Investigations on the Toxic Action of *Amanita phalloides* Fr. on the Hepatic Cell

Interest in the mode of action of mushroom poisoning has grown steadily over the past few years. This applies particularly to *Amanita phalloides* poisoning, which is so often fatal. Of the 2 principal phytotoxins isolated from this fungus, amanitine is thought to be more toxic than phalloidine (WIELAND[1]). The latter seems to exert its action on the ribosomal membrane; according to some workers the injurious agent is not phalloidine itself but its metabolic product[2]. Amanitine, on the other hand, attacks the nucleus but not the cytoplasm or the organelles within it. For these investigations we used the total mushroom extract in order to keep as close as possible to the conditions of natural poisoning and, instead of giving the lethal dose, we gave the LD_{50}. Male Wistar strain albino rats weighing about 200 g were used. The LD_{50} in our experimental conditions proved to be 35 mg of lyophilized extract/kg body weight by i.p. route. The study consisted of electron microscopy examination and assay of several enzymes 30 min, 3, 12, 24 and 48 h and 5 days after injection of the extract. The electron microscopy observations were made on livers fixed in osmic acid according to PALADE and in glutaraldehyde/osmic acid.

Marked changes in the nuclear and nucleolar morphology were already present at 30 min whereas the modifications in the cytoplasm had only just begun and were very slight (Figures 1–4). In the nucleolus the formation of 'nucleolar caps' was noted: this ultrastructural change is marked by a decrease in nucleolar volume and the separation of the granular from the filamentous component of the nucleolus. There was also a groove between nucleolus and nucleolus-associated chromatin. The formation of 'nucleolar caps' is a characteristic feature of poisoning with agents that interfere with the metabolism of the nucleic acids and after irradiation of the nucleolus with UV-rays (MONTGOMERY et al.[3]). However, this change is

[1] TH. WIELAND and O. WIELAND, Pharmac. Rev. *11*, 87 (1959).
[2] L. FIUME, Lancet *1*, 1284 (1965).
[3] P. O. MONTGOMERY, R. C. REYNOLDS and J. E. COOK, Am. J. Path. *49*, 555 (1966).

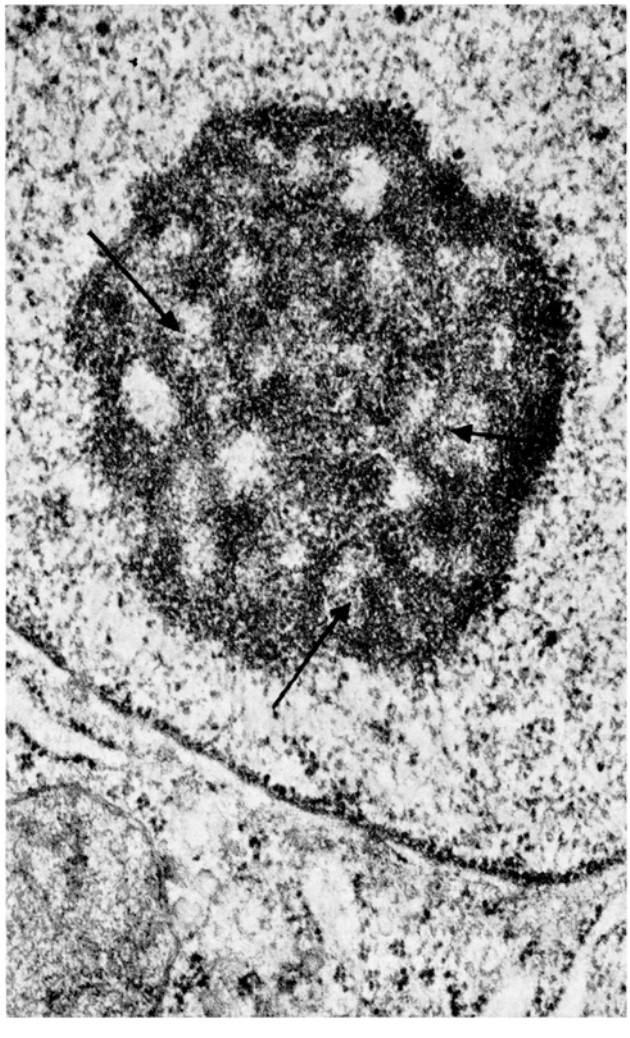


Fig. 1. Control animals. Nucleolus; the nucleolonema and 'pars amorpha' (arrows) are intimately mixed to form a rather compact structure. OsO_4 fixation. × 41.400.

