

## OBSERVATIONS ON THE CONDITIONS OF DIETARY HEPATIC INJURY (NECROSIS, CIRRHOSIS) IN RATS\*

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It was reported in 1939 (2) that hepatic injury, mainly in the form of acute focal or diffuse necrosis combined with fat infiltration, occurred irregularly in young rats fed a diet devoid of vitamin B (casein 18 per cent, cane sugar 68, melted butter fat 8, cod liver oil 2, salt mixture 4) and supplemented with thiamine, riboflavin, and pyridoxine. In a group of more than 300 rats, 48 exhibited hepatic changes of the type described. In livers of 4 of the rats there was diffuse periportal fibrosis.

Admittedly, even when hepatic injury was observed under apparently identical experimental conditions, it was found that it "could not be produced at will and was not a regular occurrence." No proof for an infectious or toxic origin of the pathological changes in the liver could be found and the evidence favored, rather, a nutritional basis (2). In this connection it is particularly noteworthy that injury to the liver was never encountered in rats which, for from 3 to 6 months, had been fed a diet deficient in the vitamin B complex and supplemented with thiamine, riboflavin, and 0.5 gm. of yeast daily. Nor was hepatic injury found in investigations on riboflavin deficiency (3, 4) involving approximately 500 rats which were fed the same basal diet supplemented with thiamine and yeast extract in the form of Peters' eluate (5). As this hepatic disease was not prevented by the administration of thiamine, riboflavin, and pyridoxine, it was assumed (2) that the hypothetical factor which protected the liver must be one that was different from these components of the vitamin B complex.

The incidence of hepatic injury remained practically unchanged in later groups of at least 250 rats<sup>1</sup> fed a vitamin B free diet, with daily supplements of 20 micrograms of thiamine, 20 to 25 micrograms of riboflavin, and 2 mg. of choline per gm. of diet (or 10 mg. of choline daily as a separate supplement)

\* A preliminary report of this work has been made (1).

<sup>1</sup> In this number is included the series of 169 rats referred to in footnote 1 of an earlier report (6).

and with or without the further addition of pyridoxine. Injury to the liver was also observed in rats which had received pantothenic acid because manifestations of pantothenic acid deficiency had become apparent on a diet supplemented with the factors mentioned. If the rats treated with pantothenic acid, however, survived the first 4 weeks of medication with distinct gain in weight, they remained permanently free from hepatic injury.

Another group of 25 young rats, weighing between 30 and 35 gm., which had received from the start all the supplements in question (vitamin B<sub>1</sub>, riboflavin, pyridoxine, pantothenic acid, and choline), exhibited no specific hepatic changes even for as long as 8 months after the beginning of the experiment.

One common denominator emerged as a conceivable etiologic factor out of the multitude of these variable observations. This was the possibility of low food intake with, at the same time, a sufficient supply of several or all the known members of the vitamin B complex. Limitation of hepatic injury to rats suffering from or convalescing from deficiency of one or more members of the vitamin B complex is consistent with this assumption, as the intake of food is probably especially low and variable under these conditions. On the other hand, the fact that hepatic injury is lacking in rats whose fairly normal development is not interrupted by deficiency conditions also tallies well with this view. As a further logical step it could be assumed that the low food intake is associated with low casein supply and therefore, in natural consequence, with the so called lipotropic activity of casein.

As the amounts of food ingested were not determined, special investigations were needed in order to make such a tentative conclusion more plausible. In the experiments which are reported here, an attempt has been made to employ different rations that were low in protein and moderately or distinctly high in fat.

In the meantime Rich and Hamilton (7) have reported the occurrence of cirrhosis of the liver in rabbits maintained on a deficient diet and its prevention by yeast. The exact nature of the deficiency was not determined. Spellberg and Keeton (8) also observed cirrhosis of obscure dietary origin in one guinea pig and one rabbit. In this connection it should be borne in mind that herbivorous animals, especially rabbits, are not altogether satisfactory experimental subjects for the study of hepatic cirrhosis. "Reagents which produce the disease in them fail to have the same effect on dogs, and from the spontaneous cirrhosis which occurs they would seem to be peculiarly susceptible to the disease" (9).

The production of cirrhosis of the liver in 3 dogs and in 7 rats fed high fat diets has been reported by Chaikoff and Connor (10) and by Blumberg (11), respectively. These results, however, were correlated not to the phenomenon of the lipotropic activity of casein but rather to the fat infiltration of the liver.

