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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, 1951a, 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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because the circulatory derangements causing the necroses are of different types, and secondly because they are superimposed, in the one case on a normal liver, and in the other case on a liver showing signs of nutritional deficiency. Nevertheless, the experiment enabled us to ascertain whether death, through circulatory congestion, of the lobe in the living body, resulted in a liver composition similar to that which we had found in dietetic liver necrosis, especially with regard to the extremely low levels of GSH and ascorbic acid. The possibility of loss of diffusible metabolites from the necrotic liver surface into the abdominal cavity was also investigated by examining the composition of excised liver lobes, either left to autolyse in the abdominal cavity, or autolysing *in vitro* for the same time.

METHODS

Experimental animals. Wistar stock rats, body weight 200 g., bred in the Medical School, were used. All experiments were carried out with ether anaesthesia. In six rats the 'base' of the left lobe of the liver was lightly ligated so as to produce impairment of venous outflow and a visible congestion in a few minutes. The abdomens were then closed and the rats were killed 4–21 hr. after operation. In two rats, the left lobe was doubly ligated and after amputation between the ligatures it was left free in the abdominal cavity; the abdomen was then closed and the rats kept for 17 hr. before killing. When the livers were removed for analysis, the left lobes were examined separately. In one case, the rat was killed and the left lobe of the liver was excised under sterile conditions, and incubated for 17 hr. at 37° in a closed Petri dish in an atmosphere kept damp by a piece of wet cotton wool.

Analytical methods. The methods used for examination of the liver are described in the preceding communication (Lindan & Work, 1953).

RESULTS

Chemical examination. Table 1 shows results of various forms of treatment of the left lobe. They are compared (1) with those of the remaining untreated lobes examined at the time of killing, (2) with control figures obtained from adult stock rats and (3) with figures from livers of rats with dietary necrosis (Lindan & Work, 1953). When the left lobe was ligated it increased rapidly in size and sometimes doubled its weight. A marked fall in GSH occurred even within 4 hr., and after 11–21 hr., it had dropped to 14–46 mg./100 g., this level being similar to that (15–28 mg./100 g.) found in necrotic livers of dietetic origin. There was no significant change in concentration of oxidized glutathione (GSSG). The ascorbic acid values were much lowered after 4 hr., while after 11 or more hours of ligature no ascorbic acid was detected, as was the case in necrotic livers of dietary origin. Ligature caused a fall of 50 % in non-protein nitrogen (N.P.N.),

as did dietetic necrosis, and the total glutathione nitrogen was 8 % of non-protein nitrogen in ligated lobes as compared with 16 % in normal controls. Changes in water and total nitrogen contents similar to those found in dietetic liver necrosis were noted only after prolonged ligature (21 hr.), when the water level was raised and nitrogen level was lowered. Glycogen was already absent 4 hr. after ligature as indicated by clarity of the sulphosalicylic acid extracts and by histological examination.

The analytical figures for the non-ligated part of the liver cannot be regarded as normal because of the general disturbances caused by the operation, but they show small changes in comparison with those found in the ligated left lobes. The notable changes were slight falls in GSH and N.P.N. after 11 hr. and a rise in GSH after 21 hr.

The results on amputated lobes depended on whether autolysis occurred in the abdomen or *in vitro*. The lobes left in the abdomen lost weight and the GSH contents were reduced to the very low levels of 10 and 15 mg./100 g. The figures found for GSSG did not represent true GSSG as they were not confirmed by chromatography (see next section). Ascorbic acid had disappeared, N.P.N. and total nitrogen were also lowered and water content was raised. The composition of the lobe incubated *in vitro* showed no similarity to any other liver analyses. Ascorbic acid was almost unchanged (27 mg./100 g.); N.P.N. was raised to 836 mg./100 g.; the iodometric titration figures before and after reduction corresponded to levels for GSH and GSSG of 145 and 135 mg./100 g. respectively, but again chromatography revealed that they could not be relied on because of the presence of large amounts of cystine or cysteine.

