

Glutathione peroxidase (EC 1.11.1.9), glutathione-S-transferase (EC 2.5.1.13), superoxide dismutase (EC 1.15.1.1) and catalase (EC 1.11.1.6) activities in tissues of ducklings deprived of vitamin E and selenium

BY GUANG-LU XU AND ANTHONY T. DIPLOCK†

Department of Biochemistry, Guy's Hospital Medical School, London SE1 9RT

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1. Vitamin E and selenium deficiencies were produced in the Pekin duckling (*Anser cinerens*) and were characterized by the development of lesions after 14 d in gizzard, intestine, heart and skeletal muscle.
2. The activities of glutathione peroxidase (EC 1.11.1.9), using hydrogen peroxide and cumene hydroperoxide as substrates, glutathione-S-transferase (EC 2.5.1.13), superoxide dismutase (EC 1.15.1.1) and catalase (EC 1.11.1.6) were measured in homogenate supernatant fractions of liver, heart, intestine, gizzard and skeletal muscle of deficient ducklings and of control birds given vitamin E or Se or both.
3. Glutathione peroxidase activities were dramatically lower in tissues of Se-deficient ducklings, and this was unaffected by vitamin E. No adaptive changes were seen in the activity of the other enzymes, even after 21 d when the deficiency was severe.
4. It appeared likely that the variability of the enzyme activities, other than glutathione peroxidase, in the different tissues studied might explain differences among the tissues in susceptibility to peroxidative damage.

A number of enzyme systems and other factors function *in vivo* to limit or prevent free radical-initiated peroxidative damage. These include glutathione peroxidase (EC 1.11.1.9; GSHpx), glutathione-S-transferase (EC 2.5.1.13; GST), superoxide dismutase (EC 1.15.1.1; SOD) and catalase (EC 1.11.1.6). GSHpx is a selenoenzyme capable of catalysing the reduction of hydrogen peroxide and a wide range of lipid hydroperoxides (Hoekstra, 1976). GST, which is present in large amounts in liver, catalyses the conjugation by glutathione of a wide range of xenobiotics; however, it is also capable of acting as a peroxidase to catalyse the reduction of lipid hydroperoxides, but not of H₂O₂, and it is sometimes referred to as 'Se-independent glutathione peroxidase' (Prohaska & Ganther, 1977). SOD is a group of metalloenzymes containing manganese, iron, or copper and zinc at their active centres which catalyse the disproportionation of the superoxide anion (McCord & Fridovich, 1969), and catalase catalyses the disproportionation of H₂O₂ (Aebi, 1974). Vitamin E and, in some instances, synthetic antioxidants function *in vivo* to limit proliferation of free-radical damage by scavenging lipid peroxy radicals (Tappel, 1962) and vitamin E may also play a structural role in stabilizing biological membranes (Diplock & Lucy, 1973).

Keshan disease is an endemic human cardiomyopathy that affects populations in a large area of the People's Republic of China, stretching in a wide band from the Northeast to the Southwest; it has been shown to be prevented effectively by the administration of a prophylactic oral dose of 1-4 mg sodium selenite every 10 d (Xi'an Medical College, Research Laboratory of Keshan Disease, 1979; Chen *et al.* 1980). In a number of animal species also, cardiomyopathy is part of a complex vitamin E and Se deficiency syndrome. The Pekin duckling (*Anser cinerens*) was chosen in the present study because it had been shown by Van Vleet (1977*a, b*) that vitamin E and Se deficiency resulted in development

† For reprints.

of myopathies in cardiac and skeletal muscle and in the smooth muscle of the gizzard and intestine; the period of time required was conveniently short (2–3 weeks), and the animals present no problems of husbandry and housing that are experienced with larger domestic farm animals. It was hoped that this study would provide an animal model for Keshan disease that could be used in subsequent studies in the People's Republic of China.