#### *Experimental Method*

Rats with an initial weight of 130 gm. or more, in groups of 10 or more, were fed seven different rations, the composition of which is summarized in Table I. In all

but a few groups the diets were supplemented, as a matter of daily routine, with 20 micrograms of thiamine, 25 micrograms of riboflavin, 20 micrograms of pyridoxine, and 100 micrograms of calcium pantothenate.<sup>2</sup> The daily intake of food was not determined. In a few groups 0.5 gm. of dried brewer's yeast (Anheuser Busch, Inc.) was substituted for the vitamin supplements, three times a week. The vitamin solutions and the yeast, as well as the other special supplements, were given separately, that is, they were not mixed with the diet.

The feeding period of all the different groups was extended to 150 days or, exceptionally, a few days longer. Pathological examination of rats that died before the termination of the experiments followed as soon after death as possible.

In the overwhelming majority of the animals the diagnosis was made only after macroscopic and microscopic examination of the liver. As stated in the previous report (2), in a few rats the excited, hyperactive behavior might hint the presence of disease of the liver. Visible jaundice (yellow ears, bile stained urine) was observed

TABLE I  
*Composition of the Diets*

Constituent	Diet S I	Diet L II	Die L III	Diet L IV	Diet L V	Diet C I
Casein smaco.....	18	10	6	18	5	8
Lard.....	..	20	23	20	20	..
Crisco.....	..	..	..	..	..	38
Melted butter fat.....	8	..	..	..	..	..
Cane sugar.....	68	64	15	56	69	48
Corn starch.....	..	..	50	..	..	..
Cod liver oil.....	2	2	2	2	2	2
Salt mixture (12).....	4	4	4	4	4	4

even less frequently. In the rats in which cirrhosis was found at autopsy, greasy, sparse, matted fur, and brown seborrheic adherent scales on the skin chiefly over the back were often observed in life. Clinical ascites was suspected in only one rat of all the groups under investigation. At autopsy, however, ascites alone and, in the presence of especially advanced cirrhosis, ascites with pericardial and pleural effusion were observed several times, although still rarely. The effusion fluid was found to be frequently bloody. Hematemesis and consequent anemia were equally exceptional findings. In these rats the anemia was hypochromic; for instance, in rat 6473 the blood count was: hemoglobin 45 per cent, red blood cells 3,990,000, white blood cells 6,900, polymorphonuclear cells 11 per cent, small mononuclear cells 85 per cent, large mononuclears 4 per cent.

The growth curve of rats suffering from hepatic injury (necrosis or cirrhosis) exhibited varying tendencies, although as a rule, at least in the last stages of the disease,

<sup>2</sup> Thiamine chloride, riboflavin, pyridoxine, and calcium pantothenate were obtained from Merck & Co., Inc., Rahway, New Jersey; purified casein, choline chloride, *l*-cystine, and tyrosine from the S. M. A. Corporation, Chagrin Falls, Ohio, and *dl*-methionine from both of these sources.

retardation and even suppression with final loss of weight seemed to prevail. In many animals, however, milder forms of cirrhosis were not incompatible with progressive gain in weight.

The investigations reported here have been carried out during the last 3 years on over 360<sup>3</sup> rats. These experiments are being continued with special emphasis placed on the problem whether or not cirrhosis of the liver once produced can be influenced therapeutically. Thus far the special dietary factors have been tested only for the purpose of determining interference with the development of the lesions.

#### *Pathological Findings*

In the livers of the rats in the various groups that received different percentages of protein and different supplements there is a great variety of pathological changes. Fat infiltration in some degree is almost invariably present. Less frequent is parenchymatous or fatty degeneration with variable degrees of fat infiltration. The most significant changes are diffuse or focal necrosis, with or without accompanying hemorrhage, and variable degrees of cirrhosis. As a rule, the necrosis is recognizable in the gross but a considerable degree of cirrhosis may exist without being obvious in the gross. The necrosis is mainly central and midzonal, but frequently a variable number of entire or almost entire lobules is the seat of this change. In some livers large portions of a lobe are necrotic, but thrombosis, which might also account for such massive necrosis, was not observed. In Tables II to IV, under the heading "necrosis" are included those livers which showed only this change. Under the heading "cirrhosis," however, is included a variable remnant of necrosis. Almost invariably, enmeshed in the fibrous connective tissue of the cirrhotic livers, there is still recognizable, in some form, a large or small remnant of the previously necrotic parenchyma of the liver. This varies from definitely recognizable partly or completely necrotic cells to globules of variable size and shape, most of which have a light yellow or greenish-yellow color in paraffin sections stained with hematoxylin and eosin. In frozen sections stained with Sudan IV these globules are pink or red and are also peroxidase positive. The exact nature of these globules has not yet been determined. Treatment of frozen sections for as long as 18 hours with absolute alcohol, methyl alcohol, acetone, ether, petroleum ether, xylene, dioxane, or pyridine does not dissolve out the constituent which takes the stain with the usual Sudan IV (Herxheimer), naphthol Sudan IV (Goldmann), naphthol Sudan black (Lison),<sup>4</sup> and the globules remain in their original form. They are presumably necrotic protoplasmic remnants of the

<sup>3</sup> Not all this number are included in this report.

