Br. J. Nutr. (1967), **21**, 147

Lysosomal enzymes and vitamin E deficiency

3. Liver necrosis and testicular degeneration in the rat

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(*Received* 6 *June* 1966—*Accepted* 19 *September* 1966)

1. The activities of several lysosomal hydrolases have been measured in tissues of rats with nutritional liver necrosis. Incipient or actual liver necrosis did not alter total, free or unsedimentable activities of cathepsin, β-glucuronidase, β-galactosidase or acid phosphatase of liver and kidney. Free hydrolytic activity towards *p*-nitrophenyl phosphate was slightly raised in liver, and serum β-galactosidase and β-glucuronidase were moderately elevated. These results suggest that lysosomal hydrolases are not directly implicated in the causation of liver necrosis.

2. Testicular degeneration was studied with reference to changes in β-glucuronidase activity. This enzyme activity, total, free and unsedimentable, was raised in deficient rat testis at 6 months old and did not decline even after a year. Raised values were also found in serum.

Dietary necrotic liver degeneration in the rat has been studied extensively by Schwarz and his colleagues (e.g. see Schwarz, 1954, 1965). Selenium was discovered to be an essential trace element, protective against liver necrosis in the rat (Schwarz & Foltz, 1957) and exudative diathesis in the chick (Schwarz, Bieri, Briggs & Scott, 1957). Since that time, these two deficiency diseases, especially liver necrosis, have become of particular importance in the elucidation of the relationship between the two nutritional factors that prevent them, vitamin E and Se. In view of this similarity in nutritional function and in accord with the theory that vitamin E acts in vivo as a lipid antioxidant, some authors claimed that Se forms antioxygenic compounds in vivo (e.g. Bieri, Dam, Prange & Søndergaard, 1961). However, a marked lack of correlation between prevention of these deficiency diseases by Se and peroxidation of tissues in vitro has been found by Schwarz (1961), Corwin (1962) Bunyan, Diplock, Edwin & Green (1962) and Bunyan, Green & Diplock (1963).

The antioxidant properties of vitamin E were also invoked to explain the increase in lysosomal hydrolase activity and rupture of lysosomal membranes detected in nutritional muscular dystrophy of the rabbit (Zalkin, Tappel, Caldwell, Shibko, Desai & Holliday, 1962). Therefore, in view of the disputed antioxidant role of Se, discussed above, it seemed important to us to make a similar study of lysosomal hydrolases in liver necrosis in the rat.

The only previous work on nutritional liver necrosis and lysosomal enzymes was in a study by Beaufay, van Campenhout & de Duve (1959) on the effects of various hepatotoxic treatments. The typical histological picture of liver degeneration as described by Schwarz (1965) was not found by these authors and so their results may not be relevant to the particular question in hand, as discussed below.

Testicular degeneration in vitamin E-deficient rats was described by Mattill & Stone (1923) shortly after the discovery of foetal resorption by Evans & Bishop (1922) and was later used as the basis for a biological assay of vitamin E (Herraiz & Radice, 1949). Enzyme activities in degenerate testes were first investigated by Arata & Pecora (1962) and Arata, Santoro, Severi & Pecora (1962*a*, *b*). They found that hyaluronidase activity of degenerate testis was decreased to one-tenth of normal by 6 months of age, whilst acetyl esterase was raised thirtyfold and β-glucuronidase threefold. However at 11 months of age these high values had decreased to normal, or below for β-glucuronidase. Since Harris & Mason (1956) were able to classify testicular degeneration as one of those vitamin E deficiency diseases that does not depend upon polyunsaturated dietary fat for its development, lipid peroxidation might be expected to be implicated less in this disease than in others. Consequently if lipid peroxidation is the cause of weakened lysosomal membranes, there should be no marked liberation of lysosomal hydrolases in testicular degeneration. It was decided therefore to study the subcellular distribution of β-glucuronidase during the development of this disease.

EXPERIMENTAL

Dietary ingredients. Dried baker's yeast was purchased from United Yeast Co, Ltd, Croydon, Surrey, and was heated at 100° for 2 h and ground before use. Torula yeast and α-protein were as described by Bunyan, Green, Diplock & Robinson (1967*a*).

