Age-Dependent Intestinal Absorption of Valproic Acid in the Rat[1]

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The absorption of valproic acid (VPA) across isolated perfused segments of jejunum, ileum and colon was examined *in situ* in 14-day- to 24-month-old Fischer-344 rats. Within each age group, the intrinsic absorptive clearance (Cl_a) of VPA at a perfusate concentration of 1 mg/ml was highest in the jejunum, lowest in the colon, and intermediate in the ileum. When intestinal Cl_a was normalized for the dry weight of the segment, within-group variability decreased. In all segments, VPA Cl_a normalized by dry weight decreased during development (≤20 to 90 days) and remained relatively constant during aging (90 days to 24 months). The mechanism of valproate absorption (active *vs.* passive) was examined across age in everted intestinal sacs prepared from each of the three segments. Data were consistent with active transport of VPA in the jejunum and ileum of rats of all ages, and in the colon of pre-weanling animals. Colonic absorption of VPA appeared to occur by passive diffusion in adult rats. In contrast, colonic absorption of *d*-glucose occurred only by passive diffusion in all age groups. These data indicate that, during development, significant alterations in the rate of VPA absorption occur throughout the rat intestine. Furthermore, while active transport of VPA by the small intestine was present throughout the age range investigated, active transport by the colon became negligible by the time of weaning.

KEY WORDS: Valproic acid; postnatal development; gastrointestinal absorption; age-dependent absorption; Fischer-344 rats.

INTRODUCTION

Age-dependent rates of intestinal absorption in the rat have been reported for many endogenous and exogenous compounds, including cholesterol (1), calcium (2), vitamin A (3), trypsin (4), aromatic amino acids (5), glucose (6), and biotin (7). Previous studies have suggested that passive absorption does not change with age, while active absorption may be age-dependent (6,8). Site-specific absorption of some compounds [*e.g.*, bile acids (9), calcium (10), magnesium (11), hexose, iron, and L-proline (12)] also has been documented. Information regarding age-related changes in site-specific intestinal absorption of xenobiotics is lacking. However, site- and age-dependent intestinal absorption of compounds may have a significant impact on the disposition of xenobiotics, particularly those which undergo enterohepatic recirculation (ER).

ER affects the disposition of numerous xenobiotics, including valproic acid (VPA) (13). In the case of VPA, the parent compound is glucuronidated in hepatocytes and the conjugate (VPA-G) is excreted into bile and expelled into the gastrointestinal (GI) tract. VPA-G is hydrolyzed by β-glucuronidase, which is present throughout the intestinal contents and mucosa but is found in particularly high concentrations in the mucosa of the jejunum (14). Following hydrolysis, regenerated VPA is available for reabsorption. ER conserves endogenous compounds, such as bile acids, and prolongs the residence time of xenobiotics (14,15). Changes in the rate, extent, or site of GI absorption may result in significant alteration of systemic exposure to a xenobiotic that undergoes ER.

Previous experiments in this laboratory have shown that the degree of ER of VPA changes with age in the rat during postnatal development (16). The age-dependent changes appear to be due in part to changes in the preferential site of intestinal glucuronide hydrolysis during development (17). However, the age-related change in ER may not be due exclusively to changes in the site of hydrolysis of the conjugate. Site- and age-related differences in the rate or extent of intestinal absorption of xenobiotics also may affect the kinetics or degree of ER and, consequently, alter the disposition profile during development and aging.

The purpose of this investigation was to examine the effects of site and age on the intestinal absorption of VPA in the Fischer-344 rat model of aging. A technique based on previous *in situ* absorption studies (18–22) was developed to quantitate the intrinsic absorptive clearance of VPA from the jejunum, ileum and colon simultaneously. A secondary objective of this study was to examine the mechanism(s) of VPA absorption (*e.g.*, active *vs.* passive) in each intestinal segment by measuring the uptake of VPA in everted sacs of rat intestine.

MATERIALS AND METHODS

Chemicals. VPA and *d*-glucose were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the highest purity available.

