EARLY STAGES OF INTESTINAL ABSORPTION
OF SPECIFIC ANTIBODIES IN THE NEWBORN

An Ultrastructural, Cytochemical, and Immunological
Study in the Pig, Rat, and Rabbit

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ABSTRACT

In mammals, passive immunity is transferred from mother to offspring by transplacental
passage or by intestinal absorption. The rabbit receives antibodies exclusively across the
placenta, whereas intestinal absorption is the principal source of antibodies for the new-
born pig. In the rat, passive immunity is transferred by both pathways. The role of the
jejunal absorptive cells was investigated in these three species, by the use of specific immune
globulins as tracers of protein absorption. Rabbit anti-peroxidase and anti-ferritin anti-
bodies were injected into the jejunum of newborn pigs, rats, and rabbits, and absorption
was studied over the first 2 hr. The specific antibodies were detected in glutaraldehyde-
fixed tissues after in vitro treatment with the antigens, and in sera by immunological meth-
ods. Intact antibodies are transferred into the circulation of the pig and the rat, but not
into that of the rabbit. In the three species, the jejunal absorptive cells take up antibodies
by endocytosis. In the pig, the antibodies are transported across the epithelium in vacuoles.
In the rabbit, the endocytosis of antibodies triggers a lysosomal response and all absorbed
antibodies are trapped in lysosomes. In the rat, both situations are found; there is no evi-
dence of transfer of antibody fragments into the circulation.

INTRODUCTION

In mammals, passive immunity against infection
is transferred from mother to offspring before birth
or in the newborn; passage of antibodies follows
two pathways and occurs across the placenta or by
intestinal absorption (7, 8, 10).

In the pig, in the horse, and in ungulates, pas-
sive immunity is transmitted principally by means
of intestinal absorption of antibodies from colos-
trum or milk (4, 10, 37, 68). At birth the pig
possesses only traces of antibodies (44, 46, 60), the
main source of which is colostrum (20, 47, 48, 53).
Absorption across the intestinal mucosa occurs
only during the first hours of life (32, 55). During
this period, the pig absorbs homologous and
heterologous gamma-globulins (33), other lacto-
serum or serum proteins (58), and foreign proteins
such as polyvinyl-pyrrolidone (26). There are
marked differences between the absorption rates
of individual proteins (58).

When passive immunity is transferred across the
placenta, as in the rabbit, the guinea-pig, and the
human, the mechanism of intestinal absorption of
antibodies does not occur (6, 12, 42, 43, 65, 66).

The mouse, rat, dog, and cat represent an inter-
mediate group in which antibodies are transmitted
both across the placenta and by intestinal absorp-
tion after birth (14, 15, 17, 41, 84). The rat re-
ceives before birth about one-third of its passive
immunity (9, 24). The rate of intestinal absorption
in the newborn rat is very high for homologous
antibodies, and moderate for mouse and rabbit
antibodies; cow antibodies are not absorbed (2, 3,
25). Antibody absorption in the rat decreases
gradually after the first day and stops completely
at the age of 21 days (25).

In the pig, rat, and rabbit, striking and ap-
parently similar histological changes occur in the
intestinal columnar absorptive cells during protein

absorption. A vesicular and vacuolar apparatus develops after birth in the absorptive cells when they are in contact with colostrum or proteins (11, 30, 31, 67, 81, 82). Animals which have not been suckled show only few vacuoles (55). These morphological features seem in contradiction to the differences of protein absorption capacities in the three species. To investigate this apparent contradiction, the initial steps of intestinal absorption of well characterized proteins, i.e. rabbit anti-peroxidase and anti-ferritin immunoglobulins G (IgG)[1] were studied by morphological and immunological methods. Preliminary results were reported recently.[2]

MATERIALS AND METHODS

Experimental Procedure

The animals in these experiments were six newborn piglets (Sus crofa domesticus L.) which had been separated from the sow immediately after birth and before any ingestion of colostrum; 40 newborn albino rats (Rattus norvegicus Berk., Wistar strain), 10 of which were used under the same conditions as the pigs for morphological investigations; and 25 newborn rabbits (Oryctolagus cuniculus L.), six of which were obtained by uterotomy at term.

The rats and rabbits were anesthetized by hypothermy in a cold room at 4°C. The piglets received an intraperitoneal injection of 40 mg of sodium barbiturate (Eunarcon, Riedel, Berlin) per kg of body weight. A 7% solution of purified rabbit anti-peroxidase or anti-ferritin IgG, 2 ml per 100 g of body weight, was injected into the first part of the jejunum during laparotomy. Jejunal tissues were fixed 15, 30, 45, 60, 90, and 120 min after IgG administration, and blood samples were taken at the same intervals. Blood samples were obtained in rats and rabbits by decapitation, and in piglets by umbilical vein cannulation.

Immunological Procedures

ANTIGENS

1. Horse spleen ferritin (Pentex, Kankakee, Illinois).

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[1] Abbreviations used in the text: AcPase, acid phosphatase; DEAE-c, diethyl-diamino-ethyl-cellulose; Fab, antigen binding fragment; Fc, crystallizable fragment; IgG, immunoglobulin G; PAB-c, para-amino-benzyl-cellulose.

[2] J. P. Kraehenbuhl et M. Campiche. Absorption intestinale des immunoglobulines G chez trois espèces animales. Bull. Schweiz. Akad. Med. Wiss. In press.

2. Horseradish peroxidase (Fluka AG, Buchs, Switzerland) at 200 U/mg for immunization and 90 U/mg for immunohistological and immunological procedures.

3. Rabbit Fab and Fc fragments, which were obtained according to the method of Porter (59) and purified by exclusion chromatography on G 100 and G 200 Sephadex.

ANTISERA AND IMMUNIZATION PROCEDURES

Anti-peroxidase and anti-ferritin sera were produced by repeated injection into rabbits of 10 mg of peroxidase and 5 mg of ferritin, respectively; rabbit anti-pig and anti-rat sera by injection of 25 mg of pig or rat total serum proteins; and rat anti-rabbit serum by injection of 1 mg of rabbit total serum proteins. Sheep anti-rabbit Fab and Fc fragments sera were prepared by immunization with 2 mg of purified Fab or Fc fragment per injection. Before use, the anti-rabbit Fc fragment serum was absorbed with Fab fragment and the purity was tested by immunoelectrophoresis.

Rabbits were immunized with an emulsion of antigen in complete Freund's adjuvant. The first injection was given into the footpads of the hindlegs, and 2–5 further injections were given into the subcutaneous tissue of the neck at weekly intervals. 1 week after the last injection, the rabbits were exsanguinated by catheterization of the carotid artery. Rats were immunized by one injection of an emulsion of antigen in complete Freund's adjuvant in the four footpads and two further injections without adjuvant at weekly intervals. The rats were sacrificed 1 week after the last injection and blood was obtained by cardiac puncture. Sheep were immunized by three intramuscular injections of an emulsion of antigen in complete Freund's adjuvant at intervals of 2 weeks. Bleeding was started 1 week after the last injection.

PURIFICATION OF RABBIT ANTI-PEROXIDASE AND ANTI-FERRITIN IgG

Globulins of rabbit anti-peroxidase and anti-ferritin sera were precipitated by addition of 18 g of Na_2SO_4 per 100 ml of serum. The precipitate was washed twice in 18% Na_2SO_4 (29). The globulins were concentrated by pervaporation, dialyzed against a phosphate buffer, and then purified by one of the two following ways.

DEAE-c: About 5 g of globulins dialyzed against a pH 7.4, 0.0175 M phosphate buffer was placed on a 100 × 4 cm column containing 150 g of diethyl-diamino-ethyl-cellulose DE 11 (Whatman, Balston, England). The column had been previously equilibrated with a pH 7.4, 0.0175 M phosphate buffer. Elution was performed with the same buffer, fractions were collected in 5 ml samples, the optical density was read at 280 mμ in a Beckman DB spectro-

photometer, and IgG was identified by immuno-electrophoresis. Approximately 1.5 g of IgG was obtained.

PAB-C: The method used has been described by Webb and Lapresle (83). 20 g of para-amino-benzyl-cellulose (Serva, Heidelberg) was activated in 0.1 N HCl. 400 mg of peroxidase or ferritin was added to the PAB-c suspension. About 180 mg of peroxidase or 200 mg of ferritin was fixed after diazotation on PAB-c. The unreacted diazo groups were blocked with glycine. About 1 g of anti-peroxidase or anti-ferritin IgG was stirred for 2 hr in the diazotized PAB-c suspension. The suspension was washed and filtered on a Buchner funnel until the optical density of the supernatant at 280 mμ was less than 0.010. The specific antibodies were eluted with pH 2.5, 0.2 M glycine-HCl buffer by briefly stirring the suspension at 4°C and then filtering it on a Buchner funnel. The effluent was immediately dialyzed against a pH 7.5, 0.2 M phosphate buffer. After concentration by pervaporation, the solution was dialyzed against physiological saline. Recoveries were approximately 20 mg of anti-peroxidase and 50 mg of anti-ferritin antibodies per batch.

Since the volume of the tracer was critical in new-born rats, the PAB-c purified antibodies were used alone because of their very high specific activity. In pigs and rabbits, these antibodies were used to enrich the IgG separated on DEAE-c. All the solutions used as tracers had IgG concentrations of approximately 7%. Ultracentrifugal analysis of the final IgG solutions at concentrations of 10, 5, 2.5 mg/ml in pH 7.4, 0.01 M phosphate buffer in 0.15 N NaCl was carried out with a Spinco model E ultracentrifuge and revealed a single symmetrical peak with a sedimentation coefficient of $s_{w,20}^{0} = 6.96$. The immuno-electrophoretic analysis of these antibodies against rat anti-rabbit serum displayed one single line of IgG with anti-ferritin or anti-peroxidase activity.

