significant amounts of lower molecular weight apoB and apoA-IV in contrast to corresponding normal lipoprotein fractions. The results of immunodiffusion studies (Fig. 2) indicate the absence of apoE in the proband's plasma, and its presence in normal plasma and in the plasma of the proband's offspring. The proband's plasma contained apolipoproteins A-I, A-II, B, C-I, C-II, and C-III as tested by radial immunodiffusion with a mean apoC-II plasma concentration of 6.0 mg/dl (normal, 2.5 ± 1.5 mg/dl).

Chylomicrons released by intestinal epithelial cells contain apolipoproteins B, A-I, A-II, and A-IV (*12, 14, 15*). Most lymph chylomicron apoB is comprised of lower molecular weight apoB or "B-48" (*12*). Chylomicrons in lymph appear to acquire significant quantities of the C apolipoproteins, presumably as a result of transfer from HDL which has filtered from plasma into lymph (*16*). After entry into plasma, lymph chylomicrons are acted on by lipoprotein lipase, resulting in the hydrolysis of triglyceride and the transfer of apoA-I, apoA-II, the C apolipoproteins, and lipid to HDL (*15*), and the formation of chylomicron remnants within the VLDL and IDL density region (*17*). Chylomicron remnants contain both apoB and apoE (*5*). On the basis of studies with radioactively labeled chylomicrons in man, only a very small fraction of chylomicron apoB is transferred to LDL (*15*). The chylomicron remnants are rapidly removed from the circulation by the liver, and this catabolic process appears to be mediated in part by an apoE receptor in the rat. The importance of this receptor-mediated process in man is unknown (*5*). This uptake phenomenon is enhanced by estrogen administration (*18*). Estrogens have been reported to ameliorate the hyperlipidemia in type III HLP female subjects in contrast to other forms of hyperlipidemia (*19*). Most patients with type III HLP have increased plasma concentrations of an abnormal apoE as demonstrated by IEF (*6*), and this protein abnormality results in a delayed catabolism of triglyceride-rich lipoproteins because of a decreased hepatic uptake (*6, 7*).

Members of the kindred with type III HLP described herein, because of their lack of detectable plasma apoE, differ from other known type III HLP kindreds, all of which have shown increased amounts of an abnormal apoE. In addition, these subjects had only mild hypertriglyceridemia, increased LDL cholesterol, and a much higher ratio of VLDL cholesterol to plasma triglyceride than reported in other type III HLP subjects. Apolipoprotein A-IV and lower molecular weight apoB (B-48), two lymph chylomicron apolipoprotein constituents generally not found in normal plasma IDL and LDL, were present in significant quantities in the IDL and LDL of these patients with apoE deficiency. These data are consistent with the following concepts: (i) apoE is important for the catabolism of chylomicron remnants; (ii) apoE deficiency results in the accumulation of chylomicron remnants in plasma, type III HLP, tubo-eruptive xanthomas, and premature coronary artery disease; and (iii) apoE deficiency represents a new disease entity.

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Intestinal Diffusion Barrier: Unstirred Water Layer or Membrane Surface Mucous Coat?

Abstract. *The dimensions of the small intestinal diffusion barrier interposed between luminal nutrients and their membrane receptors were determined from kinetic analysis of substrate hydrolysis by integral surface membrane enzymes. The calculated equivalent thickness of the unstirred water layer was too large to be compatible with the known dimensions of rat intestine. The discrepancy could be reconciled by consideration of the mucous coat overlying the intestinal surface membrane. Integral surface membrane proteins could not be labeled by an iodine-125 probe unless the surface coat was first removed. The mucoprotein surface coat appears to constitute an important diffusion barrier for nutrients seeking their digestive and transport sites on the outer intestinal membrane.*

Before a solute in the small intestinal lumen can interact with receptors for hydrolysis or transport, it must pass through a diffusion barrier, which modifies the kinetics of nutrient assimilation (*1*). It has been suggested that this barrier is an unstirred water layer located at the intestinal lumen–membrane interface (*1*).

