The effect of low dietary manganese intake on rainbow trout (*Salmo gairdneri*)

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1. Rainbow trout (*Salmo gairdneri*) of mean initial weight 15 g were given either a low-manganese or control diet containing 1·3 and 33 mg Mn/kg dry diet respectively.
2. Weight gains over a 24-week feeding period were the same for both groups of trout.
3. Hepatosomatic index, blood packed cell volume and haemoglobin concentration, plasma protein and the activities of aspartic aminotransferase (*EC* 2.6.1.1) and alanine aminotransferase (*EC* 2.6.1.2) were unaffected by dietary Mn intake.
4. Plasma potassium and iron levels were increased in the trout given the low-Mn diet.
5. The hepatic levels of magnesium, sodium, K, zinc, copper, Mn and phosphorus were significantly reduced in the fish given the low-Mn diet.
6. In those trout given the low-Mn diet the levels of Mn and calcium in the vertebral ash were significantly reduced.
7. The hepatic activity of Cu-Zn superoxide dismutase (*EC* 1.15.1.1; Cu–ZnSOD) and of Mn superoxide dismutase (*EC* 1.15.1.1; MnSOD) in cardiac muscle and liver was reduced in the group of trout given the low-Mn diet. The fall in Cu–ZnSOD and MnSOD activities coincided with reduced tissue levels of their respective metal components.

Manganese deficiency in animals and man results in a wide variety of disorders which include growth retardation, skeletal deformities, impaired glucose tolerance, ataxia and defective egg shell formation (Leach, 1974). Lall & Bishop (1977) demonstrated a dietary requirement for Mn in Atlantic salmon (*Salmo salar*) grown in sea water. More recently, while the present experiments were in progress, Ogino & Yang (1980) showed that both carp (*Cyprinus carpio*) and rainbow trout (*Salmo gairdneri*) require dietary Mn to ensure normal growth.

The present experiment was conducted to examine the effect of dietary Mn deficiency on rainbow trout. Growth, tissue mineral levels and the activity in liver and heart of the copper–zinc superoxide dismutase (*EC* 1.15.1.1; Cu–ZnSOD) and of the manganese superoxide dismutase (*EC* 1.15.1.1; MnSOD) were examined.

Dietary Mn deficiency had no effect on the growth of rainbow trout. However, tissue Mn levels and the activity of MnSOD in heart and liver were significantly reduced. The activity of Cu–ZnSOD was also lowered in liver.

MATERIALS AND METHODS

Rainbow trout of mean weight approximately 15 g were obtained from Dornoch Fisheries, Dornoch, Scotland, and randomly distributed among four circular glass-fibre tanks of diameter 1 m, depth 0·6 m and each containing 400 l water (30 fish per tank). The water from the tanks was partially recirculated, with a constant bleed-in of fresh tap-water (75 l/tank per h) from the city of Aberdeen domestic supply. The Mn concentration in the water during the experimental period was 3·3 μg Mn/l. The tanks were housed in an aquarium room with an ambient temperature averaging 15°.

The fish were given the control diet until they had acclimatized to, for example, their surroundings, feeding routine, diet and light cycle (approximately 6 d). Initial weight

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Table 1. *Composition (g/kg dry diet) of the diets given to rainbow trout* (Salmo gairdneri)

Ingredient	Basal manganese diet	Control diet
Casein	500	500
Precooked starch	200	200
Capelin oil	120	120
Vitamin mix*	28	28
Mineral mix†	114·1	114·1
Cod liver oil	30	30
α Cellulose	2	2
Ascorbyl palmitate	0·4	0·4
BHA mix‡	0·5	0·5
Cystine	5·0	5·0
$MnSO_4 . 4H_2O$	0	0·12
Measured Mn level (mg/kg dry diet)	1·3	33·0

* Supplied (/kg dry diet): riboflavin 200 mg, pyridoxine hydrochloride 40 mg, nicotinic acid 800 mg, calcium pantothenate 280 mg, *myo*-inositol 4 g, biotin 6 mg, pteroylmonoglutamic acid 15 mg, *p*-aminobenzoic acid 400 mg, choline chloride 8 g, ascorbic acid 2 g, DL-α-tocopheryl acetate 400 mg, menaphthone 40 mg, cyanocobalamin 90 μg, thiamin hydrochloride 50 mg in α cellulose.

