkaline pH. A sharp decline (over 90%) in the affinity of the ligands for each other occurred over a relatively small ΔpH of 0.5 units between pH 6.5 and 7.0 ($\Delta(H^+)$ = 0.4 mequiv./l) (Fig. 4).

The formation of the complex does not damage or denature the protein since activation was equally effective whether the proenzyme was activated after release from the complex at pH 8.0, after mixing the reagents together at pH 8.0, or alone.

The effect of ionic strength on complex stability. The chymotrypsinogen · inositol phosphatide complex was sensitive to the presence of ions. Both NaCl and KCl, at either 0.15 M or 1.0 M concentrations, effectively disrupted the precipitate (Table IV).

Discussion

Chymotrypsinogen A, a water-soluble secretory protein, was almost quantitatively extracted from aqueous solution with inositol phosphatides at relatively low concentrations of both ligands. Calcium, at concentrations of the order of 10^{-4}—10^{-5} M, facilitated the phospholipid-protein interaction. A specific complex that is insoluble in water appears to have been formed. The specificity of the interactions is suggested by the following:

(1) The ligands associated with considerable avidity and formed precipitates at relatively low concentrations.

(2) The precipitate contained relatively few moles of phospholipid per mole of enzyme (about 3 to 1). That is, interactions with a relatively small number of phospholipid molecules (or relatively few "masking" interactions) dramatically reduced the protein's solubility in water. This suggests that the surface of the protein may be specifically ordered for such interactions. In this regard, over 50% of the aromatic amino acid side-chains, usually thought of as being buried within the hydrophobic core of water-soluble globular proteins such as

chymotrypsinogen, are situated on or close to its surface [20].

(3) There appears to be a constant stochiometric relationship between the ligands with the mean molar ratios approximating integers (7 moles phosphorus : 3 calcium : 1 protein) (Table II).

(4) Small changes in protein structure can prevent complex formation. α-Chymotrypsin, an active form of chymotrypsinogen A which is identical to the "parent" molecule except that it lacks 4 amino acid residues (Nos. 14, 15, 147, and 148) and has a slightly altered tertiary structure, does not form complexes under the conditions described above. On the other hand, a wholly different, although homologous, secretory protein from the pancreas, trypsinogen, with approximately 50% of its amino acids in congruent positions, complexes effectively.

(5) Complex formation is dependent upon critical concentrations of Ca^{2+} and H^+, i.e., complexing increased from minimal to maximal when the calcium concentration was increased slightly ($0.5 \cdot 10^{-4}$ M from 1.0 to $1.5 \cdot 10^{-4}$ M), and conversely, the complex almost completely dissociated when H^+ concentration was increased by 0.5 pH unit from pH 6.5 to 7.0. The latter is consistent with deprotonation of a single (type of) amino acid residue on the protein.

Since the complexes are insoluble in water, are readily disrupted by ions, and are not formed with non-polar phospholipids, the amphipathic or amphiphilic nature of the effective phospholipids appears to be central to their action, viz., electrostatic bonds are formed between their polar head groups and the surface of the protein and the non-polar aliphatic chains, facing outward, provide an hydrophobic covering or hydrophobic microenvironment for the protein.

The existence of the complex suggests a relatively simple hypothesis for the movement of chymotrypsinogen, and perhaps other digestive enzymes, through biological membranes. Briefly, interactions with an amphipathic molecule, such as a polar phospholipid within the membrane (or free in the cytoplasm) could favor the entrance of the macromolecule into the membrane's hydrophobic core. In this way, single molecules or small groups of molecules could pass through the membrane by a type of facilitated or reaction-mediated diffusion. Since the complexes form spontaneously, the phase transition would be energetically favorable and represent the lowest free energy state for the molecules involved. Thus, the movement of special, large, ostensibly water-soluble molecules, such as secretory proteins, through membranes, could in a special and limited sense, be a relatively simple matter of diffusion. In its simplest form, this hypothesis would predict that different proteins cross the membrane in the same undifferentiated manner. Specificity would be imparted solely by differences between proteins in the association and dissociation constants for the phospholipids, and the transfer or reorientation constant for the complex in the membrane. Thus, while each molecule would be transported independently and have a characteristic k_t, its transport capacity would not necessarily be constant, but would vary as a function of the relative availability of the different transportable proteins for membrane sites, and the availability of phospholipids to carry out the transfer. Of course, the presence of protein-specific recognition sites could impart independent transport maxima for the individual proteins.

Dissociation of the protein from the outer (secretory) surface of the

membrane must be more likely than dissociation from its inner (intracellular) surface in order to produce a net secretory flux at the steady-state. Although such a differential dissociation could be produced in numerous ways, one possibility is suggested by the fact that pancreatic secretion is alkaline. Thus, a proton gradient probably exists across the apical membrane of the acinar cell which could account for the net flux of protein. That is, association would be more likely at the relatively acidic intracellular membrane surface, while dissociation would be more likely at the slightly alkaline pH of the duct lumen; much the same as seen with the complex in vitro. Thus, a net flux would be produced as long as the secretory product were removed from the site of its secretion, as it is by the flow of fluid.

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