*Br. J. exp. Path.* (1969) **50**, 270.

# THE EFFECTS OF AMANITIN POISONING ON MOUSE KIDNEY

L. FIUME, V. MARINOZZI AND F. NARDI

*From the Istituto di Patologia Generale dell'Università di Bologna, and Istituto di Anatomia e Istologia Patologica dell'Università di Pisa, Italy*

Received for publication December 23, 1968

**SUMMARY.—The effect of α- and β-amanitin on mouse kidney and rat kidney have been studied.**

**The experiments on adult male mice show that (1) an MLD of α- or β-amanitin always produces necrosis in the kidneys but never in the liver; (2) with the doses used (up to 3 MLD) necrosis of the kidneys never appears less than 3 days after injection; (3) necrosis of the liver only appears with doses above MLD and generally within 2 days.**

**No lesions were found in rat kidney after injection of amanitin.**

**Mice injected with the conjugate of β-amanitin with albumin from rabbit serum all died from necrosis of the liver without any lesions in the kidney, 3 days after i.p. injection. It has been deduced that amanitin poisoning in mouse kidney depends on reabsorption in the tubules, that either the liver or the kidney can be the target organ of amanitin (depending on the dose) and that in rats nephrosis is prevented because there is no reabsorption of amanitin in the kidney tubules.**

**The earliest ultrastructural changes occur in nuclei. First there is fragmentation of nucleoli and segregation of its granular and fibrillar components. At a second stage these fragments fall in number and then tend to disappear. At the same time there is a temporary increase in the number of perichromatin granules. Chromatin condensates at the borders of the nucleus and there is a big increase in numbers of interchromatin granules at the centre.**

**Changes in the cytoplasm appear just before necrosis sets in.**

THE main toxin in the toadstool *Amanita phalloides* is α-amanitin (Wieland, 1968). It kills mice in only a few days by damage done to the liver and kidneys. The earliest histological and ultrastructural changes in the liver are those in the nucleus (Fiume and Laschi, 1965). Here the nucleolus has been seen to break up. It has been shown biochemically that RNA synthesis in the liver stops very soon, falling by 50 per cent after only 30 min. (Stirpe and Fiume, 1967).

The experiments reported here were done to deepen knowledge about the damage done to mouse kidney by α-amanitin. The starting-point for this project was the observation that when adult male mice are killed by a minimum lethal dose (MLD) of α-amanitin, necrosis is always present in the kidney but never in the liver.

Research has also been done on β-amanitin, another toxin present in *Amanita phalloides*. Its chemical formula is identical with that of α-amanitin, except that there is a free carboxyl group instead of a carboamide group. The MLD of the 2 toxins are roughly equal, but there is more α-amanitin than β-amanitin in the fungus (Wieland, 1968).

Only the proximal tubules in mouse kidney are affected by necrosis from α- and β-amanitin. So we thought necrosis might be due to reabsorption of toxins filtered

through the glomeruli. To test this idea a conjugate was used. It was produced by linking β-amanitin and a protein with a covalent bond.

Lastly, the action of α-amanitin on rat kidney and liver was studied because rats, unlike mice, rabbits and guinea-pigs, are very resistant to it (Wieland, 1957; Fiume and Laschi, 1965).

## MATERIALS AND METHOD

Swiss albino male mice weighing 25–30 g. and male Wistar rats weighing 200–220 g. were used. α- and β-amanitin (kindly given by Professor T. Wieland) were administered by i.p. injection. They were dissolved in physiological saline. For all doses of amanitin injected the volume of the solution was 0·1 ml. per 10 g. body weight.

The β-amanitin conjugate was obtained by Cessi and Fiume (1969), using a soluble carbodiimide (ethyl CDI) to combine β-amanitin and albumin from rabbit serum according to Permutt, Parker and Utiger (1966). This conjugate contained 2·3 moles of β-amanitin for every mole of albumin. With i.p. injection the MLD for adult male mice was 8 μg. per 10 g. body weight.

β-amanitin after conjugation was roughly 10 times more toxic than before. This must be due to the much slower elimination of the toxin when it has been combined with a molecule which only filters in tiny quantities through the glomeruli (Cessi and Fiume, 1969).

The conjugate was administered by i.p. injection after being dissolved in physiological saline. The volume of solution injected was 0·1 ml. per 10 g. body weight.

For light microscopy liver and kidneys were removed from dead mice or mice killed as they were dying and fixed in Bouin's solution. Sections were cut from paraffin embedded blocks and stained with haematoxylin and eosin.

For electron microscopy specimens were obtained under ether anaesthesia. The samples were immediately fixed in one of the following 3 solutions standing in an ice-bath: (a) 4 per cent formaldehyde and 2·5 per cent glutaraldehyde; (b) 4 per cent formaldehyde and 1 per cent acrolein; (c) 1 per cent osmium tetroxide.

In all 3 cases the fixatives were dissolved in a 0·1 M Sorensen phosphate buffer.

In some cases the kidneys were fixed by perfusion with mixture (a) at room temperature. Some of the samples already fixed with (a) were postfixed with (c). All the samples fixed in $OsO_4$ alone or double-fixed were embedded in an araldite-epon mixture according to Mollenhauer (1964).

The samples fixed with (a) only were embedded in glycolmethacrylate (Leduc, Marinozzi and Bernhard, 1963).

