Phalloidin-Induced Proliferation of Actin Filaments within Rat Hepatocytes

Visualisation by Electron Microscopy and Immunofluorescence

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Confirming our previous ultrastructural and biochemical studies, the combined electron microscopy and immunofluorescence investigations show that phalloidin causes a characteristically striking increase of actin filaments within rat hepatocytes. Filaments are localized mainly at the level of special junctional complexes between contiguous cells and around bile canaliculi.

Evidence has been accumulating that contractile proteins, similar to muscle actin, myosin and tropomyosin, are also basic components of many non-muscle cells (*cf.* for review l. c. 1–5). They are usually organized into a filamentous network or bundles of microfilaments beneath the plasma membrane and are thought to be involved in the various motile activities of the cell like cytokinesis, membrane ruffling, endo- and exocytosis, intracellular transport, etc. Numerous studies have clearly shown that actin is the major constituent of the contractile apparatus of non-muscle cells and closely resembles the biochemical and structural properties of actin from muscle (*cf.* l. c. 1–2). So far, however, little is known about the mechanism of formation of actin filaments in non-muscle cells.

In the last few years, it has been shown in this laboratory that phalloidin, one of the main toxic components of poisonous fungus *Amanita phalloides* [6], is capable of producing a characteristically striking increase of actin filaments in plasma membrane preparations of rat liver [7–11] as well as in its parenchymal cells [10, 12]. Thus, phalloidin became an useful tool to investigate the mechanism of actin formation and the localisation of the contractile apparatus within hepatocytes. Correlated biochemical, ultrastructural and immunocytochemical observations clearly indicate that phalloidin-induced proliferation of actin filaments occurs first close to – or in – the plasma membrane at the level of the so called junctional complexes, mainly at the macula adherens and zonula adherens (*cf.* l. c. 13), between contiguous hepatocytes and around bile canaliculi, *i. e.* in cell portions which in control hepatocytes also display a well developed microfilamentous web (*cf.* l. c. 14–15).

It was originally observed that negatively stained plasma membrane preparations from rat liver poisoned *in vivo* with phalloidin as well as plasma membrane preparations poisoned *in vitro* show a few min after poisoning a greatly increased frequency of actin like filamentous structures [7]. When ^{3}H-labelled desmethylphalloin [16] is applied, it is bound mainly to the fraction enriched with these filaments [8]. This indicates that interaction occurs between the drug and filaments. The identification of the filaments as actin has been proven [11] by their reaction with heavy meromyosin (HMM), a specific proteolytic fragment of muscle myosin which is known to interact with actin (*cf.* l. c. 17–18). Thereafter, the observation that phalloidin is capable *in vitro* of polymerizing [11] globular actin (G-actin) from rabbit skeletal muscle to filamentous actin (F-actin) led to the conclusion that proliferation of actin filaments in the rat liver after phalloidin poisoning is directly caused by the drug. It is likely that actin, not occuring in microfilamentous structures, usually exists in other forms within the cells, probably in the depolymerized state, which is not easily detectable by electron microscopy. Polymerisation of cytoplasmic actin into microfilaments an *vice versa* depolymerisation of microfilaments to smaller structures probably occur very

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Abbreviations: HMM, heavy meromyosin; F-actin, filamentous actin; G-actin, globular actin; IgG, immunogammaglobulin.

fast within the cell, depending on need for various physiological motile activities. After phalloidin poisoning polymerisation of G-actin to F-actin is strongly enhanced so that actin filaments can be observed in the electron microscope.

An appropriate analysis of the site of origin within the hepatocyte of phalloidin-induced actin filaments could be made by observation in the electron microscope of sections through pellets of poisoned plasma membrane preparations of liver tissue. In sectioned pellets the microfilaments [10] are close to or break off from the cytoplasmic leaflet of the plasma membrane, the trilamellar appearance of which is not obviously altered. A similar topographic relationship between filaments and plasma membrane was observed also on sectioned liver tissue. On comparison with control hepatocytes, the filamentous network close to the inner leaflet of the plasma membrane appears to be widened [10, 12] and a very prominent web of filamentous material can be observed at the level of special junctional complexes – especially at desmosomes – and around bile canaliculi (Fig. 1 *). In suitably oriented sections, separate microfilaments (f) with a diameter up to 80 Å – *i. e.* actin filaments – and bundles of microfilaments (f_1) can also be observed free in the cytoplasma. We are no certain, however, under these conditions, about the presence of thick filaments (f_2). They may simply correspond to unsuitably sectioned and poorly resolved bundles of microfilaments rather than represent myosin filaments.

