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EFFECT OF DIETARY SALT LOAD ON TRANSEPITHELIAL Na^+ EXCHANGE IN FRESHWATER RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Summary

Dietary Na^+ loads (0.5–70 mmol kg^{-1} fish) were almost completely absorbed within 7 h, and branchial Na^+ excretion commenced within 1 h. Na^+ loads of less than 1 mmol kg^{-1} were lost through the gills through a significant decrease in Na^+ influx with unaltered Na^+ efflux rate (compared with Na^+ fluxes in unfed fish). At higher salt loads (>18 mmol kg^{-1}), Na^+ loss increased as a result of significantly higher Na^+ efflux rates, with no further decrease in Na^+ influx rate. Tissue Na^+ concentrations were unchanged, apart from a significant increase in blood plasma Na^+ concentration in fish loaded above 18 mmol kg^{-1}. The results show that branchial Na^+ fluxes may be rapidly adjusted in response to prevailing conditions, and possible control mechanisms are discussed.

Key words: transepithial Na^+ exchange, dietary Na^+, gills, freshwater rainbow trout, *Oncorhynchus mykiss.*

Introduction

In freshwater fish, Na^+ is lost *via* the gills, kidney and gut and is replaced by branchial absorption from the water and from the diet. Branchial Na^+ fluxes have been reviewed (Eddy, 1982; Evans, 1984; Payan *et al.* 1984; Wright, 1991), and recent work suggests that an outwardly directed proton pump results in a negative potential within the gill epithelial cell, allowing inward diffusion of Na^+ (Avella and Bornancin, 1989; Randall and Lin, 1993). Branchial Na^+ uptake is an active process, conforming to saturable enzyme kinetics (Maetz, 1971; McDonald and Rogano, 1986; Goss and Wood, 1990*a*,*b*), while Na^+ loss from the gills is thought to occur by diffusion (Maetz, 1971; Kirschner, 1979). The influences of exchange diffusion, gill perfusion and ventilation on branchial Na^+ diffusion rates have been discussed previously (Goss and Wood, 1990*a*,*b*). Most studies have used unfed fish.

Branchial Na^+ uptake and and Na^+ from the diet may exceed the requirements for homeostasis, and absorption of excess Na^+ results an excretion requirement. In feeding fish, excess Na^+ could be excreted by increased branchial loss, and by reducing Na^+ influx or by increasing Na^+ efflux rate, or both (Smith *et al.* 1989). In rainbow trout fed on freshwater shrimp *Gammarus pulex*, digestion and absorption proceeded rapidly, with 60 % of the ingested Na^+ being absorbed within 5 h (Smith *et al.* 1989), while brook trout (*Salvelinus fontinalis*) fed on a diet of gelatin capsules containing 2.9–14.7 % NaCl (500–2534 mmol kg^{-1}) responded similarly (Phillips, 1944). Rainbow trout fed salt-enriched diets showed a limited capacity to excrete Na^+ and Cl^- through the kidney or the gut (Salman and Eddy, 1988*a*), indicating that the gills are the main effectors for whole-body Na^+ homeostasis.

Dietary salt loading offers a new approach to studying gill ionic exchanges, since the method is non-invasive, the fish feed freely and they are virtually stress free. In this study, the effects of dietary Na^+ loads on branchial Na^+ fluxes were investigated and compared with fluxes in unfed fish, in an attempt to reveal short-term homeostatic mechanisms.

Materials and methods

Experimental animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)], mass 40–60 g, were obtained from a local trout hatchery (College Mill Trout Farm, Almondbank, Perthshire) and held in 200 l tanks supplied with running fresh water, temperature 15±1.0 °C, ionic concentrations (in mmol l^{-1}); Na^+, 0.19; K^+, 0.02; Ca^{2+}, 0.15; Mg^{2+}, 0.05; Cl^-, 0.13; pH 8.2. Fish were held under these conditions for at least 2 weeks and were fed at 2 % body mass per day on commercial trout pellets (Special Diets Services Ltd).

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Normal and high-salt diets

Two diets differing in salt content were employed: commercial trout pellets (Special Diets Services Ltd) containing 2.1 % NaCl (360 mmol $Na^+ kg^{-1}$) and the same diet, reconstituted to contain 12 % NaCl (2100 mmol $Na^+ kg^{-1}$) as described by Salman and Eddy (1988*b*).