Ultrathin sections were stained with uranyl acetate or double-stained with uranyl acetate and lead citrate (Reynolds, 1963).

Some of the sections embedded in glycolmethacrylate were digested for 1 hr at 37° with a 0·1 per cent ribonuclease dissolved in a phosphate buffer at pH 6·2; and others with a 0·5 per cent pepsin solution in 0·1N HCl. Afterwards both groups were stained with uranyl acetate.

## RESULTS

### *Light microscopy*

*Experiments with α- and β-amanitin.*—Fifteen mice were injected i.p. with an MLD of α-amanitin (3·5 μg. per 10 g. body weight). Seven died and 8 were killed while dying. The time between injection and death was 3–5 days.

In all 15 mice there was necrosis of the proximal convoluted tubules in the kidney (Fig. 1). None of the 15 had necrosis of the liver. In some of the mice, however, there was slight steatosis in hepatocytes together with nuclear lesions (condensation and margination of chromatin, apparently empty nucleoplasm and fall in volume of nucleolus). These gave nuclei a vesicle-like appearance.

Ten mice were injected with 5 μg. of α-amanitin per 10 g. body weight (about 1·5 MLD). Two mice died and 8 were killed while dying. The time between injection and death was 2 days for 2 mice and 3–5 days for 8.

The 2 mice which died or were killed after 2 days had no necrosis of the kidneys. But the nuclei of epithelial cells in the proximal convoluted tubules looked vesicle-like (Fig. 2) because of condensation and margination of chromatin, apparent thinning of nucleoplasm and fall in volume of nucleolus. These 2 mice had marked steatosis of the liver with big areas of necrosis.

Of the other 8 mice in the group (surviving 3–5 days after injection) all had necrosis of the proximal tubules in the kidney but none necrosis of the liver. In a few of them there was slight steatosis in hepatocytes and the same changes in the nuclei as described above.

Ten mice were injected with 10 $\mu$g. of $\alpha$-amanitin per 10 g. body weight (about 3 MLD). Five mice died and 5 were killed while dying. The time between injection and death was 1 day for 1 mouse, 2 days for 6, 3 days for 1 and 4 days for 2. There was no necrosis of the kidney in the 7 mice dying within 2 days, but the nuclei in the epithelium of the proximal convoluted tubules had the vesicle-like appearance typical of the early stages of $\alpha$-amanitin poisoning. In the 3 mice dying after 3–4 days there was necrosis in these tubules.

All these mice had very severe steatosis and necrosis of the liver except for the 2 mice dying after 4 days. These had but slight steatosis.

Ten mice were injected with 4 $\mu$g. of $\beta$-amanitin per 10 g. body weight (given as MLD by Wieland, 1968). Two mice died, 4 were killed while dying and 4 survived. The 6 that died lasted 4–5 days. All of these had necrosis of the proximal tubules, but none of them had necrosis of the liver. In 3 there was apparent emptiness in the nuclei of some hepatocytes.

*Experiments with the conjugate of $\beta$-amanitin with rabbit albumin.*—Ten mice were injected with the MLD of the conjugate. Six died and 4 were killed while dying. The time between injection and death was 3–4 days. All the mice had necrosis of the liver but none had necrosis of the kidneys or any other renal lesions. Five mice were injected with 4 MLD. All died from necrosis of the liver without any lesions in the kidneys.

*Electron microscopy*

*Mouse kidney.*—Fifteen mice were injected i.p. with $\alpha$-amanitin. The dose was 5 $\mu$g. per 10 g. body weight (about 1·5 MLD). The time between injection and death was 15 min. for 1 mouse, 30 min. for 1, 1 hr for 2, 3 hr for 2, 15 hr for 1, 24 hr for 1, 37 hr for 1, 2 days for 1 and 3 days for 5. Two mice were given 2 doses with a 1 hr interval between. They were killed 1 hr later. Two other mice were given 4 doses with 1, 3 and 3 hr intervals. Both were killed 2 hr later.

Electron microscopy confirmed that lesions from $\alpha$-amanitin are limited exclusively to the epithelium of proximal convoluted tubules. The earliest ultrastructural changes are in nuclei.

Before going ahead with a description of these lesions a short description will be given of how a normal nucleus looks in the epithelium of these tubules.

Chromatin is grouped in small masses. It is found mainly in 2 areas: at the borders of the nucleus (touching the nuclear membrane) and round the nucleolus (" nucleolus associated chromatin "). It is also, however, distributed fairly evenly right through the nucleoplasm (Fig. 3).

The nucleolus, which is roughly spherical, consists of trabecular structures which anastomose as a reticulum. In this granules and fibrils can be distinguished

(Marinozzi, 1964). The granules, which are mixed with the fibrils, are sometimes distributed right through the nucleolus. More often they occupy only part of it.

Many perichromatin granules (Watson, 1962) can be seen. They are always very near the masses of chromatin, whether these are at the borders of the nucleus, round the nucleolus or scattered in other parts of the nucleus. In spaces between masses of chromatin small clusters of electron dense granules can be seen. These are called interchromatin granules (Swift, 1959).