Other Methods

Protein concentrations were determined by the use of biuret or Folin reagents.

Passive hemagglutination was performed according to the bis-diazotized benzidine micromethod of Stavitsky and Arquilla (71).

Microzone electrophoresis was performed on cellulose acetate with pH 8.6 veronal buffer at $\Gamma = 0.05$ and strips were read with a Beckman Analytrol apparatus.

The double *immunodiffusion* technique of Ouchterlony (54) on microscope slides was used with 2% agar in pH 8.2 veronal buffer at $\Gamma = 0.05$.

Immunoelectrophoresis was performed on microscope slides as described by Scheidegger (63). The gel was the same as for immunodiffusion. A field strength of 3.5 v/cm for 90 min was applied across the agar.

Exclusion chromatography of pig sera was performed on Sephadex G 200. Samples of 2 ml were dialyzed against pH 7.4, 0.01 M phosphate buffer in 0.15 N saline, and placed on a column measuring 2 × 100 cm equilibrated with the same buffer. Fractions of 3 ml were collected and the optical density was read at 280 mμ. The column was calibrated with 2 ml of a solution of rabbit IgG.

Techniques for Morphological Studies

Fixation

Glutaraldehyde (61) was purified by vacuum distillation (1, 18). A 2.2% solution was buffered with pH 7.4, 0.08 M sodium cacodylate. The final osmolarity was checked with a Knauer osmometer and adjusted to 380 milliosmol/liter.

The rat jejunum was fixed for 3 hr by immersion in the fixative solution. The pig and rabbit intestines were fixed by vascular perfusion of the fixative solution for 10–20 min, preceded by a 1–5 min perfusion of an oxygenated Ringer's solution containing 0.1% procain and adjusted with NaCl to 320 milliosmol/liter (19). In rabbits, artificial respiration at a maximum pressure of 8–12 cm H_2O through a tracheostomy was initiated and the thorax was opened. The perfusion solutions were introduced through a glass cannula into the left ventricle at 40 mm Hg maximum pressure and drainage was ensured by sectioning the umbilical vein. A flow of 10–20 ml/min was achieved. In piglets, the entrance cannula was placed in one of the carotid arteries and perfusion was performed at 65–80 mm Hg maximum pressure. Drainage was effected by cannulating the portal vein through the umbilical vein. A flow of 80–100 ml/min was obtained. After fixation, tissues were washed in several changes of pH 7.4, 0.2 M cacodylate buffer and stored at 4°C.

Preparation of Nonfrozen Sections

Nonfrozen sections were cut on a Sorvall TC-2 tissue sectioner (69) at 10–20 μ for immunohistological treatments and at 40–60 μ for histochemical incubations.

Immunohistological Techniques

DEMONSTRATION OF SPECIFIC ANTIBODIES: Anti-ferritin or anti-peroxidase antibodies were demonstrated by exposing nonfrozen sections to 0.5% ferritin or 0.1% peroxidase in pH 7.4, 0.2 M sodium cacodylate buffer at 4°C with continuous stirring. After this treatment, which was continued for as long as 3 days, the tissues were washed in the same buffer for prolonged periods of up to 5 days with frequent changes and continuous stirring. Sections which had been treated with peroxidase were immersed for 60 min in a solution of 5 mg of 3,3'-diaminobenzidine

tetrahydrochloride (Fluka) per 10 ml of pH 7.5, 0.05 M Tris-HCl buffer adjusted to 320 milliosmol/ liter with NaCl. This incubation medium was then replaced by a solution of similar composition but containing, in addition, 0.02% H_2O_2, and incubation was continued for 10–30 min. As controls, sections were incubated in diaminobenzidine without previous peroxidase incubation, other sections were incubated without H_2O_2, and jejunal tissues from animals injected with anti-ferritin antibodies were incubated with peroxidase, diaminobenzidine, and H_2O_2, under the conditions previously described.

EFFECT OF GLUTARALDEHYDE ON ANTIBODY ACTIVITY. An in vitro test based on a gel diffusion technique on agar plates (36) was developed. Plates containing 0.05% of purified rabbit anti-ferritin IgG were immersed for 2 hr in a 2.2% glutaraldehyde solution and washed for 4 days in pH 7.4, 0.2 M sodium cacodylate buffer. Holes were cut in the agar and filled with a series of dilutions of ferritin. After 18 hr of diffusion, the plates were rinsed for 24 hr in physiological saline and stained with Prussian blue to demonstrate the precipitation rings. Plates without glutaraldehyde treatment were prepared similarly. Plates containing control pig serum were immersed in 2.2% glutaraldehyde and treated in the same manner.

HISTOCHEMICAL TECHNIQUES

ACID PHOSPHATASE DEMONSTRATION: Nonfrozen sections were incubated at 37°C for 30 to 90 min in pH 5.0 Gomori medium with β-glycerophosphate (Eastman Organic Chemicals, Rochester, N.Y.) as substrate; the medium was modified according to Barka and Anderson (5). Controls consisted of incubations without glycerophosphate or with addition of 0.01 M NaF or with a pH 7.3 medium.

SCHIFF'S STAINING: Nonfrozen sections were treated by Schiff's reagent prepared by direct bubbling with SO_2.

PREPARATIVE PROCEDURES FOR LIGHT AND ELECTRON MICROSCOPY

The sections were rinsed in the corresponding buffer and postfixed for 60 min in cacodylate-buffered 2% OsO_4. Some sections incubated with glycerophosphate were treated for 45 min with 0.5% uranyl acetate before dehydration (70). The sections were dehydrated in acetone and embedded in Durcupan ACM (Fluka). Suitable orientation was obtained by flat embedding. Sections 1 μ thick were stained with a solution of 1% Azur II and 1% methylene blue in 1% borax and examined by light microscopy. Thin sections, cut on a Porter-Blum microtome with a diamond knife, were picked up onto grids covered with a Parlodion (Mallinckrodt Chem. Work) film reinforced with carbon. Some

sections were examined unstained, others were stained with lead citrate (80) or double stained with uranyl acetate followed by lead citrate. Micrographs were taken on a Zeiss EM 9 A electron microscope at 60 kv with a 50 μ platinum aperture, or on a Hitachi HU 11 A operated at 75 kv with a thin metal 30 μ aperture in the objective.

RESULTS

Rabbit IgG in Experimental Sera

ELECTROPHORESIS: The electrophoretic patterns of pig, rat, and rabbit sera are illustrated in Fig. 1. In the newborn pig, before protein ingestion, the serum protein profile was characterized by the absence of immunoglobulins (Fig. 1 a). A low peak appeared in the γ-G-globulin region, 120 min after injection of rabbit IgG into the jejunum (Fig. 1 b). This peak was slightly shifted as compared to the adult pig γ-G-globulin peak (Fig. 1 c), and its location corresponded to that of rabbit γ-G-globulins. In the rat, a low peak of γ-G-globulins was present at birth (Fig. 1 d). This peak was apparently unchanged 2 hr after injection of rabbit IgG. In the rabbit, the newborn (Fig. 1 f) and adult (Fig. 1 g) serum protein profiles showed similar γ-G-globulin peaks.

HEMAGGLUTINATION: The titration results for anti-ferritin activity of the purified IgG and of the experimental sera are grouped in Table I. No titre could be demonstrated in control pig, rat, and rabbit sera. After anti-ferritin IgG administration, a low activity (1:8) was already found in the first available samples of pig and rat sera. The titre increased to a maximum value of 1:256 at 120 min in both species. In contrast, no activity could be detected in the rabbit sera at any time, including 120 min.

IMMUNODIFFUSION: The absorbed rabbit anti-ferritin IgG was detected in experimental sera by use of a rat anti-rabbit serum, and the antibody activity was tested with ferritin. In pig and rat sera, single lines were obtained against anti-rabbit serum (Fig. 2) and against ferritin. In rabbits, even after 120 min, no lines were detected against ferritin.

