Experimental Liver Necrosis in Rats

1. CHANGES IN LIVER, BLOOD AND SPLEEN GLUTATHIONE AND ASCORBIC ACID LEVELS IN DIETETIC LIVER NECROSIS

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Leaf & Neuberger (1947) reported that feeding rats with diets low in protein or sulphur-containing amino acids produced a marked and rapid fall in the reduced glutathione level of the liver to 20–30 % of the normal value. The authors stated that the original purpose of their work was 'to find out whether the acute massive necrosis of the liver observed in rats reared on diets containing only traces of cystine and methionine is caused by a change in the composition of the liver with respect to sulphur'. As the diets failed to produce liver necrosis, the question could not be answered. Subsequent to this work it became evident that a deficiency, not only in sulphur-containing amino acids but also in tocopherol, was required to produce dietetic liver necrosis (Himsworth, 1950).

In this laboratory, much work has been carried out on the conditions necessary for rapid and consistent production of dietetic liver necrosis in rats (Himsworth & Glynn, 1944*a, b*; Himsworth & Lindan, 1949; Lindan & Himsworth, 1950). In the standard diet (called hereinafter a 'necrogenic diet') all the protein (7 % of the diet) was provided by baker's yeast, shown chemically to be deficient in cystine and methionine (Lindan & Work, 1951*a*), and the tocopherol content was about 1 mg./100 g. diet. This diet produced liver necrosis in young rats in 12–55 (mean 28) days, provided that the rats had been subjected to preliminary tocopherol depletion prior to weaning. When supplemented with cystine or with tocopherol, the diet ceased to be necrogenic (Lindan & Himsworth, 1950).

This paper reports the effect of this yeast diet on the glutathione levels of livers, examined both before and after the development of massive necrosis. Estimations were made of both reduced glutathione (GSH) and oxidized glutathione (GSSG), since the obvious connexion between liver necrosis and tocopherol (an anti-oxidant) suggested that changes in the proportions of GSH and GSSG might occur in tocopherol deficiency. Ascorbic acid, another oxidizable compound, was also estimated.

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Water, nitrogen (total and non-protein), fat and glycogen estimations were also carried out. The interchangeability of tocopherol and sulphur-containing amino acids as preventive factors in liver necrosis led us to investigate liver glutathione levels in rats given the yeast diet supplemented with one or other of these factors. It was also of interest to find out whether the biochemical changes in necrotic livers were specific to that organ, or were a more general result of the illness and common to other tissues; accordingly spleen and blood were also examined. Preliminary figures have been reported already (Lindan & Work, 1951*b–d*).

EXPERIMENTAL

Animals and diets. The animals used were Wistar albino weanling rats (wt. 34–46 g.) bred in the Medical School. Except for controls reared on stock cube diet (group I, Table 1), the rats were depleted of tocopherol prior to weaning by feeding the mother a low-tocopherol diet during the last 10 days of lactation (Lindan & Himsworth, 1950). The diet given from weaning time had the following composition: dried baker's yeast 18 %, corn starch 73 %, lard 5 %, cod-liver oil 1 %, salt mixture 3 %. To this diet were added daily per rat the following amounts of vitamins: thiamine, 20 µg.; riboflavin, 25 µg.; pyridoxine, 20 µg.; calcium pantothenate, 100 µg. This was the necrogenic diet used in previous experiments on liver necrosis (Lindan & Himsworth, 1950) except that lard was substituted for olive oil. Rats of groups III and VI were given this diet *ad lib.* unsupplemented; additional α -tocopherol acetate was given to group IV (2 mg./rat/day for the first 5 days of experiment) and to group V (200 mg./rat/day throughout the experiment). The effect of supplements of sulphur-containing amino acids (Table 2) was tested either by adding to the diet L-cystine and DL-methionine to concentrations of 0.13 and 0.11 % respectively (thus doubling the intake of these amino acids, Lindan & Work, 1951*b*) and restricting food intake to 8 g. daily when it reached this level, or by adding L-cystine to a concentration of 0.63 % and feeding *ad lib.* Some rats were killed at the beginning of the experiments (group II, Table 1) and others at various times after 7 days. The rats which developed liver necrosis when fed the unsupplemented diet (group VI) were used for experiment if moribund or when found shortly after death (still warm).

Analytical methods. The rats were killed by breaking the neck and were bled immediately by cutting either the throat or (when blood was required for analysis) the heart.

