Mitochondrial Swelling Induced by Unconjugated Bilirubin in vitro

Bilirubin encephalopathy (Kernicterus) is usually associated with irreversible nuclear damage induced in the brain by unconjugated bilirubin. On the other side, in a high percentage of rats of the Gunn strain, which lack glucuronyl-transferase and therefore present high levels of unconjugated bilirubin in their blood, cerebral itssue damage is present[1,2].

The mechanism by which unconjugated bilirubin exerts its toxic effect is only partially known. Bilirubin, in fact, uncouples oxidative phosphorylation in isolated mitochondria[3] and inhibits, at high concentration, the electron transport in the respiratory chain in these isolated sub-cellular particles[4]. Besides, these biochemical changes are not limited to isolated mitochondria, since bilirubin uncouples oxidative phosphorylation and inhibits protein synthesis in cells of Ehrlich ascites[5].

Unconjugated bilirubin also induces structural changes in mitochondria. In fact, it has been demonstrated with the aid of the electron microscope that rat liver mitochondria are induced to swell by unconjugated bilirubin[6]. This biliary pigment suppresses respiratory control, uncouples oxidative phosphorylation and induces the swelling in mitochondria isolated from rat liver, bovine heart and from brain of chick, rabbit and monkey. The swelling induced by unconjugated bilirubin is of large amplitude, requires energy and is irreversible. Bovine serum albumin prevents both the mitochondrial swelling and the uncoupling of cytochrome-linked phosphorylation[4].

Studies of the mode of action of unconjugated bilirubin on the structure and function of the mitochondria have been carried out mainly on these isolated particles, whose biological response to the bilirubin can be very different from that of the whole cell. The cell cultures constitute a suitable substrate for the study of the action of unconjugated bilirubin on the mitochondria, as they represent a biological system free from hormonic, nervous, circulatory and immunological influences.

It therefore appeared of interest to study the morphological behaviour of mitochondria of cells cultured in vitro and subjected to the action of unconjugated bilirubin. This study was carried out by means of a phase-contrast microscope and also by means of an interference-contrast microscope. This latter instrument is an extension of the differential interference microscope[7] yielding at the same time a good resolution, a good contrast and allowing the observation of the cell surface.

Materials and methods. Cell cultures. HEp-2 cells (derived from human laryngeal carcinoma), obtained from American Type Culture Collection and serially propagated in our laboratory by routine procedures, were used. The cells were cultured at 37 °C in Eagle basal medium (Difco) supplemented with 10% inactivated calf serum, in Leighton tubes containing a coverslip.

Reagents. Unconjugated bilirubin (from gallstones) was supplied by Sigma Chemical Co., St. Louis, Missouri, USA. Glutaraldehyde was supplied by Fischer Scientific Co., Fair Lawn, New Jersey, USA. All other reagents were supplied by Merck, Darmstadt, Germany. Only triple distilled water on Pyrex glass apparatus was used.

Experiments. The experiments were carried out as described elsewhere[8]. The cell cultures were incubated

1 R. SCHMID, J. AXELROD, L. HAMMAKER and R. L. SWARM, J. clin. Invest. *37*, 1123 (1958).
2 W. A. BLANC and L. JOHNSON, J. Neuropath. exp. Neurol. *18*, 165 (1959).
3 R. ZETTERSTROM and L. ERNSTER: Nature, Lond. *178*, 1335 (1956).
4 M. G. MUSTAFA, M. L. COWGER and T. E. KING, J. biol. Chem. *244*, 6403 (1969).
5 J. H. QUASTEL and I. J. BICKIS, Nature, Lond. *183*, 281 (1954).
6 G. B. ODELL, J. Pediat. *68*, 164 (1966).
7 R. D. ALLEN, G. B. DAVID and G. NOMARSKI, Z. wiss. Mikrosk. *69*, 193 (1969).
8 F. PARADISI, L. GRAZIANO and F. DE RITIS, Res. exp. Med. *161*, 224 (1973).

