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The toxic action of orellanine and other dipyridyles on different epithelial cell cultures (LLC-PK_1, CaCo-2 and OK)

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Abstract: Orellanine, the toxic compound of *Cortinarius orellanus* and *orellanoides* was isolated from the latter for this study. In experiments with natural and synthetic orellanine and 2,2'-dipyridyl and 4,4'-dipyridyl monolayers of two renal epithelial cell lines (LLC-PK_1 and OK) and a colon carcinom cell line (CaCo-2) were damaged already after 24 hours and more pronounced after 48 hours by all four toxins at concentrations of 10^{-3} M/l. While two cell lines show strong vacuolisation, the OK line (without production of alkaline phosphatase) does not. Orellanine affects particularly the alkaline phosphatase activity.

Zusammenfassung: Orellanin, das Hauptgift von *Cortinarius orellanus* und *orellanoides* wurde aus letzterem isoliert. In Experimenten mit natürlichem und synthetischem Orellanin und 2,2'-Dipyridyl und 4,4'-Dipyridyl wurden Monolayer Zellkulturen von zwei renalen Epithelzellinien (LLC-PK_1 und OK) und einer Colon Karzinom Zellinie (CaCo-2) bereits nach 24 Stunden, viel deutlicher nach 48 Stunden geschädigt und zwar durch alle vier Toxine bei einer Konzentration von 10^{-3} M/l. Während zwei Zellinien eine starke Vakuolisierung zeigen, ist dies bei der OK Linie, die keine alkalische Phosphatase besitzt, nicht der Fall. Orellanin beeinflußt u. a. spezifisch die Aktivität der alkalischen Phosphatase.

INTRODUCTION

The 3,3',4,4-'tetrahydroxy-2,2'-dipyridyl-1,1'-oxid structure of the toxic compound of *Cortinarius orellanus* Fr. and *C. orellanoides* Hry. was confirmed by several authors (Andary & al. 1986, Antkowiak & Gessner 1975, Prast & al. 1988) and the synthesis by Dehmlow and Schulz (1985) and Tiecco et al. (1986) proved the correctness of the structure formula of Antkowiak and Gessner (1979). For toxicological experiments cell cultures represent a suitable model. The mode of action of the toxins can be controlled and studied under reproducible and constant conditions. For our purpose three established epithelial cell lines were used: LLC-PK_1, CaCo-2 and OK. Their differentiated and polarized cells form monolayers with an active vectorial transport and after confluency typical domes. The LLC-PK_1 a renal epithelial cell line which retains morphological and enzymatic characteristics of the proximal tubule and the human colon carcinoma CaCo-2 cell line exhibit on their apical surface

high activities of brush border membrane enzymes, for instance alkaline phosphatase (Gstraunthaler & al 1985, Pinto & al. 1983). The OK, an established cell line from the kidney of American opossum *(Didelphus virginiana),* has no alkaline phosphatase activity (Koyama & al. 1978). Human alkaline phosphatases are a family of three isoenzymes, the placental, intestinal and liver/bone/kidney form which are coded by different gene loci. They differ in thermostability, in inhibitor sensitivity, in electrophoretic mobility and in immunological characteristics (Goldstein & al. 1980).

MATERIAL and METHODS

The toxic substance, orellanine, was isolated of *C. orellanoides* R. Hry. (= *C. speciosissimus* Kühn . & Romagnesi), dried with a Dörrex dryer (between 40 - 60 °C). *C. orellanoides* was collected 1979 in Femsjö (Sweden). The synthetic orellanine was kindly provided by Dehmlow and Schulz. The 2,2'-dipyridyl and 4,4'-dipyridyl were from Sigma Chemical Co. Pure alkaline phosphatases (lyophilized powders): Sigma P4653: Type XXXII from bovine kidney, Sigma P3772: Type VII-L from bovine intestinal mucosa and Sigma, P3765: Type XV from bovine placenta.

