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Original Contributions

Effect of diazinon on freeze-fracture images of microvilli of intestinal epithelial cells of *Tilapia nilotica*

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Summary: The effect of the organophosphate insecticide, diazinon on the intramembranous particles (IMPs) of the microvilli of the intestinal epithelial cells of *Tilapia nilotica* fish was studied using freeze-fracture technique. Exposing fish to different repeated concentrations of diazinon ($\frac{1}{2}LC_{50}$) caused a significant decrease in population density of IMPs in P- and E-faces. IMPs of microvilli found in intestinal epithelial cells are thought to represent many kinds of proteins including enzymes. In the present work, it is suggested that diazinon induced a reduction in enzymatic content of the membrane which was accompanied by a decrease in IMPs density of the microvilli.

Zusammenfassung: Der Einfluß des organischen Phosphatinsektizids Diazinon auf die membranständigen Partikel (IMP) der Mikrovilli in intestinalen Epithelzellen von *Tilapia nilotica* wurde unter Anwendung der Gefrierätztechnik untersucht. Fische, die wiederholt unterschiedlichen Konzentrationen von Diazinon ausgesetzt wurden, zeigten eine signifikante Abnahme der Populationsdichte der IMPs auf P- und F-Flächen. Man nimmt an, daß die IMPs von Mikrovilli in intestinalen Epithelzellen viele Arten von Proteinen einschließlich Enzymen darstellen. In der vorliegenden Arbeit wird gefolgert, daß Diazinon eine Reduzierung des Enzymgehalts der Membran verursacht, die mit einer Abnahme der IMP-Dichte von Mikrovilli einhergeht.

Key words: Intestine; microvilli; intramembranous particles; insecticide; diazinon; fish

Schlüsselwörter: Eingeweide; Mikrovilli; membranständige Partikel; Insektenvertilgungsmittel; Diazinon; Fische

Introduction

Freeze-fracture electron microscopy provides a high-resolution visualization of the internal structure of biological membrane (3). The technique is thought to cleave membranes along their hydrophobic internal planes, thereby revealing intramembranous particles (IMPs) which are thought to represent integral protein constituents, on two fractured membrane faces (25, 24, 26, 1).

Interest in the environmental impact of pesticides had lead to numerous studies concerning the toxicological (2), biochemical (11) and histopathological (22, 16, 5) effects of exposure of fish to such pesticides. The

ultrastructural alterations of intestinal epithelial cells in a fresh water fish, *Tilapia nilotica*, exposed to diazinon and neopybuthrin have been described using transmission electron microscopy (7). A detailed study of the plasma membrane of epithelial cells or the intercellular contacts between adjacent cells has not, thus far, been carried out utilizing freeze-fracture replication techniques. In the present work, we describe and quantitate the distribution of microvillus intramembranous particles (IMPs) in the intestinal cells of the fish *Tilapia nilotica* exposed to the organophosphate insecticide, diazinon.

Materials and methods

Experimental animals: Living specimens of *Tilapia nilotica* were collected from the High Dam Lake, each weighing 200–300 gm. Fishes were kept in specially equipped aquaria which were continuously aerated by air pumps. The water temperature was 25 ± 2 °C and its pH was 7.2 ± 0.1; the oxygen level was 6–7 mg/l. Fish were provided twice daily with earth worms as food; they were acclimatized for 3 days in the aquaria under the above conditions before the experiment.

Chemical insecticide used: Diazinon, an organophosphate insecticide, was used in the present work. The 96-h LC_{50} level of diazinon was found to be 20 mg/l, as obtained from the lethal curve constructed for this purpose in a previous investigation performed by El-Elaimy et al. (7).

Fish groups and exposure: Four groups of five fish each were used. These groups were exposed to one treatment of $\frac{1}{2}LC_{50}$ of diazinon, two treatments of $\frac{1}{2}LC_{50}$, three treatments of $\frac{1}{2}LC_{50}$, and four treatments of $\frac{1}{2}LC_{50}$, respectively. The exposure time for each treatment was 24 h. The recovery period between repeated exposures was also 24 h. Another group of five fish were used as control.

