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THE ACUTE TOXICITY OF RETRORSINE, AFLATOXIN AND STERIGMATOCYSTIN IN VERVET MONKEYS

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SUMMARY.—The 10 day LD_{50} values of retrorsine, aflatoxin and sterigmatocystin in vervet monkeys were determined. The values were 46, 3·7 and 32 mg./kg. body weight, respectively. All 3 of these naturally occurring compounds are hepatotoxins and they give rise to varying degrees of bile duct proliferation, central and midzonal hepatocellular degeneration and haemorrhagic necrosis. Furthermore, their toxic effects are manifested in the kidney, adrenal, testis and at high doses on the endothelium of small blood vessels. Dimethylsulphoxide, the organic solvent used for dissolving sterigmatocystin in these experiments, causes centrilobular fatty changes at low doses and diffuse hepatocellular fatty changes at higher doses.

FOR many years there has been speculation about the causes of the exceptionally high incidence of malignant hepatoma in certain Bantu tribes of Africa. Kennaway (1944) suggested that the aetiology is associated with some extrinsic rather than hereditary factors. Subsequently it was postulated that the indiscriminate medicinal use and consumption of Senecio plants, which may contain carcinogenic pyrollizidine alkaloids, is responsible for liver cancer in these tribes (Cook, Duffy and Schoental, 1950). More recently attention was focused on the possible role of carcinogenic mycotoxins in the aetiology of this disease (Oettlé, 1964).

As a result of these hypotheses, we decided to study the acute toxic effects of a pyrollizidine alkaloid and two carcinogenic mycotoxins in vervet monkeys. Retrorsine was selected as a representative alkaloid because it is amongst the most toxic of the senecio alkaloids and occurs in at least 9 *Senecio* spp. (Sapeika, 1952). Aflatoxin B_1, a metabolite of *Aspergillus flavus*, is considered to be the most carcinogenic compound yet discovered (Butler, 1964). Sterigmatocystin, a structurally related compound, produced by *Aspergillus nidulans*, *Aspergillus versicolor* and *Bipolaris* sp. (Holzapfel, Purchase, Steyn and Gouws, 1966) is also a potent hepatocarcinogen (Purchase and Van der Watt, 1968) Both of these toxins were included in the study.

MATERIALS AND METHODS

Thirty male vervet monkeys (*Cercopithecus aethiops*) were randomly divided into 3 groups of 8 monkeys each and a control group consisting of 6 monkeys. The monkeys were housed singly in metal cages in air-conditioned rooms (25° and 45 per cent ± 5 per cent humidity) with 16 air changes per hour. Every morning all monkeys received the standard monkey diet used in this laboratory which consists of cooked yellow maize meal, minced liver, milk powder and a powdered vitamin supplement. During the afternoon uncooked fruit and vegetables were supplied to the monkeys. Water was available *ad libitum*.

The retrorsine used for this experiment was crystalline, and was dissolved in a mixture of distilled water and concentrated hydrochloric acid (50 : 1), giving a final concentration of 10 mg./ml. Crystalline aflatoxin was produced in this laboratory, and was 70 per cent pure ($B_1 : B_2 : G_1 : G_2 = 71 : 14 : 10 : 5$). It was added to a 1·5 per cent solution of methyl cellulose in water and a suspension prepared by ultra-sonification of the mixtures. The final concentrations of aflatoxin in 4 different suspensions were 1·16 mg./ml., 2·5 mg./ml., 5·4 mg./ml., and 11·6 mg./ml. The sterigmatocystin, also produced in this laboratory, was 80 per cent pure. Due to the low solubility of this toxin in non-toxic solvents, dimethylsulphoxide (DMSO) was used as a vehicle. The required amount of toxin for each dosage level was dissolved in 2, 5, 9 and 9 ml. of DMSO respectively

The dosage of toxin given to each monkey is given in Table I. On the day of dosing all the monkeys were sedated with an i.m. injection of phencyclidine hydrochloride (Sernylan, Parke-Davis and Company, Hounslow, Middlesex, England), at a rate of 1 mg./kg. body weight, after overnight starvation.