The purpose of this work was to investigate: (1) the possible association between loss of the Se-dependent GSHpx and the appearance of lesions in five tissues, (2) the possible appearance of adaptive changes in the other enzymes associated with the defence mechanism, (3) whether vitamin E had any effect on these factors.

EXPERIMENTAL METHODS

Animals, diets and housing

Twenty-four 1-d-old Pekin ducklings were divided at random into four groups and housed together in the groups in stainless-steel wire-bottomed cages with controlled heating and lighting. They were given the designated diets and distilled water, or distilled water containing 0.1 μg Se as sodium selenite/l, *ad lib.* during a 2–3 week experimental period. Group 1 was given the basal diet (Diplock *et al.* 1971), which was deficient in vitamin E and Se, and distilled water to drink, group 2 was given the basal diet and the Na_2SeO_3 solution to drink, group 3 was given the basal diet, to which was added 140 mg DL- α -tocopherol/kg, and distilled water to drink, group 4 was given the vitamin E-supplemented basal diet and the Na_2SeO_3 solution to drink. The ducklings were weighed daily; they were not pair fed, but a check was maintained to ensure that the intake of food, and water or sodium selenite solution, was similar in each dietary group. Some loss of appetite was noticed in the ducklings given the vitamin E- and Se-deficient diet after approximately 16 d of dietary treatment. In order to investigate enzyme levels in ducklings with severe lesions, the experiment was also repeated using the same dietary treatments as groups 1 and 2 only, and the ducklings were killed after 21 d.

Storage, sampling and homogenization of tissues

Ducklings were killed by breaking their necks 15 or 21 d after beginning the experiments and tissues for enzyme determinations were excised, trimmed, washed with ice-cold 0.25 M-sucrose solution, blotted dry and stored at -20° for subsequent assay. In a preliminary experiment it was shown that the enzymes studied were stable during storage and subsequent thawing of the tissue; Table 1 gives the results of one such experiment with liver and similar results were obtained with other tissues.

For the enzyme assays, portions of the frozen tissues were weighed and the weighed pieces chopped with scissors into ice-cold 0.25 M-sucrose which was then made to 4 vol. (w/v) with 0.25 M-sucrose and homogenized for 10 s using a Polytron Homogenizer (Northern Media Supply Ltd). The crude homogenates were centrifuged for 60 min at 100 000 *g* in a Beckman Model L-70 refrigerated centrifuge at 5° and the supernatant fraction was decanted and used to determine the activities of GSHpx, GST and SOD.

For catalase assay, the homogenates were centrifuged at 700 *g* for 10 min. Ethanol was added to the supernatant fraction to a final concentration of 0.17 M to decompose the inactive stable enzyme–substrate complex of catalase– H_2O_2 . The mixture was placed on ice for 20 min, then 100 μl phosphate buffer, pH 7.0, containing 100 ml Triton X-100/1 was added to 1 ml of the mixture (Cohen *et al.* 1970; Aebi, 1974).

Analytical methods

GSHpx activity was measured by the coupled assay of Paglia & Valentine (1967), using 0.2 mM- H_2O_2 and 0.44 mM-cumene hydroperoxide as substrates at 30° . These substrate

Table 1. Effect of storage at -20° on duckling liver enzymes*

(Portions of liver from an adequately-fed duckling were frozen at -20° ; at the stated time intervals, a piece of liver was allowed to thaw at 4° and the enzyme activities measured by the methods described on pp. 438–439 and given as units/mg protein, except for catalase which is given as units/g wet weight of tissue)

Time interval (d) after freezing...	0	7	14	17	22	43
Enzyme						
GSHpx (cumene)	0.204	0.191	—	0.181	0.212	0.205
GSHpx (H_2O_2)	0.194	0.175	0.226	0.175	0.216	0.189
GST	0.167	0.153	0.178	0.144	0.157	0.149
SOD	46.7	50.0	42.6	—	47.0	41.1
Catalase (EC 1.11.1.6)	324	—	399	346	320	—

* GSHpx (cumene), glutathione peroxidase (EC 1.11.1.9) with cumene hydroperoxide as substrate. GSHpx (H_2O_2), glutathione peroxidase with hydrogen peroxide as substrate. GST, glutathione-S-transferase (EC 2.5.1.13). SOD, superoxide dismutase (EC 1.15.1.1).

concentrations were lower than those we have used with rat tissue fractions, but it was shown in separate preliminary experiments that the enzyme was saturated at these concentrations of substrate. One unit of enzyme activity was defined as the amount of enzyme capable of oxidizing $1 \mu\text{mol}$ NADPH/min.

