121, 345 (1980); L. R. Hagey and S. K. Krisans, *Biochem. Biophys. Res. Commun.* **107**, 834 (1982); F. Kase, I. Bjorkem, J. I. Pedersen, *J. Lipid Res.* **24**, 1560 (1983).
27. S. Goldfischer, *J. Histochem. Cytochem.* **27**, 1371 (1979); _____ and H. Sobel, *Gastroenterology* **81**, 196 (1981).
28. Review of published electron micrographs of a case described as hyperpipecolatemia [P. D. Gatfield *et al.*, *Can. Med. Assoc. J.* **99**, 1215 (1968)] reveals lamellated inclusions identical to those seen in neonatal and X-linked ALD and identified as representing very long chain fatty acids.
29. J. Vamecq and F. Van Hoof, *Biochem. J.* **221**, 203 (1984).
30. H. Kindl and P. B. Lazarow, *Ann. N.Y. Acad. Sci.* **386**, 1 (1982); H. Kindl, *Int. Rev. Cytol.* **80**, 193 (1984); P. B. Lazarow, H. Shio, M. Robbi, in *Biological Chemistry of Organelle Formation*, T. Bucher, W. Sebald, H. Weiss, Eds. (Springer-Verlag, Berlin, 1980), pp. 187–206.
31. P. B. Lazarow and C. de Duve, *J. Cell Biol.* **59**, 507 (1973); M. Robbi and P. B. Lazarow, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4344 (1978); B. M. Goldman and G. Blobel, *ibid.*, p. 5066; H. Azasa, S. Miyazawa, T. Osumi, *J. Biochem.* **94**, 543 (1983); R. A. Rachubinski, Y. Fujiki, R. M. Mortensen, P. B. Lazarow, *J. Cell Biol.* **99**, 2241 (1984); Y. Fujiki, R. A. Rachubinski, P. B. Lazarow, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7127 (1984).
32. The levels of plasma hexacosanoic acid reported here are consistent with levels observed in 282 patients having X-linked ALD and 20 patients having neonatal ALD (H. W. Moser, A. E. Moser, I. Singh, B. P. O'Neill, *Ann. Neurol.*, in press).
33. Supported in part by grants from the National Institutes of Health (AM-17702, N5-03356, AG-01468, BRSG-507, RR-05399-22, AM-19394), National Science Foundation (PCM82-08315), and the Gail I. Zuckerman Foundation, and by an Established Fellowship from the New York Heart Association (P.B.L.). B. Bangaru and J. Dancis of New York University and N. F. LaRusso of the Mayo Clinic generously supplied control samples used for determining hepatic catalase. We are grateful to J. Dancis for pipecolic acid determinations.

6 July 1984; accepted 2 October 1984

Abrupt Induction of a Membrane Digestive Enzyme by Its Intraintestinal Substrate

Abstract. *The regulation of amino-oligopeptidase (AOP), an intestinal brush border hydrolase essential for the surface digestion of peptide nutrients, was examined in rats in vivo. Short-term (30-minute) intraintestinal perfusion of a tetrapeptide substrate, Gly-Leu-Gly-Gly, or a synthetic substrate, leucyl-β-naphthylamide, induced a doubling in the incorporation of [^{3}H]leucine into the AOP in association with intracellular membranes. The subsequent conversion of AOP from nascent to mature enzyme and its membrane-associated transport to the brush border occurred at normal rates.*

Amino-oligopeptidase (AOP), an integral digestive enzyme of the intestinal brush border surface (*1*), hydrolyzes the oligopeptides produced by luminal pancreatic proteases at the intestinal membrane surface in preparation for the intestinal transport of the final dipeptide and amino acid products. This hydrolase plays an essential role in the assimilation of dietary protein in mammals (*2*).