<sup>4</sup> The Sudan black method of Lison, modified by Miss Ethel Lieb, technician at the Institute of Pathology.

original hepatic cells in which lipoidal material is probably in some form of intimate combination with the protein which interferes with its removal by lipoid solvents. The globules, although undoubtedly of cellular origin but not present within cells, give a positive reaction for oxydase (alpha naphthol-crystal violet method of Loele). Enmeshed also in the fibrous connective tissue are single cells or small islands of regenerating hepatic cells. These exhibit variation in size of nuclei, some large deeply basophilic nuclei and frequent mitoses. In the fibrous connective tissue of an occasional liver there is some golden yellow pigment, either within the cytoplasm of large mononuclear cells or in the form of granules of amorphous extracellular deposits. Most of this pigment gives the reaction for iron and is probably hemosiderin.

The gross and microscopic pictures of diffuse necrosis of the liver, with or without hemorrhage, have been described in detail and illustrated in a previous paper (1). The changes in these livers are exactly similar and resemble human acute or subacute diffuse necrosis of the liver.

Livers with cirrhosis often present in the gross a rough, nodular surface and, in severe cases, a typical "hobnail" appearance (Fig. 1), but not infrequently the surface of the liver is relatively smooth and gives no indication of the degree of fibrosis.

Although the necrosis is usually central and midzonal, yet the cirrhosis is mostly periportal. However, the alteration of the natural architecture is usually so great that central veins are frequently not easily recognizable. The bands of connective tissue are usually very wide and frequently include more than one lobule (Fig. 2). The probability is great that the diffuse fibrosis that occurs is a combination of condensation fibrosis as well as a definite increase of periportal connective tissue replacing the degenerated and necrotic parenchyma, but exactly how the fibrosis frequently becomes periportal is not yet quite clear. What is clear is that the necrosis evidently precedes the development of the cirrhosis. In this series of animals the multiplication of bile ducts has not been frequent or striking, but in an occasional liver this change has been definite. Although the precirrhotic changes are more like those of so called "toxic cirrhosis" or the precirrhotic stage of so called "acute yellow atrophy," yet the final picture is more like that of Laënnec's cirrhosis of both the multi-lobular and monolobular varieties. There is no exact correlation between the degree of fat infiltration and the degree of cirrhosis in the livers.

The kidneys of all rats were not examined, but in those that showed the most severe injury to the liver diffuse necrotizing nephrosis is frequent. The pathological changes in these kidneys differ somewhat from those previously reported (6) in much younger rats fed a choline deficient diet, in which the process was more severe. In these animals the change amounts to diffuse cortical hemorrhagic necrosis, and resembles human bilateral symmetrical cortical necrosis, but in the present series the change is best described as a necrotizing nephrosis.

## EXPERIMENTAL OBSERVATIONS

*Diet L II.*—Twenty rats were put on diet L II in which the casein ratio was reduced from the 18 per cent generally used in investigations on the vitamin B complex (2) to 10 per cent, and 20 per cent of lard was substituted for the usual 8 per cent of

TABLE II  
Results of Feeding 184 Rats Diet L II\*

Special supplements						Total No. of rats treated	No. of rats with no hepatic injury	Hepatic injury present			
L-Cystine daily	Choline daily	dl-Me-thio-nine daily	Liver extract daily	Yeast daily	Yeast 3 times a wk.			No. of rats	Necrosis	Cirrhosis	Necrosis and cirrhosis
mg.	mg.	mg.	gm.	gm.	gm.						
—	—	—	—	—	—	20	5	15	2	9	4
—	20	—	—	—	—	17	9	8	6	—	2
—	—	20	—	—	—	6	1	5	1	1	3
—	—	40	—	—	—	8	8	—	—	—	—
—	—	—	0.2	—	—	9	3	6	6	—	—
—	20	—	0.2	—	—	6	2	4	4	—	—
—	—	—	—	—	0.5	14	12	2	2†	—	—
100	—	—	—	—	—	11	—	11	—	11	—
50	—	—	—	—	—	15	1	14	3	8	3
25	—	—	—	—	—	4	1	3	—	3	—
12.5	—	—	—	—	—	1	—	1	—	1†	—
50	20	—	—	—	—	8	6	2	2†	—	—
25	10	—	—	—	—	11	9	2	2	—	—
50	—	20	—	—	—	5	5	—	—	—	—
25	—	20	—	—	—	11	9	2	2†	—	—
25	10	40	—	—	—	6	6	—	—	—	—
50	—	—	0.2	—	—	11	10	1	—	1†	—
50	—	—	—	0.5	—	5	4	1	—	1	—
50	10	—	—	0.5	—	5	5	—	—	—	—
12.5	—	—	—	—	0.5	6	4	2	—	1	1
12.5	10	—	—	—	0.5	5	5	—	—	—	—

\* All animals except those receiving 0.5 gm. of yeast three times weekly were fed diet L II routinely supplemented daily with 20 micrograms of thiamine, 25 micrograms of riboflavin, 20 micrograms of pyridoxine, and 100 micrograms of calcium pantothenate. The rats in the group receiving 0.5 gm. of yeast three times weekly were not fed any further vitamin supplements.