GST activity was measured by the method of Habig *et al.* (1974) with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The assay mixture contained 1 mM -CDNB, 1 mM -glutathione and 0.1 M -potassium phosphate buffer, pH 6.5, and the rate of increase in absorbance was monitored for 5 min at 340 nm at 30° . One unit of enzyme activity was defined as the amount of enzyme capable of conjugating $1 \mu\text{mol}$ CDNB/min.

SOD activity was measured by the method of McCord & Fridovich (1969). The reaction was carried out in 0.05 M -potassium phosphate buffer, pH 7.8, containing 10^{-4} M -EDTA at 30° . The reaction mixture contained 10^{-5} M -ferricytochrome c, $5 \times 10^{-5} \text{ M}$ -xanthine and sufficient xanthine oxidase (EC 1.2.3.2) to produce a rate of reduction of ferricytochrome c equivalent to a change in absorbance of 0.02 units/min at 550 nm. The amount of SOD required to inhibit the rate of reduction of ferricytochrome c by 50% was defined as one unit of enzyme activity.

Catalase activity was measured by the method of Aebi (1974). Tissue supernatant fractions were incubated at 30° with 0.1 M -sodium perborate, pH 7.0, and 0.05 M -phosphate buffer, pH 7.0, for 30–60 s. The reaction was stopped by adding 6 M -sulphuric acid; KI solution (100 g/l) and saturated molybdic acid solution were then added and the iodine liberated titrated with 0.1 M -sodium thiosulphate solution using starch as indicator. One unit of enzyme activity was defined as the amount of enzyme catalysing the reduction of $1 \mu\text{mol}$ H_2O_2 /min.

Protein concentrations were measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Statistical analysis

All the determinations were made on pieces of tissue from individual animals. The six results obtained were expressed as mean values and standard deviations, and these values are given in the figures. The 15-d experiment was repeated twice and the 21-d experiment was repeated once and the results were similar to those given in the figures. Tests of significance of differences were performed using Student's *t* test, taking $P = 0.05$ as the limit of significance.

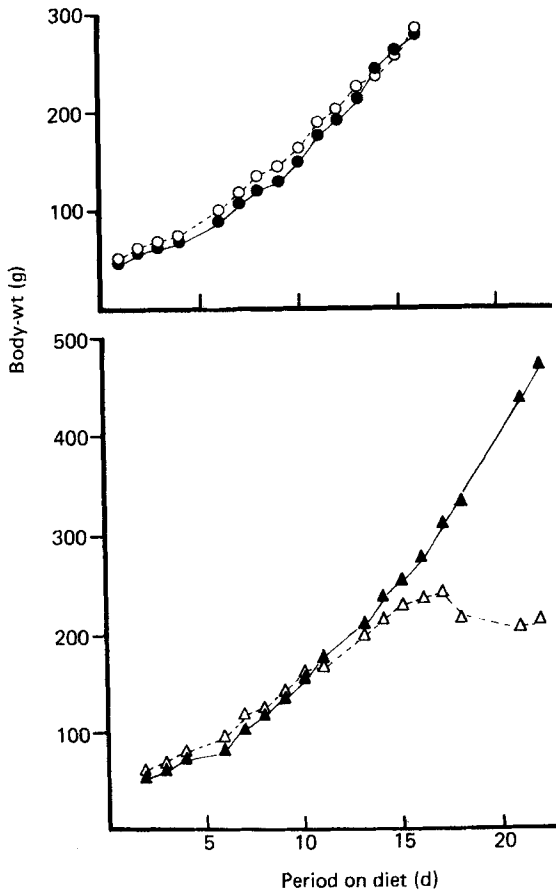


Fig. 1. Body-weight (g) of ducklings given the experimental diets for 15–21 d. (●), +Vitamin E (E) + selenium; (○), +E - Se; (▲), -E + Se; (△), -E - Se. Mean values only are given. The standard deviations were less than 10 g, except for the ducklings given the -E - Se diet for more than 18 d.