IMMUNOELECTROPHORESIS: Pig and rat sera, 120 min after anti-ferritin IgG administration, showed a single arc against anti-rabbit serum in a location corresponding to that of IgG in control rabbit serum patterns (Fig. 4 a and b). A single line of precipitation was found against ferritin in the same location. In pig sera, there was no IgG line against anti-pig serum (Fig. 4 a),
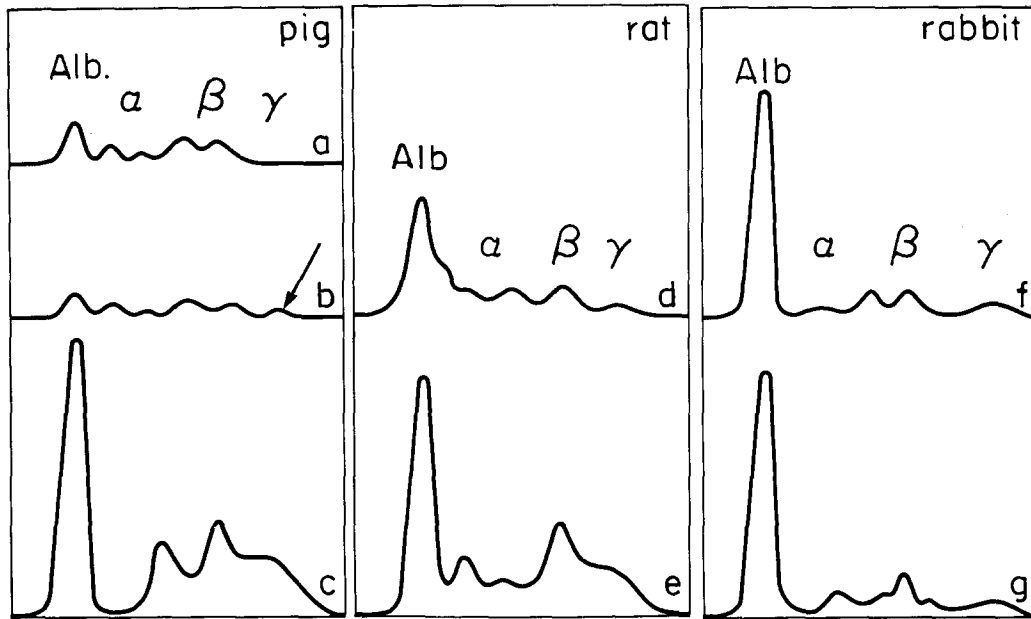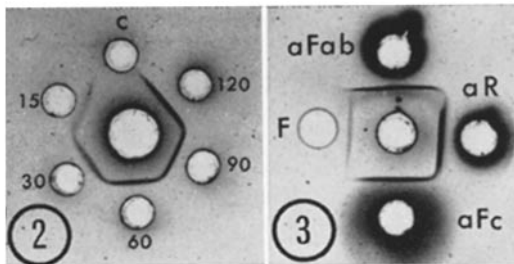
FIGURE 1 Electrophoresis patterns of newborn (a, b, d, f) and adult (c, e, g) pig, rat and rabbit sera. No γ-globulin peak is present in the profile of control newborn pig serum (a). 120 min after injection of rabbit IgG, a small peak appears $(b,$ arrow$)$ which is slightly shifted to the right when compared to that of adult pig γ-globulins (c). In newborn control rat and rabbit sera, the profiles show a small γ-globulin peak (d, f). The patterns are unchanged 120 min after IgG injection into the jejunum. In adult rat serum (e), the γ-globulin peak is higher than in the newborn, whereas in rabbit sera the size of the adult γ-globulin peak (g) is comparable with that of the newborn.

TABLE I

Anti-ferritin Activity of Anti-ferritin Serum, Anti-ferritin Antibodies, and Experimental Sera Tested by Passive Hemagglutination

	Reactions with test sample diluted to											
	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$	$\frac{1}{256}$	...	$\frac{1}{32,768}$	...	$\frac{1}{524,288}$
Rabbit anti-ferritin serum	+	+	+	+	+	+	+	+		+		−
Purified anti-ferritin antibodies	+	+	+	+	+	+	+	+		+		+
Pig sera												
control	−	−	−	−	−	−	−	−		−		−
15 min	+	+	+	−	−	−	−	−		−		−
30 min	+	+	+	+	−	−	−	−		−		−
45 min	+	+	+	+	+	+	−	−		−		−
120 min	+	+	+	+	+	+	+	+		−		−
Rat sera												
control	−	−	−	−	−	−	−	−		−		−
30 min	+	+	+	−	−	−	−	−		−		−
120 min	+	+	+	+	+	+	+	+		−		−
Rabbit sera												
control	−	−	−	−	−	−	−	−		−		−
120 min	−	−	−	−	−	−	−	−		−		−

FIGURES 2 AND 3 Immunodiffusion patterns of newborn pig serum after injection of IgG into the intestine. FIGURE 2. Central well: rat anti-rabbit serum. Peripheral wells: control pig serum (c) and pig sera taken 15, 30, 60, 90, and 120 min after IgG administration. A precipitation line is present at all time intervals. FIGURE 3. Central well: pig serum at 120 min. Peripheral wells: sheep anti-rabbit Fab fragment serum (aFab), rat anti-rabbit serum (aR), sheep anti-rabbit Fc fragment serum (aFc) and ferritin (F). Four precipitation lines are seen. The arcs between aR and aFab and aFc show a complete fusion indicative of complete immunological identity. Spurs are present between F and aFab and aFc.*

whereas rat sera showed one line against anti-rat serum. In the rabbit sera no line against ferritin was found.

Attempts at Demonstrating Rabbit IgG Fragments in Pig and Rat Experimental Sera

IMMUNODIFFUSION: In both species, after intestinal administration of rabbit IgG, single lines were obtained against anti-rabbit serum, anti-rabbit Fab fragment serum, anti-rabbit Fc fragment serum and ferritin (Fig. 3). The line against anti-rabbit serum showed complete identity with the lines against anti-Fab or anti-Fc sera. On the other hand, the line against ferritin showed partial identity with the lines against anti-Fab or anti-Fc sera.

IMMUNOELECTROPHORESIS: In pig and rat sera, single lines were found against rabbit anti-Fab and anti-Fc sera after intestinal administration of rabbit anti-ferritin IgG. They corresponded

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* Key to symbols used in the micrographs: BC, blood capillary; CC, connective tissue cell; CP, cytoplasmic process; DB, dense body; E, erythrocyte; ER, endoplasmic reticulum; G, Golgi complex; GC, goblet cell; L, lymphatic capillary; Mb, multivesicular body; Mv, microvilli; N, nucleus; V, vacuole.
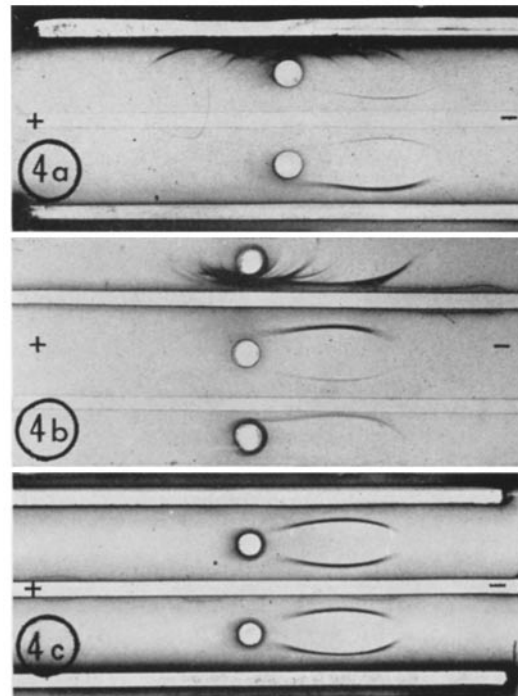

FIGURE 4 Immunoelectrophoresis patterns of pig serum 120 min after injection of rabbit anti-ferritin IgG into the intestine. All wells contain pig serum except for upper and lower wells of Fig. 4 b, which contain rabbit anti-ferritin serum.

FIGURE 4 a. Upper trough: rabbit anti-pig serum. Middle trough: ferritin. Lower trough: rat anti-rabbit serum. Precipitation lines against anti-rabbit serum and ferritin denote the presence of absorbed rabbit antibodies. Note the absence of pig IgG. 4 b. Upper trough: rat anti-rabbit serum. Lower trough: ferritin. The precipitation lines demonstrated in Fig. 4 a correspond to the IgG line of the control rabbit anti-ferritin serum. 4 c. Upper trough: sheep anti-rabbit Fab fragment serum. Middle trough: rat anti-rabbit serum. Lower trough: sheep anti-rabbit Fc fragment serum. A single line of precipitation is obtained against both sheep anti-sera. Their location corresponds to the rabbit IgG line, and no fragments are seen.

to the arc formed against anti-rabbit serum (Fig. 3 c).

EXCLUSION CHROMATOGRAPHY: Pig sera, before and after injection of rabbit anti-ferritin antibodies into the jejunum, and rabbit IgG were fractionated by gel filtration on Sephadex G 200. The elution pattern of rabbit IgG was characterized by a single peak with a maximum at approximately 140 ml. Pig sera had elution patterns show-
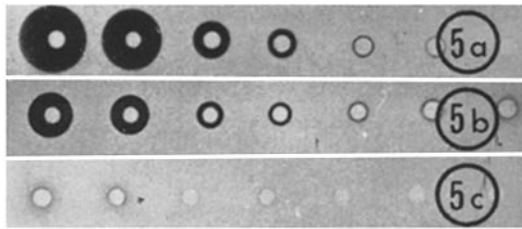
Figure 5 Immunoplates of purified rabbit anti-ferritin IgG and control pig serum. In the wells, dilution series of ferritin (from left to right: 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001%). Precipitation rings were stained with Prussian blue. 5 a and b. Rabbit IgG. After treatment with 2.2% glutaraldehyde (Fig. 5 a), the precipitation rings are about twice as large as the rings of non-treated plates (Fig. 5 b), indicating that immunological activity is still present, although decreased. 5 c. No precipitation ring and no adsorption of ferritin are observed in pig serum treated with glutaraldehyde.

ing peaks at 100, 160, and 220 ml. After IgG injection into the jejunum, an additional shoulder was observed at 140 ml on the ascending part of the second peak (Fig. 6). The fractions obtained from these sera were grouped into five pools which were tested by immunoelectrophoresis with rabbit anti-pig serum, ferritin, anti-Fab, anti-Fc, and anti-rabbit sera. Pool I contained α_2-macroglobulins. Pig globulins and the absorbed rabbit IgG were detected in pools II and III; single lines were observed against ferritin, anti-Fab, anti-Fc, and anti-rabbit sera. Pools IV and V contained only pig globulins and albumin.

Influence of Fixative on Antibody Activity

Immunoplates containing purified anti-ferritin IgG are shown in Figs. 5 a and b. Precipitation rings with ferritin of identical dilution were about twice as large when the plates had been previously exposed to glutaraldehyde. No precipitation rings and no nonspecific adsorption of ferritin was seen with a control pig serum treated with glutaraldehyde (Fig. 5 c).