Diets for liver necrosis. In early experiments the basal diet contained dried baker's yeast as described by Bunyan, Green & Diplock, (1963). In later tests the percentage composition of the basal necrogenic diet was changed to: torula yeast 30, sucrose 46·4, glucose 15, lard 5, salt mixture 3·2, vitamin mixture 0·4. The salt mixture supplied (g/kg), $CaCO_3$ 17·5, $NaH_2PO_4.2H_2O$ 6·5, KCl 3·5, $MgSO_4.7H_2O$ 4·0, ferric citrate 0·15, $MnSO_4.4H_2O$ 0·2, $ZnSO_4.7H_2O$ 0·06, KI 0·0003, NaF 0·00025, $(NH_4)_6Mo_7O_{24}.4H_2O$ 0·002, $CoSO_4.7H_2O$ 0·01, $Al_2SO_4.K_2SO_4.24H_2O$ 0·0007, $CuSO_4.5H_2O$ 0·02. This vitamin mixture supplied (mg/kg): thiamine 9, riboflavine 19, nicotinic acid 90, pyridoxine 9, calcium pantothenate 90, folic acid 2, cyanocobalamin 0·3, inositol 90, *p*-aminobenzoic acid 90, choline dihydrogen tartrate 900, menaphthone sodium bisulphite 0·28, together with vitamin A, 10·8 i.u./g and vitamin D_3, 1·6 i.u./g, both added in the form of a stabilized powder. For purposes of comparison, non-necrogenic diets were also made by supplementing these basal diets with either Se, 0·05 ppm, as sodium selenite, or with D-α-tocopheryl acetate, 100 ppm.

Diets for testicular degeneration. The deficient diets A 10 Y 3 (Bunyan, McHale & Green, 1963) or α 3 (Bunyan *et al.* 1967*b*) were used. Control diets were prepared by supplementing these deficient diets with D-α-tocopheryl acetate, 100 ppm.

Rats. Norwegian hooded rats about 28 days old were allocated at random to the experimental groups described below, with equal distribution of sexes and litter-mates. In Expt 1 the dam and litter were given diet A 10 Y 3 when the litter was 14 days old and then, from 28 days, diets based on the baker's yeast basal diet. In Expt 2, the torula yeast basal diet was given from days 14 to 28 and then the necrogenic or normal diets containing torula yeast.

Testicular degeneration. Diet A10Y3 was given to each dam when her litter was born. Then, at about 28 days, the male rats from each litter were allocated at random to two groups to receive either A10Y3 or A10Y3 + vitamin E. After 3 or 4 months these diets were changed to $\alpha 3$ and $\alpha 3$ + vitamin E, respectively.

Tissue homogenates and enzyme assays. The relevant procedures have been described by Bunyan *et al.* (1967*a*).

Protein determination. Proteins were estimated by the Kjeldahl method or the optical density method of Warburg & Christian (1941). In the latter method the results were corrected with reference to the Kjeldahl values in order to compensate for differences in the nature of the tissues.

RESULTS

Liver necrosis. Rats were killed in three different stages of the disease: (1) in the terminal hypoglycaemic coma, (2) when apparently normal, but showing severe necrosis *post mortem*, or (3) in the latent or pre-necrotic phase described by Schwarz & Mertz (1959). Because some rats were killed before liver necrosis had developed, the number found to have necrotic lesions underestimates the necrogenicity of the basal diet. Even so this apparent incidence was 75% (between 36 and 80 days old) with the torula yeast diet and 50% (50–130 days old) with the baker's yeast diet. No tests were done on rats that had died of necrosis. Dietary supplements of Se or vitamin E always protected against liver necrosis.

Table 1 shows the enzyme activities in liver, kidney and serum. In liver, the pathological state did not affect the total activities of cathepsin, β-galactosidase, β-glucuronidase, β-glycerophosphatase or *p*-nitrophenyl phosphatase.

Subcellular fractions of normal and necrotic livers were tested to see if there was any difference in the enzyme activity of the lysosomal fraction. No subcellular redistribution of cathepsin, β-galactosidase or β-glucuronidase was found. The free activities of β-glucuronidase and β-glycerophosphatase were also unaltered in whole sucrose homogenates of necrotic and pre-necrotic livers, but the free activity of *p*-nitrophenyl phosphatase was increased from 47 to 59%. No changes due to the vitamin E-deficient state were found in kidney. We examined serums of deficient rats for β-glucuronidase and β-galactosidase and found rises of 50% and 25% respectively, compared to normal rats. Liver necrosis did not significantly alter the weight of the rats or of their livers and kidneys, nor did it alter the protein content of their tissues.