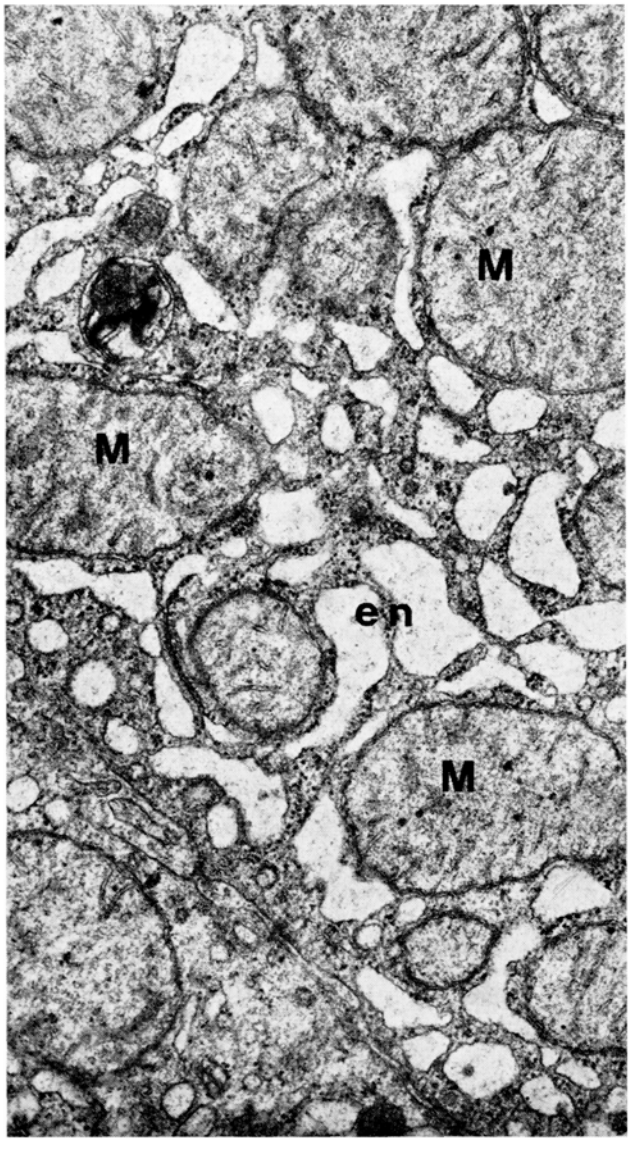


Fig. 2. Control animals. Several cytoplasmic organelles of normal appearance can be observed. OsO_4 fixation. × 32.000.

Mean values ± S.E. of lactic dehydrogenase (LDH), malic dehydrogenase (MDH), glutamic-oxalacetic (GOT) and glutamic-pyruvic transaminases (GPT), alkaline phosphatase (AP) and γ-glutamyl-transpeptidase (γ-GT) activity and DNA content in rat liver before and after *Amanita phalloides* intoxication

	Controls	30 min	3 h	12 h	1 day	2 days	5 days
LDH (μmol/min/g)	227 ± 53.8 (8)	233 ± 14.4 (4)	218 ± 58.9 (10)	256 ± 18.7 (9)	298 ± 58.5 (4)	236 ± 55.1 (6)	229 ± 27.9 (7)
MDH (μmol/min/g)	426.5 ± 46.7 (6)	466.0 ± 18.0 (4)	511.5 ± 86.5 $p < 0.05$ (7)	388.0 ± 25.2 (4)	397.5 ± 9.6 (4)	314.0 ± 56.0 $p < 0.01$ (5)	264.9 ± 9.7 $p < 0.001$ (4)
GOT (μmol/min/g)	101.00 ± 23.45 (8)	101.50 ± 27.05 (4)	105.75 ± 28.95 (10)	104.75 ± 16.10 (9)	99.38 ± 17.75 (4)	54.50 ± 44.20 $p < 0.001$ (6)	78.35 ± 12.50 $p < 0.05$ (7)
GPT (μmol/min/g)	15.100 ± 4.775 (8)	9.250 ± 1.775 $p < 0.01$ (4)	13.505 ± 3.180 (10)	19.500 ± 5.950 (9)	8.080 ± 3.670 $p < 0.02$ (4)	7.035 ± 1.085 $p < 0.001$ (6)	9.080 ± 1.950 $p < 0.01$ (7)
AP (10^3 Bessey Units/g)	11.57 ± 2.71 (7)	7.65 ± 2.65 $p < 0.05$ (4)	19.32 ± 9.35 $p < 0.05$ (10)	28.40 ± 2.30 $p < 0.001$ (9)	30.35 ± 2.56 $p < 0.001$ (4)	30.40 ± 12.80 $p < 0.02$ (4)	17.30 ± 3.80 $p < 0.02$ (6)
γ-GT (μmol/2 h/g)	1.520 ± 0.252 (6)	3.615 ± 2.110 $p < 0.05$ (6)	6.100 ± 3.640 $p < 0.001$ (10)	2.700 ± 0.424 $p < 0.001$ (9)	2.435 ± 1.065 (5)	4.210 ± 1.690 $p < 0.01$ (6)	4.380 ± 2.090 $p < 0.001$ (5)
DNA (mg/g)	2.02 ± 0.156 (6)	1.85 ± 0.218 (4)	1.54 ± 0.276 $p < 0.01$ (6)	1.77 ± 0.669 (4)	1.70 ± 0.60 (5)	2.09 ± 0.371 (4)	2.25 ± 0.276 (7)