Cell cultures: LLC-PK_1, OK and CaCo-2 cells were grown as monolayers in Dulbecco's MEM (Milieu Essential Minimum de Eagle modified by Dulbecco, Sigma) supplemented with 10% fetal calf serum (Biological Industries), 100 U/ml penicillin, 100 µg/ml streptomycin (Eurobio Laboratories) and 3.7 g/l $NaHCO_3$ (Sigma). The CaCo-2 cells require the same medium supplemented with 3.5 g/l glucose. The Petri dishes were incubated in a humidified 5% CO_2, 95% air mixture at 36.6° C. The cells were fed with fresh medium 2-3 times per week.

The extraction procedure followed with slight modification Andary & al. (1986). The toxin was extracted from 40 g dried and powdered fungus, *C. orellanoides* R. Hry., with 700 ml 20% methanol for 48 hours at room temperature. After filtration of the fragments, centrifugation of the solution, and evaporation in vacuo to 100 ml the lipid compounds were removed with hexan and petroleum ether. The orellanine in the solution was precipitated twice after standing 48 hours at 4° C. The cristalls formed were filtred, resuspended in aqua bidest. and treated with ammonium (10%, pH 9). After filtration and removal of impurities the pH was adjusted to 5 with 3.5 M acetic acid. The orellanine was again precipitated (48 h at 4° C). The purity of the cristalls was examined by thin layer chromatography on cellulose plates and with n-butanol-HCl-acetic acid-H_2O (40-20-15-3.8) as solvent system.

Thin layer chromatography: The purified orellanine, the synthetic orellanine, 2,2'-dipyridyl and 4,4'-dipyridyl were checked by thin layer chromatography. Using n-butanol-HCl-acetic acid-H_2O (40-30-2-3.8) as solvent

system and cellulose layers (Merk 5716) it was possible to identify the fractions with UV-light (366 and 294 nm respectively). Different spray reagents were also tested and particularely Fe(III)sulfate, which indicates the 2,2'-dipyridyl skeleton, proved important (Tab. 1).

Tab. 1: Reaction of spray color reagents on thin layer cellulose plates. (1) orellanin, (2) synthetic orellanine, (3) 2,2'-dipyridyl and (4) 4,4'-dipyridyl.

	1	2	3	4
Ferric(III)sulfate	+	+	+	–
Ferric(III)chloride	+	+	–	–
Potassium permanganate	+	+	–	–
Folin reagent	–	–	–	–
Ninhydrin	–	–	–	–

The **effect of the toxins** on the morphology of LLC-PK_1, CaCo-2 and OK cells. Confluent monolayers (6 - 8 days old) were exposed to 10^{-3}, 10^{-4} and 10^{-5} M/l orellanine, 2,2' dipyridyl, and 4,4' dipyridyl, which were added to the culture medium. The synthetic orellanine was tested only on LLC-PK_1 cells. The cells were washed twice with saline solution (PBS without Ca^{++} and Mg^{++} -Eurobio Laboratories) after 48 hours incubation, examined by light microscopic observation and photographed. After 24 hours only slight changes were observed.

The **effect of orellanine** on alkaline phosphatase and γ-glutamyltransferase. Confluent monolayers of LLC-PK_1 and CaCo-2 were exposed to 10^{-3}, 10^{-4} and 10^{-5} M/l orellanine for 24 and 48 hours. After washing with PBS and centrifugation at 1000 rpm for 5 minutes a crude cell homogenate was obtained with aqua bidest. The alkaline phosphatase activity was determined according to Bergmeyer (1974) at pH 9.8 with p-nitrophenylphosphate as substrate. γ-glutamyltransferase was measured according to Glossman and Neville (1972) at pH 8.2 using γ-glutamyl-p-nitroanilide as substrate. Proteins were assayed by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Inhibition of the activity of the three alkaline phosphatase isoenzymes by orellanine in vitro. The alkaline phosphatase activity of LLC-PK_1 and CaCo-2 cells, the kidney and intestinal forms respectively, and the activity of three pure isoenzymes (kidney, intestinal and placental form) was investigated. To study the enzyme inhibition, orellanine was added to diethanolamine buffer to adjust the final concentration in the substrate to