The effects of $\alpha$-amanitin poisoning can be seen after only 15–30 min. The nucleolus breaks up (Fig. 4) and the granular and fibrillar components in it segregate—separating and becoming redistributed in different areas (Fig. 5 and 6). One single nucleolus can become 4–5 fragments. These fragments usually consist of granules only or fibrils only. But occasionally you can find a fragment containing 2 zones, one granular and one fibrillar. The nucleolus-associated chromatin seems to break down too. It clusters round each of the new fragments (Fig. 5). These fragments get smaller and fewer. At the same time a lot of electron dense granules appear near them. These are single or clustered in groups of 3 or more round a central homogeneous substance (Fig. 7). This is less electron dense than them and can be digested with pepsin. The granules seem to come out of the substance like buds.

The granules range between 300 Å and 400 Å in diameter and this, together with the clear space about 200 Å wide which always separates them from chromatin, makes them look like perichromatin granules. Identical granules and granule-clusters appear near the chromatin at the borders of the nucleus.

Only 30 min. after injection of $\alpha$-amanitin there is a clear tendency for chromatin to condensate at the borders of the nucleus. At the same time bigger and bigger clusters of electron dense granules about 200 Å in diameter appear in the central area (Fig. 8). They are morphologically identical with interchromatin granules.

At the later stages there are even fewer fragments of nucleolus, and those containing granules disappear. Twenty-four hr after 1 dose or 9 hr after 4 doses no granule-containing fragments are left. The few small residues of nucleolus look almost homogeneous and no fibrillar structures can be made out either. Digestion with ribonuclease of sections from blocks embedded in glycolmethacrylate leaves their electron density almost unchanged. This strongly suggests their RNA content is virtually nil.

The other changes described above—condensation of chromatin at the borders of the nucleus and the accumulation of interchromatin granules at the centre of the nucleus—continue. In the end chromatin is found exclusively near the borders, in compact masses, and the centre is taken up by residues of nucleolus and lots of interchromatin granules.

Changes in the cytoplasm appear late, and evolve rapidly towards necrosis. Forty-eight hr after 1 injection the number and size of dense bodies increase. Many autophagic vacuoles appear too. In some cells mitochondria swell up, and a lot of electron dense material appears in the matrix.

Seventy-two hr after 1 injection of $\alpha$-amanitin almost all the epithelial cells in the proximal tubules are totally necrotic. The basement membrane is sometimes completely bare (Fig. 9) and sometimes covered by a thin layer of cytoplasm, only a few micron thick, belonging to a flattened cell which has lost its brush borders (Fig. 10). Necrotic fragments of epithelial cells are found in the most distal

tubules of the nephron. They are often mixed with an amorphous substance which looks like coagulated plasma.

*Rat liver and kidney.*—Eight rats were injected i.p. with α-amanitin. The dose was 5 μg. per 10 g. body weight. One rat was killed after 15 min., 1 after 30 min., 2 after 1 hr, and 1 each after 3, 10 and 24 hr.

One rat was given 4 doses with 3-hr intervals. It was killed 4 hr later.

No morphological changes—in nuclei or cytoplasm—appeared in any of rat kidney.

In the liver changes appear only 15–30 min. after injection. They are exactly like those described above for cells in proximal tubules in mouse kidney. In rats given 1 injection of α-amanitin lesions were worst after 1–3 hr. They began to improve after 10 hr. After 24 hr hepatocytes looked absolutely normal.

In the rat given 4 doses damage to nuclei was as bad as that usually seen in mouse liver 12–24 hr after 1 injection.

Lesions in rat and mouse liver will be described in more detail in another paper specially devoted to changes in nuclei produced by α-amanitin (Marinozzi and Fiume, unpublished).

---

## EXPLANATION OF PLATES

FIG. 1.—Mouse killed 72 hr after 1 injection of α-amanitin. Necrosis of proximal convoluted tubules is complete. Glomeruli and distal tubules are normal. × 160.

FIG. 2.—Mouse killed 48 hr after 1 injection of α-amanitin. The apparent emptiness at centre of nuclei in epithelium of proximal convoluted tubules is produced by condensation of chromatin at borders of nuclei. × 1100.

FIG. 3.—Normal nucleus in epithelial cell from proximal convoluted tubules. Fairly well-defined masses of chromatin (Ch) are arranged mainly round the nucleolus (n) and touching the inner surface of the nuclear membrane. Thin tongues or offshoots of chromatin can be seen throughout the nuclear sap. Arrows point to perichromatin granules. Staining—uranyl acetate and lead citrate. × 24,000.

FIG. 4.—Mouse killed 30 min. after 1 injection of α-amanitin. Nucleus on left is from epithelium in a distal convoluted tubule. Nucleus on right from a proximal one; the nucleus here is lighter, obviously because of condensation of chromatin at borders: note also fragmentation of nucleolus. Staining—uranyl acetate and lead citrate. × 6000.