Ultrastructure of Jejunal Villi Before and After Injection of Anti-peroxidase IgG into the Intestine

PIG

In newborn controls (Fig. 7), the absorptive epithelial cells were separated by narrow intercellular spaces bridged in both the apical and basal areas

by small desmosomes. The epithelial basement membrane was interrupted at places by short cytoplasmic processes of the absorptive cells. Nuclei were round and occupied a central or slightly apical position. At the base of the microvilli, a few invaginations of the plasma membrane were observed, and the apical cytoplasm under the brush border contained vesicles and numerous narrow tubules. Golgi complexes were infranuclear and consisted of small stacks of cisternae and few vesicles. Some coated vesicles were located in the Golgi area. After incubation for AcPase activity, lead phosphate deposits were observed in the brush border. No significant activity could be demonstrated in the Golgi areas. The lamina propria of the villi contained a central lymphatic vessel and small blood vessels at the periphery. All lumina were open and devoid of plasma. Occasionally erythrocytes were seen in the blood vessels. The interstitial space was wide and contained spider-like connective tissue cells with elongated cytoplasmic processes.

30 min after IgG administration, dense granular material was located in the intestinal lumen. Invaginations of the plasma membrane were very numerous at the base of the microvilli; the apical cytoplasm, immediately under the brush border, was filled with tubules and vesicles which contained homogeneous dense material. Some vacuoles and vesicles with dense material were found in the supranuclear area. At 60 min the vacuoles and vesicles were more numerous and also occupied the paranuclear cytoplasm (Fig. 8). Vacuoles of similar appearance were frequently observed in the endothelium of lymphatic vessels and in connective tissue cells, and only occasionally in the endothelium of blood capillaries or venules (Fig. 9). Vacuoles and vesicles were observed also in the vicinity of Golgi complexes (Fig. 10). At 120 min the number of vacuoles containing dense material had further increased. The vacuoles were located mainly in the para- and infranuclear cytoplasm (Fig. 11). After exposure to peroxidase, typical granular reaction product was seen in many vesicles and vacuoles (Figs. 11 and 13). It was recognized more readily when double staining was avoided because of the strong density which this procedure conferred on the contents of the vacuoles (Figs. 8 and 12). Peroxidase reaction product tended to be restricted to the edge of the nonfrozen sections. Even in areas where vacuoles were labeled, the distribution of reaction product
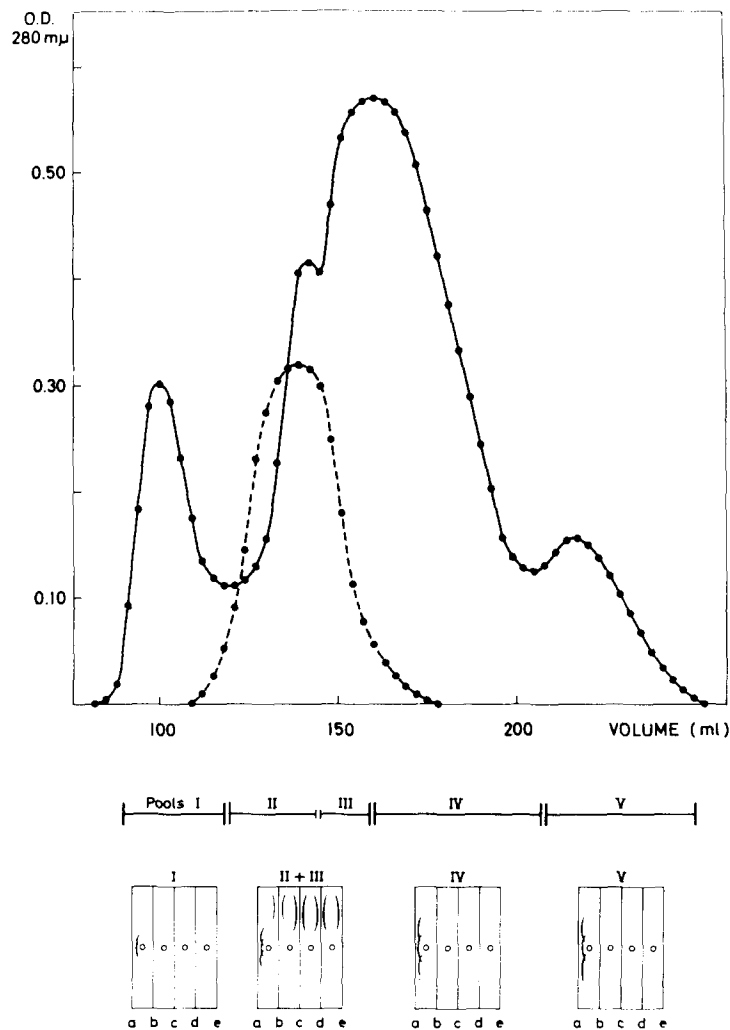
FIGURE 6 Patterns of elution from a Sephadex G 200 column of pig serum 120 min after injection of rabbit anti-ferritin IgG (———) and of purified rabbit IgG (--------). Fractions were collected in 5 pools, which were tested by immunoelectrophoresis with rabbit anti-pig serum (a), ferritin (b), rat anti-rabbit serum (c), sheep anti-rabbit Fab (d) and Fc (e) fragments sera. For a detailed comment, see Results.

was irregular and varied considerably from one vacuole to the next (Fig. 11). The AcPase activity did not increase after IgG administration. In particular, no lead deposits were found in the vesicles and vacuoles containing dense material, nor in the Golgi complexes.

RAT

In newborn control animals, the width of the intercellular spaces between the absorptive epithelial cells varied widely, and the basal surface

of the epithelial cells was smooth. The ovoid nuclei were located in the basal region of the epithelium. Apical cell membrane invaginations, vesicles and tubules were infrequent. Golgi complexes consisting of numerous cisternae and vesicles were located in the para- and supranuclear areas. Some of the Golgi elements and some dense bodies exhibited AcPase activity. The lamina propria was collapsed, blood vessels were filled with blood cells, and lymphatic vessels were very difficult to identify. This appearance was due to
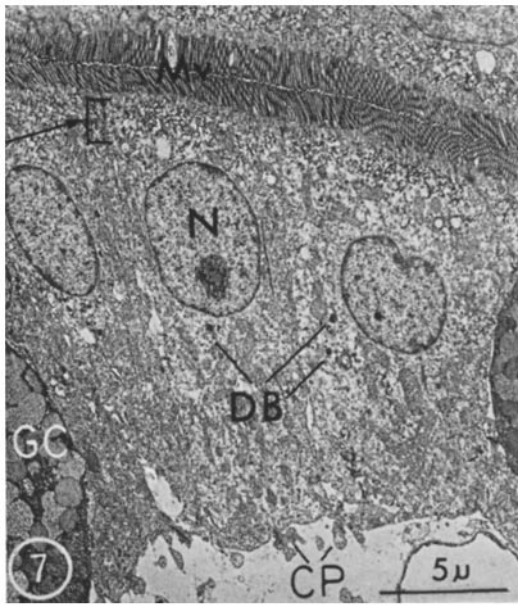
Figure 7 Control newborn pig. Absorptive cells contain numerous tubules and vesicles (arrow) in the apical area. A few small dense bodies are scattered in the cytoplasm. Uranyl acetate and lead citrate. × 3400.

the fixation by immersion and contrasted with the appearance of the lamina propria in pig and rabbit intestine fixed by perfusion.

30 min after injection of antibodies, apical invaginations and vesicles were more numerous than in the tissues of control animals; they contained dense material which was found also in the lumen. At 60 min, round vacuoles containing some moderately dense material were observed frequently in the apical cytoplasm (Fig. 14). At 120 min, vacuoles were more numerous and frequently were clustered in the apical region. Larger vacuoles were surrounded by smaller vacuoles, vesicles and multivesicular bodies containing moderately dense material, and by Golgi complexes (Fig. 15). A few vacuoles with the same contents were located in the basal part of the epithelium and in the lamina propria (Fig. 14). No vacuoles were found in the endothelium of lymphatics or blood vessels. After immunohistological treatment irregular aggregates of peroxidase reaction product were seen in the vesicles and vacuoles previously described. AcPase activity increased after administration of IgG into the intestine. Lead phosphate deposits were observed in some of the vacuoles, in multi-

vesicular bodies and in some autolytic vacuoles (Figs. 16 and 17). AcPase activity remained unchanged in Golgi complexes.

Rabbit

The absorptive epithelial cells of control animals were separated by wide intercellular spaces except in the apical and basal regions. Nuclei were ovoid and located in the basal part of the cells. Invaginations of the apical plasma membrane were frequent and numerous narrow tubules occupied the apical cytoplasm under the brush border. Golgi complexes were located in the para- and supranuclear regions. A few Golgi cisternae and vesicles, some dense bodies, and the brush border showed AcPase activity. Blood and lymphatic vessels had open lumina and the interstitial space of the lamina propria was wide.