The blood (0.3–1.0 ml.) lying in the thorax was collected in a syringe and transferred to a weighed Potter homogenizer containing 3 ml. of 2.3% (w/v) sulphosalicylic acid. The abdomen was opened quickly and a piece of liver (0.8–1.0 g.) was dropped into 8 ml. sulphosalicylic acid in a weighed homogenizer. If required, other portions of the liver were cut off for estimation of glycogen, N and water, the remainder was then removed from the abdomen and weighed; portions were used as desired for other examinations. This method of sampling livers was used for non-necrotic livers and necrotic ones in which the whole organ was of uniform macroscopic appearance. When the liver did not appear uniform, the different types of tissue were analysed separately; thus in some cases it was found either that certain lobes, usually the left, were haemorrhagic and the rest of the organ was congested but pale, or that a post-necrotic scarring had affected only part of the liver (Glynn & Himsworth, 1944). After removal of the liver the whole spleen was transferred to 3 ml. of sulphosalicylic acid in a weighed homogenizer.

The tissues were minced immediately they were put into the sulphosalicylic acid, then the whole homogenizers were re-weighed to determine the weight of tissue used. Sufficient cold sulphosalicylic acid was added to bring the tissue/acid ratio to 1:10 (w/w) in the case of blood and liver. Spleens of the smaller rats often weighed less than 0.1 g.; since it was not practicable to maintain the same ratio, no further liquid was added. Precipitated protein was removed from the mince by centrifugation followed by filtration. During manipulations the extracts were kept when possible at 2°; their glutathione content was estimated within 2 hr.

GSH was estimated by a modification of the iodometric titration method of Leaf & Neuberger (1947). Ascorbic acid was first removed from the tissue extract (1 or 2 ml.) by titration with a solution of dichlorophenol indophenol (equiv. to 0.02 mg. ascorbic acid/ml.) until a pink colour lasting 10 sec. was obtained; the titration figure gave a direct measure of the ascorbic acid content of the solution. Potassium iodate (0.005 M in 2% sulphosalicylic acid containing 2%, w/v, potassium iodide) was added in excess (1–2 ml.), and back-titrated with thiosulphate solution (0.005 M) using starch indicator. Standard solutions were prepared daily by diluting stock 0.1 M solutions.

GSSG in liver extracts (3–5 ml.) was reduced electrolytically by the method of Dohan & Woodward (1939) in a beaker (4 cm. diameter) immersed in ice-cold water; a current of 30 ma., 30 v., was passed for 20 min. The difference between the iodate titration figures obtained before and after reduction gave the amount of GSSG present; dichlorophenol indophenol titrations were unaffected by reduction.

Non-protein nitrogen (N.P.N.) levels in blood and liver were determined by estimating N in the sulphosalicylic acid extracts by the micro-Kjeldahl procedure. Water content of livers was estimated after drying weighed slices at 105° to constant weight. Fat was determined by the method described by Leathes & Raper (1925) which assays fatty acids and unsaponifiable lipids. Glycogen was estimated by the method described by Evans, Tsai & Young (1931) and by Murphy & Young (1932).

Paper chromatography. Glutathione was identified in liver extracts by paper chromatography (Lindan & Work, 1951a), using the technique of Awapara (1948) for preparation of extracts. A weighed portion of liver was minced in ten parts (w/v) of 75% (v/v) ethanol; the liquid, separated

by centrifugation, was shaken with 3 vol. CHCl_3 , and two layers were separated by centrifugation. The top (aqueous) layer (300 μl ., approx. equiv. to 0.1 g. fresh liver) was applied to filter paper (46 $\times$ 57 cm.) and subjected to two-dimensional chromatography, first in phenol:water (NH_3 atmosphere) and then in collidine:lutidine:water. Duplicate chromatograms were run, one on untreated extract, the other on extract oxidized with H_2O_2 using ammonium molybdate catalyst (Dent, 1948). As a basis for comparison of the amount of glutathione present, the intensity and size of the H_2O_2 -treated glutathione spot were compared with those of the glutamic acid spot.

Histology. A sample of each liver was fixed in formaldehyde (4%, w/v, solution in physiological saline) and stained with haematoxylin and eosin. Samples were also examined for fat after freezing and staining with Sudan III or IV. Other samples were fixed in absolute ethanol and stained for glycogen with Best's carmine.

Criteria for significance. Differences between means greater than 3 times the standard error of the difference were taken as significant.

RESULTS

Effect of feeding necrogenic diet on the levels of glutathione, ascorbic acid and non-protein nitrogen in liver before development of necrosis. The results of feeding necrogenic diet to weanling rats are summarized in Table 1, which gives the mean values for liver glutathione, ascorbic acid and N.P.N. estimations.

