this could only be done if arrangements can be made to collect bile and urine simultaneously over a long period. The increased spontaneous nicotinamide methochloride output reported in Table 6a during the first day following administration of poison is probably due to an increase of available nicotinamide caused by tissue destruction and the liberation of coenzymes. Such tissue disintegration is proved by the increased output of protein nitrogen. No indication of a decreased destruction of nicotinamide methochloride can be found and the temporary increase in urinary elimination of ingested nicotinamide methochloride after carbon tetrachloride poisoning found by Perlzweig, Huff & Rosen (1946), can be fully explained by the increase of the circulating nicotinamide from tissue breakdown and the diminished elimination of the methylated compound in the bile from the damaged liver.

SUMMARY

1. Experiments carried out *in vitro* produced no evidence that destruction of nicotinamide methochloride occurred in the liver.
2. It could be shown that a considerable portion of nicotinamide methochloride formed in the liver or

administered to a rat, was eliminated with the bile into the intestinal tract. Whether this accounts for the total amount, not eliminated in the urine, could not be decided.

3. An emulsion of intestinal bacteria destroyed a large part of the nicotinamide methochloride present in the medium.

4. The faecal elimination of nicotinamide methochloride depends on the elimination in the bile, its destruction and synthesis by intestinal bacteria and perhaps on reabsorption from the intestines. Faecal output affords no conclusive evidence regarding the nicotinamide state of the body.

5. Liver poisons such as chloroform, carbon tetrachloride or yellow phosphorus cause a temporary rise in nicotinamide methochloride output, probably due to tissue destruction, but they also reduce temporarily the efficiency of the methylating mechanism of the liver.

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The Cystine and Methionine Contents of Liver Protein in Acute Hepatic Necrosis

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Acute necrosis of the liver has been produced in rats by many workers using diets deficient in the sulphur-containing amino-acids. This work has recently been reviewed by Glynn, Himsworth & Neuberger (1945). All have found that there is a delay of 20–100 days, during which the animal appears normal, before the sudden onset of the

disease. The condition has been attributed to a simple deficiency of cystine, although methionine can replace cystine in preventing the disease. It has been suggested that during the latent period the animal is living on endogenous stores of cystine and methionine and that the onset of disease follows their final depletion. It is possible that an unknown

positive factor is also involved. An infection seems to have been excluded by Weichselbaum (1935) and by Himsworth & Glynn (1944a). Hock & Fink (1943) favour on histological grounds an explanation involving the action on the liver of a poisonous substance from the gut which requires cystine for its detoxication. This is supported by the work of Weichselbaum (1935) who was able quickly to revive rats dying from acute hepatic necrosis by feeding 100 mg. of cystine by stomach tube, or by an intraperitoneal injection of 150 mg. of glutathione. It is difficult to imagine the cystine being used to form protein under these conditions, as relatively large amounts of other amino-acids, including methionine, would also be simultaneously required, and could not be immediately available.

Clearly the changes in sulphur content of the liver that may be taking place during the latent period of the disease and after it has become established may provide information as to its mechanism. The question of the possible 'plasticity' of the liver proteins arises. This subject has been reviewed by Luck (1937) who considers that the matter is not yet proved either way. Several protein fractions have been isolated from the liver and a change in their relative proportions as well as in their individual composition could cause gross changes in the content of sulphur-containing amino-acids. No work on this subject has yet been reported with regard to acute hepatic necrosis produced by dietary deficiencies. Some attention has, however, been paid to the problems arising from directly administered liver poisons.

The histological appearance in the latter case is usually that of a zonal necrosis of liver cells, i.e. it is limited to particular zones of each lobule throughout the liver, as in the centrilobular necrosis following chloroform poisoning. In dietary necrosis on the other hand the necrosis tends to be massive in type, i.e. it affects entire lobules in certain parts while leaving them comparatively unharmed elsewhere. However, in spite of these histological differences the two problems resemble one another strongly in that both diseases are favoured by dietary deficiencies in the sulphur-containing amino-acids. Hence, there is good reason to suppose that there is in both cases a similar protective mechanism involving sulphur.