No. of animals indicated in brackets

relatively aspecific and we cannot say whether the toxic action is exerted exclusively at RNA or DNA level (SVOBODA et al.[4]) (Figure 1).

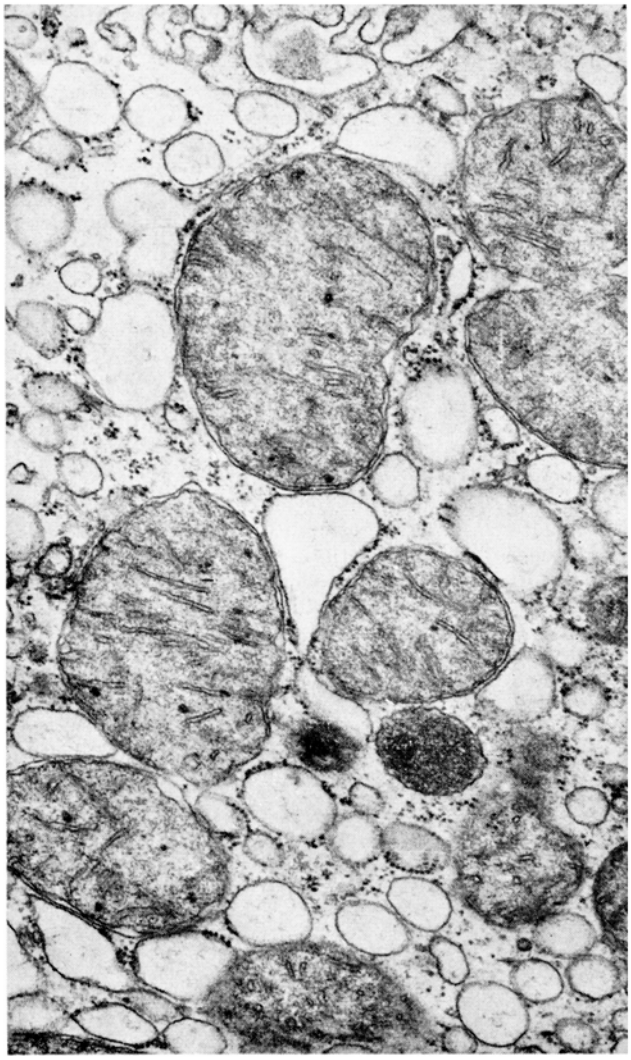
The changes described above became more marked later (specimens examined 3 h after intoxication), but at 12 and at 24 h there were some signs of attenuation and regression. Reorganization of nucleolar structure was evident in the specimens examined 2 and 5 days after the poisoning.

The behaviour of the hepatic DNA and of the considered enzymes (see Table) proved to be consistent with the changes in the organelles.

The significant decrease of DNA content 3 h after the poisoning correlates with the damage of the nuclear structures observed at the beginning of the intoxication. The fact that no changes were observed in lactic dehydrogenase activity, which is located in the cytoplasm, clearly correlates with the very small morphological damage of the cytoplasm. The increased activity of gamma-glutamyltranspeptidase, observed after 30 min and 3 h, probably comes from the detachment of this enzyme from the ribosomal structures. The modifications of the malic dehydrogenase, glutamic-pyruvic- and glutamic-oxalacetic-transaminase, γ-glutamyltranspeptidase activities at the second and fifth day after the poisoning could not be

[4] D. SVOBODA and J. SOGA, Am. J. Path. *48*, 347 (1966).