Animals. Fischer-344 male and female breeders, male 40- and 90-day-old rats and male 12- and 24-month-old rats were obtained from Charles River (Raleigh, NC). Rat pups were housed with their dams in polypropylene cages. All animals were maintained on a 12-hr light/dark cycle. Weaned rats (older than 20 days) were housed in wire-mesh cages and allowed free access to food and water; food was withheld 15 to 20 hr prior to initiation of experiments (except for the 14- and 20-day-old pre-weanlings).

In situ intestinal absorption. Rats (n = 5–6) of each age group were anesthetized with ethyl carbamate (1 g/kg i.p.) and the intestine was exposed through a midline abdom-

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inal incision. Temperature in the abdominal cavity was maintained at 37°C by means of a heating pad, temperature controller, and a temperature probe placed beneath the intestine. The proximal cannula for the colon was established immediately distal to the *ampulla coli*. Polyethylene tubing (o.d. = 1.52 mm for 14- and 20-day-old groups and o.d. = 2.42 mm for all other groups) was placed into the segment through a 2-mm incision in the gut wall. Suture (size 00) was tied around the gut wall to secure the cannula, avoiding large blood vessels. Normal saline (37°C) was used to clear solid material from the colon. A 2-cm length of outflow tubing was placed 1 cm into the rectum for collection of colon samples.

The proximal cannula for the jejunum was anchored as described above 2 to 3 cm distal to the ligament of Treitz. The jejunal outflow and the ileal inflow cannulae were established at the end of the jejunum and beginning of the ileum, respectively, with the jejunal outflow cannula placed 2 cm proximal to the ileal inflow. The jejunal outflow and ileal inflow approximately bisected the length of intestine from the duodenum to the cecum. The ileal outflow cannula was placed 2 cm proximal to the cecum. Prior to inserting and securing outflow cannulae, all three segments were cleared gently of solid material with normal saline (37°C).

The small intestine segments were situated carefully to avoid constrictions. In order to optimize reproducibility of the *in situ* perfusion technique, the segments were placed in an S pattern to maintain consistent perfusate hydrodynamics (20). Warm, saline-soaked gauze covered exposed sections, and 37°C normal saline was applied frequently to maintain constant temperature and moisture. The segments were flushed with an additional 5 ml of normal saline to ensure patency of the outflow cannulae, and were cleared by flushing with air. Care was taken at all times to avoid excessive luminal pressure. All segments were loaded rapidly with perfusate (4.56 mM KCl, 145 mM NaCl, 1.25 mM $CaCl_2$, and 5 mM NaH_2PO_4 titrated with concentrated NaOH to pH 6.4 for the small intestine segments or pH 7.2 for the colon; both solutions were ~300 mOsm) containing 1 mg/ml VPA. A single pass, constant flow (2 ml/min/kg body weight) of perfusate (37°C) was established through each segment. An aliquot of perfusate was collected from small intestine and colon inflow for each rat. The effluent from each segment was collected at timed intervals (every 5 min for 40 min) and frozen (−20°C) until analysis. After collection of the final sample, the outflow end of each segment was ligated next to the cannula and perfusion was terminated. The inflow end was ligated, and each segment was dissected from the mesentery and blood vessels. The contents of each segment were expressed gently and weighed. Each empty segment was weighed, a 2-gm weight was attached to one end, and the length was measured. Segments were allowed to dry at room temperature for 3 days to determine dry tissue weights.

Everted intestinal sacs. Rats (n = 4 to 10 per age group) were anesthetized with ethyl carbamate (1 g/kg i.p.) and the intestine was exposed through a midline incision. Abdominal cavity temperature was maintained as described above. Warm saline was allowed to flow into both the colon and jejuno-ileal segments until all particulate matter had been cleared. A modification of the procedure described by Binks and Dobrota (23) was used to prepare the everted intestinal sacs. Ice-cold perfusate (pH 6.4) was used to cool the jejuno-ileal segment. The blood supply to the segment was ligated and the section was excised rapidly, everted over a smooth glass rod, and placed in ice-cold buffer (pH 6.4) gassed continuously with 95% O_2, 5% CO_2. Segments were removed from the animal and used immediately in order to prevent time-dependent deterioration of intestinal epithelial cells (24). Two 3- to 5-cm lengths of intestine (depending on the age, but held constant for each group) were removed from the jejunal and ileal sections and were filled with approximately 1 ml/cm/kg body weight of oxygenated, chilled perfusate and returned to ice-cold, continuously oxygenated medium until all segments were prepared.