10^{-3} M/l. The influence of synthetic orellanine, 2,2'-dipyridyl and 4,4'-dipyridyl was tested only on LLC-PK_1 cell homogenate. The enzyme activity was measured photometrically by the release of p-nitrophenol from p-nitrophenyl-phosphate at pH 9.8 and 25° C over a period of 10 minutes.

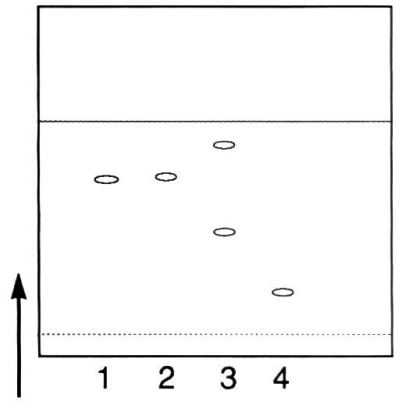

Fig. 1: Chromatographic analysis of orellanine, Rf-value 0,69 (1), synthetic orellanine Rf-value 0,69 (2), 2,2-dipyridyl, Rf-values 0,48 and 0,77 (3) and 4,4'-dipyridyl, Rf-value 0,29 (4) on cellulose thin layer plates and n-butanol-HCl-acetic acid-H_2O as solvent system.

RESULTS

The Rf-values of the extracted orellanine and synthetic orellanine are identical (Rf 0,64) and both fractions show after UV-irradiation the typical delayed bright turquoiseblue fluorescence. The other Rf-values are given in Fig. 1. When treated with Fe(III)sulfate solution both react positively. Also 2,2'-dipyridyl shows a positive colour reaction, but not the 4,4'-dipyridyl fraction (Tab. 1). All three cell lines are damaged already after 24 hours, but the changes were more evident after 48 hours with all four toxins at a final concentration of 10^{-3} M/l. The confluent monolayers of LLC-PK_1 and CaCo-2 are disrupted, many cells float in the culture medium and no domes can be observed. The cells appear strongly damaged and show numerous vacuoules (Fig. 7, 8). Also OK, the cell line without alkaline phosphatase activity, is affected by the toxin, however no vacuoles are visible (Fig. 9). The 10^{-4} M/l concentration has a similar effect as the 10^{-3} M/l. In the confluent monolayers incubated with 10^{-5} M/l no damage was recognizable after 48 hours. Orellanine affects strongly the alkaline phosphatase activity of LLC-PK_1 and CaCo-2 cells (Fig. 2, 3). To the contrary, the activity of γ-glutamyltransferase of the LLC-PK_1 ist not significantly reduced by any of the concentrations of orellanine used (Fig. 4).

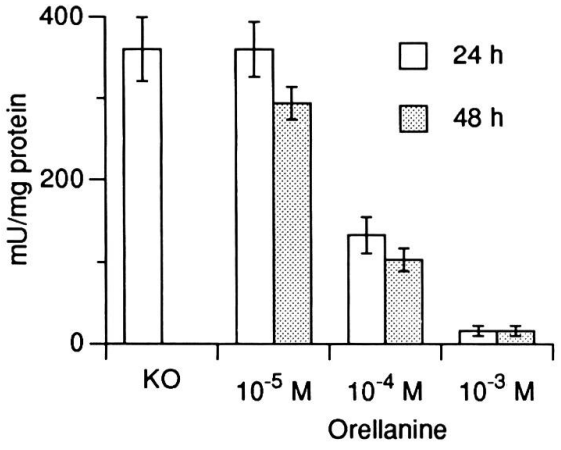

Fig. 2: Effect of orellanine on alkaline phosphatase activity of LLC-PK_1 cells after 24 and 48 hours incubation. Enzyme activities are expressed as nanomoles of substrates converted per minute per milligramm protein at 25°C. Values are means + SD of three separate tests for each of the five samples.

