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LYSOSOMAL CHANGES IN THE GUT OF RATS AFTER CHRONIC TREATMENT WITH PHENOBARBITAL*

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Abstract—Significant increases in lysosomal enzyme activities in the intestine of rats were observed 1 and 2 months after daily oral administration of 100 mg/kg of phenobarbital. As early as 1 month after daily treatment, α-glucosidase, β-galactosidase, β-*N*-acetyl-galactosaminidase, β-*N*-acetyl-glucosaminidase, β-xylosidase, acid phosphatase, and cathepsin-like and trypsin-like activities were increased, while α-mannosidase and α-fucosidase activity increased only after the second month. Ultrastructural changes—increased agranular endoplasmic reticulum and ribosomal particles and increase in acid phosphatase-positive dense bodies and coated vesicles in the absorptive cell of the intestine—were observed in the phenobarbital-treated rats. Mucosal transport of ^{14}C-methionine and ^{14}C-D-xylose was not altered.

Previous studies have shown that chronic oral treatment of rats with L-dopa produces morphological and biochemical changes in the lysosomes of the intestines, with altered absorption of D-xylose [1]. In the present study, the questions of whether the increased lysosomal activity in the gut is a specific effect of L-dopa, since chronic treatment with chlorpromazine, neomycin and imipramine did not produce such changes in the gut, and whether the altered absorption of the nutrient D-xylose was related to the lysosomal changes were further investigated using the drug phenobarbital. There have been conflicting reports on the effect of phenobarbital on the lysosomes of the liver. Mulder [2] suggested that the decrease in lysosomal enzyme activity/g of liver in rats treated with phenobarbital resulted from an increase in liver weight without concomitant increase in the total amount of lysosomal enzymes. This suggestion seemed to be contradicted by the results of morphometric studies by Staübli *et al.* [3], who found an increase in the volume of liver occupied by lysosomes in rats treated for 5 days with phenobarbital. Furthermore, Platt and Katzemeier [4] and Hornef [5] have showed evidence of increases in some of the lysosomal enzyme activities in the liver after phenobarbital treatment.

No studies have been reported on the effect of long-term oral phenobarbital treatment on the lysosomes of the gut. In the present study, long-term treatment of rats with orally administered phenobarbital produced a significant increase in the lysosomal enzyme activity of the intestines without effects on absorption of D-xylose and methionine.

MATERIALS AND METHODS

Groups of eight male Sprague–Dawley rats (160–180 g), housed in individual cages and maintained on regular laboratory diet and water, were treated with phenobarbital at a dose of 100 mg/kg body weight, daily for 8 weeks. Phenobarbital, as the sodium salt (USP), was dissolved in distilled water and administered daily in a single dose by intragastric tube. Matched controls were similarly treated daily with an equivalent volume of distilled water. After the phenobarbital treatment, intestinal transport of ^{14}C-methionine and ^{14}C-D-xylose in everted jejunal sacs, ultrastructural analysis of the jejunum, and assay of lysosomal enzyme activity of the same tissue were performed. During analytical and morphologic procedures, treated and control rats were randomly assigned code numbers, and the codes were revealed only after data collection.

Electron microscopy. After a 12-hr fast and under ether anesthesia, jejunal sections from the intestine were removed from eight phenobarbital-treated rats and an equal number of matched controls, and were prepared for both light and electron microscopic observations. The tissues were trimmed of connective tissue and immersed in either *para*-formaldehyde-glutaraldehyde [6] or glutaraldehyde [7], cut into 1-mm thick slices while in fixative, post-fixed in 1% OsO_4 for 1 hr, dehydrated in increasing concentrations of ethanol and embedded in Dow epoxy resin [8]. For orientation purposes, semi-thin (1 μm) sections of epoxy-embedded tissues from each animal were stained with toluidine blue and observed in the light microscope. Sections (400–800 Å thick) were cut on an LKB Ultratome, stained with uranyl acetate and lead citrate and observed in an RCA EMU 3-H microscope. Corresponding tissues from control rats

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were processed as above for each experimental situation. Approximately 60 grids from experimental animals and 30 grids from control animals were observed.