Miller & Whipple (1942) claimed that they could raise the gross nitrogen/sulphur (N/S) ratio of dog's liver from 11-12:1 to 14-15:1 by the giving of a low protein diet. In this condition of relatively low sulphur content the livers were easily damaged by chloroform. Giving methionine lowered the ratio to normal, or even below normal (i.e. increased the relative amounts of sulphur) and increased the resistance to chloroform. Several amino-acids not containing sulphur were tried without any similar

effect. Cystine had a weaker protective action. The high resistance of the liver of the newborn pup towards chloroform was ascribed to its unusually low N/S ratio. Miller & Whipple (1942), however, determined only the total sulphur and nitrogen in the dried livers, so they could not have detected any change in the relative contributions of protein or non-protein sulphur to these figures, nor any change in the relative cystine or methionine contents.

More recently Shaffer & Critchfield (1945) have analyzed the livers of rats on a sulphur-poor diet after several days of carbon tetrachloride inhalation. They found no change in the N/S ratio of dried fat-free liver.

It seemed desirable therefore to study the possible changes in the cystine and methionine contents of pure total liver protein in dietary acute hepatic necrosis. Suitable material from rats on a synthetic diet in which mixtures of pure amino-acids were used instead of protein was available from experiments being carried out by Glynn, Himsworth and Neuberger (1945). Analyses were also carried out on the liver protein obtained from a strain of black-hooded rats which had appeared to be more resistant than other strains to the disease, for comparison with the corresponding figures for susceptible white Wistar rats.

EXPERIMENTAL

Analytical methods. Cystine and methionine were determined by the gravimetric method of Lugg (1938). This requires three separate operations followed in each case by precipitation and weighing of the liberated sulphur as BaSO_4 . Total sulphur (*A*) is determined after fusion of the protein with sodium peroxide and carbonate, cystine plus sulphate sulphur (*B*) after refluxing with fuming nitric acid, and sulphate sulphur (*C*) after complete acid hydrolysis of the protein. Clearly $A-B$ = methionine sulphur, while $B-C$ = cystine sulphur. The last determination (*C*) was omitted in the second experiment. Lugg's method was tedious, but gave good results with pure methionine and cystine, and duplicates on proteins agreed well. Its accuracy is subject to the assumption that there is no source of sulphur in the protein apart from methionine, cystine and free sulphate. Nitrogen was determined throughout by the micro-Kjeldahl method.

The baker's yeast used in the first experiment had on analysis the following composition: total sulphur 0.53, 0.49, 0.52%; cystine plus sulphate sulphur 0.19, 0.19%; sulphate sulphur 0.03, 0.03%; whence methionine = 1.49%, cystine = 0.60%, Total N = 7.02%. Total N/Total S = 13.8:1. The yeast contained a higher proportion of its sulphur as methionine than was expected (Himsworth & Glynn, 1944b, footnote p. 137). Block & Bolling (1945), however, recently obtained only slightly lower figures than these for both amino-acids. In the usual average daily intake of 8 g. total food, containing 1.3 g. of crude yeast, each rat received 19.4 mg. of methionine and 7.8 mg. of cystine.

Animals and diets. In the first experiment livers from white Wistar and black-hooded male rats, were used. These animals had been fed in groups of eight on a diet consisting essentially of the low fat, 8% yeast protein diet described

by Himsworth & Glynn (1944a). They averaged 100 g. in weight at the beginning of the experiment.

In the second experiment, material from white Wistar male rats was used. These averaged 80 g. in weight at the beginning of the experiment. Except for the control group on casein, the dietary nitrogen was entirely supplied by mixtures of pure amino-acids (see Table 1). Full details of the diets are reported elsewhere (Glynn *et al.* 1945). The rats were separately fed and the protein, or amino-acid, intake was kept constant by reducing only the carbohydrate intake if the appetite was poor. Adequate vitamin and mineral supplements were given in all experiments.

Table 1. *Protein and amino-acid composition of diets in second experiment*

Group 1	3 rats (I/1-I/3), casein 0.86 g.
Group 2	4 rats (II/1-II/4), sulphur-free amino-acid mixture 1.3 g. + methionine 80 mg.
Group 3	3 rats (III/1-III/3), sulphur-free amino-acid mixture 1.3 g. + cystine 64 mg.
Group 4	3 rats (IV/1-IV/3), sulphur-free amino-acid mixture 1.3 g.
Group 5	4 rats (V/1-V/4), sulphur-free amino-acid mixture 1.3 g. + methionine 8 mg.

