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ORIGINAL CONTRIBUTION

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Vitamin A exerts its activity at the transcriptional level in the small intestine

Summary *The aim of this study* was to examine the effects of vitamin-A deficiency on the small intestinal morphology and on brush-border enzyme function and expression. *Methods* Weanling male rats were fed a vitamin-A deficient (VAD), sufficient (VAS), or supplemented (VASUP) diet, or were pair-fed (PF) with the VAD rats. Average food intakes were not different among the groups. *Results* From days 35 to 42, the body weight of VAD rats began to plateau, whereas the other groups, including the PF rats, continued to gain weight. At days 48 to 51, the final mean body weight of VAD rats was significantly lower than that of PF, VAS and VASUP rats ($P < 0.05$). Serum and liver retinol levels were lower in VAD rats (by 85 % and 99 %, respectively) and higher in the VASUP group (by 126 % and 160 %, respectively) compared to the VAS group ($P < 0.01$). Histological examination of the jejunum revealed that in VAD rats the villi were shorter and thicker and there was an elevation in crypt depth relative to the other treatment groups. Infiltration of inflammatory cells was also observed in the jejunum of most of the VAD rats, but not in rats from other groups. Biochemical assays revealed that in VAD rats, alkaline phosphatase (ALP) and sucrase-isomaltase (SI) activities are significantly decreased in the jejunum, compared to PF, VAS and VASUP groups ($P < 0.01$). ALP activity was decreased in the duodenum of VAD rats as well. By comparison, amino-peptidase (AP) activity per mg protein in the jejunum and ileum of VAD rats was significantly increased compared to VAS and VASUP rats ($P < 0.01$), but was not different from PF rats. In all of the small intestinal sections, mRNA expression of all three brush-border enzymes relative to β-actin were significantly lower in VAD rats than in the other treatment groups. SI was similarly expressed in all of the small intestinal organs, whereas AP and ALP expression varied. *Conclusions* Our results suggest that vitamin-A deficiency modifies the maturation and differentiation processes of the small intestinal mucosa at the transcriptional and post-transcriptional levels respectively. This in turn may be one explanation for the alteration or elimination of nutrient digestion and absorption during VAD.

Key words vitamin-A deficiency – brush-border enzymes – gene expression – rats

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Abbreviations

PBS	phosphate-buffered saline
PF	pair-fed
VAD	vitamin-A deficient
VAS	vitamin-A sufficient
VASUP	vitamin-A supplemented
ALP	alkaline phosphatase
SI	sucrase-isomaltase
AP	amino-peptidase

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Introduction

Vitamin A is essential for normal cell growth, differentiation and the maintenance of epithelial tissues [1], and retinoic-acid receptors have been shown to regulate the expression of a variety of growth factors involved in cellular differentiation [2]. One of the epithelial tissues exhibiting rapid cell proliferation and differentiation is the small intestine and therefore, this tissue is prone to be more susceptible to the effects of vitamin-A deficiency.

Homeostasis and integrity of the intestinal epithelium are largely dependent on the continuous proliferation, migration and differentiation of crypt cells [3]. Through the expression of brush-border membrane (BBM) enzymes, these cells acquire differentiated functions, at which point they are considered to be mature and active [4].

Vitamin-A deficiency has been found to cause morphometric changes and decreased functionality in the small intestine of chickens [5], mice [6] and rats [7, 8]. The present study extends previous reports on the effect of vitamin-A deficiency on the rat small intestine by measuring the activity and gene expression of three key enzymes responsible for protein and carbohydrate digestion and absorption in the small intestine.

To evaluate the effects of vitamin-A deficiency on the two important functions of the small intestine, digestion and absorption of peptides and carbohydrates, we measured the activities of amino-peptidase N (EC 3.4.11.2; AP) and sucrase-isomaltase (EC 3.2.1.48; SI), respectively. Alkaline phosphatase (EC 3.1.3.1; ALP) was used as a biochemical marker for cell differentiation. To better localize the effects of vitamin-A deficiency, on the small intestine's differentiation and function, the expressions of these three enzymes were compared in the different parts of the small intestine: duodenum, jejunum and ileum.