To elucidate the identity of the dense bodies that were frequently encountered in experimental tissues, thick sections (100 μm) and blocks (1 mm^3) of tissue from each animal were fixed in glutaraldehyde and incubated in a medium designed to demonstrate acid phosphatase activity [9, 10]. Similar tissue samples were incubated in a medium lacking only substrate to serve as controls for the histochemical procedures. No further staining with lead or uranyl salts was employed. Adjacent areas of the gastrointestinal tract were used for the biochemical analyses of lysosomal and proteolytic enzyme activities.

Lysosomal enzyme assay. The enzymes were extracted as previously described [11, 12]. To extract the enzymes from the intestine, three times saline-flushed intestine (~ 1 g) was homogenized at 4° with 30 vol. of 0·1% Triton X-100 by 30 strokes in a Dounce homogenizer. This material was tested for glycosidase and proteolytic activity. Total protein was determined by the method of Lowry *et al.* [13]. Crystalline bovine serum albumin was used as a standard.

Glycosidase, acid phosphatase or β-glucuronidase activity at pH 3·4 in each of the cell extracts was determined with the appropriate *p*-nitrophenyl derivative as substrate [14–16] in the following manner. Twenty-five μl (in some instances of high activity, 10 μl) of the enzyme extract was incubated with 1·5 μmoles of the *p*-nitrophenyl derivative (the final volume was 2 ml, 0·05 M in citrate, adjusted to pH 4·3) for 1 hr at 37°. The reaction was terminated with 2 ml of 0·5 M glycine–NaOH buffer, pH 10·5, and the optical density of the released *p*-nitrophenol was measured at 420 nm. From these data, the amount (nmoles) of the *p*-nitrophenyl derivative hydrolyzed/hr was calculated. For each substrate tested, certain reactions (with either control or phenobarbital-treated tissue as enzyme source) were terminated at 10-min intervals to ensure the linearity of the reaction up to 3 hr. The substrates used were: *p*-nitrophenyl *N*-acetyl-β-D-glucosaminidase, *p*-nitrophenyl α-D-glucoside, *p*-nitrophenyl β-D-galactoside, *p*-nitrophenyl β-D-xylopyranoside, *p*-nitrophenyl β-D-glucoside, *p*-nitrophenyl *N*-acetyl-β-D-galactosaminide, *p*-nitrophenyl α-D-mannopyranoside, *p*-nitrophenyl α-D-fucopyranoside, *p*-nitrophenyl β-D-fucopyranoside, *p*-nitrophenyl β-D-mannopyranoside, *p*-nitrophenyl β-D-glucuronide, *p*-nitrophenyl phosphate and *p*-nitrophenyl β-D-galactoside. *p*-Nitrophenol was used as standard. Parallel substrate blanks were determined with each estimation.

The amount of proteolytic activity was measured by the method of Bernacki and Bosmann [17, 18] utilizing ^{3}H-acetylated hemoglobin as substrate [19]. ^{3}H-acetylated hemoglobin was prepared with ^{3}H-acetic anhydride to a specific activity of 162 cpm/pmole based on a hemoglobin molecular weight of 68,000. Cathepsin-like activity (EC 3.4.4.23) was measured at pH 3·4 by adding 115 μg ^{3}H-acetylated hemoglobin and 50 μl enzyme extract (100 μg as protein) to a solution of 1·35 M acetic acid and 0·02 M ammonium sulfate, pH 3·4, in a final volume of 0·20 ml. This mixture was incubated for 1 hr at 37° in a Dubnoff metabolic shaker. The reaction was terminated by placing the assay tubes in an ice-water bath, adding 100 μl of 2·5% hemoglobin and 50 μl of 60% trichloroacetic acid. The precipitated protein was removed by centrifugation at 5000 *g* for 5 min and an aliquot of the supernatant fluid was plated on a glass fiber filter; radioactivity was determined as given elsewhere [20]. Activity is expressed as pmoles hemoglobin degraded/hr/mg of protein. Trypsin-like activity (EC 3.4.4.4) was analyzed as above except that 100 μl of 0·1 M phosphate buffer, pH 7·6, was substituted for the buffer.