30 min after injection of antibodies into the jejunum, homogeneous dense material filled numerous apical invaginations, vesicles, and tubules. Numerous vacuoles of irregular shape were grouped in the apical cytoplasm and contained the same material. At 60 and 120 min, clusters of vacuoles were located closer to the nuclei (Fig. 18). Complex vacuolar structures with dense homogeneous contents were seen in the immediate vicinity of the nuclei (Fig. 20) and appeared to be the result of the partial fusion of the membranes of individual vacuoles (Fig. 19). No vacuoles were found in the basal cytoplasm or in the lamina propria. The contents of these tubules, vesicles, and vacuoles showed peroxidase activity after immunohistological treatment. No peroxidase reaction product was found in the Golgi elements. At 30 min, Golgi complexes had more numerous cisternae and vesicles than in comparable areas of the control preparations. There was a further increase in the number of Golgi elements after 60 and 120 min. AcPase activity was strongly increased already after 30 min and continued to rise at later intervals. Lead phosphate deposits were abundant in most of the vacuoles of the apical pole (Fig. 21) and heavy deposits were observed in the complex vacuolar structures located near the nuclei (Figs. 21 and 22). Reaction product was present in the Golgi complexes, especially in the cisternae nearest to the nuclei (Fig. 23) and in vesicles (Fig. 26) and smooth reticulum saccules (Fig. 25) in the vicinity of the Golgi complexes. Deposits were also found in autolytic vacuoles and in multivesicular bodies (Fig. 24).
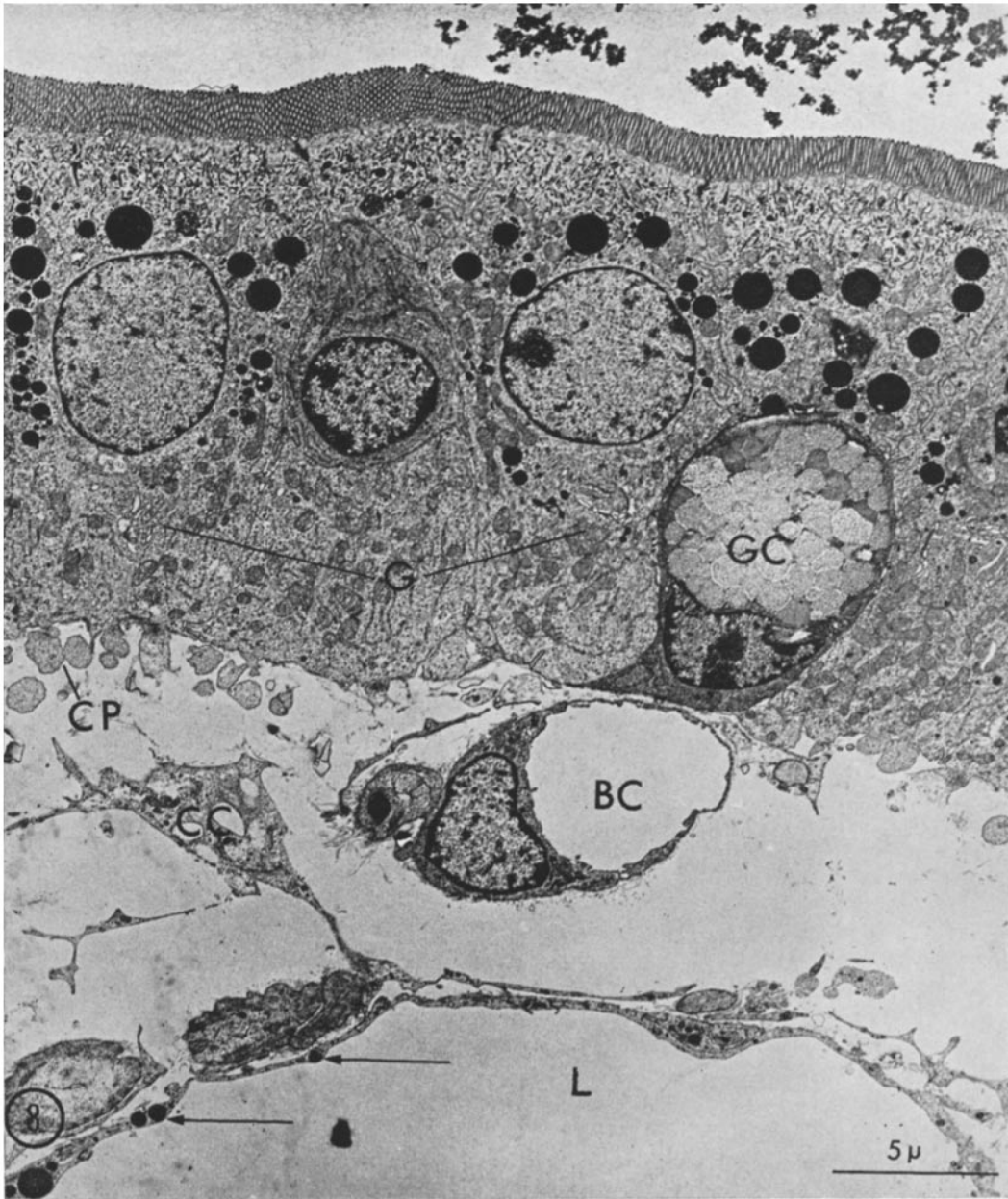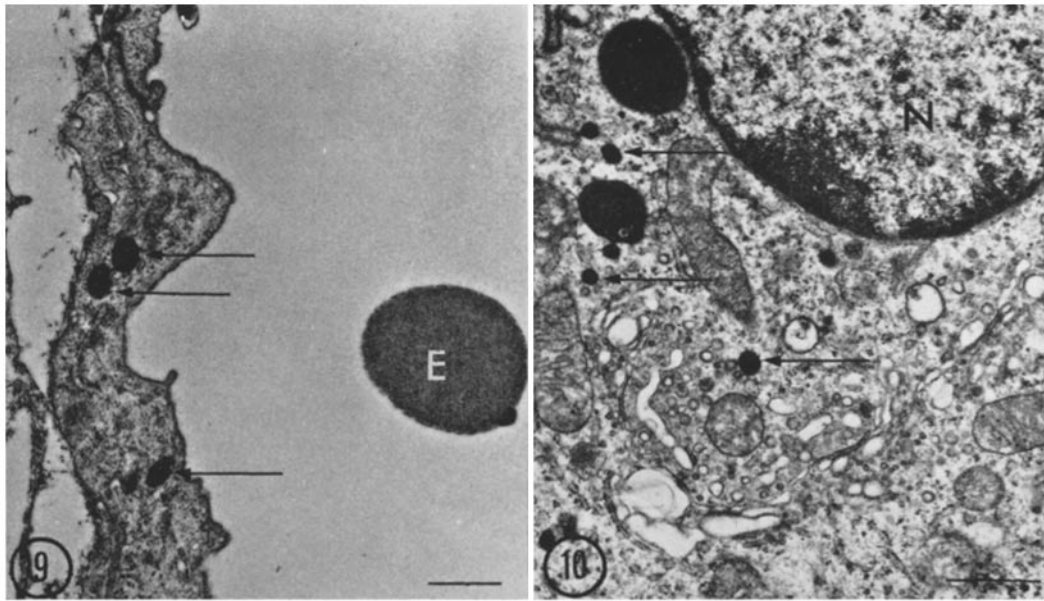
FIGURE 8 Pig. 60 min after injection of IgG. The lumen contains dense material. Numerous round dense and homogeneous vacuoles and vesicles are seen in the epithelial cytoplasm; some are present also in lymphatic endothelium (arrows). Uranyl acetate and lead citrate. × 4500.

DISCUSSION

Use of Specific Antibodies as Tracers of Protein Transport

Numerous applications of immunotracers have been reported for light and electron microscopy. In most cases, antibodies labeled by various methods have been used for the in vitro demonstration and localization of tissue antigens (45, 72). In other instances, antigens such as ferritin (56, 57) or enzymes (34, 62) have been given in vivo in order to stimulate antibody production by immunocompetent cells; the antibodies were then revealed by in vitro introduction of the corresponding antigens.

In the present study, specific anti-ferritin and anti-peroxidase antibodies were used as tracers to investigate protein absorption by the intestine. This technique has the advantage of using physiological proteins which can be detected in the tissues and in the circulation by their immunological properties. The antigens used to reveal the antibodies have characteristics which may be compared.

FERRITIN: Ferritin has the advantages of a

higher antigenicity than peroxidase and of staining properties which allow its easy demonstration by Prussian blue; neither fixation nor antigen–antibody reaction interferes with this staining. On the other hand, because of its high molecular weight (650,000), ferritin undergoes slow diffusion. In our experimental conditions, specific labeling on sections was weak and the background prominent. Therefore we preferred the use of ferritin fo· immunological procedures.

