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Electron Microscopic Studies on the Effects of Amanitin in Mice: Liver and Heart Lesions*

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Elektronenmikroskopische Untersuchungen über die Wirkung von Amanitin auf die Maus: Veränderungen in Leber und Herz

Zusammenfassung. Die Leberzellen zeigen Veränderungen der sinusoidalen Mikrovilli, große Vacuolen, die von einer mit der Plasmamembran kontinuierlich zusammenhängenden Membran begrenzt sind und Unterbrechungen der Plasmamembran. Im Skeletmuskel sind selten leichte Veränderungen zu sehen. Die Frühveränderungen im Herzen scheinen in den Capillaren lokalisiert zu sein, welche Schwellung der Endothelzellen und Fehlen von Pinocytosebläschen zeigen. Schritt für Schritt treten dann auch schwere Veränderungen in den Myokardzellen auf, wie endocelluläres Ödem, Ruptur der Plasmamembran mit Austritt von Zellorganellen. Man kann daraus schließen, daß das Amanitin wahrscheinlich seine Giftwirkung an der Plasmamembran entfaltet.

Summary. In the liver, lesions of the hepatocytes, Kupffer and endothelial cells are present. The hepatocytes show alterations of sinusoidal microvilli, large vacuoles delimited by a membrane continuous with the plasma membrane, and interruptions of the plasma membrane.

In the skeletal muscle only rare and mild alterations are observed. In the heart the early lesions seem to be located in the capillaries, which present swelling of endothelial cells and absence of pinocytotic vesicles. Successively severe lesions (consisting of endocellular edema and rupture of the plasma membrane with extrusion of the cellular organelles) also appear in the myocardial cells.

It may be deduced that amanitin probably exerts a noxious action at the level of the plasma membrane.

The toxic action of the mushroom Amanita phalloides is due to its six cyclopeptidic cytotoxins; these may be divided into two groups: the phalloidin group (including phalloidin, phallacidin and phalloin) and the amanitin group (including alpha, beta and gamma amanitin) (T. Wieland and O. Wieland, 1959; O. Wieland, 1965). The toxins of the phalloidin group act rapidly, producing degenerative lesions almost exclusively located in the liver (T. Wieland and O. Wieland, 1959; Fiume and Laschi, 1965). The amanitins, on the other hand, have a more delayed action. A mouse receiving an injection of 1 MLD_{100} of alpha-amanitin dies after 4—5 days and presents degenerative lesions of several organs, such as the liver, kidney, heart, skeletal muscle, adrenals and pancreas (T. Wieland and O. Wieland, 1959; Fiume and Laschi, 1965).

* The preliminary results of this study have been presented at the "Colloque annuel de la Société Française de Microscopie Electronique", Bordeaux, May 23rd—25th, 1966 [Meldolesi, J., G. Pelosi et F. Clementi: Aspects ultrastructuraux du foie et du cœur de la souris après intoxication expérimentale par l'amanitine. J. Microscopie (5, 65a 1966)].

In the present investigation, the ultrastructure of the liver, heart and skeletal muscle of mice treated with a mixture of purified alpha and beta-amanitin (kindly provided by Prof. T. Wieland) was studied. Animals were sacrificed at various intervals after administration of the poison.

Materials and Methods

To investigate the ultrastructure of the liver fifty-six Swiss female mice weighing 20 to 25 g were used. They were divided into 4 groups. The first three groups, composed of 16 animals each, were injected intraperitoneally with a mixture of alpha and beta-amanitin at doses of 100, 200 and 400 gamma/kg, respectively. The 8 animals of the fourth group were used as controls. Four animals of each of the first three groups and two controls were sacrificed by decapitation, on the first, third, sixth and tenth day of the experiment.

For the study of the heart and skeletal muscle twelve additional animals were injected intraperitoneally with a single dose of 200 gamma/kg of a mixture of purified alpha and beta-amanitin. Six animals were taken as controls. Four treated animals and two controls were sacrificed by decapitation, on the first, third and sixth day of the experiment.

Immediately after sacrifice, several small samples of tissue were taken from different parts of the liver or from the heart and the quadriceps femoris muscle and fixed with the following methods: 1% OsO_4 in phosphate buffer for 3 hours, or 3% glutaraldehyde for 4 hours, followed by 1% OsO_4 for 3 hours, both in phosphate buffer, and embedded in Westopal W. The ultrathin sections, obtained with an LKB "Ultrotome" microtome, were stained with uranyl acetate and lead citrate and observed with a Hitachi HU 11 electron microscope.

Results

Liver. The lesions induced in the liver by amanitin with the various doses used are qualitatively analogous, while their extension increases with the dose of the poison.

Twenty-four hours after administration of amanitin most of the liver cells are normal or present only minimal alterations (mild steatosis and slight swelling of sinusoidal microvilli). In these cells glycogen seems well preserved; the nucleus, mitochondria, endoplasmic reticulum and bile canaliculi have a normal aspect. By contrast, a few liver cells have a clearly pathological aspect. Numerous large vacuoles are very often observed, expecially in the sinusoidal region of the cell (Figs. 1, 2). They are limited by a single membrane (which, at least in some cases, is continuous with the plasma membrane) and contain a clear, finely granular substance. After double fixation with glutaraldehyde and osmium tetroxide, dense lamellar figures appear within them. The microvilli of the vascular surface of the liver cells often disappeared; in other cells they are swollen or fused into voluminous, roughly rounded, peduncolated projections protruding into the space of Disse (Fig. 3, 4). These formations may detach themselves from their insertions and become free in the space of Disse and in the lumen of the sinusoids. Nuclei are almost always normal; only in a very few cells a partition of the nucleolar components is observed (Fig. 1).

Kupffer and endothelial cells also show serious alterations. Endothelia are swollen and often separated from the hepatocytes by a greatly enlarged space of Disse containing cellular debris, erythrocytes and thrombocytes (Fig. 4). Mesenchymal cells containing lipid droplets are very numerous (Fig. 5). Many Kupffer cells are swollen; sometimes they show signs of necrosis. The sinusoids are enlarged and contain erythrocytes, leukocytes and thrombocytes. By contrast bile canaliculi are always normal.