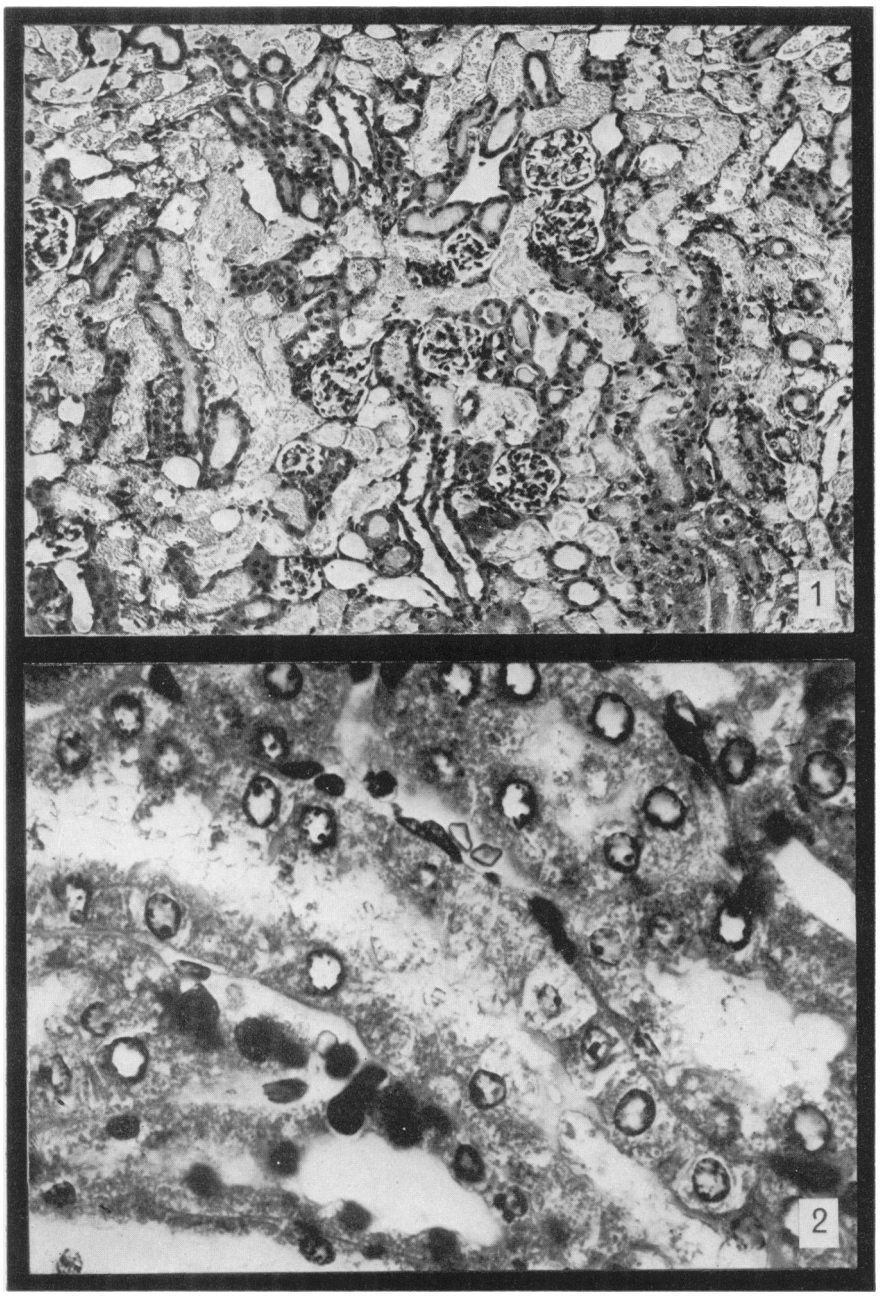
FIG. 5.—Mouse killed 30 min. after 1 injection of α-amanitin. On right 5 fragments of nucleolus can be clearly seen. Some show clear segregation into granular zone (gz) and fibrillar zone (fz). Nucleolus-associated chromatin too seems fragmented and redistributed round nucleolar remnants. Clusters of interchromatin granules (ig) are visible in the central zone of the nucleus. Staining—uranyl acetate and lead citrate. × 30,000.

FIG. 6.—Mouse killed 1 hr after 1 injection of α-amanitin. Four nucleolar fragments are visible, but only 1 contains granular components. The other 3 are predominantly fibrillar in structure. Clusters of granules can be seen round these fragments, seeming to break away from outer edges of nucleolar fragments. Staining—lead citrate. × 60,000.