† Supplied (/kg dry diet): calcium β-glycerophosphate 92·3 g, $(CH_3COO)_2$ Mg.4·47 g, KH_2PO_4 6·6 g, $NaH_2PO_4 . 2H_2O$ 7·5 g, NaCl 2 g, $FeSO_4 . 7H_2O$ 1 g, $ZnSO_4 . 7H_2O$ 0·13 g, $CuSO_4 . 5H_2O$ 39 mg, $CoSO_4 . 7H_2O$ 35 mg, $Al_2 (SO_4)_3 . 16H_2O$ 7 mg, KI 7 mg.

‡ Butylated hydroxyanisole 200 g/l, propyl gallate 60 g/l, citric acid 40 g/l in propylene glycol.

measurements were then made as previously described (Cowey *et al.* 1977), thereafter the fish were weighed every 4 weeks throughout the experiment which lasted for 24 weeks. The trout were fed to satiation four times daily (6 d/week) food pellets being put sparingly into each tank only so long as they were actively consumed.

The composition of the two diets used is shown in Table 1. The dry dietary ingredients were thoroughly mixed and made to a moist paste by the addition of distilled water (300 ml/kg dry ingredients) after which they were extruded through a commercial mincing machine from which the cutting blades had been removed. The resultant spaghetti-like diet (3 mm diameter) was freeze-dried and broken into small pieces for feeding. Duplicate tanks of fish were given either the control or the Mn-deficient diet at a level of 20 g diet/kg biomass of fish per d. The amount of food given to each tank was adjusted every 4 weeks in accordance with the measured biomass.

Chemical methods

Plasma from blood removed from the caudal vein was obtained by using tubes containing the lithium salt of heparin as anti-coagulant (LIP Ltd, Shipley, West Yorks). Liver and heart were excized from the fish, weighed and then quickly frozen in liquid nitrogen before storage at −20° until required. Vertebral samples were obtained by cutting off the tail of each trout at a point anterior to the adipose fin. The tail-back bone sample was then dropped into boiling water for 90 s; removal of the flesh adhering to the vertebral column was thus facilitated. After drying the vertebral sample overnight at 100° it was ashed and analysed. The mineral analyses of plasma, liver and vertebrae were carried out as described previously (Cowey *et al.* 1977).

Blood packed cell volume was determined as described by Blaxhall & Daisley (1973). Haemoglobin levels were measured using a commercial assay kit with methaemoglobin as a standard (Sigma Chemical Company, Poole, England). Plasma aspartic aminotransferase (*EC* 2.6.1.1; GOT) and alanine aminotransferase (*EC* 2.6.1.2; GPT) were measured

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using the optimal assay conditions for trout described by D'Apollonia & Anderson (1980). Plasma protein level was measured using the Coomassie blue method described by Spector (1978).

Liver superoxide dismutase (SOD) was determined by the method of Heikkila *et al.* (1976). The liver was homogenized in 9 vol. 0·05 M-phosphate buffer, pH 7·2, containing Triton X-100 (10 g/l). The supernatant fraction obtained after centrifugation for 5 min at 12000 *g* was used in the assay. Total SOD activity i.e. Cu–ZnSOD+MnSOD was determined in the absence of cyanide. The activity of the MnSOD was measured in the presence of 5 mM-potassium cyanide. Preliminary work showed that, at this concentration of KCN, Cu–ZnSOD activity was inhibited, thus measurement of the MnSOD activity alone was then possible by virtue of its insensitivity to KCN.