^{14}C-methionine absorption test. After a 10- to 12-hr fast, phenobarbital-treated rats with matched controls were given orally a 1-ml solution of 5 mg (3 μCi; sp. act. 55 mCi/mmole) ^{14}C-methionine (Amersham–Searle Laboratories, Des Plains, Ill.) by intragastric tube. At 2 and 6 hr after treatment, 1 ml blood was drawn from the tail into heparin-prepared tubes. Six hr after the dose was given, the rats were anesthetized with ether and an abdominal incision was made. Ligatures were placed at the cardioesophageal junction of the stomach and at the ileocecal junction, and the intervening portion of gut was cut out, dissected free of mesenteric tissue, and washed with saline solution. The gut was blotted dry, opened up by a longitudinal incision and washed with saline three times, and the washings were collected. The stomach and intestine were homogenized separately in 4 vol. of 0·01 M HCl, and 1 ml homogenate was dissolved in NCS tissue solubilizer (Amersham–Searle Laboratories). Samples (0·2 ml) of plasma, 0·5 ml stomach and intestinal washings, and 1 ml dissolved homogenate were added to 10 ml Triton X-100–toluene scintillation liquid [21] and assayed for radioactivity in a Packard Tri-Carb liquid scintillation spectrometer. Counting efficiency for ^{14}C was 85–88 per cent. All values for radioactivity were corrected for quenching and background.

Intestinal transport of ^{14}C-D-xylose and ^{14}C-methionine by everted sacs. After a 12-hr fast, eight control and eight phenobarbital-treated rats were killed by a blow on the head and an immediate abdominal incision was made. A 5-cm segment of proximal jejunum was excised, tied at one end, and everted, as described by Wilson and Wiseman [22] and Rivera-Calimlim [23]. The everted sac was then filled with 0·5 ml modified Krebs-bicarbonate buffer (pH 7·4) and securely closed by ligation. The ionic composition of the medium was as follows (mM): K^+, 5; Ca^{2+}, 1; Mg^{2+}, 0·5; Na^+, 146; Cl^-, 129; and HCO_3^-, 25. The buffer was gassed with 5% CO_2 and 95% O_2 for 30 min before use. The everted sacs were separately incubated in 10^{-4} M (0·2 μCi) ^{14}C-methionine or ^{14}C-D-xylose at the same concentration in Krebs-bicarbonate buffer. Incubation for 15 and 60 min was carried out under 95% O_2 and 5% CO_2 at 37° in a Dubnoff metabolic incubator at 100 oscillations/min. After incubation the everted sacs were blotted on tissue paper. The serosal fluid was recovered by allowing the fluid to drain out into a tube for 10 min through a slit made at one end of the sac. The sacs were then opened and washed three times with 0·1 N HCl. The tissues were dried and weighed. Then the tissues were cut into small pieces and dissolved in 1 ml NCS tissue solubilizer. Samples from the incubation medium (0·5 ml), serosal fluid (0·2 ml), and the dissolved tissue were added to 10 ml

Triton X-100–toluene scintillation liquid, and the radioactivity was assayed in a Packard Tri-Carb scintillation spectrometer. Counting efficiency for ^{14}C was 85–88 per cent. All values were corrected for quenching and background. Serosal transfer and tissue content were calculated as μmoles/g of tissue/hr.

RESULTS

Electron microscopy. No morphological changes induced by chronic administration of phenobarbital were observed in the light microscope. The predominant fine structural changes were in the endoplasmic reticulum and coated vesicles. No major alterations of the mitochondria, terminal web, microvillus projections, Golgi complex, or nucleus were noted in absorptive cells (Fig. 1). The morphology of the goblet cells also appeared to be unchanged.