We have examined the intestinal diffusion barrier by means of a kinetic analysis of the surface membrane hydrolases, sucrase, lactase, and aminooligopeptidase. These enzymes are known to operate at the luminal-cell interface of the intestinal mucosal cell (*2, 3*); when the kinetics of rat jejunum enzymes in vivo are compared to those of the isolated, pure enzymes (*4–7*), the effective thickness of the overlying water diffusion barrier can be calculated (*8*).

Carbohydrates were made up to 0.3*M* in deionized water and diluted with buffer (0.14*M* NaCl and 0.01*M* sodium, po-

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Table 1. Kinetic and physical parameters for three substrates, and values calculated for the thickness of the unstirred water layer from Eq. 1.

Physical parameters*	Substrate		
	Sucrose	Lactose	Gly-Leu-Gly-Gly
V_{max} (mole sec^{-1} cm^{-2})	1.6×10^{-8}	1.6×10^{-9}	1.9×10^{-9}
K_{ob} (m*M*)	96	27	9.0
K_m (m*M*)	20 (*5*)	20 (*4*, *6*)	0.5 (*7*)
$D \times 10^{-6}$ (cm^2/sec)	7.4 ± 0.1	7.3 ± 0.1	6.9 ± 0.2
δ (mm)	0.70	0.64	0.62

*K_{ob} is the observed half-saturation constant for intact intestine in vivo; K_m is the true K_m for appropriate pure isolated intestinal hydrolase; D is the diffusion coefficient; and δ is the unstirred layer thickness assuming it consists of water at a viscosity of 0.695 centipoise.

tassium phosphate, *p*H 7.4) to maintain osmolality at 300 mosmole/kg. From male Sprague-Dawley rats (150 to 200 g) we prepared 10-cm jejunal segments. We performed the perfusion studies on these segments at 0.4 ml/min (*9*), except when we measured lactose. Because of the relatively low capacity of brush border lactase for surface hydrolysis, we increased the segment for the measurement to 20 cm and decreased the perfusion rate to 0.2 ml/min. Peptide concentration was determined by an automated ion exchange system (*9*). Disaccharide concentration was determined by scintillation counting of ^{14}C-labeled glucose disaccharides (New England Nuclear) corrected for free luminal glucose determined by a glucose oxidase method (*4*).

Since disaccharide substrates are not absorbed intact and disappear only by virtue of surface hydrolysis by their appropriate brush border hydrolase, the rate of substrate disappearance from the lumen can be equated with hydrolytic rate of the enzyme (*9*).

Kinetic parameters were calculated from six concentrations of each substrate. Individual hydrolysis rates for each concentration, based on disappearance of substrate from the lumen, represent the mean of three consecutive collections following a 20-minute equilibration perfusion in each of four animals (N = 12). Hydrolysis of sucrose, lactose, and Gly-L-Leu-Gly-Gly displayed typical hyperbolic kinetics, and values for the Michaelis constant and maximum velocity were determined from Lineweaver-Burk plots (*10*) by least-squares fitting (r = .9 or higher).

Diffusion coefficients (D) were determined in perfusion buffer at 37°C by a modification of the agar gel diffusion method (*11*) at substrate concentrations which resulted in half maximum hydrolysis in vivo. In this system, disaccharides were determined by scintillation counting and peptides by the fluorescamine reaction (*12*). The D values (Table 1) closely approximated those calculated by the Stokes-Einstein equation or, for lactose and sucrose, values determined by others under similar conditions (*13*).