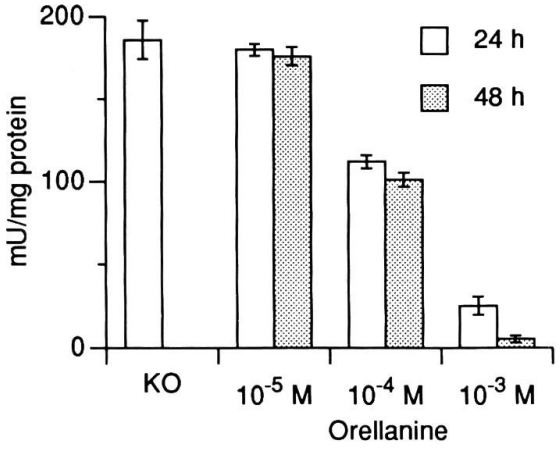

Fig. 3: Effect of orellanine on alkaline phosphatase activity of CaCo-2 cells after 24 and 48 hours incubation. Results are expressed as in Fig. 2. The values are means + SD of two separate tests for each of the five samples.

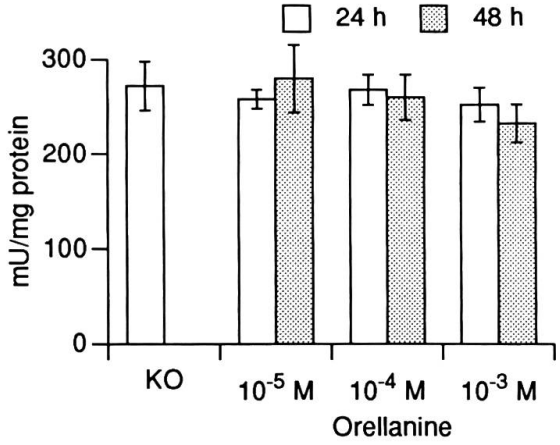

Fig. 4: Effect of orellanine on γ-glutamyltransferase activity of LLC-PK_1 cells after 24 and 48 hours incubation. Results are expressed as in Fig. 2. The values are means + SD of three separate tests for each of the five samples.

The synthetic orellanine provokes the same decrease in alkaline phosphatase activity of the LLC-PK_1 homogenate, while 2,2'-dipyridyl and 4,4'-dipyridyl have no effect on this enzyme activity (Fig. 5). The alkaline phosphatase activity of LLC-PK_1 and CaCo-2 cells and the activity of the three pure isoenzymes are inhibited in vitro by 10^{-3} M/l orellanine (Fig. 6).

DISCUSSION

Several *Cortinarius* species exhibit nephrotoxic properties with latency periods from two to more than 14 days. This contrasts strongly to *Amanita* poisonings (Flammer 1980). With the applied extraction method it was possible to obtain a pure toxin from *C. orellanoides* R. Hry., which shows the same chromatographic and toxic characteristics as the fraction extracted from *C. orellanus* Fr. (Kürnsteiner & Moser 1981).

Both the extracted and synthetic orellanine (von Dehmlow and Schulz 1985) have identical properties: the Rf-values, the positive tests with spray reagents, the fluorescence reactions under UV, the morphological effect on

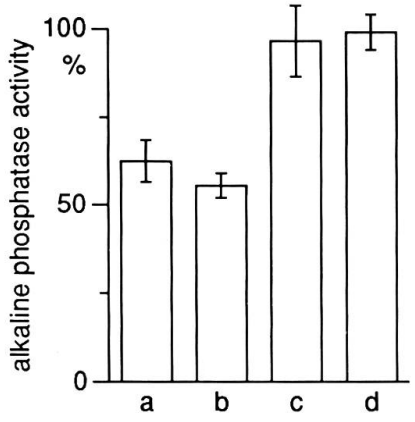

Fig. 5: Effect of orellanine (a), synthetic orellanine (b), 2,2'-dipyridyl (c) and 4,4'-dipyridyl (d) on the alkaline phosphatase activity of the crude homogenate of LLC-PK_1 cells in vitro. Values are expressed as percentage of untreated cultures.