Hearts were homogenized in buffer and centrifuged as described previously. Since factors present in the heart homogenate stimulated the auto-oxidation of the substrate 6-hydroxydopamine hydrobromide used in the liver SOD assay the method of Panchenko *et al.* (1975) was used. In both heart and liver SOD assay systems the protein concentration used had been shown by previous work to be directly proportional to the enzyme activity measured. The mineral content of the heart supernatant fraction used in the SOD assay was measured after deproteinization with an equal volume of trichloroacetic acid (100 g/l). The analysis was carried out in the same way as for plasma.

Analyses were carried out on 10 fish selected by random methods from each of the four tanks of trout i.e. 20 fish from each dietary treatment. The statistical significance of the experimental data was established by Student's *t* tests (Fisher, 1950).

RESULTS

No gross manifestations of deficiency were observed in any of the experimental trout; growth, mortality rate and food efficiency ratio were also unaffected by the level of dietary Mn (Table 2). Similarly, the hepatosomatic index, blood packed cell volume and haemoglobin level, plasma protein concentration and the activities of plasma GOT and GPT were unaltered by the feeding of the Mn-deficient diet (Table 3).

No significant changes were found in plasma calcium, magnesium, phosphorus, sodium or zinc concentrations. However, those trout given the Mn-deficient diet had significantly elevated levels of potassium and iron in the plasma (Table 4). The plasma Mn concentrations were too low for measurement by atomic absorption spectrophotometry.

In the liver of the Mn-deficient trout levels of Ca and Fe were unchanged, but there were significant reductions in the hepatic concentrations of Mg, Na, Zn, Cu, P, K and Mn when compared with the levels found in the livers from the fish given the control diet (Table 4).

Vertebral levels of Mn, Ca and Na were significantly lower in the Mn-deficient trout than in the control fish. Although there were small changes in the concentrations of the other ions measured, in particular P and Fe, the differences found between deficient and control group trout were not statistically significant (Table 4).

Mineral analysis of the supernatant fractions used in the assay of heart SOD showed that, although Zn and Cu levels were the same in both deficient and control fish, the level of Mn was significantly reduced in the Mn-deficient trout (Table 4).

Table 5 shows the SOD activities found in livers and hearts of the experimental trout. In the livers of trout given the Mn-deficient diet the activities of the Cu–ZnSOD and the MnSOD were significantly lower than those found in the control group of fish. In the heart only the MnSOD activity was reduced in those trout given the Mn-deficient diet.

Table 2. *Total number, mean initial and final weight (g), food conversion ratio (body-weight gain (g)/food intake (g) and mortalities of rainbow trout* (Salmo gairdneri *given diets containing different amounts of manganese for 24 weeks*

Dietary Mn content (mg/kg dry diet)	Total no. of fish	Mean initial wt (g)	Mean final wt (g)	Food conversion ratio	Mortalities
1·3	30	15·5	180·9	1·11	2
1·3	30	16·3	184·0	1·16	0
33·0	30	15·1	170·6	1·20	0
33·0	30	15·7	189·3	1·05	3

Table 3. *Hepatosomatic index ([liver weight g/body-weight g] × 10^2), packed cell volume, haemoglobin (g/l blood), plasma protein (g/l), plasma aspartic aminotransferase* (EC *2.6.1.1; GOT*) *and alanine aminotransferase* (EC *2.6.1.2; GPT*) *(U*/l) in rainbow trout* (Salmo gairdneri) *given diets containing different amounts of manganese*†

(Mean values with their standard errors for 20 fish/treatment)

	Mn-deficient diet		Control diet	
	Mean	SE	Mean	SE
Hepatosomatic index	1·42	0·03	1·33	0·04
Packed cell volume	44·2	1·0	42·8	0·9
Haemoglobin	84·5	2·1	79·5	2·2
Plasma protein	49·5	1·0	49·5	1·3
GPT	11·0	1·6	11·1	1·6
GOT	245·8	21·8	236·8	23·1

* μmol NADH oxidized/min per/l plasma.
† For details of diets, see Table 1.