When the kinetic values of isolated pure brush border enzymes and those determined from intact intestine in vivo are known, the equivalent thickness of an aqueous unstirred diffusion layer, which would reconcile differences in the Michaelis constants for the two systems, can be calculated by rearranging Winne's equation (*8*):

$$\delta = \frac{D\,(K_{ob} - K_m)}{0.5\,V_{max}} \qquad (1)$$

where K_m is the Michaelis constant of the pure enzyme; K_{ob}, that of the surface membrane enzyme in vivo; V_{max} is the maximum rate for the surface-bound enzyme; and δ is the effective thickness of the unstirred layer.

Table 1 shows the calculated values of δ for each of the three substrates. These enzymes reflect three distinct combinations of kinetic parameters; high K_{ob}, high V_{max}; high K_{ob}, low V_{max}; and low K_{ob}, low V_{max}. Yet, in each instance, substitution of the experimental K_{ob}'s and known K_m's into Eq. 1 yielded an equivalent aqueous unstirred layer of 0.6 to 0.7 mm. This value is somewhat greater than that determined by nonenzymatic techniques in vitro where vigorous agitation was used to minimize stasis over the intestinal membrane (*14–16*); but our results agree closely with a recent study performed in human jejunum in vivo (*17*). Notably, an unstirred layer of 0.65 mm requires that more than one-quarter of the internal diameter and nearly one-half of the volume of the rat intestinal lumen is occupied by the diffusion barrier. This implies more intestinal fluid stasis than is generally believed to exist when fluid flows through a hollow cylin-

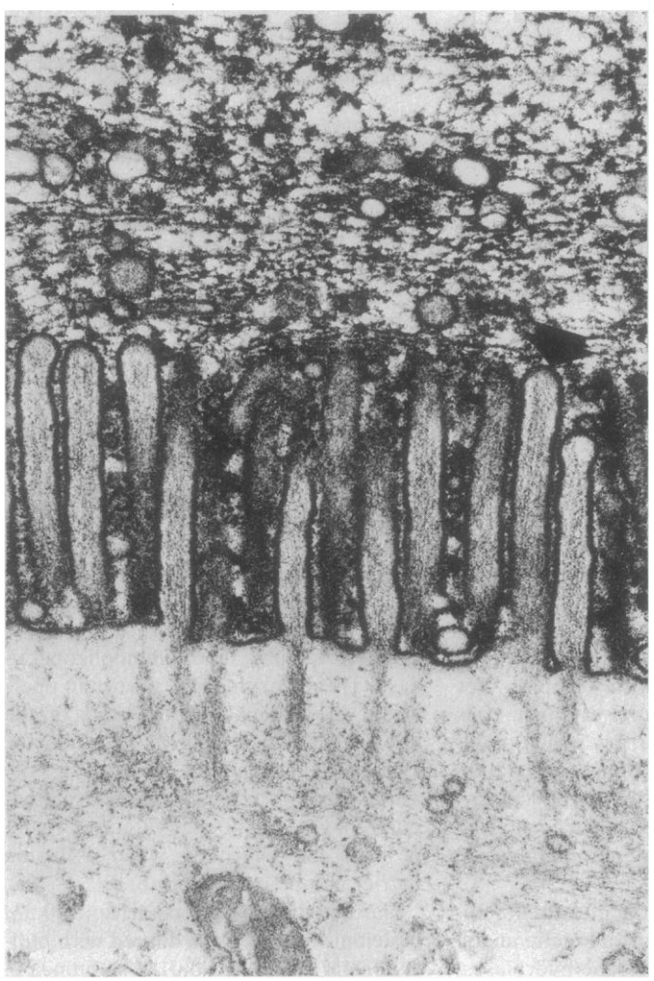

Fig. 1. An electron micrograph of the surface region of a rat jejunal cell. The lighter area in the lower half of the photograph is the cell cytoplasm with its fingerlike microvillus projections. The preparation was first stained with ruthenium red (*27*) (prior fixation in osmium tetroxide and staining with uranyl acetate lead citrate) to identify the glycocalyx at the microvillus surface (arrow) and the thick overlying surface coat (4.3 ± 1.0 μm; mean ± standard error). The dimensions can be appreciated by comparing with the microvillus length (1.6 ± 0.2 μm) (×40,000).