RESULTS

Body-weight. The changes in body-weight during the experiment are recorded in Fig. 1. Depletion of vitamin E and Se resulted after 14 d in a restriction of weight gain and, after 18 d, in a small loss of weight. When either Se or vitamin E, or both nutrients, were added to the diet, the ducklings grew normally.

Clinical and pathological alterations. The ducklings given the vitamin E- and Se-deficient diet developed clinical and gross pathological symptoms similar to those described by Van Vleet (1977a). The clinical symptoms that developed 14 d after beginning the dietary treatment included weakness and loss of appetite, a reluctance to stand or move about, a preference to rest on their hocks rather than to stand up and, after approximately 18 d, lateral recumbency and inability to rise. Gross pathological lesions consisting of the white patches or striations usually associated with the muscular atrophy of Se and vitamin E deficiency were observed consistently in gizzard and intestine, and frequently in heart and skeletal muscle. Care was taken to avoid taking tissues affected with gross pathological lesions for the enzyme determinations.

GSHpx activity. Se deficiency caused a significant decrease in the GSHpx activity in all

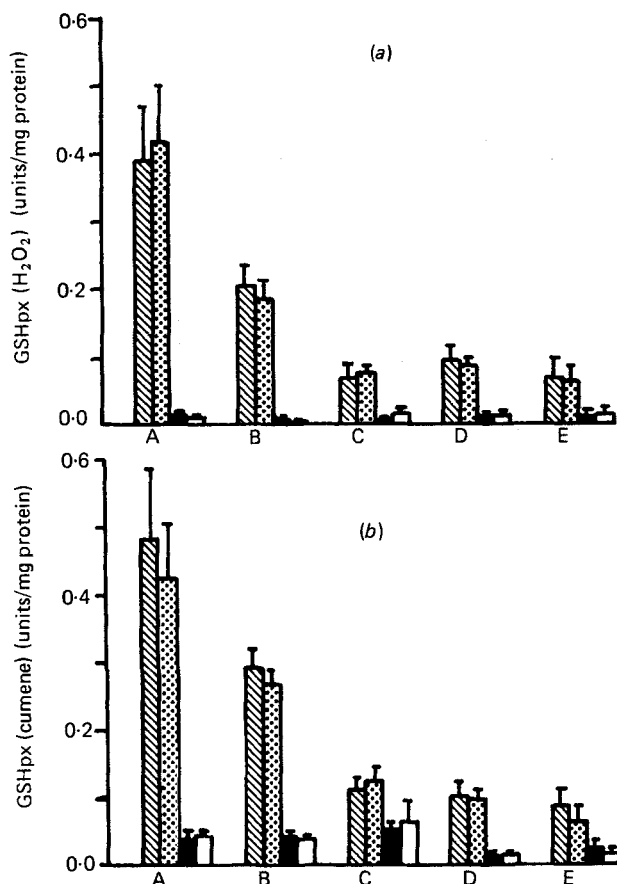


Fig. 2. Activity of glutathione peroxidase (EC 1.11.1.9) with hydrogen peroxide and cumene hydroperoxide as substrate in tissues of ducklings given the experimental diets for 15 d. (▨) + vitamin E (E) + selenium, (▩) -E + Se, (■) +E - Se, (□) -E - Se. Details of the assay methods are given on pp. 438-439. A, liver; B, heart; C, intestine; D, gizzard; E, skeletal muscle. Mean values and standard deviations (represented by vertical bars) for six determinations.

