Some effects of selenium deficiency on glutathione peroxidase (*EC 1.11.1.9*) activity and tissue pathology in rainbow trout (*Salmo gairdneri*)

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1. Two duplicate groups of rainbow trout (*Salmo gairdneri*; mean weight 27 g) were given diets of differing selenium content (deficient 0·025 mg Se/kg; supplemented 1·022 mg Se/kg) for 30 weeks.

2. There were no significant differences between treatments in weight gain but packed cell volume, liver vitamin E and liver and plasma Se concentrations were all significantly lower in the Se-deficient trout.

3. Ataxia occurred in about 10% of the Se-deficient trout and histopathologies were evident in nerve cord (damage to axon sheath) and liver (loss of integrity in endoplasmic reticulum and mitochondria with appearance of increased vesiculation).

4. Glutathione peroxidase (*EC* 1.11.1.9) activity was significantly reduced in liver and plasma of Se-deficient fish but there was no indication, from differential assay, of any non-Se-dependent glutathione peroxidase activity. Glutathione transferase (*EC* 2.5.1.18) activity was significantly increased in Se-deficient trout.

Although selenium has long been recognized as an essential dietary nutrient in a variety of domestic animals (Schwarz & Folz, 1957; Oh *et al.* 1976; Siddons & Mills, 1981; Xu & Diplock, 1983), few pathological or metabolic changes have been observed in rainbow trout (*Salmo gairdneri*) depleted of Se. Diets containing 0·06–0·07 mg Se/kg (Hilton *et al.* 1980; Bell *et al.* 1985) and fed continuously over a prolonged period (up to 40 weeks) did not adversely affect growth rate and although hepatic and plasma glutathione (GSH) peroxidase (*EC* 1.11.1.9) activities were greatly reduced, tissue indices of antioxidant capacity, such as erythrocyte fragility, were not significantly affected. In particular, hepatic glutathione transferase (*EC* 2.5.1.18) activity was not altered by this Se depletion. This enzyme is responsible for the Se-independent GSH peroxidase activity present in rats (Pierce & Tappel, 1978), an activity that has been shown to increase during Se deficiency (Lawrence *et al.* 1978).

While GSH transferase purified from rainbow trout liver did not show GSH peroxidase activity, it did inhibit lipid peroxidation in trout liver microsomes in vitro (Bell *et al.* 1984).

The lack of effect of Se depletion in rainbow trout in these experiments (Hilton *et al.* 1980; Bell *et al.* 1985) may have been because dietary concentrations of 0·06–0·07 mg Se/kg do not induce a sufficiently-marked deficiency. In the experiments reported here, dietary Se was reduced to 0·02 mg Se/kg by replacing casein in the diet with a mixture of free amino acids of similar composition in an attempt to induce a more marked Se deficiency.

MATERIALS AND METHODS

Animals and diets

Rainbow trout were obtained from Selcoth Fisheries, Moffat; they had a mean weight of approximately 27 g and they were randomly distributed (thirty fish/tank) between four fibre-glass tanks of diameter 1 m, depth 0·6 m, and containing 500 litres water. The water, from Aberdeen city domestic supply, passed through an activated-charcoal filter to the tanks with a total flow to each tank of 10 litres/min. The ambient temperature in the aquarium room averaged 15° and the photoperiod was 12 h light – 12 h dark.

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Table 1. *Composition of the basal diet (g/kg dry diet)*

Torula yeast	350
Cod-liver oil*	100
Vitamin mix†	28
Mineral mix‡	40
Starch	157·1
Antioxidant mix§	0·4
Amino acid mixture‖	324·5

* Super Solvitax; British Cod Liver Oils Ltd, Hull.

† Supplied (/kg diet): thiamin hydrochloride 50 mg, riboflavin 200 mg, pyridoxine hydrochloride 50 mg, nicotinic acid 750 mg, calcium pantothenate 500 mg, *myo*-inositol 2 g, biotin 5 mg, folic acid 15 mg, choline bitartrate 9 g, ascorbic acid 1 g, menaphthone 40 mg, cyanocobalamin 0·09 mg, DL-α-tocopheryl acetate 400 mg.