A pair-fed group was included in the design to control for the known confounding effects of lower food consumption on the outcome measurements [9]. A vitamin-A-supplemented group was added to the experimental design in order to check whether a small overdose of vitamin A affects the small intestine.

Materials and methods

Animals

Male weanling Wistar rats, specific pathogen-free, were obtained from the Harlan Laboratory at the Weizmann Institute of Science, Rehovot, Israel. They were housed in metabolic cages in a room with controlled temperature (25 ± 2 °C), relative humidity (65 ± 5 %) and light (08:00–20:00). Ethical approval was obtained for the study and all the procedures were conducted in full compliance with the strict guidelines of the Hebrew University Policy on Animal Care and Use.

Experimental design and diets

Male weanling rats (40–45 g) were distributed randomly into four groups of 15 rats each. The first group, VAD, was fed a pelleted version of the vitamin-A deficient diet described previously [10] (cat. # 960220, ICN Nutritional Biochemicals, Costa Mesa, CA; Table 1). The second group, VAS, was fed the custom control diet for vitamin-A deficiency, containing retinyl palmitate (1200 µg/kg diet) (3000 IU/kg diet) (ICN Nutritional Biochemicals). The third group, VASUP, was fed the same custom control diet; however, during the last 4 weeks of the experiment, rats in this group were intubated daily with 300 µg (750 IU) retinyl palmitate in 0.25 mL 1 % glycerol. Rats in the fourth group, PF, received the VAS diet, but were pair-fed with those in the VAD group. The rest of the groups were fed ad libitum. The diets contained (on a dry weight basis): 5 % fat, 20 % protein, 60 % carbohydrate and optimal amounts of the other essential nutrients. Food intake was monitored daily and the rats were weighed every other day to compare the growth-curve plateaus in the VAD versus VAS group, as a measure of the VAD state. Serum retinol was measured weekly in three rats from each study group. The feeding of VAD diet lasted 7 weeks until vitamin A levels decreased in both serum and liver of a representative animal.

Table 1 Composition of vitamin-A deficient diet[1]

Ingredient	Quantity g/kg diet
Vitamin free casein	180
DL-methionine	3
Corn starch	298.5
Sucrose	298.5
Alphacel, non-nutritive bulk	50
Cottonseed oil	50
Brewers yeast	80
Viosterol	0.011
Minerals[2]	6.534
Salt mixture No. 2, U. S. P. XIII[3]	40

[1] Catalogue no: 960220, ICN Biomedicals, Costa Mesa, CA

[2] Minerals (g/kg diet): calcium carbonate, 6.250; manganese sulphate·H_2O, 0.180; zinc carbonate, 0.050; cupric sulphate·$5H_2O$, 0.025; chromium potassium sulphate, 0.022; sodium fluoride, 0.005; potassium iodide, 0.001; sodium selenite, 0.001

[3] Components (g/kg diet): calcium biphosphate, 5.43; calcium lactate, 13.08; ferric citrate (16–17 % Fe), 1.18; magnesium sulphate, 5.48; potassium phosphate dibasic, 9.60; sodium biphosphate, 3.49; sodium chloride, 1.74

Tissue sampling

Rats from each group (VAD, PF, VAS and VASUP) were killed by cervical dislocation when the VAD group rats reached a plateau in growth. Blood was collected from the portal vein. Liver and small intestinal (duodenum, jejunum and ileum) tissues were quickly removed, washed in cold phosphate-buffered saline (PBS), frozen in liquid nitrogen and stored at –80 °C until analysis.

