

# Influence of triiodothyronine on the polypeptide composition of the intestinal brush border membrane during amphibian metamorphosis

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**ABSTRACT** Brush border fragments (BBF) were isolated from homogenates of intestinal epithelium prepared from four groups of tadpoles: premetamorphic larvae, thyrostatic larvae, spontaneously metamorphosed larvae, and triiodothyronine (T3)-induced froglets. Isolation was accomplished by a combination of both  $\text{Ca}^{2+}$  precipitation and differential centrifugation methods. These preparations were routinely enriched seven-to elevenfold for the two amphibian brush border marker enzymes,  $\gamma$ -glutamyltransferase and maltase. Comparison by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining revealed the presence of a polypeptide of Mr 27000 only after spontaneous and T3-induced metamorphosis. One-dimensional SDS-PAGE together with lectin staining showed six strongly concanavalin A reactive polypeptides (Mr 52000, 57000, 65000, 80000, 130000 and 150000) in both preparations examined. Immunoblot analyses allowed us to detect in both preparations the presence of villin (Mr 105000), a cytoskeletal component of microvilli. Two-dimensional isoelectric focusing IEF/SDS-PAGE together with silver staining showed the polypeptides of Mr 41500, 43000, 60500 and 101000 to be specific components of the primary intestinal epithelium brush border. In contrast six polypeptides of Mr 27000, 52000, 58000, 58500, 59000 and 95000 were only detected in intestinal BBF after spontaneous and T3-induced metamorphosis. Their presence is under the control of the thyroid hormone. The results provide new insight regarding the subcellular localization of polypeptides whose synthesis changes during spontaneous (Figiel *et al.*, 1987) and T3-induced metamorphosis (Figiel *et al.*, 1989).

**KEY WORDS:** *amphibians, metamorphosis, triiodothyronine, intestinal microvillous polypeptides*

## Introduction

The intestine of anuran amphibians undergoes a remarkable transformation during metamorphosis, essentially from a long, relatively simply organized, longitudinal tube with brush border larval epithelial cell lining, few muscle cells, little connective tissue and a serosa, to a typical multilayered and folded vertebrate gut. The most conspicuous event occurring in the metamorphosing intestine is the development of the adult type epithelium. This tissue is at climax a kaleidoscope of cell destruction, proliferation and differentiation. Following a characteristic pattern of epithelial lysosomal activity and change, the larval (primary) epithelium degenerates and the remains of the tissue are extruded into the lumen and ejected (Hourdry, 1969). On the other hand, the secondary epithelium is formed by proliferation of stem cells at the base of the primary epithelium. At the end of metamorphosis, cell nests give rise to a folded secondary epithelium (Dauça and Hourdry, 1978a). In a preliminary work we have shown that the different modes of feeding by larvae, postmetamorphic froglets and

adults are reflected by molecular changes in the protein, glycoprotein and enzyme composition of the intestinal microvillous membranes (Dauça *et al.*, 1981). However, such an analysis was attempted with the application of one-dimensional polyacrylamide gel electrophoresis, a limited resolving technique.

The pronounced histological and cytological changes that occur in the intestine of anuran tadpoles during metamorphosis are controlled primarily by thyroid hormones (TH). Degeneration of the primary epithelium and development of the secondary tissue can be induced through treatment of intact animals with TH. About 10 days after immersion of *Alytes obstetricans* larvae in thyroxine (T4), the secondary intestinal epithelium replaces the primary tissue (Dauça

*Abbreviations used in this paper:* BBF, brush border fragments; CFH, calcium-free Hofreiter solution; 2D-gel, two-dimensional gel; EDTA,  $\text{Na}_2$ -ethylenediamine tetraacetate;  $\gamma$ -GT, gamma-glutamyltransferase; IEF/SDS-PAGE, isoelectric focusing/sodium dodecylsulfate polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; T3, 3,3',5'-triiodo-L-thyronine; T4, thyroxine; TH, thyroid hormones.

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TABLE 1

## ISOLATION OF INTESTINAL BRUSH BORDER FRAGMENTS

Enzyme	Stages	Fraction	Specific activity	Enrichment
Maltase	Spontaneous metamorphosis	Premetamorphic larva (20)	H 88.82±7.83	1.0
		P <sub>2</sub> 575.55±46.21	6.48	
	Juvenile (14)	H 168.19±16.99	1.0	
		P <sub>2</sub> 1830.47±79.74	10.88	
	T <sub>3</sub> -treatment	Thyrostatic larva (11)	H 68.96±13.24	1.0
		P <sub>2</sub> 489.18±24.53	7.09	
17 day-T <sub>3</sub> -treated larva (16)	H 95.57±16.86	1.0		
	P <sub>2</sub> 680.45±57.12	7.12		
γ-glutamyl-transferase	Spontaneous metamorphosis	Premetamorphic larva (20)	H 42.48±8.62	1.0
		P <sub>2</sub> 581.32±55.67	13.68	
	Juvenile (14)	H 21.45±3.52	1.0	
		P <sub>2</sub> 231.09±49.76	10.77	
	T <sub>3</sub> -treatment	Thyrostatic larva (11)	H 11.31±1.75	1.0
		P <sub>2</sub> 132.89±55.15	11.75	
17 day-T <sub>3</sub> -treated larva (16)	H 11.68±1.87	1.0		
	P <sub>2</sub> 80.97±11.91	6.93		

Data are expressed as specific activity in IU/g protein (mean±SE). IU= 1 μmole substrate hydrolysed per min.