TABLE I.—*Toxicity of Retrorsine, Aflatoxin, Sterigmatocystin, DMSO and Methylcellulose*

Treatment	Monkey no.	Weight (kg.)	Dose/kg. body wt.	Survival time (days)
Retrorsine	44	4·8	10·0 mg.	10
	35	4·4	10·0 mg.	10
	22	4·7	21·0 mg.	10
	28	4·2	21·0 mg.	10
	38	4·2	46·0 mg.	10
	29	4·0	46·0 mg.	1
	24	4·4	100·0 mg.	3
	17	4·4	100·0 mg.	3
Aflatoxin	137	2·5	1·2 mg.	10
	146	2·0	1·2 mg.	10
	142	5·3	2·5 mg.	10
	140	4·3	2·5 mg.	10
	141	5·0	5·4 mg.	8
	143	4·8	5·4 mg.	6
	144	5·1	11·6 mg.	5
	148	4·2	11·6 mg.	1
Sterigmatocystin	12	5·2	15·0 mg.	10
	15	4·4	15·0 mg.	10
	6	3·8	32·0 mg.	10
	8	3·3	32·0 mg.	4
	14	5·1	70·0 mg.	7
	26	4·9	70·0 mg.	2
	3	2·5	150·0 mg.	6
	4	4·2	150·0 mg.	2
DMSO	10	4·4	0·4 ml.	10
	2	4·7	2·0 ml.	10
Methylcellulose	50	2·2	5·0 ml.	10
	58	1·2	5·0 ml.	10
	52	2·9	5·0 ml.	10
	21	6·0	5·0 ml.	10

In the experimental animals which received retrorsine or aflatoxin, and the controls which received methyl cellulose, disposable nelaton catheters 5 mm. in diameter were used for gastric intubation (Rüsch, West Germany). Sterigmatocystin was administered by i.p. injection after applying sterile precautions. After administration of the suspensions or control solutions both the intragastric tubes and i.p. needles were flushed with 4 ml. of sterile saline.

All animals that survived for a period of 10 days after dosing were killed by rapid i.v. pentobarbitone sodium administration at a dose rate of 180 mg./kg. body weight.

Specimens of liver, heart, lung, spleen, kidney, adrenal, testis, intestines and stomach were

obtained at post mortem from all animals and fixed in 10 per cent buffered formalin. Blocks of tissue were embedded in paraffin wax, and 0·5 μ sections were cut and stained with haematoxylin and erythrocin. When necessary sections were stained with Oil Red O, PAS or Wilder's reticulum stain.

Weil's technique (Weil, 1952) was applied to the data given in Table I for the determination of the LD_{50} values.

RESULTS

The LD_{50} values with 95 per cent confidence limits, are given in Table II.

TABLE II.—*LD_{50} Values for Retrorsine, Aflatoxin and Sterigmatocystin*

Toxin	LD_{50} mg./kg. body wt.	95 per cent confidence limits
Retrorsine	46	21·5 –100
Aflatoxin	3·7	2·5 – 5·4
Sterigmatocystin	32	15·0 – 70·0

Three monkeys had 1–2 cm. fluid filled tapeworm cysts (*Echinococcus* sp.) situated in the parenchyma of either the lungs (No. 148) or the livers (Nos. 146 and 52). Granulomata due to schistosomiasis were seen in the livers of 3 monkeys (Nos. 8, 28 and 140). These lesions were all periportal in location.

Control animals.—No macro- or microscopic alterations were observed in the animals dosed with methyl cellulose.

After i.p. administration of DMSO, the livers showed distinct centrilobular fatty changes at 0·4 ml./kg. body weight and diffuse hepatocellular fatty change at the level of 2 ml./kg. body weight.

Animals dosed with retrosine.—No macroscopic abnormalities were seen in monkeys killed after 10 days (Table I).

Histological examination of the kidneys obtained from the monkeys which received retrorsine (10 mg./kg.) showed a slight glomerulo-nephritis. In the livers single cell necrosis and a few small foci of midzonal necrosis could be observed. Haemorrhage occurred into these necrotic areas. Hepatocellular pleomorphism and Kupffer cell prominence was present. Oedema was seen in the perivascular and periductular stroma of the portal tract. Around a few large central veins subendothelial or perivenous oedema was present (Fig. 1).

The kidneys of the animals which received 21 mg./kg. had more severely damaged glomeruli, many of which were shrunken leaving a large Bowman's space. The livers showed bile duct reduplication and focal midzonal necrotic areas which frequently extended to include the centrilobular areas (Fig. 2). Many of these necrotic areas were filled with blood and resulted in the mottled effect seen macroscopically in one liver (No. 28). Bile stasis was prominent in the necrotic foci. Portal tract oedema and sub-endothelial oedema of portal veins with disruption of the endothelium could be seen.