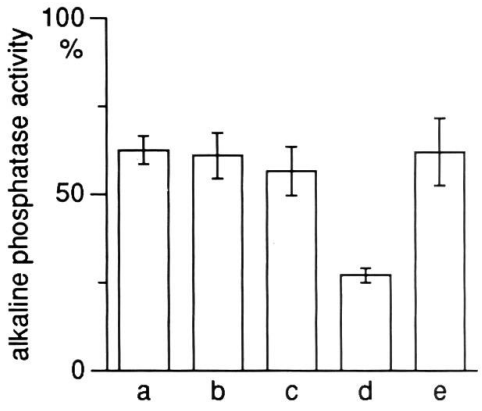

Fig. 6: Effect of orellanine on the three alkaline phosphatase isoenzymes in vitro: (a) crude cell homogenate of LLC-PK_1, (b) pure isoenzyme-kidney form, (c) crude cell homogenate of CaCo-2, (d) pure isoenzyme-intestinal form, (e) pure isoenzyme-placental form. Values are expressed as percentage of the control.

LLC-PK_1 cells and the inhibition of alkaline phosphatase activity in vitro. A cyclic polypeptid nature of the toxic component, which was described by Tebett and Caddy (1984) could not be corroborated. Gstraunthaler and Prast (1983) suggest, that 2,2'-dipyridyl and 4,4'-dipyridyl, compounds which resemble in structure to orellanine, have a similar mechanism of toxic action. The effect on the three cell lines is identical: the cells are strongly damaged and show numerous vacuoles. But with orellanine the symptoms are more pronounced. The cytotoxicity of the fungus toxin is 12 times higher than that of 2,2'-dipyridyl (Heufler et al. 1986). 10^{-3} M/l 2,2'-dipyridyl and 4,4'–dipyridyl inhibit only about 30% of the alkaline phosphatase activity of LLC-PK_1 cells after 24 hours incubation (Gstraunthaler & Prast 1983, Heufler & al. 1987), the same concentration of orellanine, however, more than 90%. From this strong decrease we may presume a direct and specific mode of action of orellanine on the alkaline phosphatase activity (Prast & Pfaller 1988). The experiments in vitro seem to confirm this assumption: orellanine only inhibits the alkaline phosphatase activity, but not the two dipyridyles. All three investigated isoenzymes were affected by orellanine and enzyme kinetics measurements demonstrated two different inhibition patterns. The inhibition of the kidney form appears to be noncompetitive and the other two isoenzymes, the placental and intestinal form, were inhibited competitively (Ruedl & al. 1989). More recent unpublished data suggest that this may also be influenced by the concentration of the toxin. Orellanine is, like gentamycin

(Takahashi & al 1987) levamisole (Jahan & Butterworth 1986) and L-bromotetramisole (Brunette & Dennis 1981) a specific inhibitor of alkaline phosphatase activity.