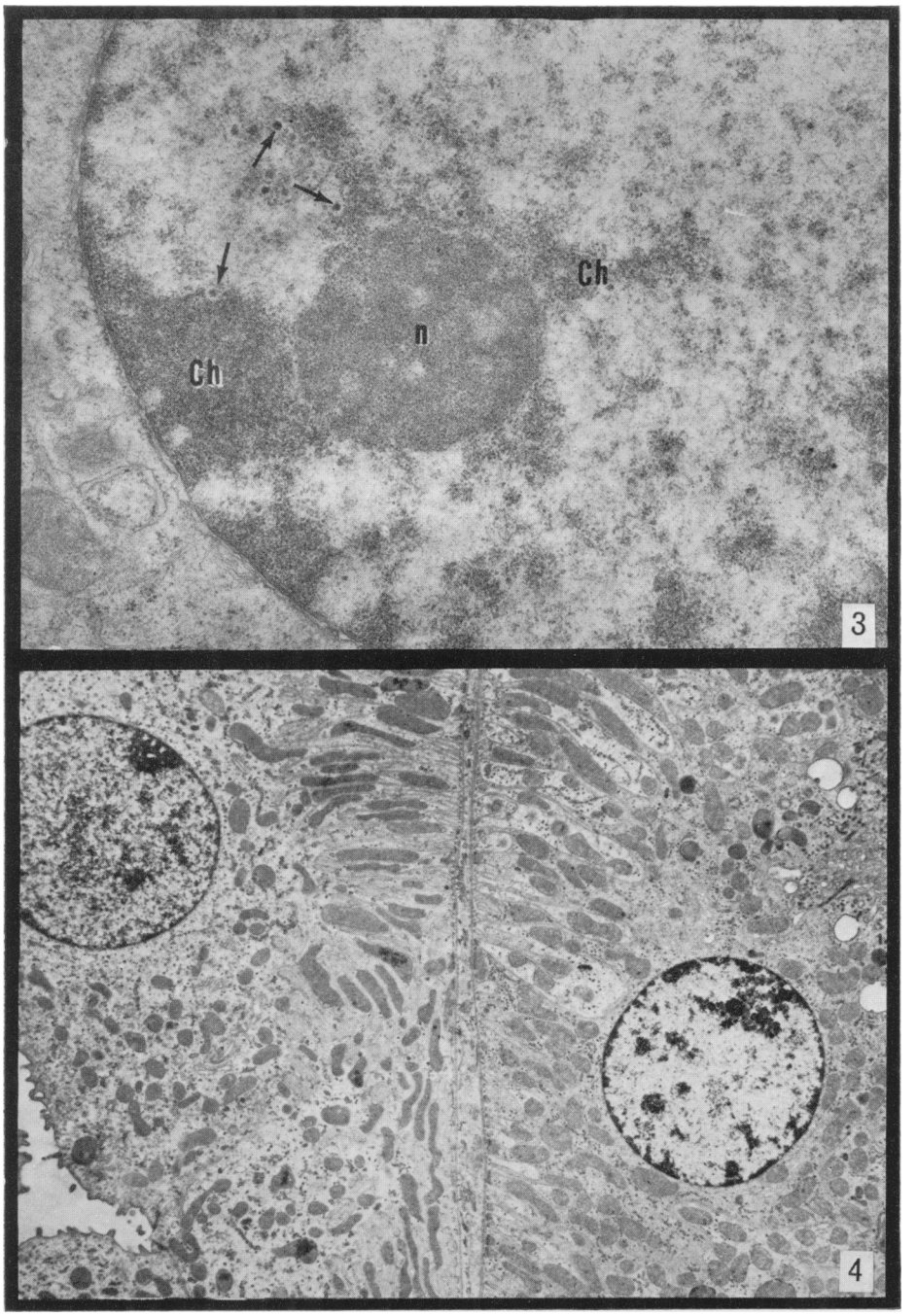
FIG. 7.—Mouse given 4 doses of α-amanitin and killed 9 hr after 1st injection. Round nucleolar residue (n) many large electron-dense granules (arrows) can be seen. They are grouped in a crown formation round polymorphous masses of an amorphous substance (as), which is less electron-dense. A clear thin halo separates these granules from the surrounding chromatin (ch). Staining—uranyl acetate and lead citrate. × 120,000.

FIG. 8.—Mouse killed 48 hr after 1 injection of α-amanitin. Epithelial cells in proximal convoluted tubules. Very marked condensation of chromatin, which seems concentrated exclusively at borders of nuclei, touching nuclear membrane. Clusters of interchromatin granules (ig) are easily seen at centre. At this stage there are still no important changes in cytoplasm. Staining—uranyl acetate and lead citrate. × 6000.