It should be noted, however, that myosin filaments could not be found in negatively stained preparations and do not increase after phalloidin poisoning. On the other hand, electron microscopy has *per se* a limited capability to identify different chemical types of filaments.

This restriction has been avoided by immunocytochemical studies using specific antibodies. Thus, both major contractile proteins, actin [19–21, 4] and myosin [21, 3] and also tropomyosin [21, 5] could be visualized in various non-muscle cells and in hepatocytes. Immunofluorescence studies have shown that a characteristic polygonal pattern of staining of the hepatocyte borders [22] can be observed when anti-actin antibodies [19] obtained from patients with chronic aggressive hepatitis [23] or when antibodies [21] obtained from rabbits immunized with various contractile proteins from human uterus (actomyosin, actin, myosin, heavy and light meromyosin, and tropomyosin), are applied to liver cryostat sections. This is illustrated in Fig. 2 ** with a control liver after incubation with fluorescent antiserum against actomyosin from chicken stomach [24]. The topographic coincidence of the filamentous network observed by electron microscopy close to the inner leaflet of the hepatocyte plasma membrane with the distribution of fluorescent staining in the light microscope provides reasonable evidence that the contractile proteins are localized in the filamentous network. As expected, a similar cytotopochemical coincidence between localisation of filamentous material in the electron microscope and distribution of immunofluorescent staining could be observed also on sections of poisoned livers. Comparison of Figs 3 and 4 with Fig. 1 indicates that increased immunofluorescence along the hepatocyte borders corresponds to the striking proliferation in the same cell portions of microfilaments, their actin nature, suggested on the basis of their structural appearance [10], having been definitely proven by their reaction with heavy meromyosin [11].

Further studies are necessary to investigate whether or not phalloidin interacts first with a membranal protein or with a protein close to the inner leaflet of the cytoplasmic membrane. It looks, however, as if the sensitive protein is originally a part of the membrane.

Because of its direct effect on actin, which is a major constituent of contractile apparatus of the cell, phalloidin provides a useful tool to investigate various cellular motile activities under pathophysiological conditions. Change in membrane permeability and ruffling due to phalloidin may be the cause, at least in part, of the endocytotic vacuolisation of the hepatocytes observed after phalloidin poisoning (*cf.* l. c. 12).

* Fig. 1 see Table on page 794 a.

** Figs 2–4 see Table on page 794 b.

Fig. 1. Electron micrograph of two contiguous rat hepatocytes 40 min after phalloidin injection displaying a prominent network of filamentous material close to the plasma membrane at the level of special junctional complexes (arrows) and around bile canaliculi (bc). f, separate thin filament. f_1, bundle of thin filaments. f_2, thick filament (?). mv, microvilli of bile canaliculi, containing transversely cut filaments. db, dense bodies. m, mitochondria. 2 μg phalloidin/g bodyweight were injected intravenously. Animals were killed by decapitation and liver fragments were removed and fixed with glutaraldehyde-formaldehyde and osmium. Embedding in Epon 812. Uranyl acetate and lead citrate stain. X 60.000.

