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GASTROINTESTINAL ABSORPTION OF INTACT PROTEINS

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INTRODUCTION

It is commonly assumed either (*a*) that dietary proteins are digested completely to free amino acids within the lumen of the gastrointestinal tract before absorption occurs, or (*b*) that only trace amounts of macromolecular fragments enter the circulation and that these are of absolutely no nutritional, physiological, or clinical relevance. The first of these assumptions is blatantly untrue. It is now known that intestinal peptide transport is a major process, with the terminal stages of protein digestion occurring intracellularly after transport of peptides into the mucosal absorptive cells (68–71). Also, there now is irrefutable evidence that small amounts of intact peptides and proteins do enter the circulation under normal circumstances (31, 33). The second assumption is a gross simplification, but it does highlight two areas in which current knowledge is seriously deficient, namely the actual quantitative sig-

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nificance of intact protein absorption and the biological and medical relevance of this process and of abnormalities in it. These two aspects are particularly emphasized in this review.

Intact protein absorption must now be regarded as a normal physiological process in humans and animals. There is a good deal of indirect evidence suggesting that augmented absorption of intact proteins into the circulation may be pathologically significant, and there are numerous diseases for which the pathophysiology is poorly understood and for which hypotheses implicating enhanced permeability of the gastrointestinal tract to macromolecules have been postulated (98, 103–107). But, while there is no doubt that intestinal permeability is increased in a wide range of diseases, it must be stressed that there is not yet enough evidence to prove a causal link between enhanced intact protein absorption and disease. There also are grounds for believing that decreased absorption of intact protein may be disadvantageous but this too is not proven.

Understanding of these areas is in its infancy and needs to be encouraged vigorously. It is timely to do so now as there have recently been noteworthy advances in several aspects of gastrointestinal function. These are discussed in the following sections.

THE GUT AS A "BARRIER" It is no longer tenable to regard the gut as an impenetrable "physical" barrier, since many particles and large molecules previously regarded as "nonabsorbable" are in fact absorbed (18, 31); indeed, some of these are now used diagnostically to measure intestinal permeability or "leakiness" (37, 76). Information about gut leakiness in various diseases, both gastrointestinal and systemic, is accruing and is proving helpful in understanding factors that reduce the barrier function of the gastrointestinal tract. (See the section below on measuring intestinal permeability.)

PEPTIDE TRANSPORT Peptide transport systems, distinct from free amino acid carriers, have been characterized in intestinal brush-border membranes and are now thought to play a major role in protein assimilation (68–71). Intracellular hydrolysis of small peptides thus becomes an important terminal stage of protein digestion. The concept that significant amounts of small peptides can escape total digestion to amino acids and enter the circulation in intact form is a new one, but it is gaining acceptance (31, 33); one potentially important consequence of this is that biologically or pharmacologically active peptides arising during protein digestion may reach peripheral tissues (including central nervous system) in active form and the effects may be profound (32, 35). While, in general, the effects of intact biologically active peptides entering the circulation are likely to be deleterious (31), there is interest in enhancing the process so as to permit oral administration of peptide drugs (21)

and similar considerations can be applied to the development of oral vaccines and other protein drugs. Enhancement of intestinal permeability, interference with gastrointestinal hydrolysis, and reduction in protein/peptide digestibility by chemical modification are potential approaches.

THE GUT AS AN IMMUNE AND A NEURAL ORGAN The small and large intestines are now regarded as important immune organs; macromolecular antigen transport appears to be a normal and essential part of immune function, and specific cells adapted for this function are now recognized (see below). Also, it is now clear that there is a highly developed enteric nervous system ("a brain in the bowel") (29), and recent evidence shows that electrolyte and fluid movements are under local neural control (87). Hence, the possibility that similar interrelationships exist to control or modulate macromolecular transport should not be neglected.

Evidence for Intact Protein Absorption

Probably the most compelling single item of evidence showing that intact proteins or macromolecular fragments of them are absorbed is provided by the demonstration, repeatedly made by numerous independent workers, that antibodies to many food proteins and their immune complexes occur in the circulation of healthy individuals—probably all individuals (e.g. 6, 19, 40, 41, 60, 82, 90). Furthermore, increased levels of antibodies are found in individuals with various diseases likely to promote intestinal "leakiness," such as celiac disease (27, 40), although Pitcher-Wilmott et al disagreed (85). While it is theoretically possible that such antibodies might arise through the intestinal immune system responding to luminal proteins rather than absorbed ones, analyses of plasma by radioimmunoassay now show the presence of orally administered proteins, such as ovalbumin in blood (55, 58): these show a time-course or tolerance curve generally similar to that for absorbed amino acids or peptides. Hence, it is impossible to escape the conclusion that immunologically significant amounts of intact protein (or immunologically identifiable large fragments thereof) have been absorbed.