Retinol determination

Serum and liver retinol were assayed by reverse-phase HPLC with UV detection. For serum retinol determinations, 200 µL of serum were deproteinized with 200 µL ethanol [containing 0.4 µg retinyl acetate (Sigma Chemicals, St. Louis, MO) as an internal standard]. Then 1 mL petroleum ether was added, the mixture was vortexed twice, and centrifuged for 10 min at 1000 × g. The upper phase was collected, dried under nitrogen and reconstituted with 100 µL methanol. Retinol was assayed by reverse-phase HPLC (Merck C_{18}, 5 µm, 4.6 × 150 mm, Merck, Darmstadt, Germany), using methanol/acetic acid [99:1] as the mobile phase and UV detection (Multiwavelength detector model Md-910, Jasco, Japan) at 325 nm [11]. For tissue analysis of retinol contents, 3 mL ethanol and 0.75 mL KOH (50 % in water) were added to 1 g of sample, stirred and kept at 60 °C for 30 min. The samples were cooled to room temperature and extracted three times in 3 mL petroleum ether and 2 mL deionized water. The upper phase was collected, and 2 mL of 1 % HCl in water and 10 µg retinyl acetate (Sigma Chemicals), as an internal standard, were added. The solvent was evaporated under N_2 and the sample was dissolved in 1 mL of methanol. HPLC analysis was as described above for serum retinol.

Histological examination

Fresh intestinal specimens were placed in PBS and fixed overnight in 4 % paraformaldehyde in PBS at 4 °C. Serial 5-µm sections were prepared after the samples had been dehydrated in graded ethanol solutions, cleared in chloroform and embedded in paraffin. For morphometric analysis, sections were stained with hematoxylin and eosin (H&E) and were evaluated by light microscopy, by a pathologist who was unaware of the experiment being performed.

RNA analysis

Total tissue RNA was isolated from tissues using TRI REAGENT (1 mL/100 mg tissue) according to the manufacturer's protocol (MRC, Molecular Research Centre, Cincinnati, OH). The integrity of the RNA was verified by ethidium bromide staining, and the RNA concentration was determined spectrophotometrically. Total RNA (30 µg) was separated on a 1.5 % agarose gel containing 1.2 % formaldehyde, using 1 X MOPS running buffer. After electrophoresis, RNA was transferred overnight by capillary transfer to a nylon Hybond-N filter (Amersham Pharmacia Biotech, Amersham, U.K) using 10XSSC, and cross-linked to the membrane by UV irradiation.

Preparation of cDNA probes

The cDNA probes used in the northern blot analysis were as follows: a 522-bp cDNA fragment from Homo sapiens intestinal AP [12]; a 786-bp cDNA fragment from rat intestinal SI [13]; a 345-bp cDNA fragment from rat intestinal ALP [14], and a 602-bp fragment from rat β-actin (GenBank GI 55574). The cDNA's were generated by reverse-transcription (RT)-PCR using rat small intestinal RNA as a template.

Hybridization

The probes were labeled with ^{32}P-dCTP by the random prime labeling method (Biological Industries, Kibbutz Beit Haemek, Israel). The blots were prehybridized at 42 °C for 4 h and hybridized at 42 °C overnight; a high-stringency wash [0.1X saline sodium citrate (SSC)/0.1 % sodium dodecyl sulfate (SDS) at 60 °C] was perfomed according to the procedures recommended by Amersham for Hybond N. Blots were exposed for 24 h at –80 °C to Kodak XAR 5 film in the presence of an intensifying screen. The specific mRNA signals were quantified using an image analyzer (Gelpro 3 analyzer for Windows 95, Media Syberletics, 1993–1997) and the signals were standardized to the β-actin mRNA signal.

Enzyme activity assay

Enzyme activity was determined in homogenized small intestinal tissues (50 mg tissue/1 mL of 50 mM sodium phosphate buffer, pH 7.2). SI activity was assayed colorimetrically using sucrose as a substrate [15, 16] and was expressed as mmole glucose released/1 min per g of jejunal protein. AP activity was determined by hydrolysis of L-leucine-p-nitroanilide to p-nitroanilide and L-leucine for 15 min at 37 °C. The p-nitroanilide was determined spectrophotometrically at 405 nm according to [17], and 1 unit of AP activity was defined as the production of 1 µmol p-nitroanilide/min per g jejunal protein. ALP activity was determined by measuring the hydrolysis of p-nitrophenol spectrophotometrically

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(Sigma kit 104 [6, 18]). One unit of ALP activity was defined as the production of 1 µmol nitrophenol/min per g jejunal protein. Total protein was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) following detergent solubilization.