The 1.3 g. of amino-acid mixture contained pure amino-acids in the same quantities as in the 0.86 g. of casein except for methionine and cystine. The individual rats are designated as shown in brackets.

Two animals from each of the Groups 1 and 2 (namely I/2, I/3, II/3 and II/4) were killed at about 100 days. The remaining animals from these groups (namely I/1, II/1 and II/2) were then given the basal sulphur-free diet (Group 4, Table 1).

Technique of sampling of livers. In the first experiment, several rats happened to die on the same day with acute necrosis of the liver. They had been 118 days on the diet. This enabled the bulking of the livers so as to provide ample amounts of protein for analysis. It also made possible the perfusion with saline before working up as usual of another group of necrotic livers from animals in the same experiment. This was considered important. Even in normal livers the blood unavoidably left behind makes a variable contribution to the protein content. In acute necrosis of the liver there is good evidence (Himsworth & Glynn, 1945) of additional infiltration of the liver with fluid, probably plasma, containing protein. After removal of necrotic livers considerable oozing of serum from the inferior vena cava was commonly seen. Losses in weight of as much as 1.5 g. in a liver weighing 5.8 g. when first removed were caused in this way. The oozing appeared to be greatest when the onset of the disease was most acute. Clearly if this extra protein differed markedly from liver protein in its sulphur-containing amino-acid content it would be impossible to distinguish analytical differences due to the liver necrosis from those due to this variable amount of adventitious protein. In two of the liver perfusions the amount of haemoglobin washed out was roughly estimated in the Haldane apparatus and the total nitrogen determined. In the first liver (wt. 11.7 g.) the haemoglobin corresponded to the amount contained in 0.75 ml. of normal blood (Hb 100%) while the amount of nitrogen washed out was

15% of the total nitrogen remaining in the liver. In the second liver (wt. 7.3 g.) the figures were 0.5 ml. and 20% respectively. Some loss of actual liver protein would also be expected under the conditions of operation.

On the following day livers were obtained from some apparently normal rats surviving on the same diet, which were killed as controls. Some black-hooded rats, also normal, were killed and their livers analyzed. The results are given in Tables 2 and 3.

These experiments showed that perfusion of the liver lowered the non-protein nitrogen but did not affect the cystine or methionine content of the protein obtained (Table 2). No further perfusions were therefore considered necessary.

Isolation of liver protein. The rats were killed by stunning, quickly opened and bled into the pleural cavities by cutting the inferior vena cava. When the heart had stopped beating the liver was quickly removed, weighed and dropped into 50 ml. of boiling water. After 15 min. gentle boiling it was removed and ground with 20 times its weight of 10% (w/v) trichloroacetic acid. It was then centrifuged in a 250 ml. bottle, the supernatant liquid poured off, and the residue washed successively with hot water, hot ethanol containing 10% (v/v) ether and light petroleum (b.p. 40-60°). The aqueous washings were combined with the water used for boiling the liver and analyzed for non-protein nitrogen by the Kjeldahl method. The ethanol and ether washings were evaporated to dryness and the residue extracted with light petroleum. The residue from evaporation of this extract was weighed as fat. The insoluble liver protein, usually a fine buff powder, was dried at 100° to remove solvents, finely ground and then left exposed for several days till it had attained constant weight. It was then ready for analysis. In the three rats IV/1, IV/2 and V/4 (marked † in Table 4) the livers were not worked up in this way. They were rapidly dried *in vacuo* at 100°, then finely ground and thoroughly extracted with ether. The dried residue was analyzed as such. It probably contained a little remaining non-protein material.

The livers of rats I/2 and I/3 were combined for analysis, the growth curves of the rats having agreed very closely. Similarly the livers of II/3 and II/4 were combined. The sulphate sulphur was not determined for any of these rats, owing to the small amount of protein available. It was assumed throughout to equal 0.04%.

Liver perfusions. These were carried out immediately after death. The procedure was as above except that before removal of the liver from the abdomen, a small needle was tied into the portal vein. The liver was then removed with the portal vein intact and the needle *in situ*. Isotonic saline was allowed to pass at room temperature and at a few cm. pressure for about 10 min. by which time the effluent from the cut ends of the vena cava was clear and colourless and the liver had become noticeably paler although the red patchy subcapsular 'haemorrhages' were not washed out. The liver bulged evenly during the perfusion and the lobular pattern was evenly exaggerated throughout, suggesting adequate perfusion of the saline in the vascular pathways.