Enrichment is the ratio of specific activity of the brush border fragments to that of the homogenate.

The values in parentheses represent the number of independent experiments.

H= mucosal homogenate.

P<sub>2</sub>= isolated brush border fragments

and Hourdry, 1985). Both the time frame and the sequence of events are similar to spontaneous metamorphosis when the level of plasma circulating TH is increased. TH-induced metamorphosis provides a useful event for the detection of the intestinal microvillous proteins which are under the control of triiodothyronine (T<sub>3</sub>), a more potent hormone than T<sub>4</sub> (Kistler *et al.*, 1977). Indeed following division of basal stem cells, the daughter cells programmed to differentiate into absorptive cells develop a highly organized structure, the brush border. This organelle is composed of tightly arranged microvilli. The

latter are characterized by membrane exhibiting digestive hydrolase activities. The brush border cytoskeleton is mainly composed of actin and villin. Villin, an actin-binding protein has been recently detected in the intestinal brush border of amphibians (Figiel *et al.*, 1987). In addition, although the specific activities of the digestive hydrolases have been extensively studied *in vivo* (Dauça *et al.*, 1980b; Ben Brahim *et al.*, 1987) and *in vitro* (Pouyet and Hourdry, 1988) during TH-treatment of anuran larvae, the effects of T<sub>3</sub> on the polypeptide composition of the amphibian intestinal brush border are still not well defined.

Therefore the purpose of the present study was to map polypeptides and glycoproteins of brush border fragments (BBF) isolated from intestines of spontaneously metamorphosed and T<sub>3</sub>-treated tadpoles using one- and two-dimensional gel electrophoresis, silver-staining or concanavalin A overlay techniques. The resulting electrophoregrams have been compared to those obtained for intestinal BBF of premetamorphic and thyrostatic larvae, enabling us to define the influence of T<sub>3</sub> on differentiation of the amphibian intestinal brush border.