The microscopic findings of the animal surviving the dose of 46 mg./kg. (No. 38) included hepatocellular midzonal and central necrosis, bile stasis, mild bile duct proliferation, congestion and megalocytosis. Portal tract oedema was seen and portal vein walls were distended and disrupted by oedema and haemorrhage, especially into the subendothelial and medial layers. The endothelial lining of these vessels was fragmented. Similar alterations were seen in the large central veins.

The liver of the monkey that died at this dosage level (No. 29) was macroscopically pale red-brown and friable with dark red foci diffusely distributed through the substance of the liver. Bile stasis and massive hepatocellular necrosis with haemorrhage resulting in distinct " blood pools " was observed microscopically.

Both monkeys (Nos. 24 and 17) died after administration of retrorsine (100 mg./kg.), and the macroscopic changes observed were petechial haemorrhages in the lungs, pericardium, duodenum and jejunum and diffuse mottling of a firm swollen liver. The caecae and colons contained uncoagulated dark red-black fluid. The microscopic changes consisted of massive haemorrhages into the wall of the small intestine between the lamina propria and the tunica muscularis, necrosis of duodenal epithelium, necrosis of the islets of Langerhans (Fig. 3) and focal necrotic areas affecting many acini in the pancreas. The kidneys exhibited a severe toxic nephritis and the parietal layers of the glomeruli were distinctly thickened (Fig. 4). Hyaline degeneration and necrosis of tubular epithelium together with a few hyaline casts within the tubules were seen. Haemorrhage occurred throughout the parenchyma and especially into necrotic areas. Zenker's hyaline degeneration was seen in the myocardium. The adrenal glands exhibited extensive haemorrhage into both cortex and medulla. The medullary cells were diffusely affected by fatty changes. The nuclei of some cells in the fascicular zone were karyorrhectic with 4 even sized fragments forming tetrads. In the testis interseminiferous tubular oedema with obliteration of interstitial tissue was seen and the sertoli cells and primary spermatogonia were detached from the basement membrane. The liver showed extensive haemorrhagic necrosis and, in less extensively affected areas, diffuse fatty changes and hepatocellular degeneration and necrosis were prominent (Fig. 5). Bile pigment could be seen intracellularly, mainly in areas where fat had been removed during the preparation of paraffin sections.

Monkeys dosed with aflatoxin.—In the monkeys receiving aflatoxin (1·16 mg./kg.) no macroscopic tissue changes were observed. Microscopically glomerular

EXPLANATION OF PLATES

FIG. 1.—Oedema in the wall of a central vein in the liver of a monkey which received retrorsine (10 mg./kg.) 10 days previously. H. and E. × 460.

FIG. 2.—Midzonal necrosis in the liver of a monkey which received retrorsine (21 mg./kg.) 10 days previously. H. and E. × 90.

FIG. 3.—Necrosis of an Islet of Langerhans in a monkey that died 3 days after receiving retrorsine (100 mg./kg.). H. and E. × 460.

FIG. 4.—Thickening of the parietal layer of Bowman's capsule 3 days after receiving retrorsine (100 mg./kg.). H. and E. × 145.

FIG. 5.—Liver of monkey 24 which died 3 days after receiving retrorsine (100 mg./kg.) showing haemorrhagic necrosis, fatty changes and hepatocellular degeneration. H. and E. × 90.