Studies of the intact rat have shown that rapid synthesis of AOP as a nascent protein occurs within 15 minutes after an intraintestinal amino acid "pulse-chase" (short-term perfusions with labeled and unlabeled substrate) in association with the endoplasmic reticulum (ER). Within 30 to 45 minutes, apparent post-translational glycosylation and transfer to Golgi membranes (the site of fucosylation) takes place. Final vectorial transfer to the luminal brush border (BB) surface is not achieved until 3 hours after the intraluminal amino acid pulse (*3, 4*). Although the activity of the BB aminopeptidase in rats is decreased by starvation and increased by a high protein diet (*5*), the mechanism of dietary protein or peptide action on regulation of the aminopeptidase is unknown. We examined the regulatory role of high-affinity specific substrates of the AOP, the tetrapeptide Gly-Leu-Gly-Gly, and leucyl-β-naphthylamide (LeuNA), in the synthesis, intracellular assembly, and transport of AOP to the BB surface.

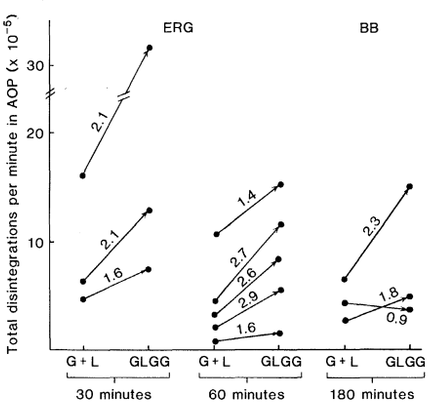

Fig. 1. Comparison of the incorporation of [^{3}H]leucine into AOP in ERG and BB fractions of paired rats receiving intraintestinal 2 m*M* Gly-Leu-Gly-Gly (GLGG) or its constituent amino acids (G+L). Rats were perfused with tetrapeptide or amino acid solution for 30 minutes and then pulse-labeled with [^{3}H]leucine intraluminally and chased with the peptide or amino acid solution for 30, 60 or 180 minutes. AOP was specifically immunoprecipitated from the Triton-solubilized ERG and BB fractions, and the total amount of label in the enzyme was determined by allowing for the fractional solubilization by Triton X-100 and the yield of the marker enzyme (*3*). The arrows connect paired animals at times after the [^{3}H]leucine pulse; ratios of the amount of label in AOP from rats perfused with tetrapeptide to that from rats perfused with amino acid (control) are given over each arrow. Perfusion with Gly-Leu-Gly-Gly significantly increased incorporation of label into AOP in the ERG (see text). Similarly, in two of the three experiments, the total amount of label in AOP in the BB from the substrate-perfused rats at 180 minutes was twice that in control, and the specific amount of label in AOP (disintegrations per minute per milliunit) in the BB fraction was increased significantly (see text). Statistical analysis was by the paired *t*-test; data are reported as the mean ± standard error.

Male Sprague-Dawley rats, paired by age and weight (200 to 300 g) and fed a protein-deficient diet (*6*) for 12 days, were anesthetized, and jejunal loops (25 cm long) were isolated and cannulated as described (*2*). The intestinal loop of the paired rats was simultaneously perfused for 30 minutes with 150 m*M* NaCl containing either 2 m*M* L-Gly-Leu-Gly-Gly or the equivalent amino acid solution (6 m*M* glycine and 2 m*M* L-leucine; control). After a 5-minute intraluminal "pulse" with L-[^{3}H]leucine (2.5 mCi) and a 30-, 60-, or 180-minute "chase" with the substrate or amino acid solution, BB and ER-Golgi (ERG) membranes were prepared (*3*) and their purity assessed by use of sucrase (as a marker for the BB) and aryl esterase (for the ERG). The average purification factors were 13 for the BB and 4.1 for the ERG; the fractional yields were 0.40 for the BB and 0.08 for the ERG. Leucyl-β-naphthylamide hydrolase, sucrase, aryl esterase, and protein were assayed as described (*3*). After solubilization of the BB and ERG membranes with 0.5 percent Triton X-100, AOP and sucrase were isolated by specific immunoprecipitation with monospecific rabbit antibodies to AOP, and the total and specific amount of label in the immune precipitates obtained by centrifugation was determined by scintillation counting (*3, 4*). Purification factors, fractional yields, and percent solubilization of the membrane fractions were similar to those reported (*3, 4*) and were not different for animals perfused with either substrate or amino acid. Several experiments were done at each time after the pulse (see Fig. 1).

