

## THE TELEOST GUT PERSORBS MICROPARTICULATES

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### Summary

The ability of the teleost gut to absorb microparticulate material was examined following rectal intubation ( $3.5 \text{ g kg}^{-1}$ ) of commercial grade cornstarch ( $\cong 21 \mu\text{m}$  diameter), or potato starch ( $\cong 43 \mu\text{m}$  diameter). Tissue samples were taken from the mid — and hind-gut of control and treated fish 18 h postintubation. Collected samples were processed using standard plastic and staining protocols and resultant photomicrographs examined by computer-assisted image analysis. Cornstarch particles ( $8\text{--}14 \mu\text{m}$ ), were observed to pass from gut lumen to the *lamina propria* via a paracellular or persorptive route only. No evidence for the like passage of potato starch was found.

*Key words:* bioencapsulation, gastrointestinal tract, Herbst effect, microparticulates, paracellular uptake, persorption

### INTRODUCTION

The fish (gastro)intestinal tract is generally considered to express the typical vertebrate plan for the processing, transport and absorption of ingested nutrients (see: Smith 1988). Several studies have demonstrated that the teleost gut, in common with a variety of other Phyla, also retains the capacity to absorb protein macromolecules by well-defined vesicular mechanisms (e. g., Noaillac-Depeyre & Gas 1973, 1979; Rombout *et al.* 1985). And, the

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potential application of the latter phenomenon, in the control of production-related processes, such as reproduction, growth and vaccination, has been extensively reviewed (McLean & Ash 1987; Sire & Vernier 1992; McLean *et al.* 1999). In higher vertebrates, including man, the passage of microparticulates (5–120  $\mu\text{m}$ ) from gut lumen to intestinal *lamina propria* has also been reported (*e. g.*, Kumagaya 1922; Volkheimer 1974, 1993). In mammals, the mechanisms available for the translocation of microparticulates from gut lumen to *milieu interior* include transport by wandering macrophages (O'Hagan 1996), endocytotic uptake by absorptive enterocytes, the M-cell and its mimetics (Morris *et al.* 1994; Hodges *et al.* 1995), and at villus tips (O'Hagan 1996). The latter pathway, which is generally referred to as »persorption«, or the »Herbst effects« (Herbst 1884), is manifest at mucosal sites which are lined by a single cell layer (Volkheimer 1977). The precise means by which microparticulates are persorbed by the intestine remain ambiguous. However, persorption is generally considered an abrasive process. A focal component of the mechanism appears to be the peristaltic movements of the gut. In mammals, when combined with rhythmic contractions of intestinal villi and the pulsatile nature of the circulatory system, microparticulate material may be forced between epithelial cells or enter inter-epithelial spaces following microscopic damage (Volkheimer 1972).

It has been reported (Volkheimer & Schulz 1968), that forced access of microparticulates may be particularly prominent during cell sloughing processes, when the integrity of the intestinal mucosa may be compromised due to the loosening of the junctional complex. In addition, microparticulates may gain access to the *milieu interior* following intestinal or gastric insult (McLean & Donaldson 1990). In fishes, gut lacerations may be caused by malnutrition (Segner *et al.* 1987), exposure to pollutants (Banerjee & Bhattacharya 1995) or following parasitic invasion (Meguid & Eure 1996). The ingestion of particulate material, including small pebbles and stones (Baird 1872; Wickes 1908), bones and scales, may also damage the absorptive epithelia; thereby presenting portals through which particulate material may gain access to *lamina propria* and thence the circulation.

While there is little doubt that microparticles breach the physical, chemical and immunological barriers of the mammalian gut, no direct evidence for a similar mechanism in fish exists. In previous studies, the translocation of microparticulates, such as bacterial cells, from the gut lumen to sub-epithelial spaces, has been inferred by immunohistochemical techniques (*e. g.*, Olafsen 1984; Vigneulle & Baudin Laurencin 1991), with general consensus that the transcellular pathway predominates. The present study was undertaken to investigate whether the absorptive process of the fish gut includes a persorptive pathway.