The initial values are represented by the mean figures obtained from livers of five tocopherol-depleted weanling rats (group II); GSH + GSSG was 227 mg./100 g. fresh liver with 2 mg./100 g. as GSSG. Livers of six normal weanlings reared on stock diet (group I) gave essentially similar values, the higher figure for GSSG (16 mg./100 g.) did not represent a significant difference. Feeding necrogenic diet, unsupplemented with tocopherol, caused a rapid fall in the liver GSH level, a value between 42 and 66 mg./100 g. being reached by the 13th day. The rate of fall to this level was variable; in two out of seven livers examined between the 7th and 13th day of experiment, GSH had already reached the basal level, in four others it showed intermediate subnormal values, while one liver examined on the 7th day showed the nearly normal value of 160 mg./100 g. Since no further changes were found in the analyses of livers examined after the 13th day, it appeared that the basal level for maintenance on the necrogenic diet had been reached by this time; all figures obtained after the 13th day were therefore averaged and are presented in Table 1, group III for 'pre-necrotic' livers. No figures in this group were obtained after 34 days of experiment, because all the remaining rats had developed liver necrosis by this time. The mean level of GSH was 53 mg./100 g. The values for GSSG were very variable, ranging from 9 to 48 (mean 21) mg./100 g., but showed a significant rise from the

Table 1. *Effect of diet and of necrosis on mean glutathione, ascorbic acid and N.P.N. contents of rat livers*
(Figures in brackets indicate standard deviation of the series.)

Group	No. of rats	Diet	State of liver	Glutathione				N.P.N. (mg./100 g. liver)	T.G.N.* × 100 N.P.N.	Ascorbic acid (mg./100 g. liver)	Ascorbic acid (mg./100 g. body wt.)
				(mg./100 g. liver)	GSH + GSSG	GSH	GSH + GSSG				
I	6	Stock	Normal (weanlings)	4.26 (±0.394)	214 (±19.4)	9.12 (±1.046)	9.94 (±0.903)	182 (±16.1)	17.3 (±0.98)	50 (±9.0)	21.6 (±0.360)
II	5	25% Casein low toc.†	Normal (toc.-depleted weanlings)	4.46 (±0.167)	225 (±23.2)	10.06 (±4.320)	10.14 (±1.250)	166 (±22.6)	18.7 (±1.14)	38 (±4.1)	1.70 (±0.239)
III	10	Necrogenic no toc.	Pre-necrotic‡	5.32 (±0.612)	53 (±9.3)	2.79 (±0.368)	3.95 (±0.716)	150 (±24.2)	6.7 (±0.47)	27 (±4.7)	1.51 (±0.395)
IV	6	Necrogenic; 2 mg. toc. for first 5 days	Pre-necrotic‡	5.50 (±1.400)	72 (±15.0)	3.86 (±0.869)	5.58 (±0.739)	193 (±24.8)	7.3 (±0.60)	29 (±6.0)	1.57 (±0.381)
V	5	Necrogenic; 200 mg. toc. daily	Pre-necrotic‡	5.56 (±0.945)	63 (±5.9)	3.56 (±0.827)	5.21 (±1.040)	186 (±45.2)	6.9 (±0.37)	26 (±3.6)	1.42 (±0.209)
VI	8	Necrogenic	Necrotic	7.94 (±1.960)	22 (±2.9)	1.85 (±0.360)	3.98 (±1.330)	94 (±27.2)	6.8 (±1.50)	0	0

* T.G.N. = total glutathione nitrogen.

† toc. = tocopherol.

‡ 'Pre-necrotic' signifies mean of all figures on non-necrotic livers obtained after more than 13 days on necrogenic diet with or without tocopherol.

initial level of 2 mg./100 g.; GSH + GSSG was much below the normal level. No significant change occurred in the N.P.N. levels during the experiment, and the proportion of N.P.N. represented by glutathione nitrogen was reduced to 6.7% from the value of 18.7% found for weanlings. The ascorbic acid contents of the livers dropped slightly (from 38 to 27 mg./100 g.) during the first 13 days, and thereafter showed no change.

These pre-necrotic livers were larger than normal, calculated on 100 g. body weight basis; accordingly, to eliminate the effect of liver enlargement, all figures have been calculated both as mg./100 g. liver and as mg./100 g. body weight. The small drop in ascorbic acid was found to be without significance on 100 g. body-weight basis; the individual variability of the N.P.N. levels was so great that no significant changes could be detected in this fraction. The falls in both GSH and GSH + GSSG were highly significant on 100 g. body-weight basis.

Effect of supplementing necrogenic diet with tocopherol or sulphur-containing amino acids. The presence of tocopherol had no detectable effect on the liver changes produced by the necrogenic diet. The falls in levels of GSH and ascorbic acid between the 7th and 13th day of experiment resembled those occurring with unsupplemented diet and had ceased by the 13th day. The mean figures obtained after 13 days for animals given five small doses of tocopherol at the beginning of the experiment and none thereafter (group IV, Table 1) are not significantly different from those of the unsupplemented group III; the mean and the standard deviation for GSH figures are high because the group (six rats) includes one rat with a GSH level of 100 mg./100 g. at 14 days. Massive dosage with 200 mg. daily of tocopherol throughout the experiment also had no significant effect on the analytical figures (group V). Since tocopherol protected the animals against the development of liver necrosis, experiments of longer duration could be carried out in groups IV and V; they showed that even after 53 or 63 days on the diet, the liver GSH was no lower than after 14 days.