‡ Supplied (g/kg diet): $Ca(H_2PO_4)_2.H_2O$ 27·6, $CaCO_3$ 2·1, $MgCO_3$ 3·6, $FeSO_4.7H_2O$ 1·2, KCl 2·0, NaCl 3·2, $Al_2(SO_4)_3.16H_2O$ 0·008, $ZnSO_4.7H_2O$ 0·16, $CuSO_4$ 0·04, $MnSO_4.4H_2O$ 0·14, KI 0·008, $CoSO_4$ 0·04.

§ Contained (g/l): 200 butylated hydroxyanisole, 60 propyl gallate and 40 citric acid dissolved in propylene glycol.

‖ Supplied (g/kg diet): lysine 32, histidine 12·2, arginine 21·6, threonine 14·6, valine 15·8, methionine 6, isoleucine 13·6, leucine 28·3, phenylalanine 13·2, tryptophan 4·3, aspartic acid 32·1, serine 15·9, glutamic acid 50, proline 12·2, glycine 14·9, alanine 21·7, tyrosine 11·3, cystine 4·8.

The fish were weaned from a commercial diet onto the basal diet (Table 1) and about 1 week later initial weight measurements were made on individual fish which had been anaesthetized with MS222 (ethyl *m*-aminobenzoate methane sulphonate; Sigma Chemical Co. Ltd, Poole, Dorset; 0·2 g/l). Fish were fed at a rate of 20 g/kg biomass per d (four or five times per d), 6 d each week. Any food uneaten from the daily ration was weighed and recorded. Fish were weighed at 28-d intervals and the ration adjusted accordingly. The experiment lasted 30 weeks.

The basal diet (Table 1) was formulated to meet the nutritional requirements of trout other than for Se (diet 1). The complete diet had Se added as sodium selenite to 1 mg/kg (diet 2). The dry dietary components were mixed in a Hobart commercial mixer (Model A200; Hobart Manufacturing Co. Ltd, London), then formed into a paste by adding 300 ml water/kg powder. The paste was extruded through the mincer using an appropriate die to provide pellets of suitable size for the fish. After freeze-drying, the material was broken into pellets of approximately 5 mm length. Each of the two diets was fed to duplicate groups (tanks) of fish.

Analytical methods

Each analytical measurement was carried out on six individuals from each treatment, i.e. three individuals were selected at random from each tank. Blood samples were taken from the caudal vein into tubes containing sodium EDTA as anticoagulant.

Se in water, diets and tissues was measured as described by Hasunuma *et al.* (1982). Vitamin E in diets was extracted by the method of McMurray *et al.* (1980) and from tissues as described previously (Cowey *et al.* 1981). In both cases measurement of vitamin E was performed by high-performance liquid chromatography (Hung *et al.* 1980) with fluorometric detection (model LS4 fluorescence spectrometer; Perkin-Elmer, Beaconsfield, Bucks.). Liver microsomes were prepared as described by Bell *et al.* (1984) and Fe^{2+} NADPH-stimulated oxidation of microsomal lipids was as described by Bell *et al.* (1984). All enzyme assays were performed at 20°. Livers were homogenized in 9 vol. 20-mM-Tris-hydrochloric acid (pH 7·4), 1 mM-dithiothreitol and Triton X-100 (10 mg/g) and the resulting homogenate used in the assay of GSH peroxidase and GSH transferase. For GSH peroxidase the rate

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Table 2. *Selenium and vitamin E contents of the experimental diets together with values for weight gain of rainbow trout* (Salmo gairdneri) *given the diets for 30 weeks*
(Mean values with their standard errors for thirty fish per tank)

Diet*...	1				2			
	Mean		SE		Mean		SE	
Measured Se (mg/kg)	0·025		0·004		1·022		0·041	
Measured vitamin E g/kg	0·63		0·06		0·56		0·08	
Tank...	A		B		C		D	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Initial wt (g)	25·70	1·24	26·34	1·74	26·57	2·01	28·12	2·11
Final wt (g)	144·46	8·47	155·94	12·76	159·37	10·63	163·87	9·62
Feed intake:wt gain	1·71		1·61		1·54		1·50	

