Uptake and Transepithelial Transport of Nerve Growth Factor in Suckling Rat Ileum

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Abstract. Nerve growth factor (NGF) is necessary for the development of sympathetic and some sensory neurons. Milk may be a source of NGF for suckling young, but sites of intestinal absorption of the protein have not been identified. To determine whether NGF is transported across the absorptive epithelium of suckling rat ileum, we assessed binding, uptake, and transport of ^{125}I-NGF by light microscopy and EM autoradiography. Blood and tissue extracts were analyzed by biochemical and immunological methods to determine whether NGF was taken up structurally intact. NGF binding sites were identified on microvilli and apical invaginations of ileal absorptive cells in vitro. Injected into ileal loops in vivo, NGF radioactivity retained by fixation was evident after 20 min in apical regions of absorptive cells, in endocytic tubules (which mediate the uptake of membrane-bound ligands), in vesicles (which mediate nonspecific endocytosis), and in the supranuclear lysosomal vacuole. At 1 and 2 h, radiolabel in these compartments increased and silver grains were evident at the basal cell surface, and in cells, matrix, and vessels of the lamina propria. In blood and liver, radiolabeled molecules that were immunologically and electrophoretically indistinguishable from NGF and that co-eluted with NGF on gel filtration columns were detected, confirming that some NGF was transported across the epithelium structurally intact. Thus, absorptive cells of suckling rat ileum can take up NGF by both receptor-mediated and nonspecific endocytosis, and direct NGF either to the lysosome for degradation, or into a transepithelial transport pathway.

NERVE Growth Factor (NGF)[1] is a protein essential for the development of sympathetic and some sensory neurons (25). Parenteral administration of NGF during the first two postnatal weeks leads to hypertrophy of both classes of neurons, while deprivation of NGF, through the administration of NGF antibodies, leads to neuronal atrophy.

Proteins immunologically related to NGF have been detected in milk (2, 15, 19, 28, 37) and NGF administered orally to neonatal mice induces hypertrophy of sympathetic neurons (2). Milk may therefore be a source of NGF for suckling young. The site of absorption of NGF within the intestine, the mechanism of transport across the intestinal epithelium, and the extent to which the protein is degraded during transport have not been investigated.

A possible site of NGF uptake is the ileum. In the suckling rat, the ileal epithelium consists of absorptive cells that are specialized for uptake and degradation of milk macromolecules (7, 8, 14, 22). We have previously reported that these cells transport fluid-phase molecules and membrane-bound molecules to a large apical lysosome via separate prelysosomal pathways (11). Soluble tracers were transported in vesicles and delivered directly to the lysosome. Membrane-bound tracers, including cationized ferritin and certain lectins, first entered an apical tubulocisternal membrane system before passing into endosomal vesicles that ultimately fused with the lysosome. In addition, small amounts of cationized ferritin escaped lysosomal degradation and were transported in vesicles to the basolateral cell surface for release by exocytosis. These data raised the possibility that ileal absorptive cells may be a site of transcellular transport of physiologically important molecules.

Data in this study show that absorptive cells of the suckling rat ileum take up NGF by both receptor-mediated and nonspecific endocytic mechanisms, and that some NGF is transported across the epithelium to the lamina propria and circulation. Most NGF is degraded after uptake, but small amounts of immunoreactive and structurally intact NGF are detectable in portal and systemic blood and in liver.

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Materials and Methods

NGF Binding to Isolated Epithelium

2.5S NGF was prepared by the method of Mobley et al. (27) with an additional carboxymethyl cellulose column as previously described (36). NGF

1. *Abbreviations used in this paper*: EGF, epidermal growth factor; NGF, nerve growth factor.

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was radioiodinated by the procedure of Young et al. (38), except that 24 μg of NGF was used rather than 6 μg. Specific activity was 17 μCi/μg.

The method of Bjerknes and Cheng (4) as modified by Phillips et al. (31) was used to obtain epithelial sheets from rat ileum. Sprague-Dawley rats (12-d-old) were perfused with 30 mM EDTA in calcium and magnesium-free Hank's balanced salt solution (HBSS) buffered with 20 mM Hepes. Some epithelial sheets were quickly resuspended in HBSS/Hepes containing calcium and magnesium at 4°C and lightly fixed for 10 min in 1% glutaraldehyde in 0.1 M cacodylate buffer at 23°C. Tissues were rinsed briefly in cacodylate buffer, incubated in HBSS/Hepes containing 0.1 M glycine at 4°C (to block free aldehydes), and rinsed in HBSS/Hepes (without glycine) at 4°C. Epithelia were transferred to 0.5 ml PBS at 4°C containing 300 ng/ml ^{125}I-NGF, and gently agitated for 30 min. Unfixed epithelial samples were equilibrated at 4°C, then incubated in 0.5 ml PBS at 4°C for 15 min before being exposed to ^{125}I-NGF (300 ng/ml). 30 min later, tissues were rinsed and fixed. In all cases, control tissue samples were incubated in buffer containing unlabeled NGF (100 μg/ml) before and during exposure to ^{125}I-NGF. NGF binding was assessed by EM autoradiography after a 10-wk exposure. Radiolabel on apical cell surfaces was quantitated in paired EM autoradiographs (presence or absence of excess unlabeled NGF) by counting total silver grains associated with microvilli and apical invaginations over a standard length (182 μm) of epithelial surface.

Administration of ^{125}I-NGF In Vivo

12-d-old Sprague-Dawley rats were anesthetized by an intraperitoneal injection of 0.1–0.2 ml of a 25% urethane solution in PBS. A 3-cm segment of ileum was ligated, the proximal ligature initially being left loose to allow entry of a 27-gauge needle. The ligature was tightened around the needle and 50–60 μl of ^{125}I-NGF (26 μg/ml) in 0.1 M potassium phosphate, pH 7.0, containing 1 mg/ml BSA (buffer A) was injected into the lumen. (BSA was included in buffers to reduce nonspecific binding of NGF; 29, 30.) As the needle was withdrawn, the ligature was further tightened to minimize backflow of radiolabeled protein. Intestines were returned to the abdominal cavity and the pups were maintained at 37°C. One animal was killed immediately after receiving the radiolabel and tissues were collected within 5 min. Three other animals were killed at 20, 60, and 120 min.

Collection of Tissues

At death, 20–30 μl blood was withdrawn from the portal vein, the animal was decapitated, and trunk blood was collected. The ileum was cut just outside the ligatures and the loop was removed intact. The lumen was rinsed with 2 × 5 ml PBS (4°C) containing 0.5 mg/ml phenylmethylsulphonyl fluoride (PMSF), 10 mM ε-amino-*n*-caproic acid, 0.1 mM EDTA, 1.0 mM EGTA, and 0.5 mg/ml *p*-toluenesulfonyl-L-arginine methyl ester, and the rinses were collected. The loop was then divided into two portions; one was immediately frozen for biochemical analysis, and the other immersed in fixative and processed for microscopy and autoradiography. The brain, submandibular glands, heart, lungs, liver, spleen, remaining gastrointestinal tract from stomach to rectum, kidneys, and bladder were collected and frozen. Radioactivity was immediately counted in a gamma counter (75% efficiency; Packard Instrument Co., Downer's Grove, IL.) and tissues were stored at −20°C. Blood was allowed to clot at 4°C, was centrifuged at 49,000 *g* for 30 min, and serum was collected and stored at −20°C.