The most notable change in drug-treated animals was the marked increase in the amount of membranous profiles of the agranular endoplasmic reticulum, especially in the apical portion of the cytoplasm (Figs. 2 and 3). Frequently these structures were in the form of vesicles and occasionally in tubular configurations, the former probably resulting from the fragmentation of tubules. Some of the vesicles contained electron-opaque homogeneous material. In the supranuclear region, long tubular profiles of the agranular endoplasmic reticulum also contained this homogeneous substance (Figs. 2 and 3). The numbers of ribosomal particles existing singly or in polysomal clusters were more prominent in the nuclear and intra-nuclear regions of cells of drug-treated animals (Figs. 2 and 3). Dense bodies were occasionally seen in the apical portion of the cytoplasm, although these structures were not increased in number among cells

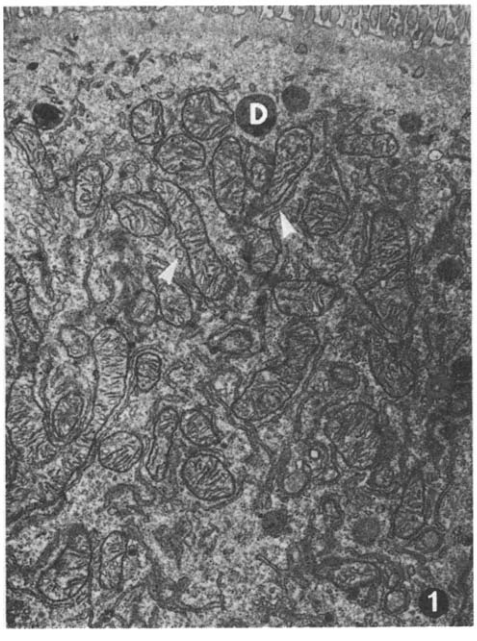

Fig. 1. Portion of jejunal columnar absorptive cell of a control animal. Tubular profiles of the endoplasmic reticulum, both agranular and granular, are rather common (arrowheads). Heterogeneous dense bodies (D) are variable in number from cell to cell (17,532).

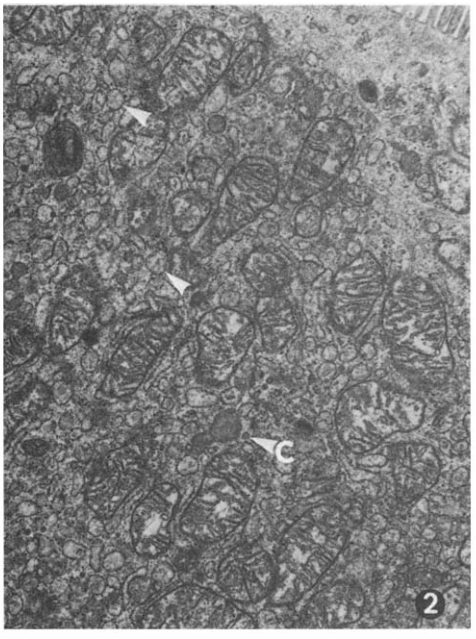

Fig. 2. Part of columnar absorptive cell from phenobarbital-treated animal. A prominant feature is the increase in spherical profiles of the agranular endoplasmic reticulum (arrowheads), some of which contain electron-dense material. Coated vesicles (C) are also increased in cells of drug-treated animals (17,532).

of the terminal portion of the villus and, as in control tissues, were highly variable from cell to cell. Incubations of these tissues to demonstrate acid phosphatase revealed enzyme activity in the dense bodies, identifying them as lysosomal in nature. Small coated vesicles were more prominent in intestinal cells of phenobarbital-treated animals (Figs. 2 and 3) and were often found in association with the poorly developed Golgi complex. The coated vesicles did not appear to be linked with the plasmalemma.

Occasionally in phenobarbital-treated animals, large double membrane-bound vesicles were noted, which exhibited a relatively clear internum (Fig. 3). These organelles were thought to be interdigitation of adjacent cells rather than degenerating mitochondria. No association of these structures with lysosomes or autophagic vacuoles was noted.