Relationship between stomach content, salt load and food ingested

In the Na^+ flux experiments (see below), it was not always possible to measure the quantity of food ingested by individual fish. To overcome this problem, additional experiments were conducted to establish the relationship between the mass of the stomach contents recovered after 7 h (the standard duration of the experiments) and the accurately determined quantity of food supplied to individual fish.

A known number of preweighed pellets (2.1 % NaCl) of uniform mass were fed to 10 trout in a 500 l tank and a similar experiment was performed on seven trout receiving the high-salt diet (12 % NaCl). After 7 h, the fish were killed by terminal anaesthesia (100 mg l^{-1} benzocaine) and the number of pellets consumed by each individual was usually evident in the stomach contents. Where there was doubt, the number could reliably be determined by relating individual and group stomach content masses to the total number of pellets fed. This relationship allowed accurate measurements of food consumed and Na^+ load absorbed to be made for individual fish, from the mass and Na^+ content of the stomach contents after 7 h. Solid material was seldom found beyond the stomach. These methods follow well-established procedures for calculating food intake from gut contents (e.g. Talbot, 1985).

Dietary salt loading prior to Na^+ flux experiments

A group of 10 trout was placed in a 100 l tank and remained unfed for 48 h to allow the gut to empty. After feeding, excess food was removed from the water. This procedure was adopted since single fish seldom fed whereas groups readily showed feeding activity. At this stage, it was impossible to identify those fish that had fed or the quantity of food consumed by individuals.

A fish (which may or may not have fed) was randomly chosen from the group and gently ushered into a transparent tubular flux chamber (17 cm×4.5 cm) situated within the 100 l holding tank. On most occasions, the fish entered the chamber voluntarily and, once inside, remained quiescent and stress-free throughout the experimental period. All experiments were conducted at 15±1 °C.

Na^+ net flux, influx and efflux measurements

Once occupied by a fish, the chamber was immediately connected to a 300 ml recirculating system driven by a peristaltic pump delivering aerated water at 32.4 l h^{-1}, which allowed removal of water samples without disturbing the fish. 7.4 kBq of $^{22}Na^+$ (Amersham International, Amersham, UK) was added to the water to give a specific activity of about 6500 cts min^{-1} μmol^{-1} Na^+. After 5 min to allow for even distribution of the radiolabel, a 2.0 ml water sample was taken, followed by hourly samples for 7 h. Net Na^+ gain or loss was calculated from changes in Na^+ content of the medium using a Pye Unicam SP9 absorption spectrophotometer, Na^+ influx rate from changes in water $^{22}Na^+$ specific activity and Na^+ efflux rate from the difference between Na^+ influx and Na^+ net flux according to the method of Maetz (1956) and Wood *et al.* (1984). $^{22}Na^+$ activity of water and tissue samples was determined (counting error <1 %) using an LKB gamma counter. Corrections for 'backflux' were not necessary, since the specific activity of Na^+ in the tissues was always less than 10 % of that in the medium (Maetz, 1956; Wood *et al.* 1984).

This procedure was repeated for both fed and unfed fish, identified after examination of stomach contents. Results were obtained from 13 fish feeding on normal diet (2.1 % NaCl), six feeding on the high-salt diet (12 % NaCl) and 14 fish which had not fed, serving as the control group. The mass of ingested food was subsequently determined.

These experiments measured whole-body Na^+ fluxes, which are principally branchial Na^+ exchanges. Urinary Na^+ efflux is about 10–15 % of the total Na^+ efflux (i.e. branchial + urinary) in fish fed normal or high-salt diets (Salman and Eddy, 1988*b*). If the urinary component is accounted for, a relatively small adjustment to the branchial flux values is required, which would not be significant apart from when small differences between values are important. This minor disadvantage is strongly outweighed by the stress incurred by fitting urinary cannulae and the reduced possibility of cannulated fish feeding. Our experimental design, i.e. starving for 48 h prior to experimentation, ensured no faecal production during the experiment.