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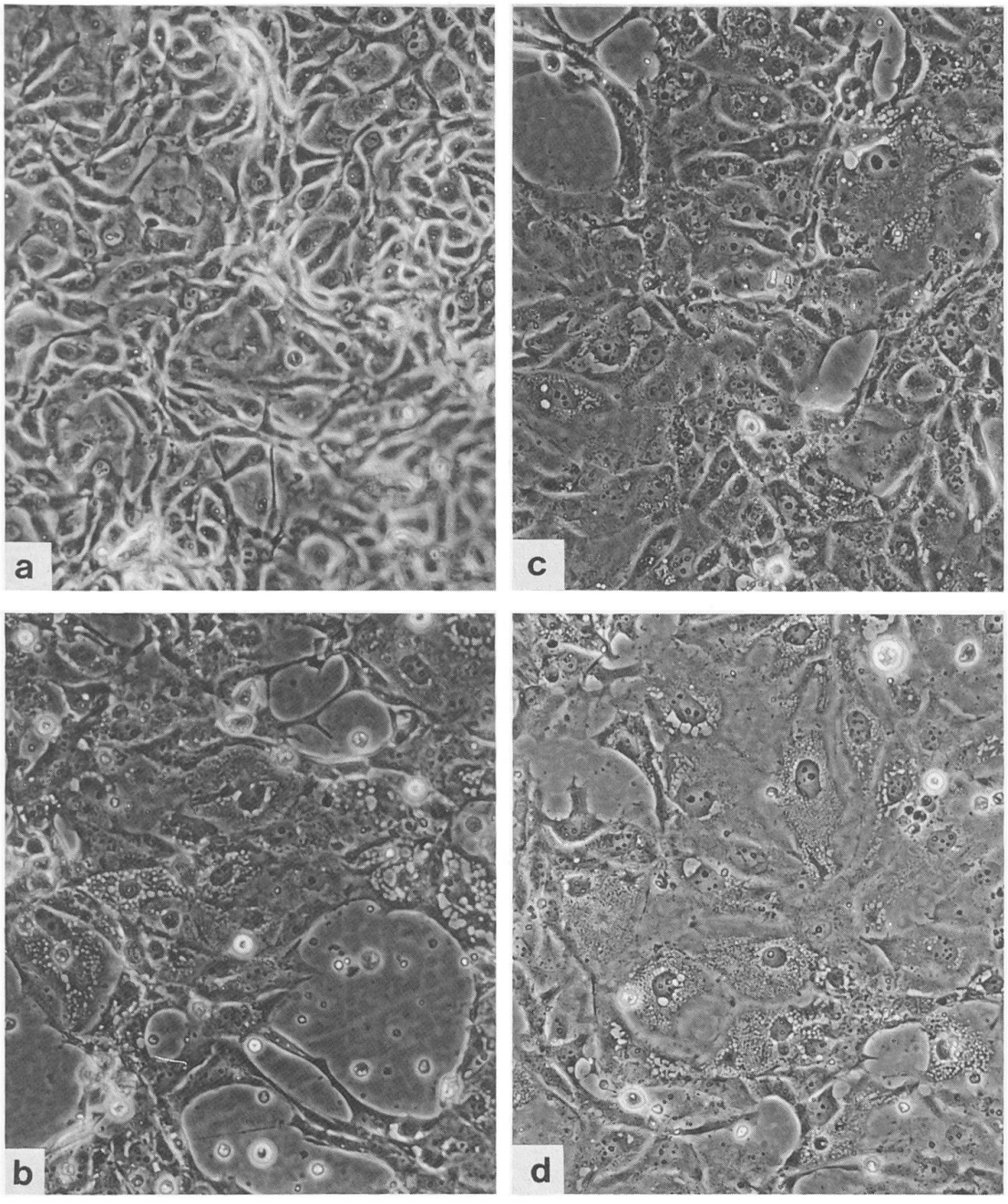

Fig. 7: LLC-PK_1 cells 48 hours after intoxication (phase contrast microscopy).
a: control, b: orellanine, c: synthetic orellanine, d: 2,2'-dipyridyl.