## MATERIALS AND METHODS

### Experimental animals

Twenty female rainbow trout (*Oncorhynchus mykiss*;  $30 \pm 2.3$  g wet weight) obtained from a commercial fish hatchery (Blaesborg Fiskeri, Denmark) were transferred to experimental aquaria and allowed to acclimatise for a 2 week period prior to experiment start. Animals were maintained in custom-made flow-through circular ( $5 \text{ m}^3$  water volume) fibreglass tanks. Inlet freshwater, supplied by the municipal water works, was heated and oxygenated (Hede Nielsen A/S Denmark) in a buffer tank before distribution. Water temperature ( $15.0 \pm 1.8$  °C) and oxygen ( $10 \pm 1.0$  mg/l) levels were controlled by an OxyguardR 8 system (Oxyguard A/S, Denmark), with profiles being logged (M+S logger; Møller & Sørensen, Denmark) downloaded and analysed weekly (Nina Soft A/S, Denmark). A 12 h photoperiod (dimmed light, artificial fluorescent tubes) was applied throughout the study period and animals were hand fed to satiation once daily (08:00 h) with 3 mm pellets (Ecolife 19; BioMar A/S, Denmark).

### Experimental treatments

In order to examine the effect of particle size and shape upon the persorptive process corn- and potato starches were used. To enhance visualisation, starches were pre-stained with iodine for 5 min. Particle size distribution was assessed using a Leeds & Northrup Microtrac II size analyser (Fig. 1).

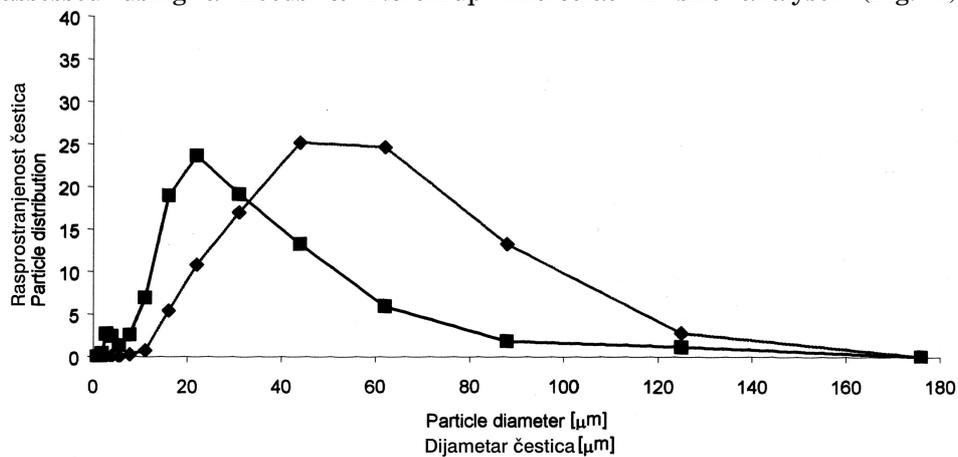


Fig. 1. Size distribution of cornstarch (■) and potato starch (◆) particles employed in the present investigation was evaluated by size analyser. Each point represents the mean value of three measurements.

Slika 1. Rasprostranjenost veličine čestica kukuruznog (■) i škroba krumpira (◆) uporabljenih u istraživanju određena je s pomoću analizatora veličine. Svaka točka predstavlja srednju vrijednost između triju mjerenja.

Particles were added to the analyser until a refraction of 0.85 was attained. Three measurements were made for each particle type. Solutions of each corn and potato starch were made using distilled water as the diluent at concentrations of 3.5–mg starch/g body weight. Test solutions were rectally intubated (200 µl volume) into anaesthetised (0.04 g/l benzocain, Sandoz, Switzerland) trout using a 5 cm long heat-smoothed plastic catheter connected to a 25G x 16 mm luer needle (Terumo Europe N. V., Belgium) attached to a 1 ml tuberculin syringe (Becton Dickinson S. A., Spain) over a 10 s period. Since preliminary time-course (1–22 h) studies (n = 18) indicated that all stages of the persorptive process could be visualised 18 h post-intubation, experimental fish were sacrificed, using an overdose of anaesthetic, only at this time point. Following removal, the intestine was flushed 4 times with demineralised water to remove contents and subsequently dissected into 3–4 mm sections for the mid, and hing-gut regions, based upon gross morphological differences between segments.