Although the rats' dietary protein intake was restricted, the AOP activity in the BB (1260 ± 120 mU per milligram of protein) was similar to that for animals fed a regular diet (1290 ± 90 mU per milligram). The total amount of label incorporated into AOP in the ERG fractions from rats perfused with Gly-Leu-

Gly-Gly was greater than that from control animals by 89 ± 17 percent ($P < 0.025$) at 30 minutes and by 124 ± 14 percent ($P < 0.01$) at 60 minutes. The mean ratios of the total amount of label in AOP from animals perfused with tetrapeptide to that from animals perfused with amino acid were 1.9 (30-minute ERG fraction) and 2.2 (60-minute ERG fraction). In two of the three experiments, the total amount of label in AOP from tetrapeptide-perfused rats was twice that from control animals in the BB fraction after 180 minutes, a time when newly synthesized AOP reaches a significant concentration in this fraction (*3*). Also, the specific amount of label in the BB at 180 minutes was significantly increased by perfusion of substrate (tetrapeptide-perfused, 178 ± 32 dis/min per milliunit of AOP; amino acid–perfused, 128 ± 23 dis/min per milliunit; $P < 0.05$). The total AOP activity in the BB did not change detectably in peptide-perfused animals in these short-term studies, probably reflecting a relatively stable large pool of this hydrolase in the surface membrane.

Several features of this increase in the incorporation of [^{3}H]leucine indicate that it represents a true increase in AOP synthesis rather than artifact. (i) No significant difference in incorporation of [^{3}H]leucine into sucrase, an unrelated jejunal BB enzyme, was found in the two groups; the mean ratios of the total amount of label in sucrase from tetrapeptide-perfused animals to that from amino acid–perfused animals were 1.2 (30-minute ERG fraction) and 1.0 (60-minute ERG fraction). (ii) There was no statistical difference in incorporation of [^{3}H]leucine into total protein (10 percent trichloroacetic acid precipitates) in the ERG or BB fractions of tetrapeptide-perfused animals compared to amino acid–perfused animals ($P > 0.1$). (iii) The specific amount of label in the leucine precursor in the 30-minute cytosol fractions, determined by amino acid analysis, was not different from the two groups [$(1.2 \pm 0.5) \times 10^4$ and $(1.1 \pm 0.3) \times 10^4$ dis/min per nanomole of leucine for the peptide-perfused and control animals, respectively; $P > 0.25$)]. (iv) The amino acid–perfused rats had a similar total amount of label in AOP [9.0×10^5 dis/min at 30 minutes in the ERG (Fig. 2)] as reported in a previous study from our laboratory for rats on a regular diet [9.1×10^5 dis/min (*7*)]. Thus, the doubling of the incorporation of [^{3}H]leucine into AOP appears to be best explained by a twofold increase in its de novo synthesis induced by intraintestinal peptide substrate.

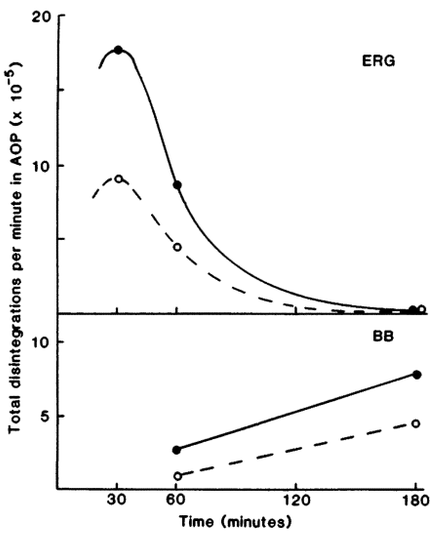

Fig. 2. Kinetics of incorporation of [^{3}H]leucine into AOP in ERG and BB membranes. The mean values taken from data in Fig. 1 for the total amount of label in AOP in rats perfused with GLGG (●) or G+L (○) are plotted as a function of the duration of chase. In the ERG, both groups of rats showed maximum incorporation of label into AOP at 30 minutes, which reached significant concentrations in the BB at 180 minutes. This sequence of intracellular transport of AOP mirrored the kinetics found under normal conditions (*3*). Notably, incorporation of label into AOP was two times greater in the tetrapeptide-perfused rats than in the amino acid–perfused rats.