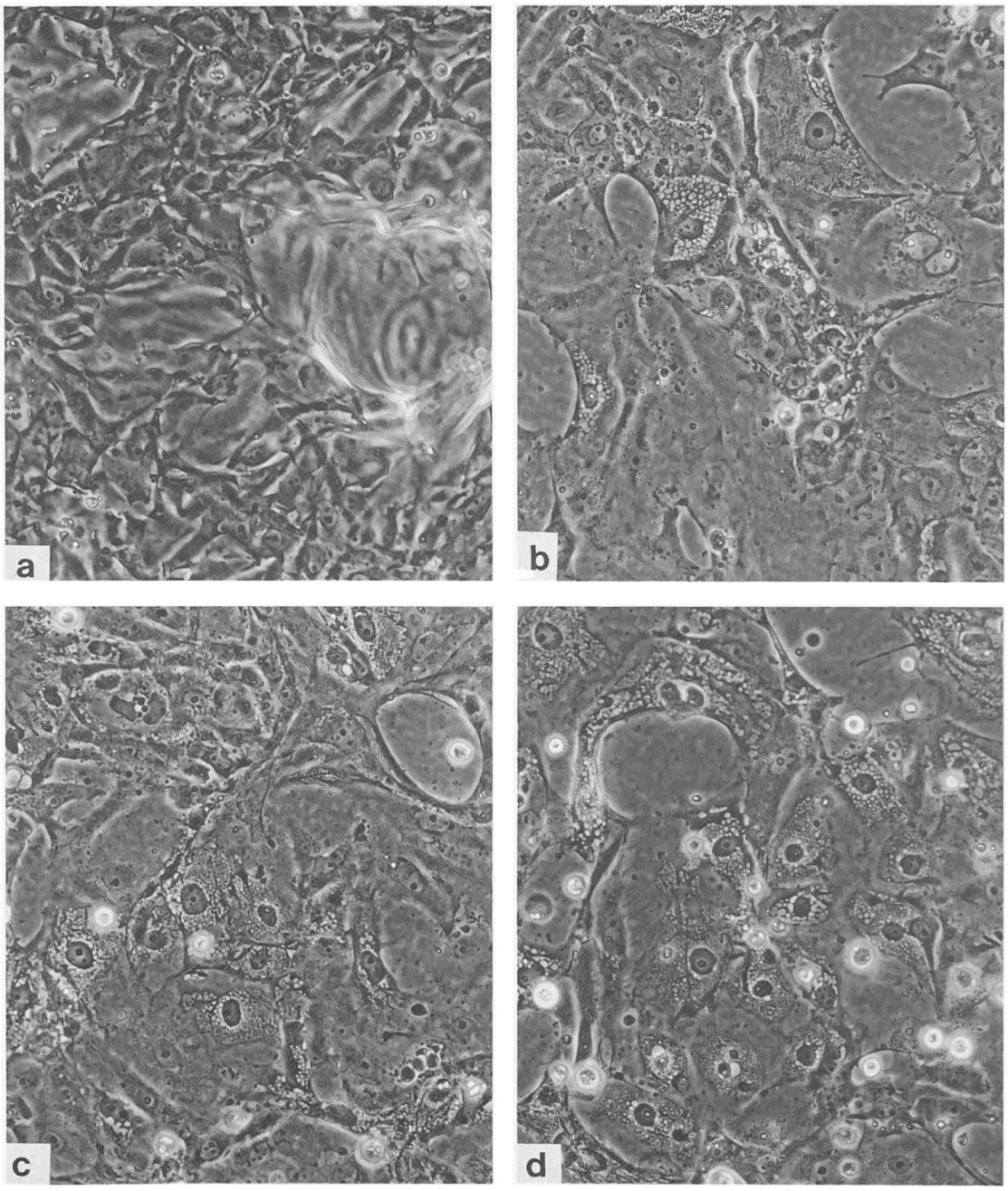

Fig. 8: CaCo-2 cells 48 hours after intoxication (phase contrast microscopy).
a: control, b: orellanine, c: 2,2'dipyridyl, d: 4,4'-dipyridyl.