PEROXIDASE: Peroxidase has a low molecular weight (38,900) which allows a relatively fast diffusion. As a result of its enzymatic properties, a cumulative effect can be expected to increase the amounts of reaction product; however, the antigen –antibody reaction causes a 40% loss of enzymatic activity (79). Nonspecific in vitro adsorption of peroxidase on tissue structures has been reported by Straus (77, 78); we have seen only a few small deposits of this type on external mitochondrial membranes. Peroxidase reaction product was unevenly distributed and found predominantly at the periphery of nonfrozen sections treated according to the original technique of Graham and Karnovsky (23). Results were very much improved
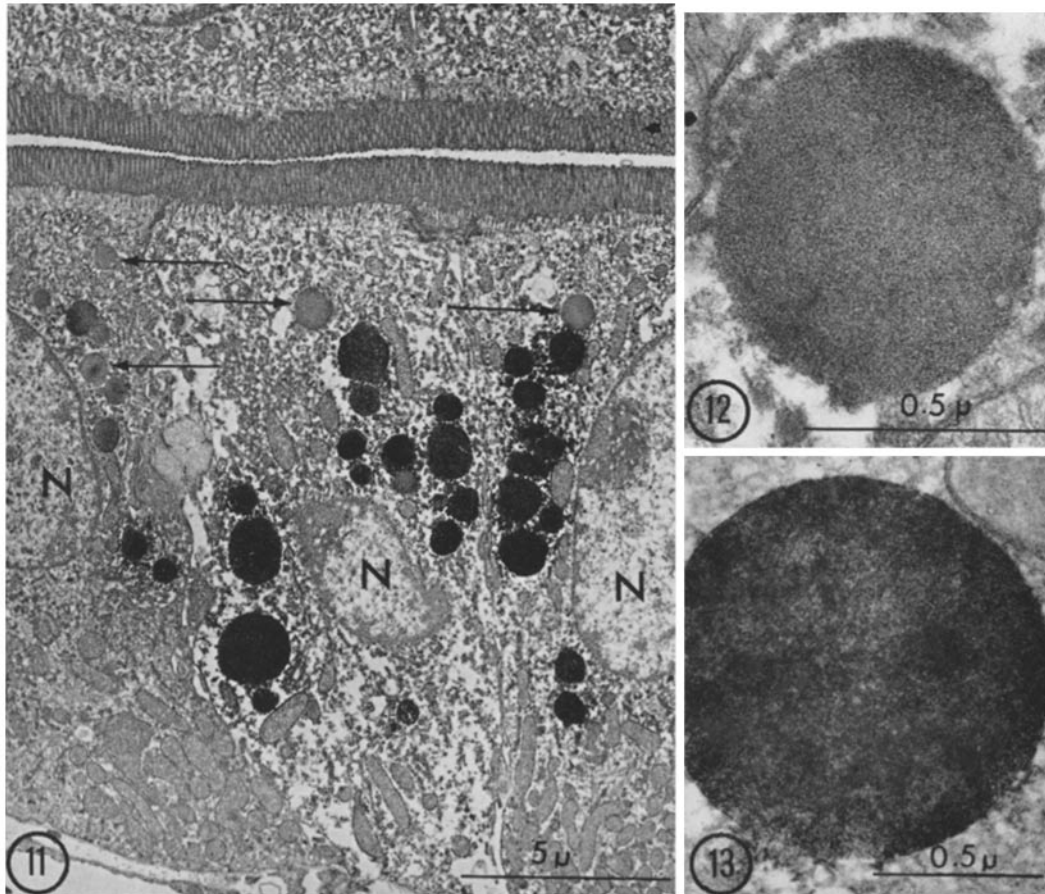
Figures 11 to 13   Pig. 120 min after IgG injection. Dense vacuoles in epithelial cells. Sections were stained with lead citrate only.

Figures 11 and 13 are from specimens exposed to peroxidase and treated with diaminobenzidine and H_2O_2. Reaction product is dense and granular (Fig. 13). Some vacuoles do not show peroxidase activity (Fig. 11, arrows).

Figure 12   Control without immunohistological treatment. Fig. 11, × 5500; Fig. 12, × 65,000; Fig. 13, × 45,000.

Figure 14   Rat. 60 min after IgG injection. Immunohistologically treated specimens. Numerous vacuoles of irregular size contain peroxidase reaction product. One vacuole with reaction product is located in the lamina propria (arrow). Endogenous peroxidase-like activity is exhibited by a red blood cell. Uranyl acetate and lead citrate. × 2800.

Figure 15   Rat. 120 min. Immunohistologically treated specimen. A large supra-nuclear vacuole containing reaction product of peroxidase at the periphery is surrounded by smaller vacuoles (arrows) and multivesicular bodies loosely filled with reaction product. Uranyl acetate and lead citrate. × 14,000.

Figures 16 and 17   Rat. 90 min. After incubation with glycerophosphate, AcPase reac-tion product is located in vacuoles containing dense material and in smooth endoplasmi reticulum saccules (Fig. 17, arrow). Lead citrate. Fig. 16, × 16,500; Fig. 17, × 20,000c
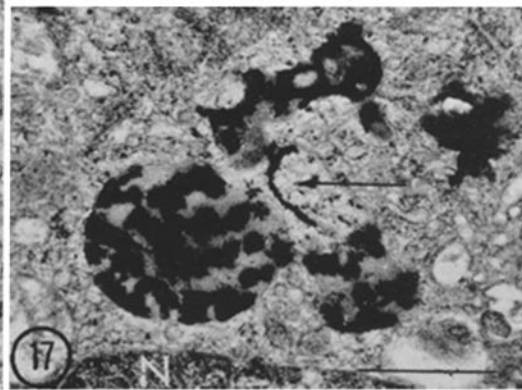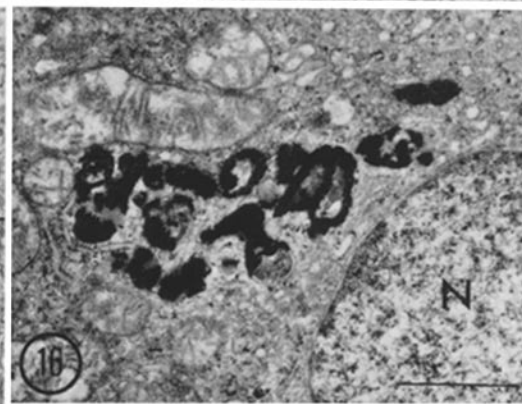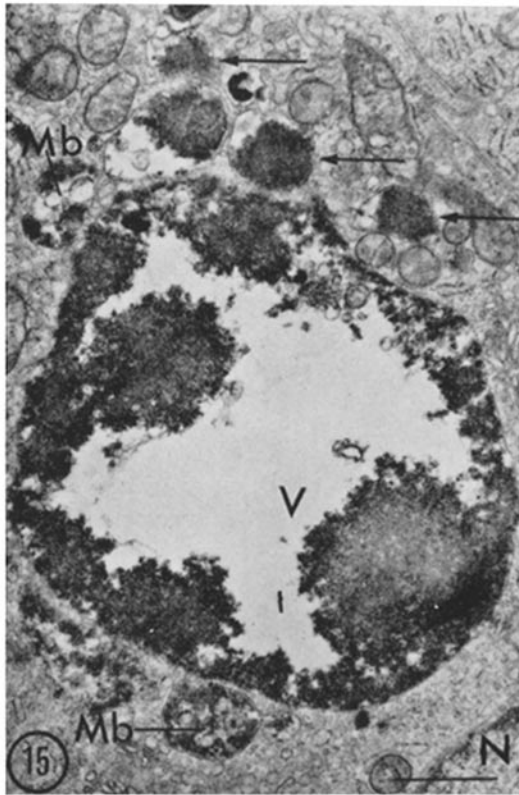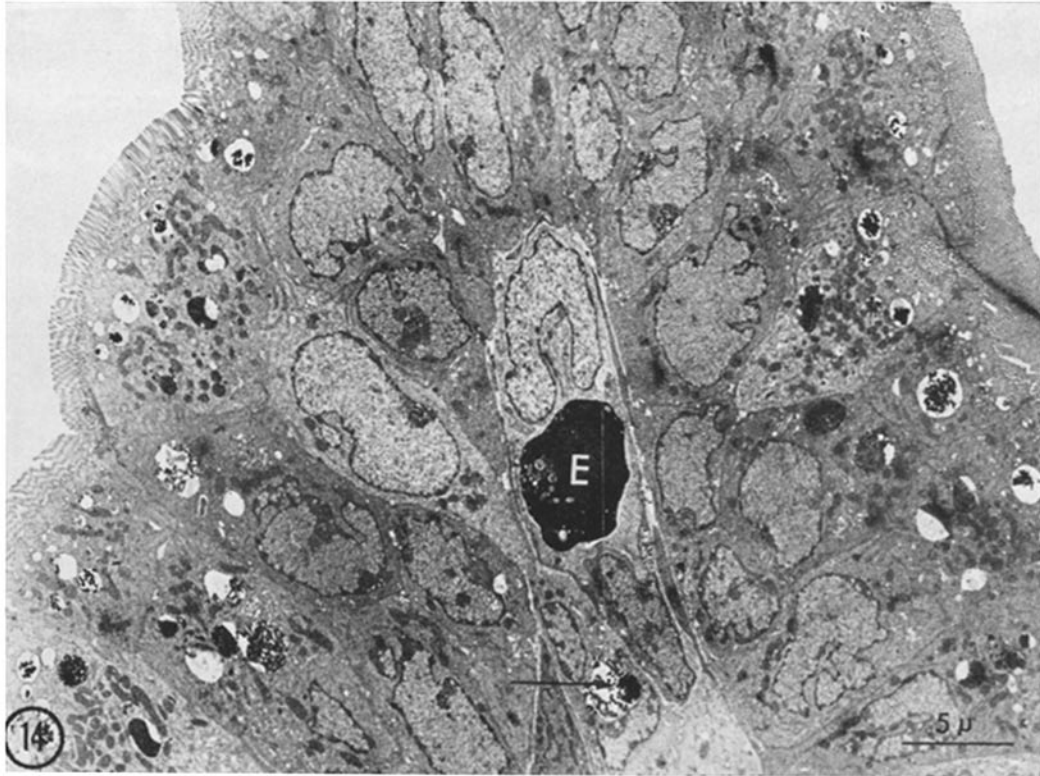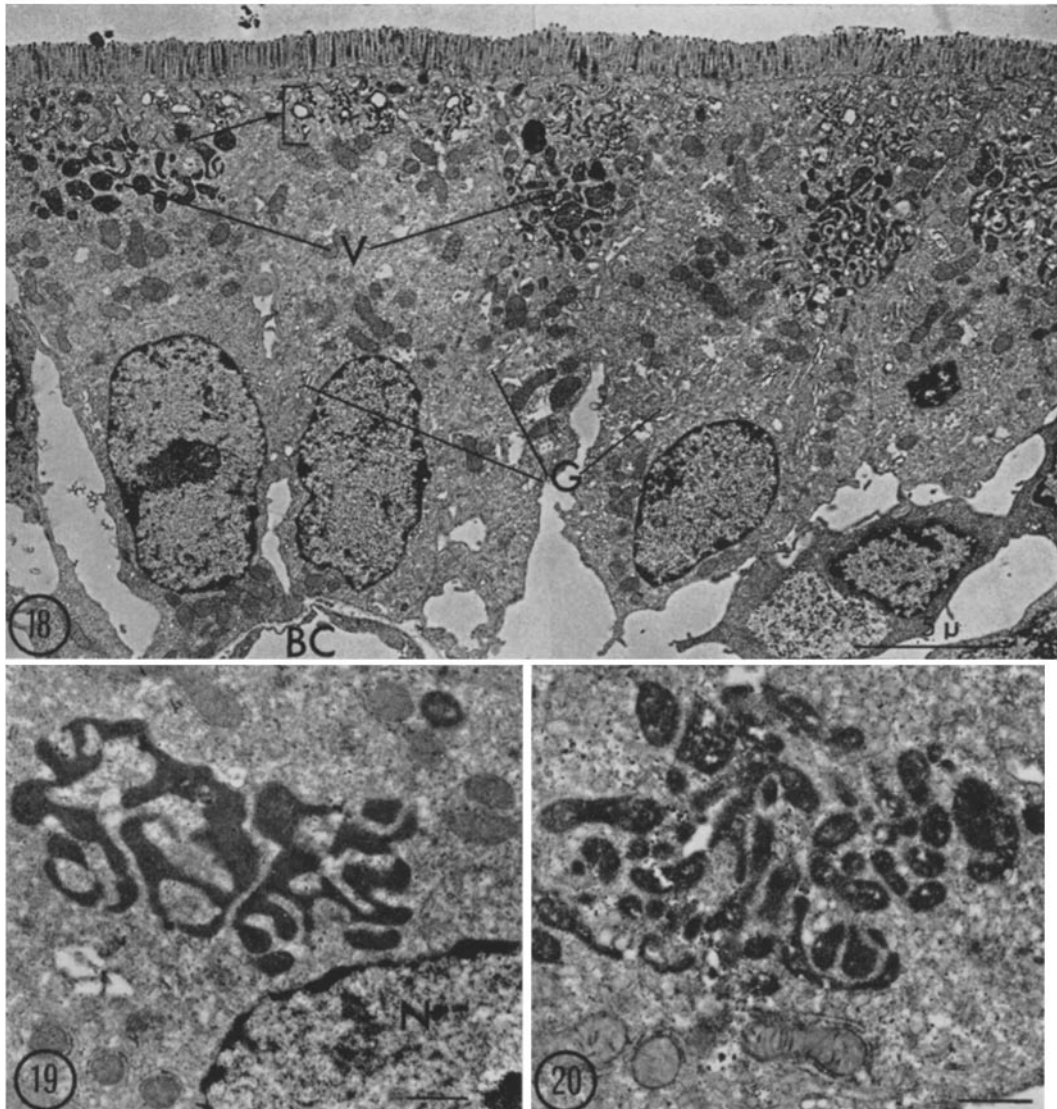
FIGURE 18   Immunohistological treatment. Peroxidase reaction product labels the contents of vesicles and tubules under the brush border (arrow) and of vacuoles of irregular shape grouped in the apical cytoplasm. Several Golgi complexes are seen. × 4000.