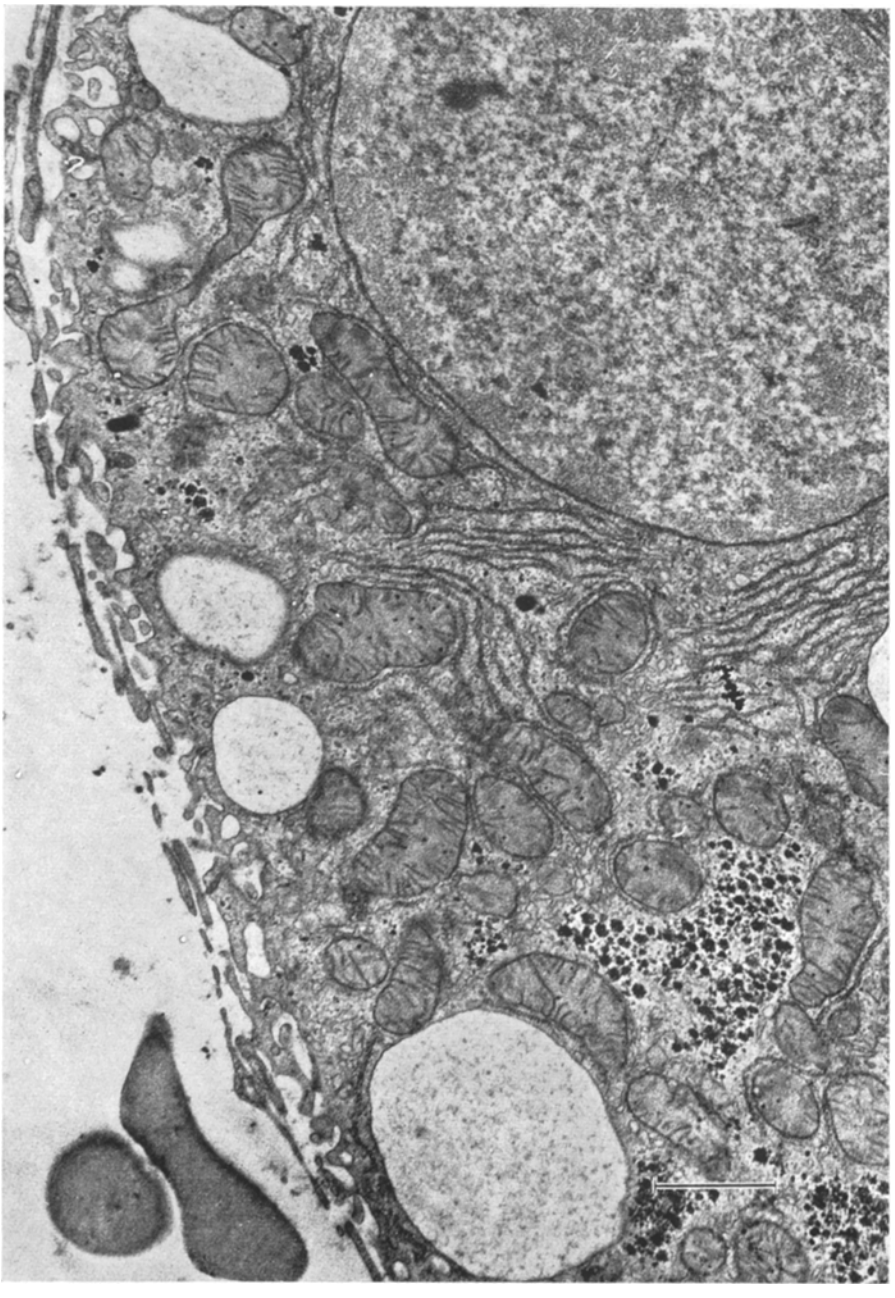

Fig. 1. Mouse liver 3 days after injection of amanitin (200 gamma/kg). Along the sinusoidal surface of an hepatocyte vacuoles delimited by a single membrane are evident. The nucleus, mitochondria and endoplasmic reticulum have a normal aspect. The nucleolus exhibits a partition of its components

On the third day after injection of the toxin the lesions described are much more extensive.

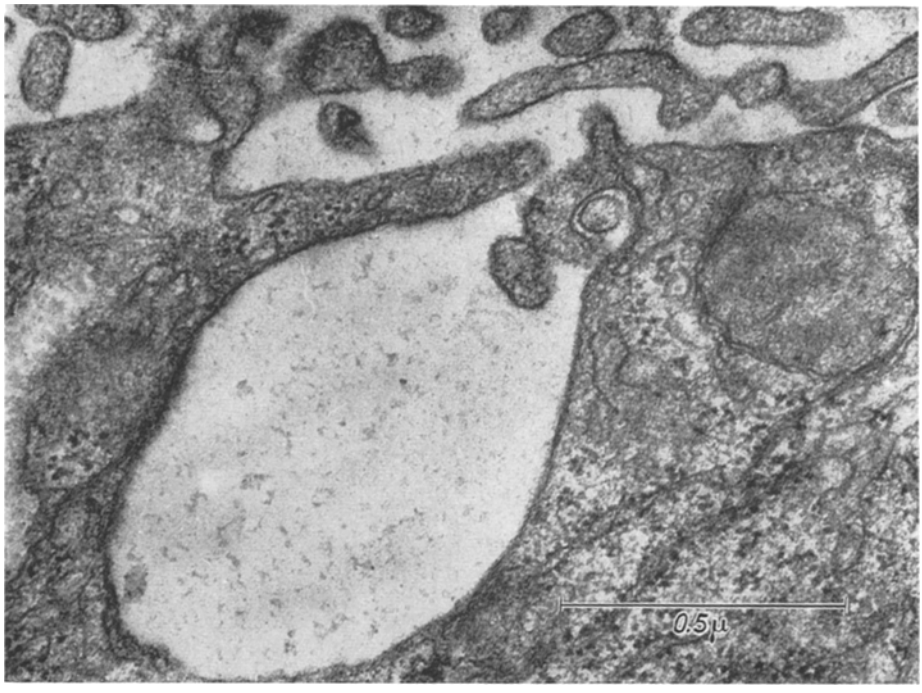

Fig. 2. Mouse liver 24 hours after the injection of amanitin (400 gamma/kg). In one hepatic cell there is a large vacuole delimited by a single membrane continuous with the plasma membrane. Mitochondria and ergastoplasm have a normal aspect