After preparation of the small intestine segments, the colon was flushed with ice-cold buffer (pH 7.2) until cooled, and the rat was exsanguinated. A 5- to 10-cm section of the transverse colon was excised rapidly, everted, washed, and a 4-cm segment was prepared in a manner identical to the small intestine, with the exception that the serosal fluid was pH 7.2. Everted sacs, segregated by segment, were placed in 125-ml Erlenmeyer flasks containing 75 ml of the 37°C perfusate solution (mucosal fluid) containing 0.2 mg/ml VPA and 10 mM *d*-glucose at pH 7.2 (colonic) or pH 6.4 (all others), and oxygenated continuously. Transport of *d*-glucose was used to test the viability of the everted sacs. During the incubation period, flasks containing the test mixture were circulated in a constant temperature (37°C) orbital shaker water bath (100 oscillations/min). Everted sacs were incubated for 30 min, which was shown in preliminary experiments to be an adequate period to demonstrate active absorption of VPA in the various segments. After incubation, the serosal fluid (inside) was removed and serosal and mucosal (outside) samples were frozen (−20°C) until analysis for VPA and *d*-glucose concentrations.

Sample analysis. VPA concentrations were determined by a sensitive and specific gas chromatographic assay with flame ionization detection based on the procedure developed by Löscher (25). Samples (0.05 mL) were acidified with 0.5 N HCl (0.1 mL) and extracted with ethyl acetate (0.2 mL) containing cyclohexanecarboxylic acid (100 μg/mL) as the internal standard. After mixing by vortex and centrifuging, a 1-μL aliquot of the organic layer was injected onto a free fatty acid phase wide-bore (0.53 mm i.d.) fused silica capillary column (15 m) with helium (10 mL/min) as the carrier gas. Isothermal chromatography was conducted at 120°C; injector and detector temperature were 150°C and 250°C, respectively. The assay was linear ($r > 0.999$) for concentrations <1000 μg/mL, with a coefficient of variation <5% and a detection limit of 0.5 μg/mL. The concentrations of *d*-glucose in the serosal and mucosal fluid collections were determined with a glucose enzymatic assay kit (no. 115-A, Sigma) adapted for 10-μL sample volumes.

Data analysis. For the *in situ* intestinal perfusion experiment, data collected for each group included inflow and outflow VPA concentrations, segment length, and the following weights: segment full, segment empty, fluid expressed, and perfused intestine wet and dry. At least two perfusate

samples from 5–6 rats per group were collected between 5–35 min after initiation of the perfusion. Extraction (E) of VPA by the segments was estimated as

$$E = \frac{C_i - C_o}{C_i} \quad (1)$$

where C_i is the perfusate inlet concentration. C_0, the outlet concentration, was corrected gravimetrically for water flux by multiplying the measured outlet concentration by the ratio of outlet to inlet perfusate volumes during each collection interval. Fluid loss was 4–9%.

The intrinsic absorptive clearance (Cl_a) was calculated as

$$Cl_a = -Q \cdot \ln(1 - E) \quad (2)$$

where Q is the flow rate of perfusate through the intestinal segment. Methods used to reduce variability in Cl_a due to inherent variability in the segments included dividing by length (L), dry weight, segment volume (V), surface area ($2 * \sqrt{V * \pi * L}$), and mean transit time (V/flow rate). The mean and coefficient of variation were determined for each method of normalization for all segments in each age group in order to identify the method that minimized variability. A two-way analysis of variance table was constructed to examine the effects of segment (jejunum, ileum, colon) and age (14-, 20-, 40-, and 90-day-old, and 12- and 24-month-old) on VPA Cl_a. There was a significant interaction between segment and age, so analysis of variance was performed by segment with age as the factor. A Dunnett's test was used to perform all possible pairwise comparisons between age groups. In all cases, the criterion for statistical significance was $p < 0.05$, adjusted for the number of comparisons performed.