† Only mild changes were observed.

butter (Table I). To this modified basal diet the routine daily supplements of B complex factors were added, as described. In the group of 20 rats hepatic injury became an almost regular complication (Table II). Necrosis without cirrhosis was observed in 2 rats, with cirrhosis in 4 rats, and cirrhosis without necrosis in 9 rats that died between the 92nd and the 140th experimental days or were killed on the 150th day.

Addition of 20 mg. of choline daily reduced the incidence of hepatic injury (Table II) which, in this series of 17 animals, manifested itself almost exclusively by necrotic

changes, whereas cirrhotic changes, when present, were of a very mild degree and even then were combined with necrosis. Necrosis alone was observed in the livers of 6 of the 17 rats and necrosis with beginning cirrhosis in 2 animals; the incidence, therefore, was still almost 50 per cent as compared with 75 per cent in the control group which received no choline.

In view of the known antagonistic effect of cystine on choline, as well as on casein, in experiments dealing with the lipotropic activity of casein (13), the influence of adding cystine to the diet on the production of hepatic injury was studied in another group of rats (Table II). Daily supplements of 100 mg. of *l*-cystine were given to 11 rats. In the livers of all these animals signs of more or less severe cirrhosis were seen in the gross specimen and on microscopic examination. Thus it has been proved that cystine is a very potent factor in the accentuation of cirrhosis of the liver in rats.

To another series of 15 rats supplements of 50 mg. of *l*-cystine were given daily. Pathological changes of the liver, mainly cirrhosis, were manifested by 14 of these rats. Acute diffuse cortical necrosis of the kidneys (6) was found in 4 of these animals.

Cirrhosis of the liver was observed in 3 of 4 rats fed a daily special supplement of 25 mg. of *l*-cystine for 150 days. The liver of 1 rat which received only 12.5 mg. of *l*-cystine daily for this period showed slight cirrhosis.

In conclusion, *l*-cystine appears to exert a definitely injurious effect on the livers of rats maintained on a diet low in casein, high in fat and low in choline. With larger doses of *l*-cystine the hepatic injury seemed to become more accentuated.

The effect of *l*-cystine on the liver was neutralized to a large extent by the daily addition of 10 and 20 mg. of choline to diet L II (Table II). Only 4 of the 19 rats in this group exhibited necrotic changes and all 19 remained free from cirrhosis even up to 150 days of the experiment. It is noteworthy that the 4 rats with necrosis of the liver died before the 120th day of the experiment. Cortical necrosis of the kidneys did not occur in this series of animals.

A daily special supplement of 0.5 gm. of yeast neutralized the effect of 50 mg. of *l*-cystine as well as that of the modified basal diet L II (Table II). The liver of only 1 rat of the 5 rats fed the special supplement exhibited slight cirrhosis during the 150 days of the experiment. Excellent growth and completely normal livers were observed in another 5 rats fed daily a combination of 50 mg. of *l*-cystine, 0.5 gm. of yeast, and 10 mg. of choline as a special supplement (Table II).

The preventive effect of a supplement of yeast alone on dietary hepatic injury produced by diet L II became apparent even when a low dose of yeast (0.5 gm.) was given three times a week instead of daily (Table II); of a group of 14 rats maintained on the modified basal regime hepatic injury was manifested in only 2. When a daily special supplement of 12.5 mg. of *l*-cystine, in addition to 0.5 gm. of yeast three times a week, was fed to 6 rats, evidence of cirrhosis was found in the livers of only 2 rats and of necrosis in 1 rat; the further addition of 10 mg. of choline to the regime of 5 rats prevented the appearance of pathological changes in the liver.

A daily special supplement of 20 mg. of *dl*-methionine left the incidence of hepatic injury practically unaltered in a group of 6 rats but slightly allayed its severity (Table II). In contrast, special daily supplement of 40 mg. of *dl*-methionine had the same prophylactic effect that was produced by cystine plus choline. It is especially interesting that the combined administration of 50 (or 25) mg. of *l*-cystine and 20 mg. of

*dl*-methionine daily proved to be beneficial to the liver, although when these supplements were given separately *l*-cystine was injurious and 20 mg. of *dl*-methionine was practically without effect. The effect of *dl*-methionine given in combination with *l*-cystine is similar to that produced by the simultaneous administration of *l*-cystine and choline.