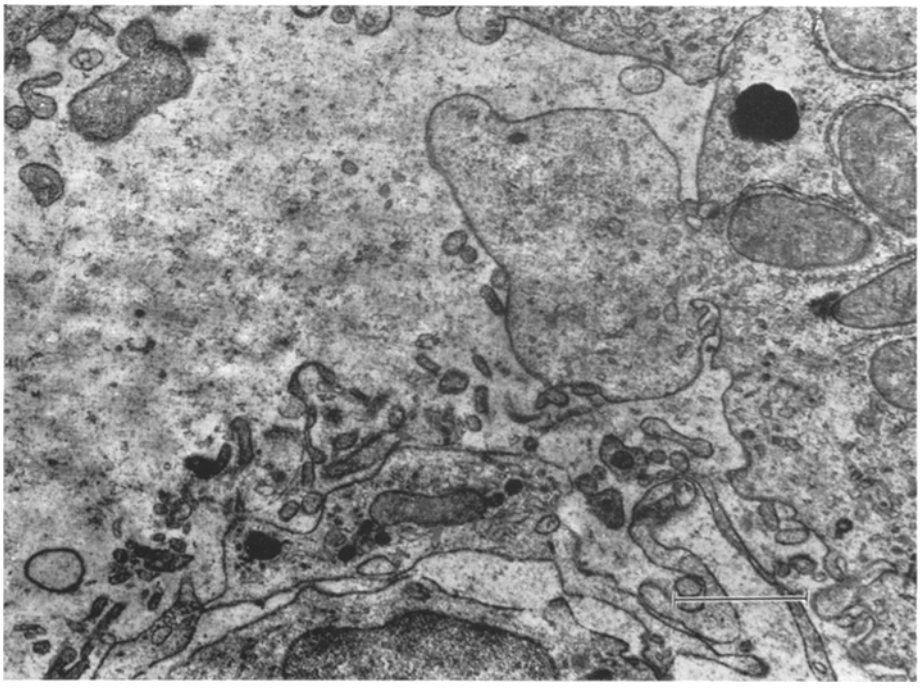

Fig. 3. Mouse liver 3 days after injection of amanitin (400 gamma/kg). The space of Disse is obviously dilated. Sinusoidal microvilli are swollen and fused to form large rounded cytoplasmic projections

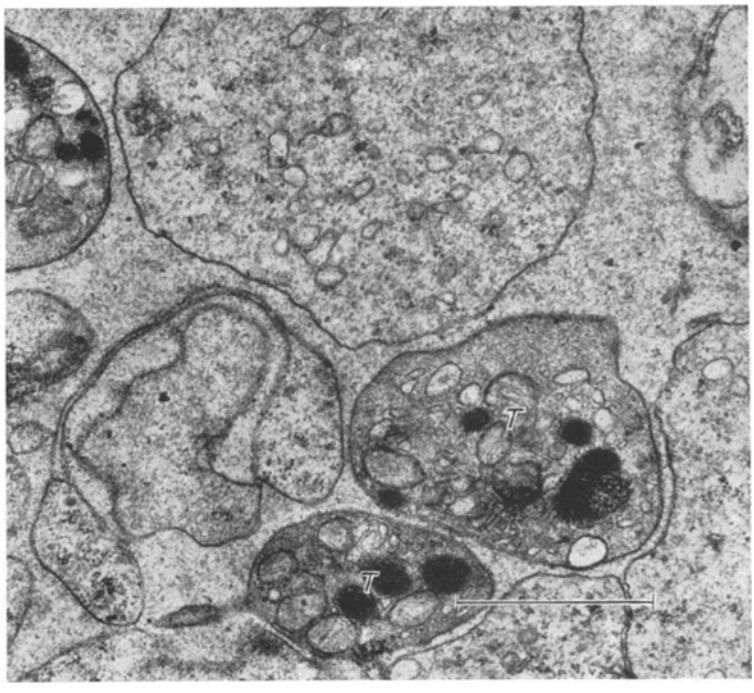

Fig. 4. Mouse liver 24 hours after injection of amanitin (400 gamma/kg). Several irregular cytoplasmic projections probably of hepatocytic origin and three thrombocytes (*T*) are present in a space of Disse

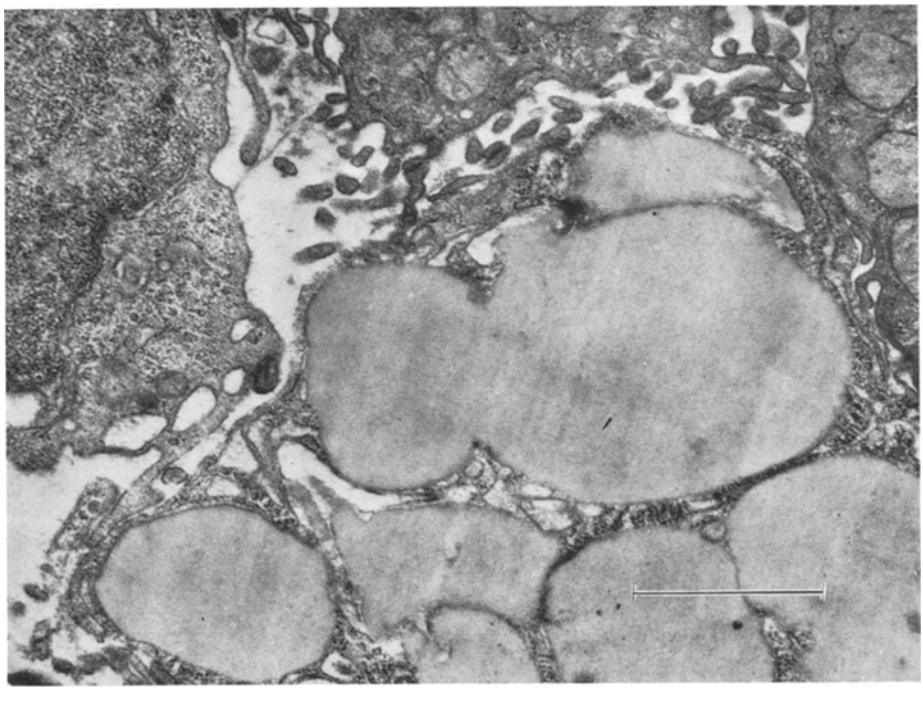

Fig. 5. Mouse liver 24 hours after injection of amanitin (400 gamma/kg). A mesenchymal cell containing various large lipid drops is observed