#### *Tissue processing and histological evaluation*

Intestinal samples were fixed in 4% formaldehyde (Bie & Berntsen, Denmark), dehydrated in ethanol (Danisco Distillers A/S, Denmark), and embedded in plastic using standard procedures (Heraeus Kulzer, Germany). Resultant blocks were then frozen at –20 °C prior to sectioning (3 µm thickness; 2.4 ° cutting angle) on an Shandon AS325 microtome. Section were placed in demineralised water to aid unfolding prior to transfer to coated superfrost slides (Menzel Gläser, Germany). Preparations were subsequently stained either with eosin (yeast) or H&E (cornstarch) using standard methods (Heraeus Kulzer), and coverslips mounted using DePeX medium (Merck Denmark A/S) and allowed to dry overnight. Stained sections were examined using an Olympus BH2 microscope connected to a Sanyo colour CCD hi-resolution video camera. Sections of interest were taken by frame grabber (Matrox Meteor PCI grabber, Matrox, USA), and saved in non-compressed file format (tif). Resultant images were processed using TEMA software (ver. 1.0; ScanBeam, Denmark) precalibrated for particle size measurement for each microscope lens employed. Adobe PhotoShop (ver. 3.0, Adobe Systems Inc., USA) was used to modify contrast and brightness and to crop saved images.

## **RESULTS**

Various stages of the paracellular transit of cornstarch particles from gut lumen to lamina propria, 18-h post-rectal intubation of juvenile rainbow trout, are depicted in Fig. 2. That the particles visualised illustrated true persorption of starch was determined by various procedures. First, the persorbed particles fell within the predetermined size range as characterised during particle size analysis (Fig. 1). Second, the visual definition of the particles within histologi-

