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HEPATIC LESIONS IN PROTEIN-DEFICIENT ADULT RATS

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Summary.—Four groups of 10 young adult Wistar male rats were fed *ad libitum* on a protein-free diet for periods of 7, 28, 56 and 84 days. Control groups were fed on a 20% casein diet. Food intake and body weights of rats were registered. Plasma protein levels and liver weight and fat content were determined. Sections of the caudate lobe were studied histologically. Fatty changes were classified in three grades.

Protein-deficient rats exhibited loss of body weight and had low levels of plasma protein concentration.

Liver lost weight after 7 days of protein deficiency; there was a gradual reduction in liver weight as periods of protein deprivation were longer. After 7 days, liver fat concentration was not significantly higher than in the respective control group; it was significantly higher in all the other malnourished animals. As periods of protein deprivation were longer, fatty changes became more severe.

Other hepatic lesions were found in 5 of the 10 rats submitted to the longest period of protein deficiency. One of the rats showed a diffuse cellular atrophy, 2 animals showed an extensive haemorrhagic necrosis, another showed a focal area of reticulum collapse and the last exhibited a distortion of the normal architecture of the liver due to diffuse reticulum collapse and early nodular regeneration; these 2 last rats showed early fibrosis in portal areas.

The findings suggest that other deficiencies may complicate the protein deficiency when rats are given a protein-free diet over prolonged periods. Even if the protein-deficient diet has protective nutrients, it may be that, when rats eat less food, as occurs in prolonged experiments, deficiency of one or all of these elements can occur, depending on their relative amount in diet.

HUMAN protein-calorie malnutrition is usually complicated by vitamin and mineral deficiencies, intercurrent infections and infestations (Deo, Sood and Ramalingaswami, 1965). These multiple factors make difficult the assessment of the role of protein deficiency *per se* in the pathogenesis of the biochemical and structural changes. So there are obvious advantages in the study of experimental models in which the dietary conditions are known and intercurrent factors prevented (Kirsch, Saunders and Brock, 1968*b*). Recent models of protein-calorie malnutrition closely resemble human syndromes such as kwashiorkor and marasmus (Kirsch *et al.*, 1968*b*). Data obtained from dogs, monkeys and pigs are more likely to apply to man (Deo *et al.*, 1965; Kirsch, Brock and Saunders, 1968*a*); however, both syndromes can be produced in rats (Edozien, 1968; Kirsch *et al.*, 1968a; McCance and Widdowson, 1966).

Porta and Hartroft (1970) claim that the liver is a very responsive index of good nutrition and that deviations from its morphofunctional integrity reflect with sensitivity nutritional abnormalities. Dietary hepatic lesions have been utilized as experimental models of hepatic necrosis (Machado *et al.*, 1971; Porta, de la Iglesia and Hartroft, 1968), hepatic cirrhosis

(Hoffbauer, 1959) and as evidence of protein deficiency (Campana *et al.*, 1975; Madi *et al.*, 1970; Porta and Hartroft, 1970). However, the relationship between protein deficiency and hepatic fibrosis and cirrhosis is controversial. Variations of the relative amount of nutrients in *ad libitum* diets, apart from the proteins and presence of contaminants in nutrients, may alter lesions inducing errors in the interpretation of the pathogenetic mechanisms of findings (Newberne, 1975). On the other hand, when animals are fed on very low-protein diets, they eat less food (Kirsch *et al.*, 1968*b*), a situation which may induce deficiency of some nutrients when their relative amounts in diets are not high enough. Then in prolonged protein deficiency studies, other hepatic lesions, apart from fatty infiltration, may occur.

This report describes the hepatic changes observed in rats submitted to a protein-free diet for periods of 7, 28, 56 and 84 days. The livers of rats submitted to the longest period of protein deprivation showed lesions, accompanied or not by fatty infiltration; these were tentatively related to the composition of the experimental diet. Serum proteins and fat content of the liver were also determined.

MATERIALS AND METHODS

Eighty young adult male rats of the Wistar strain, 123–124 days old, were grouped in respect of initial body weights and allocated at random to 8 groups of 10 animals. Four groups were fed a 20% casein diet *ad lib.* (control groups = C) and the other 4 groups a protein-free diet also *ad lib.* (protein-deficient groups = D). After experimental periods of 7, 28, 56 and 84 days, the animals of one of the control groups and those of one of the protein-deficient groups were killed.