A liver concentrate,<sup>5</sup> which represented the fraction of an aqueous liver extract soluble in 95 per cent alcohol, has been tested as a special supplement to diet L II (Table II). This concentrate was considered by Woolley (14) to be a good source of members of the vitamin B<sub>2</sub> complex other than riboflavin, pyridoxine, and pantothenic acid, as they are needed by the rat. Administration of a daily dose of 0.2 gm. of this concentrate, alone to 9 rats or together with 20 mg. of choline to 6 rats, proved to be completely ineffective in preventing hepatic injury. From the additional result that it had a neutralizing effect on *l*-cystine given in doses of 50 mg. daily to 11 rats, it could be concluded that in this combination the liver extract acts through its content of choline (or a choline-like substance). Thus these experiments with liver extract furnish new indirect evidence of the importance of the combined administration of cystine plus choline in the dietary management of hepatic injury in rats.

*Diet S I.*—If it is assumed that the pathogenesis of dietary necrosis and dietary cirrhosis of the liver is comparable to that of fatty metamorphosis of the liver, as shown by the results of experiments on the lipotropic activity of casein (13), an increase in the content of casein in the experimental diet should have a preventive effect on hepatic injury caused by *l*-cystine similar to that caused by the addition of choline or *dl*-methionine. In accordance with this expectation, a special supplement of 50 mg. of *l*-cystine daily was given with diet S I, which had a casein content of 18 per cent (Table I), but failed to provoke the slightest pathological change in the livers of 11 rats, even though it was administered for 179 days. The diet was routinely supplemented daily with 20 micrograms of thiamine, 25 micrograms of riboflavin, 20 micrograms of pyridoxine, and 100 micrograms of calcium pantothenate.

*Diet L IV.*—In order to show that it was not the low content of fat in diet S I which was responsible for the results obtained, a special diet (L IV) was devised with the same relative amount (20 per cent) of fat (lard) as in diet L II but with 18 per cent of casein as in diet S I. The routine vitamin B complex supplements were added (Table I). The results obtained with diet L IV were again unequivocal. The high intake of casein prevented the appearance of hepatic injury in spite of the simultaneously high intake of lard, regardless of whether no special supplement (10 rats) or a special daily supplement of 50 mg. of *l*-cystine (11 rats) was added to the diet.

*Diet L III.*—Decreasing the content of casein in the diet below 10 per cent met with some difficulties. Experiments with diet L V, with a casein content of 5 per cent (Table I), showed that it contained apparently too little protein to permit survival of the rats for a period long enough to allow hepatic injury to develop. Substitution of corn starch for part of the content of cane sugar and increasing the casein level from 5 to 6 per cent resulted in diet L III (Table I), a slight modification of the ration recommended for special purposes by White and Jackson (15). The experiments performed with diet L III are summarized in Table III. The results were essentially

<sup>5</sup> Kindly supplied by Dr. David Klein of the Wilson Laboratories, Chicago.



the same as those obtained when rats were fed diet L II, with the distinction that the addition of *l*-cystine in a dose as low as 12.5 mg. daily had a markedly aggravating effect on the incidence of hepatic injury in 6 of the 7 rats that received this dosage. In all 5 rats that received 25 mg. of *l*-cystine daily as a special supplement not only was severe cirrhosis manifested but in 4 of these animals acute necrotizing nephrosis

TABLE III  
*Results of Feeding 44 Rats Diet L III\**

Special supplement			Total No. of rats treated	No. of rats with no hepatic injury	Hepatic injury present			
<i>l</i> -Cystine daily	Choline daily	Yeast 3 times a wk.			No. of rats	Necrosis	Cirrhosis	Necrosis and cirrhosis
mg.	mg.	gm.						
—	—	—	13	3	10	5	4	1
25	—	—	5	—	5	—	5	—
12.5	—	—	7	1	6	—	6	—
25	10	—	6	4	2	2‡	—	—
12.5	10	—	5	4	1	1‡	—	—
—	—	0.5	8	8	—	—	—	—

\* For routine daily supplements see note\* Table II.

‡ Only mild changes were observed.

TABLE IV  
*Results of Feeding 62 Rats Diet C I\**

Special supplement			Total No. of rats treated	No. of rats with no hepatic injury	Hepatic injury present			
<i>l</i> -Cystine daily	Choline daily	Yeast 3 times a wk.			No. of rats	Necrosis	Cirrhosis	Necrosis and cirrhosis
mg.	mg.	gm.						
—	—	—	12	2	10	1	8	1
25	—	—	11	1	10	1	9	—
25	10	—	10	7	3	—	3	—
—	—	0.5	9	7	2	—	2	—
25	—	0.5	10	5	5	—	5	—
25	10	0.5	10	10	—	—	—	—

\* For routine daily supplements see note\* Table II.

was also observed. The beneficial effect of the combined administration of cystine plus choline, as well as of 0.5 gm. of yeast three times a week, could again be clearly demonstrated.