* For details, see Table 1.

of oxidation of NADPH was followed at 340 nm by the coupled reaction with GSH reductase (NAD(P)H) (*EC* 1.6.4.2). The reaction mixture contained 1 mM-GSH, 1·5 mM-cumene hydroperoxide or 0·25 mM-hydrogen peroxide, 0·1 mM-NADPH, 1·0 unit GSH reductase (Sigma Chemical Co. Ltd; 1 unit oxidizes 1 μmol NADPH/min), 1 mM-EDTA, 2 mM-sodium azide and 50 mM-potassium phosphate buffer, pH 7·4. GSH transferase activity was assayed by following the conjugation of 1-chloro-2,4-dinitrobenzene with GSH at 340 nm (Habig *et al.* 1974). The assay contained, final concentrations, 100 mM-potassium phosphate (pH 6·5), 2 mM-GSH and 1 mM-chloro-2,4-dinitrobenzene. Protein was measured by the method of Lowry *et al.* (1951). Plasma pyruvate kinase (*EC* 2.7.1.40) was measured as described by Bell *et al.* (1985).

Histology

Small portions of liver, muscle and nerve cord were fixed overnight in glutaraldehyde (25 g/l), 0·1 M-phosphate buffer (pH 7·6), saline (5 g sodium chloride/l, giving an osmolarity of 650 mosmol/l) and washed in 0·1 M-phosphate buffer, NaCl (15 g/l). After dehydration, blocks were embedded in 'Emix' resin (Emscope Laboratories Ltd, Ashford, Kent). Sections (0·5 μm) were stained with toluidine blue (10 g/l) sodium tetraborate (10 g/l) at 70° for 1 min before examination under a light microscope. Sections (15 nm) were stained with uranyl acetate and lead citrate and examined in a Jeol 100CX electron microscope operating at 60 kV.

Statistical analysis

The differences in growth due to experimental treatments were determined by analysis of variance (at the 99% probability level) of the initial and final weights of individual fish. Significance of differences between treatments for packed cell volume, liver vitamin E, liver and plasma Se, liver and plasma GSH peroxidase, liver and plasma GSH transferase and plasma pyruvate kinase was assessed by Student's *t* test.

RESULTS

Initial and final weights of trout given the two diets are shown in Table 2. Good growth rates were obtained on both treatments and there were no significant differences in weight

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gain in any of the tanks. Feed intake: weight gain values (Table 2) were comparatively high but this has frequently been the case when trout are given diets containing large quantities of free amino acids (Walton *et al.* 1982); there were no significant differences between treatments. No mortalities occurred nor were any gross pathologies evident in fish given the control (Se-supplemented) diet. About 10% of fish fed on the Se-deficient diet developed ataxia, i.e. an abnormal swimming action in which they swam either on their sides or in a totally-inverted fashion.

No signs of pathology were discernible by light microscopy in the tissues of Se-deficient fish. Electron microscopy of liver from control trout (Plate 1(*a*)) gave a conventional picture, the cells being characterized by parallel rows of rough endoplasmic reticulum bordering the cell membrane and surrounding the nucleus in which the chromatin was dispersed. Mitochondria were in close association with the rough endoplasmic reticulum and scattered throughout the cytoplasm but excluded from the large glycogen pools which occupied up to 30% of the cell volume. Vesicles, some containing glycogen and fine electron-dense material, others with distinct particles – possibly low-density lipoproteins, occurred frequently. Lysosomes and residual bodies were present toward the bile canaliculus edge of the cells.