the tissues studied ($P < 0.01$ or 0.001) (Figs. 2(a and b) and 4(a and b)) and this decrease was independent of and unaffected by the vitamin E status of the ducklings. The total GSHpx activity of the tissue (i.e. Se-dependent and Se-independent due to the GST activity) measured with cumene hydroperoxide as substrate was consistently higher than the Se-dependent activity, measured with H_2O_2 as substrate, in all the tissues. The percentage losses of Se-dependent GSHpx activity due to Se-deficiency were 97, 96, 85 and 80 respectively in liver, heart, gizzard, intestine and skeletal muscle, and the percentage losses of total GSHpx activity caused by Se deficiency were 91, 84, 88, 50 and 77 in the same tissues. In the Se-supplemented ducklings the order of specific activity of the Se-dependent enzyme was: liver > heart > gizzard > intestine > skeletal muscle.

GST, SOD and catalase activities. Se and vitamin E deficiency were without effect on the GST activity of all the tissues studied (Figs. 3(a) and 4(d)) and in the Se- and vitamin E-supplemented ducklings the order of specific activity of GST was: liver > intestine > heart > gizzard > skeletal muscle.

The activities of SOD and catalase (Figs. 3(b and c) and 4(c and e)) were unchanged

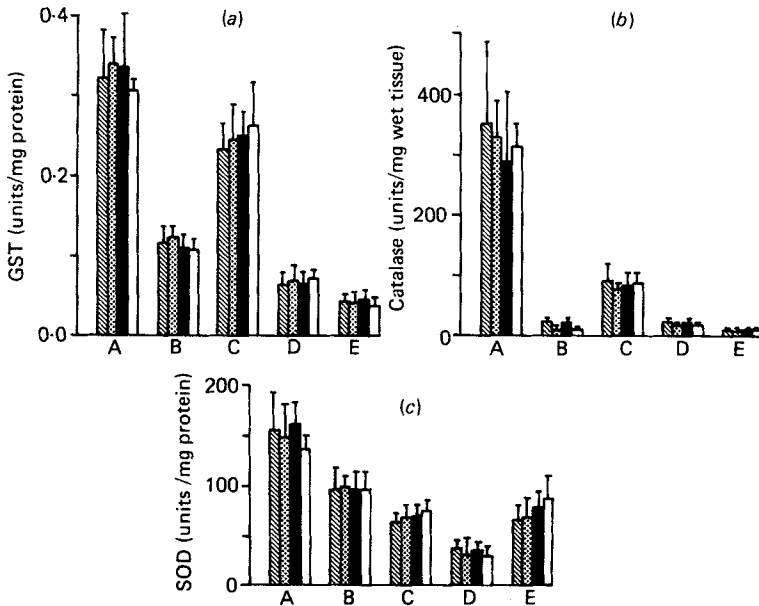


Fig. 3. Activities of glutathione-S-transferase (*EC* 2.5.1.13; GST), superoxide dismutase (*EC* 1.15.1.1; SOD) and catalase (*EC* 1.11.1.6) in tissues of ducklings given the experimental diets for 15 d. (■) + vitamin E (E) + selenium, (▨) -E + Se, (■) + E - Se, (□) -E - Se. Details of the assay methods are given on p. 439. A, liver; B, heart; C, intestine; D, gizzard; E, skeletal muscle. Mean values and standard deviations (represented by vertical bars) for six determinations.