Freezing and fracturing: The intestines of control fish, as well as of exposed fish were removed and small pieces were immediately fixed with 2.5 % glutaraldehyde solution for 2 h. After equilibrating with 30 % glycerol buffered with 0.1 M cacodylate, tissue pieces (150 μm) were rapidly frozen in liquid freon 22 and transferred into liquid nitrogen. Subsequently, materials were fractured and replicated with platinum and carbon at −110 °C in an Eiko FD-2A freeze-fracture device. Replicas were cleaned in sodium hypochlorite, rinsed in distilled water, mounted on 400 mesh copper grids and examined in a Philips EM 200 electron microscope.

Quantitative analysis

For quantitation of microvillus IMPs, replicas of well-oriented microvilli were photographed and printed at a magnification of 100 000.

For each treatment, eight to 20 electron micrographs were analyzed and particle density was measured by counting the number of particles within a template which outlined a 100 by 50 nm area at the mid point of well-oriented microvilli (1).

The following morphometric parameters were determined: the number of IMP per μm^2 on P- and E-faces, the total number of IMP per μm^2 (P + E) and the partition coefficient (Kp) obtained by dividing the number of IMP per μm^2 on the E-face. Statistical comparisons of IMP density between control and experimental groups were made using the two-tailed Student's *t*-test.

Results

The epithelium of the intestine of *T. nilotica* consists of numerous columnar cells and a few, interspersed goblet cells. The columnar cells are characterized in thin sections by well-developed cell organelles. The lumi-

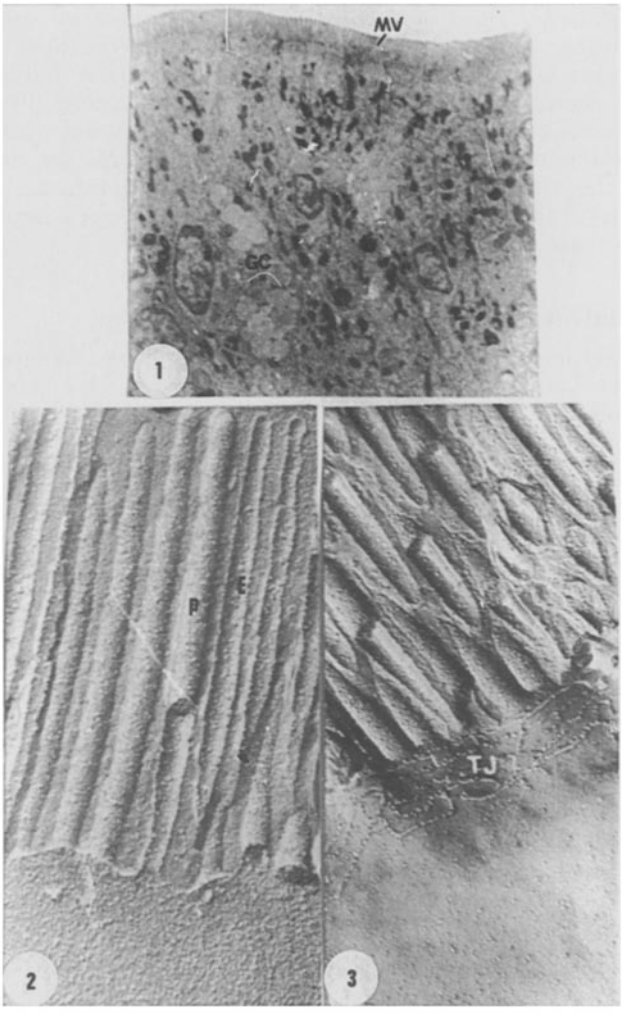
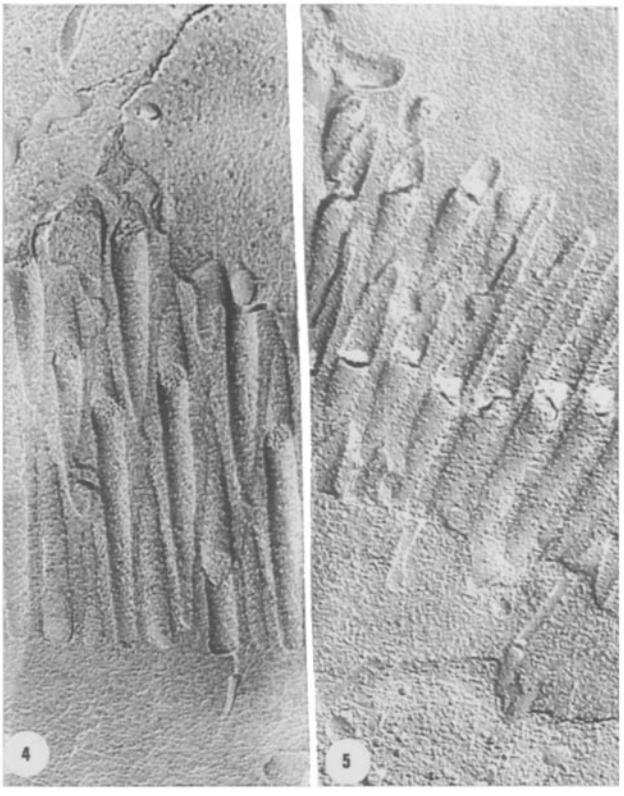