The rats were placed in individual cages and maintained in a temperature controlled room (21–23°), with a 12-h light period. Tapwater was freely available.

The 20% casein diet consisted of casein—20 g, salt mixture—4 g, cottonseed oil—5 ml, codfish liver oil—1 ml and corn starch—70 g; the salt mixture was prepared according to the method of Hegsted *et al.* (1941), plus the addition of cobalt chloride; 1000 g of diet contained the following amounts of salts: NaCl—5·572 g; KI—0·0318 g; KH_2PO_4—15·559 g; $MgSO_4 . 7H_2O$—2·2919 g; $CaCO_3$—15·2559 g; $FeSO_4 . 7H_2O$—1·0799 g; $MnSO_4 . H_2O$—0·1604 g; $ZnSO_4 . 7H_2O$—0·0219 g; $CuSO_4 . 5H_2O$—0·0203 g; $CoCl . 6H_2O$—0·0009 g. Five ml of vitamin mixture (Manna and Hauge, 1953) were also added to 100 g of diet, plus the addition of vitamin B_{12}; 1000 ml of this mixture contained the following amounts of vitamins: thiamin hydrochloride—0·6 g; riboflavin—0·6 g; pyridoxine hydrochloride—0·6 g; calcium pantothenate—3·0 g; nicotinic acid—1·0 g; choline chloride—30·0 g; biotin—2·0 mg; folic acid—4·0 mg; inositol—8·0 g; *p*-aminobenzoic acid—6·0 g; vitamin K—0·2 g; vitamin B_{12}—0·6 mg.

The protein-free diet consisted of: corn starch—90 g; salt mixture—4 g; cottonseed oil—5 ml; codfish liver oil—1 ml. Vitamin mixture was added in the same way as in the 20% casein diet.

The 20% casein diet and the protein-free diet were isocaloric and the food intake was registered so that the intake of control and protein-deficient groups during consecutive periods of 7 days was known. The body weight of each animal was recorded weekly.

At the end of each experimental period, the rats were killed by decapitation. The blood was collected in a heparinized tube and plasma protein concentration estimated according to the method of Henry (1964).

The liver was removed and weighed; the caudate lobe was excised and fixed in 10% formalin. Liver minus the caudate lobe was again weighed, dried to constant weight at 105° and then ground to powder. Specimens of 100 mg of this powder, in duplicate, were placed in Soxhlet thimbles and fat was extracted for 8 h with ethylic ether (Hawk and Elvehjem, 1953) in the Soxhlet apparatus (Cheek and West, 1955) suited for micromethod.

Fragments for histological studies were obtained from the caudate lobe, so as to prevent interferences of variations of hepatic circulation on morphological and biochemical observations (Himsworth, 1954*a*). Paraffin sections were stained with haematoxylin and eosin (HE); when indicated, Masson's trichrome, Gomori, Oil-Red-O, Ziehl–Neelsen, PAS and ultra-violet autofluorescence were also utilized.

According to the severity of involvement, diffuse fatty changes were classified in 3 grades (Campana *et al.*, 1975): Grade I—few hepatic cells showed minute intracytoplasmatic vacuoles without nuclear displacement; Grade II—a greater number of cells with vacuolation often without nuclear displacement; Grade III—a massive vacuolation was present, all the cells assuming a "clear" appearance, often with nuclear displacement (Fig. 1).

Significance levels were tested using Student's *t* test.

TABLE I.—*Body Weight, Plasma Protein Concentration, Weight and Fat Content of the Liver of Rats*