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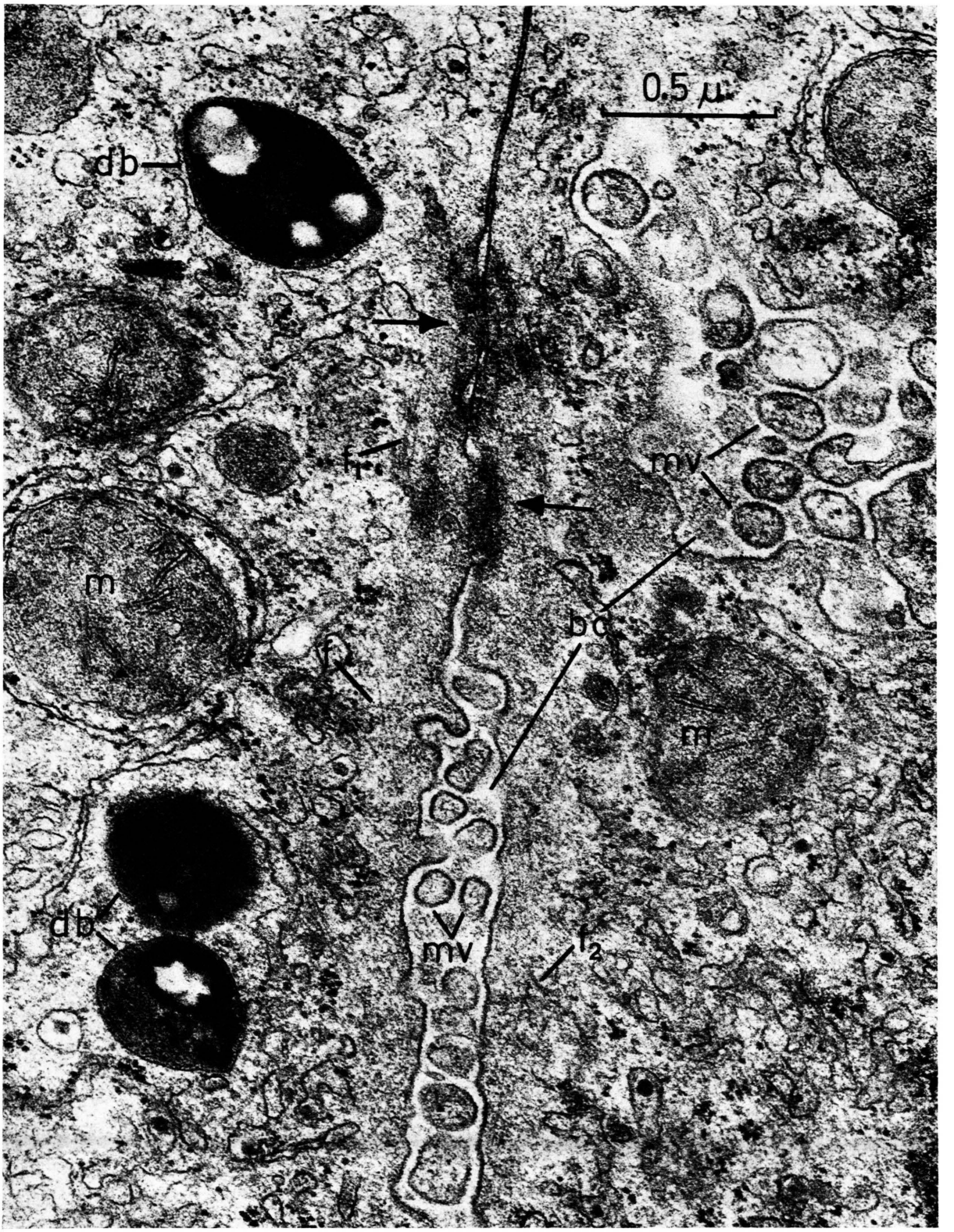