Fig. 1. Electron micrograph of normal intestine of *T. nilotica* showing goblet cells (GC) interspread between the columnar cells. Note numerous long microvilli (MV); × 9000.

Fig. 2. Freeze-fracture image of microvilli of columnar cell of control fish intestine. Note the numerous membrane particles on the P-face (P) compared to the E-face (E); × 60 000.

Fig. 3. Freeze-fracture replica of a columnar cell of control fish intestine. At the base of the microvillus border, the tight junction (TJ) appears as a meshwork of fibrils; × 60 000.

nal surfaces of these cells are densely covered with slender and closely packed microvilli (Fig. 1). The microvilli of freeze-etched columnar cells of the intestine are most frequently cleaved in such a way as to reveal



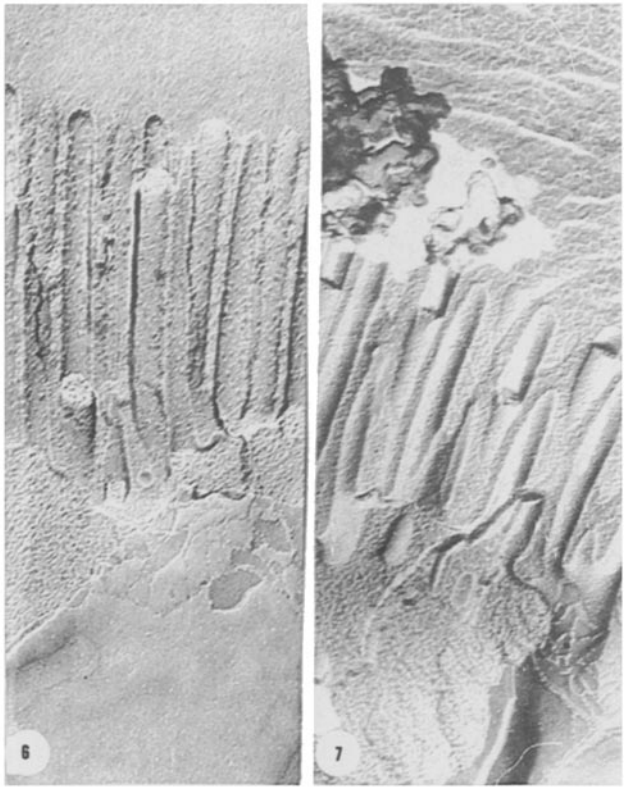
Figs. 4–7. Freeze-fracture replicas of microvilli of columnar cells of intestine of fish exposed to different repeated concentrations of diazinon ($\frac{1}{2}LC_{50}$) showing a decrease in IMPs density; × 50 000.

surfaces of their limiting plasma membrane. When individual microvilli are obliquely cleaved, two different surface views of their surrounding membrane are seen. The convex surfaces (P) facing towards the external space are characterized by a ridge of etch-resistant material at their bases. The concave surfaces (E) facing towards the microvillous core can be seen to be continuous with and identical to the inner surface of the ridge

Table 1. Population density of membrane particles in microvillus per μm^2 of columnar cells, in intestine of fish exposed to the insecticide diazinon.