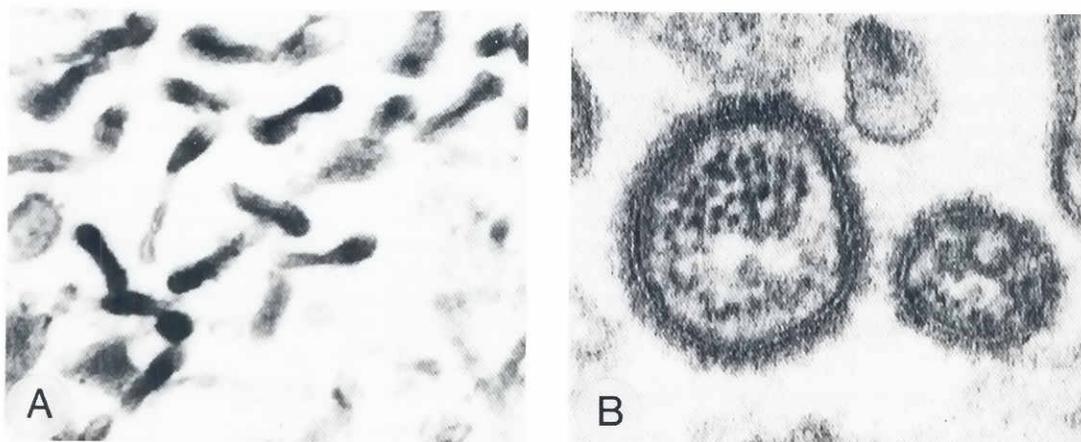
## Results

### Brush border isolation

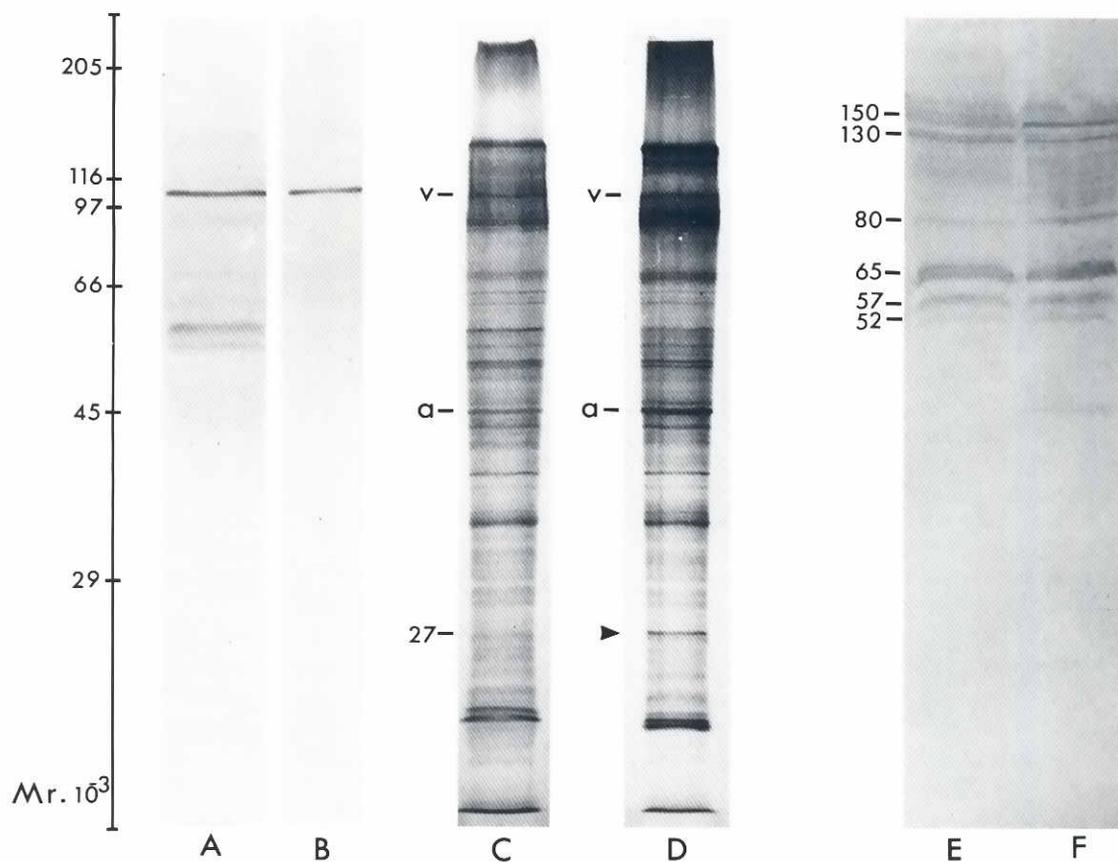
Enrichment of brush border fragments was estimated by comparing specific activities of two amphibian microvillous enzymes gamma-glutamyl-transferase (γ-GT) and maltase (Dauça *et al.*, 1979, 1980a) in the homogenates and P<sub>2</sub> pellets. All data are summarized in Table 1. Enrichment factors of γ-GT were 13.7-, 11.8-, 10.8- and 6.9-fold for premetamorphic tadpoles, thyrostatic larvae, spontaneous juveniles and 17 day-T<sub>3</sub>-treated larvae, respectively. Maltase was enriched about 7-fold in P<sub>2</sub> fractions from premetamorphic, thyrostatic and T<sub>3</sub>-treated larvae. Enrichment of this enzyme in the final preparation was about 11-fold when the intestines of spontaneous juveniles were used. The recoveries for γ-GT and maltase range between 85-93%.

### Electron microscopy

Electron microscopy of P<sub>2</sub> pellets revealed a homogeneous preparation composed of fuzzy-coated membrane vesicles characteristic of brush border fragments or intact microvilli (Fig. 1A). At high magnification the fine structure of the vesicle membrane is seen to be covered with a granular fuzzy coat. Vesicles are often



**Fig. 1. Electron microscopy of isolated intestinal brush border fragments (Fraction P<sub>2</sub>).** (A) Fraction P<sub>2</sub> is mainly composed of membranous fragments exhibiting a vesicular or tubular shape. Some vesicles contain electron dense material presumed to be the microvillous core. x30000. (B) At higher magnification, the trilaminar structure of the membrane and its fuzzy coat are clearly seen. x290000.



**Fig. 2. One-dimensional SDS/polyacrylamide-gel electrophoresis profiles of intestinal brush border proteins during natural metamorphosis.** Tadpole (lane C) and juvenile (lane D) proteins were compared after silver staining. The arrow indicates the position of the polypeptide of Mr 27000. (Lanes A and B) Villin immunodetection with monoclonal antibodies directed against pig intestinal villin in larval (A) and juvenile (B) intestinal brush border. (Lanes E and F) Identification of concanavalin A-binding glycoproteins in larval (E) and juvenile (F) intestinal brush border. The molecular weight standards used were myosin (205000),  $\beta$ -galactosidase (116000), phosphorylase b (97400), serum albumine (66000), ovalbumine (45000) and carbonic anhydrase (29000). a, actin; v, villin.

filled with electron dense material which presumably originated from the core of the microvilli (Fig. 1B).

#### One-dimensional gel electrophoresis

Figs. 2 and 3 show the electrophoregrams of BBF polypeptides from premetamorphic tadpoles (2C), spontaneous juveniles (2D), thyrostatic (3C) and 17 day-T<sub>3</sub>-treated (3D) larvae. In all cases examined, the silver-stained electrophoregrams indicate a wide range of molecular relative masses for BBF polypeptides from about 205000 through 23000.

There is a good correspondence between the proteins separated from the intestinal BBF of premetamorphic and thyrostatic tadpoles (Figs. 2C and 3C). However several polypeptides show differential staining. The polypeptide of Mr 45000 was presumed to be actin. In parallel experiments, glycoprotein components were identified by concanavalin A overlay technique. The treatment of premetamorphic tadpoles with propylthiouracil has no effect on the glycoprotein composition of the intestinal brush border. Six strongly concanavalin A reactive bands corresponding to polypeptides of Mr 52000, 57000, 65000, 80000, 130000 and 150000 were systematically detected (Figs. 2E and 3E). Several weakly reactive or minor glycoproteins could also be distinguished in the molecular weight domain of 80000 to 130000. A slightly positive smear was also noted in the upper region of the gel. After 1D-gel electrophoresis, immunoblot analyses were performed either with polyclonal (data not shown) or monoclonal (Figs. 2A and 3A) antibodies prepared against pig intestinal villin. A polypeptide of Mr 105000 immunologically related to villin was detected in BBF preparations