In other pulse-chase experiments, jejunal loops were perfused with either 1.5 m*M* LeuNA (another highly specific substrate for AOP) in 150 m*M* NaCl or 150 m*M* NaCl alone. The experimental design was the same as that described above except that the animals were treated with a pulse of [^{35}S]methionine (1 mCi). The total amount of label incorporated into AOP in the ERG fraction was greater by a factor of 1.8 (at 30 minutes) and 3.7 (at 60 minutes) in the rat perfused with LeuNA than in the control animal. Thus, substrates such as the high-affinity tetrapeptide Gly-Leu-Gly-Gly or the peptide analog LeuNA appear to act rapidly and specifically, presumably by virtue of their interaction with AOP at the intestinal lumen–membrane interface to enhance the intracellular de novo synthesis of AOP.

Studies of Gly-Leu-Gly-Gly hydrolysis in the rat jejunum have shown that its hydrolytic products in vivo are glycine, leucine, and the dipeptide Gly-Gly (*2*). Because the dipeptide product may be the inducer of AOP synthesis, pulse-labeling experiments were carried out as described except that rats were perfused with a mixture of glycine, leucine, and Gly-Gly (2 m*M* each). In two experiments, the ratio of the total amount of label incorporated into AOP in the 60-minute ERG fraction from rats perfused with amino acid plus Gly-Gly to that from control rats perfused only with glycine plus leucine was 1.05 ± 0.2 ($P > 0.2$). Thus, the physiological products of Gly-Leu-Gly-Gly hydrolysis appear to be incapable of inducing AOP synthesis.

In identifying possible regulatory steps in the intracellular assembly and transport of AOP to the BB, the kinetics of [^{3}H]leucine incorporation into AOP in ERG and BB fractions was examined. The mean total amount of label incorporated into AOP in the ERG fractions in rats perfused intraluminally with the tetrapeptide or amino acid solutions before the [^{3}H]leucine pulse and chase was maximum at 30 minutes (Fig. 2). Newly synthesized, labeled AOP transported to the BB was detectable at 60 minutes and increased further at 180 minutes. Although the total incorporation of [^{3}H]leucine was quantitatively increased by short-term perfusion of the rats with tetrapeptide substrate, the kinetics of intracellular assembly and transport of AOP in both groups of protein-deficient rats was qualitatively similar to that which we previously found in rats fed a normal diet (*3*).

Possible changes in the assembly of the enzyme molecule were further assessed by sodium dodecyl sulfate (SDS) electrophoresis and autoradiography of AOP specifically immunoprecipitated from the 30- and 60-minute ERG fractions and from the 60-minute BB fraction in rats luminally perfused with tetrapeptide or the comparable amino acid mixture (Fig. 3). The AOP doublet was visi-

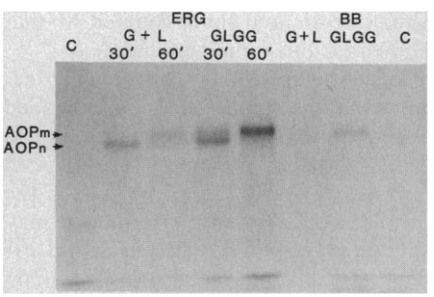

Fig. 3. SDS acrylamide autoradiography of the AOP immunoprecipitates obtained from pulse-chase experiments described in Figs. 1 and 2. The amount of AOP applied was 4 to 8 mU (ERG) and 100 mU (BB). The 6 percent gel with 2 percent cross-linking was impregnated with EN^{3}HANCE (New England Nuclear) and exposed to Kodak X-AR film (*3*). Despite the greater incorporation of label in the tetrapeptide-perfused (GLGG) rat, the sequence and timing of conversion from nascent (AOP_n) to the mature (AOP_m) enzyme and transfer to the BB surface membrane was the same as observed for normal rats (*4*).