*Diet C I.*—The most severe hepatic changes (necrosis, cirrhosis), often accompanied by ascites and pleural and pericardial effusion, were found in rats fed diet C I (Table I), which had a low content of casein (8 per cent) and high content of fat (crisco, 38 per cent). The increased intake of fat certainly aggravated the manifestation of hepatic injury (Table IV). The effects of the administration of *l*-cystine, on the one hand, and of *l*-cystine plus choline, on the other hand, were fully consistent with the

results characteristic of diets L II and L III. Acute necrotizing nephrosis was seen only in the group of rats that received the special supplement of *l*-cystine alone.

The results obtained with the use of diet C I, therefore, are a direct confirmation and corroboration of conclusions already firmly established.

#### DISCUSSION

The experiments reported here show conclusively that in the production of dietary hepatic injury (necrosis and cirrhosis, with or without ascites and pericardial and pleural effusion) the determining factors are known to be connected with the lipotropic action of casein (13). In short term experiments identical conditions produce fatty metamorphosis in the liver, whereas experiments of longer duration, up to 150 days, lead to necrosis or cirrhosis of the liver in rats.

At the same time, independently of these investigations and the preliminary publication (1) of their results, Webster (16) arrived at similar deductions. Results of experiments likewise independently conducted, which were published later by Blumberg and McCollum (17) as well as by Sebrell and his collaborators (18), are in essential agreement with those of the present and preliminary (1) series. Fat infiltration of the liver has been considered earlier by Connor (19) a prerequisite of cirrhosis, without correlating this assumption to the phenomenon of the lipotropic activity of casein and to its mechanism.

The lipotropic action of casein is generally (13) believed to be in direct proportion to its content of methionine and to the presence of choline in the diet, but it is reversely influenced by administration of *l*-cystine and supplements of fat, especially cholesterol. Identical conditions seem to regulate also the occurrence of cortical hemorrhagic necrosis of the kidneys as it is observed mainly in young rats (6, 20). A thorough review of the experimental data given here reflects mirror-like all the conditional factors in their relation to necrosis and cirrhosis of the liver. Thus, the prophylactic effect in rats of a high intake of casein or of adequate supplements of methionine and choline is noted, the latter mainly in combination with cystine. Striking is the fact that hepatic injury is caused by supplements of *l*-cystine alone and by a high fat ratio in the diet, which appears to operate here in the same way as in the mechanism of fat infiltration of the liver.

One minor discrepancy between the conclusions reached by Blumberg and McCollum (17) and those reported in this and the preliminary paper (1) refers to the rôle of choline in the prevention of hepatic injury. According to Blumberg and McCollum addition of choline to the diet (10 mg. per gm. of diet) resulted in normal livers, whereas in the studies reported here when choline (20 mg. per day) was added as a separate supplement to a slightly different form of diet, low in protein and moderately high in fat, hepatic injury, especially in the form of necrosis, still occurred in about 50 per cent of the animals (Table II). It is noteworthy that choline, even when it is given in combination with liver extract (Table II), proved to be ineffective in the prevention of hepatic

injury. In contrast, the combined administration of *l*-cystine and choline has been found to be very beneficial in all experiments of the present series. As a matter of fact, this latter result has been confirmed by Blumberg and McCollum (17) as well as by Daft, Sebrell, and Lillie (18).

That choline seems to act through the intermediation of *l*-cystine is also apparent from investigations (21) on hepatic injury in rats following ingestion of dimethylaminoazobenzene (butter yellow). It has been definitely established that choline or *l*-cystine, given separately, exerted no effect on the course of hepatic injury in rats maintained on diet L III when it was supplemented with butter yellow. Administration of *l*-cystine *plus* choline, however, afforded definite protection.

The emphasis placed on the combined effect of cystine plus choline is at variance not only with the conclusion of Blumberg and McCollum (17) but also with that of Griffith (20) concerning the rôle of choline in the prevention of cortical necrosis of the kidney. Furthermore, the importance of the combined administration of cystine plus choline has hardly been stressed in past work dealing with the phenomenon of the lipotropic activity of casein (13). The discrepancy is, of course, not a real one, in view of the fact that in all cases in which a beneficial effect was claimed from choline alone the diet used always contained cystine, even if only in small amounts.