Tissue and stomach content analysis

At the end of the experiment, fish were rapidly removed from the apparatus with minimal stress and killed by a blow to the head, a procedure occupying no more than 10–20 s. Blood from caudal vessels (about 100 μl) was taken into a heparinised syringe while samples (approximately 100 mg) of muscle, liver and kidney were rapidly excised for radioactivity determination. Blood samples were centrifuged at 12000 revs min^{-1} for 10 s and blood plasma and tissue samples were frozen (−30 °C) and stored for subsequent analysis of Na^+ levels. Tissue samples were incubated in 0.2 mol l^{-1} nitric acid for at least 1 week at 20 °C, then centrifuged at 1000 revs min^{-1} and, following dilution of the supernatant, Na^+ concentration was determined as before using a Pye Unicam SP9 absorption spectrophotometer. During the course of the experiment (7 h), isotopic equilibration within well-perfused tissues such as liver and kidney was probably complete, but in poorly perfused tissue such as muscle it was probably incomplete.

The stomach contents of fish which had fed were removed, weighed and placed in an oven (60 °C) for about 15 h before reweighing to determine dry mass. Food pellets similarly treated contained 10±1 % (mean ± S.E.M., N=6) water. Na^+

concentrations of stomach contents or food were determined as for tissues. Total ammonia (NH_3+NH_4^+) in the water was determined using the indophenol method of Solorzano (1969), to obtain hourly ammonia excretion rates (μmol kg^{-1} h^{-1}) for each fish.

Statistical analysis

Data were compared using Student's *t*-tests, one-way analysis of variance (ANOVA) or linear regression (Minitab), and differences were considered to be significant at $P<0.05$. Mean values ± S.E.M. are presented unless otherwise indicated.

Results

Relationship between food ingested, food recovered and absorbed Na^+ load

For fish fed a normal diet (2.1 % NaCl), the relationship between stomach contents (*s*) and food ingested (*I*) based on dry mass values (in mg ± S.D.; $N=10$) was:

$$s = 0.97(\pm0.036)I + 45.4(\pm10.1)\ (r^2=0.99;\ P<0.001)\,;$$

while for fish ($N=7$) fed the high-salt diet (12 % NaCl), the relationship was:

$$s = 4.11(\pm0.19)I + 5.96(\pm17.1)\ (r^2=0.99;\ P<0.001)\,.$$

Thus, using these regression lines, the food ingested by each fish could be back-calculated (to within ±5 %) and the Na^+ load was calculated as the difference between the Na^+ content of ingested food and the Na^+ content of the stomach contents:

$$A = I[Na^+]_I - s[Na^+]_s\,,$$

where *A* is the absorbed salt load, *I* is the mass of food ingested (in g), *s* is the mass of the stomach contents (in mg) and $[Na^+]_I$ and $[Na^+]_s$ are the Na^+ concentrations of the ingested food and stomach contents respectively.

Branchial Na^+ fluxes over a 7 h period following Na^+ ingestion

All salt-loaded fish (Table 1) showed significant suppression of Na^+ influx rates compared with unfed fish, but the rate was independent of the salt load and seldom fell below about 0.05 mmol kg^{-1} h^{-1}. Na^+ efflux rate was significantly increased in fish loaded with NaCl at over 18 mmol kg^{-1}, although it remained unaltered at the lower Na^+ load (Table 1). Thus, salt loading caused a net Na^+ loss through suppression of Na^+ influx and, at higher salt loads, an increase in Na^+ efflux (compared with values for unfed fish). Hourly determination of branchial Na^+ flux rates showed that Na^+ fluxes changed within 1 h of feeding, indicating that fish responded rapidly to the salt load and altered Na^+ exchange rates across the gill epithelium appropriately. There were no further significant changes in Na^+ flux rates during the 7 h experiment (see Table 1).

Na^+ content and $^{22}Na^+$ activity of tissues

Na^+ loads of approximately 0.5–1 mmol kg^{-1} had little effect on plasma Na^+ concentration, and the values were not significantly different from those for unfed fish. However Na^+ loads exceeding 18 mmol kg^{-1} increased plasma Na^+ levels significantly above those measured for unfed fish (Fig. 1).