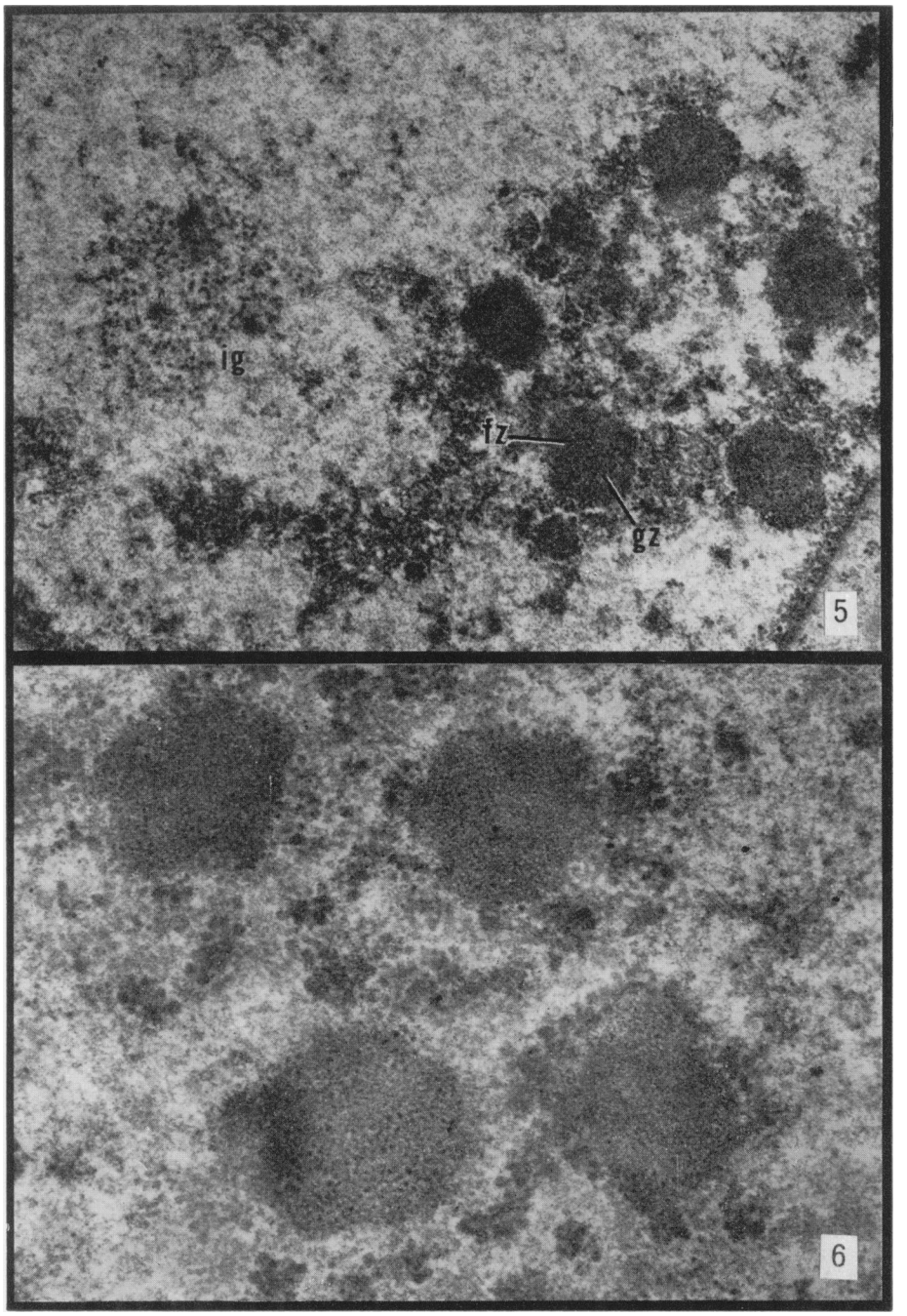
FIG. 9 and 10.—Mouse killed 72 hr after 1 injection of α-amanitin. Fragments of necrotic epithelium are visible in the lumina of proximal convoluted tubules (PCT), long stretches of whose walls consist of only basal membrane (bm). Fig. 10 shows a residual epithelial cell (Ep). It is flattened and has no brush border. It covers much of the circumference of the tubule. There are no big changes either in the epithelium on the walls of Bowman's capsule (bottom of Fig. 9) or in that in the collector tubules (bottom of Fig. 10). Staining—uranyl acetate and lead citrate. Fig. 9, × 6000; Fig. 10, × 12,000.