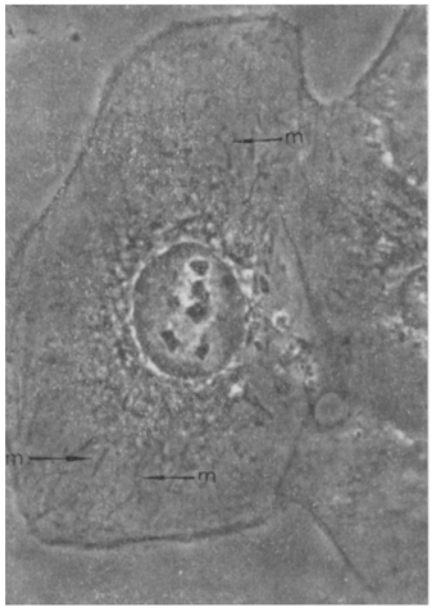

Fig. 1. A normal HEp-2 cell, with some filamentous mitochondria (m). Phase-contrast microscope. ×900.

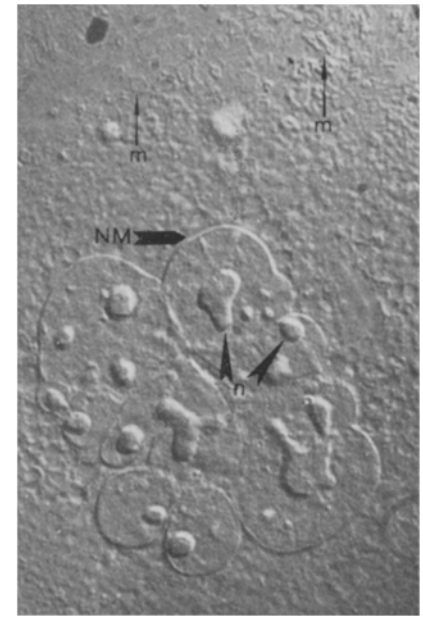

Fig. 2. A normal multinucleated HEp-2 cell. Some rod-like mitochondria (m) are visible at the cell surface. NM = nuclear membrane. n = nucleoli. Interference-contrast microscope. ×900.

with bilirubin solution at 37 °C for 15, 30, 60 and 180 min respectively. After incubation, the coverslips were removed from the Leighton tubes, twice washed with Hanks' BSS, fixed in glutaraldehyde and stained as reported elsewhere[9]. The specimens were then observed and photographed with a Leitz Orthoplan microscope equipped with a Heine's phase-contrast device or with a Normarski's interference contrast equipment.

Some experiments were carried out with unfixed cells in order to control the effect of fixation on the mitochondrial morphology. As a control, cell cultures were treated in the same way, but bilirubin solution was substituted with plain Eagle's basal medium, diluted or not as described elsewhere, always without calf serum[8].

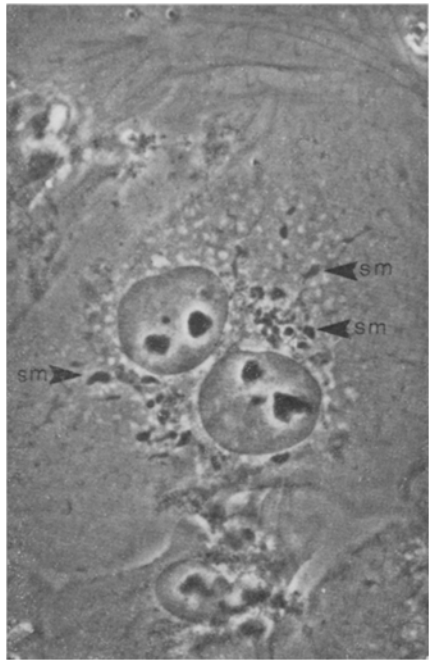

Fig. 3. HEp-2 cell after 60 min of contact with bilirubin solution. A remarkable mitochondrial swelling (sm) is present. Phase-contrast microscope. ×900.