Feeding the fish a diet containing 12 % NaCl constituted a mean Na^+ load of 36.2±8.8 mmol kg^{-1} ($N=6$), of which 85.7±2.3 % was absorbed within 7 h at a rate of 5.2±1.2 mmol kg^{-1} h^{-1}, resulting in a significant increase in blood plasma Na^+ concentration (Table 2), without any effect on the Na^+ concentration of muscle, liver or kidney. $^{22}Na^+$ absorbed across the gills showed highest activity (Bq μmol^{-1} Na^+) ($P<0.05$) in blood plasma of unfed fish, although these fish showed lower activity ($P<0.05$) in liver, muscle and kidney compared with salt-loaded fish (Table 2). The average hourly ammonia excretion rate over 7 h for unfed fish was 0.35±0.5 mmol kg^{-1} h^{-1} ($N=14$), which was not significantly different from values for both groups of salt-loaded fish.

Table 1. *Na^+ flux rates for rainbow trout which were unfed (no salt-loading), had absorbed NaCl loads of 0.5–1 mmol kg^{-1} or loads greater than 18 mmol kg^{-1}*

	NaCl load		
	0.5–1 mmol kg^{-1} ($N=13$)	>18 mmol kg^{-1} ($N=6$)	Unfed ($N=14$)
Influx (μmol kg^{-1} h^{-1})	123±26*	158±17*	443±45
Efflux (μmol kg^{-1} h^{-1})	−254±30	−1652±175*	−315±24
Net flux (μmol kg^{-1} h^{-1})	−130±45*	−1280±110*	125±25

Changes in Na^+ flux rates occurred within 1 h and hourly determinations over 7 h indicated no further significant changes; therefore, mean values are presented.

A negative value indicates Na^+ loss from the fish.

*Significantly different at $P<0.05$ from unfed flux rates.

Values are means ± S.E.M.

Discussion

Na^+ enters freshwater fish by branchial uptake from the water and through the diet, while Na^+ loss is through the gills, the kidney, the gut and possibly *via* mucus (Handy and Eddy, 1990); balance is achieved when Na^+ gain matches Na^+ loss (Smith *et al.* 1989). Na^+ gain may be varied by changes in the rate of branchial Na^+ influx or possibly by altering dietary intake, although feeding to satisfy nutritional requirements far outweighs any Na^+ regulatory aspects. Ingested Na^+ is rapidly absorbed by the gut of rainbow trout (Table 2), while faecal material, even of fish fed high-salt diets, has a low Na^+ content (Salman and Eddy, 1988*a*).

The teleost kidney absorbs Na^+ from the filtrate, producing urine of low Na^+ content, but its role in ionic and acid–base regulation is minor compared with that of the gills (McDonald and Wood, 1981; Wood, 1988), even in trout fed high-salt diets

(12 % NaCl). Thus, the principal mechanism for Na^+ homeostasis is variation in branchial Na^+ influx and branchial Na^+ efflux rates. Reduction of the Na^+ influx rate (without changing Na^+ efflux rate) is of advantage, since Na^+ gain by the fish is decreased, so aiding excretion of a modest salt load, as discussed below. However, the main mechanism for excretion of large salt loads is to increase Na^+ efflux rate (see below).

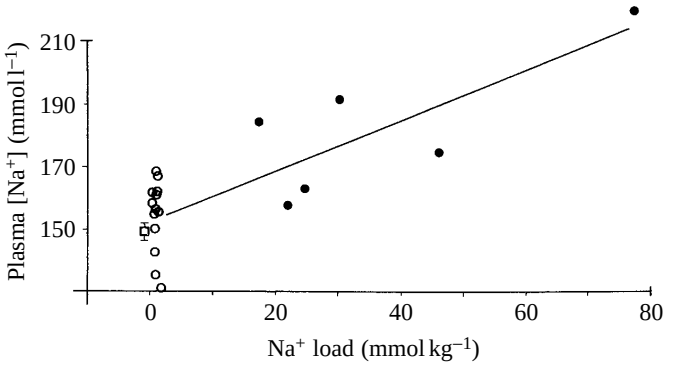

Fig. 1. Relationship between absorbed Na^+ load (mmol kg^{-1}) and blood plasma Na^+ concentration (mmol l^{-1}) for rainbow trout. Open circles, values for trout fed normal salt diet (2.1 % NaCl), Na^+ load of 0.5–1 mmol kg^{-1}. Filled circles, values for fish fed a high-salt diet (12 % NaCl), Na^+ load of over 18 mmol kg^{-1}. Each symbol is for a separate fish. Open square, mean value (± S.E.M.) for unfed fish (N=14), not included in regression analysis. The regression equation (± S.D.), where x is the Na^+ load (μmol kg^{-1}) and y is the blood plasma [Na^+], is: y=0.82(±0.15)x+154(±3.4), r^2=0.63, P=0.003.