Hepatocytes from Se-deficient fish (Plate 1 (*b*)) were characterized by condensed chromatin in the nucleus and a prominent nucleolus. The endoplasmic reticulum was swollen and spaces between cisternae enlarged with apparent loss of ribosomes, giving the cells an appearance of increased vesiculation. Glycogen was plentiful but there was loss of integrity in the mitochondria with the cristae often being ill-defined. Residual bodies with heterogeneous contents of varying electron density occurred more frequently than in control fish. Electron microscopy of livers of the ataxic Se-deficient trout (Plate 1 (*c*)) showed mitochondria that were internally disintegrated and nuclei that were distorted; large vacuoles were present in the glycogen pools.

No gross structural changes were seen in electron micrographs of muscle tissue, myofibrillar structure being intact in both control and Se-deficient fish. Nerve cord from control fish (Plate 2(*a*)) had normally myelinated axons with typical neurotubules, neurofilaments, vesicles and occasional mitochondria within the axoplasm. Considerable damage to the axon sheath occurred in Se-deficient fish (Plate 2(*b*)) and vacuolation of the axoplasm was evident with loss of organelles.

The greater degree of Se-deficiency indicated by tissue Se levels (Table 3) probably led to a reduction in packed cell volume that had not been observed previously (Bell *et al.* 1985). No thiobarbituric-acid-positive material was found when microsomes from the livers of control or Se-deficient fish were incubated under oxidizing (Fe^{2+}-NADPH) conditions in vitro. This was probably due to the high levels of vitamin E present in livers of both Se-deficient and control fish (Table 3). These levels reflected the high dietary intake. Vitamin E is known to inhibit malondialdehyde formation in the in vitro microsomal system (Bell *et al.* 1984; Cadenas *et al.* 1984).

Se concentrations in liver and plasma were very greatly reduced by the Se-deficient diet and values were markedly lower than in a corresponding treatment of earlier experiments (Bell *et al.* 1985). Even so the levels attained are still more than double those reported by Oh *et al.* (1976) for Se-deficient lambs.

The activities of liver and plasma GSH peroxidase are shown in Table 4. While the decrease in activity in the Se-deficient state is in line with all previous results, the differential assay with hydrogen peroxide and organic peroxides again does not reveal any convincing evidence for the presence of a non-Se-dependent GSH peroxidase activity (cf. Bell *et al.* 1984, 1985). For example the ratio, activity measured with cumene hydroperoxide: activity measured with H_2O_2 can be shown to be 210 in the livers of Se-deficient rats and 2·5 in

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Table 3. *Packed cell volume, liver vitamin E concentration (μg/g wet tissue) and liver and plasma selenium concentrations (μg/g wet tissue or μg/ml) in rainbow trout* (Salmo gairdneri) *given the experimental diets**

(Mean values with their standard errors for six fish per treatment)

	Diet 1		Diet 2		Statistical significance of difference:
	Mean	SE	Mean	SE	$P <$
Packed cell volume	0·298	0·025	0·418	0·070	0·001
Liver vitamin E	227·4	30·89	342·4	41·64	0·01
Liver Se	0·122	0·019	1·321	0·258	0·001
Plasma Se	0·031	0·006	0·219	0·038	0·001

* For details of diets, see Table 1.

Table 4. *Activities (nmol NADPH oxidized/min per mg protein or per ml plasma) of glutathione (GSH) peroxidase* (EC 1.11.1.9) *in livers and plasma of rainbow trout* (Salmo gairdneri) *given the experimental diets**

(Mean values with their standard errors for six fish per treatment)

	Diet 1		Diet 2		Statistical significance of difference:
	Mean	SE	Mean	SE	$P <$
Plasma GSH peroxidase†	57·35	4·74	402·15	30·79	0·001
Liver GSH peroxidase‡	2·50	0·68	14·38	1·83	0·001
Liver GSH peroxidase§	5·05	0·85	18·60	1·72	0·001

* For details of diets, see Table 1.
† Assay conditions as Bell *et al.* (1985).
‡ 0·25 mM-hydrogen peroxide as substrate.
§ 1·5 mM-cumene hydroperoxide as substrate.

the livers of Se-supplemented rats (Lawrence *et al.* 1978). The ratio in trout given Se-deficient diet 1 was 2·20 (SE 0·48) compared with 1·31 (SE 0·06) for trout given the control diet. These values, although dissimilar, are not significantly different and are not good evidence either for or against the presence of a non-Se-dependent GSH peroxidase.