Tissue Processing and Autoradiography

For microscopy, segments of ileal loop were immersed in 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at 22°C. After 30 min, samples were cut into 0.5-mm slices and fixed for an additional 2 h. Isolated epithelia were fixed by flooding with the same fixative solution at 4°C. After 30 min, epithelial samples were warmed to 23°C and fixed 2 h more; subsequent solution changes were done after tissues were loosely pelleted in an Eppendorf microcentrifuge. All tissue samples were rinsed in cacodylate buffer at 4°C, postfixed in 1% OsO_4 in cacodylate buffer, en bloc-stained with 1% uranyl acetate in sodium acetate buffer, pH 6.0, dehydrated, and embedded in Epon-araldite. For light microscopy autoradiography, 1-μm sections were stained with iron hematoxylin, coated by dipping in emulsion (Ilford, Knutsford, England) diluted 1:1, exposed for 3 wk, and developed. Thin sections were mounted on Formvar-coated nickel grids, stained with uranyl acetate and lead citrate, and coated with carbon. Grids were then placed on glass slides and coated with a thin film of emulsion (L4; diluted 1:4 with distilled water; Ilford) by the loop method (5, 6). EM of undeveloped grids confirmed that the emulsion coat consisted of a monolayer of silver halide crystals. After exposure at 4°C for 13 wk, autoradiographs were developed for 45 s at 18°C in Kodak D19 (diluted 1:9 with distilled water) and were fixed in 24% sodium thiosulfate for 3 min. Sections were examined and photographed with a JEOL 100CX electron microscope.

Tissue Extraction

Tissues were homogenized in 0.1 M potassium phosphate, pH 7.0, containing PMSF, EDTA, EGTA, ε-amino-*n*-caproic acid, and *p*-toluenesulfonyl-L-arginine methyl ester in a Dounce homogenizer. All tissues were homogenized in 2.0 ml except for the gastrointestinal tract (4.5 ml) and brain (2.5 ml). Homogenates were centrifuged at 49,000 *g* for 30 min. The efficiency of radiolabel extraction ranged from 75 to 95%, except for brain (58–63%), gastrointestinal tract (46–67%), and lung (45–64%). Extraction was not significantly improved by repeated homogenization.

Immunoaffinity Chromatography

Antiserum against NGF was raised in rabbits and characterized as previously described (36). Immune serum (3.0 ml) was applied to a 4-ml column of protein A Sepharose (Sigma Chemical Co., St. Louis, MO). The column was washed with buffer A, and IgG was eluted with 1.0 M acetic acid (yield, 13.8 mg protein). IgG was coupled to 1.0 ml cyanogen bromide-activated Sepharose 4B (Sigma Chemical Co.) according to the manufacturer's instructions, and poured into a column (1.0 × 1.3 cm).

Tissue extracts in volumes up to 1.0 ml were applied to the affinity column and washed with 15 ml of buffer A. Bound protein was eluted with 20 mM HCl, 0.15 M NaCl (50 ml). Levels of immunoreactive protein were determined by dividing the amount of radioactivity in the HCl eluate by the total radioactivity recovered in the HCl and phosphate washes. Recovery of radioactivity ranged from 52 to 85%. Nonspecific binding was estimated by eluting ^{125}I epidermal growth factor (EGF) from the column under conditions identical to those used for ^{125}I-NGF–containing tissue extracts. Total recovery of EGF was 69–75%, 4% of which eluted in the HCl wash. The later (4%) was considered nonspecific background and was subtracted from all measurements of the retention of ^{125}I-NGF on the column.

Gel Filtration

Tissue extracts in volumes up to 1.0 ml were applied to a Sephadex G-50 column (0.9 × 35 cm) equilibrated in buffer A, and were eluted in the same buffer. Flow rate averaged 6 cm/h. Fractions (1 ml) were collected and radioactivity in each was counted. The column was calibrated with blue dextran, cytochrome C (mol wt 12,300), ^{125}I-EGF (mol wt 6,045), $^{3}H_2O$, ^{125}I-NGF, and tryptically degraded ^{125}I-NGF. Tryptically degraded NGF was prepared by incubating 100 ng ^{125}I-NGF and 10 μg BSA with 25 μg trypsin in 120 μl 0.1 M potassium phosphate, pH 7.0, for 4 h at 37°C. Several samples were chromatographed in buffer containing urea. The Sephadex G-50 column was equilibrated with 6 M urea in buffer A. Tissue samples were diluted 1:1 with 12 M urea in buffer A, applied to the column in volumes up to 1.0 ml, and eluted at a flow rate of 2.5 cm/h.

SDS Gel Electrophoresis

Tissue extracts and serum were electrophoresed on 13–22% acrylamide gradient SDS gels (23). Dried gels were autoradiographed using Kodak XAR5 film with a Cronex Lightning-Plus intensifying screen (DuPont) at −80°C. Molecular weight markers were BSA (mol wt 66,000), ovalbumin (mol wt 45,000), glyceraldehyde 3-P dehydrogenase (mol wt 36,000), carbonic anhydrase (mol wt 29,000), trypsinogen (mol wt 24,000), trypsin inhibitor (mol wt 20,100), and α-lactalbumin (mol wt 14,200) (Sigma Chemical Co.).

Competition Experiments In Vivo

To determine the effect of excess NGF on the uptake and transepithelial transport of radiolabeled NGF, ^{125}I-NGF (50 μl, 2.8 μg/ml) was injected into ileal loops (3 cm) in the presence or absence of unlabeled NGF (2.8 mg/ml). 60 min later, blood was collected by cardiac puncture, and TCA-precipitable radioactivity in serum was determined. Loops were removed and prepared for light microscopy autoradiography. The distribution of radioactivity in well-oriented epithelial areas was assessed by counting total grains over lengths of epithelium containing 50 epithelial cells, subdivided into three compartments: (*a*) apical (including microvilli and the endocytic

complex), (*b*) vacuolar (lysosomal vacuoles and cytoplasm between the lysosome and lateral cell membrane), and (*c*) basal (including all subvacuolar cytoplasm, nuclei, and basolateral membranes).

Results

Autoradiography

To determine whether ileal epithelial cells possess membrane receptors for NGF, unfixed or lightly fixed isolated epithelial sheets were exposed to ^{125}I-NGF at 4°C. Cells in unfixed epithelial sheets showed significant degeneration during the 45–60-min incubation period, as reported previously (31); for that reason, we used lightly fixed epithelia for analysis. Radiolabel was associated with microvilli and with apical invaginations of absorptive cells (Fig. 1 *A*). In the presence of excess unlabeled NGF, binding was reduced to 22% of control values (Fig. 1 *B*). These results suggest that binding sites specific for NGF are present on the luminal membranes of ileal cells and on the intermicrovillus membrane domain, a site from which endocytosis occurs (11, 12).

When ileal loops were exposed in vivo to ^{125}I-NGF for 20, 60, and 120 min, increasing amounts of radiolabel were evident within ileal absorptive cells and in the lamina propria. EM autoradiographs were assessed qualitatively to identify the cellular compartments containing radiolabeled molecules. Ileal absorptive cells are specialized for the uptake of molecules from the intestinal lumen, a function that is reflected in their morphology (Figs. 2–4). Coated pits are abundant on membrane invaginations between microvilli. The apical cytoplasm is filled with a complex system of endocytic compartments including an anastomosing tubulovesicular system and large, clear endosomal vesicles. Other vesicles containing dense material and multivesicular bodies are located below the tubular and vesicular endosomes. The center of the cell is dominated by a large lysosomal vacuole in which milk molecules and tracer proteins are degraded (7, 8, 11, 14).