Some of the Na^+ regulatory mechanisms used in salt-loaded trout can be considered by following the route of Na^+ from ingestion to excretion. Once dietary salt enters the stomach, it is moistened, and 7 h later most of the ingested Na^+ has been absorbed, as indicated by the low Na^+ levels of trout stomach contents (Table 2). In fish receiving a high salt load, absorption from the gut increased blood Na^+ levels, compared with levels in unfed fish (Fig. 1; Table 2) and, within 1 h, the branchial Na^+ efflux rate had increased and remained high for 7 h, suggesting that excretion of the Na^+ load was not complete at the end of this period. Ammonia excretion rates over 7 h remained unchanged over this period, indicating that ionic regulatory events precede excretion of dietary nitrogen.

Unfed fish showed a higher $^{22}Na^+$ specific activity in the blood and lower activity in tissues compared with salt-loaded fish (Table 2), suggesting that $^{22}Na^+$ activity absorbed from the medium by the gills did not accumulate in tissues but remained in the extracellular compartment. The lower $^{22}Na^+$ specific activity in the blood of salt-loaded fish could be attributed to reduced Na^+ influx rates (Table 1) and dilution of activity by Na^+ absorbed from the gut. The higher $^{22}Na^+$ activity in tissues could possibly be explained by redirection of blood to the tissues and increased Na^+ exchange. Redirection of Na^+ to the tissues may reflect enhanced uptake and distribution of nutrients (e.g. amino acids, glucose) for growth in fish after feeding, but not in unfed fish. This merits further study.

The effects of an ingested salt load on blood plasma Na^+ levels in rainbow trout (Table 2) are similar to those obtained

Table 2. *[Na^+] in blood plasma, tissue and stomach contents for rainbow trout following absorption of a salt load*

	NaCl load		
	0.5–1 mmol kg^{-1} (N=13)	>18 mmol kg^{-1} (N=6)	Unfed (N=14)
Fish mass (g)	49.1±2.6	46.7±4.5	43.6±2.6
[Na^+] ingested (mmol kg^{-1})	1.26±0.25	42.6±11*	–
[Na^+] stomach contents (mmol kg^{-1})	0.41±0.1	6.56±2.2*	–
Na^+ load (mmol kg^{-1})	0.73±0.17	36.2±8.8*	–
Na^+ absorption rate (mmol kg^{-1} h^{-1})	0.1±0.02	5.2±1.2*	–
% Na^+ absorbed	65.4±3.4	85.7±2.3*	–
Blood plasma [Na^+] (mmol l^{-1})	155.1±3.4	183.5±9.8*	149.3±2.6
Blood plasma $^{22}Na^+$ activity (Bq μmol^{-1} Na^+)	2.1±0.5	0.42±0.17*	5.3±1.2**
Muscle [Na^+] (mmol kg^{-1})	13.3±0.84	18.6±2.4	14.1±0.78
Muscle $^{22}Na^+$ activity (Bq μmol^{-1} Na^+)	9.1±1.3	4.3±1.1*	3.7±0.3**
Liver [Na^+] (mmol kg^{-1})	41.8±3.3	44.4±2.9	39.4±1.5
Liver $^{22}Na^+$ activity (Bq μmol^{-1} Na^+)	9.2±1.3	5.57±1.3*	1.12±0.03**
Kidney [Na^+] (mmol kg^{-1})	54.9±3.7	63.1±5.4	51.9±1.7
Kidney $^{22}Na^+$ activity (Bq μmol^{-1} Na^+)	8.5±1.1	4.5±1.0*	1.0±0.33**

Values have been grouped into unfed fish (N=14), fish absorbing a Na^+ load of 0.5–1 mmol kg^{-1} (N=13) and those absorbing more than 18 mmol kg^{-1} (N=6).