Testicular degeneration. In contrast to the general lack of effect of liver necrosis on hydrolytic enzymes, testicular degeneration was accompanied by some striking and significant changes in the β-glucuronidase activity of the testis (Table 2). At 7 weeks old, the testes had not yet degenerated, as judged by their weights, and β-glucuronidase activity was not affected. However, at 6 and 13 months old the weights of the deficient rat testes were about one-third of normal. β-Glucuronidase (m-units/g) was raised threefold in these testes but this difference from normal disappeared when the activity was expressed as m-units/testis, because of the threefold decrease in weights. Examination of free and supernatant activity revealed increases in the deficient tissue, but

Table 1. *Liver necrosis and hydrolytic enzymes in rats*

(Results are given as means with standard deviations. The number of tests is shown in parentheses)

Tissue	Expt no.	Age (days)	Severity of liver necrosis in deficient rats (no. of rats): None	Slight	Severe	Terminal coma	Enzyme activity: Enzyme		Deficient rats	Normal rats
Liver	2	36–43	—	5	1	1	Cathepsin	Total activity (units/g)	87 ± 16 (5)	78 ± 10 (8)
								Lysosomal activity (%)*	86 ± 20 (2)	87 ± 18 (2)
	2	38, 39	1	—	1	1	β-Galactosidase	Total activity (units/g)	0·32 ± 0·10 (3)	0·28 ± 0·06 (4)
								Lysosomal activity (%)*	90 ± 14 (2)	88 ± 17 (2)
	1, 2	38–130	1	—	7	1	β-Glucuronidase	Total activity (units/g)	1·13 ± 0·20 (5)	0·93 ± 0·38 (7)
								Free activity (%)†	64 (1)	66 ± 4 (2)
								Lysosomal activity (%)*	75 ± 13 (2)	85 ± 6 (3)
	1, 2	42, 58	2	—	1	—	β-Glycerophosphatase	Total activity (units/g)	3·6 ± 1·1 (2)	4·8 ± 0·7 (2)
								Free activity (%)†	23 (1)	17 ± 5 (2)
	1	70–78	3	—	1	1	p-Nitrophenyl phosphatase	Total activity (units/g)	11 ± 2·7 (5)	13·2 ± 1·6 (8)
								Free activity (%)†	59‡ ± 11·3 (5)	47 ± 2·6 (8)
Kidney	2	38–45	1	—	2	—	Cathepsin	Total activity (units/g)	163 ± 11 (2)	173 ± 18 (3)
	2	42	—	—	1	—	β-Glycerophosphatase	Total activity (units/g)	3·2 (1)	3·7 (1)
	1	64	—	2	—	—	p-Nitrophenyl phosphatase	Total activity (units/g)	9·4 (1)	9·1 ± 0·6 (2)
								Lysosomal fraction (units/g)	3·2 (1)	2·9 ± 0·2 (2)
Serum	2	40	—	—	1	1	β-Glucuronidase	Total activity (units/g)	0·22 (1)	0·31 (1)
	2	40–46	—	—	4	1	β-Glucuronidase	m-units/ml	1·8‡ ± 0·22 (3)	1·2 ± 0·11 (3)
	2	44, 45	1	1	—	—	β-Galactosidase	m-units/ml	3·4‡ ± 0·43 (2)	2·7 ± 0·03 (2)

* Activity of lysosomal fraction expressed as percentage of activity of whole homogenate after removal of a 'nuclear' fraction.

† Activity of whole homogenate in 0·25 M-sucrose solution, without added Triton X-100, expressed as a percentage of the total activity.

‡ Significantly higher than normal value, $P < 0·05$.

even so, most of the β-glucuronidase activity remained bound within the lysosomal fraction. Serum β-glucuronidase in these vitamin E-deficient rats was about 50% higher than in normal rats.