Statistical analysis

Data were expressed as means ± SEM. Treatment-dependent changes were analyzed using one-way analysis of variance (ANOVA). Statistical differences among means were considered significant at $p < 0.05$.

Results

Food intake and body weight

There were no differences in the average daily food intake among the groups throughout the experiment (data not shown). At days 35 to 42, the body weight of VAD rats began to plateau, whereas the other groups, including the PF rats, continued to gain weight. Mean body weight was significantly less ($P < 0.05$) in the VAD group than in the VAS, PF and VASUP groups as of day 43. At days 48 to 51, the final mean body weight of VAD rats (256.1 ± 14.5 g) was significantly lower ($P < 0.05$) than those of PF rats (298.3 ± 9.3 g), VAS rats (293.8 ± 8.3 g) and VASUP rats (294.0 ± 11.3 g).

Retinol concentrations

Serum and liver retinol concentrations are presented in Table 2. Consumption of the VAD diet for 48 to 51 days led to a marked reduction (of 73 % and 99 %, respectively) in serum and liver retinol in comparison to the control VAS rats ($P < 0.01$). There were no significant differences between the PF and VAS groups at any time point. Both liver and serum retinol were significantly higher (by 160 % and 126 %, respectively) in the VASUP group than in the VAS group ($P < 0.01$).

Table 2 Serum and liver retinol concentrations of rats fed vitamin-A deficient (VAD), pair-fed (PF), vitamin-A sufficient (VAS) or vitamin-A supplemented (VASUP) diets for 48–51 days[1]

	Serum retinol µmol/L	Liver retinol nmol/g
VAD	2.1 ± 0.4^{b}	0.35 ± 0.35^{b}
PF	7.1 ± 1.3^{a}	55.3 ± 8.1^{a}
VAS	7.8 ± 0.8^{a}	52.9 ± 10.8^{a}
VASUP	17.6 ± 3.6^{c}	137.5 ± 33.5^{c}

[1] Data are expressed as means ± SEM, n = 15 per group. Within each column values with a different superscript letter are significantly different ($p < 0.01$)

Morphology of the small intestine

To detect the effects of vitamin A on jejunum morphology, H&E-stained sections of the jejunum of rats fed the different diets were analyzed by light microscopy by an independent observer. Representative sections of the jejunum of rats fed the different diets are shown in Fig. 1. In the jejunum of VAD rats, the villous architecture was abnormal and the villi were short and massive in comparison to the other treatment groups. In contrast, the intestinal crypts appeared unchanged (Table 3). Mucosal infiltration of inflammatory cells was also observed in the jejunum of the VAD rats, but not in rats from other groups (Fig. 1).

Biochemical analysis of brush-border enzyme

ALP specific activity was significantly lower in the VAD group compared to PF, VAS and VASUP rats in both the jejunum and duodenum (Fig. 2a). No significant diffe-