Table 2 shows some analyses of livers from rats given the necrogenic diet supplemented with additional sulphur-containing amino acids. In one experiment the dietary level of these amino acids was doubled; fed *ad lib.* from weaning, this diet maintained better growth and appetite than did the unsupplemented diet; in 35 days, average growth was 0.5 g. daily and food consumption rose from 4 to 8 g. daily; after this time, when food consumption was restricted to 8 g. daily, growth ceased. No necrosis developed during 87 days. Normal analytical figures were obtained on the liver of one rat killed after 55 days on the diet, showing that the small supplement was sufficient to make good the deficiency of the necrogenic diet which caused the

Table 2. *Effect of cystine and methionine supplementation of necrogenic diet on liver analysis*

Supplement to diet (g./100 g. diet)	No. of rats	Days on diet	Liver wt. (g./100 g. body wt.)	Glutathione (mg./100 g. liver)		N.P.N. (mg./100 g. liver)	T.G.N.* × 100 N.P.N.	Ascorbic acid (mg./100 g. liver)
				GSH	GSH + GSSG			
0.13 L-cystine and 0.11 DL-methionine	{1 3}	55 85	5.17 5.04	223 146	228 155	200 206	15.6 10.4	35 32
0.63 L-cystine	1	38	6.96	182	188	165	15.6	18

* T.G.N. = total glutathione nitrogen.

Table 3. *Analysis of individual heterogeneous necrotic livers and post-necrotic livers*

Condition of liver	Wt. of whole liver (g./100 g. body wt.)	(mg./100 g. liver)				T.G.N.* × 100 N.P.N.
		GSH	GSH + GSSG	Ascorbic acid	N.P.N.	
Necrotic { red portion pale portion }	6.0	{ 21 21 }	{ 55 58 }	{ 0 0 }	{ 95 92 }	{ 7.6 8.3 }
Necrotic { red portion pale portion }	4.1	{ 28 87 }	{ 48 125 }	{ 0 18 }	{ — 100 }	{ — 6.5 }
Post-necrotic { normal lobe scarred lobe }	4.8	{ 192 90 }	{ 220 135 }	{ 11 0 }	{ 204 145 }	{ 14.2 12.3 }
Post-necrotic scarring	6.6	190	208	31	—	—
Post-necrotic scarring	6.9	101	126	55	100	16.6
Post-necrotic scarring	3.4	178	197	45	310	9.0

* T.G.N. = total glutathione nitrogen.

fall in GSH. Three other rats killed after receiving 8 g. diet daily for another 30 days showed slight lowering of GSH, probably due to the prolonged food restriction.

A daily supplement of L-cystine (625 mg./100 g. diet) for 38 days also maintained a normal liver GSH level.

Effect of necrosis on liver composition. The mean results on necrotic livers of uniformly haemorrhagic appearance, developing in rats fed necrogenic diet are given in Table 1 (group VI). Because the composition of necrotic livers was the same whether the organ was removed from a moribund rat or from a rat found dead but still warm, the analytical figures for the two types of livers are presented as one group. GSH had fallen to 22 mg./100 g., which is less than half the pre-necrotic level (group III). There was no change in the amount of GSSG present (24 mg./100 g.) and as a consequence the ratio GSSG:GSH had changed to 1:1 from the pre-necrotic value of 1:2.5. Necrotic livers were considerably larger in relation to body size than the pre-necrotic livers (7.94 g./100 g. body weight, as compared with 5.32 g./100 g. body weight); when this was allowed for by calculating the results on 100 g. body-weight basis, the level of GSH + GSSG was found to be unaltered by necrosis, but GSH level was significantly lower in necrotic than in pre-necrotic livers. Although the resultant mean for

GSSG/100 g. body weight, was apparently raised in necrosis (2.13 compared with 1.16 mg. for pre-necrotic), the standard deviation (± 1.13) was so great that the difference had no statistical significance.

The N.P.N. figure was reduced by necrosis from 150 to 94 mg./100 g., but when calculated on 100 g. body weight basis, the pre-necrotic and the necrotic levels were similar, as in the case of GSH + GSSG. It follows that the proportion of N.P.N. represented by total glutathione nitrogen was unchanged by the development of necrosis. Extracts of necrotic livers appeared to contain no ascorbic acid, according to our method of titration; but added ascorbic acid titrated normally in these extracts. The extracts from all necrotic livers examined were water-clear, in contrast to the cloudy appearance of extracts of normal or pre-necrotic livers. This difference was shown histologically to be due to the absence of glycogen from necrotic livers.

When the liver was not uniformly haemorrhagic, samples of different appearance were analysed separately. Eight such necrotic livers were examined; in four cases the analytical figures for the different types of tissue were similar, both being at the 'necrotic level'. In the other four livers, only the haemorrhagic lobes showed 'necrotic levels' of GSH, N.P.N. and ascorbic acid; the pale portions had 'pre-necrotic levels' of GSH, but the ascorbic

acid was very much lowered and had even disappeared in some cases. This suggests that in necrosis the fall in ascorbic acid content preceded that of GSH. Examples of some of these results are given in Table 3.