Table 2. *Testicular degeneration and lysosomal enzymes*

	Vitamin E-deficient rats			Normal rats			
	7 weeks	6 months	13 months	7 weeks	4 months	6 months	13 months
Testis weight (g)*	0·64 ± 0·18 (2)	0·48 (1)	0·45 ± 0·08 (4)	0·41 ± 0·03 (2)	1·16 ± 0·23 (3)	1·35 ± 0·03 (3)	1·15 ± 0·28 (2)
Testis protein (mg/g)	64	—	143	53	83	—	145
β-Glucuronidase in testis							
Total activity (m-units/g)	24 (2)	61 (1)	60 (2)	22 (2)	22 (2)	18 (1)	25 (2)
Total activity (m-units/testis)	15 (2)	29 (1)	27 (2)	9 (2)	25 (2)	24 (1)	38 (2)
Free activity† (%)	14 (2)	21 (1)		18 (2)		14 (1)	
Supernatant-fraction activity‡ (%)	3 (2)	14 (1)	18 (2)	8 (2)	12 (2)	10 (1)	12 (2)
Serum β-glucuronidase (m-units/ml)	—	1·9 (1)	2·1 (4)	—	—	1·5 (1)	1·4 (2)

Figures in parentheses indicate the number of rats.
* Results are given as means with standard deviations.
† Activity of the whole homogenate in 0·25 M-sucrose solution, without added Triton X-100, expressed as a percentage of the total activity.
‡ Activity of supernatant fraction, expressed as a percentage of the activity of the whole homogenate, after removal of a nuclear fraction.

DISCUSSION

The effects of a necrogenic diet on lysosomal enzymes in rat liver have been studied by Beaufay *et al.* (1959) as part of an investigation of several hepatotoxic treatments. Although some of their deficient rats died and others were found in a comatose state, no histological signs of liver necrosis were seen. The treatment decreased liver weight and protein content and the activities of cytochrome oxidase and glucose-6-phosphatase. Total and soluble activities of acid phosphatase, ribonuclease, cathepsin and acid deoxyribonuclease were increased, the effects being more pronounced in comatose than non-comatose rats. In comatose rats, total β-glucuronidase activity was raised, whilst soluble activity increased even in non-comatose rats. Beaufay *et al.* (1959) considered the release of lysosomal enzymes to be a feature common to all injured tissues, whether necrotic or likely to become necrotic. It has been suggested that toxic or dietary factors produce obstruction of the hepatic circulation (Himsworth, 1947). De Duve & Beaufay (1959) studied ischaemic livers, but the results were not entirely similar to those for deficient livers since the latter concentrated rather than lost their hydrolases. The lack of histological change in these deficient rat livers was attributed by Beaufay *et al.* (1959) to enzymic disorganization preceding the structural changes. In our tests, however, a situation nearly the reverse of this was found. About three-quarters of our deficient rats showed clear signs of liver necrosis, but in none was there much change in hydrolase activity, nor did necrosis affect liver protein. In this study and in previous work (Green, Edwin, Bunyan & Diplock, 1960; Bunyan, Green & Diplock, 1963), the development of liver necrosis in our rats always followed the

pattern described by Schwarz (1958); this consisted of a latent phase, during which liver slices displayed decline of respiration in vitro, followed by a rapid terminal phase when the characteristic macroscopic and microscopic changes occurred in the livers. Beaufay *et al.* (1959) may have failed to produce true dietary necrotic liver degeneration for two reasons. First, they started with rather large rats (100–150 g) that might have had considerable reserves of vitamin E if previously given a normal diet and, secondly, we have calculated that their necrogenic diet, which included maize and arachis oil, probably contained about 9 μg α-tocopherol/g, a concentration that we have found to be marginally protective against liver necrosis (Bunyan, Green & Diplock, 1963).

In a study of the electron microscopy of liver necrosis, Piccardo & Schwarz (1958) described vacuolization of the endoplasmic reticulum at an early stage, followed by disruption of the nuclear membrane. Later, mitochondria swelled and degenerated, leading to the appearance of degenerative microbodies. According to Schwarz (1958), the metabolic lesion, a decline in respiration of liver slices in vitro, does not appear until after the first change in the cytoplasm, at about the time when the degenerative microbodies appear. This latent phase of about 8 days, and longer in some strains of rat, is followed by massive necrosis and death.