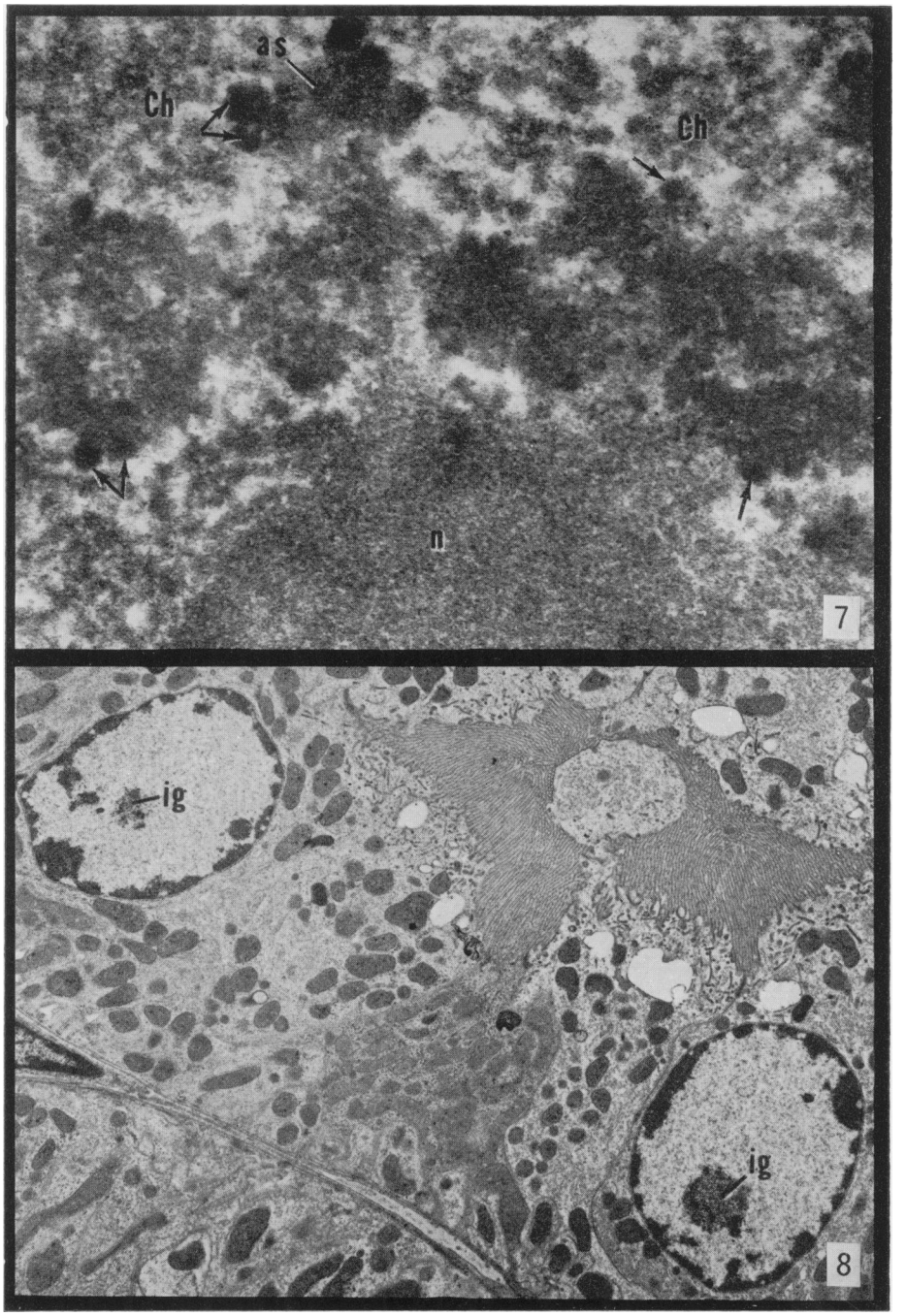

Fiume, Marinozzi and Nardi.



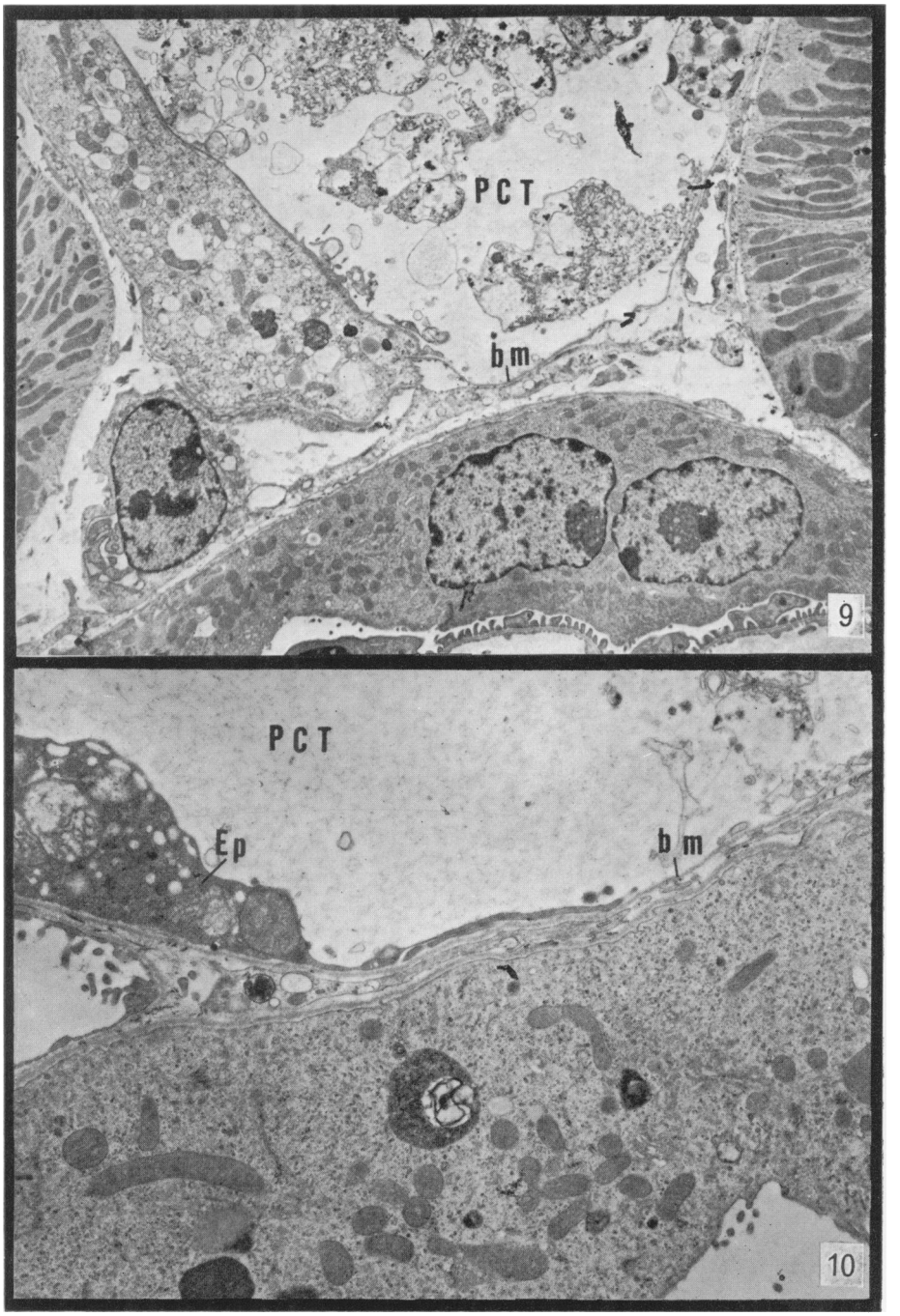

Fiume, Marinozzi and Nardi.




Fiume, Marinozzi and Nardi.




Fiume, Marinozzi and Nardi.




Fiume, Marinozzi and Nardi.