The combined effect of *l*-cystine plus choline is reminiscent of a similar phenomenon encountered lately in assays of the growth promoting activity of the essential amino acids which are linked with choline. It has been shown by du Vigneaud and his collaborators (22) that homocystine would support growth on a methionine free diet only in the presence of choline or related substances and that methionine, on the other hand, can replace homocystine plus choline. According to du Vigneaud and his coworkers, "The explanation presented as the most probable one for this observed relationship of choline to homocystine was that choline had acted as a donor of methyl groups for the synthesis of methionine from homocystine" (23). In these experiments on growth, cystine could not be substituted for homocystine. It has recently been demonstrated, however, that methionine may be a precursor of choline in so far as the methyl groups are concerned (22) and that cystine is capable of stimulating growth only when methionine is present in suboptimal amounts (24).

Without being able to give a satisfactory explanation for all these reactions, particularly for the apparent lack of interchangeability of cystine and homocystine, we can say, however, that their relationship to the conditions that determine hepatic injury is evident. In this connection it is significant that methionine in adequate doses prevents development of hepatic injury (Table II)<sup>6</sup> just as well as it is prevented by *l*-cystine plus choline, and that suboptimal doses of methionine have the same detoxifying effect on cystine that choline

<sup>6</sup> See also Daft, Sebrell, and Lillie (18).

has, methionine being, probably, a precursor of choline, as stated by du Vigneaud and his collaborators. It should be especially pointed out that, whereas with special supplements of liver extract alone (0.2 gm. daily) to diet L II the average loss in weight in 150 days in 5 animals was found to be 36 gm. and the average gain for 8 rats that received 50 mg. of *l*-cystine daily for 150 days was 9 gm., an average gain of 99 gm. occurred in a group of 11 rats that received liver extract plus *l*-cystine as special supplements. Similar differences were not noted in groups of rats fed special supplements of choline, *l*-cystine and choline plus *l*-cystine, respectively.

In conclusion, the impression is gained that the combined administration of choline plus *l*-cystine is necessary for the synthesis of a third substance (methionine?) which in its turn is concerned in the prevention of hepatic injury.

It is even more difficult to explain the hepatic injury caused by administration of *l*-cystine alone. Griffith (20) attributes it to some unspecific activation of the metabolism. This assumption it is not easy to reconcile with the fact that large toxic doses of *l*-cystine may, in a few days or weeks, produce necrosis of the liver (25) and, according to recent investigations, also the specific picture of Laënnec's cirrhosis (26). It should be emphasized that in the present experiments and in those of Blumberg and McCollum, as well as of Daft, Sebrell, and Lillie, the amounts of cystine administered as supplements were within the physiological limits of a normal diet. The identical response to toxic doses in short term experiments and to lower doses in investigations of longer duration speaks in favor of a specific effect.

The lipotropic action of casein is generally considered (13) to be completely determined by the interaction of cystine, methionine, and choline. Dissenting from this conception, Channon and his collaborators (27) seem to assume the presence of further, until now unidentified, and admittedly less important factors in casein. The experiments here reported have not taken into consideration the amount of cystine and choline administered sufficiently to render any conclusive answer to this problem. It should, however, be mentioned that, at least in the amounts chosen, supplements of *l*-cystine plus choline plus yeast or of *dl*-methionine in large doses were more effective in completely suppressing hepatic injury than was a supplement of *l*-cystine plus choline.

Application of the results of these experiments on rats to conditions in man is allowable only within the limitations of a conclusion *per analogiam*. Nevertheless, as circumstantial evidence, the unquestionable similarity of the etiological conditions that prevail in the most important form of human cirrhosis, namely, that observed in alcoholics, should be pointed out. Low intake of protein combined with insufficient supply of the vitamin B complex (including choline) is a prominent feature of the daily diet of persons addicted to alcohol and tallies with the leading conditions of dietary cirrhosis in rats. Thus, the assumption of a specific injurious effect of alcohol here becomes superfluous

just as it does in pellagra or the beriberi of alcoholics. The recent claim of the beneficial effect of a "highly nutritious diet" supplemented with concentrates of the vitamin B complex (28) on cirrhosis in man is suggestive corroboration of the experiments presented here.

It is perhaps also permissible to call attention to the identical nutritional etiology of hemorrhagic cortical necrosis of the kidneys and of necrosis of the liver, and to the occurrence of both pathological lesions in pregnant women, hepatic injury (hemorrhagic necrosis) being often found in eclampsia.

From the point of view of general pathology it is of great interest that identical etiological conditions may lead, in rats, either to necrosis or to cirrhosis of the liver or to both.

The experiments reported here have been, thus far, exclusively of a prophylactic nature. Their scope needs to be extended to include therapeutic investigations. In a few animals in which hepatic injury was ascertained by biopsy, therapeutic administration of yeast or liver powder for a short time (up to 2 months) proved to be insufficient. These experiments are being continued on a larger scale and for prolonged periods.