Again GSH peroxidase activity in the Se-deficient fish was reduced by 73% compared with control fish when cumene hydroperoxide was used as substrate. The corresponding value with H_2O_2 as substrate was 83%. These values do not support the view that a Se-independent GSH peroxidase is present in trout.

There were significant increases in the activities of GSH transferase in both liver and plasma of Se-deficient fish (Table 5). This result contrasts with our earlier findings on trout depleted of Se (Bell *et al.* 1985). The increased activity is similar to that reported in rats given a Se-deficient diet (Lawrence *et al.* 1978).

The mean activity of plasma pyruvate kinase was enhanced in Se-deficient trout (Table 5). Even higher values occurred in those fish showing the abnormal swimming action (115·23 (SE 43·4) nmol NADH oxidized/min per mg protein). This may be due to leakage from tissues suffering structural damage into plasma.

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Table 5. *Activities of glutathione (GSH) transferase* (EC 2.5.1.18) *from plasma and liver and pyruvate kinase* (EC 2.7.1.40) *from plasma of rainbow trout* (Salmo gairdneri) *given the experimental diets**

(Mean values with their standard errors for six fish per treatment)

	Diet 1		Diet 2		Statistical significance of difference: $P <$
	Mean	SE	Mean	SE	
Liver GSH transferase†	0·713	0·072	0·311	0·034	0·001
Plasma GSH transferase‡	2·79	0·22	0·66	0·14	0·001
Plasma pyruvate kinase§	1·38	0·23	0·76	0·08	0·05

* For details of diets, see Table 1.
† μmol thioester bond formed/min per mg protein.
‡ nmol thioester bond formed/min per mg protein.
§ nmol NADH oxidized/min per mg protein; values were log transformed before analysis.

DISCUSSION

In previous studies (Hilton *et al.* 1980; Bell *et al.* 1985) no frank Se-deficiency symptoms have been found in trout when diets contained an adequate amount of vitamin E. In the present study dietary Se levels were lower than those attained previously in experiments with fish (0·025 μg Se/g diet) and it is evident that, when Se deficiency is sufficiently severe, tissue pathologies do arise even when diets contain high concentrations of vitamin E.

It is of interest that while the concentration of Se in the livers of trout in the present experiment was very much lower than that in an earlier experiment (Bell *et al.* 1985), the activity of GSH peroxidase was very similar in the two experiments. This suggests that Se present in GSH peroxidase is retained much more tenaciously in Se deficiency than is Se present in other proteins or in other states.

Recently Gatlin & Wilson (1984) demonstrated that the growth of channel catfish (*Ictalurus punctatus*) is affected by dietary Se level. A basal diet containing 0·06 mg Se/kg dry diet caused growth depression and a requirement level of 0·25 mg Se/kg dry diet was inferred from growth data and liver and plasma 'Se GSH peroxidase' activity levels. This suggests that channel catfish are more demanding in their Se requirement than rainbow trout.

Gatlin & Wilson (1984) provided information on 'Se GSH peroxidase' and 'non-Se GSH peroxidase' in livers and plasma of channel catfish given different dietary concentrations of Se. The basis for these findings is by no means clear, in particular, whether the 'non-Se GSH peroxidase' is a measurement with cumene hydroperoxide or a differential assay with H_2O_2 and cumene hydroperoxide as substrates. If 'non-Se GSH peroxidase' does in fact represent such an activity it is difficult to see, for example, why values should be much lower at low Se intakes than at high intakes; if on the other hand 'non-Se GSH peroxidase' is actually total GSH peroxidase activity it is difficult to see why values obtained at high-Se dietary intakes are the same as values for 'Se GSH peroxidase' activity.

It remains the case that there is as yet no unequivocal evidence of a Se-independent GSH peroxidase activity in fish.