ble in the ERG fraction in both animals. At 30 minutes the smaller, immature form predominated, whereas at 60 minutes it had been converted to a larger form that comigrated with the mature 130,000-dalton glycoprotein isolated from the BB. Neither the molecular forms nor the kinetic sequence of conversion from the nascent to the mature enzyme in ERG in protein-deficient rats differed from that observed for those fed a normal diet (*3, 4*). Hence, despite the abrupt increase in synthesis induced by luminal peptide substrate, the intracellular membrane–associated assembly and transport processes do not appear to be altered.

These pulse-labeling experiments in protein-depleted rats show that luminal exposure of substrate to the AOP at the intestinal membrane abruptly and specifically induces the synthesis of AOP on intracellular membranes. The subsequent post-translational conversion of AOP from the nascent to mature enzyme in the ERG and the kinetics of intracellular transport from the ERG to the cell surface remain unchanged. Because the amino acid and dipeptide products released from AOP hydrolysis of the tetrapeptide substrate did not induce synthesis, the signal is probably generated by virtue of the substrate as it interacts with the AOP.

In a study of sucrase, another intestinal BB digestive enzyme, incorporation of intraluminal [^{3}H]leucine into the cytosolic and BB enzyme increased 3 hours after simultaneous administration of sucrose and the amino acid tracer to rats (*8*). Although parameters of intracellular synthesis were not assessed at earlier times in that study, sucrase synthesis may be precisely regulated by luminal substrate in a manner similar to that shown for the aminopeptidase.

Because only a short hydrophobic segment of the aminopeptidase is embedded within the BB membrane whereas most of its domains are exterior to the cell surface, transduction of a signal to the intracellular synthetic machinery may require a secondary messenger; however, it is clear that the absorbable amino acid and peptide products do not carry the information for aminopeptidase induction. Whatever final mechanism of signaling is responsible for the induction of synthesis of the membrane aminopeptidase, the abrupt enhancement of digestive enzyme synthesis by its luminal substrate constitutes a precise local mechanism for regulation of intestinal nutrient digestion. The question of whether this is a general phenomenon of substrate-hydrolase regulation for nutrients presented to the intestinal surface must await analysis of other digestive and absorptive processes.

ANN M. REISENAUER*
GARY M. GRAY†
Department of Medicine, Stanford University School of Medicine, Stanford, California 94305

References and Notes

1. J. B. Rhodes, A. Eichholz, R. K. Crane, *Biochim. Biophys. Acta* **135**, 959 (1967); G. M. Gray and N. A. Santiago, *J. Biol. Chem.* **252**, 4922 (1977); R. K. Kania, N. A. Santiago, G. M. Gray, *ibid.*, p. 4929; Y. S. Kim and E. J. Brophy, *ibid.*, **251**, 3199 (1976); ———, J. A. Nicholson, *ibid.*, p. 3206.
2. K. W. Smithson and G. M. Gray, *J. Clin. Invest.* **60**, 665 (1977).
3. D. J. Ahnen, N. A. Santiago, J.-P. Cezard, G. M. Gray, *J. Biol. Chem.* **257**, 12129 (1982).
4. D. J. Ahnen, A. K. Mircheff, N. A. Santiago, C. Yoshioka, G. M. Gray, *ibid.*, **258**, 5960 (1983).
5. Y. S. Kim, D. M. McCarthy, W. Lane, W. Fong, *Biochim. Biophys. Acta* **321**, 262 (1973); J. A. Nicholson, D. M. McCarthy, Y. S. Kim. *J. Clin. Invest.* **54**, 890 (1974).
6. Protein-free test diet composition (percent): Celufil, 15; cod liver oil, 1; corn starch, 70; vegetable oil, 10; salt mixture (USP XVII), 4; vitamin supplement (U.S. Biochemicals).
7. Converted from 3.2×10^5 count/min by dividing by the tritium-counting efficiency [see figure 5 in (*3*)].
8. J.-P. Cezard, J.-P. Broyart, P. Cuisinier-Gleizes, H. Mathieu, *Gastroenterology* **84**, 18 (1983).
9. This work was supported by NIH grants AM 15802 and AM 11270. A.M.R. was a postdoctoral trainee under gastroenterology training grant AM 07056 and membrane pathobiology training grant GM 07026.
* Present address: Division of Clinical Nutrition, School of Medicine, TB 156, University of California, Davis 95616.
† To whom requests for reprints should be sent.