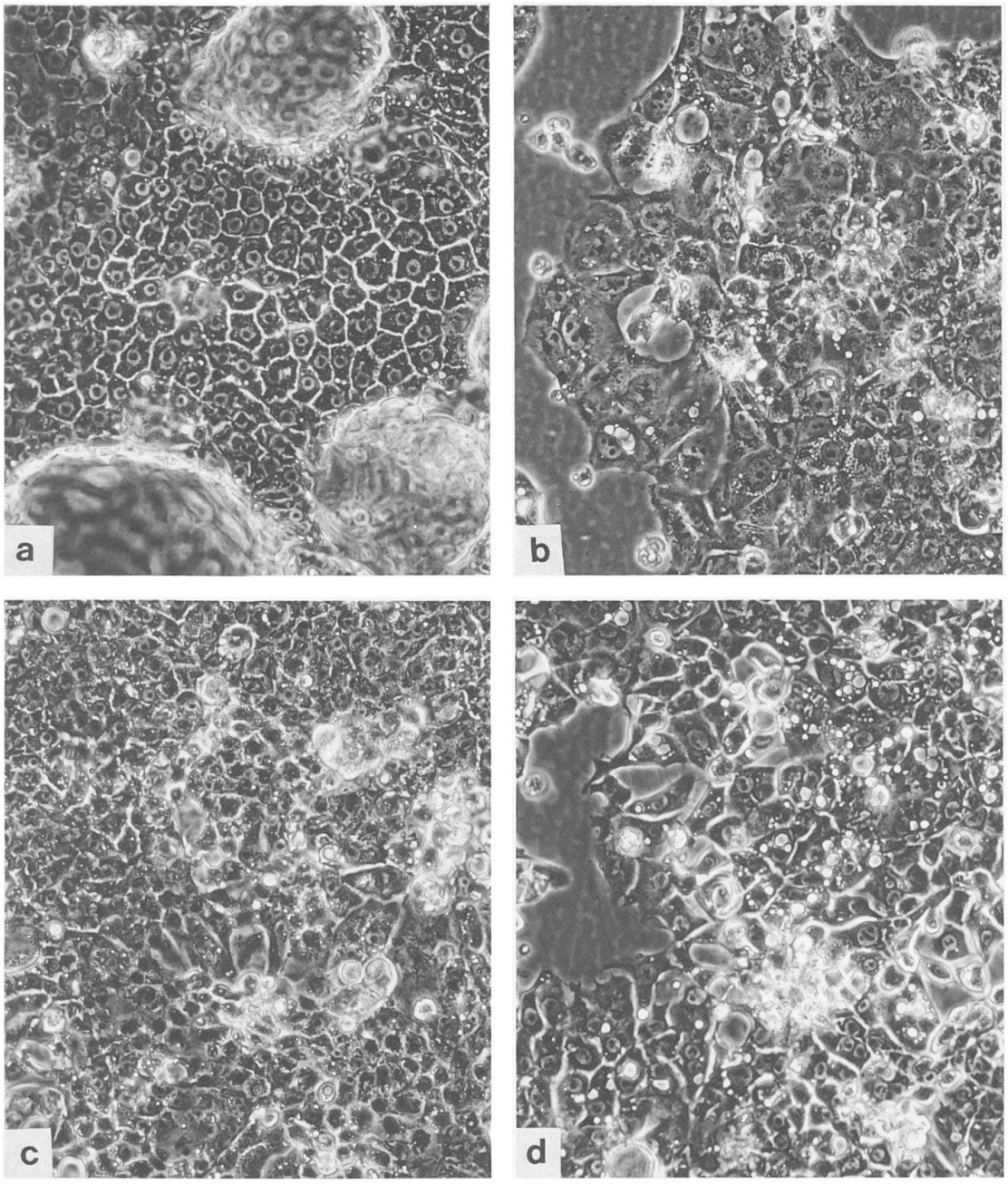

Fig. 9: OK cells 48 hours after intoxication (phase contrast microscopy).
a: control, b: orellanine, c: 2,2'-dipyridyl, d: 4,4'-dipyridyl.