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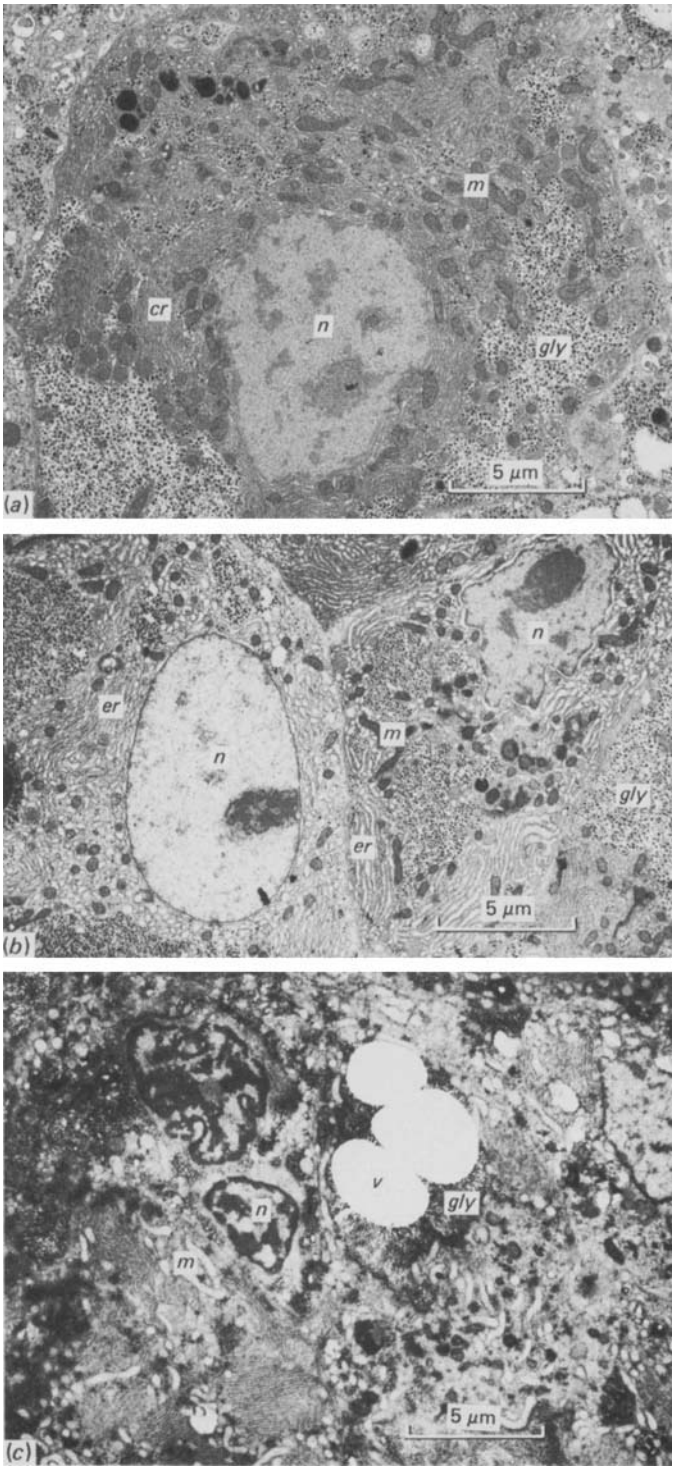