Large vacuoles are almost always present (Fig. 1); the sinusoidal microvilli are swollen or disappear (Fig. 1, 3). By contrast the cytoplasmic organelles have a normal aspect; only in a few cells the ergastoplasm is dilated and the ground substance has a very low density. At the vascular pole of a great number of hepatic cells the plasma membrane is obviously interrupted. In this case cytoplasmic organelles of normal aspect are released into the space of Disse and the sinusoids (Fig. 6).

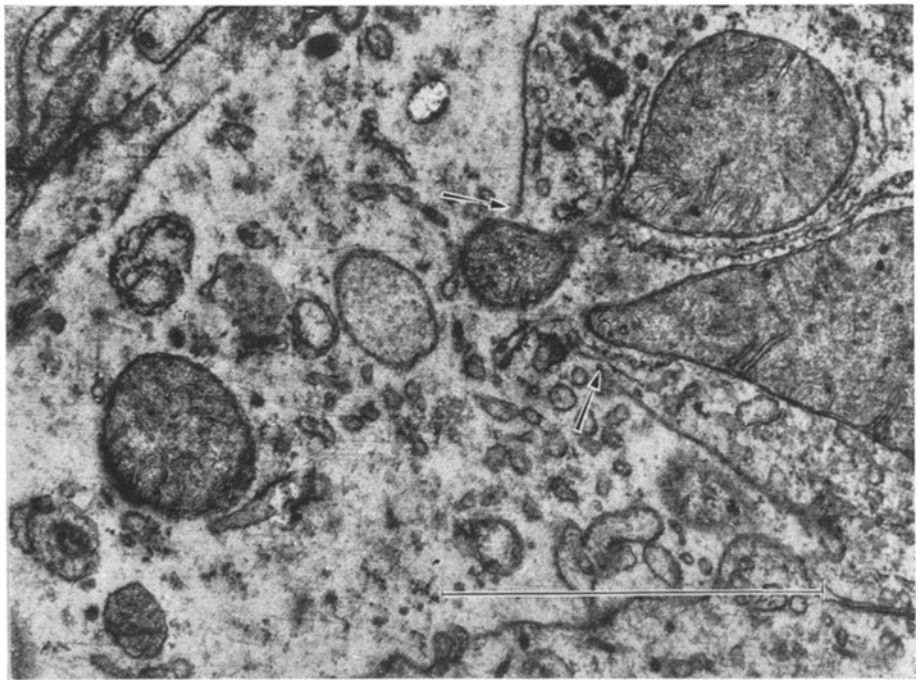

Fig. 6. Mouse liver 3 days after the injection of amanitin (400 gamma/kg). The plasma membrane of an hepatic cell is interrupted at the vascular pole (arrows). Numerous apparently unmodified cytoplasmic organelles lie in the space of Disse, which is greatly dilated. Microvilli are completely absent from the sinusoidal surface of the hepatocytes

Six and ten days after administration of the toxin, the lesions already described are still present. Interruption of the plasma membrane is very frequently seen; the sinusoidal lumen may be completely filled by cytoplasmic organelles (Fig. 7). Sometimes endothelial cells lying on a basal membrane are observed (capillarization of sinusoids) (Fig. 7).

Heart. 24 hours after the injection of amanitin, a considerable number of cardiac capillaries present significant alterations: the endothelial cells appear swollen, at times slightly and at times to such an extent that their lumen is almost completely occluded (Fig. 8). There are a few pinocytic vesicles or none at all, although they are normally very numerous at this site (Figs. 8, 9). In some cases the plasma membrane of the endothelial cells is interrupted.

In the cardiac cells sometimes large rounded vacuoles, more often peripherally located, are found. They contain a finely granular material and are sometimes

delimited by a double membrane. At other times they appear as clear spaces, within other part of the cell, without a limiting membrane being apparent (Fig. 10).

The sarcoplasmic reticulum appears dilated. Mitochondria are completely normal in some fields, while in others they show swelling or alterations of the cristae, which tend to become homogenized.

The myofibrils are widely separated from each other, disordered and pushed away from the cellular surface. A clear space completely free of organelles is