DISCUSSION

The absence of any gross disorders or abnormal growth in those trout given the Mn-deficient diet is in contrast to the findings of Ogino & Yang (1980). These authors showed that when rainbow trout of mean initial weight 1·5 g were given a diet containing 4 mg Mn/kg dry diet over a period of 12 weeks the growth rate was lower than that of trout given the same diet containing 13 mg Mn/kg diet. The authors also described deficiency symptoms in the Mn-deficient trout which included abnormal growth of the tail and a shortening of the body length. The Mn-deficient diet used by Ogino & Yang (1980) contained an Mn concentration threefold that used in the present experiment. Since neither growth rate nor body length was affected by feeding this diet for 24 weeks, it may be inferred that small fish are much more sensitive to Mn deficiency than are larger fish and so have a greater dietary requirement per unit body-weight. This greater sensitivity to Mn deficiency on the part of small fish may reflect a heightened metabolic need (e.g. for bone formation) during a period of rapid growth. In the experiment described by Ogino & Yang (1980) a ninefold increase in body-weight of small rainbow trout was achieved after 12 weeks of feeding (food conversion ratio 0·91). In the present experiment the increase in body-weight over a similar period was approximately fourfold with a food conversion ratio of 1·1. It is noteworthy that in liver, heart and vertebrae from Mn-deficient trout the concentrations of Mn were significantly lower than those found in tissues of control fish. By analogy, the requirement

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Table 4. *Concentrations of minerals in plasma (mmol/l), livers (mmol/kg wet tissue), hearts (mmol/kg wet tissue) and vertebrae (mol/kg ash) of rainbow trout* (Salmo gairdneri) *given diets containing different amounts of manganese*†

(Mean values with their standard errors for 20 fish/treatment)

	Plasma				Liver				Heart				Vertebrae			
	Control diet		Deficient diet		Control diet		Deficient diet		Control diet		Deficient diet		Control diet		Deficient diet	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Calcium	3·36	0·08	3·32	0·06	2·11	0·17	1·86	0·10					9·33	0·04	9·09*	0·09
Magnesium	0·37	0·02	0·41	0·03	7·70	0·50	6·36*	0·13					0·11	0·004	0·12	0·01
Phosphorus	3·91	0·15	3·78	0·15	94·98	3·28	76·12***	1·19					5·67	0·12	5·52	0·07
Sodium	116·54	2·62	118·24	1·35	47·52	2·34	40·61**	0·93					0·52	0·02	0·46*	0·02
Potassium	2·83	0·21	3·73**	0·23	98·16	4·93	76·36***	1·15					0·45	0·03	0·45	0·02
Zinc	0·32	0·01	0·34	0·01	0·41	0·02	0·33**	0·01	0·22	0·01	0·24	0·01	5·22‡	0·14	5·11‡	0·13
Iron	0·024	0·001	0·028**	0·001	0·74	0·05	0·82	0·05					1·13‡	0·08	1·31‡	0·09
Manganese					0·021	0·001	0·016***	0·001	0·0018	0·0007	0·0014***	0·0005	1·35‡	0·07	0·24‡***	0·01
Copper					1·34	0·17	0·80**	0·08	0·0021	0·0007	0·0021	0·0007				

Mean values for Mn-deficient group were significantly different from those for the control group * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$.

† For details of diets, see Table 1.

‡ Values expressed as mmol/kg ash.