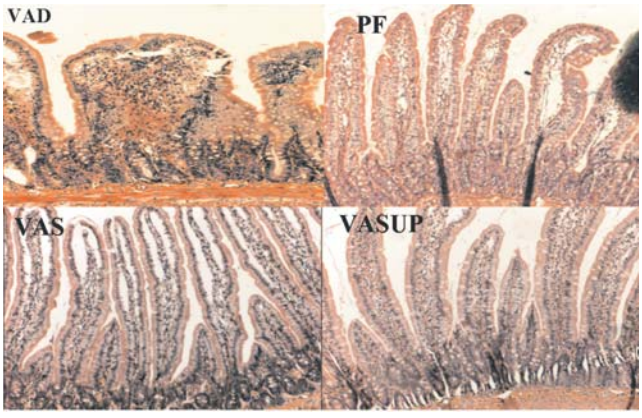

Fig. 1 Representative sections of jejunum from vitamin-A deficient (VAD), pair-fed (PF), vitamin-A sufficient (VAS) and vitamin-A supplemented (VASUP) rats fed diets for 48–51 days, stained with hematoxylin and eosin. Original magnification: X 100. A histological section of normal jejunum is seen in the lower left hand panel (VAS). With the dissecting microscope these villous have the appearance of that seen in the lower right hand panel (VASUP) and upper right hand panel (PF). Histologic sections as well as a dissecting microscopic view of vitamin-A deficiency reviled decrease in villous height together with architectural dystrophy, are shown in the upper left

Table 3 Semi quantitative evaluation of the morphological changes observed in the jejunum of rats fed vitamin-A deficient (VAD), pair-fed (PF), vitamin-A sufficient (VAS) or vitamin-A supplemented (VASUP) diets for 48–51 days[1]

	VAD	PF	VAS	VASUP
Width	6.3 ± 1.1^{a}	1.6 ± 0.3^{b}	1.7 ± 0.1^{b}	1.7 ± 0.2^{b}
Height	6.0 ± 0.3^{a}	$8.7 \pm 0.4^{b,c}$	9.5 ± 0.4^{b}	$8.6 \pm 0.2^{b,c}$
Crypt depth	2.8 ± 0.3^{a}	2.7 ± 0.1^{a}	2.6 ± 0.1^{a}	2.5 ± 0.1^{a}

[1] Data are expressed as means ± SEM, n = 5 per group. Numerical values were assigned to the observer's impression of the specific tissue variables

rences in ALP specific activity were observed in the ileum of rats in the different treatment groups. SI specific activity was significantly lower in the VAD group compared to PF, VAS and VASUP groups in the jejunum. In the duodenum and ileum, there were no significant differences among the treatment groups (Fig. 2b). AP specific activity in the jejunum and in the ileum was significantly higher in the VAD group compared to the other experimental groups. In the duodenum there were no significant differences among the treatments (Fig. 2c).

Analysis of brush-border enzyme gene expression

mRNA expression of the brush-border enzymes ALP, SI and AP was measured by northern blot analysis and compared to β-actin mRNA expression (Fig. 3). The expression of all three brush-border enzymes relative to β-actin was significantly lower in VAD rats than in the other treatment groups in all parts of the small intestine. SI was similarly expressed in all the small intestinal organs, whereas AP and ALP were differentially expressed. ALP was highly expressed in the duodenum and jejunum in comparison to much lower expression in the ileum. On the other hand AP exhibited the lowest expression in the duodenum.

Discussion

The primary purpose of this study was to investigate the effects of vitamin-A deficiency on the morphology and the function of the small intestine. Vitamin-A deficiency was induced in rats by feeding them a VAD diet for 48 to 51 days. Although food intake was not affected by the