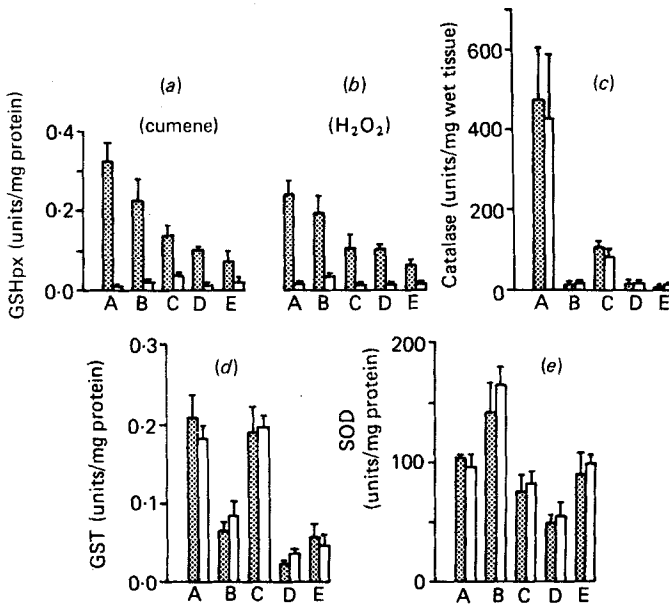


Fig. 4. Activities of glutathione peroxidase (*EC* 1.11.1.9; GSHpx) with H₂O₂ and cumene hydroperoxide as substrates, glutathione-S-transferase (*EC* 2.5.1.13; GST) superoxide dismutase (*EC* 1.15.1.1; SOD) and catalase (*EC* 1.11.1.6) in tissues of duckling given the experimental diets for 21 d. (▨) -vitamin E (E) + selenium, (■) +E + Se, (□) -E - Se. Details of the assay methods are given on pp. 438-439. A, liver; B, heart; C, intestine; D, gizzard; E, skeletal muscle. Mean values and standard deviations (represented by vertical bars) for six determinations.

by Se and vitamin E deprivation. In the Se- and vitamin E-supplemented ducklings, the order of specific activity for SOD was: liver > heart > intestine > skeletal muscle > gizzard, and for catalase it was: liver > intestine > heart and gizzard > skeletal muscle.

DISCUSSION

The body-weight changes recorded and the similarity of the pathological findings to those of Van Vleet (1977*a*) indicate that the deficient animals were indeed in the early stages of the deficiency after 15 d and *in extremis* after 21 d of the dietary treatment.

Consideration of the results of the GSHpx measurements indicates that Se deficiency resulted in a very large fall in the activity of this enzyme and that in liver, heart and gizzard of Se-supplemented ducklings most of the activity was due to the Se-dependent enzyme. However, in the skeletal muscle, and particularly in the intestine, this was not so and a much larger amount of the enzyme activity was Se-independent. The presumption that this Se-independent activity was largely due to GST is borne out by the results of the GST measurements, although in the rat it has been found that the enzyme activity measured with CDNB as substrate contains substantial contributions from forms of the enzyme without peroxidase activity (A. Mehlert and A. T. Diplock, unpublished results). The order of activity of the Se-dependent GSHpx is the same as that given by Van Vleet (1977*a*) for the Se contents of those tissues. The absence of any effect of vitamin E status on the level of activity of GSHpx is in agreement with the results of others using different animal species: Godwin *et al.* (1975) and Whanger *et al.* (1977) in sheep, Cowey *et al.* (1981) in rainbow trout (*Salmo gairdneri*), and Su & Csallany (1977) in mice.

It has been reported by Lawrence & Burk (1978) and Lawrence *et al.* (1978) that, in rats, Se deficiency is accompanied by a compensatory rise in GST activity. No such change in the activity of GST was observed in the present study, indicating that the duckling lacks a mechanism for compensating for acute Se and vitamin E deficiencies. Similarly, there was no change in the present study in the activities of SOD and catalase in the ducklings with Se and vitamin E deficiency, in agreement with the work of Lee *et al.* (1981) on rat hepatic catalase and of Serfass & Ganther (1976) on rat phagocyte SOD.

The high level of specific activity of all the four enzymes measured in liver may explain the absence of hepatic lesions in this species, and to some extent the order of the levels of these enzymes in the other tissues studied reflects the order of severity of the lesions in those tissues reported by Van Vleet (1977*a*), which is presumably also a reflection of the peroxidative damage occurring in them.

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