For the everted intestinal sac experiment, serosal (S) and mucosal (M) samples were analyzed for *d*-glucose and VPA concentrations. Paired t-tests were used to determine whether serosal concentrations were statistically different from mucosal concentrations.

RESULTS AND DISCUSSION

Potential time-dependent changes in VPA Cl_a were investigated in initial experiments to optimize the sampling intervals. Representative Cl_a *vs.* time profiles in the jejunum, ileum and colon for 14- and 90-day-old rats (Fig. 1) indicated that VPA Cl_a in isolated segments of jejunum, ileum and colon stabilized within 10 min and remained constant for at least 40 min. Based on these results, the Cl_a in subsequent experiments was calculated as the mean of the samples collected 10–35 min after initiating the perfusion. For all groups, mean VPA Cl_a was highest in the jejunum, intermediate in the ileum, and lowest in the colon (*e.g.*, mean Cl_a values of 1.49, 1.13, and 0.56 mL/min/kg, respectively, in 14-day-old rats).

One of the difficulties inherent in quantitating Cl_a from a given segment of intestine is the size (length and cross-sectional area) of the segment. Larger segments would be expected to extract more substrate than smaller segments due to the greater surface area available for absorption. In order to minimize within-group variability and to compare absorption among groups, constant intestinal segment lengths may be used. However, in examining the influence of age on intestinal absorption, selection of a specific segment length is problematic; the size of intestine differs extensively between rat pups and adult animals. To facilitate comparisons of Cl_a across age, body weight-normalized

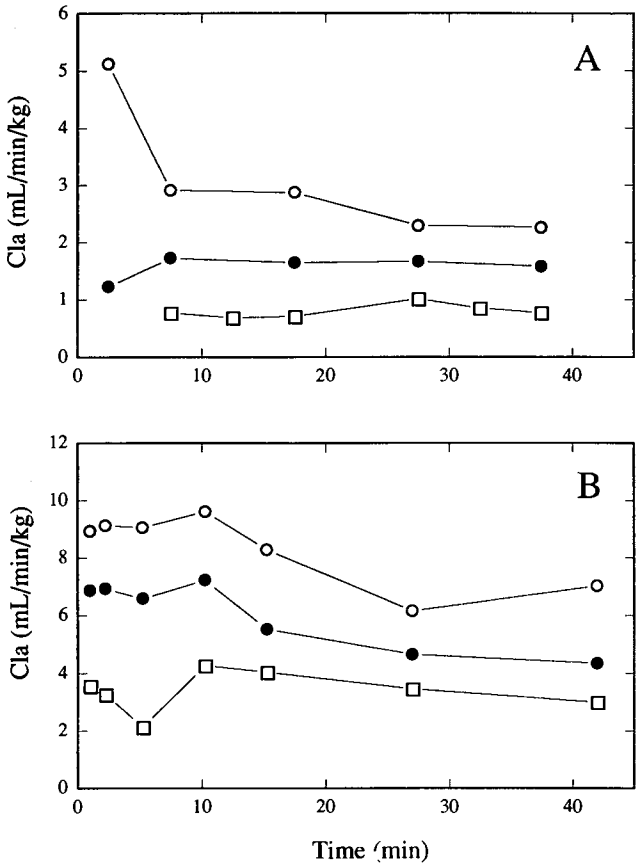
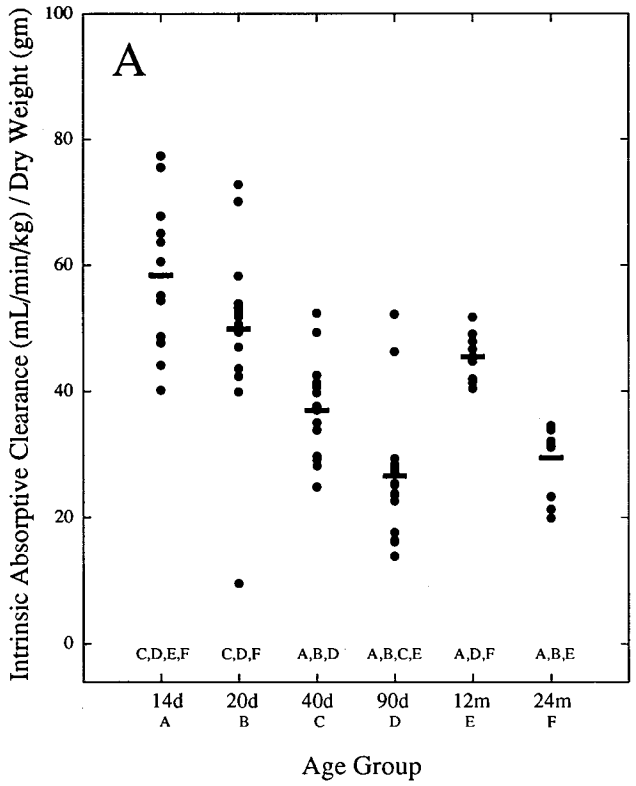