Chromatographic examination. Chromatograms from peroxide-treated extracts of the left lobe which had been ligated 4 hr. showed a glutathione spot about equal to the glutamic acid spot, and also a definite cysteic acid spot. The livers ligated for longer periods were not examined chromatographically. In the case of the amputated lobes autolysing in the abdominal cavity for 17 hr., no glutathione or cysteic acid spots were visible; the high glutathione values found by titration in the remaining liver were confirmed, as the glutathione spot was unusually large and intense, while the cysteic acid spot was negligible.

Examination of extracts of the liver which had been autolysed *in vitro* had to be carried out on one-fifth of the normal amount of extract, because of an enormous excess of free amino acids. Even at this low level, the glutathione spot was apparent, but was only about one-third the strength of the cysteic acid spot, showing that cystine or cysteine was present in excess.

Table 1. *Composition of livers rendered necrotic by different means*

(Figures represent values for individual rats except where otherwise stated.)

Group Treatment of lobe	Normal*		Circulatory congestion						Autolysis		Dietetic necrosis†
	None	None	Ligated 4 hr.	None	Ligated 11 hr.	None	Ligated 21 hr.	None	Excised and left in abdomen 17 hr.	None	
Lobe	Left	Others	Left	Others	Left	Others	Left	Others	Left	Others	All
Wt. of lobes (g./100 g. body wt.)	1.16	2.74	2.00	3.00	2.54	2.51	1.34	1.72	0.86	2.72	7.94
	—	—	1.57	3.43	—	—	1.96	2.72	0.61	3.77	—
	—	—	—	—	—	—	2.00	3.77	—	—	—
GSH (mg./100 g. lobes)	195	195	88	190	21	119	33	287	15	273	22
	—	—	78	153	—	—	14	273	10	268	—
	—	—	—	—	—	—	46	268	—	—	—
GSSG (mg./100 g. lobes)	10	10	12	8	—	—	11	9	37†	—	24
	—	—	30	10	—	—	9	—	47†	—	—
	—	—	—	—	—	—	11	10	—	—	—
GSH (mg./100 g. body wt.)	2.26	5.35	1.76	5.70	0.53	2.83	0.44	4.94	0.13	7.80	1.85
	—	—	1.22	5.25	—	—	0.27	7.42	0.06	7.20	—
	—	—	—	—	—	—	0.52	10.02	—	—	—
Ascorbic acid (mg./100 g. lobes)	37	37	9	47	0	21	0	31	0	26	0
	—	—	2	29	—	—	0	34	0	23	—
	—	—	—	—	—	—	0	35	—	—	—
N.P.N. (mg./100 g. lobes)	178	178	102	246	76	122	72	191	73	—	94
	—	—	158	212	—	—	—	—	80	—	—
	—	—	—	—	—	—	92	260	—	—	—
N.P.N. (mg./100 g. body wt.)	2.06	4.87	2.04	7.38	1.98	3.06	0.96	3.28	0.63	—	7.56
	—	—	2.04	7.26	—	—	—	—	0.49	—	—
	—	—	—	—	—	—	1.84	9.70	—	—	—
§T.G.N. × 100	15.8	15.8	13.5	11.0	—	—	8.3	21.2	9.8	—	6.8
N.P.N.	—	—	9.4	10.5	—	—	—	—	9.8	—	—
	—	—	—	—	—	—	8.3	14.5	—	—	—
Water (g./100 g. lobes)	70	70	—	—	70	73	—	71	83	72	80
	—	—	68	71	—	—	83	71	—	—	—
	—	—	—	—	—	—	81	72	—	—	—
Total N (g./100 g. lobes)	3.30	3.30	—	—	3.50	—	—	3.85	2.30	4.00	1.94
	—	—	—	—	—	—	2.60	3.85	—	—	—
	—	—	—	—	—	—	2.73	—	—	—	—
Water (g./100 g. body wt.)	0.81	1.91	—	—	1.78	1.83	—	1.22	0.72	1.96	5.40
	—	—	1.07	2.45	—	—	1.63	1.93	—	—	—
	—	—	—	—	—	—	1.62	2.72	—	—	—
Total N (g./100 g. body wt.)	0.04	0.09	—	—	0.09	—	—	0.07	0.02	0.11	0.13
	—	—	—	—	—	—	0.05	0.10	—	—	—
	—	—	—	—	—	—	0.04	—	—	—	—

* Mean of three rats.

