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INCREASE IN EFFLUX OF INORGANIC PHOSPHATE DURING ELECTRICAL ACTIVITY IN SMALL NON-MYELINATED NERVE FIBRES

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SUMMARY

1. The movements of labelled phosphate were measured in garfish olfactory and in rabbit vagus nerves at rest and during activity.

2. In garfish olfactory nerve kept in solutions with 120 mM-sodium and 0·2 mM-phosphate the fractional loss of ^{32}P was $9{\cdot}82 \times 10^{-4}$ min^{-1}. Lowering the sodium concentration of the washing fluid decreased the efflux; lowering the phosphate produced a transient increase with subsequent return towards the efflux in 0·2 mM-phosphate.

3. Stimulation at 0·50 sec produced an extra fractional loss of 12×10^{-6} impulse^{-1}. At 1/sec the effect was larger; at 5/sec it was about the same as at 0·5/sec.

4. After stimulation the effect of activity disappeared exponentially with a time constant of 4·4 min.

5. Lowering the sodium decreased the extra efflux with stimulation, whereas changing the phosphate concentration did not much affect the extra efflux.

6. In rabbit vagus nerve kept in 154 mM-sodium and 0·2 M-phosphate the fractional loss of ^{32}P was $4{\cdot}91 \times 10^{-4}$ min^{-1}. Lowering the sodium or the phosphate decreased the resting efflux.

7. Stimulation of the vagus nerve at 15/sec produced an extra fractional loss of $0{\cdot}87 \times 10^{-6}$ impulse^{-1}.

8. The extra efflux with stimulation seems to result predominantly from an increase in intracellular inorganic phosphate resulting from increased break-down of ATP after activity.

INTRODUCTION

In small non-myelinated nerve fibres a short period of electrical activity gives rise to a series of metabolic events, probably triggered by a substantial increase in the intracellular concentration of sodium ions (Rang & Ritchie, 1968) which causes the Na-K pump to work faster. As a result, ATP is broken down at an increased rate so that its intracellular concentration falls (Greengard & Straub, 1959; Chmouliovsky, Schorderet & Straub, 1969), the concentration of inorganic phosphate increases (Baker, 1965; Chmouliovsky *et al.* 1969; McDougal & Osborn, 1976), and there is an increase in the heat production and oxygen consumption of the nerve (see Ritchie, 1973).

There should also be an increased efflux of orthophosphate with electrical activity. For recent experiments have shown that there is a steady efflux of phosphate from nerve that is mediated by a sodium-dependent transport system (Anner, Ferrero, Jirounek, Jones, Salamin & Straub, 1976), which should transiently increase as a result both of the increase in intracellular sodium and the increase in inorganic phosphate. Indeed, such an increase has already been reported after *long* periods of stimulation in myelinated nerve (Mullins, 1954; Abood, Koketsu & Miyamoto, 1962). Unfortunately, in these experiments the collection of the phosphate efflux was not restricted to the region of conducted action potentials, but involved also the region underneath the stimulating electrodes, which may have led to artifactual transmembranal ion fluxes (see Keynes & Ritchie, 1965).

We have therefore examined the phosphate fluxes in nerve using a method that excludes such an artifact, and using a more suitable preparation. Very small non-myelinated nerve fibres, such as those of the olfactory nerve of the pike (see Howarth, Keynes, Ritchie & von Muralt, 1975) or garfish (Easton, 1971), with their much greater area of axonal membrane per unit weight, might be expected to give more pronounced effects on stimulation so that *short* periods of stimulation could be used with a consequent better temporal resolution of the activity-induced increase in phosphate efflux. For comparison rabbit vagus nerves were used in some experiments.

METHODS

The technique used was essentially similar to that of Ritchie & Straub (1975). Olfactory nerves of garfish (or in a few experiments rabbit desheathed vagus nerves) were isolated and soaked for 2 hr in [^{32}P]orthophosphate-labelled garfish Ringer with 0·2 mM total phosphate at room temperature (22 °C). The nerves were then mounted in the apparatus described by Ritchie & Straub (1975), and perfused with label-free solutions at a constant rate (0·15 or 0·3 ml/min) by a motor-driven syringe or peristaltic pump. The influx into the apparatus was divided into two parts. About a quarter was directed over the portion of the nerve that lay underneath the stimulating electrodes and was discarded. The remainder perfused the rest of the nerve and was collected and counted. For stimulation supramaximal shocks of 1 msec duration were used. The conducted action potential was monitored.

The radioactivity in the effluent, which was collected for periods of 2 or 5 min in scintillation vials to which scintillant was subsequently added, was determined in a scintillation counter, correction being made for quenching by the external channels ratio method. At the end of the experiments the nerve was removed, homogenized in 1 ml. distilled water and centrifuged for 10 min at 3000 *g*. Aliquots of the supernatant were used for the determination of the radioactivity and the total phosphate in the water soluble fraction of the nerve. The counts in the individual vials were recorded on punched tape and fed into a PDP 11/70 computer, programmed to calculate the rate constant of loss (i.e. fraction loss per minute) of the labelled phosphate.