This conclusion is reinforced by numerous animal and isolated-tissue experiments. For example, McLean & Ash (73, 74) have reported the time-course of appearance of intact (or largely intact) horseradish peroxidase in blood and several peripheral tissues in fish in vivo: approximately 0.001% (rainbow trout) or 0.7% (carp) of the oral dose of 20 mg was detected in intact form in the tissues examined, which did not include muscle or brain. Several studies, notably those by Walker and his associates (96, 97, 108, 109) and by Desjeux and his coworkers (25, 50, 52) have demonstrated passage of high-molecular-weight fragments of protein across isolated animal jejunum.

There also have been numerous demonstrations that inert particles [e.g. carbon particles in Indian ink (102)] and viruses (e.g. 112) can cross healthy intestine. The cells responsible have been identified as M cells (see below). Additionally, we have rather dramatic evidence provided by the drastic consequences of botulism, in which a high-molecular-weight fragment ($\sim 10^6$ daltons) of protein has been shown to cross the intestine (references cited in 31).

While all these techniques have limitations, the concordance between results obtained by independent workers using different experimental approaches is now so strong that we cannot fail to accept that intact proteins and high-molecular-fragments thereof do cross the gastrointestinal tract in humans and animals (both neonates and adults).

Main Shortcomings in Current Knowledge

It is suggested that the main shortcomings needing most immediately to be critically addressed lie in five areas.

1. While we know enough to conclude that macromolecular absorption is not a large-scale process in adults, it is not possible yet to state with reliable accuracy what fraction of the protein in a nutritionally complete meal will enter the circulation in macromolecular form. Yet this quantification is a most important question.
2. What physiological factors regulate intestinal "closure" in humans? How complete is "closure" under normal circumstances? How can therapeutic intervention or infant feeding practices alter the process of "closure," and can objective benefits thus be gained?
3. Do the currently used probes of intestinal permeability reflect permeability to intact proteins?
4. Fuller understanding is needed of the possible role of increased intestinal permeability and/or of intact protein absorption in diseases, including food sensitivities (and putative "food allergies"), celiac disease, inflammatory bowel disease (including Crohn's disease), irritable bowel syndrome, eczema, rheumatoid arthritis, and schizophrenia. (Wherever possible physiological parameters should be assessed and subjective observations eliminated.)
5. Can intact macromolecule absorption be enhanced sufficiently to permit oral administration of peptide and protein drugs and vaccines?

Methodological Difficulties

Inadequacies in methods for quantification of intact protein absorption, possibly coupled with some reluctance hitherto to recognize the importance of such quantification, is probably primarily responsible for the deficiencies in our knowledge already mentioned. Some technical difficulties are identified below, but a novel technical approach to the problem would be welcome.

RADIOISOTOPE TRACING OF MACROMOLECULES Radiolabelling affords a convenient method for following the progress of a molecule, or part of it, but it must be recognized that degradation of the parent molecule followed by rapid reincorporation of the label can occur and thereby lead to false interpretations of experimental results. Likewise, small fragments produced by partial degradation of a labelled molecule may bind to endogenous proteins and thus create an erroneous impression of the molecular size of the absorbed fragments; this was shown by Udall et al (95) to have led to led to exaggerated estimates of the extent of macromolecular absorption. Also, Skogh (92) showed that serious overestimation of absorption of high-molecular-weight fragments of ^{125}I-labelled protein would arise but that it could be minimized by simultaneously giving a large dose of nonradioactive iodide. Recycling of the label can be excluded by confirming the immunological identity of the labelled species, but this is not infallible because relatively small fragments can be immunologically cross-reactive; hence, size measurements also need to be made. Proof that the label is still attached to the original molecule in substantially unchanged form is remarkably hard to provide and has often been neglected. Limm & Rowley (64) also encountered problems with radio-iodinated protein, and they advocated use of ^{3}H-dinitrophenyl labelling for studies in vivo, although this too is not exempt from the problems discussed by Udall et al (95). Use of the ELISA (enzyme-linked immunosorbent assay) technique has advantages since it requires both the antigenic determinant site and the enzyme active site to be retained in native form; fortunately, an ELISA technique is available for horseradish peroxidase (73), which is often used as a molecular probe for macromolecular absorption.