## DISCUSSION

The experiments in adult male mice show that:

(1) An MLD of $\alpha$- or $\beta$-amanitin always produces necrosis in the kidneys but never in the liver;
(2) with the doses used (up to 3 MLD) necrosis of the kidneys never appears less than 3 days after injection;
(3) necrosis of the liver only appears with doses above MLD;
(4) where there is necrosis of the liver, it can result in death before necrosis of the kidney has had time to develop, so in all mice given more than an MLD and dying within 2 days there was necrosis of the liver but not of the kidneys.

In mice, therefore, the target organ can be the kidney or the liver, depending on the dose used.

The only kidney cells damaged by amanitin are those in the proximal tubules. As with the liver (Fiume and Laschi, 1965) the earliest ultrastructural changes occur in nuclei. Changes in cytoplasm begin after 48 hr, and then evolve rapidly towards necrosis.

The changes in nuclei, which are identical with those in hepatocytes (Marinozzi and Fiume, unpublished) are the fragmentation nucleoli, with the segregation and, later, disappearance of ribonucleoprotein components, the margination and condensation of chromatin, temporary increase in the number of perichromatin granules, and the accumulation of interchromatin granules in the central part of nuclei.

The segregation of granules and fibrils in nucleoli is the most typical morphological result of poisoning with actinomycin D (Schoefl, 1964; Jezequel and Bernhard, 1964) and with a wide variety of other antimetabolites (Simard and Bernhard, 1966; Bernhard and Granboulan, 1968). These are chemically heterogeneous but do have one thing in common with actinomycin D—they block the synthesis of RNA by combining with DNA and so stopping transcription by RNA polymerase.

$\alpha$-amanitin strongly inhibits the activity of RNA-polymerase activated by $Mn^{2+}$ and $(NH_4)_2SO_4$, but, unlike actinomycin D, it hardly inhibits the activity of RNA-polymerase activated by magnesium (Stirpe and Fiume, 1967).

The only morphological effect which makes $\alpha$-amanitin and actinomycin D look similar is the segregation they produce in nucleoli. Fragmentation of the nucleolus and early condensation of chromatin—2 of the most striking effects of $\alpha$-amanitin—have never been reported as relevant findings for actinomycin D.

Within the kidneys only the proximal convoluted tubules are damaged by amanitin. Probably, therefore, nephrosis is due to amanitin reabsorption in these tubules. Both $\alpha$- and $\beta$-amanitin have low M.W. (about 1000), so they probably filter through the glomeruli with preurin. They would then be reabsorbed from preurin in the cells of the proximal convoluted tubules. These cells would, in that case, contain higher concentrations of amanitin than any other part of the nephron.

With an MLD of amanitin damage to the kidneys develops as far as necrosis, whereas damage in the liver is reversed. This is easy to explain if it is supposed that concentrations in the proximal tubules remain high, after—or long after—levels in the liver have fallen below what is necessary for a cytopathic effect.

Confirmation of the hypothesis that tubular necrosis from amanitin poisoning in mouse kidney is due to the absorption of amanitin by preurin is provided by

experimenting with the conjugate of $\beta$-amanitin with a protein which only filters through the mouse glomeruli in minute quantities. Necrosis is much worse in the liver but completely prevented in the kidneys.

If these hypotheses are correct the fact that $\alpha$-amanitin produces no lesions at all in rat kidney and that damage is very quickly reversed in the liver would be explained by an incapacity of epithelial cells in rat kidney tubules to reabsorb amanitin from preurin.

This research was supported by grants from the C.N.R. (National Research Council) of Italy.

We would like to thank Prof. T. Wieland for his generous gift of amanitins. Also we wish to express our gratitude to the translator Dr. A. Johnson of Pisa University.

## REFERENCES

BERNHARD, W. AND GRANBOULAN, N.—(1968) In ' The Nucleus ' (Eds. Dalton, A. J. and Haguenau, F.), New York (Academic Press), p. 81.
CESSI, C. AND FIUME, L.—(1969) *Toxicon*, in press.
FIUME, L. AND LASCHI, R.—(1965) *Sperimentale*, **115**, 288.
JEZEQUEL, A. M. AND BERNHARD, W.—(1964) *J. Microscopie*, **3**, 279.
LEDUC, E. H., MARINOZZI, V. AND BERNHARD, W.—(1963) *J. roy. microscop. Soc.*, **81**, 119.
MARINOZZI, V.—(1964) *J. ultrastruct. Res.*, **10**, 433.
MOLLENHAUER, H. H.—(1964) *Stain Technol.*, **39**, 111.
PERMUTT, M. A., PARKER, C. W. AND UTIGER, R. D.—(1966) *Endocrinology*, **78**, 809.
REYNOLDS, E. S.—(1963) *J. Cell Biol.*, **17**, 208.
SCHOEFL, G. I.—(1964) *J. ultrastruct. Res.*, **10**, 224.
SIMARD, R. AND BERNHARD, W.—(1966) *Int. J. Cancer*, **1**, 463.
STIRPE, F. AND FIUME, L.—(1967) *Biochem. J.*, **105**, 779.
SWIFT, H.—(1959) Studies on nuclear fine structure; *Brookhaven Symp. Biol.*, **12**, 134.
WATSON, L. W.—(1962) *J. Cell Biol.*, **13**, 162.
WIELAND, T.—(1957) *Exposés ann. Biochem. Med.*, **19**, 107.—(1968) *Science*, **159**, 946.