Water content of tissues. In all the rats in the second experiment a slice of the liver was taken for drying at 105° to constant weight and the residue analyzed for total nitrogen by the Kjeldahl method. Loss of weight at 105° of a slice of kidney and of adductor muscle was also determined as a matter of routine, as a check on generalized

oedema. These routine liver analyses confirmed the gross chemical changes found by Himsworth & Glynn (1945). The necrotic livers were heavier than normal, the ratio, liver weight/body weight $\times 100$ being constantly greater than 5. It was less than 4 in the non-necrotic livers. An increase in g. water/100 g. liver was also found in the necrotic livers and also a rather slighter increase in the total liver protein ($N \times 6.25$)/100 g. body weight. The fat content of the livers varied from 0.6–7.0% and bore no relation to the presence of necrosis.

RESULTS

The results are given in Tables 2–5.

Table 2. *Gross liver analyses in first experiment with yeast diet*

Group	No. of rats grouped	Body wt. when killed (g.)	State of livers	Whether perfused	Fat (g./100 g. fresh liver)	NPN (g./100 g. fresh liver)	Protein obtained from livers (g.)	N (g./100 g. liver protein)
A. White Wistar rats	3	130, 108, 84	Acute necrosis	No	5.7	0.12	3.65	14.1
B. White Wistar rats	4	112, 100, 161, 91	Acute necrosis	Yes	7.1	0.08	3.92	14.3
C. White Wistar rats	2	165, 174	Normal	No	5.2	0.17	2.15	13.8
D. Black-hooded rats	2	124, 133	Normal	No	5.4	0.13	1.85	13.6

Table 3. *Methionine and cystine content in g./100 g. of protein isolated from livers in the first experiment with the yeast diet*

Group	Total S	Cystine + SO ₄ -S	SO ₄ -S	$\therefore$ Methionine	$\therefore$ Cystine	Total N/Total S
A. White Wistar rats	1.33	0.43	0.04	4.16	1.48	12.0
B. White Wistar rats	1.33	0.43	0.04	4.18	1.44	12.0
C. White Wistar rats	1.46	0.43	0.06	4.77	1.38	11.0
D. Black-hooded rats	1.47	0.44	0.05	4.76	1.45	10.9

The figures are all calculated on the basis of g./100 g. of protein containing 16% N.

DISCUSSION

The object of this investigation was to secure evidence in favour of the theory that acute hepatic necrosis is a simple deficiency disease due to lack of the two sulphur-containing amino-acids in the diet. Strong evidence would be obtained if it could be shown that the latent period in the development of the disease and the final diseased condition of the liver were closely related to decreases in the total quantity of one or other amino-acid. An 'ideal' experiment could be devised in which rats would be separately fed on synthetic diets with pure amino-acids in place of protein. Some would then be killed in groups during the latent period of the disease. These livers, as well as those obtained after death from acute necrosis, would be analyzed. Even under these conditions, however, the rats would be expected to die of acute necrosis of the liver at widely varying times owing to the existence of unknown factors in the pathogenesis of the disease (see Table 4; larger variations and even complete failure to produce the disease have also been found with yeast and casein diets). Large numbers of animals would therefore be required to average out these variations. The cost of the experiment would be prohibitive.

However, it is believed that some definite conclusions can be drawn from the relatively few experiments described here. In the first experiment with the yeast diet, the detailed analyses (Table 3) show, no significant difference in cystine and methionine contents between the normal black-hooded and the Wistar rats such as might have accounted for the different resistance of their livers to the disease. The cystine contents of the liver proteins were very constant and were independent of the presence or absence of necrosis of the liver. The methionine content, however, was a little higher in

the normal animals than in those with necrosis. The gross differences in N/S ratio were much smaller than those found by Miller & Whipple (1942) in their dogs. As the rats were group-fed there may have been wide variations in individual food intake especially during the day before the normal animals were killed, as there was available to them the food left over by the rats dying from acute necrosis. It is not believed, therefore, that any further conclusions can be formed from this experiment.