After 20 min of continuous uptake, radiolabel was concentrated in apical regions, particularly in membrane invaginations and endocytic tubules (Fig. 2). Smaller amounts were detected in large clear endosomes, in vesicles containing dense material, and in the supranuclear lysosomal vacuole. Occasional silver grains were present in basolateral regions and in the vicinity of basolateral cell surfaces. After 1 h, increased numbers of silver grains were associated with all components of the endocytic pathway, including the apical cell surface and endocytic tubules (Fig. 3), clear endosomes, dense and multivesicular bodies, and the giant lysosomal vacuole. Increased label was also associated with lateral and basal cell surfaces and over extracellular matrix, cells, and vessels of the lamina propria. After 2 h, radiolabel associated with microvilli, invaginations, and apical tubules was increased over that observed at 20 min, but comparable to the amount at 1 h. Numbers of silver grains were significantly greater than at earlier time points in clear endosomal vesicles and the giant lysosomal vacuole (Fig. 4) as well as at lateral and basal cell surfaces (Fig. 5) and throughout the lamina propria.

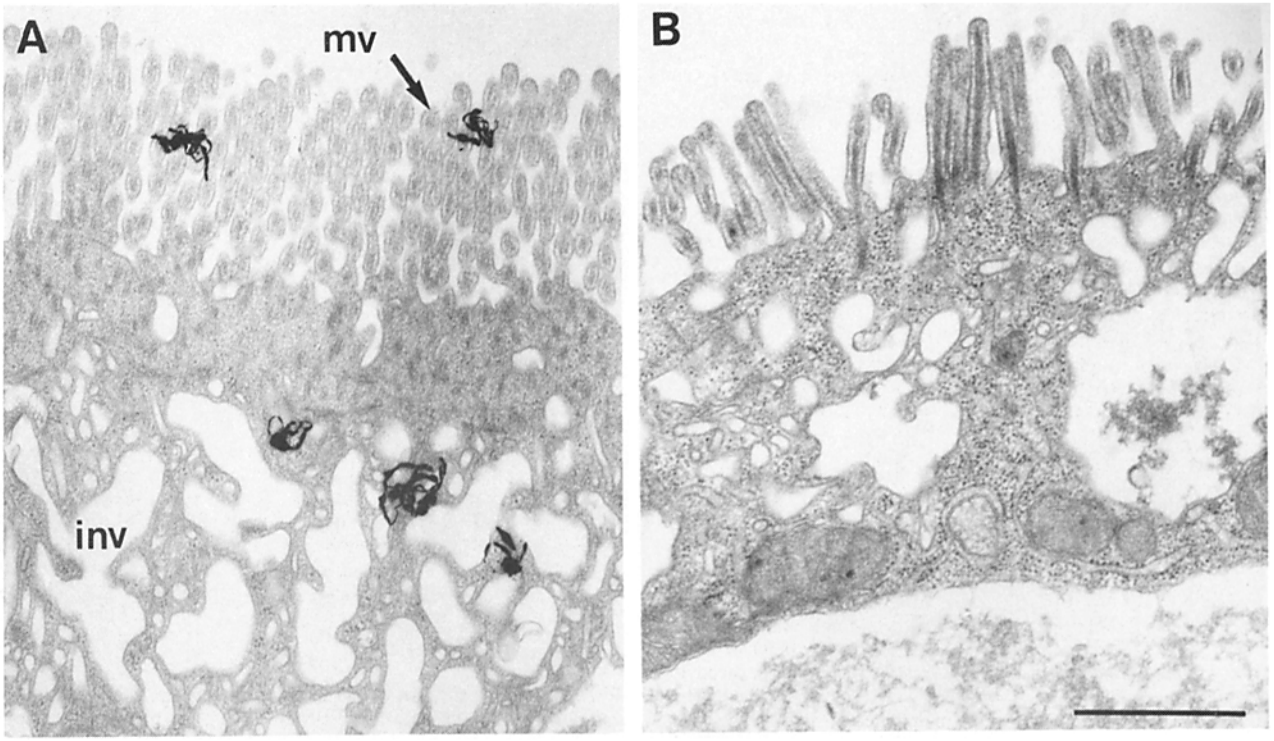

Figure 1. EM autoradiographs of the apical borders of representative absorptive cells in isolated fixed epithelial samples, exposed in vitro to ^{125}I-NGF (300 ng/ml) at 4°C. (*A*) Microvilli (*mv*) and the deep invaginations of apical plasma membrane (*inv*) are sectioned obliquely. The locations of silver grains suggest that radioactive NGF is associated with both membrane domains. Deeper regions of the cell showed no label. (*B*) In the presence of excess unlabeled NGF (100 μg/ml), the number of apical silver grains was reduced by 78%. On some cells, as shown here, no label was detected. Bar, 1 μm.