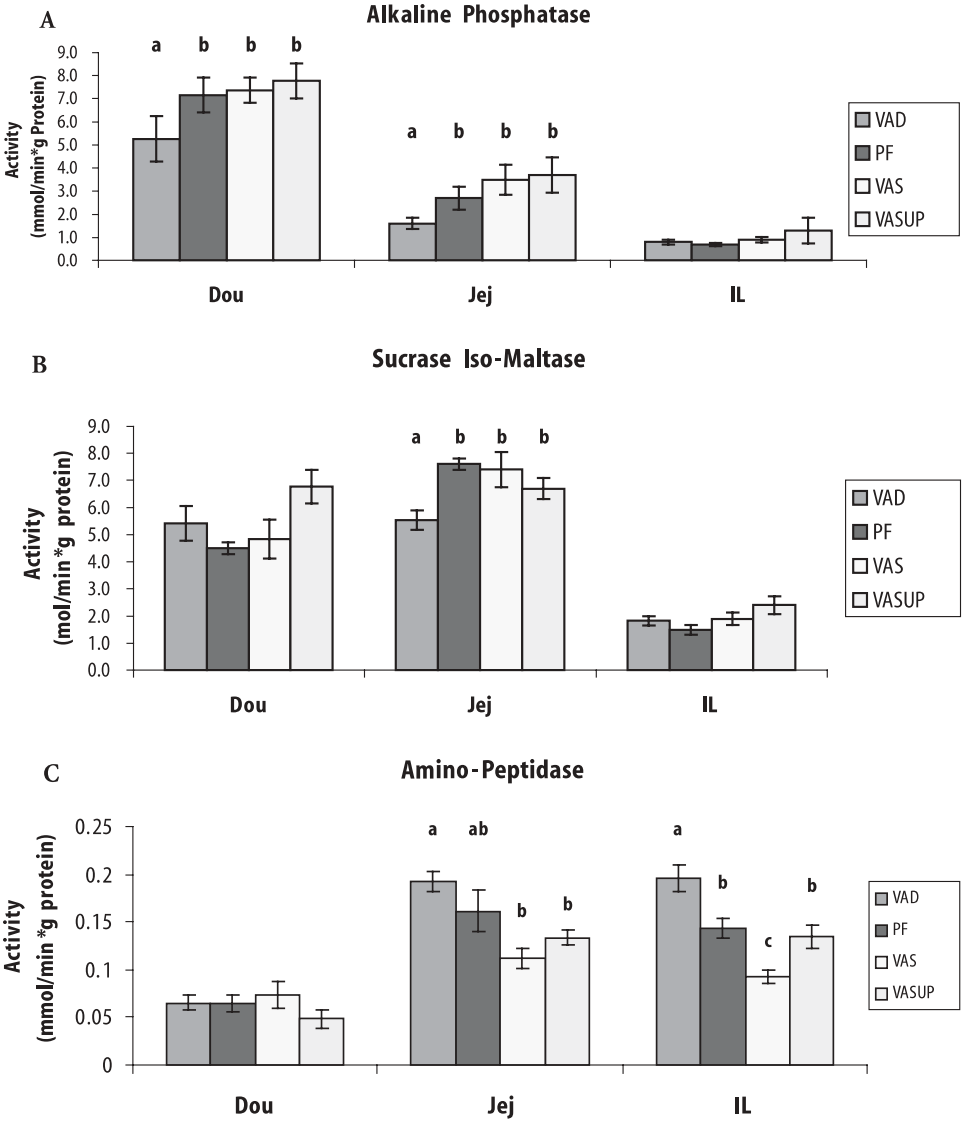

Fig. 2 Intestinal brush border enzyme activity of vitamin-A deficient (VAD), pair-fed (PF), vitamin-A sufficient (VAS) and vitamin-A supplemented (VASUP) rats fed diets for 48–51 days: alkaline phosphatase (**a**), sucrase iso-maltase (**b**), and amino-peptidase (**c**). Data are expressed as means ± SEM; n = 10–15 per group. For each panel, bars with a different superscript letter are significantly different ($p < 0.01$)