Blood plasma, tissue and stomach content Na^+ concentrations were determined 7 h after feeding, as were the $^{22}Na^+$ activities of these tissues. The quantity of food ingested and [Na^+] absorbed (Na^+ load) were calculated as in Materials and methods.

* indicates values significantly different from the 0.5–1 mmol kg^{-1} group at P<0.05.

** indicates values for unfed fish significantly different from the >18 mmol kg^{-1} group at P<0.05.

Values are means ± S.E.M.

for brook trout fed gelatin capsules containing NaCl, although the number of fish and the salt load acquired were not recorded (Phillips, 1944). Blood chloride levels were unchanged in brook trout fed a NaCl load of 15.3 mmol kg^{-1} fish, but ingestion of 46 mmol kg^{-1} fish elevated blood Cl^- levels, which peaked at about 40 % above the normal value 7 h after feeding, although by 24 h, concentrations approached normal levels (Phillips, 1944). Higher levels of NaCl ingestion (77 mmol kg^{-1} fish) led to a prolonged increase in plasma chloride levels, oedema and in many cases death, presumed to be due to a breakdown in tissue osmotic equilibrium (Phillips, 1944).

As in other vertebrates, blood Na^+ levels are normally controlled about an optimum level, and when deviations occur, the appropriate regulatory mechanisms are effected (e.g. Laverty and Wideman, 1989; Laragh, 1986). In freshwater fish, very little is known about these mechanisms, especially the control of branchial Na^+ diffusion, which is believed to be a function of gill permeability and, to a lesser extent, transepithelial potential (Maetz, 1971; Kirschner, 1979; Gonzalez and McDonald, 1992). Thus, branchial Na^+ efflux rates should alter proportionately with variations in blood Na^+ levels, provided that the electrical potential between blood and water remains relatively constant. In fish with a high NaCl load, the elevated blood plasma Na^+ levels are associated with increased branchial Na^+ efflux rates (Table 1; Fig. 1). However, this relationship may be more complex, since fish exposed to acid water showed increased Na^+ efflux rates, associated with decreasing blood Na^+ levels (McDonald, 1983), which could be due to opening of paracellular channels (Wood and McDonald, 1987), together with impairment of transport mechanisms. The effects of H^+ and Ca^{2+} on Na^+ diffusion through branchial transcellular and paracellular routes are discussed by McDonald *et al.* (1983). Na^+ efflux rates are altered in response to acid–base disturbances, even though blood plasma Na^+ levels remain more or less unchanged (Goss and Wood, 1990*b*), and stimulation of Na^+ efflux may result from increased gill perfusion and ventilation (Goss and Wood, 1990*a*; Gonzalez and McDonald, 1992; Nilsson, 1986). The proposals that Na^+ diffusive efflux (as opposed to exchange diffusion) through paracellular passages may be an actively controlled process and the role of differential efflux rates for Na^+ and Cl^- in acid–base regulation are discussed by Goss and Wood (1990*a*). The nature and control of branchial ionic diffusion deserve further research.

Results for fish which had ingested lower salt loads (0.5–1 mmol kg^{-1}; Table 2) are in some ways more interesting than those for fish taking a large Na^+ load. The lower Na^+ load was still a significant increase, about 5 % of the body Na^+ content, but was insufficient to elevate the blood Na^+ concentration determined at the end of the experiment (Fig. 1). Branchial Na^+ efflux rates remained unaltered, compared with those of unfed fish, and Na^+ balance appeared to be effected through suppression of the Na^+ influx rate throughout the 7 h experiment (the high Na^+ load group also showed suppressed Na^+ influx rates; Table 1). A possible explanation is that a small increase in plasma Na^+ (or Cl^-) concentration, as suggested in Fig. 1 and Table 2, exerted an inhibitory influence on the Na^+ (or Cl^-) uptake process in the branchial epithelial cells (Avella and Bornancin, 1989; Wright, 1991). One possibility is an increase in intracellular Na^+ concentration, which would tend to depolarise the cell and decrease the electrochemical gradient for Na^+ entry from the water. Another is the action of hormones involved in volume regulation (e.g. the renin–angiotensin system, atrial natriuretic peptides; Olson, 1992); these areas require further study.

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