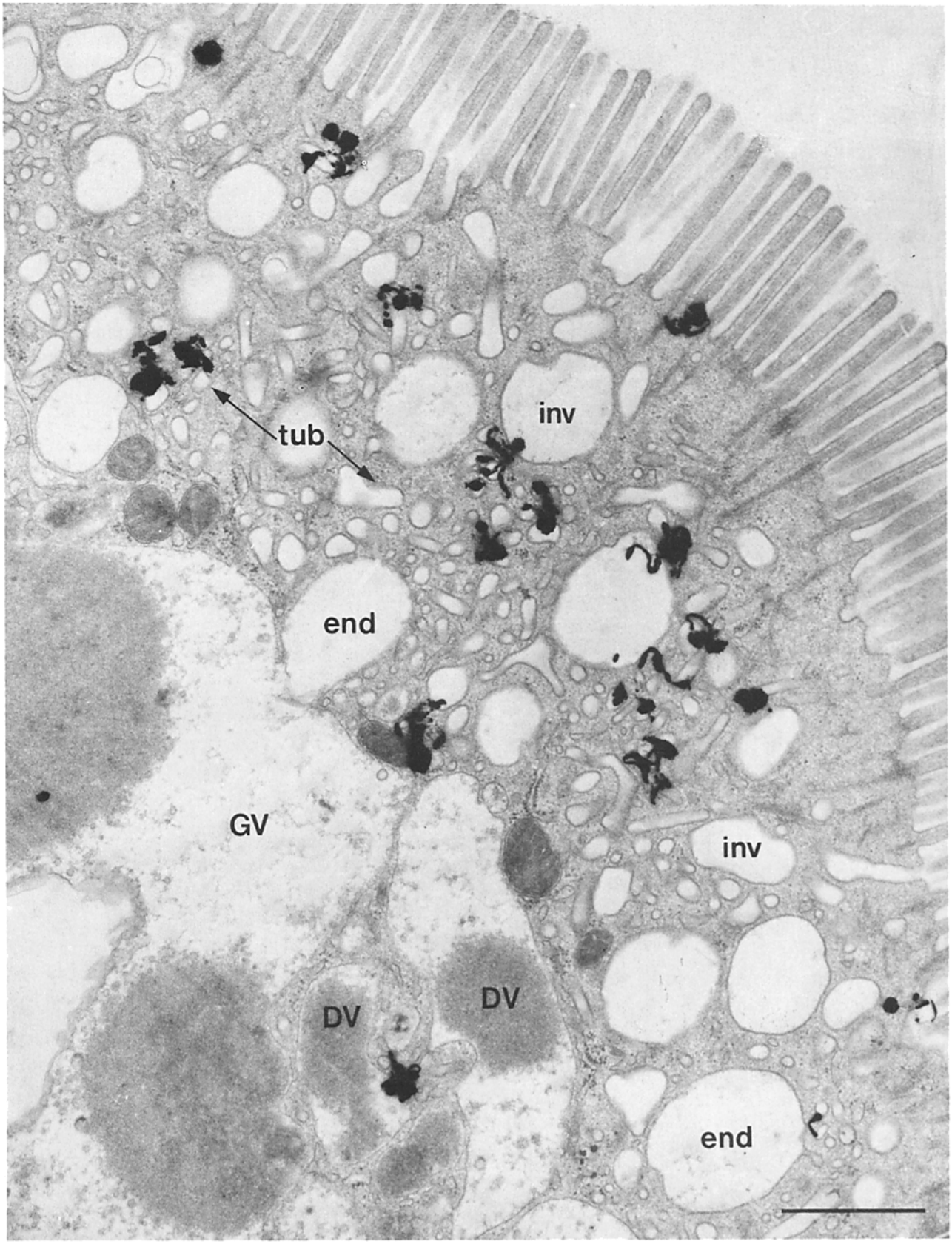

Figure 2. EM autoradiograph of an ileal absorptive cell 20 min after intraluminal injection of ^{125}I-NGF. Silver grains are located primarily over apical tubules (*tub*); some grains are also associated with apical plasma membrane invaginations (*inv*) and clear endosomal vesicles (*end*). *GV*, Giant vacuole. *DV*, Dense vesicle. Bar, 1 μm.

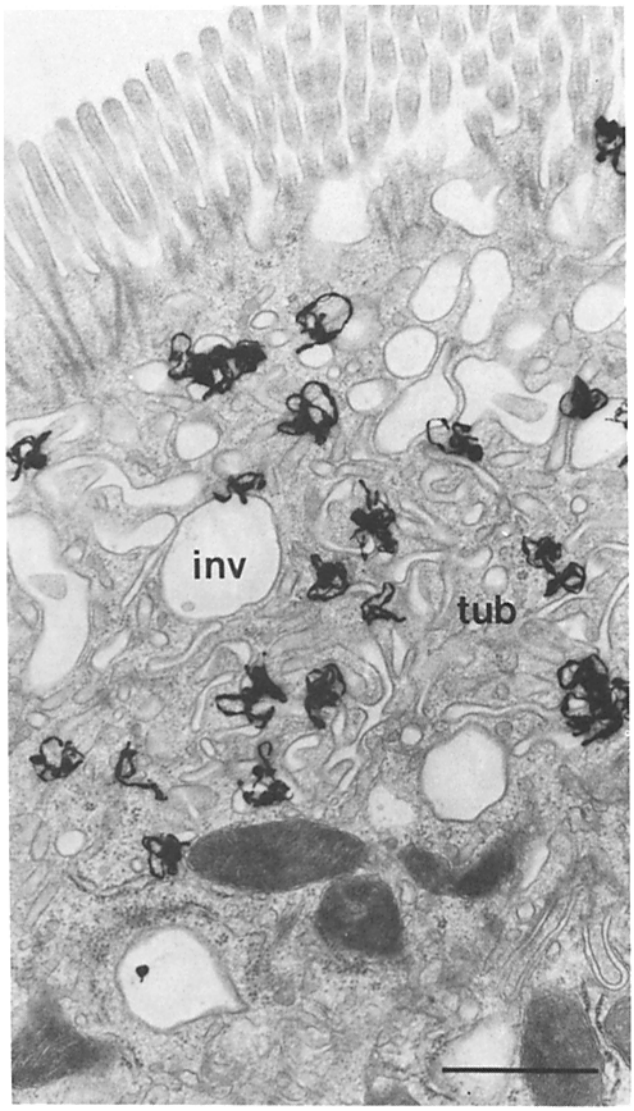

Figure 3. EM autoradiograph of the apical region of an ileal absorptive cell 1 h after intraluminal injection of ^{125}I-NGF. Silver grains are concentrated over apical invaginations (*inv*) and tubules (*tub*). Numbers of grains over endosomal vesicles and the lysosomal vacuole were increased at this time, and label was consistently detected at or near basolateral cell surfaces (not shown). Bar, 1 μm.

Tissue Distribution of Radioactivity

At all time points (5, 20, 60, and 120 min) after injection of ^{125}I-NGF, most recoverable radioactivity was found within the ileal lumen (26–35% of the injected dose; Table I) and remained relatively constant over 120 min. Levels of radioactivity in extracts of the ileal loop wall also remained steady over the course of the experiment, ranging from 10 to 15% of the applied radiolabel. Nonloop portions of the gastrointestinal tract contained radioactivity, ranging from 0.12% at 5 min to 6.58% at 120 min. Much of this material was intact NGF (see below) and probably arose from backflow out of the loop into adjacent ileal segments. In control experiments, leakage from the loop extended 1–2 cm on either side (data not shown), and was therefore restricted to the ileum.

Tissues other than the gastrointestinal tract contained no radioactivity at 5 min, but accumulated radioactivity between 20 min and 2 h. Some of this may represent radiolabel in blood within the organ vasculature rather than in the tissue itself. Appreciable amounts of radiolabel were detected in brain, kidneys, liver, and lung (Table I), but other tissues analyzed contained <0.5% of the total at 120 min. At each time point, systemic and portal serum contained equal amounts of radioactivity per volume (Fig. 6). The appearance of radiolabel in serum was biphasic, with radioactivity accumulating more quickly at early time points (before 20 min), and at a slower rate between 20 min and 2 h. In contrast, free ^{125}I was taken up at an initial rapid rate, but showed no further rise after 20 min (data not shown).

Affinity Chromatography

Serum, tissue extracts, and samples collected from the ileal lumen were applied to affinity columns containing anti-NGF IgG to determine whether radioactivity in the samples was immunologically reactive (Table II). In stock solutions of ^{125}I-NGF, 88–92% of the protein was retained on the column (after adjustment for nonspecific binding; see Materials and Methods). The column retained lower amounts of radioactivity retrieved from the lumen of the ileum (78–87%). Radioactivity in extracts of ileal loop walls taken at 20 and 60 min was retained on the column in amounts similar to that of lumen contents (76–78%), but by 2 h the amount was reduced (46%). Appreciable radioactivity in extracts of nonloop portions of the gastrointestinal tract taken at 20 and 60 min was also retained (19–39%), but none adhered at 120 min. In portal and systemic blood, up to 9 and 15% of radioactivity, respectively, bound to the column. Significant amounts of immunoreactive material were also present in liver at 20 min (7%) and in brain at 60 min (5%). Less than 5% of the radioactivity in all other tissues bound to the column and at 120 min values were no greater than background.

Gel Filtration Chromatography

To assess the molecular size of radioactive molecules in tissue extracts and serum, samples were chromatographed on a Sephadex G-50 column (Table III and Fig. 7). Control samples of ^{125}I-NGF eluted in four peaks: 13% of the activity eluted at the void volume and most likely represents aggregates of NGF generated by chloramine T oxidation; 62% of the activity eluted in a volume indistinguishable from unlabeled 2.5S NGF; 19% eluted at the total column volume and is likely free iodine or small radiolabeled peptides; and 6% of the activity was retarded and eluted in a volume greater than the column volume. Tryptically degraded radiolabeled NGF eluted primarily in the last peak (73%).

Most of the radiolabel retrieved from the lumen of the ileal loop eluted in a volume indistinguishable from NGF (67–84%). In extracts of ileal loop wall, the amount eluting in the NGF peak decreased from 61% at 1 h to 39% at 2 h, indicating degradation of the protein. Significant amounts of intact NGF were detected in nonloop portions of the gastrointestinal tract (50–57%), in portal and systemic serum (up to 13 and 10%, respectively), and in liver (5%), brain (3%), and bladder (6%) at single early time points (20 or 60 min). Material eluting in the NGF peak was not detected in other tissue samples.