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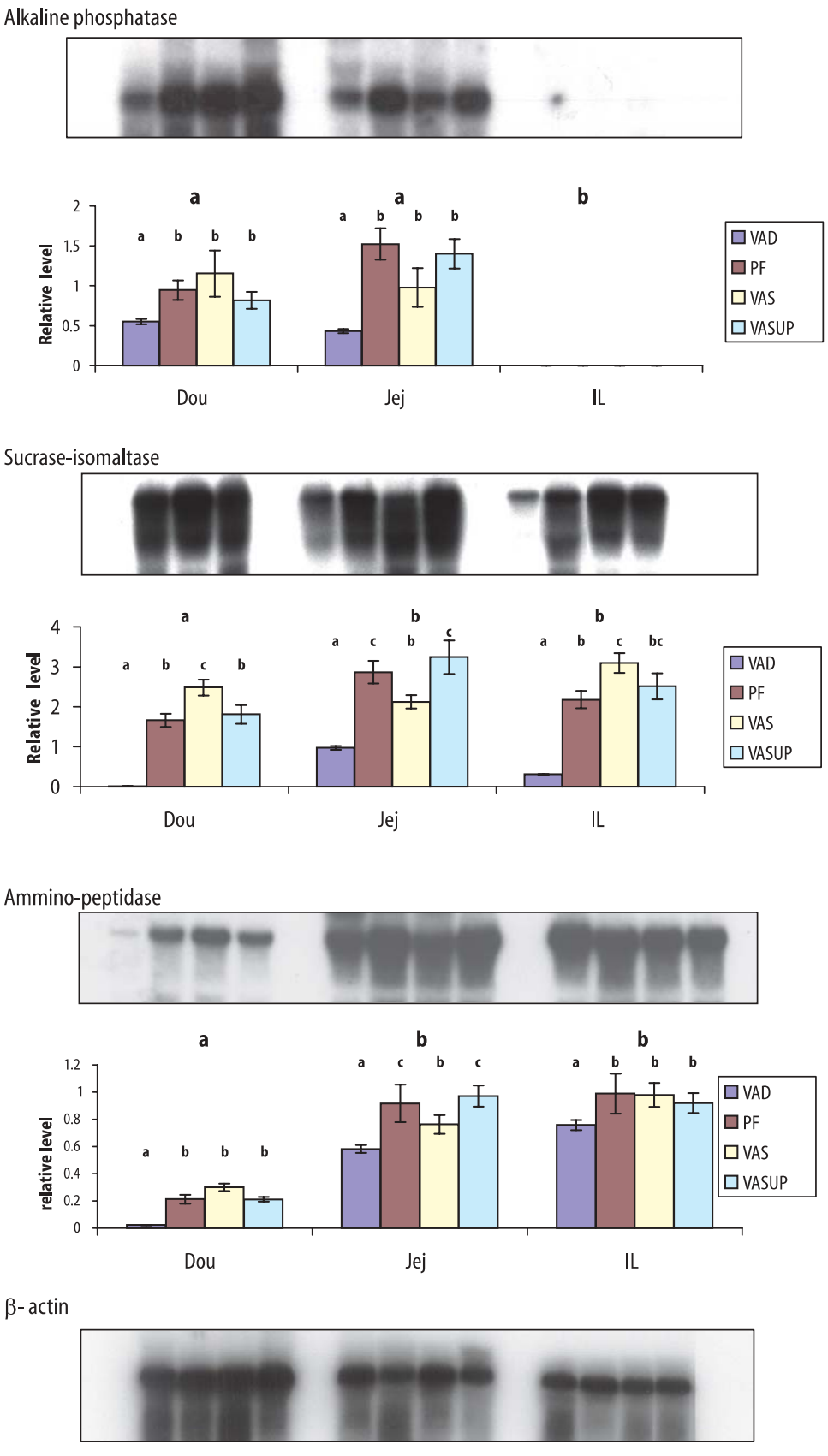

Fig. 3 Northern blot analysis of intestinal brush border enzyme mRNA levels of vitamin-A deficient (VAD), pair-fed (PF), vitamin-A sufficient (VAS) and vitamin-A supplemented (VASUP) rats fed diets for 48–51 days: **a** alkaline phosphatase (ALP); **b** sucrase iso-maltase (SI); and **c** amino-peptidase (AP). β-actin mRNA **d** level was used to quantify the mRNA level of ALP, SI and AP by densitometric analysis. Data are expressed as means ± SEM. For each panel, bars with a different superscript letter are significantly different ($p < 0.01$)