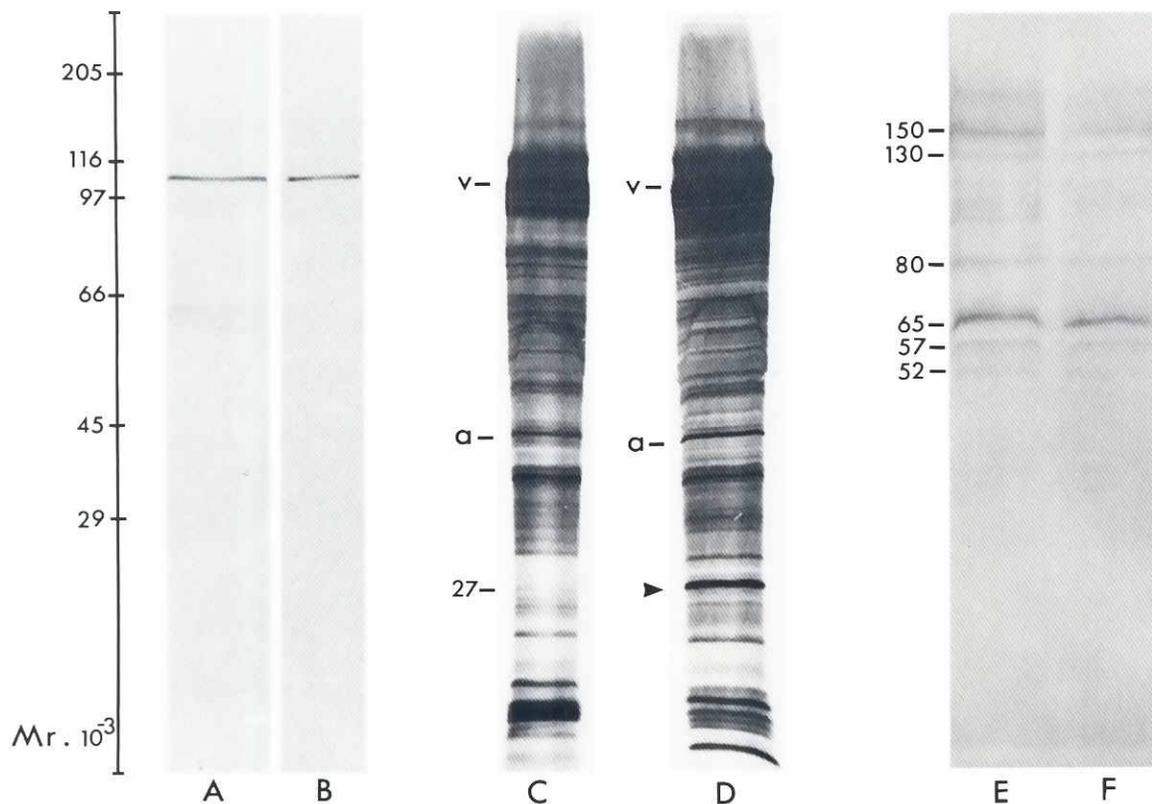
of premetamorphic (Fig. 2A) and thyrostatic (Fig. 3A) larvae intestines.

Comparison of the polypeptide compositions of the BBF purified from the primary (Figs. 2C and 3C) and secondary (Figs. 2D and 3D) intestinal epithelia reveals some quantitative and qualitative differences. The most characteristic feature is more numerous and intense bands in the molecular weight region of 60000 to 150000. Another striking difference is the presence of polypeptide of Mr 27000 in the intestinal BBF prepared from spontaneous juveniles (Fig. 2D) and 17 day-T<sub>3</sub>-treated tadpoles (Fig. 3D). On the other hand, no differences are noted in the glycoproteins (Figs. 2F and 3F) and villin (Figs. 2B and 3B) expression in the newly formed secondary intestinal epithelium during spontaneous and T<sub>3</sub>-induced metamorphosis.

#### Two-dimensional gel electrophoresis

Because of the complexity of the polypeptide compositions of larval and juvenile intestinal BBF, comparisons were also undertaken by two-dimensional gel electrophoresis. This technique allows a much more detailed analysis of each developmental stage and makes possible a much clearer appreciation of the differences and similarities between the changing polypeptide constituents of the BBF.

In the present study, we were mainly interested in detecting representative components of the anuran intestinal BBF which display qualitative and quantitative changes in response to spontaneous and T<sub>3</sub>-induced metamorphosis. Judgments regarding these changes were made after superposition of the distinct spots present on the four silver stained gels carried out for each experi-



**Fig. 3. One-dimensional SDS/polyacrylamide-gel electrophoresis profiles of intestinal brush border proteins during T3-induced metamorphosis.** Comparison after silver staining of thyrostatic larvae (C) and T3-induced juvenile (D) brush border polypeptides. (Lanes A and B) Villin immunodetection with monoclonal antibodies directed against pig intestinal villin in intestinal brush borders of thyrostatic larvae (A) and newly metamorphosed animals after a 17 day-T3-treatment (B). (Lanes E and F) Identification of concanavalin A-binding glycoproteins in thyrostatic larvae (E) and induced juveniles (F). The molecular weight standards used are the same as in Fig. 2. a, actin; v, villin.

mental case studied. The results of two-dimensional electrophoretic analysis of polypeptides extracted from intestinal BBF, before and after spontaneous and T3-induced metamorphosis, are shown in Figs. 4 and 5. In both cases investigated, about 100 major polypeptides are continuously present. Their relative molecular masses are mostly between 200000 and 250000. Isoelectric points are situated in the region of acidic to neutral pH, between pH 4 and 7.4. Under our experimental conditions, no basic proteins are observed.