In brain and liver extracts, appreciable amounts of radio-

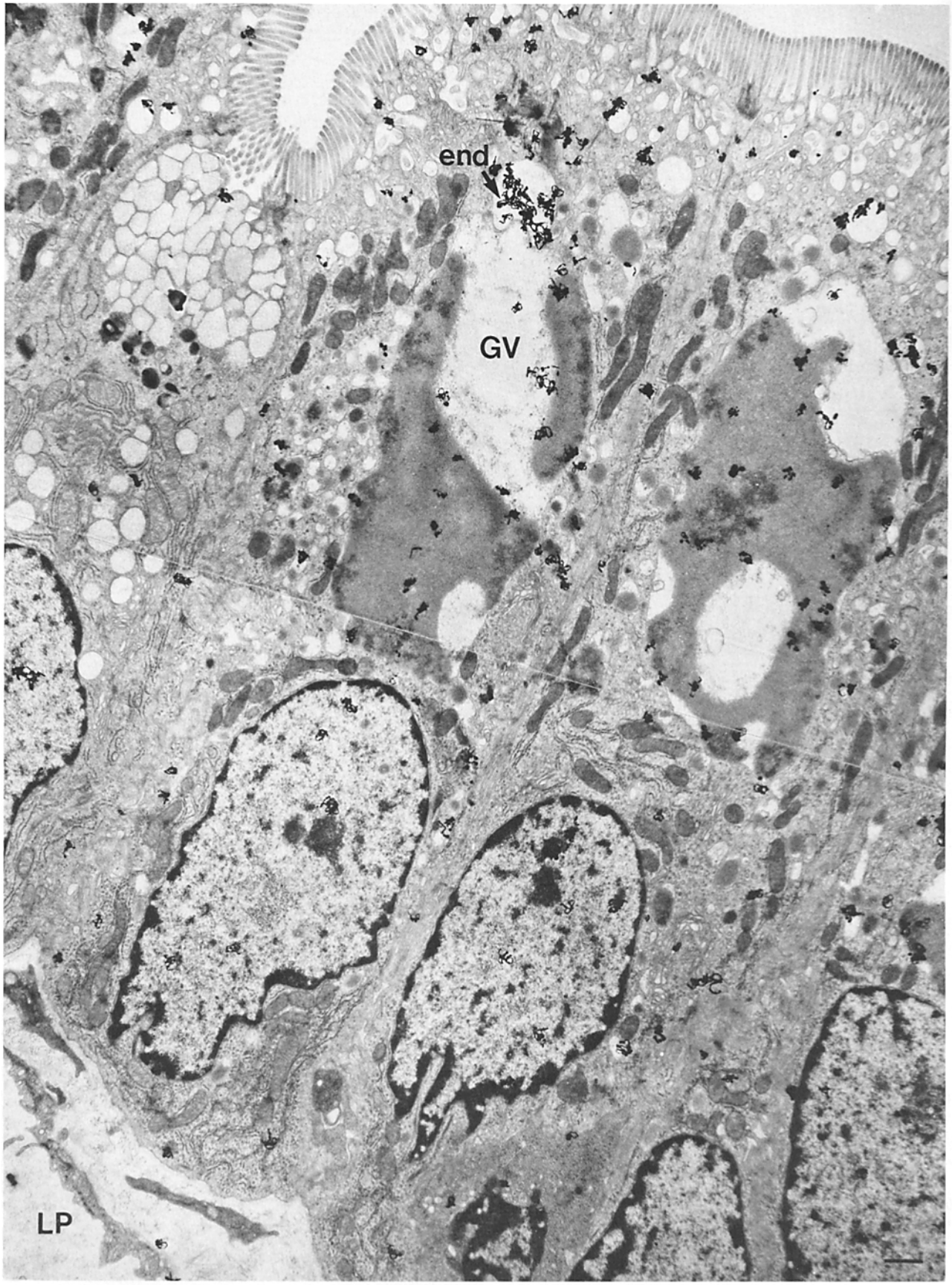

Figure 4. EM autoradiograph of the ileal epithelium 2 h after continuous exposure to intraluminal ^{125}I-NGF. Label associated with microvilli, apical invaginations, and tubulocisternae suggests continued uptake of NGF. Label is concentrated in some clear endosomal vesicles (*end*), in dense vesicles, and in the giant lysosomal vacuole (*GV*). Basal cell regions, lateral cell surfaces, and the lamina propria (*LP*) show increased radioactivity. Bar, 1 μm.

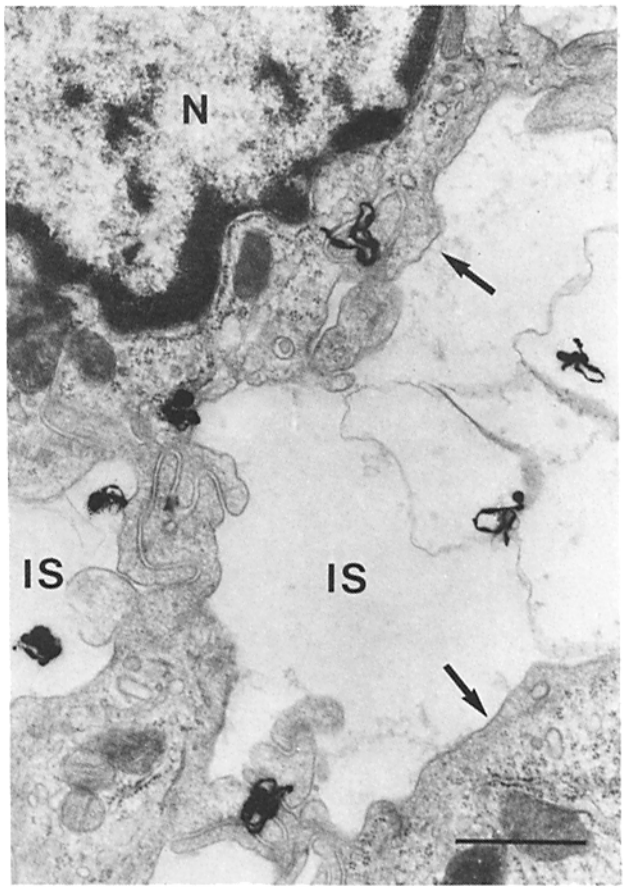

Figure 5. Lateral cell surfaces (*arrows*) of three adjacent cells at the level of nuclei (*N*) are separated by wide intercellular spaces (*IS*). After 2 h of uptake, silver grains in this region are primarily located on or near cell surfaces and in the intercellular spaces. Bar, 1 μm.

activity eluted in the void volume of the Sephadex column, accounting for as much as 43 and 44% of total activity, respectively. However, when chromatographed in buffer containing 6 M urea, the material eluted near the total column volume (Fig. 8), suggesting that it represents small fragments of NGF, rather than intact NGF, noncovalently bound to other molecules.

Gel Electrophoresis

Serum and tissue extracts were electrophoresed on SDS polyacrylamide gels and analyzed by autoradiography. Radiolabeled bands comigrating with intact NGF were detected in systemic and portal serum at 20, 60, and 120 min (Fig. 9) and in extracts of liver sampled at 20 and 60 min (not shown). Radiolabeled bands with similar mobility were not detected in other tissues tested, and no bands migrating ahead of NGF were seen in any sample.