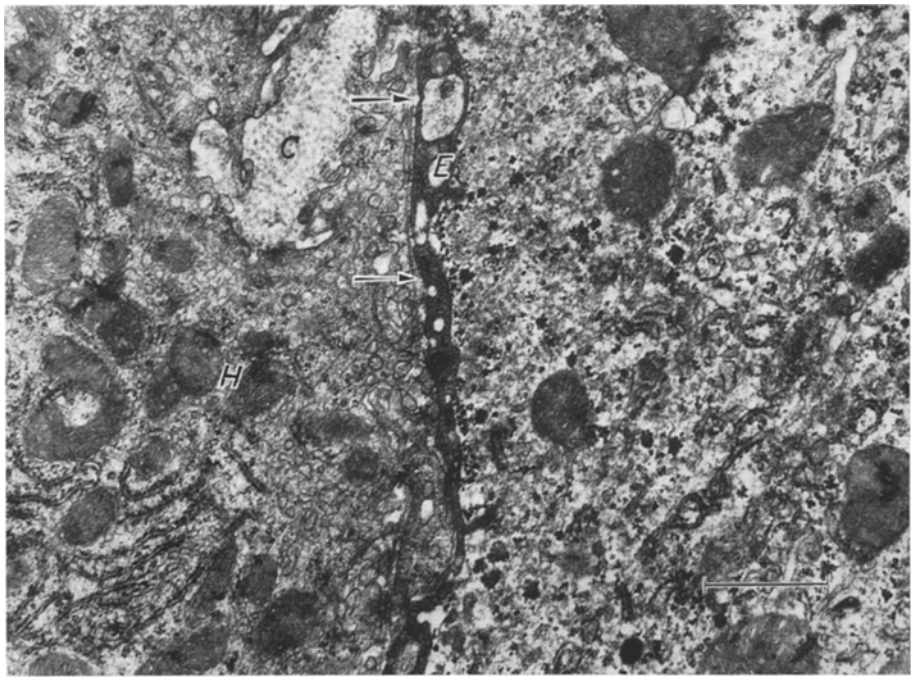

Fig. 7. Mouse liver 6 days after injection of amanitin (400 gamma/kg). A sinusoid is completely occupied by free cytoplasmic organelles of normal aspect originating from hepatic cells. The hepatocyte on the left (*H*) is nearly normal. The endothelium (*E*) lies on a basal membrane (arrows)

thus formed between the myofibrils and the plasma membrane (Fig. 11). These alterations seem to be due to endocellular edema.

In some cells small interruptions of the plasma membrane are present.

The alterations of the cardiac capillaries are still present three days after the injection of the poison. Signs of small hemorrhagic extravasations, with presence of erythrocytes in the interstitial space, are often observed. In the myocells dilatation of the sarcoplasmic reticulum is evident, while endocellular edema and myofibrillar alterations seem to be less frequent.

The above-described alterations are still evident after 6 days. In several myocells the myofibrils are disarrayed, widely separated from each other, reduced in number and interrupted at several points (Fig. 12). Some superficial myocells

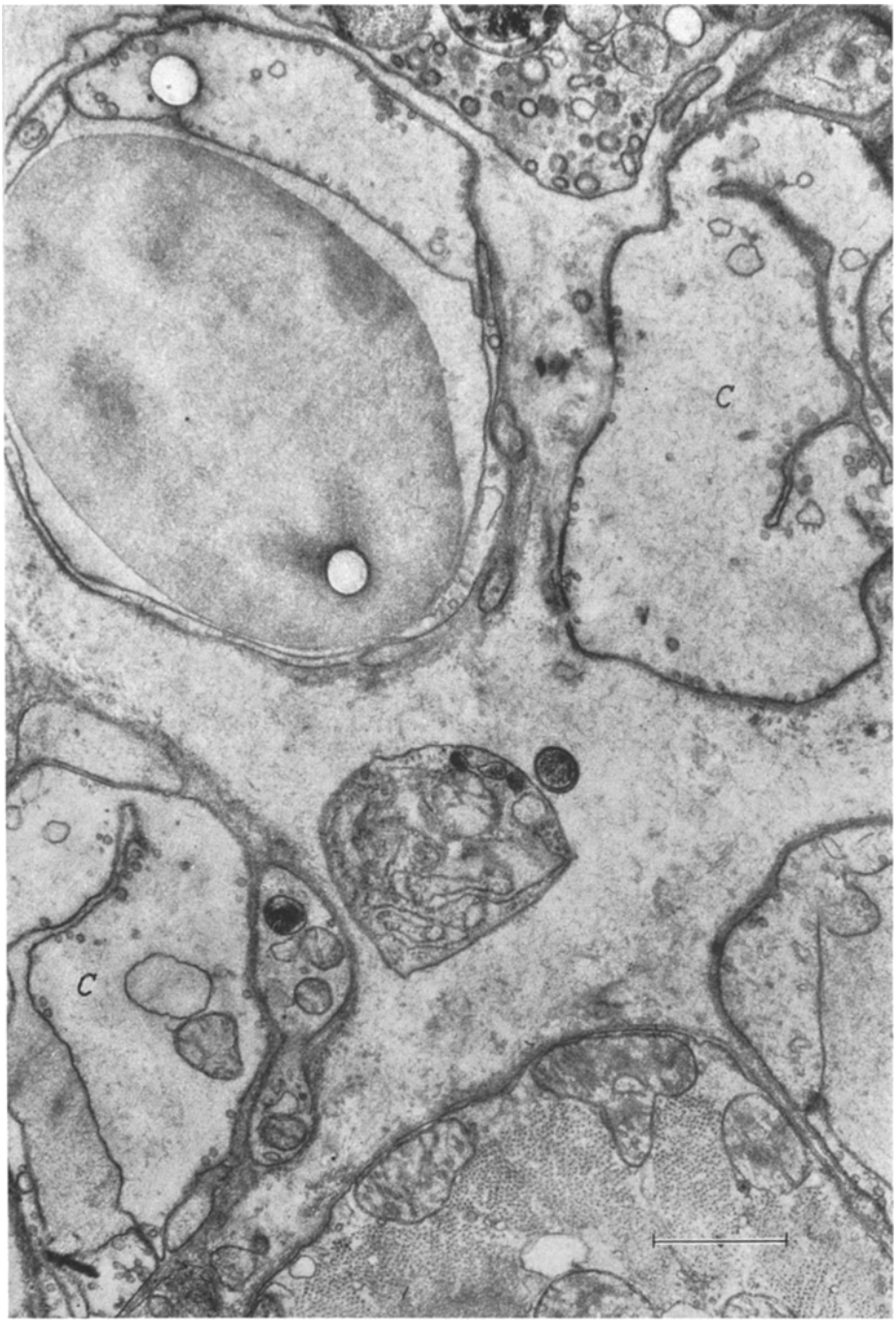

Fig. 8. Mouse heart 24 hours after injection of 200 gamma/kg of amanitin. Two capillaries (top right and bottom left) are occluded as a result of endothelial edema (*C*). Pinocytic vesicles of endothelium are very rare

only contain residues of the Z band in the form of canaliculi perpendicular to the long axis of the myofibrils. The sarcoplasmic reticulum is moderately dilated.