Amongst these polypeptides, only 10 exhibit differential developmental patterns. They have been assigned numbers when they correspond to polypeptides previously detected in *Alytes obstetricans* intestinal epithelium during spontaneous (Figiel *et al.*, 1987) or T3-induced (Figiel *et al.*, 1989) metamorphosis. Polypeptides that have been assigned letters had not yet been identified in the previous works.

Polypeptides 3, A, B and 24 of Mr 101000, 60500, 43000 and 41500 respectively, are only detected in intestinal BBF of premetamorphic (Fig. 4A) and thyrostatic (Fig. 5A) larvae. It may be added that strongly silver-stained polypeptides are observed in the region of acidic pH 4.8-5. They exhibit high relative molecular masses from 80000 through 100000 of 2D-gels. In contrast, many of the smaller polypeptides are present as discrete spots.

On the other hand, six polypeptides appear in the intestinal BBF

of spontaneous juveniles (Fig. 4B) and 17 day-T3-treated tadpoles (Fig. 5B). These polypeptides are 4, 14, 15, 16, 18 and C of Mr 95500, 59000, 58500, 58000, 52000 and 27000 respectively.

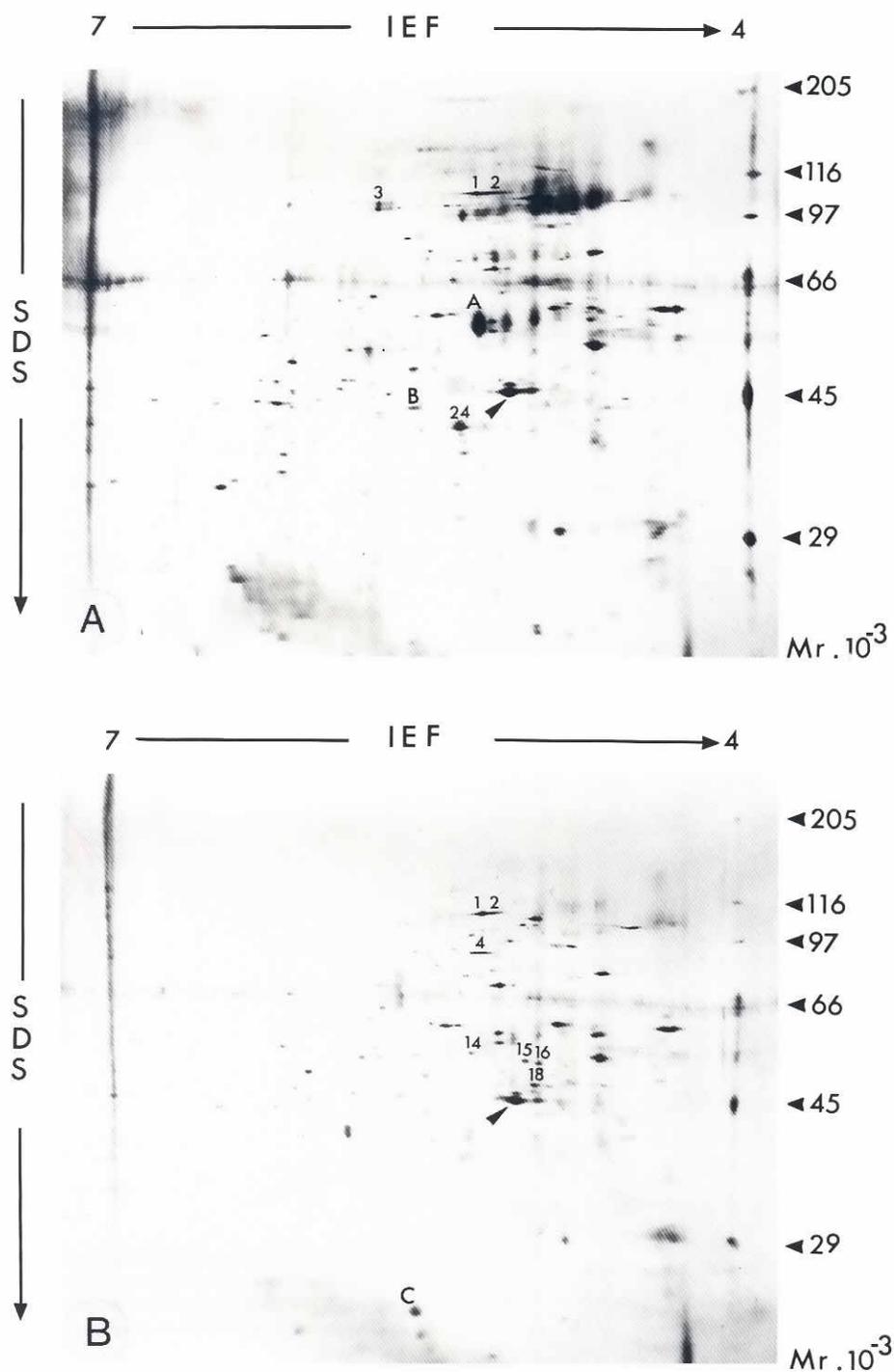
Polypeptides 1 and 2 were identified as isoelectric variants of villin in previous works (Figiel *et al.*, 1987, 1989).