The lamina propria of the villi contained more eosinophils than did these regions of control animals.

Lysosomal enzymes. The effects of phenobarbital treatment on the lysosomal enzyme activities in the intestines of the rats are shown in Tables 1 and 2. Significant increases in β-glucosidase, β-galactosidase, β-*N*-acetyl-galactosaminidase, β-*N*-acetyl-glucosaminidase, β-xylosidase and acid phosphatase were observed as early as 1 month after the beginning of treatment. No increases in α-mannosidase, α-fucosidase or α-galactosidase were observed in rats treated for 1 month with phenobarbital, but in rats treated for 2 months these enzymes were increased. Proteolytic enzymes (cathepsin-like activity and trypsin-like activity) were slightly increased after 1 month of treatment and significantly increased at the end of 2 months. Variability of these enzymes occurs with the age of the animal, metabolic state of the organ, stage of the cell cycle of the cellular population, state of

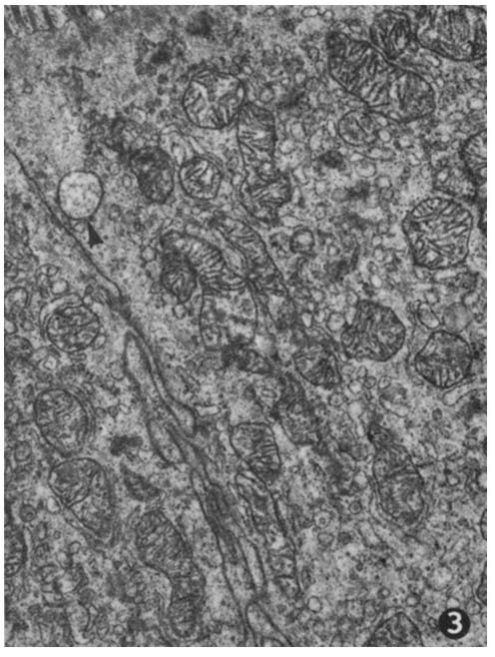

Fig. 3. Portions of two absorptive cells from a phenobarbital-treated animal. Noteworthy is the increased incidence of large, membrane-bound structures (arrowhead), thought to be interdigitations of lateral surface membranes. In some instances, mitochondria appeared to lose portions of their internal matrix (15,938).

the tissue upon assay, contributions of other components, etc. [14, 18, 20]; therefore, in each instance parallel-treated and control groups were assayed and treated identically in all experiments. Statistical significance was determined on this basis rather than on grand means or percentage of general values.

Intestinal transport of ^{14}C-methionine in vivo *in rats after chronic treatment with phenobarbital.* To determine whether the increased lysosomal enzyme activities observed in the gut of the phenobarbital-treated rats were associated with altered absorption by the intestine, we studied ^{14}C-methionine transport both *in vivo* and *in vitro*. Table 3 shows that although there was a slight but statistically significant decrease in plasma levels of ^{14}C-methionine at 2 and 6 hr after oral administration, the stomach and intestinal contents (both washings and gut tissues) were not significantly different. That the intestinal transport of ^{14}C-methionine was not greatly altered by long-term treatment with phenobarbital is further supported by the data from the everted sac experiment shown in Table 4. There were no significant differences between the serosal transfer and tissue uptake of the segments of the intestine taken from phenobarbital-treated rats and those from matched controls after 15-min and 60-min incubations. Similarly, the serosal transfer and tissue uptake of the ^{14}C-D-xylose by everted sacs were not affected by long-term phenobarbital treatment.