Analyses were also carried out on livers which showed macroscopically that an acute attack of necrosis had been overcome and the lobe was now developing post-necrotic scarring. Results on four such livers are included in Table 3. The figures were very variable but the values for GSH (90–192 mg./100 g.) were much higher than the pre-necrotic figures. There was unusual variation in N.P.N. and also in the glutathione N/N.P.N. ratio, which was always higher than the pre-necrotic figure. Ascorbic acid concentration was usually raised, except in one case where one lobe had a content of 11 mg./100 g. and the other lobe had none.

Gross chemical changes in pre-necrotic and necrotic livers. Water, total nitrogen, fat and glycogen analyses were carried out on tocopherol-depleted weanling rats and pre-necrotic rats fed the necrogenic diet. The results (Table 4) show that pre-necrotic livers had subnormal water and nitrogen concentrations, but markedly raised fat contents. The glycogen concentration was the same as in normal livers. Calculated on 100 g. body-weight basis, the nitrogen content was the same as in the weanling controls, but the water content was higher. Figures for necrotic livers show a further marked rise in water content and a slight rise in nitrogen when calculated on 100 g. body-weight basis.

Histological examination of liver. The pre-necrotic livers of rats killed after 13 days or more on the un-supplemented necrogenic diet showed the following picture. In the sections stained with haematoxylin and eosin, nuclei stained well, but the cytoplasm was paler than in normal liver cells and in some parts of the liver it hardly stained at all. As a result of this, the liver cords and sinusoids were not well defined. Küpfer cells were of normal appearance, and there was no cellular infiltration. Staining for glycogen and fat revealed normal distribution of glycogen granules and the presence of fatty infiltration (mostly mid-zonal). This picture of pre-necrotic liver agreed well with that found by Abell,

Beveridge & Fisher (1950) in their animals given a similar diet.

Tocopherol supplementation of the diet did not affect the histological picture of pre-necrotic livers. Supplementation with cystine and methionine resulted in normal staining of the protoplasm and the absence of fatty infiltration. Addition to the diet of L-cystine alone resulted in liver cytoplasm which stained normally and in increased fatty infiltration. Necrotic livers conformed to the description given by Himsworth & Glynn (1944a); no glycogen was detected.

Paper chromatography. Chromatograms of extracts from normal livers showed, in addition to a large number of spots due to amino acids, strong spots corresponding in position to glutathione (Lindan & Work, 1951a). After oxidation, the single glutathione spot was of maximum colour intensity and considerably bigger than the glutamic acid spot. In the extract from a pre-necrotic liver, the glutathione spots were weaker than normal, and after peroxide treatment the glutamic acid spot was stronger than the glutathione spot. Glutathione was hardly detectable in necrotic liver extracts, but after oxidation it gave a weak spot.

The spot due to cysteic acid was rarely seen in chromatograms of oxidized extracts from normal and pre-necrotic livers. In extracts of necrotic liver, the cysteic acid spot was more often present than in normal livers, but was weaker than the glutathione spot. One necrotic liver which did not show the usual very low titration for GSH gave a cysteic acid spot stronger than the glutathione spot; in view of this finding the case was not included in the averages for necrotic livers.

Levels of glutathione, ascorbic acid and non-protein nitrogen in spleen and blood. The effects of feeding necrogenic diet with or without supplements, on the composition of spleen and whole blood are shown in Table 5. The small size of the spleens only allowed values to be obtained for GSH and ascorbic acid. The normal group of rats is made up from three weanlings and four adult rats fed stock diet; all of them gave approximately similar figures for spleens, with means of 114 mg./100 g. for GSH and 38 mg./100 g. for ascorbic acid. Feeding necrogenic diet

Table 4. Gross chemical changes in the liver of rats fed necrogenic diet

State of liver	No. of rats	Liver wt. (g./100 g. body wt.)	(g./100 g. liver)				(g./100 g. body wt.)			Water: protein ratio
			Water	Total nitrogen	Fat	Glycogen	Water	Total nitrogen	Protein nitrogen	
Normal (tocopherol-depleted weanlings)	8	4.43	71	2.55	3.7	3.9	3.19	0.116	0.108	4.73
Pre-necrotic	8	5.91	65	1.99	16.3	3.8	3.74	0.118	0.110	5.45
Necrotic	4	6.80	80	1.94	—	0	5.40	0.131	0.119	7.34

Table 5. *Mean analytical results on spleen and blood of rats given the necrogenic diet with or without supplements*

(Figures in brackets indicate range.)