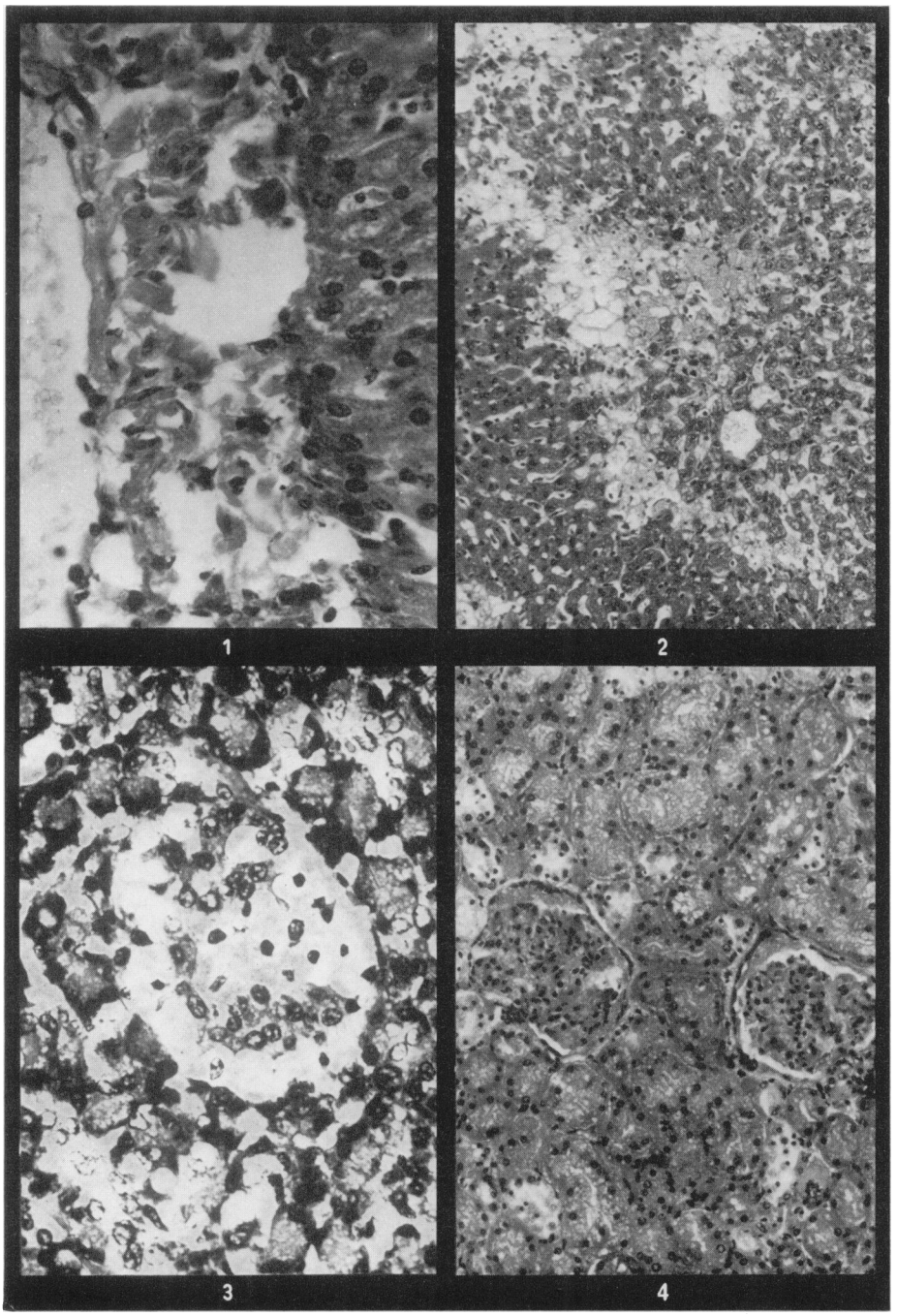
FIG. 6.—Liver of a monkey that died 8 days after receiving aflatoxin (5·4 mg./kg.) showing polygonal collapse with islands of hepatic tissue remaining around a portal tract. H. and E. × 90.

FIG. 7.—The same liver as shown in Fig. 6, illustrating distinct bile duct proliferation. H. and E. × 145.

FIG. 8.—Kidney of a monkey that was killed 10 days after receiving sterigmatocystin (15 mg./kg.) showing glomerular degeneration and oedema within Bowman's space. H. and E. × 145.

FIG. 9.—Central haemorrhagic necrosis of the liver after a dose of sterigmatocystin (70 mg./kg.). Periportal fatty changes and bile duct proliferation can also be seen. H. and E. × 90.

FIG. 10.—Glomerular degeneration and proximal and distal couvoluted tubular necrosis after a dose of sterigmatocystin (150 mg./kg.). H. and E. × 145.