Effect of Excess Unlabeled NGF on Uptake, Transport, and Degradation of ^{125}I-NGF In Vivo

After 60 min of continuous uptake of ^{125}I-NGF, 38% of the radiolabel present in serum was TCA-precipitable. After uptake in the presence of a 1000-fold excess of unlabeled NGF, only 16% of serum radioactivity was precipitated. Light microscopy autoradiographs of mucosal tissue from the loops, however, failed to reveal either a decrease in total grain counts over a standard length of epithelium area, or any selective change in relative counts over apical, vacuolar, or basal epithelial compartments.

Table I. Distribution of Radioactivity after Injection of ^{125}I-NGF into Ileal Loops

	Time after injection of radiolabel			
	5 min	20 min	60 min	120 min
Ileal loop lumen	31.1	35.1	25.7	30.2
Tissues				
Ileal loop wall	12.1	10.0	13.1	15.4
Gastrointestinal tract	0.1	1.1	3.1	6.5
Liver	0.0	0.3	0.4	1.0
Brain	0.0	0.2	0.3	1.2
Kidneys	0.0	0.1	0.3	1.1
Lungs	0.0	0.1	0.2	0.8
Spleen	0.0	0.0	0.1	0.2
Bladder	0.0	0.0	0.1	0.3
Submandibular gland	0.0	0.0	0.1	0.2
Heart	0.0	0.0	0.1	0.2
Total	43.3	46.9	43.5	57.1

Tissues were removed at the times indicated after the injection of ^{125}I-NGF into ileal loops and radioactivity was measured in a gamma counter. Radioactivity is expressed as the percentage of the total amount injected into the loop. Totals include radioactivity only in those tissues listed; tissues not listed were not examined. High values in the nonloop portions of the gastrointestinal tract represent leakage into the 1–2 cm of ileum adjacent to the loop.

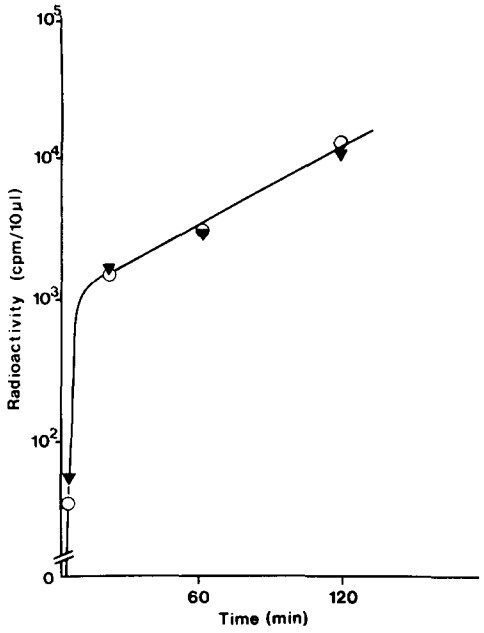

Figure 6. Radioactivity in serum from portal and systemic blood measured after injection of ^{125}I-NGF into ileal loops. Appearance of radioactivity was biphasic, the rate being more rapid before 20 min. *Open circle*, serum from portal blood; *triangle*, serum from systemic blood.

Discussion

Absorptive cells of the neonatal rodent ileum are specialized for endocytosis of luminal contents, and contain a large

Table II. Immunoaffinity Chromatography

	Time after injection of radiolabel		
	20 min	60 min	120 min
Ileal loop lumen	83	87	78
Tissues			
Ileal loop wall	78	76	46
Gastrointestinal tract	39	19	0
Liver	7	1	ND
Brain	0	3	5
Kidneys	0	2	0
Lungs	3	0	0
Spleen	ND	1	0
Bladder	ND	3	0
Submandibular gland	ND	4	0
Heart	ND	0	0
Blood			
Portal serum	ND	9	1
Systemic serum	ND	15	13

Tissue extracts and sera were applied to an anti-NGF affinity column (1.0 × 1.3 cm) and washed with 15 ml of 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mg/ml BSA. Absorbed protein was eluted with 20 mM HCl, 0.15 M NaCl. Values are expressed as the ratio of radioactivity in the HCl eluate to total recovered radioactivity, minus nonspecific values of 4%. When stock NGF was applied to the column, 88–92% of radioactivity was specifically eluted from the column. ND, not determined.

Table III. Gel Filtration Chromatography

		Peak: A	B	C
Standards				
Stock NGF		13	62	25
Trypsinized NGF		5	0	95
Ileal loop lumen	20	2	70	28
	60	6	84	10
	120	5	67	2
Tissues				
Ileal loop wall	60	11	61	28
	120	19	39	42
Gastrointestinal tract	60	5	57	38
	120	0	50	50
Liver	20	18	5	77
	60	44	0	56
	120	44	0	56
Brain	20	17	3	80
	60	43	0	57
	120	31	0	66
Bladder	60	15	6	79
	120	13	0	87
Blood				
Portal serum	60	11	5	84
	120	25	13	63
Systemic serum	60	10	7	83
	120	9	10	81

Tissue extracts and sera were applied to a Sephadex G-50 column equilibrated in 0.1 M potassium phosphate, pH 7.0, containing 1 mg/ml BSA, and eluted in the same buffer at 6 cm/h. Fractions (1 ml) were collected and radioactivity in each fraction was measured. Values are expressed as the percentage of total radioactivity recovered in the sample. Peak A, void volume; peak B, elution volume similar to that of NGF standards, peak C, sum of peaks eluting in volumes greater than the elution volume of NGF.

lysosomal vacuole that degrades milk molecules (7, 8, 14, 22). Products of lysosomal digestion diffuse out of the lysosome, across the basolateral cell membrane, and into the circulation of the suckling young. Early studies with nonspecific protein tracers suggested that endocytosis of luminal macromolecules is entirely nonselective and that none escape complete degradation (8, 14, 21, 22). In this respect the function of absorptive cells in the ileum was thought to contrast with that of jejunal absorptive cells, where Fc receptors effect receptor-mediated endocytosis of maternal IgG, sorting and withdrawal of receptor-bound IgG from an apical tubulovesicular membrane compartment, and transepithelial transport of intact IgG (1, 32, 33).

The possibility that the neonatal ileum is a second site of transepithelial transport has not been previously tested. Membrane systems generally associated with receptor-mediated endocytosis (3, 35) and receptor-ligand sorting (coated pits, tubules, and vesicular endosomes) (10) are present and are amplified in the ileal absorptive cell (11, 21). Furthermore, ileal cells sort endocytosed tracers: soluble tracers are carried in dense vesicles directly to the lysosomal vacuole, while membrane-bound macromolecules pass through the tubulocisternal system and large clear endosomes before entry into the lysosome (11). Small amounts of membrane-bound tracer are also transported to the basolateral cell surface, which is consistent with transepithelial transport. Evidence in this study suggests that the ileum transports NGF, a physiological ligand, and that the protein is delivered to the circulation intact.

It is unlikely that ileal absorptive cells use NGF and that we are witnessing the uptake of a ligand that is of direct physiological importance to the cell. The pattern of binding and uptake of radiolabeled NGF into ileal cells differs from that reported in pheochromocytoma (PC12) cells. In PC12 cells, which respond to NGF, only a small proportion of membrane-bound NGF was endocytosed and transported via tubules, endosomes, and multivesicular bodies to lysosomes; most remained associated with the cell surface (34). In ileal absorptive cells, on the other hand, large amounts of NGF were endocytosed and relatively small amounts were detected on apical cell surfaces at all time intervals.