Fig. 1. Intestinal Cl_a *vs.* time in the jejunum (○), ileum (●) and colon (□) of individual 14- (A) and 90- (B) day-old rats. Perfusate flow rate = 2 mL/min/kg; inflow VPA concentration = 1 mg/mL. Data are plotted at the midpoint of the perfusate collection period.

Table I. Comparison of Methods for Normalizing Cl_a

	Jejunum		Ileum	
	Mean	% CV	Mean	% CV
Cl_a (mL/min/kg)	1.49	29.3	1.13	15.1
Cl_a/Dry Weight (gm)	30.9	12.9	20.6	8.2
Cl_a/Length (cm)	0.145	15.6	0.087	14.7
Cl_a/Volume (mL)	14.6	67.4	5.81	33.8
Cl_a/Surface Area (cm^2)	0.393	34.2	0.197	17.7
Cl_a/Transit Time (min/kg)	36.5	64.0	14.7	33.4

Methods used to normalize absorptive clearance (Cl_a) for each segment in the group of 14-day-old rats (n = 12). The lowest coefficient of variation (% CV) for both segments was obtained by normalizing Cl_a by segment dry weight.



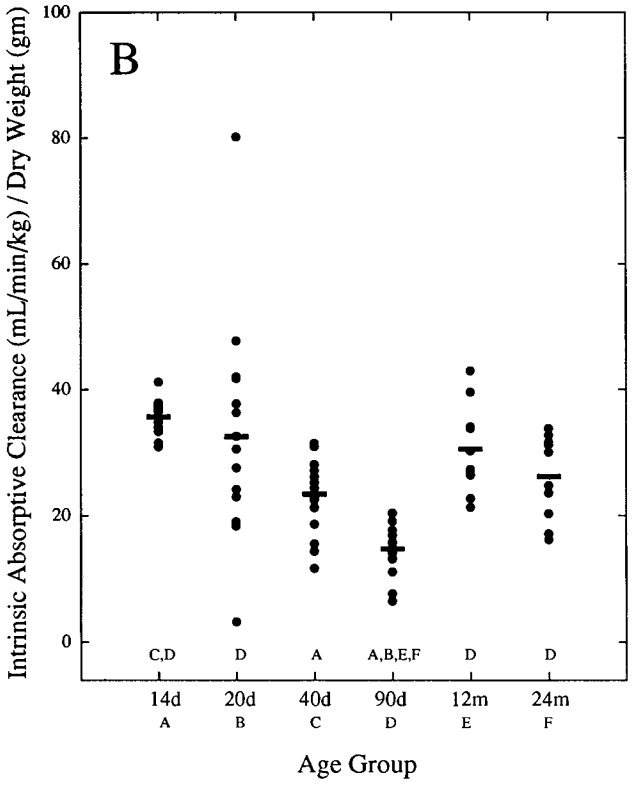


Fig. 2. VPA Cl_a normalized by segment dry weight for jejunum (Panel A), ileum (Panel B), and colon (Panel C). Lines indicate the mean within each group. For simplicity, each age group has been identified with a letter (A through F) designated below the x-axis. Letters above each age group indicate statistically significant differences from that group. For example, in the jejunum, the 40-day- through 24-month-old groups were statistically different from the 14-day-old group.

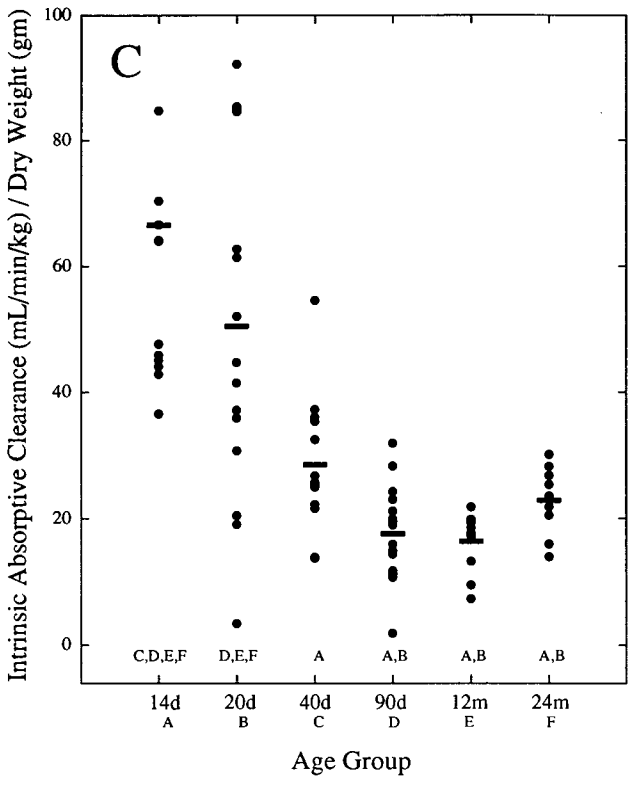


Fig. 2. Continued.

perfusion flow rate was held constant and the Cl_a in a specific segment was normalized to reduce variability within groups. Rather than measuring arbitrarily a predetermined length of intestine, this approach allowed use of the entire jejunum, ileum and colon. Different normalization schemes were evaluated for Cl_a and are compared in Table I. The coefficient of variation was lowest when Cl_a was normalized by the dry weight of the segment. Subsequent statistical comparisons were made only on the basis of weight-normalized Cl_a.