DISCUSSION

Biochemical and electron microscopic evidence of significant increase in the lysosomal enzyme activities in the intestine of rats chronically treated with oral phenobarbital was observed as early as 1 month after the beginning of treatment. The persistence and higher lysosomal enzyme activities in the intestines of rats treated with phenobarbital for 2 months suggest that these findings are not transient and appear to be drug related. Of five drugs (L-dopa, phenobarbital, neomycin, chlorpromazine and imipramine) given chronically to rats, only L-dopa and phenobarbital have been associated with increase in lysosomal enzyme activity in the intestine. Rivera-Calimlim *et al.* [1] suggested that the increase in lysosomal enzyme activity in the intestine of rats treated chronically with L-dopa was time and concentration dependent, since significant changes were observed only after 7 months of treatment and appeared to be limited to the intestine. It appears that L-dopa may

Table 1. Intestinal glycosidase and acid phosphatase activity after chronic treatment with phenobarbital*

Enzyme		Phenobarbital (1 month)			Phenobarbital (2 months)		
		Control	Treated	P	Control	Treated	P
γ-Glucosidase	(a)	121·4 ± 43	193 ± 30	> 0·05	150 ± 14	247 ± 43	< 0·025
β-Galactosidase	(a)	121·5 ± 5·3	196 ± 18·1	< 0·01	78 ± 6·0	110 ± 11	< 0·025
	(b)				112 ± 11	242 ± 38	< 0·01
β-N-acetyl-galactosaminidase	(a)	176 ± 5·3	337 ± 43	< 0·02	116 ± 9·8	170 ± 12	< 0·01
β-N-acetyl-glucosaminidase	(a)	243 ± 28	446 ± 36	< 0·01	783 ± 47	1084 ± 112	< 0·025
	(b)				669 ± 80	1222 ± 78	< 0·01
β-Xylosidase	(a)	1 ± 0·5	24 ± 5·4	< 0·01	1·7 ± 1	11·2 ± 3·9	< 0·025
Acid phosphatase	(a)	186 ± 23·4	279 ± 35	< 0·05	451 ± 21	632 ± 53	< 0·01
α-Mannosidase	(a)	163·0 ± 29·7	151 ± 10·3	> 0·5	17·7 ± 1·1	36·7 ± 8·5	< 0·05
	(b)				57·7 ± 2·5	88 ± 8·0	< 0·01
α-Fucosidase	(a)	55·0 ± 9·9	50·7 ± 13	> 0·5	4·2 ± 1·7	32 ± 3·6	< 0·01
	(b)				1·7 ± 1·1	12 ± 4·3	< 0·025
α-Galactosidase	(a)	69·7 ± 4·6	52·2 ± 91	> 0·5	64·7 ± 8·2	87 ± 5·7	< 0·05

* Values are expressed as mean ± S. E. M., nmoles/mg of protein/hr (N = eight rats in each group, but since assays were done in duplicate or triplicate the number of actual assays was always two or three times N). The significance of the difference between the control and treated rats was determined by the unpaired Student's *t*-test; P values are given to accurately ascertain statistical significance. The (a) and (b) refer to two independent experiments.

Table 2. Intestinal proteolytic activity after chronic oral treatment with phenobarbital*

Enzyme	Phenobarbital (1 month)			Phenobarbital (2 months)		
	Control	Treated	P	Control	Treated	P
Cathepsin-like activity						
(a)	904 ± 59	1176 ± 120	< 0·05	618 ± 41	998 ± 117	< 0·01
(b)				372 ± 22	742 ± 88	< 0·01
Trypsin-like activity						
(a)	18·9 ± 1·5	27·2 ± 4·5	< 0·05	29·7 ± 2·2	64 ± 10	< 0·01
(b)				10·7 ± 2·5	49·3 ± 17	< 0·05

* Values are expressed as mean ± S. E. M., nmoles/mg of protein/hr (N = four). See legend to Table 1.

be selectively taken up and sequestered within primary or secondary lysosomes, resulting in concentrations within the lysosomes high enough to directly affect the lysosomal enzymes. This does not seem to be the case with phenobarbital; its effect on lysosomal activity is not limited to the intestines but has been shown also in the liver [3–5] and at earlier times.