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Table 5. *The activities (units†/g tissue) of manganese superoxide dismutase* (EC *1.15.1.1; MnSOD*) *and copper-zinc superoxide dismutase* (EC *1.15.1.1; Cu–ZnSOD*) *in livers and hearts of rainbow trout* (Salmo gairdneri) *given diets containing different amounts of manganese*‡

(Mean values with their standard errors for 20 fish/treatment)

Tissue	Diet	MnSOD Mean	MnSOD SE	Cu–ZnSOD Mean	Cu–ZnSOD SE
Liver	Mn-deficient	36·1	3·4	686·8	26·9
	Control	56·0*	5·7	798·9*	25·9
Heart	Mn-deficient	26·1	2·8	60·5	4·6
	Control	60·2*	2·8	54·7	5·8

Mean values for Mn-deficient group were significantly different from those for the control group * $P < 0.01$.

† One unit of SOD activity was defined as the amount of enzyme needed to obtain 50% inhibition of the substrate auto-oxidation.

‡ For details of diets, see Table 1.

of growing pigs for Mn is extremely low (Underwood, 1971) growth on diets low in Mn being maintained at the expense of tissue Mn.

The proportions of Cu–ZnSOD and MnSOD activity in the hearts from trout given the control diet were very similar to those found in the carp. However, in the trout livers, the proportion of MnSOD in the total hepatic SOD activity was greater than that found in carp liver (Mazeaud *et al.* 1979).

In the livers and hearts from the Mn-deficient trout the activity of MnSOD was significantly lower than that found in the tissues of control fish. Paynter (1980) and De Rosa *et al.* (1980) reported similar findings in the heart and liver of rats and mice, however, in the chicken, only the liver MnSOD activity was reduced by dietary Mn deficiency (De Rosa *et al.* 1980). It would therefore appear that, in common with these experimental animals, the activity of MnSOD in trout liver and heart is sensitive to the availability of the metal in the diet.

The activity of the liver Cu–ZnSOD was also lowered in the Mn-deficient trout. This finding contrasts with those in rat liver and heart where the Cu–ZnSOD activity was unaffected by dietary Mn intake (Paynter, 1980) and chicken liver where there was a compensatory increase in Cu–ZnSOD as the MnSOD activity fell. This fall in Cu–ZnSOD activity may, like the MnSOD activity changes, be linked to the reduction in hepatic levels of the metal components of the enzyme, i.e. Cu and Zn. The absence of any difference between the heart Cu levels and Cu–ZnSOD activity in both Mn-deficient and control trout further supports this suggestion. Indeed Paynter *et al.* (1979) showed that in rat liver the activity of Cu–ZnSOD was positively correlated with the hepatic Cu concentrations.

The observed changes in liver and heart mineral levels, the decrease in MnSOD activity in liver and heart and the reduced activity of Cu–ZnSOD in liver of Mn-deficient trout may have adverse effects under certain conditions. These enzymes remove superoxide radicals from the cell and so protect against free radical damage, for example, Mn deficiency in mice is characterized by membrane damage, the mitochondrial membranes being especially affected (Bell & Hurley, 1973). The reduced levels of SOD found in Mn-deficient trout in the present experiment may suffice to remove superoxide radicals arising during normal metabolism; should there be any abnormal formation of these radicals (rancid fat in the diet, pesticide uptake from the water affecting the microsomal mixed-function oxidase system) then tissue damage may be initiated.

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Deformities of the bones are well known during Mn deficiency (Underwood, 1971) and such deformities have been described in Mn-deficient rainbow trout by Ogino & Yang (1980). In the present experiment, although the vertebral Ca level was significantly reduced in the deficient fish, the Ca:P value was the same in both Mn-deficient and control trout. Leach (1974) described the involvement of Mn in the activation of glycosyltransferases and it is possible that most of the bone deformities found in Mn deficiency are not due entirely to incomplete calcification but may also be directly related to a reduction in the products of the transferase reactions e.g. the mucopolysaccharides needed in the formation of chondroitin sulphate and hence a loss of rigidity in the bone connective tissue may occur. This may be the situation in trout since the vertebral Mn concentration was significantly lower in the Mn-deficient group of fish which suffered from bone deformities compared with the normal control group (Ogino & Yang, 1980).

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