TRACER DOSES OF NUTRIENT PROTEINS Numerous studies have, for sound experimental reasons, used very small doses of the protein whose absorption is being studied. However, this represents a very artificial situation, and it is impossible to extrapolate from such data to assimilation of a full meal. The controversial work of Hemmings leading to his concept of "distributed digestion" (42–45, 47), i.e. the proposal that macromolecular fragments of protein were absorbed on a large-scale so that peripheral tissues were a major site for digestion of dietary proteins, was based on the appearance in the tissues of isotopically labelled high-molecular-weight fragments from minute quantities (1–10 mg) of protein introduced intraluminally in suckling and adult rats; the protein was introduced in some experiments with a grossly hypertonic and alkaline solution (2 mol/liter $NaHC0_3$) (42). His results indicating massive-scale absorption of 40–70% of bovine IgG or gliadin as high-molecular-weight fragments in adult rats have never been confirmed independently. This remarkable estimate contrasts with the estimate of Warshaw, Walker &

Isselbacher (109) that 2% of oral ^{3}H-bovine serum albumin is absorbed in intact form, which in turn seems to be an unexpectedly high estimate.

MEASUREMENTS OF MACROMOLECULES IN BLOOD These, by virtue of their simplicity, have commonly been made. While they can provide qualitative evidence that intact protein has been absorbed and can show the time-course of the event (though few studies provide this information), they, alone, are unable to provide information about the total amount absorbed in macromolecular form. Studies on distribution of absorbed macromolecular fragments in various organs as well as in blood can be particularly illuminating (e.g. 73, 74), but these are not practicable in humans! The rapidity of uptake by peripheral tissues and the amounts that may be sequestered by, for instance, the liver are striking (73). Further, the presence of active proteases and peptidases with broad specificity in plasma is generally neglected: this has certainly accounted for some failures to detect the appearance of peptides in blood after protein or peptide meals (35), and it is possible that rapid proteolysis in the circulation has often resulted in erroneous estimates of the quantity of intact protein crossing the gastrointestinal tract.

INTESTINAL PREPARATIONS IN VITRO Much basic knowledge of gastrointestinal physiology has accrued through the use of isolated tissue preparations in vitro. However, many preparations are much more susceptible to damage during the setting-up period than is often recognized (86). Structural changes, massive leakage of intracellular peptidases, and impaired absorption can be detected when rat small intestine is perfused in vitro if the standard precaution of maintaining the blood circulation with the animal alive is neglected until *after* the luminal perfusion has been completely established (30, 34). Thus, metabolic changes occur remarkably rapidly on excision of tissue, and these may alter both qualitatively and quantitatively the complex energy-dependent processes involved in endocytosis, lysosomal fusion and hydrolysis, and exocytosis—processes that are now thought to be central to intact protein absorption (see below). Hence, even minor reductions in mucosal structural and metabolic integrity are likely to affect observations on the transmucosal passage of intact protein. Where two or more absorptive routes/mechanisms are involved, their relative importance may be altered by such in vitro artifacts. Hence, studies undertaken with everted sacs, biopsy fragments, tissue sheets in Ussing chambers, etc must be regarded circumspectly, although the possible errors introduced may be in the direction of either underestimation or overestimation of passage of intact macromolecules. Needless to say, such in vitro techniques are essential, being the only ones applicable to many studies on human tissue and to experiments following the progress of macromolecular markers through cells.

It should also be noted that Rhodes & Karnovsky (89) showed that surgical manipulation of rat intestine promoted macromolecule absorption in vivo. Quite apart from the potential relevance of their observations to surgical patients, the possibility that experimental manipulations in vivo or in vitro have unduly influenced results on macromolecular absorption has been neglected by most workers. This points to the need to pursue investigations by several different complementary approaches, including the study of absorption in intact humans and animals.

INTERSPECIES DIFFERENCES For obvious reasons, much work has been performed on animal species. However, sight must not be lost of one particular difference between humans and other mammals. Most species acquire the majority of their passive immunity via the gastrointestinal tract postpartum, and their gastrointestinal tract thus has to be able to transmit protein (selectively IgG in many species) for the first few days (21–22 days for the rat) or weeks of life: then "closure" (see below) occurs and this process ceases. Thus, in this initial neonatal period, absorption of intact protein plays a vital role. In contrast, however, humans acquire passive immunity via the placenta, and "closure" (i.e. cessation of transmission of IgG) occurs apparently abruptly at, or close to, birth. The monograph by Baintner (5) deals in valuable detail with macromolecular absorption in the context of immune transmission.

Hence, the mechanisms and routes of intact protein transport in neonatal animals may be fundamentally different from those operating in humans. However, observations on neonatal animals can provide information about the physiology of "closure" that cannot be obtained in humans.

A striking example of interspecific differences is provided by McLean & Ash (74, 75), who report a 1000-fold greater absorption of intact horseradish peroxidase by carp than by rainbow trout. They suggest that agastric species may have special requirements for maintenance of their immunocompetence, and that specially adapted enterocytes may be responsible for the augmented intact protein absorption.