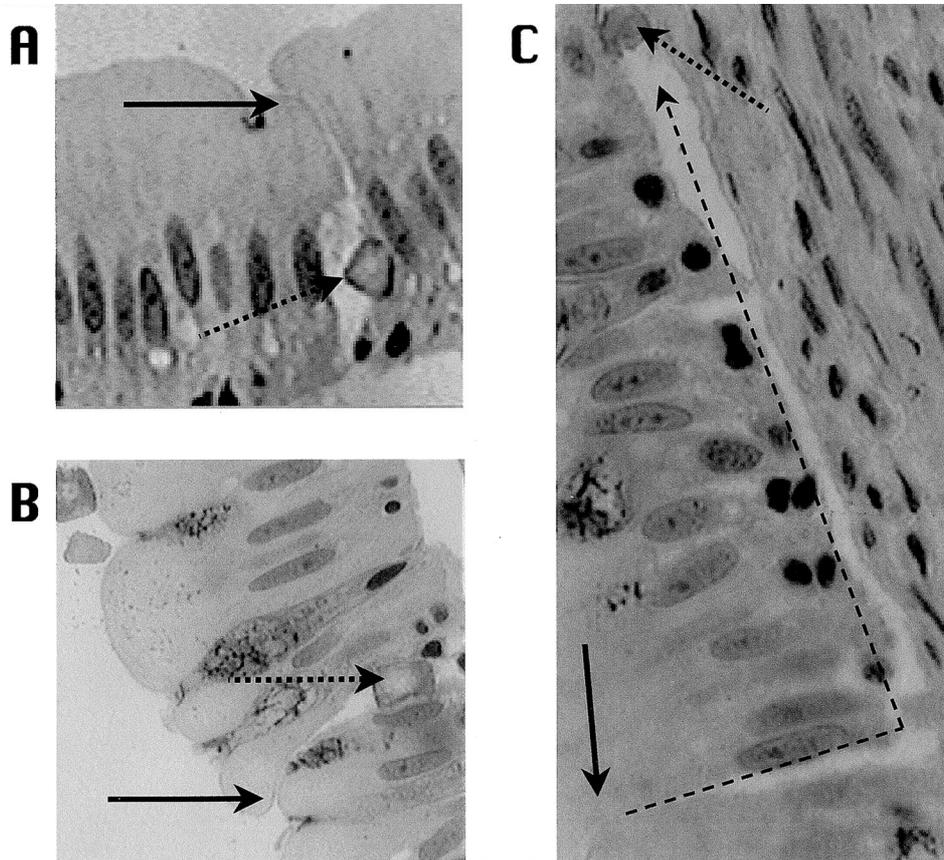


Fig. 2 A–C. Photomicrographs of cornstarch stationed between absorptive enterocytes of the anterior hind gut of rainbow trout 18 hours post-luminal introduction. The starch granules, indicated by dotted arrows were approximately  $13 \times 13 \mu\text{m}$  (Fig. 2 A, B). Solid arrows mark the point of the starch granules. Fig. 2 C illustrates the migration route of a persorbed cornstarch granule (two-dimensional dashed arrow). Granule dimensions were  $7 \times 7 \mu\text{m}$ . The presumed entry point of the granule is marked by the solid arrow. The epithelial layer of the gut was apparently separated from the lamina propria during the passage of cornstarch from the gut lumen. H&Ex400.

Slika 2 A–C. Fotomikrografije škroba kukuruza smještena između apsorptivnih enterocita prednjeg i stražnjeg dijela crijeva kalifornijskih pastrva 18 sati nakon postlumenalne introdukcije. Granule škroba, označene točkastim strelicama, bile su približno  $13 \times 13 \mu\text{m}$  (sl. 2 A, B). Pune strelice označuju točke ulaska granula škroba. Slika 2 C ilustrira put kretanja persorbirane granule kukuruznog škroba (dvodimenzionalna crtkana strelica) Granule su bile dimenzija  $7 \times 7 \mu\text{m}$ . Točka ulaska granula označena je punom strelicom. Sloj crijevnog epitela bio je zapaženo odvojeno od lamine proprije za vrijeme prolaska škroba kukuruza iz lumena crijeva. H&Ex400.

cal sections was greatly enhanced by their pre-treatment with iodine. In addition, sections illustrating persorption were examined and compared visually, and by image analysis, side-by-side with prepared slides of stained cornstarch alone.

Fig. 2 A–C illustrates the passage of iodine-stained cornstarch granules between absorptive enterocytes of an intestinal fold derived from an anterior segment of the rainbow trout hindgut, 18-h post-rectal intubation. Granule sizes were approximately 13x13  $\mu\text{m}$ . The particles were associated with small entry tracks roughly 32  $\mu\text{m}$  in length that led directly from the intestinal lumen to intercellular spaces. Fig. 2 C illustrates the migration route of a cornstarch particle, approximately 7  $\mu\text{m}$  in diameter, from intestinal lumen along the basal lamina of the absorptive epithelia. The photomicrograph demonstrates the two-dimensional character of the persorptive process. Concomitantly, due to the two-plane movement of the particle, the figure also argues against processing artefact, such as the possibilities of tissue tearing and particle movement during the slicing process.

## DISCUSSION

The present study examined whether microparticulate material breached the intestinal epithelia of the fish gut and subsequently gained access to the *lamina propria*. Two different microparticles were selected, *viz.* corn (grain) and potato (tuber) starch, such that the influence of particle size and shape upon the persorptive process could be examined. The starches used exhibited typical granular morphology, with shape varying with granule size — a distinguishing feature of starch derived from botanical sources (Frazier *et al.* 1997). All potato granules, and cornstarch granules > 20  $\mu\text{m}$ , generally exhibited a disk shape, whereas smaller cornstarch (< 20  $\mu\text{m}$ ), was more heterogeneous in form. In accordance with the reports of others (Swinkels 1985), smaller diameter cornstarch was characteristically polygonal in shape and expressed discrete angling.