Diet	State of liver	No. of rats	Spleen analysis (mg./100 g. spleen)		Blood analysis (mg./100 g. blood)	
			GSH	Ascorbic acid	GSH	N.P.N.
Stock	Normal	7	114 (76-142)	38 (28-47)	22 (17-27)	34* (33 and 35)
Neurogenic with or without tocopherol	Pre-necrotic	11	67 (40-93)	31 (21-47)	15 (7-27)	38 (25-51)
Neurogenic	Necrotic	6	77 (64-86)	31 (21-46)	15 (10-24)	93* (88 and 98)
Neurogenic with cystine (with or without methionine)	Non-necrotic	5	116 (95-131)	36 (19-44)	21 (12-25)	42* (40 and 43)

* Two rats only.

had no effect on the ascorbic acid level, but lowered the mean GSH level in pre-necrotic rats to 67 mg./100 g., there being no difference between the groups with or without tocopherol. The development of liver necrosis had no appreciable effect on either GSH or ascorbic acid levels, which were similar to those found in the pre-necrotic group. Supplementing the neurogenic diet with sulphur-containing amino acids kept the GSH of the spleen at its normal level.

The size of blood samples and the low GSH titration figures did not allow reliable figures for the GSSG of blood to be obtained. The level of ascorbic acid was too low either to be estimated by the method used or to interfere with the GSH titration figures. The results on blood GSH were more difficult to interpret than in the case of spleen because the ranges for the different groups overlapped, but they showed the same tendencies as spleen analyses. N.P.N. levels in the bloods of rats with liver necrosis were double the levels of normal or pre-necrotic rats.

DISCUSSION

Iodometric titration of deproteinized tissue extracts is admittedly not a specific method of estimating GSH, since all sulphhydryl groups, whether free or in peptide combination, will be titrated. Leaf & Neuberger (1947) found that with normal rat livers, the figure (220 mg./100 g.) for GSH obtained by iodometric titration agreed reasonably well with that (183 mg./100 g.) resulting from the more specific manometric method of Woodward (1935). However, when the GSH content was lowered to 54 mg./100 g. by protein deficiency, the difference of about 20 mg./100 g. between the two methods was significant. We have satisfied ourselves, by paper chromatography, that in liver extracts from our normal or 'pre-necrotic' rats, the amount of free cysteine and cystine present is negligible in com-

parison with the glutathione present. In necrotic livers, we detected a slight rise in the proportion of cysteine or cystine. As cysteine raises the titration figure beyond the equivalent proportion to be expected (Leaf & Neuberger, 1947), its presence in necrotic livers would increase the glutathione titration figures; it is therefore possible that the true glutathione level in necrotic livers is even lower than we have estimated it to be.

A spectrophotometric method which can distinguish between GSH, cysteine, cysteinyl-glycine and γ -glutamylcysteine has given lower liver GSH levels (127 mg./100 g.) for normal rat livers (Binkley, Christensen & Wu, 1951). Cysteine dipeptides are reported to be absent but there were 32 mg./100 g. cysteine plus cystine present. This result is hard to reconcile with our findings and those of Leaf & Neuberger and may be due to strain difference. Other levels reported in the literature for GSH lie between 170 and 220 mg./100 g. (Binet, Weller & Goudard, 1937; Turpin, Serane & Valletta, 1938; Yamamoto, 1940; Ingbar, Otto & Kass, 1951) and are affected by the strain of rat (Leaf & Neuberger, 1947). The individual variations found in normal animals are unusually great, and in view of the high metabolic turnover rate of GSH it is probable, as Leaf & Neuberger say, that 'a single determination gives only a momentary glimpse of a rapidly changing situation'.

The marked and rapid fall found by us in the levels of GSH in livers of rats fed the necrogenic diet is comparable to that obtained by Leaf & Neuberger on rats fed a diet almost devoid of protein. No further changes in GSH concentration were noted until liver necrosis occurred, when there was a further fall in GSH together with a virtual disappearance of ascorbic acid.

The results on GSSG are more difficult to interpret because of the wide variation found in the levels even of normal rats. The normal figures quoted in

the literature vary between 0 and 40 mg./100 g. (Binet *et al.* 1937; Turpin *et al.* 1938; Yamamoto, 1940); our figures for normal weanlings are in this range. Since these variable figures depend on the differences between two titrations, it seems unwise to draw any conclusions about the significance of the low mean value (2 mg./100 g.) found for the tocopherol-depleted weanlings. Nor is it possible to assess whether the mean value of 21 mg./100 g., found in pre-necrotic livers, represents a true rise over the starting figure found for the depleted weanlings. In any case, the striking fact emerges that feeding the necrogenic diet caused a change in the ratio GSSG:GSH from the normal figure of 1:11 to the pre-necrotic one of 1:2.5, development of necrosis resulted in a further change in the ratio to 1:1. Study of the N.P.N. figures showed that in necrotic livers losses had occurred, not only in glutathione and ascorbic acid, but also in the whole N.P.N. fraction.