The Gut as an Immune Organ—The M-Cell Route

The gastrointestinal tract is a major site of immunologically competent tissue—hence the expression gut-associated lymphoid tissue. Throughout the intestine, the lamina propria contains a substantial population of lymphocytes and macrophages; furthermore, the small intestine contains many Peyer's patches, clearly discernible nodules of lymphoid tissue. Two distinct functions seem to operate: immunization against antigens that have crossed the epithelium, and inhibition of antigen uptake by promotion of intraluminal (or brush-border surface) digestion.

M CELLS A major step in understanding the gut's function as an immunological organ and the significance and mechanism of macromolecular transport was taken in 1973 when Bockman & Cooper (13), followed by Owen & Jones (80, 81) in 1974, showed that Peyer's patches were covered by a special type of cell, hitherto unrecognized. This is the M cell or membranous cell [or lymphoepithelial cell (112)]. These can be identified by electron microscopy, and they are significantly different from columnar epithelial absorptive cells. It was initially thought that their apical surface had microfolds rather than microvilli, but it is now clear that they do possess irregular short and wide microvilli, although there are fewer than on columnar absorptive cells. Vesicles are particularly abundant in the cytoplasm, a reflection of their endocytotic activity, and there appear to be fewer lysosomes in the cytoplasm (111); this is consistent with a diminished rate of intracellular protein degradation as observed by Desjeux's group (25).

Transport by endocytosis into and across these M cells has now been shown for a number of proteins, viruses, and inert particles. It is hypothesized that the function of M cells is to permit direct access of luminal antigens to the subepithelial lymphocytes, which now can approach close to the intestinal lumen. Hence an immune response is elicited. For a full discussion of the properties of M cells, the reviews by Wolf & Bye (111) and Egberts et al (26) should be consulted.

One important question is that of the relative contributions made by M cells and by "ordinary" columnar epithelial absorptive cells to macromolecular absorption. Owen concluded that horseradish peroxidase entered M cells much more rapidly than columnar cells (80), but similar rates of entry were reported by Ducroc et al (25). However, the latter group observed less intracellular degradation in tissue containing Peyer's patches, so that the net transepithelial passage of macromolecules would be greater for the M cells. Keljo & Hamilton (59) also found a 3-fold increase in the passage of peroxidase across regions of piglet intestine containing Peyer's patches, which supports the quantitative importance of this route. Although Sass et al (90a) found that surgical ablation of Peyer's patches from rat intestine led to increased, rather than decreased, appearance of iodinated human gammaglobulin in the blood and thoracic lymph, such a dramatic surgical procedure is likely to have been very disruptive to normal function: hence, it is risky to conclude from these experiments that Peyer's patches were unimportant for macromolecular passage. Walker, taking an overview, suggests that the M-cell route is used preferentially at low (or physiological) loads of luminal antigen, but that all absorptive cells may participate at increased antigen levels (106).

ORAL IMMUNIZATION Several animal studies have shown that prior oral exposure to an antigen specifically reduces the absorption of that antigen in

intact form (4, 6, 63, 108). Parenteral immunization of rats also reduced protein absorption, but this route was less effective than oral immunization (108). Walker et al (108) were able to show enhanced proteolysis of antigens in intestines taken from immunized animals; this was thought to arise from enhanced binding actually on the mucosal (brush-border) surface. Pang, Walker & Bloch (83) subsequently found the presence of immune complexes in luminal washings and mucosal extracts in immunized rats, thus supporting intraluminal binding as a "protective" feature.

Other indications that intestinal immunoglobulins are normally involved in minimizing intact antigen absorption are glimpsed in the observations that deficiencies of secretory IgA are associated with various autoimmune diseases and gastrointestinal diseases which may involve increased macromolecular absorption. Thus Vaerman & Delacroix (101) state that it is well known that IgA-deficient individuals frequently display high serum titers of antibodies against dietary antigens; see also similar comments and references provided by Walker (106). Note that selective IgA deficiency is not particularly rare (2), so immunodeficiency may be a common factor in promoting absorption of intact antigens.

Measurement of Human Intestinal Permeability

Several noninvasive tests of intestinal absorptive capacity and "permeability" have recently been introduced, primarily for clinical diagnostic application but also with clear benefits for basic physiological investigation. Essentially these entail oral administration of one or, preferably, two nonmetabolizable probe substances that, once absorbed, are rapidly and efficiently excreted in the urine. One probe is chosen to be a modestly absorbed compound, so that good absorption and subsequent urinary excretion is an index of good absorptive function and a normal surface area of absorptive epithelium (a modern and much more efficient counterpart of the traditional xylose tolerance test); the other is chosen to be a scarcely absorbed molecule—indeed many were previously regarded as "nonabsorbable" compounds—so that its absorption and excretion provides an index of gastrointestinal "leakiness." The latter are thought to pass by the paracellular route (although formal proof of this is lacking), and passage is increased if a hypertonic solution (which "loosens" tight junctions, possibly via cellular shrinkage) is given concurrently (76, 110).