Table 3. ^{14}C-methionine absorption after chronic oral treatment with phenobarbital

Sample	Control	Treated	P
Plasma (μg/ml) (2 hr)	28·5 ± 1·8	23·2 ± 0·8	< 0·02
(6 hr)	23·3 ± 1·0	20·1 ± 0·7	< 0·02
Stomach homogenate* (μg)	33·2 ± 2·2	34·8 ± 1·9	NS†
Stomach washings* (μg)	36·8 ± 0·3	46·5 ± 0·5	NS
Intestinal homogenate* (μg)	77·8 ± 6·3	83·0 ± 6·0	NS
Intestinal washings* (μg)	36·8 ± 4·6	46·5 ± 4·7	NS

* Values were obtained 6 hr after oral ^{14}C-methionine and are expressed as mean ± S. E. M. Stomach and intestinal homogenates represent the whole stomach and small intestine of the rats.

† NS = not significant.

Although biochemical enzyme studies reveal a significant increase in many of the enzymes associated with lysosomes (Tables 1 and 2), the frequency with which these acid phosphatase-positive dense bodies is observed by electron microscopy is not remarkably increased in experimental tissues. However, there are increased numbers of small, Golgi-associated coated vesicles in the intestinal cells of drug-treated rats. These bodies, previously associated with the transport of hydrolytic enzymes, together with the electron-opaque material within the vesicles and tubules of the agranular endoplasmic reticulum, may be responsible for part of this enzyme increase.

Of significance was the marked increase in profiles of the agranular endoplasmic reticulum. That phenobarbital induces such organellar hypertrophy in other organs is now well established.

Whether the biochemical and fine structural changes observed in the gut were associated with altered functions of the absorptive cells was investigated in rats by absorption studies of ^{14}C-methionine *in vivo* and *in vitro*. Both studies failed to show important alteration in intestinal membrane transport of ^{14}C-methionine in rats after long-term treatment with phenobarbital. Intestinal transport of ^{14}C-D-xylose was also not altered.

Previous studies by Thomas *et al.* [24] also failed to show any effect of 3- to 5-day treatment of rats with phenobarbital given intraperitoneally on the absorption of 3-*O*-^{14}C-methyl-D-glucose, ^{14}C-leucine and ^{14}C-palmitic acid.

We present further evidence that, in the rat, chronic administration of a drug that is often administered chronically to humans causes biochemical and morphologic changes in the intestine. The clinical significance of the lysosomal changes in the gut induced by chronic administration of phenobarbital requires further investigation. Recently, Tytgat *et al.* [25] reported that 400–600 mg/day of phenobarbital had no effect on the ultrastructure of human intestine, but in this study, phenobarbital was given for only

Table 4. Intestinal transport of ^{14}C-D-xylose and ^{14}C-methionine in everted sacs from rats chronically treated with phenobarbital*

Group	^{14}C-D-xylose		^{14}C-methionine	
	Serosal transfer	Tissue uptake	Serosal transfer	Tissue uptake
After 15-min incubation				
Control	0·016 ± 0·01	0·045 ± 0·01	0·023 ± 0·001	0·081 ± 0·009
Treated	0·012 ± 0·01	0·035 ± 0·02	0·035 ± 0·01	0·15 ± 0·03†
After 60-min incubation				
Control	0·59 ± 0·02	0·48 ± 0·1	0·79 ± 0·16	0·63 ± 0·1
Treated	0·63 ± 0·17	0·57 ± 0·1†	0·89 ± 0·17	0·82 ± 0·1†

* Values are expressed as mean ± S. E. M., μmoles/g of tissue/hr (N = eight rats).

† Difference from control is not statistically significant (P > 0·5). Although values in the 15-min incubation with ^{14}C-D-xylose showed a P value of less than 0·01 when tested with Student's *t*-test, the absolute amount of difference is too small for clinical significance. Moreover, the 60-min incubation did not show any statistical difference between treated and control.

5 days, which is definitely not a chronic treatment. The dose of 100 mg/kg of oral phenobarbital used in the present study did not produce any loss of weight in the animals treated as compared to the controls. Extrapolation of this dose to man on the basis of body surface area gives a dose of around 600 mg, which is within the daily dose range in man.

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