		Groups							
		C7 (n = 10)	D7 (n = 10)	C28* (n = 9)	D28 (n = 10)	C56* (n = 9)	D56 (n = 10)	C84 (n = 10)	D84† (n = 8)
BW_i	(g)–$\bar{X}$	311·3	310·3	311·1	310·9	308·3	308·1	307·4	307·1
	s.d.	18·31	18·41	19·11	25·80	14·79	16·0	19·68	19·27
	t	0·12		0·02		0·03		0·03	
BW_f	(g)–$\bar{X}$	315·5	300·8	348·5	256·9	377·9	213·7	401·3	168·4
	s.d.	17·62	16·47	15·13	19·26	22·74	8·48	18·68	14·8
	t	1·93		11·43‡		21·30‡		28·73‡	
Total protein–$\bar{X}$ (g/100 ml plasma)		7·7	7·0	7·2	5·8	6·6	4·5	7·1	4·4
	s.d.	0·61	0·50	0·31	0·30	0·52	0·25	0·27	0·26
	t	2·81‡		10·0‡		11·41‡		21·43‡	
Liver (g)–$\bar{X}$		11·0	9·4	11·8	8·6	12·8	7·9	12·6	5·9
	s.d.	0·72	1·15	1·25	0·80	1·70	0·93	1·96	0·96
	t	3·73‡		6·72‡		7·91‡		8·82‡	
Fat content (g/kg wet weight of liver)–$\bar{X}$		30·0	34·0	24·0	60·0	24·0	93·0	23·0	50·0
	s.d.	5·3	6·7	4·7	52·4	2·7	25·3	5·0	18·2
	t	1·48		3·58‡		13·31‡		4·27‡	

* Results of two rats, one of Group C28 and other of Group C56, were discarded, the first because it suffered a marked loss of weight during the experimental period and the second because a postmortem study showed it to have a suppurative pulmonary infection.

† Material for analysis was not collected from one rat of Group D84 as the animal was found dead in the cage on the 79th day of the experiment. The results of another rat of Group D84 were discarded due to technical problems.

n = number of rats BW_i = initial body weight BW_f = final body weight $\bar{X}$ = mean value
s.d. = standard deviation *t* = value of *t* of Student's *t* test ‡ $P < 0·05$.

RESULTS

Initial mean body weights of each control group and its respective protein-deficient group were not significantly different; rats of the control groups presented gain of body weight, whereas in the deficient groups weights were significantly lower than the respective control groups with the exception of Group D7; the longer the period of protein deprivation, the more marked the loss of weight of the rats (Table I).

Food intake for the control groups averaged 17·9 g/rat/day, which was higher than the average for the protein-deficient groups (13·4 g/rat/day). However, when food intake was related to animal body weight (g/100 g), average values for the control groups and protein-deficient groups were respectively 5·0 and 5·5 g. Protein-deficient rats ate progressively less as periods of protein deprivation were longer.

In protein-deficient groups, there was reduction in plasma protein concentration and liver weight. The results indicated that plasma protein concentration and liver weight diminished as periods of protein deprivation were longer (Table I).

Values for the liver fat concentration are also recorded in Table I. Significant increases in the concentration of fat were shown in the liver after 28, 56 and 84 days of protein deficiency; this was most marked after 56 days, when the amount of fat accounted for almost 10% of the wet weight of the liver. Liver fat concentration was lower in Group D84 when compared with Group D56 (50·0 and 93·0 g/kg of wet weight of liver respectively), but was still higher than in its control group (C84—23·0 g/kg of wet weight of liver).

Table II shows data on the fatty infiltration of the liver. The liver of the majority of malnourished rats showed fatty infiltration, which involved the entire lobule. Staining by Oil-Red-O in some animals indicated that the maximum amount of fat was found around the portal

TABLE II.—*Liver—Frequency and Severity of Fatty Infiltration in Rats Submitted to Protein-free Diet*

	Groups			
	D7*	D28	D56	D84†
Without fatty infiltration	4	0	0	1
Fatty infiltration				1‡
Grade I	2	4	3	2
Grade II	2	5	5	4
Grade III	0	1	2	0

* Histological study was not performed in two rats.
† Histological study was not performed in one rat; extensive areas of haemorrhagic necrosis prevented recognition of steatosis in another rat.
‡ One rat presented extensive areas of haemorrhagic necrosis; fatty infiltration was seen around the well-preserved portal areas.

areas (Fig. 2). Fatty changes were seen in 4 of 8 animals of Group D7, in every rat of Groups D28 and D56 and in 7 animals of Group D84. Fatty changes of Grade I were seen in only 3 of the 40 control animals.