#### SUMMARY

Experimental dietary hepatic injury (diffuse or focal necrosis and cirrhosis in rats, with or without ascites and pleural and pericardial effusion) is determined by the dietary factors instrumental also in the production of fat infiltration of the liver and thus opposed to the lipotropic activity of casein. Accordingly, rats maintained on a diet low in casein with a moderately high or high content of fat and without choline regularly exhibited hepatic injury after between 100 and 150 days. Supplements of *l*-cystine had an aggravating effect on the production of cirrhosis of the liver, whereas a supplement of choline alone reduced the severity and the incidence of hepatic injury, although not decisively. The combined administration of *l*-cystine plus choline or of *dl*-methionine in adequate doses, however, proved to be highly effective in preventing injury to the liver.

These conclusions have been corroborated by the use of different modifications of the basal diet.

Rats with dietary hepatic injury exhibit, in sequence, changes that vary from diffuse necrosis resembling human acute or subacute yellow atrophy to advanced portal cirrhosis.

Diffuse necrotizing nephrosis was a frequent accompaniment of the hepatic injury. Cystine again proved to be a factor which aggravated this condition.

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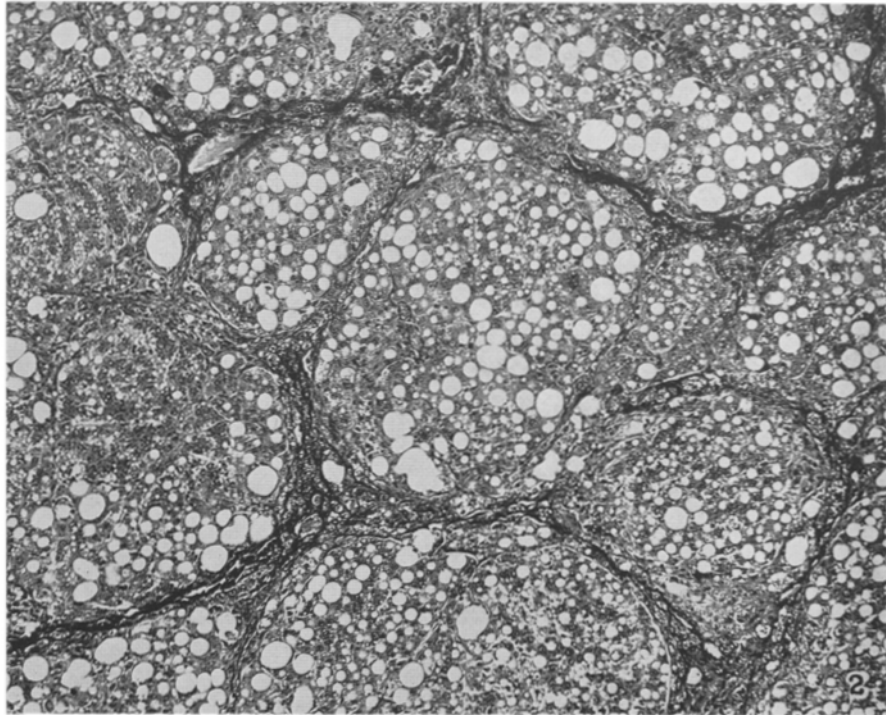
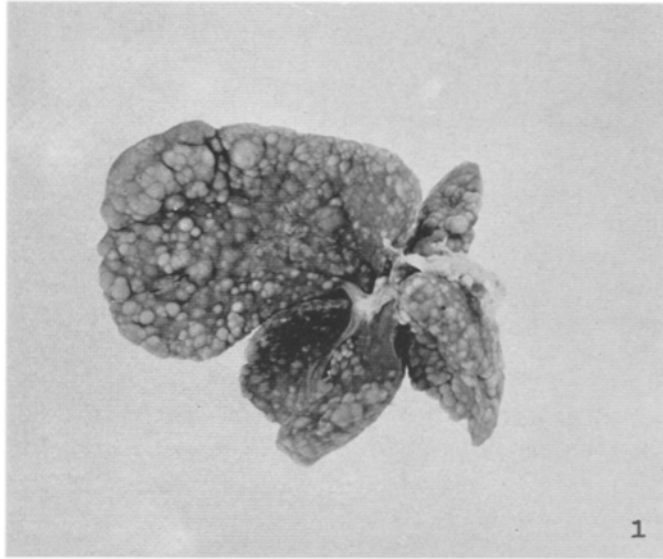
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## EXPLANATION OF PLATE 12

FIG. 1. Cirrhotic liver of rat 6568. (Natural size.)

FIG. 2. Section of liver of rat 4628, showing moderate degree of cirrhosis and fat infiltration. Masson trichrome stain.  $\times 71$ .



(György and Goldblatt: Dietary hepatic injury in rats)