Following rectal delivery of trout with the test materials, cereal starch was observed to penetrate the absorptive epithelia via a paracellular route, typifying various stages of the persorptive process (Fig. 2 A–C), 18 hours post-intubation. Persorbed cornstarch granules exhibited a diameter of between 8–14  $\mu\text{m}$ , which was below the preparations measured average (21  $\mu\text{m}$ ; Fig. 1). The present findings, therefore, suggest that the persorptive process in fish may be size restricted. In humans, persorption of particles of slightly greater diameter (15–20  $\mu\text{m}$ ) than observed in the present study, has been reported (Volkheimer 1972). A major difference between the present study and others with mammals relates to the time span of persorptive process. In humans, for example, microparticles may be detected within minutes following gavage (Volkheimer 1993). If persorption relies upon an abrasive action

for initiation of the process, the differences in the intrinsic rhythmic contraction of the small bowel in mammals (5–12 times per minute; Kalsner 1976), and intestine of teleosts (1–2 times per minute, Atlantic cod *Gadus morhua*; Karila & Holmgren 1995, *in vitro* observations; <1 time per minute, rainbow trout; E. McLean unpublished *in situ* observations), may provide a partial explanation for the observed contrariety. The failure to detect persorption of potato starch may have arisen due to the overall larger size range of the preparation employed ( $\approx$  diameter of 43  $\mu\text{m}$ ), or correlate with granule shape *viz.* uniform smoothness and lack of discrete angles that, in the case of cornstarch, was readily apparent. A conspicuous feature during the present study was the passage of starch granules between goblet cells and absorptive enterocytes. It has been suggested that the integrity of the junctional complex between these cell types may not be totally concluded (Baintner 1986); and that the gut may exhibit differences in tight junction patency, which may be modified, for example, during fasting.

Given that persorption appears to be a general feature of the vertebrate gut, one question that arises relates to whether the mechanism can be applied to regulate production-related processes in animals or as a vehicle for the oral delivery of medicaments in the clinical setting. Research with mammals indicates that persorption is dose-dependent (Volkheimer 1972) and several authors have suggested that the phenomenon may provide utility in terms of delivering bioactive materials (*e. g.*, O'Hagan *et al.* 1995; O'Hagan 1996; Seifert 1998). While to date no other study has examined the persorption approach for delivering bioactive materials to fish, Tsai *et al.* (1994), reported that striped mullet *Mugil cephalus*, fed with yeast recombinant for a fish growth hormone, yielded significant growth acceleration over a 6 week trial period when compared to control groups. Although Tsai *et al.*'s study did not examine the route of uptake of the yeast, the persorptive pathway, as described herein, may have been the preferential route of absorption, since yeast particles fall into the size range of 6–8  $\mu\text{m}$  and exhibit angular form.

Clearly, the potential exists to employ persorption for the delivery of micro-, or bio-encapsulated vaccines, growth promotants and similar materials to fish or potentially as a means of providing supplemental nutrition for larval animals. It is of note that cornstarch is employed in the production of aquafeeds and specifically with those incorporating pigments such as astaxanthin. It is also recommended as a binding agent at levels of 10% dry weight of diet (El-Sayed, 1998). High levels of granular dietary starch could thus be considered as potentially hazardous to cultured teleosts since the patency of the (gastro)intestinal tract could be continually challenged; leading to increased opportunities for bacterial invasion. It is noteworthy that the persorptive pathway may influence results from digestibility studies that use microparticulate markers. Future studies upon this process should examine the impact of a variety of biotic and abiotic factors upon the reproducibility of the mechanism.

## Sažetak

### PROBAVNI SUSTAV KOŠTUNJAČA PERSORBIRA MIKROČESTICE

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Mogućnost probavnog sustava koštunjavičastih vrsta riba da apsorbiraju sitne čestice pojedinih tvari bila je istraživana s pomoću rektalne intubacije (3, 5 g kg<sup>-1</sup>) komercijalnoga škroba kukuruza (promjera  $\cong$  21  $\mu$ m) i škroba krumpira (promjera  $\cong$  43  $\mu$ m). Uzorci tkiva uzeti su iz srednjeg i stražnjeg dijela crijeva kontrolne i tretirane skupine riba, i to 18 sati nakon intubacije. Sakupljeni su uzorci spremljeni u standardne plastike i obojeni prema protokolu, te fotomikrografski snimljeni na računalu. Čestice škroba kukuruza (8–14  $\mu$ m) zapažene su da iz lumena crijeva prolaze u laminu propriju samo paracelularnim ili persorptivnim putem. No, nije zapažen prolazak čestica škroba krumpira.

*Ključne riječi: bioenkapsulacija, probavni sustav, Herbstov efekt, mikročestice, parastanični ulaz, persorpcija*

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