Other lesions were found in 5 of the 10 rats submitted to the longest period of protein deprivation (D84). The liver of one of the rats showed a diffuse cellular atrophy, without fatty change (D84, No. 10, Fig. 3). Two animals showed extensive haemorrhagic necrosis mainly centrilobular, with preservation of portal areas (D84, No. 1 and No. 9, Fig. 4). In animal D84, No. 3 there was Grade II fatty infiltration and focal subcapsular area of collapse accompanied by minimal mononuclear infiltration, ductal proliferation, early fibrosis in portal areas and signs of hepatocellular regeneration. In Rat D84, No. 7 architecture was distorted due to bands of collapse, delimiting nodules usually smaller than one lobule (Fig. 5). Sometimes bands of collapse were seen to connect portal to central areas. Fat vacuolization was present but, in the periphery of the nodules, parenchymal cells were smaller, with hyperchromatic nuclei, and did not present vacuolization. Portal areas showed an infiltrate of mononuclear cells, ductal proliferation and collagen deposition which extended along the bands of collapse (Fig. 6A). Evidence of regeneration was also present.

Macrophages containing ceroid were seen in the portal areas and in intralobular fibrous bands of Rats D84, No. 3 and D84, No. 7 (Fig. 6B).

DISCUSSION

Loss of body weight, diminished food intake, low levels of serum or plasma proteins, loss of liver weight, fatty liver infiltration and increased content of fat in the liver, observed in our malnourished rats, have been already documented in experimental protein deficiency (Boyd, 1970; Edozien, 1968; Enwonwu and Sreebny, 1970; Heard *et al.*, 1977; Kirsch, *et al.*, 1968*b*).

Staining by HE showed that the liver fatty infiltration in the protein-deficient rats was diffuse throughout the lobule, but the distribution of fat was shown to be mainly periportal, as described in "experimental kwashiorkor" (Porta and Hartroft, 1970; Sidransky, 1976), when specific stains were used.

After 7 days of protein deprivation, liver fat concentration was not significantly higher than in control animals, although microscopic changes were already present; as periods of protein deficiency grew longer, fatty changes became severe and the amount of biochemically determined fat increased (Tables I and II). Increase in the severity of liver fatty changes as protein deficiency progresses has been reported (Deo *et al.*, 1965; Enwonwu and Sreebny, 1970; Madi *et al.*, 1970). However, the maximum amount of fat was registered in Group D56; in fact, Group

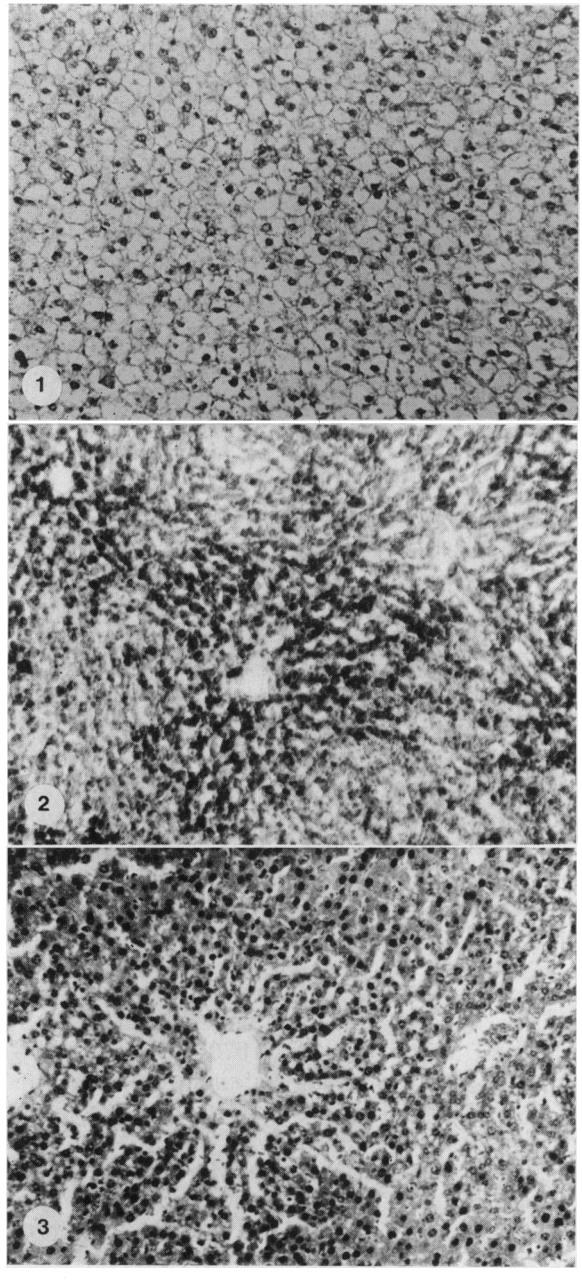

FIG. 1.—Diffuse fatty liver infiltration of grade III. H. and E. ×65.
FIG. 2.—Fatty changes predominantly involving periportal areas of the liver lobules. Frozen sections stained with Oil-Red-O ×65.
FIG. 3.—Diffuse cellular atrophy of the liver lobule. H. and E. ×65.