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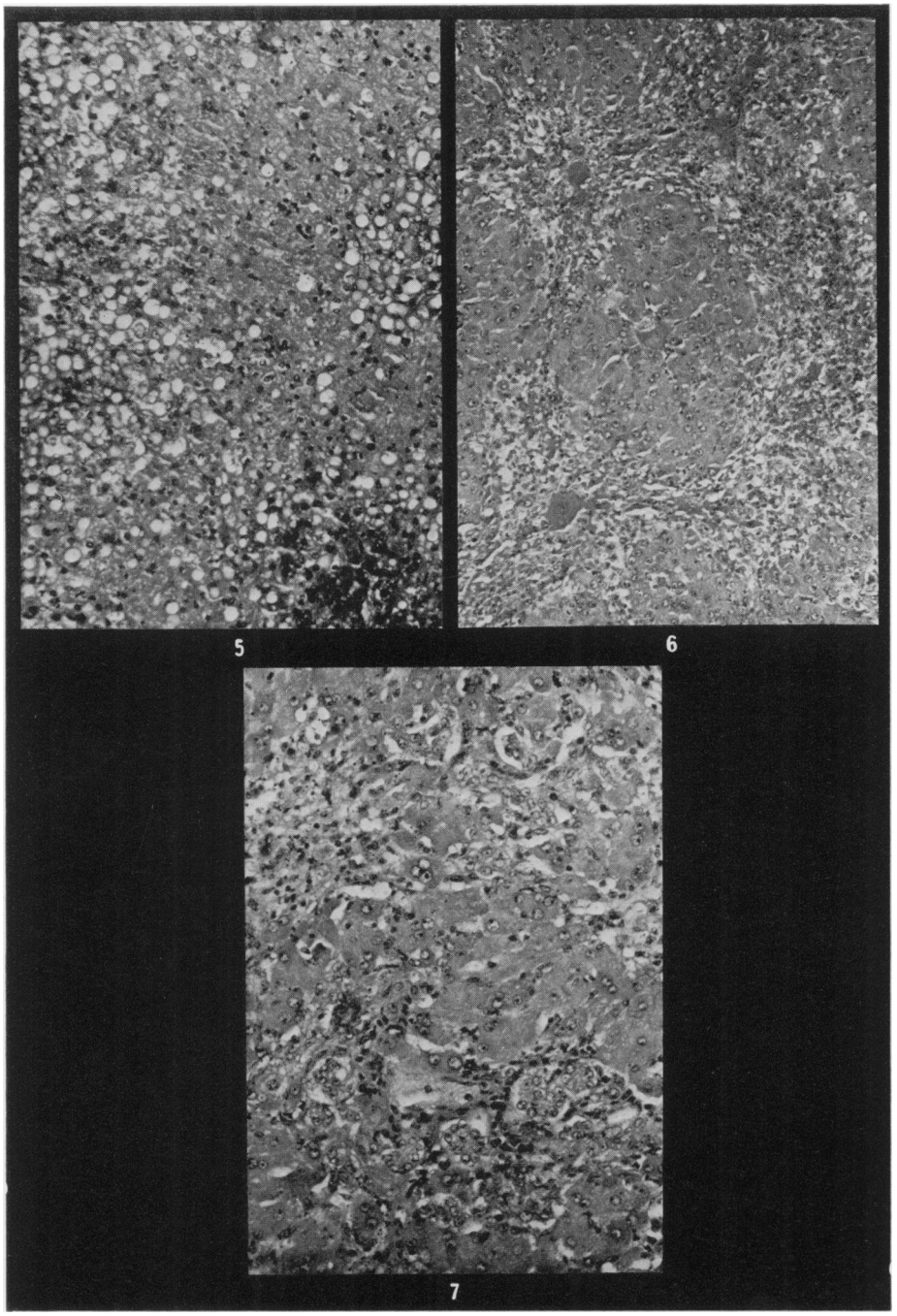