12 July 1983; accepted 3 July 1984

Enzyme Regulation in a Trypanosomatid: Effect of Purine Starvation on Levels of 3′-Nucleotidase Activity

Abstract. Crithidia fasciculata, *a nonpathogenic relative of the leishmanial and trypanosomal pathogens of humans and animals, showed a 3′-ribonucleotidase activity similar to that in* Leishmania donovani. *The level of 3′-nucleotidase activity in* Crithidia *was regulated by the availability of purines in the culture medium. Specifically, organisms obtained from culture medium depleted of purines contained elevated levels of enzyme activity compared to those grown in complete medium. The 3′-nucleotidase, located at the cell surface, may serve as a first step in purine salvage for these protozoa, which are unable to synthesize the purine ring de novo.*

Trypanosomatid protozoa, including human pathogens of the genera *Leishmania* and *Trypanosoma*, are dependent on an exogenous supply of preformed purines because they are incapable of de novo purine synthesis (*1*). Knowledge of the mechanisms by which the organisms acquire or "salvage" these essential nutrients is therefore of significance in understanding the host-parasite relation and may lead to improved chemotherapeutic strategies against these parasites of public health importance (*2*).

This dependence on purine salvage pathways has led to studies of the transport and metabolism of nucleosides and nucleobases in various trypanosomatids. Less, however, is known of salvage initiated by purine nucleotides. Evidence has accumulated for the existence of a potentially unusual 3′-nucleotidase activity in *Leishmania donovani* (*3*), African trypanosomes (*4*), and the nonpathogenic trypanosomatid *Crithidia fasciculata*. The 3′-nucleotidase activities in these related organisms share several characteristics, including substrate preference, optimum *p*H, effects of divalent cations and chelators, and resistance to fluoride, tartrate, and molybdate ions. Results from enzymatic studies with living cells and from cytochemical studies (*5*) indicate that the trypanosomatid 3′-nucleotidase is an ectoenzyme capable of hy-

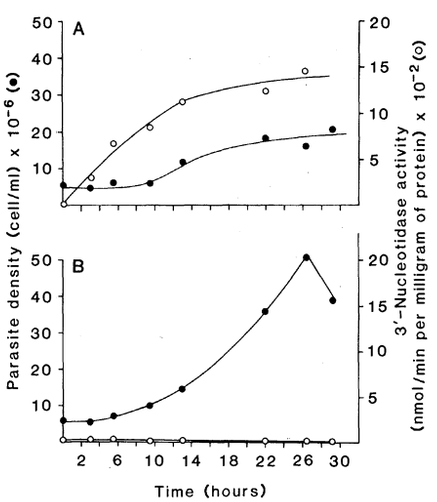

Fig. 1. Kinetics of increase of 3′-nucleotidase activity upon transfer of *C. fasciculata* to adenosine-depleted medium. Cells were harvested from medium containing 75 μ*M* adenosine and washed with tris-buffered saline. Washed organisms were resuspended in fresh medium either lacking (A) or containing (B) 100 μ*M* adenosine. Samples were removed at indicated times and cell density was determined by hemacytometry. Cells removed at these times were assayed for 3′-nucleotidase as described in the legend to Table 1. (●) Cell density; (○) 3′-nucleotidase activity.