These tests are simple to apply, involving a single oral dosage of the test substances, followed by a single 5-hour urine collection and analysis of the two probes in the urine. They are also effective in that diminished absorption of the first probe, the transcellularly "absorbable" one, is commonly seen in many gastrointestinal diseases; a concomitant increase in the absorption of the second probe, the paracellularly poorly absorbable one, is observed. The ratio between the absorptions of the two often provides clear-cut discrimination

between health and disease. The practical and theoretical benefits of the simultaneous inclusion of dual probes have been explained by Menzies (76).

Because of their success and simplicity, these tests have been applied to a wide variety of gastrointestinal diseases and other conditions in which altered intestinal function may occur but that would otherwise be difficult to identify objectively. Thus, it has become clear that a temporarily leaky gut accompanies a remarkably wide range of diseases although, of course, interpretation of the underlying causal relationships is difficult. While these tests do provide a useful means of measuring leakiness, it must be stressed that the probes being used are all relatively small: hence, leakiness to them will not necessarily be indicative of excessive permeability to larger molecules such as proteins, especially since Hamilton et al (studying probes of up to 660 daltons) found a marked inverse effect of molecular volume on permeation rate (38). While isotopically labelled polymers, such as polyvinylpyrrolidone, of up to 30,000 daltons have been used in animals (65), the fractional absorption of such large molecules is so small that it is unlikely that it could be practically measured with accuracy in humans; furthermore, potential artifacts associated with labile labels such as ^{125}I could arise (see above). Also, it must be noted that the results of such investigations, probably relating mainly to the paracellular route, are not necessarily relevant to absorption processes involving receptor-mediated endocytosis; further findings such as those of Heyman et al (49) support the view that it is augmented transcellular (not paracellular) passage of macromolecules that occurs in severely malnourished children.

Typical markers that have been used for the paracellular route are lactulose and cellobiose (both very poorly absorbed disaccharides, of 342 daltons) (38, 76), ^{51}Cr-EDTA complex (~340 daltons) (9, 11, 84), and polyethylene glycols (average molecular weight 400 or 4000) (16, 57), although the last appear to behave anomalously because of their high lipid solubility and poor urinary recovery (72). Disease states in which reversible increases in permeability to these probes have been demonstrated include celiac disease (8, 17, 78, 110), Crohn's disease (9, 36, 99) [also in first-degree relatives of Crohn's disease patients (53)], viral and bacterial gastroenteritis (28a, 79), parasitic infestations (100) [see also results for rats (12)], alcoholism (84), and some food allergies (3). Less clear-cut conclusions have made about permeability changes in other diseases such as eczema (37, 57) and schizophrenia (113) (see below), and tests have even been made in "joggers enteropathy" (84) although these last results appear not to have been published. Hence, a "leaky" gut must be regarded as a real clinical entity, and it is now important to establish what the consequences are; in particular, to what extent such increases in "permeability" are accompanied by increases in intact protein and other macromolecular transport and whether there are any clinically significant consequences.

Assessment of permeability in humans and its possible significance has been admirably reviewed by Menzies (76, 77), Peters & Bjarnason (10, 84), and Hamilton (37). See also the discussion by Cooper (18). These investigations should now be accepted as a valuable and objective measurement of gastrointestinal function suitable for more widespread application: simplification of some of the analytical techniques (e.g. those for urinary sugar analysis) might encourage more use. However, users must be constantly aware of the dangers in drawing causal inferences.

A long-standing controversial query is whether increased permeability or macromolecular absorption may be associated with (and then possibly causal in) food allergies, skin diseases such as eczema, and schizophrenia—see the section below on food allergy. Although negative findings on schizophrenia were reported (8), Mindham and Axon and their colleagues (113) have recently reported increased intestinal permeability in a subset (11 of 32) of their psychiatric in-patients; small-intestinal biopsies were normal, so that celiac disease was excluded. It would be of special interest to know whether these same patients had elevated antibody titers against dietary proteins, and whether the leakiness of their intestines (or those of the "negative" subjects) was aggravated by the inclusion of potential allergens, including gluten, in the test meal.