VPA Cl_a normalized by segment dry weight is shown in Fig. 2. Two-way analysis of variance procedures indicated that both segment and age influenced VPA Cl_a significantly. Within each segment, VPA Cl_a was maximal at 14- to 20 days-postpartum; no statistically significant differences were observed between these two age groups for any segment. Cl_a tended to decrease with further maturation (through 90 days), and increased statistically at 12 months (jejunum or ileum) or remained unchanged (colon) through 24 months.

The everted intestinal sac experiment was conducted to elucidate the mechanism(s) of VPA absorption. Everted sacs of rat intestine have been used to evaluate transport processes *in vitro* and to document the existence of active absorption (11,23–24,26–28). This technique has been used to examine the mechanisms of intestinal membrane transport for biotin (7), magnesium (11), cisplatin (23), riboflavin (27), inorganic mercury (28), and morphine (29). Although the viability of intestinal sacs has been questioned, recent studies have indicated that the experiment may be conducted in a manner that allows evaluation of transport for at least 1 hr

with good viability (24). Due to the rapid absorption of VPA, the present experiments required less than 1 hr to determine the existence of active transport. *D*-glucose, which is absorbed actively in adult rat small intestine, was used to assess viability of everted intestinal preparations. Active absorption of VPA by rat small intestine in the absence of *d*-glucose has been observed (data not shown).