der such as the gut. This relatively thick, unstirred water layer is based on an intestinal surface area given by the minimum cylinder model (1.57 cm^2 per centimeter in length) (*9*, *15*). If the villar convolutions are also considered, the surface area would be about ten times greater and the calculated equivalent thickness of the unstirred layer would then be 8 mm, a value greater than the total interior diameter of rat jejunal lumen. That the minimum cylinder model best represents intestinal microanatomy is also supported by studies of Boom *et al.* (*18*) demonstrating that osmium tetroxide, a small neutral molecule, binds to intestinal cells at the villar tips; access to cells further down the villus is limited to that occurring in the deeper regions of solid organs by virtue of slow diffusion through the tissue.

Although a change in tertiary structure and consequent alteration in enzyme kinetics might occur when a protein is separated from its membrane environment, intestinal hydrolases display the same kinetic mechanisms after being solubilized from the membrane (*19*). Furthermore, each of the intestinal enzymes yielded similar values for the unstirred layer thickness (Table 1). Thus comparative kinetic analysis of surface hydrolases of intact intestine with those purified to homogeneity appears to be a valid means of estimating the diffusion barrier.

The seeming unphysiological depth of the hypothetical unstirred water layer prompted us to consider another possible intestinal interfacial diffusion barrier. The solute most intimately juxtaposed to the intestinal cell membrane is a specialized mucous coating, a complex suspension of mucoproteins, glycoproteins, glycolipids, and probably membrane proteins in the process of being degraded (*19*). The gastrointestinal mucous coat forms a tightly bound gel that appears to severely restrict the diffusion of protein molecules (*20*), but little information is available on the role of mucus as a barrier to smaller molecules such as the final nutrient peptide or saccharide products which must diffuse to their hydrolase or transport sites on the membrane. Recently, gastric mucus has been shown to constitute a major diffusion barrier for hydrogen ions (*21*). Although the mucous coat forms a highly viscous gel (*22*), it is highly hydrated and its barrier properties probably are also a function of the effective size of the water-filled pores established by its extended carbohydrate chains.

As shown in Fig. 1, there is a generous mucous coat overlying the convoluted intestinal surface membrane that is more than twice the thickness of the microvilli. We attempted to study intact intestine devoid of its mucous coat, but removal of the surface coat from everted intestinal sacs (*23*) by treatment with neuraminidase, proteases, or detergents was unsuccessful because the underlying membrane was concomitantly damaged. However, isolated brush border membranes (*24*) with luminal surface facing outward (*25*) were found to be free of the mucous coat when examined by electron microscopy (not shown). We exposed rat everted small intestinal sacs and isolated brush border surface membrane vesicles to $Na^{125}I$-labeled lactoperoxidase (*26*) in parallel experiments. As Fig. 2 shows, many membrane proteins were readily labeled in isolated brush border membranes, but no significant labeling occurred when the intact intestine was exposed to the probe. Hence, tyrosine residues of integral intestinal membrane proteins do not appear to be available to the ^{125}I-labeled lactoperoxidase at the surface of intact intestine, probably because they are protected by the overlying mucous coat.