† Mean of four to eight weanling rats, see Lindan & Work, 1953.

‡ Values unreliable, not confirmed by chromatography.

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

Histological examination. The ligated lobes presented varying degrees of congestion and of liver-cell necrosis. Lobes examined after 4 hr. showed distention with blood both of vessels in portal tracts and the sinuses. Liver-cell cords were preserved, but the protoplasm stained pale and was finely vacuolated. Nuclei were normal in the majority of liver cells but some were pycnotic. Lobes examined after 11 and 21 hr. showed extensive haemorrhages into the liver parenchyma.

Liver cells were compressed to disintegrating bands occupying only half of the microscopic field. Protoplasm was of uniform appearance but stained very poorly and nuclei were visible only in some liver cells. There was a marked infiltration of liver parenchyma with polymorph-like cells. The lobes which were not ligated had a fairly normal appearance. Some cells showed signs of mitosis, specially in rats killed after 11–21 hr. The amputated lobe left in the abdominal cavity presented a picture of

shrunk liver cells lying in bands. Sinusoids looked, therefore, enlarged and contained homogeneous material which stained like protoplasm. Nuclei were pycnotic or showed signs of karyorrhexis.

DISCUSSION

There were many similarities between the compositions of livers rendered necrotic, either by circulatory congestion, or by dietary deficiency of sulphur-containing amino acids and tocopherol. The resemblances were particularly striking in regard to the low concentrations of GSH and the absence of ascorbic acid, but the levels of total nitrogen and N.P.N., water and glycogen were also comparable. These biochemical similarities between the two types of liver damage were apparent, despite the facts that the liver composition before the onset of necrosis was normal in one case and abnormal (pre-necrotic) in the other; that the mechanisms of production of necrosis were not identical, and that the final histological pictures were different. The findings in the two types of necrosis can be interpreted in several ways. The similarities in composition might be coincidental, resulting from two completely different pathological processes; or the final low levels of GSH and ascorbic acid might be found in several types of severe liver damage and might represent the result, either of a common biochemical lesion initiated by different means, or of general changes occurring in the dying or dead liver left in the living body. The last interpretation seemed to be more probable in the light of experiments described in this paper. When the normal liver was ligated the level of GSH dropped from 195 to about 30 mg./100 g.; when liver necrosis occurred in rats fed the necrosis-producing diet, GSH fell to 22 mg./100 g., but in this case the starting pre-necrotic level was only 53 mg./100 g. Thus the final level attained in necrosis was independent of the liver GSH level before the onset of necrosis. The fall in the ascorbic acid level in both types of liver damage preceded that of GSH, suggesting that the biochemical changes in the two types of necrosis were caused by similar mechanisms. These final changes in GSH and ascorbic acid were found only in the damaged liver lobes and were not generalized throughout the body. It should be pointed out that excised normal liver lobes left to autolyse in the abdominal cavity showed GSH and ascorbic acid levels similar to those of congestive and dietetic necrosis. Apparently, in all three circumstances there was a loss of diffusible substances from the damaged liver surface into the abdominal cavity; this was borne out by a fall in N.P.N. in all cases. The liver excised and left to autolyse *in vitro* at 37° showed a completely different picture involving a tremendous rise in

N.P.N. and almost no loss of ascorbic acid; the changes were concomitant with general proteolysis occurring under conditions where breakdown products were not removed. Drochmans (1950), investigating changes in liver phosphates and nucleic acids following ligature or autolysis *in vitro*, also came to the conclusion that ligated lobes lost diffusible metabolites in contrast to the tissue autolysing *in vitro*. We could not investigate autolysis *in situ* in the dead normal animals over a prolonged period because of general bacterial action in the abdomen, but we found no changes in liver GSH or ascorbic acid two hours after killing. This stability of liver GSH was accounted for by the work of Neubeck & Smythe (1944) showing that the enzyme which specifically hydrolyses GSH was absent from rat livers, although very active in other animals.