## Discussion

Microvillous membrane vesicles prepared from the primary intestinal epithelium of *Alytes obstetricans* tadpoles and from the secondary tissue of newly-metamorphosed froglets are analysed by one- and two-dimensional gel electrophoresis. Changes occurring in the polypeptide patterns during spontaneous metamorphosis are compared to those detected during T3-treatment.

Three main categories of microvillous polypeptides have been distinguished:

- The first group is composed of polypeptides present in intestinal brush border fragments of both *Alytes* premetamorphic tadpoles and juveniles. This major category is composed of most of the 1D- and 2D-silver stained polypeptides. Amongst them, six glycoproteins are identified by concanavalin A overlay technique, a more sensitive method than that previously used (Dauça *et al.*, 1981). Three polypeptides have been identified as cytoskeletal components. Polypeptides 1 and 2 (Mr 10500) have been related to villin



**Fig. 4.** Two-dimensional gel electrophoresis of polypeptides from intestinal brush border of *Alytes obstetricans* premetamorphic larvae (A) and spontaneously metamorphosed juveniles (B). Isoelectric focusing occurred from left to right with the acidic end to the right. The second dimension performed with sodium dodecylsulfate (SDS) separated the proteins on the basis of molecular weight. Gels were silver stained. The molecular weight standards used were rabbit muscle myosin, 205000; *Escherichia coli*  $\beta$ -galactosidase, 116000; rabbit muscle phosphorylase b, 97400; bovine serum albumin, 66000; ovalbumin, 45000 and bovine erythrocytes carbonic anhydrase, 29000 (Sigma Chemical Co). Arrow head: actin isoforms.

isoelectric variants by immunoblot analyses using polyclonal pig intestinal villin. The Mr 45000 protein is presumably actin.

- The spots of group II correspond to polypeptides that were only detected in larval microvillous extracts. Polypeptides 3, A, B and 24 are prototypes of this category.
- On the other hand, polypeptides of the third category (4, 14, 15, 16, 18 and C) are components of the juvenile intestinal microvilli and are not found in the larval extracts.

The biological models used make it possible to determine the effects of T3 upon the polypeptide composition of the intestinal secondary brush border. Spontaneous metamorphosis is characterized by several changes in endocrine glands including the thyroid glands (Regard, 1975), the interrenal cells (Rapola, 1963; Hanke and Neuman, 1972; Dodd and Dodd, 1976; Grassi-Milano *et al.*, 1979; Hsu *et al.*, 1980) and the pancreatic islets of Langerhans (Frye, 1964; Cheng-Kaung, 1983). These histological changes