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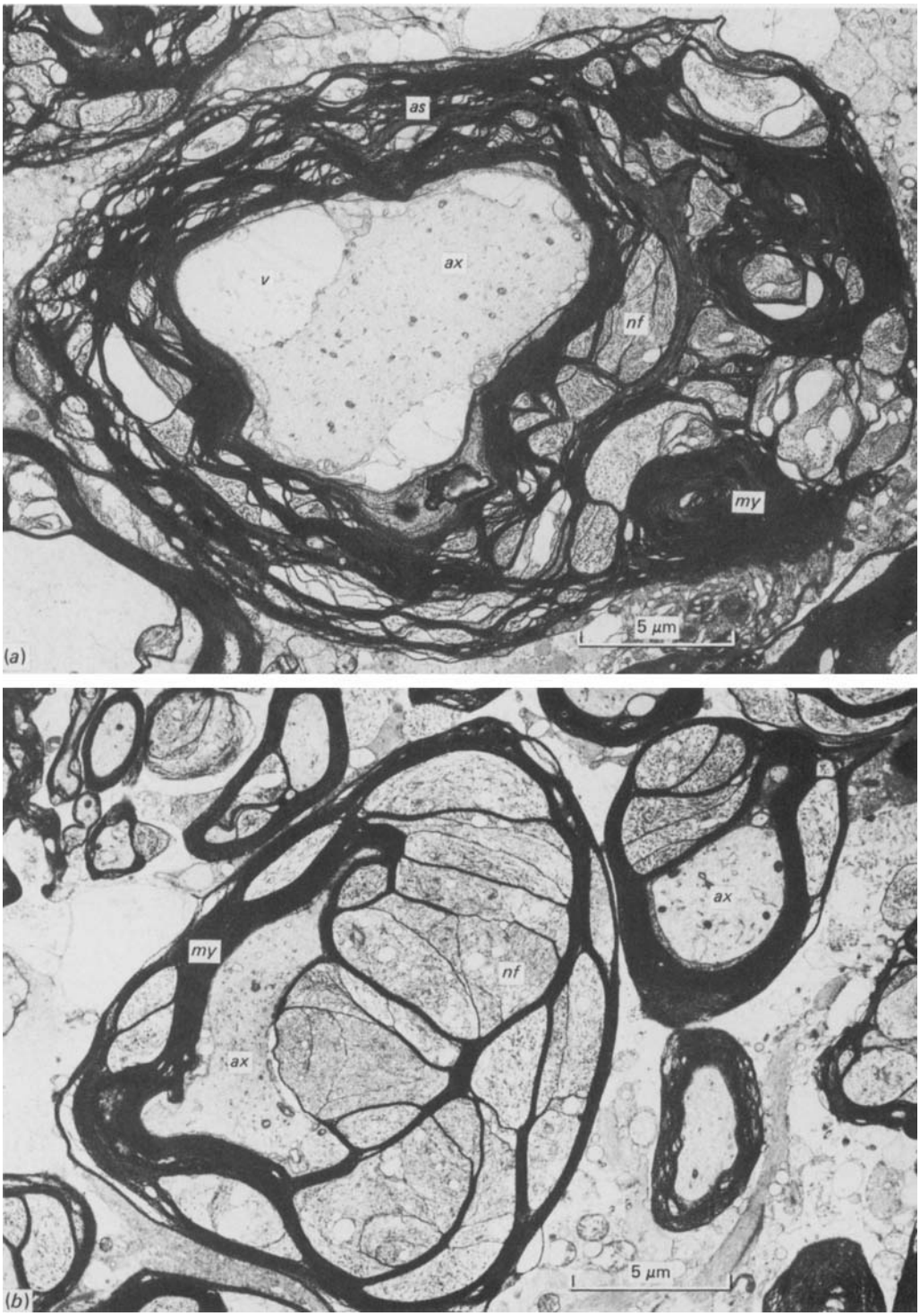

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EXPLANATION OF PLATES

Plate 1. Electron micrographs of livers from rainbow trout (*Salmo gairdneri*) fed on (*a*) selenium-supplemented diet (diet 2), (*b*) Se-deficient diet (diet 1), (*c*) Se-deficient diet and showing ataxia. er, Endoplasmic reticulum; gly, glycogen; m, mitochondria; n, nucleus; v, vacuole. For diet compositions, see Table 1.

Plate 2. Electron micrographs of nerve cords from rainbow trout (*Salmo gairdneri*) fed on (*a*) selenium-supplemented diet (diet 2), (*b*) Se-deficient diet (diet 1). as, Axon sheath; ax, axoplasm; my, myelin; nf, neurofilaments; v, vacuole. For diet compositions, see Table 1.

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