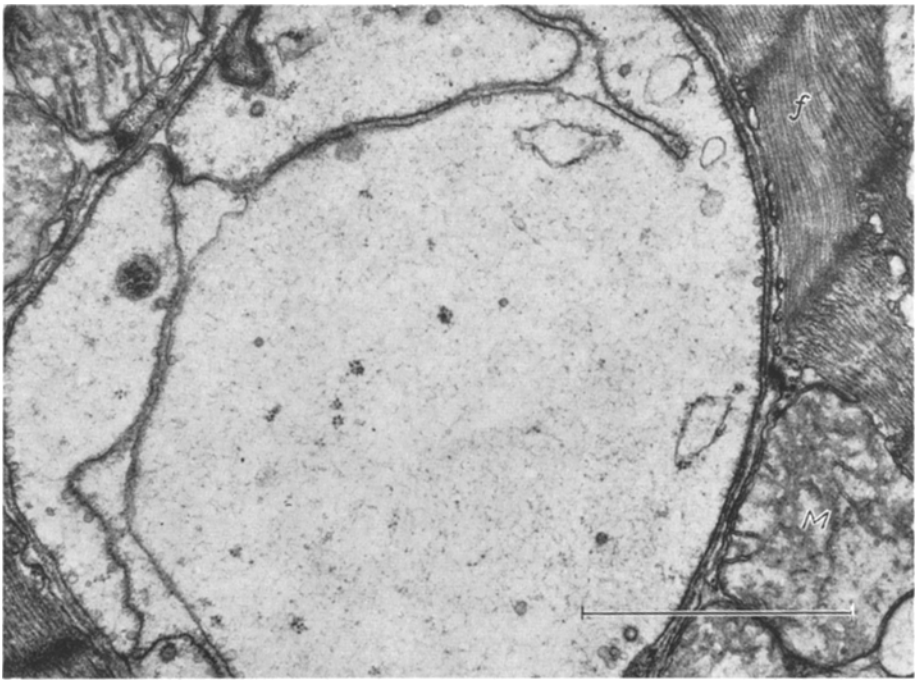

Fig. 9. Mouse heart 24 hours after injection of 200 gamma/kg of amanitin. Two cardiac cells surround a large capillary which shows a considerable endothelial edema and a few pinocytic vesicles. *f* myofibrils; *M* mitochondria

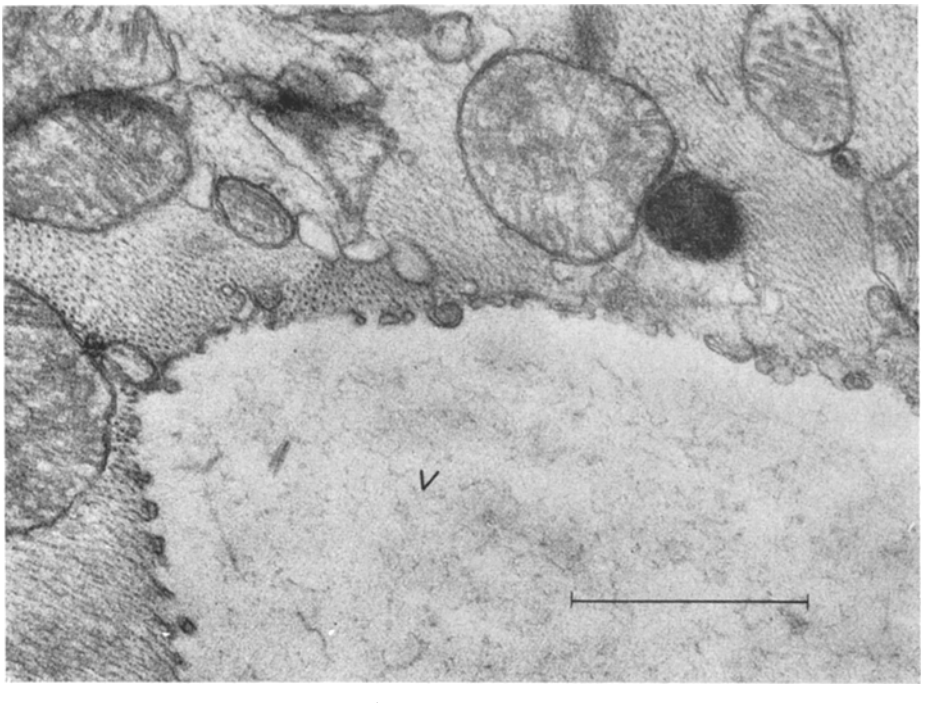

Fig. 10. Mouse heart 24 hours after injection of 200 gamma/kg of amanitin. Inside a cardiac myocell, part of a vacuole (*V*) without a delimiting membrane is noted