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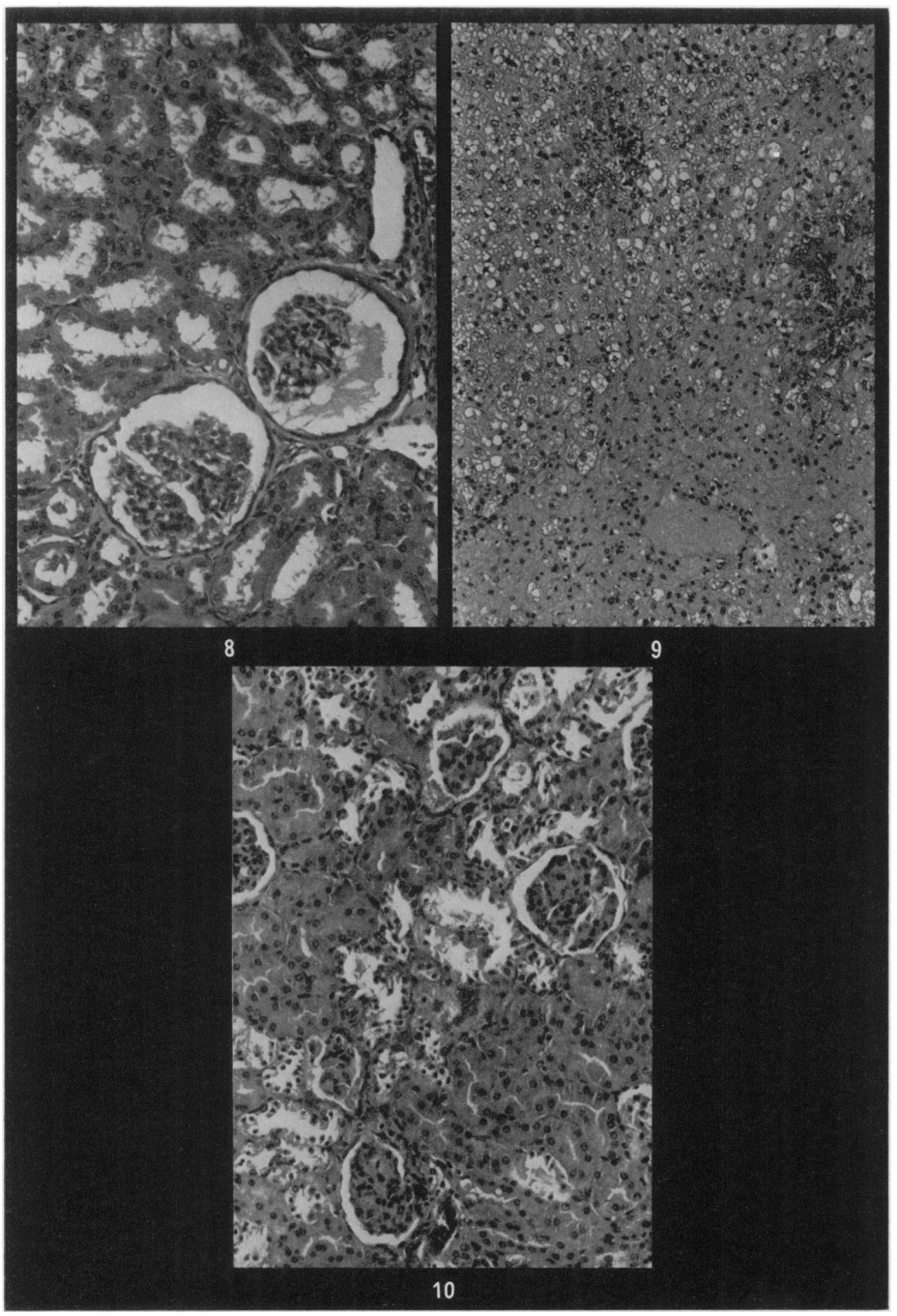

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degeneration with oedema in Bowman's space was seen together with hyalin droplet degeneration and necrosis of nephron epithelial cells. Severe fatty changes were seen in the collecting tubular epithelium. The cells of the adrenal medulla exhibited fatty changes and the medullary tissue contained dilated blood-filled spaces. In the liver periportal single cell necrosis and a few central multicellular necrotic foci were observed. The hepatocytes were vacuolated and these vacuoles contained PAS-positive material. Oedema and haemorrhage were seen in the portal tracts around the vessels and ducts and within the walls of the veins and arteries.

Both monkeys exposed to aflatoxin (2·5 mg./kg.) survived and on post-mortem examination red mottling in and on a pale red-brown liver was seen. Histologically it was found that the periportal hepatocytes were swollen and acidophilic. Many of these cells were vacuolated but did not stain with Oil Red O or PAS. In addition, foci of midzonal necrosis were irregularly distributed throughout the parenchyma. The central and midzonal sinusoids were dilated with blood and hepatocytes in central and midzonal locations had a coarse granular cytoplasm which was vacuolated in some cells. The vacuoles did not stain with Oil Red O.

At 5·4 mg./kg. the macroscopic alterations observed at post-mortem examination consisted of petechial haemorrhages in the lungs, kidneys and stomachs. The histological examination confirmed the macroscopic findings. Severe toxic nephritis was seen in the kidneys. The adrenal glands showed massive destruction of their medullae with pools of blood replacing the medullary substance. The liver of monkey 141 showed massive collapse with only islands of hepatocytes remaining. The areas of collapse occurred between and including the central veins, many of which were thus completely obliterated. Collapse occurred in a polygonal fashion with the lesser affected portal tract remaining in the centre surrounded by swollen hepatocytes (Fig. 6). Many periportal hepatocytes either had pycnotic or karryorhectic nuclei. In the collapsed areas there was an infiltration of round cells and an increase in reticular fibres. Bile stasis was prominent and severe haemorrhage occurred into many of the collapsed areas. Proliferation of bile duct epithelial cells was also observed (Fig. 7). In the case of monkey 143 there was haemorrhagic necrosis of the entire parenchyma with only a few portal tracts remaining.