The livers rendered necrotic either by dietary deficiency or by circulatory congestion increased in size, and the changes in their protein and water contents could be explained by accumulations of a protein solution derived from blood. In contrast to this, the changes in GSH and ascorbic acid could not be attributed to the dilution effect.

In conclusion, it is possible that the final falls in GSH and ascorbic acid occurring in all three types of necrotic livers investigated (circulatory congestion, dietary deficiency and excision) were due to general biochemical changes taking place in the dying or dead liver left in the living body. Since a dietary deficiency in sulphur-containing amino acids is one of the factors necessary for the production of dietetic liver necrosis, the question has arisen whether any changes found in respect of the sulphur-containing fractions of necrotic livers could explain the disease (Leaf & Neuberger, 1947; Dent, 1947; Lindan & Work, 1951b). The tempting theory that the final drop of liver GSH in necrosis of dietetic origin is due to exhaustion of reserves of sulphur-containing amino acids, and is therefore an ultimate cause of liver necrosis, has as yet no proof.

SUMMARY

1. The composition of liver lobes rendered necrotic by subjection to acute circulatory congestion was investigated and compared with that of necrotic livers of dietary origin.

2. The levels of reduced glutathione, ascorbic acid, non-protein and total nitrogen, water and glycogen were found to be similar in both types of necrosis.

3. The composition of amputated liver lobes left to autolyse in the abdominal cavity resembled that of necrotic livers, but an excised lobe autolysing *in vitro* showed no resemblance to necrotic livers.

4. As a result of the findings, the suggestion is put forward that the levels of glutathione and ascorbic acid found in dietetic liver necrosis could be the result of changes occurring in a dead or dying liver left in the living body, and were not specific consequences of the dietetic lesion.

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The Effect of Calcium on the Respiratory and Phosphorylative Activities of Heart-muscle Sarcosomes

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A long recognized difficulty in the study of the enzyme systems which bring about oxidative phosphorylation is their great instability at temperatures above about 0°. The instability is particularly noticeable if the enzyme preparation is incubated before it is added to the reaction mixture which is used to demonstrate oxidative phosphorylation.

In studies of the oxidative phosphorylation systems in heart-muscle sarcosomes, the respiratory granules of heart muscle corresponding to mitochondria in liver and kidney (Cleland & Slater, 1953*a, b*), this instability was particularly troublesome, especially when rat-heart sarcosomes were used. Paul, Fuld & Sperling (1952) have also observed this instability in heart-muscle 'cyclophorase' preparations.

The inactivation of the respiratory enzyme systems is accompanied by marked morphological changes, which have been described in another paper (Cleland & Slater, 1953*a*). In that paper, it is shown that the rate of development of these morphological changes is greatly decreased by the addition of the chelating agent, ethylenediaminetetraacetic acid (Versene). Further, as has already been reported in a preliminary communication (Slater & Cleland, 1952), the instability of the α -ketoglutaric oxidase system of the sarcosomes is due to calcium contained in the sarcosomal preparation,

and the addition of ethylenediaminetetraacetic acid greatly stabilizes the system. The present paper describes these experiments in greater detail, and also deals with the mode of action of the calcium. A study was also made of the components of the reaction mixture used in oxidative phosphorylation experiments, which, in the absence of ethylenediaminetetraacetic acid, confer limited stability on the enzyme systems.

Calcium is a normal constituent of blood serum, and since the demonstration of Ringer (1883) that it is required for the beating of frog heart, it has often been included in the medium in studies of the respiration of tissue slices. It has frequently been observed, however, that the addition of calcium depresses the respiration of minces and dispersions made with 'homogenizers'. An exception is the oxidation of succinate by 'homogenates', which was found by Axelrod, Swingle & Elvehjem (1941) to be stimulated by calcium. This was shown by Swingle, Axelrod & Elvehjem (1942) to be due to the activating effect of calcium on the enzyme hydrolysing diphosphopyridine nucleotide (DPN). In the presence of DPN, fumarate, produced from succinate, is oxidized to oxaloacetate, and this is a strong inhibitor of succinic dehydrogenase (Das, 1937; Keilin & Hartree, 1940; Pardee & Potter, 1948). The destruction of DPN, accelerated by calcium, prevents the formation of oxaloacetate