Another recent work of special interest is the demonstration by André (3) of an increase in intestinal permeability to a disaccharide probe when a suspected food allergen was administered together with the permeability test meal; the increase in permeability was not seen when the test meal with allergen was preceded by oral sodium chromoglycate. This suggests that a true intestinal allergic response was associated with the increase in permeability (probably paracellular), but we cannot determine whether the increase was a primary event or a secondary consequence. It seems to be equally plausible (*a*) that intact protein initially crossed the intestine (possibly via a transcellular route) and initiated degranulation of subepithelial mast cells, which then triggered off the increase in paracellular permeability to the much smaller probe molecules; or (*b*) that the offending protein, or other dietary constituents, damaged the mucosa initially (possibly by a lectin-type interaction with the brush-border membrane), and thus increased the permeability, which would then permit increased ingress of the allergen to the subepithelial lymphoid tissue, with a subsequent increased allergic response as the final step. Distinction between these possibilities, though difficult, is needed. Because of the key role of lysosomal digestion in intact protein absorption (see below), it must be remembered that both transport processes and intracellular metabolism/digestion contribute to the net appearance of protein at the subepithelial lymphoid tissue. Hence, possible derangements in both need to be examined.

Unfortunately, while it is now simple to measure permeability to these relatively low-molecular-weight probes, there are no corresponding simple tests for macromolecular permeability. It is essential to know whether changes in paracellular permeability to the disaccharide probes are accompanied by parallel changes in macromolecular permeability. Corresponding tests on macromolecular permeation would, however, require analytical methods of extremely high sensitivity in view of the small amounts likely to be absorbed: radioimuunoassays and ELISA assays offer potential. Ovalbumin has been proposed as such a macromolecular marker by Husby and colleagues (55, 56), human lactalbumin by Jakobsson et al (58), and Paganelli & Levinsky (82) have developed radioimmunoassays for bovine serum albumin, β-lactoglobulin, and ovalbumin for this purpose. However, as Bjarnason & Peters (10) point out, these methods probably are technically too demanding to attract routine usage in clinical gastroenterology.

It is to be hoped that there develops a surge of interest in macromolecular probes, such as was seen in the development of the lower-molecular-weight ones, but four additional complications may arise in the application of intact protein probes: (*a*) initial exposure to a probe molecule may induce oral immunization, which is likely to modify the intestine's subsequent handling of the same probe if the test is repeated; (*b*) the response may depend upon the patient's previous dietary history unless some definitely nondietary constituent is selected; (*c*) proteolysis, both intramucosal during absorption and extraintestinal, will affect the ultimate recovery of the probe so that measured permeability will represent a composite of several processes; and (*d*) the simplicity of single measurements on pooled urine will be lost.

Madara & Pappenheimer (67) recently reported that intraluminal glucose at physiological concentrations can reversibly increase intestinal permeability in vivo and in vitro, possibly through a perijunctional "muscle" ring opening up the tight junctions. These observations raise two important possibilities: (*a*) that an increase in intestinal paracellular permeability may be part of the normal physiological response to a meal, and (*b*) that net fluid movement across the intestine (which is stimulated by solute absorption) may be a driving factor for macromolecular transport—presumably operating via solvent drag through the paracellular route.

A probe that has been widely used to trace macromolecular transport in animal tissues is the enzyme horseradish peroxidase, a glycoprotein of molecular weight ~ 40,000. It can be visualized by electron microscopy since it contains iron, and it also can be localized by immunocytochemistry. Since it can be measured in plasma and other tissues by the sensitive ELISA method, which requires both antigenic and enzymic patency (73), it may prove to be applicable in humans.

Possible Routes and Mechanisms for Macromolecular Absorption

REGIONS OF GASTROINTESTINAL TRACT Having established that intact proteins do cross the gastrointestinal tract, it is pertinent to consider what region(s) are involved. Little is known about the possible involvement of regions other than the small intestine, which is certainly a major site of such absorption. Stomach and large intestine are unlikely to be important sites, but they should not be neglected. Rectal entry of intact protein has been shown in some fish species; while this is not likely to be of physiological relevance, it is a potential route for therapeutic administration of polypeptides and vaccines. One route that has been neglected is the buccal mucosa, especially sublingual tissue. The rapid response in allegedly food-allergic patients to food in the mouth and the apparent efficacy of sublingual neutralization or desensitization (88) merit investigation of the mechanisms involved and of the quantities that may enter the body via this route. To date most information on this topic seems to be empirical and subjective and in the hands of practitioners of "clinical ecology."

PARACELLULAR OR TRANSCELLULAR ROUTES Both (*a*) the paracellular pathway through the "tight" junctions—arguably inaptly named because of their permeability and their major role in fluid and ion transport—and through cell extrusion zones and areas of damaged mucosa, and (*b*) the transcellular pathway may be involved in intact protein absorption. However, most evidence favors the latter route as dominant, especially in healthy intestine, although the process is a complex one involving metabolic energy expenditure, cytoplasmic tubule formation, and lysosomal processing. Bockman & Winborn (13a) observed ferritin passing through hamster intestinal cells by pinocytosis, with none passing between the cells.