When related to body weight, necrotic livers were about one and a half times the size of pre-necrotic livers. When the increase in size of necrotic livers was allowed for by calculating the results on 100 g. body-weight basis, there was still a fall in GSH in necrotic livers, but the amount of GSH + GSSG appeared to be unaffected by the development of necrosis. Owing to the great variability in the figures for GSSG, it is not possible to say whether in the necrotic liver the final fall in GSH was due to its conversion to GSSG, for the rise in amount of GSSG per 100 g. body weight was not significant. Therefore, although the fall in the necrotic livers of the GSH + GSSG concentration (per 100 g. liver) may be only a dilution effect, the fall in GSH is not entirely caused by dilution. The drop from 30 to less than 1 mg./100 g. of ascorbic acid, could not be caused only by swelling of the liver.

In order to determine if any of the changes found in pre-necrotic livers are directly related to those occurring in necrotic livers, it is necessary to consider the effect on liver composition of dietary changes known to be associated with cause or prevention of liver necrosis. Previous experiments by Leaf & Neuberger (1947) showed that supplementing a low protein diet with sulphur-containing amino acids would prevent the usual fall in liver GSH. In our experiments, smaller supplements of cystine and methionine just sufficient to double their level in the necrogenic diet, prevented both the development of liver necrosis, the pre-necrotic fall in liver GSH, and the histological changes associated with the pre-necrotic condition. Tocopherol supplements, although protective against liver necrosis for periods varying according to their size (Lindan & Himsworth, 1950), had no effect on any of the changes found in pre-necrotic livers whatever the size of supplement. Different necrogenic diets in which the

percentage of yeast protein was raised or casein was substituted for yeast, all produced necrotic livers in which mean GSH, ascorbic acid and N.P.N. levels were similar to those reported in this communication (Lindan, unpublished observations). Insufficient data are available on the pre-necrotic analyses obtained on these diets, but it would seem that the pre-necrotic GSH levels were not as low as in the case of the 18 % yeast diet. This is due to the less marked deficiency in the sulphur-containing amino acids of these diets, a fact emphasized by the finding that spleens of rats which had developed necrotic livers on these diets showed normal GSH levels. It appears probable that the subnormal liver levels of GSH and the raised proportion of GSSG resulting from feeding the necrogenic 18 % yeast diet, although a consequence of a deficiency in cystine and methionine, are not the direct cause of liver necrosis; they may be predisposing factors for the disease when associated with some additional unknown changes resulting from tocopherol deficiency. One can regard the pre-necrotic levels and the ratios of GSSG:GSH as the lowest values compatible with life for the degree of amino acid deficiency imposed by the necrogenic yeast diet without reference to tocopherol content. Whether the final changes observed in necrotic livers are specifically due to the dietetic deficiency is discussed in the following paper (Lindan & Work, 1953).

It should be pointed out that the levels of GSH in pre-necrotic and necrotic livers may vary from one strain of rat to another. Thus, we were unable to reproduce these figures on rats bred in the Hampstead Medical Research Council Laboratories. However, the general trend of GSH changes was the same; there was an initial drop to a pre-necrotic level; this was maintained until necrosis occurred and then fell further to a necrotic level.

Information obtained from determination of the gross chemical composition of livers shows that the necrogenic diet was also deficient in lipotropic factors; variable degrees of fatty infiltration found in pre-necrotic livers confirmed the findings of Abell & Beveridge (1950). Supplementing the diet with cystine and methionine prevented both liver necrosis and fatty infiltration, while cystine alone, although preventive of necrosis, accentuated fatty infiltration. This is in agreement with the well known fact (Himsworth & Glynn, 1944*a*) that liver necrosis and fatty infiltration are two unrelated lesions. The increase in size of pre-necrotic livers is accounted for by accumulation of fat and a little water; on the other hand, the swelling of necrotic livers can be calculated as due to uptake by pre-necrotic livers of a fluid containing about 3.4 % protein. This figure is much lower than that of 9.3 % given by Himsworth & Glynn (1945) for adult rats developing necrosis.

The final changes in composition of necrotic livers appear to be directly associated with the development of necrosis and may be confined to the portions of the liver first affected by the disease. In some cases of liver necrosis, in which one liver lobe was more haemorrhagic than the others, the final changes in GSH occurred only in the more haemorrhagic lobe; the paler lobes showed the usual pre-necrotic GSH levels, although their ascorbic acid levels were at or near those of the necrotic state.

It is tempting, but unrewarding, to speculate on the inter-relationship between tocopherol, ascorbic acid, GSSG and GSH. Tocopherol is an antioxidant, and it was therefore surprising to find that it had no effect on the levels of GSH and ascorbic acid, both easily oxidizable substances. It is, however, interesting to note that Dam & Granados (1951) have obtained complete protection from dietetic liver necrosis by including methylene blue in a necrogenic diet.