VAD diet, there was a slowing of weight gain in VAD rats starting on days 35 to 42, with no parallel slowing in the PF rats. The final difference in weight between the two groups on days 48 to 51, despite equivalent intake, may have been due to impaired nutrient utilization in the VAD rats, as has been previously suggested [19] and as indicated by the morphological and biochemical data on the present study. Rats fed a VAD diet for 48 to 51 days had lower serum and liver retinol contents, but they did not exhibit any other overt clinical signs of vitamin-A deficiency, in accordance with earlier studies [20, 21]. In the present study, vitamin-A deficiency caused changes in the brush-border morphology. Partial atrophy and disorganization of the villous architecture were observed in the jejunum of VAD rats relative to the other treatment groups (Fig. 1; Table 3). These changes in brush-border morphology, which are in accordance with other studies [5–7, 10], express a decrease in the surface area responsible for digestion and absorption and thus may reduce the functionality of the small intestine and contribute to the observed reduction in growth rate in VAD rats.

Another important finding of the study was the inflammatory changes found in the intestine of the VAD rats. This phenomenon, characterized by mucosal infiltration of the inflammatory cells and hyperplasia, was not manifested in the PF group, which was vitamin-A sufficient. Hence, it could not be attributed to an insufficiency of vital nutrients other than vitamin A. These findings are consistent with other publications, which have reported inflammatory responses in VAD animals [20, 21].

To examine the specific effects of vitamin-A deficiency in the different parts of the small intestine, three key enzymes of the brush border were investigated at both the biochemical and gene expression levels. In VAD rats, ALP-specific activity was significantly lower in both the jejunum and duodenum, while SI specific activity was lower in the jejunum, compared to PF, VAS and VASUP groups. This decrease represents a reduction in the level of differentiation of the intestinal brush border in VAD rats. By comparison, AP specific activity in the jejunum and ileum of VAD rats was significantly higher compared to VAS and VASUP rats, but was not different from PF rats. The increase in AP activity in the brush border of VAD rats is consistent with recent publications demonstrating enhanced peptidase activity under conditions of malnourishment [22, 23]. It has been proposed that the up-regulation of AP activity in response to malnourishment may be a result of catabolic changes in the whole body [22].

The dissimilar responses of the different parts of the small intestine to vitamin-A deficiency are an interesting observation. Variations in the level of activity of the studied enzymes in the different parts of the small intestine were paralleled by the level of response to VAD. For example, ALP and SI, which are highly active in the proximal part of the small intestine, were subject to variations due to VAD in those sections, but not in the ileum, where their activity is relatively low. AP, in contrast, is more active in the jejunum and the ileum, and maximal variations in its activity due to VAD were observed in these sections, while the duodenum was not sensitive. These results confirm the well-documented specific behavior of the distinct parts of the small intestine under various physiological or experimental conditions [24].

In contrast to the variations in the enzyme activities, mRNA levels of all three brush-border enzymes relative to β-actin were significantly lower in VAD rats than in the other treatment groups, in all three sections of the small intestine. SI was similarly expressed in all small intestinal organs, whereas AP and ALP expression varied: while AP expression was lowest in the duodenum, ALP was not expressed at all in the ileum. Whereas the decrease in ALP and SI activities in the small intestine of VAD rats was generally paralleled by decreased mRNA content for these enzymes, this was also reported by others to occur in fetal small intestine [25]. However AP activity remained unchanged. This discrepancy may represent different mechanisms of protein-synthesis regulation – at the transcriptional and the post-transcriptional levels, respectively – and should be further investigated.

Taken together, the changes in the morphology and functionality of the small intestine in VAD rats may result in impaired nutrient utilization, which, in turn, may explain the slowed weight gain in the VAD group, despite equivalent intake. The changes noticed in the VAD rats were not manifested in the PF group and therefore can be attributed solely to the effects of vitamin-A deficiency. No significant differences were found between the VAS group and the VASUP group in either intestinal morphology or enzyme activity and expression.

Our data show that vitamin-A deficiency induces morphological and functional changes in the small intestinal mucosa and down-regulates the expression of key brush-border enzymes. This modification of differentiation processes demonstrates the important role of vitamin A in the integrity and functionality of the intestinal epithelium and may shed light on the mechanism leading to slower growth in vitamin-A deficiency.

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