Accumulation of a substrate on the serosal (interior) side of an everted sac against a concentration gradient indicates absorption by active processes. Intestinal transport of *d*-glucose by everted intestinal sacs remains constant for at least 12 min during incubation for preparations from 3- to 90-day-old rats (30). Active transport of *d*-glucose by fetal (20 days gestation) rat colon occurs at a rate similar to that in the ileum (31). However, in the postnatal rat colon, active transport of *d*-glucose is negligible (31,32). Thus, *d*-glucose would not be a good measure of active transport by colonic preparations, but would verify that the colon segments remained intact (absence of leaks) when S/M ratios remain significantly below unity after incubations.

The morphology of fetal colon differs from adult colon in both humans and rats (31). Rat fetal colon possesses well-developed villous structures which disappear shortly after birth (33). In addition to morphological changes, functional differences exist. In adult rats, calcium transport occurs predominantly in the proximal duodenum (10), hexose in the proximal small intestine (12), and L-proline in the distal small intestine (12). All three transfer mechanisms are more widely distributed at birth and are present in the colon of the newborn rat (34).

D-glucose transport profiles are shown for each age group in Fig. 3. All groups had serosal concentrations significantly higher than mucosal concentrations in the jejunal and ileal preparations, indicating that, throughout the age range studied, active transport processes for *d*-glucose prevail in the small intestine. In contrast, no evidence of active *d*-glucose transport was found in colonic preparations from any group. In addition, segments from 24-month-old rats were tested for *d*-glucose absorption over a 60-min period. Active transport of *d*-glucose appeared to persist for the entire period as mean S/M values for the jejunum and ileum increased two-fold from 30-min incubations; colonic preparations showed no active absorption, with S/M ratios of 0.22 and 0.25 for the 30- and 60-min incubations. S/M ratios < 1 suggest passive diffusion of substrate by an intact (not leaking) everted intestinal sac.

In the same preparations, jejunal and ileal VPA serosal *vs.* mucosal concentrations were statistically different (S/M ratios > 1) for all age groups, suggesting active transport of VPA (Fig. 4). Furthermore, in contrast to the lack of active transport of *d*-glucose in colonic preparations, the 14- and 20-day-old rats evidenced colonic S/M ratios consistent with active transport of VPA. For all other groups, colonic S/M ratios were not indicative of active transport, suggesting that VPA absorption by the colon in weaned animals occured by passive diffusion.

In the newborn rat, intestinal absorption changes significantly during the first three weeks (34). Transport mechanisms become localized, and rates of absorption decrease in some areas and increase in others. The absorption of glucose