At the dose of 11·6 mg./kg. monkey 144 showed clinical icterus and on post-mortem examination of both 144 and 148 the livers were enlarged, yellow-brown in colour and the incised edges everted. Petechiae and ecchymoses were present in the lungs, on the epicardia, kidneys and both internal and external surfaces of the stomachs and red-black fluid filled both caecae and colons. The haemorrhages seen on post-mortem examination were also observed histologically. Degeneration and necrosis were seen affecting all structures within the kidneys. The adrenal medullae were completely replaced by blood. The livers exhibited massive necrosis and large " pools " of blood. In the areas not obliterated by haemorrhage bile stasis was prominent and all remaining hepatocytes were distended with fat (Oil Red O positive material).

Animals dosed with sterigmatocystin.—The animals receiving a low dose of sterigmatocystin (15 mg./kg.) by i.p. injection had purulent foci on the peritoneum at the site of injection. Both livers were enlarged, yellow-brown in colour and friable.

Microscopic examination revealed that the kidneys had degenerating glomeruli

with oedema in Bowman's space (Fig. 8) and hyaline droplet degeneration in the proximal and distal convoluted tubules. In the medullary rays the tubular epithelium showed fatty changes and necrosis.

The cells of the glomerular zone of the adrenal cortex showed a complete loss of cytoplasm and only pycnotic, basophilic nuclei could be seen. Medullary architecture was disrupted by large blood filled dilatations.

The liver of monkey 12 showed predominantly centrilobular changes consisting of small foci of necrosis, fatty vacuolation of hepatocytes and ballooning degeneration. Kupffer cells were prominent and infiltration of macrophages, plasma cells and round cells were seen in the necrotic areas. Bile stasis was prominent and in the less severely affected periportal areas the cytoplasm of hepatocytes was eosinophilic and coarsely granular. Very slight fatty changes could be seen in these areas which increased in severity and extent towards the central vein. The sinusoids were dilated throughout the entire lobules.

The liver of monkey 15 was less severely affected.

On post-mortem examination the monkeys that received 32 mg. sterigmatocystin per kg. had inflammatory foci on the peritoneal surface and in the attached omentum at the site of toxin injection. The animal that survived for 10 days (No. 6) exhibited a histological picture similar to that of the lower dose group with the exception that its liver exhibited a high amitotic index resulting in numerous binucleate hepatocytes. Early bile duct proliferation could also be observed.

Monkey 8 which died on the 4th day after dosing showed generalized congestion of the liver with midzonal and centrilobular haemorrhagic necrosis.

At 70 mg. sterigmatocystin per kg. the macroscopic findings consisted of extensive peritonitis in the region of the injection site. The livers were swollen and yellow and monkey 14 was icteric. The cut surfaces of the liver everted and after incision fat could be seen on the post-mortem instruments. Tumor splenis was present.

Histologically the spleens were found to be distended with red blood cells and the splenic macrophages were filled with haemosiderin. There was haemorrhage into the kidney parenchyma and the glomeruli were either hyalinized or the capillary loops were fragmented. Hyaline degeneration, fatty changes, and necrosis were seen in the epithelial cells of the nephrons. Yellow-brown pigment deposits were observed between the proximal convoluted tubular nuclei and the basement membranes. Zenker's hyaline degeneration was seen in the myocardium. In the livers there were diffuse fatty changes, extensive single cell and central haemorrhagic necrosis and intracellular bile stasis (Fig. 9). Marked congestion was present throughout the liver and Kupffer cells appeared prominent. Bile duct epithelial proliferation was more marked than in the lower dose group.

Both monkeys which received the highest doses of sterigmatocystin had clinical icterus. On post-mortem examination severe diffuse peritonitis was found. Petechial haemorrhages were seen on all serosal surfaces and in all organs.

Microscopic examination revealed diffuse haemorrhages in all the organs. In the kidneys there was degeneration of the glomeruli and the basement membranes of some Bowman's capsules were extensively thickened. Degeneration and necrosis was seen in proximal and distal convoluted tubules of the kidneys (Fig. 10). The spleens were severely congested and contained abundant pigment. Vacuolation was observed in the central areas of many Malpighian corpuscles.