FIGURE 19   Juxtanuclear vacuolar complex containing dense material. This type of structure appears to be the result of the partial fusion of the membranes of individual vacuoles. × 8500.

FIGURE 20   Immunohistological treatment. Reaction product of peroxidase increases the density of the vacuolar contents. × 9500.
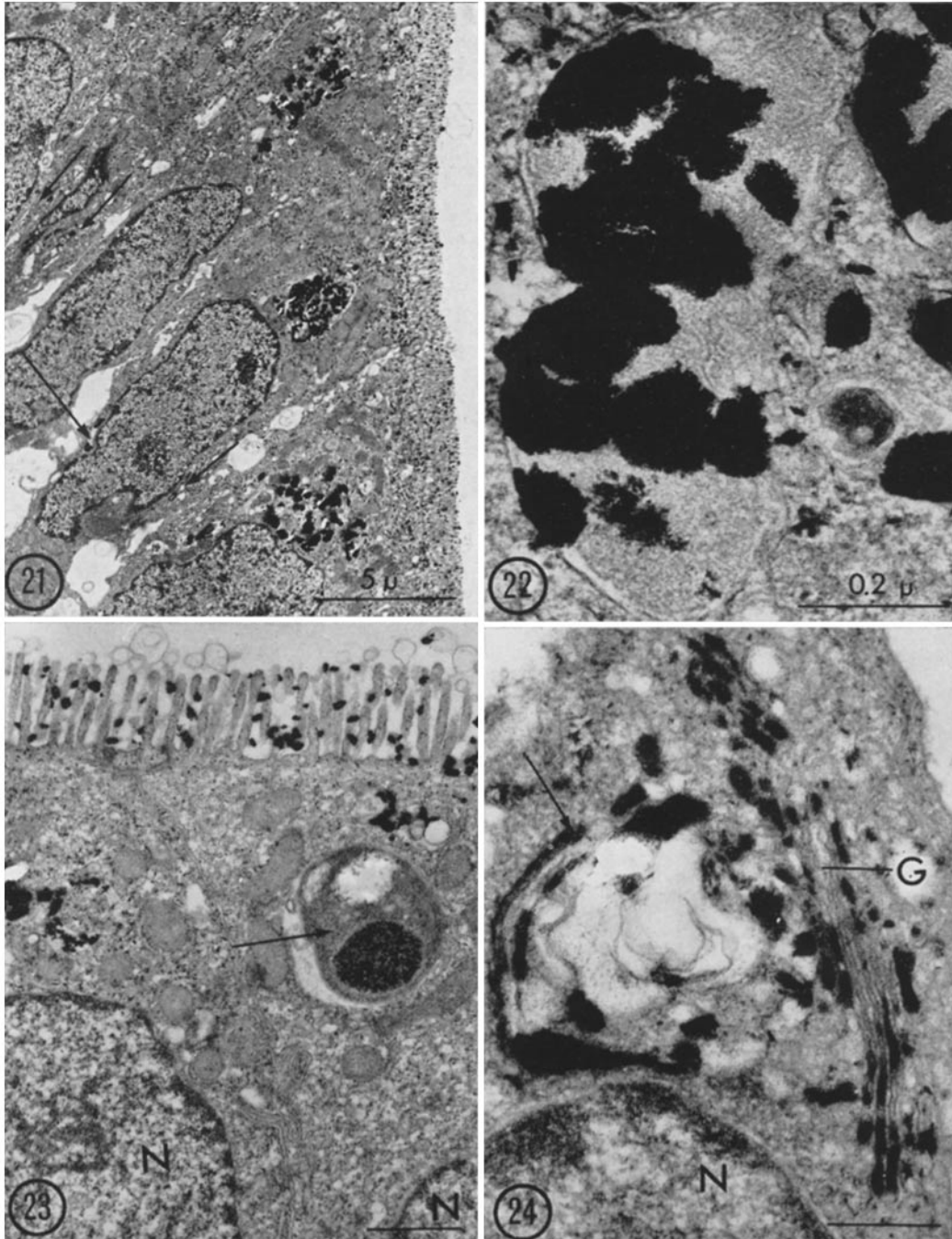
FIGURES 21 to 26   Rabbit. 120 min after antibody injection. Micrographs from specimens incubated for AcPase activity. Lead citrate.

FIGURE 21   Slight deposits of reaction product are located in the brush border. Groups of vacuoles and vacuolar complexes are marked by heavy deposits. Reaction product is seen also in Golgi elements (arrows). × 4000.

FIGURE 22   Vacuoles containing dense material are labeled by large lead phosphate deposits. × 110,000.
FIGURE 23   Reaction product is shown in the brush border, in vesicles and in an autolytic vacuole (arrow). × 13,500.