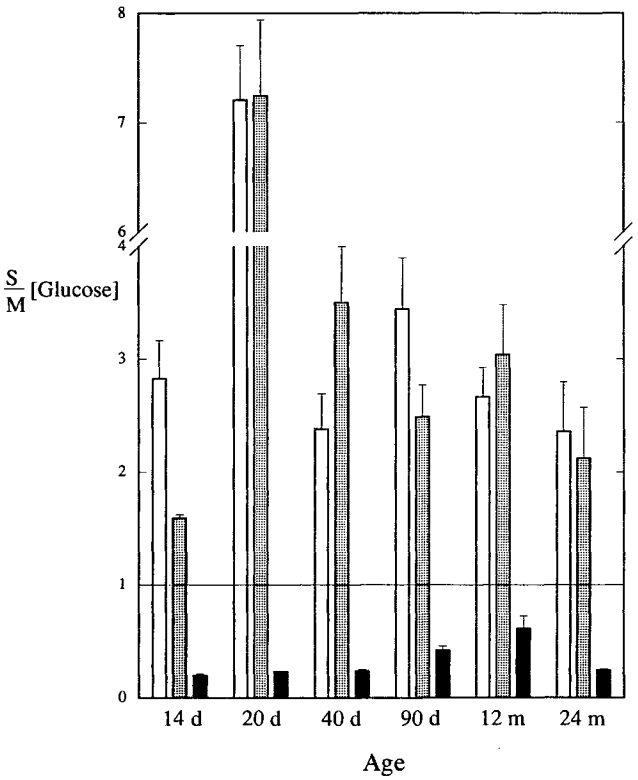


Fig. 3. Serosal-to-mucosal (S/M) concentration ratios for *d*-glucose (mean ± SE; n = 5–10 per age group) in everted intestinal sacs from segments of jejunum [open bar], ileum [shaded bar] and colon [closed bar] from 14-day- to 24-month-old rats. Serosal and mucosal concentrations were statistically different [$p < 0.05$] for all groups.

proceeds by active transport throughout the intestine in fetal rats, but occurs only in the small intestine of neonatal animals (31). Active transport of many other compounds, including calcium (10), hexose and L-proline (12), are present throughout the intestine of the newborn, but become localized in the small intestine shortly after birth. The third week is a critical period for development of intestinal function, when the adult pattern of intestinal transport mechanisms begins for many compounds (34). Active absorption of VPA was present throughout the intestine of rats for the first three weeks, but was present only in the small intestine of rats in the older age groups.

The results of this study indicate that the rate and mechanism of VPA absorption in the rat change during development. In both *in situ* isolated perfused intestine and *in vitro* everted intestinal sacs, there was a general trend towards decreasing intestinal absorption of VPA during development. The observed age-dependent changes in VPA ER during development in pre-weanlings (16) could be the result of higher rates of glucuronide hydrolysis (17) and enhanced absorption of the parent compound; the relatively rapid recycling of the parent compound resulting from these processes could mask the contribution of ER to the disposition of VPA in serum. The impact of age-dependent intestinal VPA absorption on systemic exposure to VPA, as well as on the

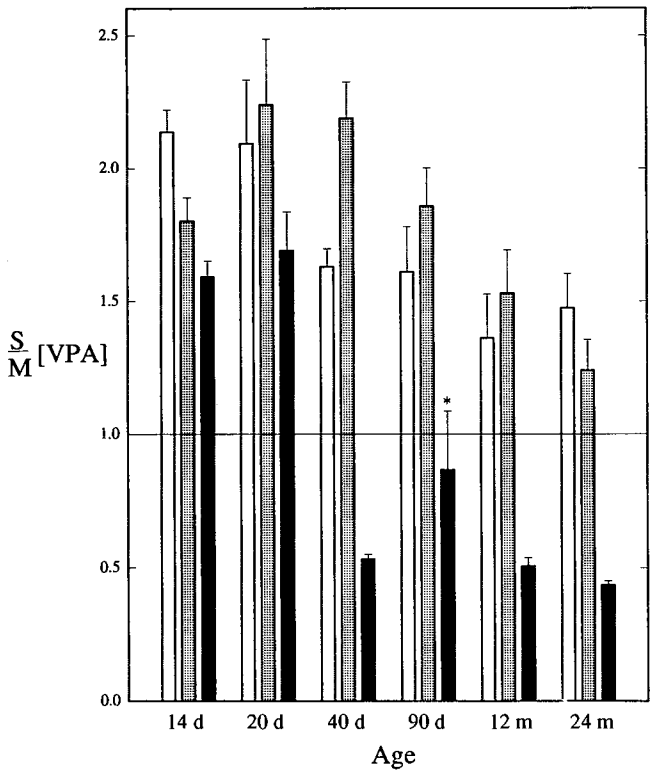


Fig. 4. VPA S/M concentration ratios (mean ± SE; n = 4–8 per age group) in everted intestinal sacs from segments of jejunum [open bar], ileum [shaded bar] and colon [closed bar] from 14-day- to 24-month-old rats. Serosal and mucosal concentrations were statistically different [$p < 0.05$] for all groups except the colon segments (*) from the 90-day-old rats.

degree of ER of this compound, is the subject of ongoing investigations.

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