The testis showed changes similar to those seen in the animals exposed to retrorsine (100 mg./kg.). Zenker's hyaline degeneration was seen in the myocardium. The livers were similar to those of the monkeys in the previous group but larger areas of haemorrhagic necrosis were seen throughout the entire parenchyma.

DISCUSSION

In rats, retrorsine is considered to produce a primary vascular lesion most probably in the branches of the hepatic veins which results in secondary hepatocellular necrosis (Davidson, 1935). Centrilobular haemorrhagic necrosis resulting from the toxic effects of retrorsine on both the liver cells and the central and hepatic veins was described by Selzer, Parker and Sapeika (1951). These effects on rats are comparable with those found in vervet monkeys in the present study. The periportal necrosis observed in *Macaca rhesus* monkeys exposed to senecionine (Wakin, Harris and Chen, 1946) differs markedly from the lesions obtained in vervet monkeys exposed to retrorsine. Retrorsine causes mainly a central and midzonal necrosis of hepatocytes followed by destruction of the wall of the central vein with resulting haemorrhage. Concurrently with these alterations the stroma of the portal tracts are infiltrated by oedematous fluid which progressively increases until the portal tracts are separated from the surrounding hepatocytes and the intrastromal vessels and ducts are isolated from their normal positions. The wall of the portal vein also becomes infiltrated first by oedematous fluid, and after distension of the media, fragmentation of the intima follows with resulting haemorrhage into the fluid filled spaces. These vascular alterations which occur over a period of 10 days or less are not comparable with those seen in veno-occlusive disease experimentally produced in *Macaca specios* monkeys by monocrotaline, where there was found to be an association of necrosis of hepatocytes and venous occlusion in the central area of the liver lobule (Allen, Carstens and Olson, 1967). Monocrotaline affects the endothelial cells of the hepatic veins with subsequent oedema within the wall of the vessel to such an extent that the lumen is completely or partially occluded (Allen, Carstens and Katagiri, 1969). In the present study megalocytes, characteristic of pyrollizidine alkaloid poisoning (Bull, 1955) were only observed in monkey 38 and here they were found to manifest as isolated cells and not as the bulk of parenchymal cells.

The LD_{50} value of aflatoxin in the vervet monkey was about half of the value found in the rat. The acute liver lesions seen in vervet monkeys differ from those seen in rats (Butler, 1964); in the monkeys no distinct periportal necrosis was seen, but instead polygonal zones of collapse extending between and including the central veins were observed. Bile duct proliferation was found to be prominent and an increase in reticular fibres in the collapsed areas was observed after 8 days. The oedematous and haemorrhagic reaction which was observed in the portal tracts of livers from animals exposed to retrorsine was similar to the changes present in the aflatoxin treated animals.

Sterigmatocystin manifests its acute toxic effects primarily on the liver and kidneys of the monkey. The LD_{50} value is approximately 10 times greater than that obtained with aflatoxin in vervet monkeys and half that obtained with sterigmatocystin in Wistar rats (Purchase and Van der Watt, 1969). In high doses sterigmatocystin produces a centrilobular haemorrhagic necrosis in monkeys, but a comparison of the lesion with that produced by other toxins or by sterigmatocystin in rats is impossible because of the unexpected effect of the solvent (DMSO)

in monkeys. The morphology of the liver lesion may also have been influenced by the peritonites resulting from intra-peritoneal injection of the toxin.

Dimethylsulphoxide was selected as a solvent because sterigmatocystin is insoluble in most non-toxic solvents and DMSO is said to have minimal effects in the primate. The only deleterious effects observed in monkeys (*Macaca mulata*) receiving between 0·4 and 4·0 g./kg. i.v. was a transient dose related haemodilution phenomenon accompanied by fluctuations in serum glutamic pyruvic transaminase, blood sugar, platelet and leucocyte counts (Feinman, Ben and Levin, 1964). In the present study, however, DMSO was found to produce severe fatty changes in the liver, which not only hampered a detailed comparison between the effects of the toxins studied, but may have influenced the accuracy of the LD_{50} estimation. In spite of the limitations introduced by the use of DMSO, it is possible to say that all 3 of the toxins used produced a central and midzonal necrosis accompanied by haemorrhage. Sterigmatocystin produced no vascular alteration in contrast to both retrorsine and aflatoxin which produced a central and periportal perivascular effect. Retrorsine was more active in this respect. Although bile duct proliferation was observed with all 3 toxins, aflatoxin produced the most pronounced effect.

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