The results of the second experiment with the synthetic diet show only slight variations in the cystine content of the liver protein and more marked changes in methionine (Table 4). At first sight the figure of 7.15% methionine in the necrotic liver of rat IV/3 contrasts markedly with those of 4.64 and 5.07% for the healthy controls I/2 + I/3 and II/3 + II/4 which were fed on a diet adequate for growth. On the other hand, similarly high methionine values were found in the liver protein from rats III/2 and III/3 which did not get necrosis but appeared to die of anaemia and hypoproteinaemia. It seems that the chief factor here is not the presence or absence of acute necrosis but the state of development of the animal. The high methionine values occurred in the small livers of the rats that had failed to grow

Table 4. Methionine and cystine content in g./100 g. of protein isolated from livers of rats in the second experiment with the amino-acid diet

Rat	Diet	Diet fed days	Weight of rat when killed (g.)	State of livers	Protein isolated from liver (g.)	N (g./100 g. protein)	Total S*	Cystine and SO ₄ -S*	∴ Methionine*	∴ Cystine*	Total N/Total S
I/1	Casein then sulphur-free	180 and 18 respectively	118	Necrosis	0.61	13.9	1.74	0.58	5.37	1.73	9.2
I/2 and I/3	Casein	108	181 and 200	Normal	2.30	14.4	1.60	0.51	5.07	1.55	10.0
II/1	High methionine then sulphur-free	118 and 22 respectively	119	Necrosis	0.59	13.8	1.76	0.57	5.48	1.70	9.1
II/2	High methionine then sulphur-free	118 and 8 respectively	178	Necrosis	1.24	13.7	1.73	0.60	5.25	1.77	9.2
II/3 and II/4	High methionine	101	222 and 225	Normal	2.75	14.7	1.51	0.52	4.64	1.61	10.6
III/1	High cystine	146	43	Normal	0.42	14.0	1.72	0.55	5.43	1.65	9.3
III/2	High cystine	98	41	Normal	0.33	14.5	2.24	0.64	7.45	2.01	7.1
III/3	High cystine	57	61	Normal	0.44	13.8	2.19	0.61	7.33	1.82	7.3
IV/1†	Sulphur-free	33	47	Necrosis	0.26	13.1	1.72	—	—	—	9.3
IV/2†	Sulphur-free	21	57	Normal	0.44	13.5	1.68	0.62	4.97	1.78	9.5
IV/3	Sulphur-free	57	55	Necrosis	0.30	13.0	2.16	0.62	7.15	1.72	7.4
V/1	Low methionine	44	74	Necrosis	0.44	12.5	2.07	—	—	—	7.7
V/2	Low methionine	103	92	Post-necrotic scarring	0.72	14.5	—	0.56	—	1.74	—
V/3	Low methionine	38	94	Necrosis	0.44	15.2	1.70	0.53	5.42	1.74	9.4
V/4†	Low methionine	34	95	Necrosis	0.88	12.3	1.79	—	—	—	8.9

* The figures for sulphur and amino-acids are all calculated on the basis of g./100 g. of protein containing 16% of N.
† Liver protein obtained by drying at 100° followed by ether extraction.

Table 5. Sulphur, methionine and cystine content in g./100 g. of protein isolated from livers of rats in the second experiment with the amino-acid diet

Rats rearranged in order of length of time on methionine-free or low methionine diets													
Rat no.	I/2 + I/3	II/3 + II/4	I/1	II/1	IV/1	V/4	V/3	V/1	IV/3	III/3	III/2	III/1	III/1
Body weight at death (g.)	181 and 200 respectively	222 and 225 respectively	118	119	47	95	94	74	55	61	41	43	43
Days on methionine-free or low methionine diet	0	0	18	22	33	34	38	44	57	57	98	146	146
Sulphur (g./100 g.)*	1.60	1.51	1.74	1.76	1.72	1.79	1.70	2.07	2.16	2.19	2.24	1.72	1.72
Methionine (g./100 g.)*	5.07	4.64	5.37	5.48	—	—	5.42	—	7.15	7.33	7.45	5.43	5.43
Cystine (g./100 g.)*	1.55	1.61	1.73	1.70	—	—	1.74	—	1.72	1.82	2.01	1.65	1.65
N/S ratio of liver protein	10.0	10.6	9.2	9.1	9.3	8.9	9.4	7.7	7.4	7.3	7.1	9.3	9.3
Cause of death	Healthy controls, killed						Acute necrosis of liver				Anaemia and hypoproteinaemia		