Fig. 3. 3 h after the poisoning. Segregation of nucleolus-associated chromatin (Ch). The nucleolus is markedly reduced in size. There is complete separation of the fibrillar (f) and granular (g) components of the nucleolus. Glutaraldehyde – OsO_4 fixation. × 42.000.



ascribed to the toxic action itself and seem to be linked to the reorganization of the cell. The behaviour of alkaline phosphatase is difficult to interpret: a cholostasis phenomenon does not seem a sufficient explanation.

These serial investigations point to some conclusions: in our experimental conditions the early evidence of structural damage from total extract of *A. phalloides* Fr. occurs in the nucleus and nucleolus and it is in these structures that the changes begin to regress; the signs of damage of the cytoplasm and its regression occur after nuclear changes. The reversibility of hepatic cell damage in the rat varies with the dose given, for there is gradual normalization of the intracellular organelles and enzymatic activity.

Riassunto. Nel ratto intossicato con estratto di *Amanita phalloide* le prime alterazioni strutturali dell'epatocita si realizzano a livello del nucleo e del nucleolo con formazioni di «nucleolar caps». Da queste stesse strutture iniziano i fenomeni di regressione delle alterazioni. Le manifestazioni sia del danno che della sua regressione a livello citoplasmatico risultano essere successive a quelle nucleari. Il comportamento del DNA e di alcuni enzimi del tessuto epatico è risultato nel complesso coerente con le alterazioni morfologiche. In rapporto con la dose usata queste alterazioni risultano reversibili poichè nelle cellule colpite entro 12 h cominciano i fenomeni di ristrutturazione.

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9 October 1967.

Fig. 4. 12 h after the poisoning. Depletion of glycogen granules. Disorganization of granular endoplasmic reticulum with dilatation of cisternae and loss of ribosomes. OsO_4 fixation. × 31.000.

Hypercoagulability and Thrombocytopenia After Platelet Factor 4 Infusion into Rabbits

Purified platelet factor 4 (PF_4) possesses several biological activities: it neutralizes heparin, it precipitates a solution of purified fibrinogen and it induces non-enzymatic clotting of soluble fibrin monomer complexes (paracoagulation). The latter reaction is strongly inhibited by citrate, oxalate and EDTA. PF_4 is rapidly released during platelet aggregation induced in platelet-rich plasma (PRP) by ADP, thrombin, adrenaline and collagen[1,2]. It is also released in vivo following thrombin infusion into rabbits[3]. PF_4 does not aggregate platelets in PRP, which is probably due to the inhibitory effect of citrate, and it only enhances platelet aggregation by ADP. For this reason it seemed of interest to study the effects of PF_4 in vivo.

PF_4 was isolated from pig platelets according to FARBISZEWSKI et al.[4]. The final product dissolved in 0.9% NaCl contained 160 μg protein/ml. In a dilution of 1:8 it shortened the heparin thrombin time of rabbit platelet-poor plasma from 75–26 sec. Purified PF_4 solution at a dose of 5 ml/rabbit was injected i.v. into the marginal ear vein of 5 rabbits (1.5–2 kg weight). Blood samples were obtained by heart puncture before and 5, 15 and 45 min after the injection. Plastic clotting time, platelet count, fibrinogen level, 'stypven' (Roussel viper venom, Borrough and Welcome, London) clotting time, and thrombin time were determined using routine laboratory methods. Platelet adhesiveness was estimated according to HELLEM[5]. Moreover, PF_4 was determined in platelet-poor plasma by

[1] S. NIEWIAROWSKI, A. POPŁAWSKI, B. LIPIŃSKI and R. FARBISZEWSKI, Conf. Platelets in Hemostasis, Miemo, Italy, Sept. 1967 (Karger, Basel in press).
[2] S. NIEWIAROWSKI, B. LIPIŃSKI, R. FARBISZEWSKI and A. POPŁAWSKI, Experientia, in press.
[3] R. FARBISZEWSKI, S. NIEWIAROWSKI, K. WOROWSKI and B. LIPINSKI, Thromb. Diath. haemorrh., in press.
[4] R. FARBISZEWSKI, S. NIEWIAROWSKI and A. POPŁAWSKI, Biochim. biophys. Acta *115*, 397 (1966).
[5] A. J. HELLEM, Scand. J. clin. Invest. *12*, 57 (1960).