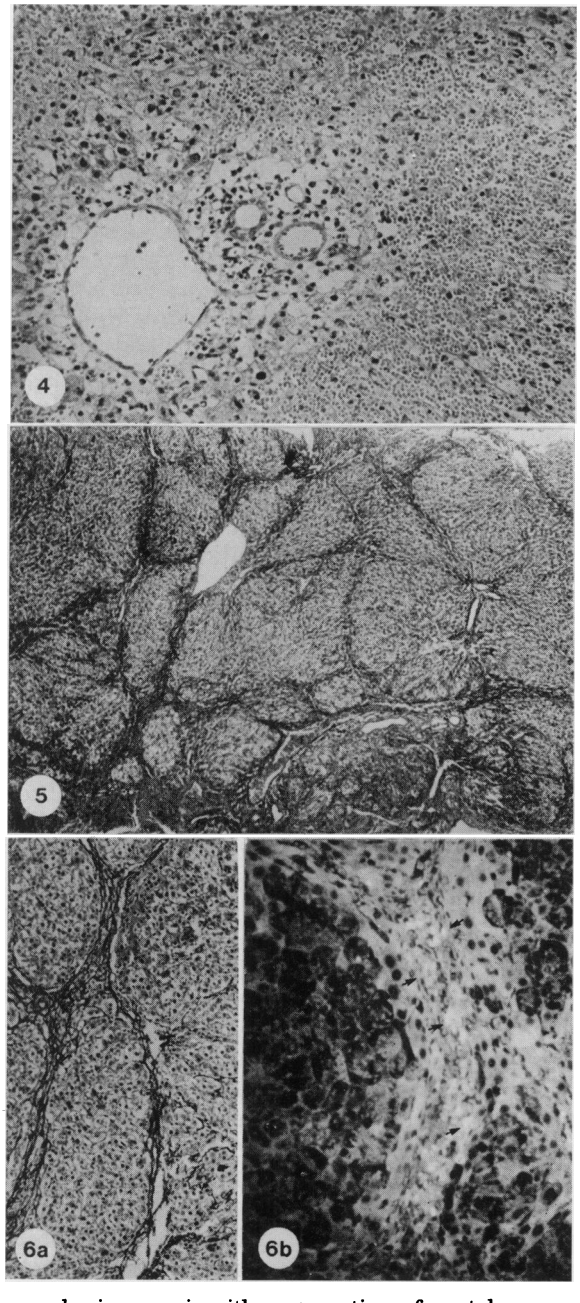

FIG. 4.—Extensive haemorrhagic necrosis with preservation of portal areas of the liver lobule. H. and E. ×65.

FIG. 5.—Section of caudate lobe of the liver of Rat D84, No. 7, showing nodular distortion of the normal architecture. H. and E. ×52.

FIG. 6.—Sections of caudate lobe of the liver of Rat D84, No. 7, showing: (A) bands of recticulum collapse; Gomori stain ×80. (B) macrophages containing ultraviolet autofluorescent ceroid (arrows) in intralobular fibrous bands; ×130.

D84 showed a lower figure for liver fat content (Table I). The origin of liver fat in protein deficiency is controversial; defective hepatic removal of triglycerides and also increased mobilization of free fatty acids from fat depots have been suggested (Flores, Seakins and Mönckeberg, 1973); both factors may be active, together with an increased rate of hepatic lipid synthesis (Hoyumpa *et al.*, 1975). As periods of protein deprivation were longer, our malnourished rats diminished their food intake, lost weight and probably most of their fat body depots, as is known to happen in protein-deficient pigs (McCance and Widdowson, 1966). So it may be presumed that one of the pathogenic factors of excess liver fat—that is, the availability of fat body depots—was less operative in rats of Group D84. This would explain the finding of a lower figure for liver fat concentration in this group when compared with group D56 (Table I). It is interesting to point out that one animal of Group D84 (No. 10) showed a diffuse cellular atrophy and no fatty changes in the liver (Fig. 3). It is likely that, as protein deficiency entails a reduction of appetite, calorie deficiency may complicate protein deficiency in very long experiments.