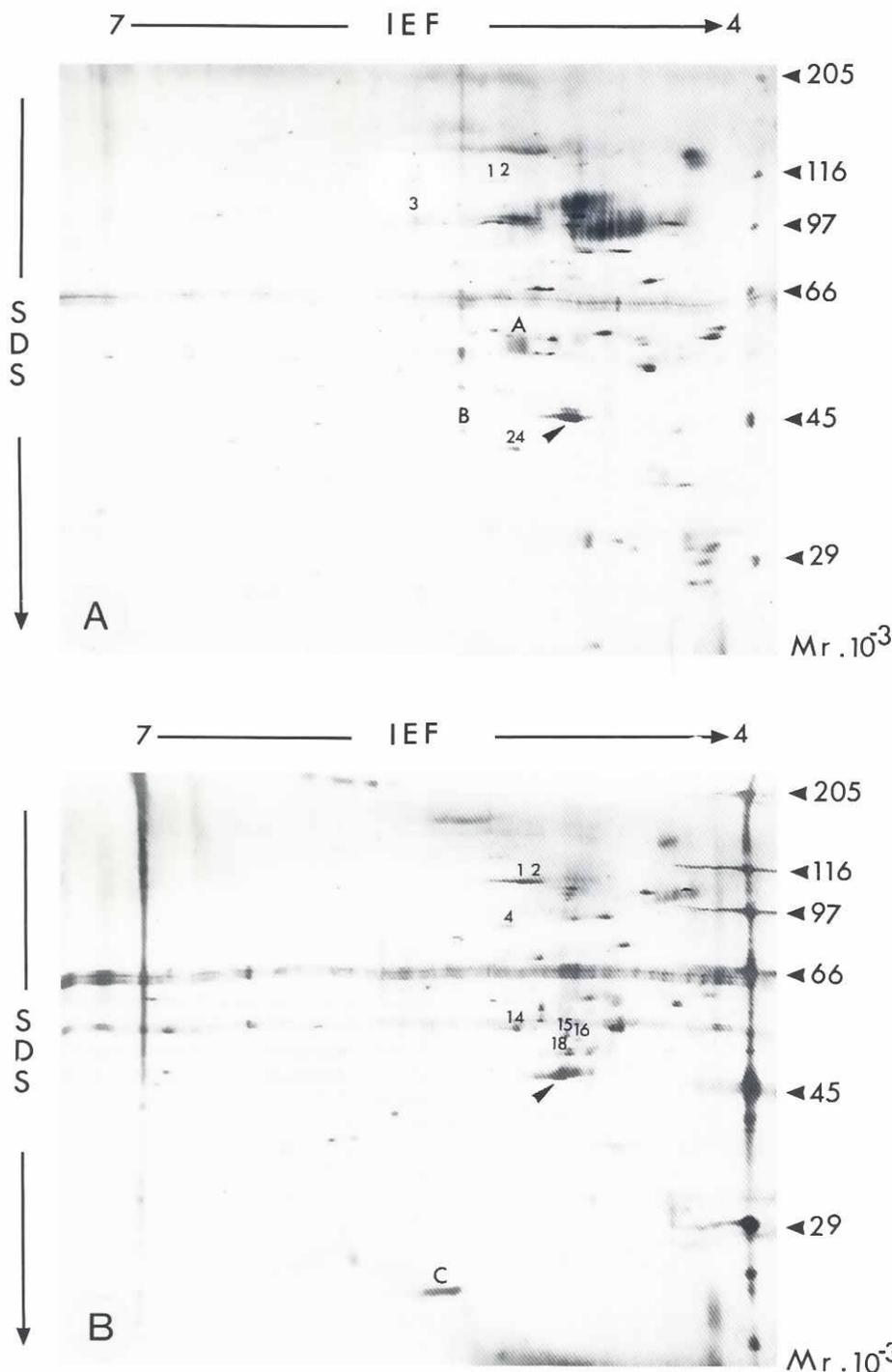


Fig. 5. Two-dimensional gel electrophoresis of polypeptides from intestinal brush border of *Alytes obstetricans* thyrostatic (A) and 17 day-T<sub>3</sub>-treated larvae (B). For legends see Fig. 4.

result in increases in plasma concentrations of both TH (Miyachi *et al.*, 1977; Regard *et al.*, 1978; Mondou and Kaltenbach, 1979), glucocorticoids (Jaffe, 1981; Krug *et al.*, 1983; Jolivet-Jaudet and Leloup-Hatey, 1984) and insulin (Hulsebus and Farrar, 1985). On the other hand, tadpoles treated with TH undergo a disharmonic development somewhat different from that of spontaneous metamorphosis, depending upon the concentration of TH administered. Activation of interrenal cells and pancreatic islets of Langerhans has not been observed during TH-induced

metamorphosis (Jolivet-Jaudet and Leloup-Hatey, 1984). Thus, it may be assumed that intestinal brush border polypeptides absent in thyrostatic larvae but detected in T<sub>3</sub>-treated tadpoles are under the control of the thyroid hormone.

Our results obtained for T<sub>3</sub>-treated larvae were very similar to, if not identical with, those that were noted for spontaneous juveniles. It was found that T<sub>3</sub> has no effect on the glycoprotein composition of the intestinal microvillous membrane as the six concanavalin A reactive bands noted in premetamorphic tadpoles and thyrostatic

larvae were also detected in juveniles as well as in T3-treated animals. Nevertheless, using 2D gel electrophoresis and silver staining we have identified six non-glycosylated polypeptides (4, 14, 15, 16, 18 and C) which were present in BBF prepared from juveniles and T3-treated tadpoles but absent in preparations from thyrostatic larvae. Our data suggest that these brush border components may be under the direct control of T3.

In several recent instances, it has been emphasized that amphibian metamorphic changes are ascribed to the TH-induced synthesis of specific proteins. In the skin, keratins of adult types have been shown to be produced in direct response to T4 (Reeves, 1977). Similarly, the changes from ammonotelism to ureotelism that occur during spontaneous or TH-induced metamorphosis, are brought about by increased synthesis of the liver urea cycle enzymes (Cohen, 1970). Moreover blockage of thyroidal function with propylthiouracil resulted in inhibition of about half the changes in protein synthesis observed in the livers of metamorphosing *Xenopus* larvae (May and Knowland, 1981). Ray and Dent (1986) have demonstrated that both treatment of tail fin explants with T4 and elevation of endogenous levels of TH during spontaneous metamorphosis increased the relative rates of synthesis of several identical proteins in the resorbing tail fin. More recently, we have investigated developmental patterns of protein synthesis at each stage of intestinal epithelium renewal during spontaneous (Figiel *et al.*, 1987) and T3-induced metamorphosis (Figiel *et al.*, 1989) of *A. obstetricans* tadpoles. Polypeptides 3 and 24 were found to be specific to the primary epithelium. Polypeptides 14, 15 and 18 were synthesized at different levels depending upon the stage. Polypeptides 4 and 16 were only synthesized when the secondary intestinal epithelium was formed. The present study provides new insight regarding the subcellular localization of those polypeptides as our results indicate that they are components of the brush border.

In conclusion, we have identified the rearrangements in the polypeptide composition of the amphibian intestinal brush border during spontaneous and T3-induced metamorphosis. Such changes have been precisely mapped using one- and two dimensional IEF/SDS-PAGE. The major glycoproteins have been revealed with concanavalin A overlaying and villin has been detected using immunoblot analyses. Moreover this is the first report dealing with the effects of T3 on the polypeptide composition of the amphibian intestinal brush border membrane. The identification of the microvillous T3-induced polypeptides is under way.

## Materials and Methods

### Animals and treatments

Tadpoles of *Alytes obstetricans* were collected from the Montpellier area (South of France), fed boiled lettuce, and staged according to the developmental table of Taylor and Kollros (1946). They were divided into groups as follows: for experiment 1, premetamorphic larvae (controls) at 12°C and spontaneously metamorphosed juveniles at 20°C; for experiment 2, thyrostatic, premetamorphic tadpoles treated with 0.58 mM propylthiouracil (PTU) (controls) changed weekly at 12°C and T3-induced froglets obtained from tadpoles in PTU solution containing 5nM 3,3',5'-triiodo-L-thyronine (T3) (change daily during the 17 days of hormonal treatment) at 20°C. PTU and T3 were purchased from Sigma Chemical Co (Saint-Louis, USA).