NGF is absorbed and processed by both of the prelysosomal intracellular pathways previously described in the ileal absorptive cell (11); radiolabel detected by autoradiography was associated with vesicles that collect soluble tracers, and with the tubulocisternal compartment that receives membrane-bound molecules. It was not possible to determine what proportion of tubule-associated radioactivity was subsequently directed to the lysosome, or what proportion of the radioactivity in the lysosome was derived from fluid-phase or absorptive endocytic pathways. In any case, large amounts of radioactivity were present in the lysosome and probably account in part for the low molecular weight breakdown products detected in some tissues (including the ileum). Smaller amounts of radioactivity in basal regions of the cell, at basolateral membranes, and in the lamina propria may in part represent the immunoreactive and structurally intact NGF that was subsequently detected biochemically in blood and liver. On the other hand, many of these grains were not eliminated by competing, unlabeled NGF and could represent NGF breakdown products small enough to pass through the lysosomal membrane, but large enough to be retained by fixation.

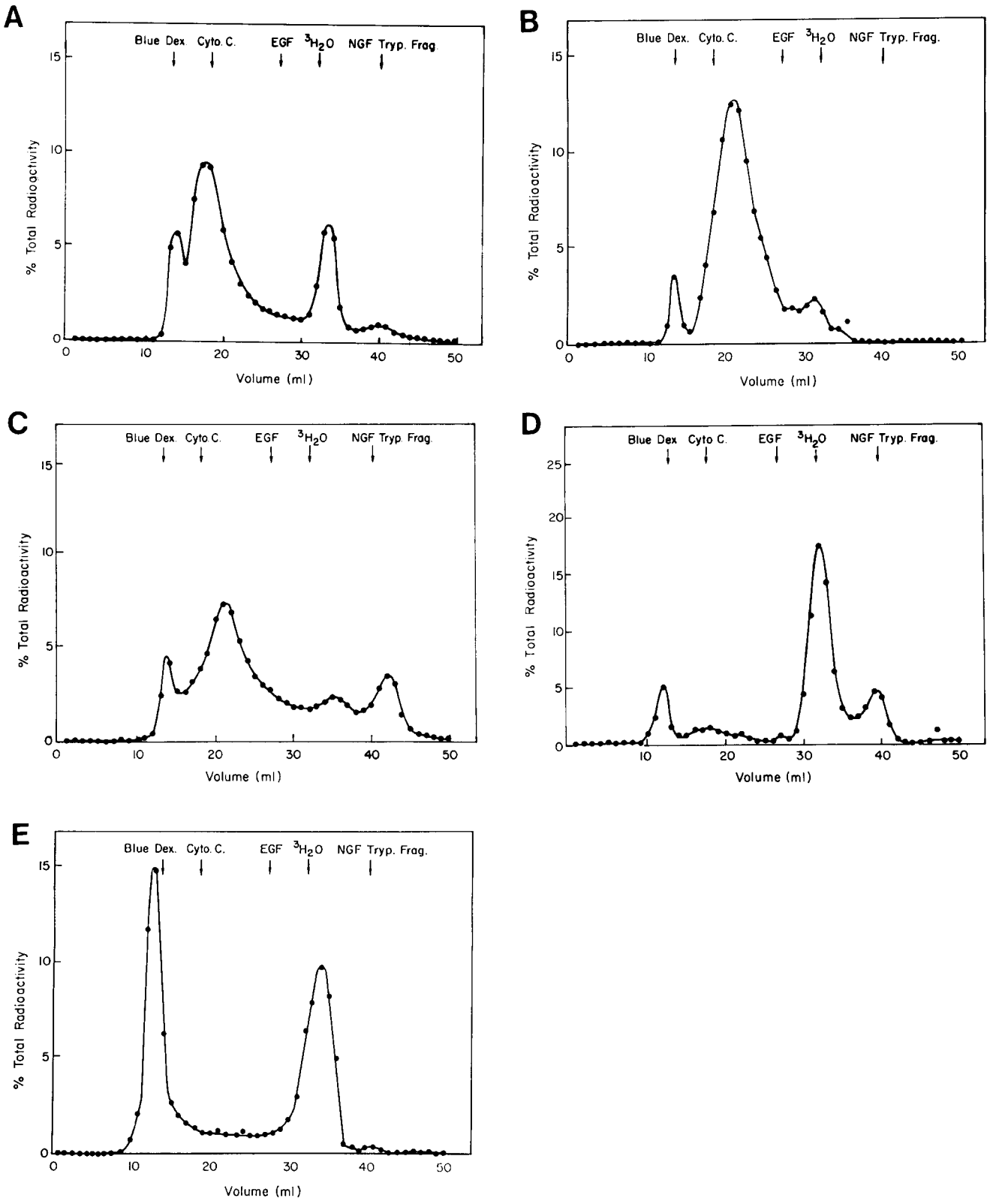

Figure 7. Tissue extracts collected 60 min after injection of ^{125}I-NGF into ileal loops were applied to a column of Sephadex G-50 equilibrated in 0.1 M potassium phosphate, pH 7.0, containing 1 mg/ml BSA, and eluted in the same buffer. Radioactivity in each fraction is plotted as the percentage of total recovered radioactivity. (*A*) Stock ^{125}I-NGF; (*B*) material within ileal loop lumen; (*C*) extract of ileal loop wall; (*D*) serum from systemic blood; (*E*) liver extract. Indicated at the top of the figure are the elution volumes of blue dextran (void volume), cytochrome C (mol wt 12,300), ^{125}I-EGF (mol wt 6,045), ^3H_2O (total volume), and tryptically degraded ^{125}I-NGF.

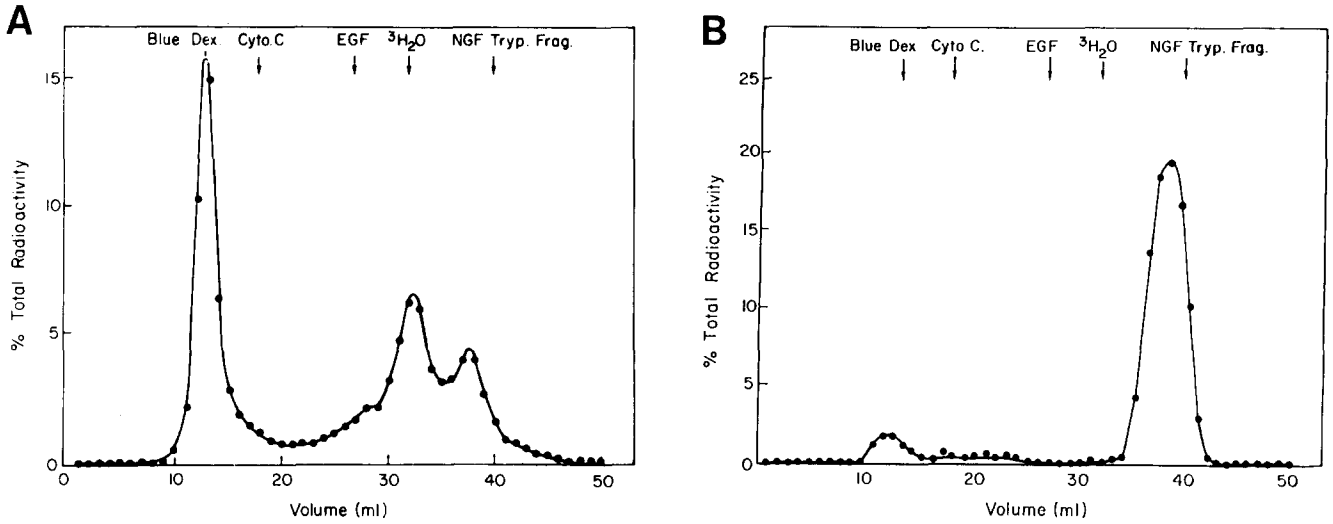

Figure 8. Brain extract collected 60 min after intraluminal injection of ^{125}I-NGF was chromatographed on a Sephadex G-50 column in the absence (*A*) or presence (*B*) of 6 M urea. Radioactivity in each fraction was measured and is plotted as the percentage of total recovered radioactivity. When run in the absence of urea, a significant amount of label eluted in the void volume. In the presence of urea, most radioactive material eluted near the total column volume, indicating that it represents degraded NGF, rather than intact NGF, noncovalently bound to other tissue components.