Our enzyme assays were done mostly during the latter part of this latent phase and a number of rats were also in the terminal coma. Moderate rises in serum β-glucuronidase and β-galactosidase and a small increase in free activity of *p*-nitrophenyl phosphatase in liver were the only changes that reflected this incipient and, sometimes, actual, massive necrosis of the liver. An elevated serum hydrolase titre can indicate changes in tissue enzymes, as shown by the work of Weissmann, Uhr & Thomas (1963) on guinea-pigs with hypervitaminosis A. The serum β-glucuronidase activity of these animals was raised threefold and there was also a twofold rise in the activity of the supernatant fraction of their livers. The moderate changes that we found in serum activity were not associated with liver changes, but may have indicated small changes in some other tissue. We found greater enzyme activity in liver with *p*-nitrophenyl phosphate than with β-glycerophosphate as substrate, in agreement with the demonstration that at least three enzymes hydrolyse the former substrate in guinea-pig and other mammalian livers (Neil & Horner, 1964). One enzyme component is confined to the supernatant fraction, explaining the higher free activity with the *p*-nitrophenyl substrate. Although the slightly greater free activity in deficient livers may be due to a disruption of the lysosomal membrane it may also possibly be due to greater penetration; penetration by another *p*-nitrophenyl substrate, the galactoside, has been shown by Furth & Robinson (1965).

The studies of Trump, Goldblatt & Stowell (1962) on necrosis of mouse liver slices in vitro are of great interest in connexion with theories about the role of lysosomal hydrolases in tissue degeneration. The earliest changes they found were an enlargement of mitochondria and changes in the endoplasmic reticulum. Lysosomal hydrolases did not initiate cell damage in this system; the breakdown of the lysosomal membrane was thought to be a secondary process. These authors referred to unpublished work by van Lancker showing early rises in unsedimentable deoxyribonuclease during auto-

lysis, but commented that such biochemical data should be interpreted with caution. Nagel & Willig (1964) studied ischaemic necrosis in rat kidney. They found that bound catheptic enzymes were inactivated during necrosis and they concluded that these enzymes did not play a part in the necrotic state, even though there was a release of enzymes from the droplets to the cytoplasm.

There is, of course, evidence of increases in hydrolytic activity in some tissues of the rat as a response to dietary changes, in addition to the studies of Weissmann *et al.* (1963). T. Moore (1966, private communication) found that, in rats given a dietary regimen that produces post-mortem renal autolysis, cathepsin activity of the kidney rises before death and there is also a rise in unsedimentable activity. Koszalka, Mason & Krol (1961) found two- to ten-fold rises in proteolytic and autolytic activity of dystrophic skeletal muscle. Dingle, Sharman & Moore (1966) showed that hypervitaminosis A and, to a lesser extent, hypovitaminosis A caused a rise in free catheptic activity of rat liver, but vitamin A status had no effect on kidney.

Our work on testicular degeneration does not entirely confirm the findings of Arata *et al.* (1962*a*, *b*). These authors reported a fall of β-glucuronidase to lower than normal values by 11 months, after the threefold rise at 5–6 months. Our results confirm the threefold rise, but not the fall in activity. We also found elevated serum values and slight rises in unsedimentable activity in testis.

In studies on the pathology of vitamin E-deficient tissues, there has been some disagreement as to whether the role of the lysosome is primary or secondary in the sequence of events leading to cell degeneration and death. Beaufay *et al.* (1959), as mentioned above, thought that enzymic alteration preceded histologically observable damage in vitamin E-deficient rat liver. Zalkin *et al.* (1962) also concluded that lysosomal enzymes were implicated in the initiation of muscle degeneration. Later work by Desai, Calvert & Scott (1964), however, showed that these enzymes were not directly implicated in the cause of muscular dystrophy but rather in the removal of degradative products from the tissue. Our studies on liver necrosis show, further, that an animal can die because of liver degeneration and yet not show any marked alteration in its pattern of hydrolases. The continued elevated levels of β-glucuronidase activity in the degenerate testis could be due to the continued need to remove incomplete products of defective spermatogenesis.

We acknowledge the technical assistance of Miss E. A. Murrell.

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Printed in Great Britain