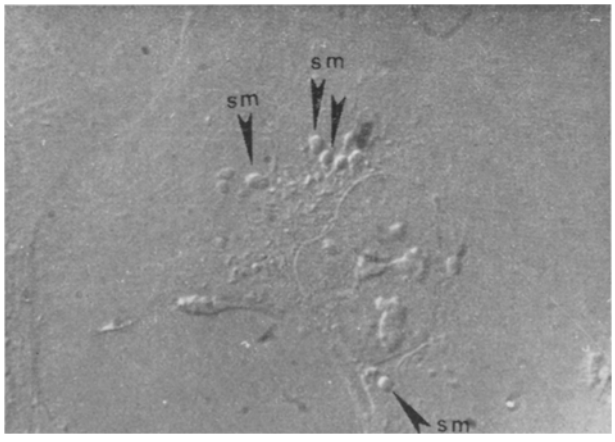

Fig. 4. HEp-2 cell after 60 min of contact with bilirubin solution. The swollen mitochondria are observable on the cell surface (sm). NM, nuclear membrane; n, nucleoli. Interference-contrast microscope. ×900.

Results. The control cell cultures show numerous rod-like mitochondria as well as some roundshaped mitochondria (Figure 1). The cells treated for 15 min with unconjugated bilirubin do not show any modification in mitochondrial morphology. After 30 min of incubation with bilirubin solution, an evident increase in mitochondrial size appears which reaches its highest extent degree after 60 min (Figures 2 and 3). After 180 min of contact with unconjugated bilirubin the mitochondria remain swollen, without further modification in size. Mitochondrial swelling is not, on the other hand, extended to all the mitochondria of the same cell; most of them are swollen but some show normal morphology. The study of unfixed specimens furnished the same results as the fixed cells. However, it was possible to observe that, already after 15 min of incubation and before the appearance of swelling, the mitochondria no longer present any active movement.

Discussion. The findings reported in this paper show that unconjugated bilirubin induces a mitochondrial swelling in cells cultured in vitro. This phenomenon, therefore, is not limited to the isolated mitochondria, but takes place also in the intact cell. This behaviour is similar to that of Ca^{++} and phosphates that induce mitochondrial swelling both in vivo and in vitro, while other well-known swelling agents in vitro, such as L-thyroxine, L-ascorbic acid and L-cysteine have no effect in vivo[10].

The mitochondria swollen by unconjugated bilirubin have a round shape, without remarkable differences in shape between one another. This appearance is similar to that of the mitochondria swollen by Ca^{++} in vivo and could be due to damage of the mitochondrial membranes. On the other hand, it is known that unconjugated bilirubin binds to the lipids of the mitochondrial membranes, which might result in alterations of the mitochondrial function[11].

This study shows, moreover, that unconjugated bilirubin rapidly penetrates into the cell, since after only 15 min the mitochondria lose their motility and after 30 min the mitochondrial swelling takes place. The fact that the swelling is not present contemporaneously in all the mitochondria of the same cell is a phenomenon common to the mitochondrial swelling induced in vivo by different substances[9] and it is probably related to the functional state of the mitochondria at the moment of penetration of the swelling agent.

Riassunto. Gli Autori hanno studiato, per mezzo del microscopio in contrasto di fase e del microscopio a contrasto di fase interferenziale, le modificazioni della morfologia dei mitocondri di cellule coltivate in vitro e sottoposte all'azione della bilirubina non coniugata. Questo pigmento biliare induce, dopo 15 min di contatto con le cellule, la perdita della motilità mitocondriale e dopo 30 min un evidente rigonfiamento dei mitocondri. Viene discussa l'interpretazione dei risultati sulla base dei dati morfologici e biochimici ottenuti da altri Autori su mitocondri isolati.

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[9] F. Paradisi, Pathologia Microbiol. *30*, 481 (1967).
[10] F. Paradisi, Experientia *23*, 752 (1967).
[11] M. G. Mustafa and T. E. King, J. biol. Chem. *245*, 1084 (1969).