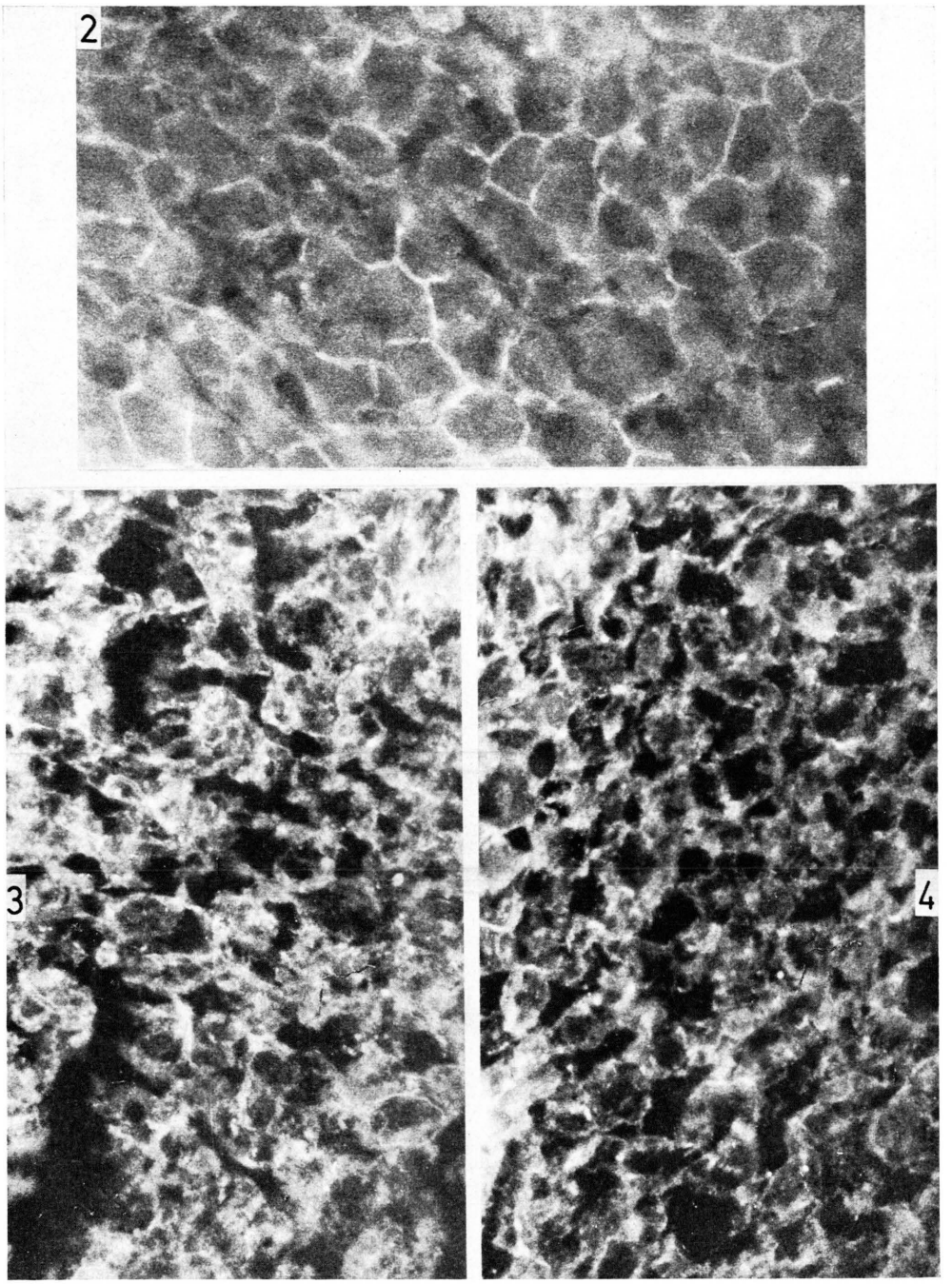

Figs 2—4. Epifluorescence micrographs of cryostat sections of rat livers incubated with labelled anti-actomyosin antibodies. Leitz Ortolux epifluorescence microscope, filter BG 480 and K 510, lamp: Hg 200 W, Ilford film HP 4 (27-29 DIN).

Fig. 2. Control liver. The characteristic polygonal staining pattern of the hepatocyte borders and some bright dots probably corresponding to bile canaliculi can be observed (*cf.* l.c. 19, 21, 22).

Fig. 3. After 40 min poisoning — the same liver as in Fig. 1 — the polygonal pattern is much less evident than in the control. Immunofluorescence is increased along the hepatocyte borders and irregularly spread to large peripheral areas of the hepatocytes. Bright irregular dots at the periphery of the hepatocytes are larger than in the control.

Fig. 4. After 60 min poisoning findings are similar to those observed after 40 min.

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