FIGURE 24   A Golgi apparatus (G) contains reaction product in most of its cisternae. In the immediate vicinity, endoplasmic reticulum saccules containing reaction product are arranged concentrically (arrow). × 16,000.
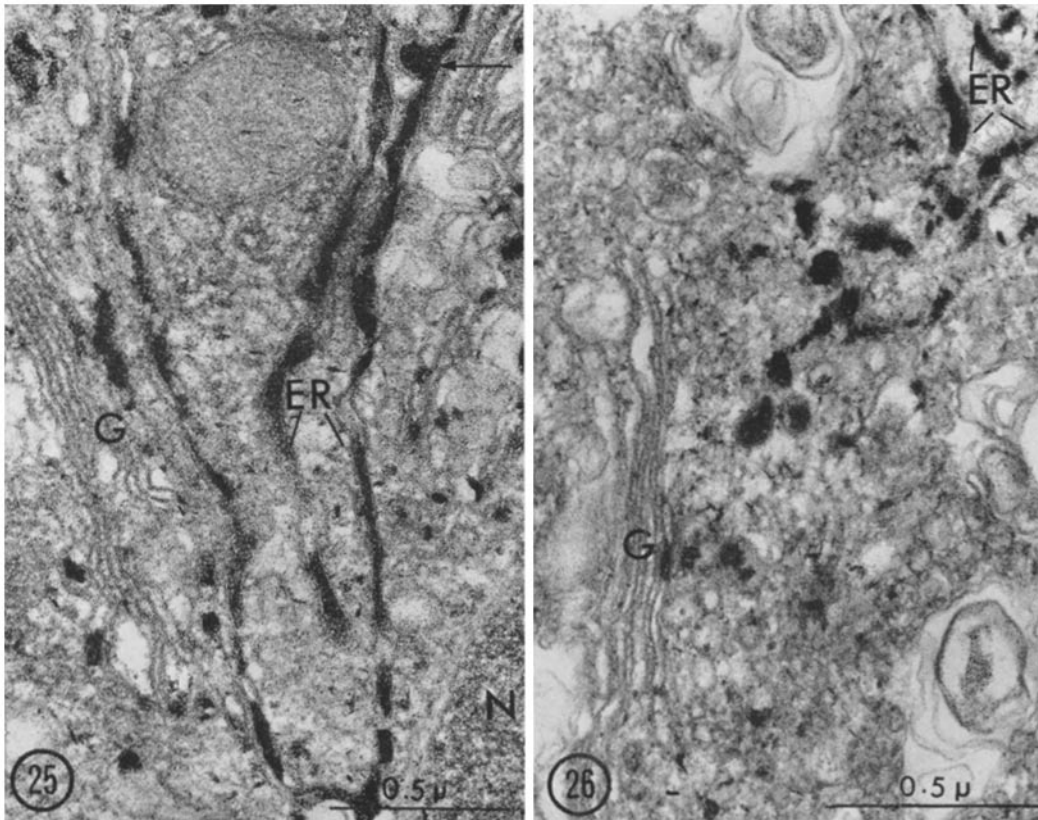
FIGURE 25 Reaction product is seen in the Golgi cisternae nearest to the nucleus. Some endoplasmic reticulum saccules are filled with reaction product. Note the vesicle-shaped localized dilatation of one of the saccules (arrow). × 50,000.

FIGURE 26 Small vesicles between a Golgi complex and endoplasmic reticulum saccules contain reaction product. × 55,000.

when tissues were first incubated in a diamino-benzidine medium without H_2O_2 (see Methods). This corresponds to the modified technique recently described by Schneeberger and Karnovsky (64). In intestinal mucosa, endogenous peroxidase-like activity is restricted to blood cells and does not create problems of interpretation. Because of these characteristics, peroxidase was used mainly for morphological studies.

INFLUENCE OF FIXATION: The immuno-plate test showed that antibody activity was still present after glutaraldehyde treatment. The increased diameter of precipitation rings indicated that glutaraldehyde was responsible for a marked decrease of activity. In the immunoplate test, prolonged rinsing was found to be necessary in order to minimize nonspecific adsorption of ferritin on glutaraldehyde-treated plates. In glutaraldehyde-fixed tissues, rinsing in buffer after fixation, peroxidase treatment, and subsequent washing were needed in prolonged periods to obtain specific labeling without background. Even under these conditions, the depth of penetration of peroxidase was small. One possible explanation of this difficulty may be the persistence, after glutaraldehyde fixation, of free aldehyde groups which interfere with the diffusion of antigens. This explanation is supported by our observations of a nonspecific staining of glutaraldehyde-fixed sections with the Schiff's reagent.

Endocytosis of Antibodies from the Intestinal Lumen

In the three species, the rabbit antibodies are taken up by pinocytosis into the jejunal absorptive
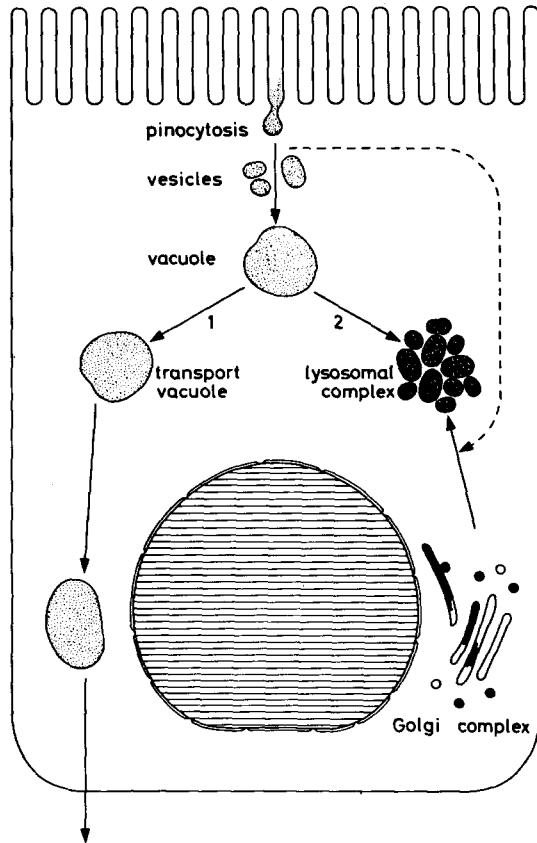
FIGURE 27 Schematic representation of the functions of jejunal absorptive cells during antibody absorption in the newborn. After uptake by pinocytosis, IgG molecules are either transported intact across the cell in vacuoles (1) or trapped in lysosomes (2); in this case, endocytosis of proteins triggers the release of lysosomal enzymes (interrupted arrow). The first mechanism is found in the pig, the second in the rabbit, and both in the rat.

cells and vacuoles are formed by the fusion of vesicles. The morphological features of endocytosis are alike in the three species, although the antibodies were homologous in one case and heterologous in the others. The fate of the vacuoles containing antibodies appears to depend on the species. In the pig, the vacuoles are progressively transferred into the basal cytoplasm without interference with other organelles. In the rabbit, the vacuoles tend to coalesce in the supranuclear area. Vacuoles of both types coexist in the jejunal cells of the rat (Fig. 27).

Transfer of Antibodies into the Circulation

Rabbit antibodies were already present in the blood of pigs and rats 15–30 min after their injection into the intestine. These results are not in agreement with the observations of Comline and Roberts (13), who reported a 60–120 min delay between the administration of colostrum into the duodenum and the appearance of colostral proteins in the thoracic duct; they did not find absorption by way of the portal vein. In our study,

the early appearance of antibodies in the portal blood of pigs indicates that they are transferred directly into the blood capillaries, possibly after discharge of the content of epithelial vacuoles into the interstitial space. It should be noted that blood capillaries are located close to the epithelium and that their endothelium has fenestrations which may be sites of passage of macromolecules. Vacuoles containing antibodies were frequently seen in the endothelium of lymphatics and in connective tissue cells, but only rarely in the endothelium of blood vessels. It is not clear by what mechanism the vacuoles are transferred from the epithelial cells to the connective tissue cells and to the lymphatic endothelium.

In rabbits, no evidence of transfer of antibodies into the circulation could be established.

Role of Lysosomes in the Absence of Antibody Transfer

In the rabbit, uptake of antibodies from the intestinal lumen into the jejunal absorptive cell is

prominent, but no antibodies are transferred into the circulation. The pathway which is followed by the antibodies is comparable to that described for proteins absorbed in other tissues (21, 23, 38, 39, 40, 75, 76). The vacuoles formed by pinocytosis are grouped in the apical cytoplasm and tend to coalesce in the supranuclear region. Immediately after the beginning of endocytosis of antibodies, AcPase activity increases markedly. It is generally accepted that AcPase is a representative lysosomal enzyme and a reliable tracer of lysosomal activity (16, 22, 49, 50). The relations between lysosomal enzymes such as AcPase and cathepsins have been extensively discussed (40, 73, 74). Cathepsins are the main proteolytic enzymes of lysosomes and their activity is increased during protein absorption (35, 73, 74). The considerable increase of AcPase activity observed in the rabbit denotes a lysosomal activation which probably includes increased proteolytic activity. In the rabbit, almost all vesicles and vacuoles containing antibodies exhibit pronounced AcPase activity. In addition, during absorption of antibodies, AcPase activity increases in the Golgi complexes which tend to become larger, and AcPase-positive vesicles and saccules appear, resembling those described as "GERL" by Novikoff (51, 52). These findings indicate that the Golgi apparatus has a role in the release of lysosomal enzymes, as previously suggested by others (21, 27). No vacuoles containing antibodies are found in the basal part of the rabbit jejunal epithelium and lamina propria. This observation, in addition to the fact that practically all vacuoles containing antibodies also contain AcPase, suggests that the absorbed antibodies are efficiently trapped by the lysosomal system. It seems likely that lysosomal activity is the means by which the jejunal cells of the newborn rabbit prevent the absorption of antibodies into the blood.

Relations between Transfer and Lysosomal Segregation of Antibodies

In the rat, the two different situations of transfer of intact antibodies into the circulation and of their segregation in lysosomes coexist. The question arises whether antibodies partially digested in the epithelial cells are subsequently transferred into the circulation. The first steps of IgG digestion are known to result in the formation of Fab and Fc fragments which can be distinguished from intact IgG by immunodiffusion and immunoelectrophoresis (28). No such fragments were detected by these methods in the sera of rats. The fragments which are formed by digestion of IgG in the lysosomes of rat and rabbit jejunal absorptive cells do not appear to be released as such. They could either be retained in the lysosomes or they could undergo further digestion and be degraded into peptides and aminoacids. In the rat, it is not clear why the vacuoles which transfer intact antibodies escape the action of lysosomal enzymes.

Pierce and Smith (58) reported the presence of fragments in the pig after oral ingestion of immune globulins. In our study, the IgG was injected directly into the pig jejunal lumen, and no fragments were detected in serum. Furthermore, lysosomal activity did not increase during absorption of antibodies. It seems likely therefore that the fragments found by Pierce and Smith resulted from gastric digestion and that they had been absorbed as such by the intestinal epithelium.

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