Some rats of Group D84 presented hepatic necrosis and distortion of the normal architecture with early fibrosis. Ditary hepatic necrosis and dietary hepatic fibrosis are considered as different types of reactions of the liver to dietetic injuries (Himmsworth, 1954*b*; Schwarz, 1965).

Hepatic necrosis in protein deficiency has been reported (Madi *et al.*, 1970; Porta and Hartroft, 1970); it may be also induced in rats by feeding them diets low in vitamin E, selenium and sulphur amino acids (Porta *et al.*, 1968; Schwarz, 1976). Schwarz (1965) claimed that there are two protective factors in dietary necrotic liver lesion—namely, tocopherol and selenium —and that supplements of sulphur amino acids modulate the final outcome by reducing the need for vitamin E. In our experiment, the polyunsaturated fatty acids present in the cottonseed and codfish liver oils (Wilson, Fisher and Fuqua, 1975), entail the need of an adequate supply of vitamin E, through the formation of lipid hydroperoxide (Hoekstra, 1975). It can be presumed that, although this vitamin was present in dietary oils, adequate amounts of it were not available when experimental periods were longer and rats ate less food. It is possible that our protein-deficient rats were also selenium-deficient, as the protein-free diet did not contain cystine, which is usually contaminated by selenium (Schwarz, 1965). So, we believe that there are sufficient grounds to accept that the hepatic necrotic lesions, seen in Rats D84, No. 1 and D84, No. 9, had a dietary basis. Actually, the findings of a focal area of collapse in Rat D84, No. 3 and of bands of collapse in Rat D84, No. 7 indicate that focal areas of hepatocellular necrosis had previously occurred (Figs. 5 and 6A).

In these 2 rats, there was evidence of hepatocellular regeneration and of early fibrosis. The relationship between protein deficiency and fibrosis and cirrhosis has been extensively discussed. Ramalingaswami (1964) submitted that fibrosis and cirrhosis may develop in man and in the rat when fatty change is severe and prolonged enough or when another injury is added to the liver. The role of choline has been emphasized: fibrosis and cirrhosis developed in rats fed for 6 months on a low-choline low-protein diet (Porta and Hartroft, 1970) but not in rats fed for 1 year on high-choline low-protein diet (Best *et al.*, 1955); in choline deficiency, fatty infiltration and architectural distortion have been pathogenetically associated with experimental nutritional fibrosis and cirrhosis (Himsworth, 1954*b*; Hoffbauer, 1959); it has been also shown that choline supplementation enhances the absorption of collagen and retards the formation of new collagen in livers of cirrhotic rats (Takata, Porta and Hartroft, 1967). In our work, after an experimental period of 3 months, the livers of the 2 previously mentioned animals had fibrosis; it is

possible that, towards the end of the experiment, choline availability was not adequate as rats ate progressively less food, favouring the increase of hepatic connective tissue.

The finding of ceroid in livers of vitamin E- and selenium-deficient animals has been reported and considered as a prenecrotic lesion (Porta and de la Iglesia, 1965). In our work, it was observed only along the bands of collapse in Rats D84, No. 3 and D84, No. 7, the livers of which showed regeneration and scarring. Among the factors pathogenetically associated with the formation of ceroid in nutritional studies, unsaturated dietary fat seems to be the most important, irrespective of dietary protein and choline content; this relationship is accentuated by vitamin E deficiency (Hartroft and Porta, 1965). Thus the finding of this pigment gives additional support to the assumption that our rats were vitamin E-deficient.

In our experiment it may be assumed that selenium was not available for ingestion; on the other hand, unsaturated fats were present in the dietary oils. So, if rats of Group D84 were vitamin E-deficient, it can be presumed that they were subjected to a necrogenic diet. But if this is accepted, it is difficult to understand why there were, at the same time, two different phases of liver reaction. It may be advanced that in 2 animals—D84, No. 1 and D84, No. 9—the injury was recent and extensive and in animals D84, No. 3 and D84, No. 7, it was older and localized, permitting the development of regeneration and scarring.

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