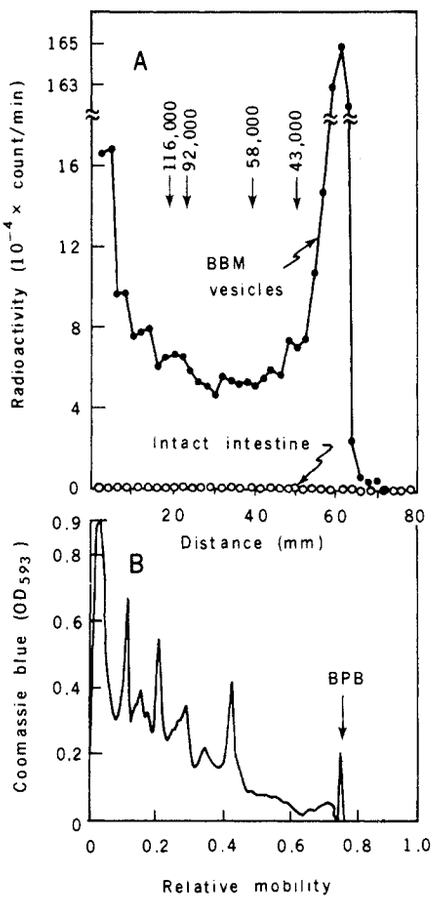

Fig. 2. (A) Sodium dodecyl sulfate acrylamide electrophoresis of intestinal brush border membrane proteins labeled with ^{125}I-lactoperoxidase. In parallel experiments, the intact everted intestinal sac (*Intact intestine*) (*23*) and isolated brush border membrane (*BBM*) vesicles (*24*) were exposed to $Na^{125}I$-labeled lactoperoxidase (*26*). Brush border membranes, prepared from the gut sac, and the directly labeled membrane vesicles were then solubilized with 0.1 mg of papain per 100 mg of tissue weight for 1 hour (*28*), and the supernatant obtained after centrifugation at 100,000*g* for 1 hour was passed through a 2.5 by 45 cm bed of Bio-Gel P-60 to remove free radioligand and small peptides. After concentration by vacuum dialysis and exposure to 1 percent sodium dodecyl sulfate at 100°C for 30 minutes a portion [80 μg of protein (*29*) in 50 μl] of solubilized membranes from the gut sac experiment (intact intestine) and from the isolated vesicle experiment (*BBM vesicles*) was applied separately to 7.5 percent gels containing 0.1 percent sodium dodecyl sulfate (*30*). After electrophoresis, 2-mm slices were cut and dissolved in 30 percent hydrogen peroxide for analysis of radioactivity. (B) A representative duplicate gel of the final membrane preparation was stained with Coomassie blue G250 and analyzed in a Gilford 250 scanning spectrometer. Positions of standard proteins (β-galactosidase, 116,000; phosphorylase, 92,000; catalase, 58,000; ovalbumin, 43,000) are shown in (A); *BPB*, bromphenol blue.

Our experiments indicate that the diffusion barrier at the intestinal surface cannot be accounted for solely by a simple unstirred water layer. Instead, on the basis of both the kinetics of surface hydrolysis and the surface protein labeling with a radioactive ligand probe, we conclude that the intestinal mucous coat is an important diffusion barrier for nutrients and other oligomers that have to be digested, transported, or bound to receptor sites on the outer intestinal membrane.

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22 June 1981

Integration and Stable Germ Line Transmission of Genes Injected into Mouse Pronuclei

Abstract. *Genetic material has been successfully transferred into the genomes of newborn mice by injection of that material into pronuclei of fertilized eggs. Initial results indicated two patterns of processing the injected DNA: one in which the material was not integrated into the host genome, and another in which the injected genes became associated with high molecular weight DNA. These patterns are maintained through further development to adulthood. The evidence presented indicates the covalent association of injected DNA with host sequences, and transmission of such linked sequences in a Mendelian distribution to two succeeding generations of progeny.*

The successful introduction of exogenous DNA into cultured mammalian cells (*1–4*) has led to the development of a novel gene transfer system that has yielded new information about gene regulation in higher eukaryotes. One difficulty with this system is that cultured cells are not capable of organismal development and differentiation. DNA sequences cloned by recombinant DNA technology can be microinjected into the pronuclei of fertilized mouse oocytes and can be subsequently located in the DNA of newborn mice (*5*). This system allows the study of transferred gene sequences in the context of normal embryonic development. Since development is a process that includes maturation to adulthood, reproduction, and senescence, it is important to examine the fate of transferred genes beyond the point of birth. We have now followed this injected material through further stages of mouse development.