Likewise, the corroboration found in more recent work by Desjeux and colleagues (50, 52) suggests that only a small fraction of absorbed horseradish peroxidase crossed by the paracellular route in their rabbit ileal experiments in vitro. Hence, the transcellular route seems to be more important than the paracellular route, although increases in it caused by disease or with excessive exfoliation may even make it a predominant route. Their observations on biopsy material from malnourished infants suggested that decreases in intracellular processing were the basis for the increased transepithelial passage of the peroxidase marker (49).

The permeability probes discussed above for use in humans appear to reflect paracellular leakiness, which undoubtedly is increased at least to small molecules in many diseases. This route also can be used by particles and

macromolecules: Volkheimer (102), who considered that motility was a driving force for particulate absorption, coined the term "persorption" for this process.

Hypertonic solutions increase the permeability of small intestine to medium/small-molecular-weight substances such as the paracellular route markers (lactulose, etc) mentioned above but not to the transcellular route markers (rhamnose, etc) (110). This effect is exaggerated in many gastrointestinal diseases, so that some investigators routinely administer hypertonic solutions together with their test meals during assessment of intestinal permeability (76). Although hypertonic solutions do promote the absorption of intact insulin (molecular weight 6000) (28), the effect of hypertonicity on absorption of larger molecules does not seem to have been investigated. This might offer a further approach to study the relative importance of the transcellular and paracellular routes.

ENDOCYTOTIC MECHANISMS The scheme described by Walker & Isselbacher (107) meets most of the histochemical and electron microscopic observations on macromolecule transport. Protein molecules bind to receptors on the surface of the apical (brush-border) membrane; the membrane invaginates to form phagosomes or vesicles encapsulating the protein. The phagosomes migrate in the cytoplasm to lysosomes via a system of cytoplasmic microtubules. Most fuse to form phagolysosomes or secondary lysosomes in which proteolysis occurs by a series of cathepsins and other acid proteases. Some apparently fail to fuse or use a separate pathway and leave the cells by exocytosis at the basolateral membrane. All these steps are energy dependent. A similar process occurs in neonatal animals before "closure" but large numbers of vacuoles are formed; at that stage IgG receptors exist on the brush-border membrane and it is thought that binding to them (and their inclusion in the vesicles) specifically protects the engulfed IgG from proteolysis in the phagolysosomes (5). In the experiments of Heyman et al (50), 97% of the peroxidase entering the cells was degraded to fragments of 2000-4000 daltons.

Hence it appears that lysosomal proteolysis is a major factor in minimizing entry of intact protein to the circulation, although the mechanism of this process has been less intensively studied in intestinal cells than in, for example, hepatocytes (48). Specific inhibitors (see 48) offer a useful approach.

"Closure"

Although the name hints at a physical process of sealing the epithelial barrier, the events of "closure" appear to relate wholly to intracellular developments

associated with intestinal maturation, rather than paracellular events, which lead to the cessation of (or substantial reduction in) intestinal transmission of large amounts of IgG in animals. After closure, brush-border receptors for IgG disappear (5).

In humans, where the intestinal route is regarded as unimportant for transmission of passive immunity (5), closure is thought to occur suddenly at birth. However, the evidence on this point is confusing and suggests that closure has largely occurred by the time of birth but that some further closure does take place in the early days of extrauterine life. Also, there is some legitimate speculation that the intestine at this stage may be particularly vulnerable to damage by some exogenous antigens, some of which may precipitate long-term gastrointestinal disease or "allergy" (93). Objective evidence on this would be welcome since manipulation of infant-feeding practices potentially offers a powerful means of reducing the incidence of disease: for example, it is suggested that the reduction in infant celiac disease observed since the 1970s was associated with an increase in breast-feeding and later introduction of cereals (15).

Evidence shows that, while permeability to macromolecules is greater in pre-term infants than in full-term ones (7, 90), it is also significantly present during the first few weeks of life of full-term infants and gradually reduces thereafter (58). Hence, closure may not be as abrupt and complete at birth as is generally presumed, and some passive immunity may also be gained by the gastrointestinal route. Further, it is suggested that exposure to human milk and to dietary antigens does affect this early postuterine maturation process. In rabbits, Udall et al (97) showed that absorption of immunoreactive bovine serum albumin by rabbits decreased markedly after the first week postpartum. Further, the absorption in the first week was greater if the animals were fed on a synthetic "milk" than when they were allowed to breast-feed (96). Thus factors in the natural milk were thought to be involved in hastening (but not essential for) the intestinal maturation. This is also consistent with the concept that exposure to specific antigens in early life may be relevant in subsequent events including, possibly, the pathogenesis of various gastrointestinal diseases; and with the idea (98) that sensitization to allergens may arise, especially in children, during gastrointestinal inflammation or infection. (See also Ref. 5, p. 106.)