* The sulphur and amino-acid contents are calculated on the basis of g./100 g. of protein containing 16% N.

because they had been on a diet lacking in the essential amino-acid methionine. This deduction is borne out also in the rats of Groups 1 and 2 (i.e. rats I/1, II/1 and II/2) which were put on the basal sulphur-free diet after they had grown normally on the adequate casein and high methionine diets respectively. Rat II/2 died of typical acute hepatic necrosis in the record time of only 8 days on the deficient diet. Its weight had only fallen from 206 to 178 g. during that time. By comparing the liver analysis for this rat with those for the control animals II/3 + II/4 we can determine the effect of the disease itself with less interference than usual by other variables such as body weight and previous dietary history. The analyses are not significantly different. In Table 5 the analyses are re-arranged in order of the length of time during which the rat had been on the methionine-free (Groups 3 and 4) or low methionine (Group 5) diets. The analyses are now roughly in order of increasing sulphur and methionine contents and of decreasing N/S ratio. There is a less marked tendency for the cystine content to rise also. The only notable exception is the remarkable rat III/1 which survived 146 days on a diet rich in cystine but completely devoid of methionine and which only lost 7 g. in weight in the last 50 days. It appears to have made no difference to the composition of the liver protein if the daily diet contained no sulphur (Group 4), 8 mg. of methionine (Group 5) or 64 mg. of cystine (Group 3). Moreover the general trend of analytical results is quite independent of whether or not the rat died of acute hepatic necrosis. The conclusion is drawn that there is no change in cystine or methionine content of the liver protein characteristic of the dietetic necrosis as such.

The possibility that there may be gross changes in the content of other amino-acids has been briefly tested. As a rough check the two liver proteins from rats II/3 + II/4 (normals) and from rat II/2 (acute hepatic necrosis) were hydrolyzed for 24 hr. with approx. 5N-hydrochloric acid under identical conditions. Equal quantities of the hydrolyzates were then run on the two-dimensional paper chromatograms of Consden, Gordon & Martin (1944) using the pairs of solvents phenol/collidine and butanol-benzyl alcohol/collidine. The former gives a better separation of the low molecular weight amino-acids and the latter distinguished best between the larger molecules. The developed chromatograms were almost identical as to the size, strength and position of all the amino-acids shown.

The results obtained here can be reconciled with those of Miller & Whipple (1942) by assuming that

the changes in total sulphur and N/S ratio they recorded are entirely referable to changes in the non-protein part of the liver. That such changes can occur has been shown by Lee & Lewis (1934) who analyzed separately for total sulphur the non-protein and protein fractions of the liver of rats who had received cystine. They found sharp rises in non-protein sulphur, but the liver protein was unchanged. The work of Brunschwig, Nichols, Bigelow & Miles (1945) also supports the existence of a similar mechanism. These workers found that in dogs liver damage due to chloroform, as measured by impaired bromsulphthalein excretion, could be lessened by injections of sodium thioglycollate as well as of methionine. They suggested that the protective action was due to the donation of —SH groups. However the thioglycollate may act, the reaction must occur in the non-protein fraction as, unlike methionine, it cannot be built up into body protein. The comparison between dogs and rats, however, is subject to criticism on the grounds of the possibility of species differences. The recent work of Leaf & Neuberger (1947), who find a marked fall in the glutathione and total —SH in the non-protein nitrogen of the liver of rats after about 10 days on a diet low in sulphur-containing amino-acids, points more directly to the same conclusion.

SUMMARY

1. Analyses for cystine and methionine of the liver protein from rats with acute hepatic necrosis are reported. The diets covered a wide range of intake of cystine and methionine.
2. It is concluded that the development of acute necrosis is not related to a change in the proportion or amount of these amino-acids in the liver protein.
3. The methionine content of the liver protein from rats maintained on diets devoid of methionine, until they lost much weight was increased, but the cystine content rose only slightly.
4. These results can be best reconciled with previous work on liver damage caused by chlorinated hydrocarbons, by assuming that the significant changes, if any, in total sulphur of the liver are referable only to the non-protein fraction.

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