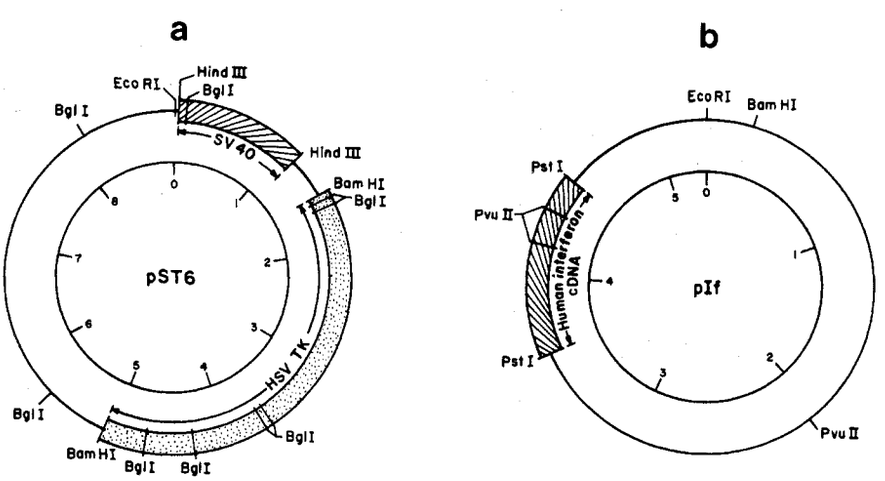

Fig. 1. Simplified diagrams of recombinant plasmids used for microinjection of mouse embryos. The Eco RI site of pBR322 is marked at 0 kb for reference.

Two recombinant plasmids were used for microinjection. The first, designated pST6 (*5*), was composed of the Hind III C restriction endonuclease fragment of SV40 virus and the herpes virus thymidine kinase (TK) genes cloned in plasmid pBR322; the second, pIf (*6*), contained human leukocyte interferon complementary DNA (cDNA) also cloned in pBR322 (*7*). A simplified diagram of each plasmid with its relevant restriction sites is shown in Fig. 1. Between 1000 and 35,000 copies of each plasmid were injected into each zygote. All microinjections were carried out as described (*5*).

The feasibility of producing such genetically transformed mice, which we call "transgenic" mice, depends upon several factors. Our experience has been that higher copy number gives a higher rate of transformation, but that the viscosity of concentrated preparations increases embryo mortality at the time of injection. Injection of 1000 copies of pST6 gave a survival rate of 50 to 70 percent with a third of the survivors eventually giving rise to live young. About 1 in 30 of such young retained transferred genes (*5*). When 30,000 copies of this plasmid were injected, embryo survival was reduced to 30 to 50 percent, but 1 in 15 mice retained the sequences. The pIf plasmid is smaller than pST6 and was therefore more easily injected. Survival of microinjection of 10,000 copies of this plasmid varied between 50 and 75 percent. Ten mice were born from 33 embryos thus far implanted, a rate which compares well with survival rates of embryos injected with pST6 (*5*). Of these ten mice, one was transgenic. This rate appears higher than that obtained from pST6 injections, but statistically significant numbers allowing a rigorous comparison of these experiments are not yet available.

Southern blot hybridization has been used to evaluate plasmid sequences in newborn and adult mice (*5, 8–10*). In the case of adults, DNA was extracted from spleens. Whether or not the donor material was integrated into the host genome was assessed by three criteria: (i) the acquisition of resriction sites in the host genome but not in the recombinant plasmids, (ii) the mobility of plasmid sequences in agarose gels when the DNA applied to the gels was undigested, and (iii) the ability of the plasmid sequences to be transmitted through the germ line to succeeding generations.

Two mice (73 and 9.02) injected with either pST6 or pST9 (pST9 is identical to pST6 except that the orientation of the SV40 insert is reversed) and one mouse

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