Leary & Lecce (61) showed that surgically bypassed segments of piglet intestine "closed" at the same time as the neighboring segments that were exposed to food. Hence, hormones (rather than luminal contents) were concluded to trigger closure. While, Svendsen et al thought that insulin played a key role (94), there is confusing evidence for involvement of various other hormones, notably cortisol: much of the evidence has been discussed by Baintner (5, pp. 44–46; 95).

Food Allergy and Intolerance

The possibility that various ailments lacking other established pathophysiological explanations may be associated with diet frequently is expressed in lay and "fringe" medical circles but also, increasingly, in professional fora. Unfortunately, much evidence has been anecdotal and subjective, and the need to provide sound analyses of the underlying pathophysiological mechanisms has too often been neglected. However, recently there has been some movement to redress this problem; see, for example, the tome by Brostoff & Challacombe (14). An understanding of intact protein absorption is central to this subject; the conclusion that some intact protein absorption does occur in health and that it may be augmented in disease inevitably provokes enquiry into the clinically relevant consequences. The possibility that, for example, inflammatory bowel diseases, are caused by dietary proteins and can be cured/treated by dietary manipulation has a plausible hypothetical background (91) and some (but not enough) supportive evidence (1, 39).

Dannaeus et al (20) reported increased absorption of ovalbumin in egg-sensitive children, and this was reduced by sodium chromoglycate: this suggests that there may have been elevated intestinal permeability (or decreased lysosomal hydrolysis) *secondary* to a mast cell response.

Dohan (22–24) has advanced the theory that schizophrenia is associated with gluten ingestion in genetically susceptible individuals—note that gluten is the wheat protein known to be causal in celiac disease. Elevated plasma levels of gliadin antibodies have been reported in schizophrenia, but only in a small number of patients. However, gluten exclusion has also been reported to be beneficial in a small subset of schizophrenics (101a) and, of particular interest in the present context, intestinal permeability has been found to be increased in some schizophrenics (113). Interpretation of the causal relationships underlying these findings is difficult, but objective tests of gastrointestinal function and of intact protein absorption may make new advances possible.

Other diseases in which food allergy and suggested enhanced macromolecular absorption have been discussed include eczema and rheumatoid arthritis, but there is no general acceptance of gastrointestinal mechanisms in the etiologies of these conditions. Also, only subsets of the populations studied have had increased intestinal permeability (37a, 57, 113), but these may of course reflect true subgroups of etiologies.

André's work (3) mentioned above provides a new stimulus and suggestions for an objective assessment of potential adverse effects of dietary proteins on intestinal function but, as always, caution is needed in drawing causal conclusions.

SUMMARY

There is now no reasonable doubt that small quantities of intact proteins do cross the gastrointestinal tract in animals and adult humans, and that this is a physiologically normal process required for antigen sampling by subepithelial immune tissue in the gut. It is too small to be nutritionally significant in terms of gross acquisition of amino-nitrogen, but since it has important implications relating to dietary composition it must receive consideration from nutritionists. The process of intact protein absorption occurs without eliciting harmful consequences for most individuals, but it appears likely that a small number of people absorbing these "normal" ammounts may react idiosyncratically; also, some individuals may absorb excessive amounts, and they may suffer clinically significant consequences. Likewise, individuals with diminished absorption of intact protein may be at risk.

Normal absorption probably occurs predominantly by transcellular endocytosis with some possible contribution by a route between cells; increased net entry of protein to the circulation may reflect (*a*) increased paracellular (intercellular) passage, (*b*) increased transcellular passage, and/or (*c*) decreased lysosomal proteolysis. Tests to distinguish among these possibilities are strongly desirable.

Intact protein absorption may be involved in the pathogenesis of inflammatory bowel disease, "food allergies," and other diseases, including even major psychiatric disorders, but the current evidence is mainly indirect and suggestive. Great caution and careful objective studies are needed to establish whether such relationships with disease do exist and to unravel the underlying basic physiological mechanisms.

Now that interest has developed in the assessment of intestinal permeability to small- and medium-sized molecules, it is hoped that equally simple methods for studying macromolecular permeability will be developed and applied. Therapeutic methods for enhancing intact polypeptide absorption would be valuable for vaccine and peptide drug administration by the oral route. Therapeutic reduction of the process may be relevant in food-sensitive patients.

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