Group	P-face	E-face	(P + E)	Kp(P/E)
Control	2927 ± 54	1859 ± 263	4786	1.58
Diazinon: $1 \times \frac{1}{2} LC_{50}$	1533 ± 155	1270 ± 186	2803	1.26
$2 \times \frac{1}{2} LC_{50}$	1518 ± 91	1289 ± 153	2807	1.21
$3 \times \frac{1}{2} LC_{50}$	2036 ± 191	1419 ± 93	3455	1.44
$4 \times \frac{1}{2} LC_{50}$	1522 ± 195	1210 ± 92	2732	1.26

Data are expressed as means ± standard deviation of five animals.
Kp is the partition coefficient.



material facing the P-surfaces. The intramembranous particles (IMPs) were randomly distributed on the P- and E-faces. The P-face contains more particles than E-face (Fig. 2). Tight junctions between cells consist of a meshwork of ridges. This meshwork (4–6 strands wide) is located immediately between the microvillus border in the tight junction region with basal loops and free ends which extend below the base of the meshwork. These extensions (0.2 μm long) were partly continuous and partly composed of incomplete particle chains (Fig. 3). The plasma membrane of the goblet cells tended to fracture in a rather patchy fusion and their tight junctions were identical with those of the columnar cells, as might be expected because their tight junctions are structures shared with neighboring cells.

Concerning the effect of diazinon, Table 1 shows the density of IMPs in the P- and E-faces, the corresponding total amount of IMPs and the partition coefficient in normal and experimental fishes. The results showed that the IMPs density on the P-face was significantly lower ($p < 0.001$) in treated fish as compared with controls (Figs. 4–7). The change in IMPs density on the E-face in fish exposed to diazinon showed a similar pattern to that in P-face. The Kp values are different in control and experimental groups.

Discussion

Freeze-fracturing has become a common and powerful technique for examining membrane architecture, since it makes the interior of the membrane accessible to direct observation. This technique is thought to cleave membranes along their hydrophobic internal plane, the plane of the weakest bonding at the center of lipid bilayer, and thereby revealing intramembranous particles (IMPs) which are thought to represent integral protein constituents on the two fractured membrane faces (1, 3, 26). The appearance of IMPs in cell membranes has been described in various animals, in chickens (27), in small mammals (1, 14), and has been analyzed quantitatively in the adult human and monkey small intestines (15). These reports consistently revealed a population of randomly distributed intramembranous particles, a majority of which are associated with the P-face. Our results in *T. nilotica* also showed that the P-face contains more particles than the E-face.

In addition, the present freeze-fracture study revealed that the population density of IMPs in microvillus membrane is significantly lowered in fishes exposed to diazinon. It is now widely accepted that most of IMPs in biological membranes represent integral protein constituents (24, 25, 26); it is easily understood that the decrease in the number of particles means a reduction of the protein molecules in the microvillus membrane.

In the small intestinal epithelium, it has been found that various enzymes involved in the later stages of digestion exist in the epithelial cells and that their activities are predominantly localized in the membrane of the microvillus (18). Miller and Crane (17) first isolated the brush border from intestinal absorptive cells of rats and demonstrated sucrase and maltase activity. Many studies have confirmed that disaccharidase such as sucrase, maltase, isomaltase and lactase, and alkaline phosphatase are localized in the membrane of microvilli (4, 10, 6, 8). In addition, proteolytic enzymes such as amino peptidase, dipeptidase and tripeptidase have been shown to present in this membrane (19, 9), although dipeptidase and tripeptidase activity are found to a large extent in the cytoplasm (20).

The correlation between membrane enzyme activity and IMP density in microvilli was noticed by Madara et al. (15). They found that the increase in activity of membrane enzymes during absorptive cell migration from the crypt to the villus was accompanied by an increase in IMPs on the P-face of the microvillus. In addition, there was a close correlation in celiac sprue between the decrease of the IMP density on the P-face and enzyme activity in the membrane of the microvillus (13). The concomitant changes in membrane enzyme activity and IMP density on the P-face of the microvillus implies that at least some of the IMPs represent protein molecules of the enzymes.

Biochemical studies in fish subjected to insecticide toxication, including diazinon, show changes in the enzymatic content and also metabolic disturbance which are parallel to the morphological changes (28, 11, 12, 21, 23). In the present work, it is considered that the lowered population density of the membrane particles in the microvillus membrane after treatment with diazinon may have resulted from the reduction in enzyma-

tic content in the microvilli of the epithelial cells, which was accompanied by a decrease in its membrane particles.

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