### Isolation of brush border fractions

Intestines were excised at 0°C, split open, washed in a cold saline solution and incubated for 9 min at 37°C in a calcium-free Holtfreter (CFH) solution containing 150 µg/ml phenylmethylsulfonyl fluoride (PMSF) and 5 mM Na<sub>2</sub>-ethylenediamine tetraacetate (EDTA). They were then transferred to

ice-cold CFH solution and the epithelium was isolated from the connective tissue and the muscle layers as previously described by Dauça and Hourdy (1978b). When spontaneously metamorphosed or T3-treated animals were used, 3-5 isolated intestinal epithelia were pooled for the assays, whereas the intestinal epithelium from a single thyrostatic tadpole gave enough material for a single assay.

Isolation of brush border fraction was performed according to the classical technique of Schmitz *et al.* (1973) adapted to the amphibian intestine by Dauça *et al.* (1979, 1980a). In brief, a 1% homogenate was made in 50 mM mannitol-2 mM Tris (pH 7.1) at 4°C. CaCl<sub>2</sub> was added to a final concentration of 10 mM for 10 min. Centrifugation of the homogenate at 3000 g for 10 min gave a supernatant fraction (S<sub>1</sub>) and a pellet (P<sub>1</sub>) which was resuspended in 50 mM mannitol-2mM Tris for further analysis. S<sub>1</sub> was centrifuged at 20000 g for 15 min and yielded a pellet (P<sub>2</sub>) containing the brush border fragments and a supernatant fraction (S<sub>2</sub>). P<sub>2</sub> was resuspended in a small volume of twice distilled water.

### Electron microscopy

Pellets (P<sub>2</sub>) of crude brush border fragments were fixed according to Graham and Karnovsky (1966) with slight modifications. They were fixed for 40 min in a mixture of 1.54% glutaraldehyde and 1.54% paraformaldehyde (final concentration) buffered at pH 7.4 according to Millonig (1961). After subsequent washes with Millonig's buffer, the fixed pellet was postfixed in 1% osmium tetroxide in the same buffer. Then, the pellet was rinsed in the buffer and dehydrated in alcohol. After two changes in propylene oxide, the pellet was cut into small pieces and embedded in Epon-araldite medium (v/v). After polymerization, ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) and visualized in a Zeiss EM9S2 microscope.

### Assay methods

Maltase activities were assayed according to a modification by Lloyd and Whelan (1969) of Dahlqvist's method (1964). Gamma-glutamyltransferase (γ-GT) activities were measured according to Naftalin *et al.* (1969). Protein concentrations were estimated by the method of Lowry *et al.* (1951).

### Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

P<sub>2</sub> pellets were lyophilized and kept frozen until used.

For one-dimensional SDS-PAGE, P<sub>2</sub> lyophilizates were resuspended in Laemmli's buffer (1970). Electrophoresis was performed using 12.5% polyacrylamide slab gels. A voltage of 200 V was applied for 1 h, then increased to 400 V, until the bromphenol blue tracking dyes reached the lower buffer reservoir.

For two-dimensional gel electrophoresis, P<sub>2</sub> lyophilizates were resuspended in O'Farrell's lysis buffer (1975). Samples containing 75 µg proteins were loaded onto gels. Isoelectric focusing (IEF) of the proteins was achieved by some modifications of the method of O'Farrell (1975). The ampholytes had a 5% final concentration, and the mixture was composed of one volume of pH 3-10 from Serva (Heidelberg, Germany) and four volumes of pH 5-7 from Serva, Pharmacia (Uppsala, Sweden) and LKB (Bromma, Sweden) products. A voltage of 650 V was applied for 15 h, then 1000 V for 1 h. The second dimension SDS-PAGE was performed on 12.5% polyacrylamide gels. Gels were run at 200 V for 1 h, then 400 V for 3 h.

For one- and two-dimensional gel electrophoresis, four independent experiments were carried out in all cases examined. Slab gels were silver-stained (Morrissey, 1981). Protein relative molecular masses were estimated by reference to the migration of standard proteins (bovine erythrocyte carbonic anhydrase, 29000; ovalbumin, 45000; bovine serum albumin, 66000; rabbit muscle phosphorylase b, 97400; *Escherichia coli* β-galactosidase, 116000 and rabbit muscle myosin, 205000) all obtained from Sigma Company.

### Glycoprotein detection

Transfer of electrophoretically separated proteins from SDS-polyacrylamide gels to nitrocellulose was performed according to Burnette's technique (1981). The subsequent identification of concanavalin A-binding glycoproteins was done using horseradish peroxidase as described by Faye and Chrispeels (1985).

**Immunoblotting analysis**

Amphibian brush border villin was detected using Western blotting procedure (Burnette, 1981). Experiments were carried out using either polyclonal (diluted 1:800) or monoclonal (20 µg/ml in PBS) antibodies to pig villin. Polyclonal and monoclonal antibodies have been characterized respectively by Robine *et al.* (1985) and Dudouet *et al.* (1987).

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