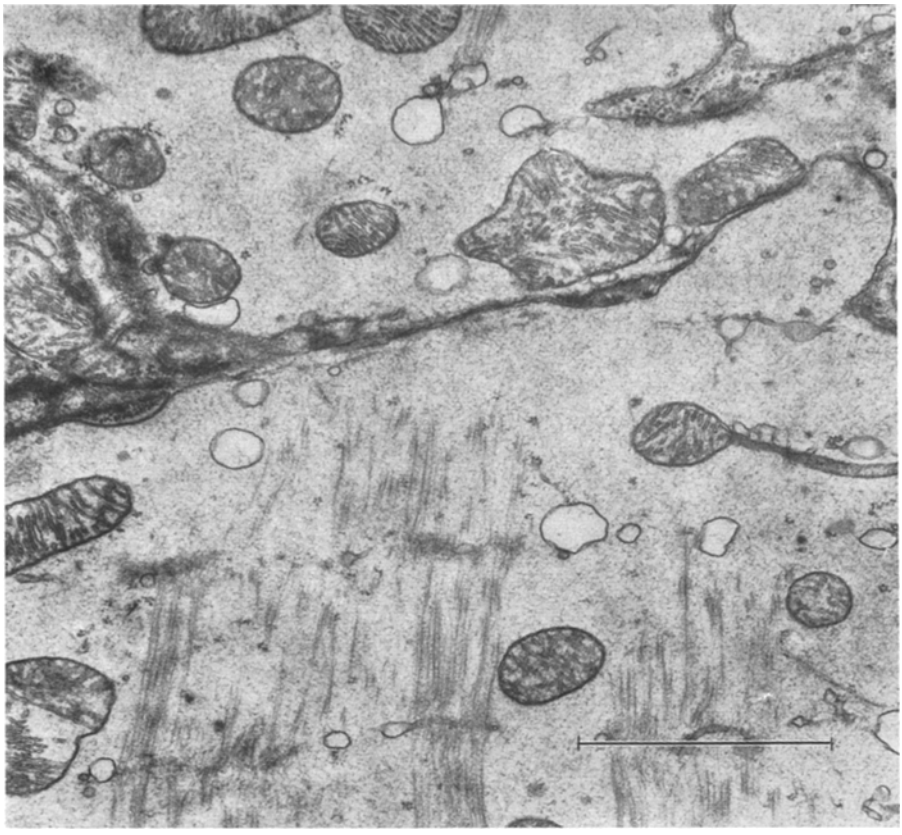

Fig. 11. Mouse heart 24 hours after injection of 200 gamma/kg of amanitin. Edematous myocells, with myofibrils interrupted and widely separated are evident. Near the cellular surface there is a clear space free of organelles

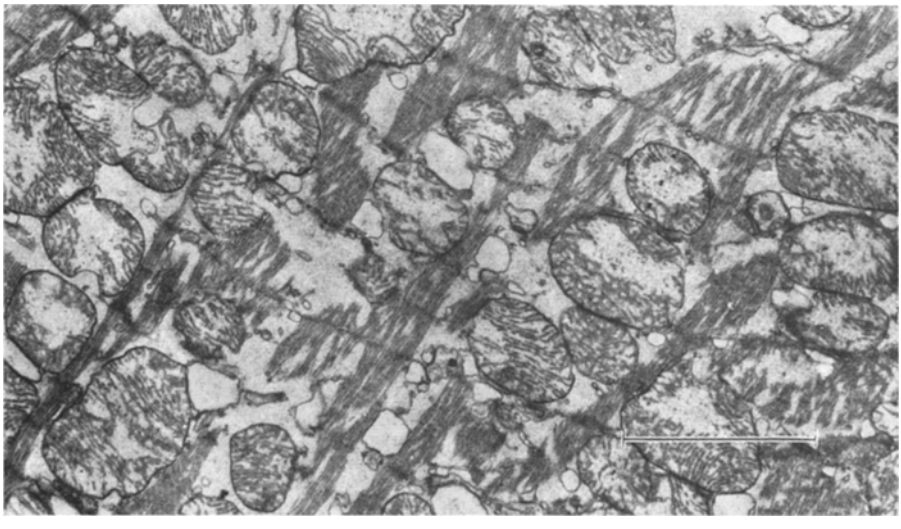

Fig. 12. Mouse heart 6 days after injection of 200 gamma/kg of amanitin. The myofibrils are disordered and interrupted. The sarcoplasmic reticulum is dilated

Rather frequently interruptions of the plasma membrane are observed. Through them apparently normal cytoplasmic organelles are extruded into the interstitial space (Figs. 13).

Skeletal Muscle. In the quadriceps muscle, moderate and infrequent endothelial swelling may be noted only on the first day following treatment. After 3 and 6 days no noteworthy alterations are observed.

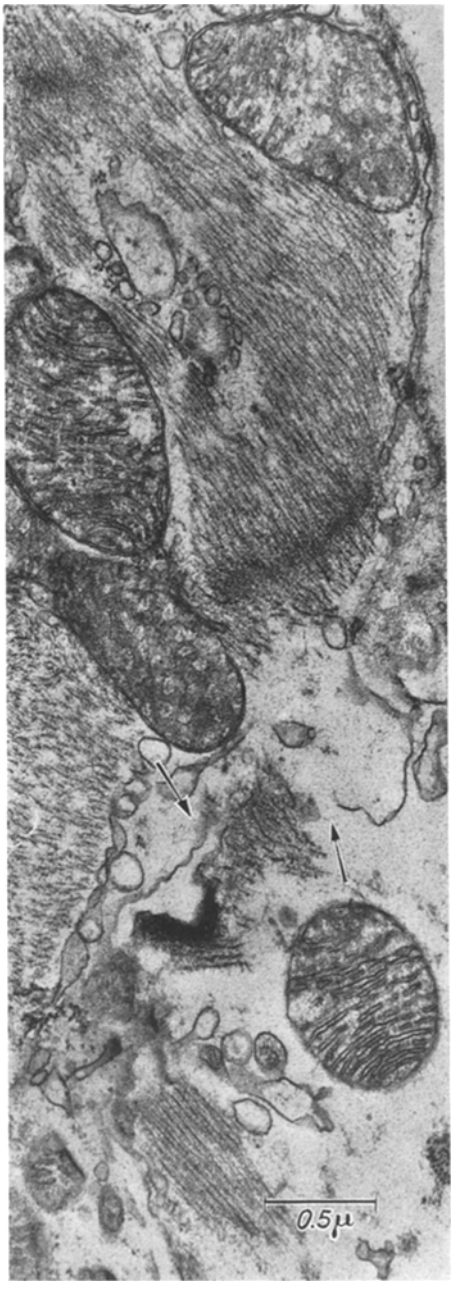

Fig. 13. Mouse heart 6 days after injection of 200 gamma/kg of amanitin. A myocell with interrupted plasma membranes is evident (arrows). Mitochondria are present in the interstitial space

Discussion

It is assumed that the mouse MLD_{50} of alpha and beta-amanitin are respectively about 350 and 400 gamma/kg (T. WIELAND, 1964). However, the various strains of mice present a certain variability of response (T. WIELAND and O. WIELAND, 1959). In the course of preliminary experiments we established that our strain is particularly resistant, since the minimum dose of our alpha and beta-amanitin mixture able to kill half of the injected mice was about 600 gamma/kg. The doses of toxin utilized in this study (100—200 and 400 gamma/kg) must therefore be considered able to produce elective lesions.

The toxic symptoms produced by amanitin are evident some days after administration of the poison (T. WIELAND and O. WIELAND, 1959). In the liver, however, ultrastructural lesions are already present after a few hours.