There is another point, apart from the problem of necrosis, which is worth noting from our results. GSH values well above the pre-necrotic figures were found in all lobes which had signs of recovery from an acute attack of necrosis (post-necrotic scarring). This is suggestive that in these lobes regeneration was occurring, since raised GSH levels are known to occur in regenerating liver (Binet, Rouvillois & Bargeton, 1939; Christensen, Rothwell, Sears & Streicher, 1948; Ferarri & Harkness, unpublished observations).

SUMMARY

1. Investigations were made on the effect of feeding a necrogenic diet, containing 18 % of baker's yeast, on the composition of liver, spleen and blood before and after development of liver necrosis.

2. Reduced glutathione was lowered in 13 days

to one-quarter of the normal value; it remained unchanged until necrosis developed, when it fell further to one-tenth of its normal value.

3. Owing to the variability of oxidized glutathione values, no conclusion could be reached as to any changes found in them. Significant changes occurred in the ratio of oxidized to reduced glutathione.

4. Ascorbic acid was slightly lowered in pre-necrotic livers and could not be detected in necrotic livers.

5. The proportion of non-protein nitrogen represented by total glutathione nitrogen was lowered in pre-necrotic livers to one-third of its normal value and remained unchanged during necrosis.

6. Supplementation of the necrogenic diet with sulphur-containing amino acids maintained the liver glutathione at its normal level, but tocopherol had no effect, although it prevented necrosis.

7. The histological picture of pre-necrotic liver showed changes commonly associated with low-protein diets, and was not changed by additional tocopherol in the diet. Cystine and methionine supplementation resulted in normal appearance of the liver.

8. The reduced glutathione level in spleen was halved in rats given the necrogenic diet and did not change further during liver necrosis; it was restored to normal by supplementation with sulphur-containing amino acids. Reduced glutathione values of blood were very variable but showed a similar trend.

9. Ascorbic acid was normal in spleen even when it was absent from the necrotic liver.

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Experimental Liver Necrosis in Rats

2. THE LEVELS OF GLUTATHIONE AND ASCORBIC ACID IN LIVERS SUBJECTED TO ACUTE CIRCULATORY CONGESTION IN RELATION TO THE LEVELS FOUND IN DIETETIC LIVER NECROSIS

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Dietetic liver necrosis in rats is produced by a double deficiency of sulphur-containing amino acids and tocopherol (Himsworth, 1950). The condition is characterized by a latent period during which the animal appears normal, although receiving the deficient diet, followed by a sudden onset of illness resulting in death within a few hours. A theory which has been suggested (Himsworth, 1950) for the mechanism of production of this liver lesion is as follows: after the latent period, acute swelling of the parenchymatous cells develops, causing obliteration of the sinusoids and impairment of circulation which subsequently leads to death and autolysis of the cells followed by haemorrhages into the organ. Biochemical changes have been noted in these necrotic livers affecting the levels of water, protein, non-protein nitrogen, glycogen, phospholipids, cholesterol, glutathione and ascorbic acid (Himsworth & Glynn, 1945; Abell & Beveridge, 1950; Lindan & Work, 1951*a, b*, 1953). The question arises as to whether these changes were specifically connected with the dietetic lesion, or were simply the result of death of the cell.

In the preceding paper (Lindan & Work, 1953) it was shown that there were two successive falls in the level of reduced glutathione (GSH) of the liver of rats fed the necrosis-producing diet. The preliminary fall was at the beginning of the latent period and was the result of the deficiency in sulphur-containing amino acids. The second fall occurred on development of liver necrosis, but there was no certainty whether it was due to exhaustion of reserves of these amino acids or whether it was the

precipitating factor for liver necrosis. As lowered GSH levels in rabbit livers were found after ligation of the bile duct or part of the blood supply (Binet, Weller & Goudard, 1937; Binet, Rouvillois & Bargeton, 1939), it was possible that various forms of liver damage unconnected with dietetic deficiencies might well result in falls in liver GSH. Further, the absence of ascorbic acid noted by us in necrotic livers of dietetic origin was not directly connected with the dietary deficiencies, since little change in ascorbic acid occurred during the latent period on the deficient diet. It seemed therefore of interest to determine whether the very low liver levels of GSH and ascorbic acid found in dietetic liver necrosis were confined to livers rendered necrotic by dietary means, or occurred also in some other type of liver damage.

This problem could be investigated if other types of acute liver necrosis could be obtained without the complication of prolonged dietary deficiency. Experimental liver necrosis can be caused by poisons or infections, but it was desirable to avoid such complicating factors. Accordingly, we produced a necrosis by means of an acute circulatory derangement in a normal liver lobe by lightly ligating its base; this operation obstructed outflow of blood more than inflow and resulted in acute congestion of the lobe followed by haemorrhages into the parenchyma and death of liver cells. The non-ligated lobes of the liver were not grossly damaged and the animal continued alive throughout the experiment, thus allowing an investigation to be made on changes taking place in the dead or dying liver remaining in the living body. It is realized that the necrotic condition produced in this way is not directly comparable to dietetic liver necrosis, firstly

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