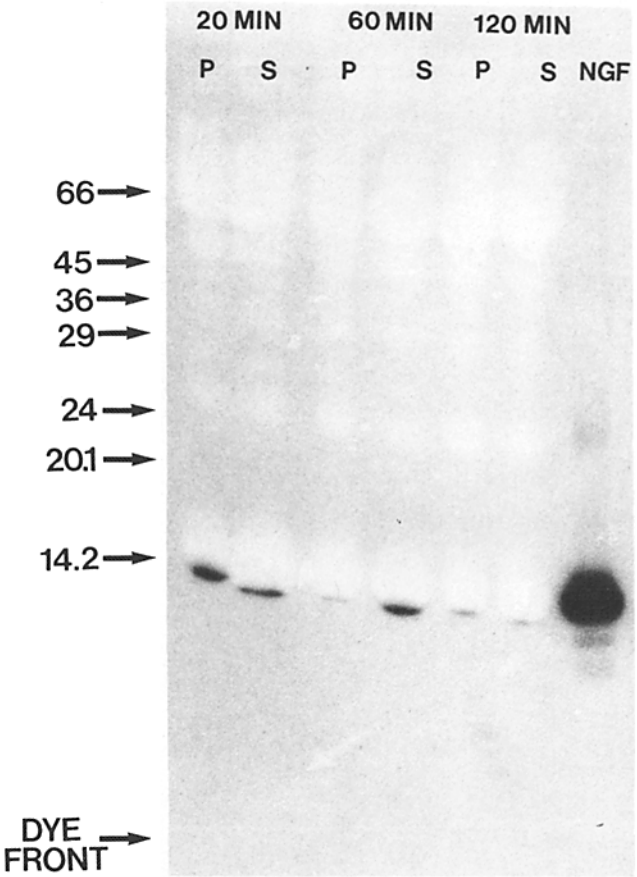

Figure 9. ^{125}I-NGF (500 cpm) and serum (4 μl) collected 20, 60, and 120 min after intraluminal injection of ^{125}I-NGF was electrophoresed on an SDS polyacrylamide gel. The gel was stained with Coomassie Blue, dried, and exposed to Kodak XAR5 film for 5 d at −80°C. The migration positions of molecular weight standards ($\times 10^{-3}$) are indicated. A radiolabeled band comigrating with NGF is apparent in each serum sample, and no other radioactive bands are visible. P, serum from portal blood; S, serum from systemic blood.

Free iodine and radiolabeled amino acids presumably would be lost during tissue processing. Indeed, autoradiographs prepared after intraluminal injection of ^{125}I not bound to protein showed no silver grains (data not shown).

Biochemical data confirm that some of the NGF was taken up and transported by the ileum intact. Radioactive molecules with electrophoretic mobilities identical to NGF were detected in portal and systemic blood 20, 60, and 120 min after the ileal load, and in liver at 20 and 60 min. A portion of the radiolabel in serum and liver was immunoreactive and eluted from gel filtration columns in volumes indistinguishable from intact NGF. It should be noted, however, that most of the radiolabel in serum and in tissue extracts was not intact NGF. Degradation of the protein could have occurred in ileal epithelial cells, in neuronal target tissues (24, 34), or in other peripheral tissues (20). These products are not necessarily biologically inactive, however, since proteolytic fragments of NGF stimulate nerve growth, reportedly at lower concentrations than the intact protein (26). In some tissues, such as brain and bladder, small amounts of radioactivity were detected that adhered to the NGF affinity column or that coeluted with NGF on gel filtration columns, but this material was not detected comigrating with NGF on SDS gels.

Although NGF in rat milk has not been measured, levels of NGF in mouse milk are reported to range from 0.032 to 1.1 μg/ml (15, 19, 28). It is therefore likely that concentrations of NGF used in this study are greater than those normally ingested by the suckling rat. However, administration of concentrations in the putative physiological range would not have allowed detection of uptake and transepithelial transport of radiolabeled NGF from a 3-cm loop of ileum using available methods. Aloe and Levi-Montalcini (2) also used high doses of NGF (10 μg/d) to demonstrate that the protein given orally stimulates development of sympathetic neurons in neonatal mice.

Over the time course of this study, there was no appreciable decline in levels of NGF radioactivity within the ileal lumen, indicating that the amount of NGF was, in fact, well

in excess of the absorptive capacity of the ileal cells. Furthermore, luminal NGF remained structurally intact, which is not unexpected, given the low levels of proteolytic enzyme activity in the suckling rat intestine (17). Therefore, ileal absorptive cells were exposed to a continuous high concentration of NGF. Given these conditions, most NGF would enter the nonspecific uptake pathway that delivers fluid-phase tracers to the lysosomal vacuole (7, 8, 11, 14). Nevertheless, a small amount of NGF was transported intact across the epithelium. At the high concentrations used, could intact NGF have been carried across nonspecifically, along with fluid contained in transport vesicles? We think not, for two reasons. First, in previous studies, concentrations of soluble proteins higher than those used here (native ferritin, 25–50 mg/ml) resulted in massive uptake into lysosomes, but there was no detectable transepithelial transport (11). Second, unlike NGF, soluble tracers generally do not adhere to ileal cell membranes or enter the endosomal tubules.

We did not establish whether binding or transepithelial transport of NGF is due to its interaction with a specific membrane receptor. Excess unlabeled NGF competed for binding sites on isolated epithelium, but NGF is a cationic protein (pI 9.3) (27) that might adhere electrostatically to anionic cell surfaces. It could thus enter tubules and transport vesicles in the same way cationized ferritin does (11). While our data suggest that NGF receptors may exist on ileal cells, further studies are needed to resolve this question.

Transepithelial vesicular transport must be relatively rapid, since silver grains were visible on the basolateral membrane and in the lamina propria, and intact NGF was detected in portal and systemic blood and several tissues, as early as 20 min after introduction of NGF into the ileal lumen. The appearance of radiolabel in serum was biphasic, with radioactivity accumulating more quickly at early time points (before 20 min), and at a slower rate between 20 min and 2 h. The initial rate may represent the rapid transport of iodinated amino acids or free iodine across the ileal cells, and the second, the release of NGF transported in vesicles and the diffusion of breakdown products from lysosomes.

NGF plays an important role in the development and function of the nervous system, but the sources of NGF in suckling and adult animals have not been conclusively identified. Several lines of evidence suggest that NGF is an ubiquitous protein that is produced locally in target tissues and taken up directly by neurons that innervate the tissue (9, 16, 18). Why then is there a cellular mechanism in the ileum of suckling rats for taking up exogenous NGF from the gut lumen? Evidence that orally administered NGF stimulates growth of sympathetic neurons in newborns (2) and that molecules immunologically indistinguishable from NGF are present in milk (2, 15, 19, 28, 37) suggests that additional sources of NGF may be required for normal postnatal neural development.

The cellular mechanism demonstrated in this study for the uptake of NGF could have general importance in mediating the uptake of other biologically active macromolecules in milk. In that regard, studies currently underway in our laboratories indicate that similar cellular pathways may be involved in the transepithelial transport of epidermal growth factor (13).

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