FIUME and LASCHI (1965) at the 15th hour observed exclusively *nucleolar lesions* in hepatocytes. Recently this finding has been confirmed by biochemical researches (FIUME et al., 1966; FIUME and STIRPE, 1966). However, the nucleolar lesions seem to be very fugacious, since we observed that 24 hours after the administration of the poison only a very few hepatic cells exhibited them. By contrast, lesions are present at the vascular pole of the hepatocytes, in the endothelial and in Kupffer cells. The vascular localization

of the lesions is also present in the subsequent phases of the intoxication, when the lesion involves most of the cells.

However, the alterations of the vascular portion of the hepatocyte are not specific. *Swelling and fusion of the sinusoidal microvilli* were first observed in allergic hepatitis (Steiner, 1961) and then in various other pathologic conditions (Lane and Baker, 1966; Hübner, 1964; Rouiller, 1965; Bassi and Bernelli, 1964; Rouiller et al., 1965; Minio and Gardiol, 1965). *Large vacuoles* delimited by a single membrane continuous with the plasma membrane, which are similar to those we observed, rapidly appear in animals exposed to low O_2 pressure (Oudea, 1963) and in acute hepatic ischemia (Hanzon, 1960; Hübner, 1961; Hübner and Bernhard, 1961; Bassi and Bernelli, 1964; Brewer and Heath, 1965). They rapidly disappear when the animals are brought back to normal conditions (Hübner and Bernhard, 1961). Similar vacuoles have also been observed in allyl-alcohol intoxication (Hübner, 1964). The presence of these vacuoles in amanitin intoxication can not be attributed to an acute alteration of the hepatic circulation, since they are present also many days after injection of the toxin. *Interruptions of the plasma membrane* have also been observed in various pathological conditions. However, severely altered cells in either necrotic (Abbot and Jézéquel, 1962) or pre-necrotic phase (Haenni, 1964) were involved. An ultrastructural pattern analogous to the one we observed is found only in several familial icteric syndromes where a congenital weakness of the vascular pole of the hepatocyte is admitted (Simon and Varonier, 1963; Minio et al., 1965a and b). In both cases the hepatocytes, while presenting interruptions of the membrane, appear only slightly altered and the cytoplasmic organelles present in the extracellular space have a normal aspect.

As far as the heart is concerned, the earliest alterations found involve the capillaries. *The edema of the endothelial cells,* together with diminished or absent pinocytosis, leads one to believe that the continuous metabolic exchanges taking place between the capillaries and the interstitial space are profoundly altered. The consequent deficit in oxygen and nutritional materials may play a rôle in the origin of the myocellular lesions. In this regard, it is interesting to note that mitochondrial alterations similar to those produced by amanitin were observed in the frog heart exhausted "in vitro". On the basis of biochemical findings these results were attributed to blockade of energy metabolism (Ryback et al., 1964). It may be hypothesized that amanitin also induces depletion of ATP in the heart, as it definitely does in the liver (O. Wieland, 1965; T. Wieland and O. Wieland, 1959).

The most evident *myocellular alterations,* i.e. intracellular edema and rupture of the plasma membrane, were observed only at a later period. These, however, do not seem to be the consequence of the previous damage to the capillaries.

In fact, the morphological affinity between the endothelial and myocellular alterations leads one to think that probably, in both cases, they are due to direct amanitin action. It is possible that the mushroom poison, which is carried by the bloodstream, would first attack the endothelia and successively exercise its noxious action on the heart-cells.

The myofibrillar alterations are probably not caused by a single mechanism. The edema may produce the widely separated myofibrils, while the disorder of the myofilaments, which occurs later, may be related to the possible lack of ATP and consequently to a deficit of energy available for contraction (CLEMENTI et al., 1963).

The finding of *vacuoles* within the myocell is difficult to interpret. Similar findings have already been noted in myocardial infarction (CLEMENTI et al., 1964), in the heart of animals poisoned with tetanus toxin (PELOSI et al., 1966) and in degenerating myoblasts (AUBER-THOMAY, 1966). In all these cases the vacuoles were surrounded by membranes. In the vacuoles that we observed a delimiting membrane was not always evident, thus making it impossible to establish whether they are zones of localized endocellular edema or the morphological expression of a phenomenon by which the cell isolates useless or toxic materials (PELOSI et al., 1966).

The endocellular edema we observed may be the expression of disturbance of ion-exchanges within the cell and between inside and outside the cell itself. In fact, a lowering of the Na/K ratio is probable in the heart, since it has been observed in the liver of amanitin-treated mice.

There is certain amount of doubt as to whether the interruption of the plasma membrane, which we observed both in the liver and in the heart, is really present "in vivo", or if the membrane is interrupted only after the death of the animals, during fixation and embedding of the tissues. It is not possible to give a definite answer to this question. In the present study the interruption of the plasma membrane has been very frequently encountered in specimen fixed with different methods (osmic fixation or double fixation with glutaraldehyde and osmium tetroxide). The frequency and the reproducibility of thes finding seem to suggest that, even if these interruptions were to take place only during tissue processing, they would still indicate that the plasma membrane is affected by the poison.

The absence of lesions in the skeletal muscle, in contrast with the results of previous light microscopical observations (T. WIELAND and O. WIELAND, 1959), is probably related to the relatively low doses of amanitin used by us. The serious cardiac alterations and, on the other hand, the absence of muscular alterations provide a further indication of the different reaction of the two contractile tissues.

The results we obtained suggest that in amanitin poisoning the plasma membrane both of the liver and heart cells is severely injured.

This conclusion seems to correlate well with previous morphological and biochemical investigations by T. and O. WIELAND. According to their results, the Amanita phalloides toxins would exercise their noxious action not by interference with one or more enzymatic systems, but rather by damaging the structure and function of the biological membranes (T. WIELAND, O. WIELAND, 1959; O. WIELAND, 1965).

On the other hand this interpretation agrees also with clinical data. In fact, electrocardiographic signs of anomalies of membrane depolarization and repolarization have been observed in subjects affected by amanitin poisoning (BONNEL, 1955).

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