The garfish nerve bathing solution had the following composition (mM): NaCl, 120; KCl, 2·5; $CaCl_2$, 0·9; $MgCl_2$, 0·5; sucrose, 60; dextrose, 24; Tris(hydroxymethyl)amino methane, pH 7·2, 10. Sodium orthophosphate was present in the concentrations indicated in the text. In some experiments morpholinopropionyl sulphonate buffer (10 mM) was used. In all experiments the pH was adjusted to 7·2. For low sodium solutions the sodium was replaced by equimolar amounts of Tris(hydroxymethyl)amino methane. In the rabbit experiments the solution described by Anner *et al.* (1976) was used, which contained (mM): NaCl, 154; KCl, 5·6; $CaCl_2$, 0·9; $MgCl_2$, 0·6; Tris, 10; glucose, 5; and sodium orthophosphate, in the concentrations indicated in the text.

The [^{32}P]phosphoric acid (carrier free) was obtained from New England Nuclear, Boston. It was used for 3 weeks at most.

Whenever possible mean values ± S.E. are given.

RESULTS

Garfish experiments

Efflux of phosphate in resting nerve

When perfusion with label-free solution was started, the fractional loss of phosphate per minute (rate constant) decreased rapidly during the first 60 min. Later the rate constant remained fairly steady with time and it was not until then that the effects of different solutions, or the effects of stimulation, were studied.

Measurements at different times during the experiments (fairly evenly distributed from 1 to 6 hr after beginning the washout) gave a mean resting efflux rate constant of $9{\cdot}82 \pm 0{\cdot}50 \times 10^{-4}$ min^{-1} in a bathing solution containing 0·2 mM-phosphate and 120 mM-sodium. This value was obtained by taking the mean of six individual efflux samples in each test in near steady state conditions and using twenty-six such means from ten different nerves.

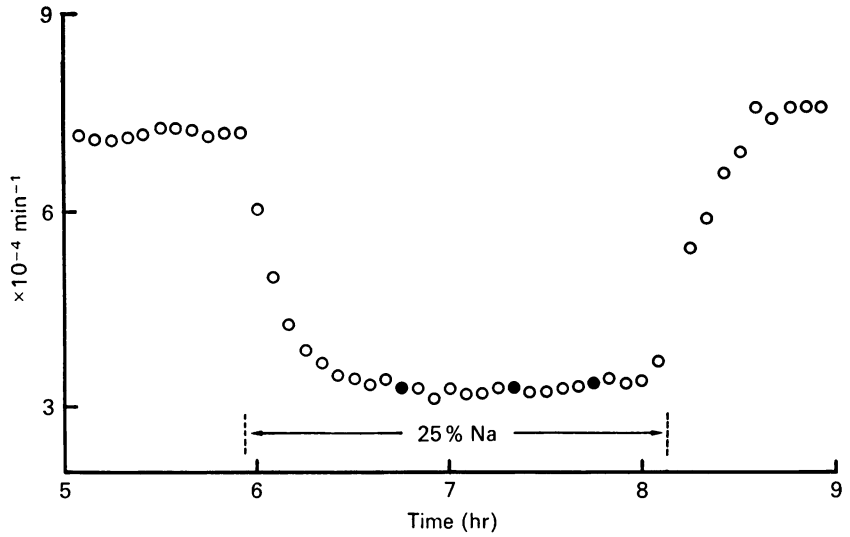

Fig. 1. Effect of changing sodium concentration on phosphate efflux in garfish olfactory nerve. The ordinate indicates the fractional loss of radiophosphate in the resting preparation (open circles), and during stimulation at 0·5/sec for 2·5, 1, and 5 min (filled circles) in a low-sodium (30 mM) solution. The abscissa is the time after beginning of washing. The sodium concentration of the washing fluid was 120 mM unless otherwise indicated. Temperature 20·4 °C.

Effect of external sodium

When the sodium concentration of the perfusing fluid was lowered by replacing sodium with Tris, the efflux of phosphate decreased rapidly (Fig. 1); a new steady state was reached within a few minutes. Returning the nerve to normal sodium containing bathing solution restored the efflux to the initial, or sometimes to a slightly higher, value. The results of measurements at different sodium concentrations are summarized in Table 1.

When sodium was replaced by lithium, the efflux also decreased.

These effects of low-sodium solutions on garfish nerve are similar to those found

in rabbit vagus nerve (Ferrero, Jirounek, Rouiller, Salamin & Straub, 1976; Straub, Ferrero, Jirounek, Rouiller & Salamin, 1977).

Effect of external phosphate

In nerves equilibrated in 0·2 mM-external phosphate an increase in external phosphate produced a small decrease in labelled efflux. On the other hand, a decrease in external phosphate to 0·02 or 0·002 mM produced a rapid increase in efflux. However,

TABLE 1. Effect of changing the external sodium concentration both on the normalized phosphate efflux at rest (K_r) and the corresponding extra efflux with stimulation (K_s) in garfish olfactory nerve

Na (mM)	120		90		60		30		0	
	K_r	K_s	K_r	K_s	K_r	K_s	K_r	K_s	K_r	K_s
	1·00	1·00	0·49	0·31	0·42	0·38	—	—	—	—
	1·00	1·00	—	—	0·50	0·53	0·40	0·14	0·40	0·07
	1·00	1·00	—	—	0·52	0·33	0·47	0·11	—	—
	1·00	1·00	—	—	0·69	0·48	—	—	0·80	0·02
Mean values	1·00	1·00	0·49	0·31	0·53	0·43	0·43	0·12	0·60	0·02

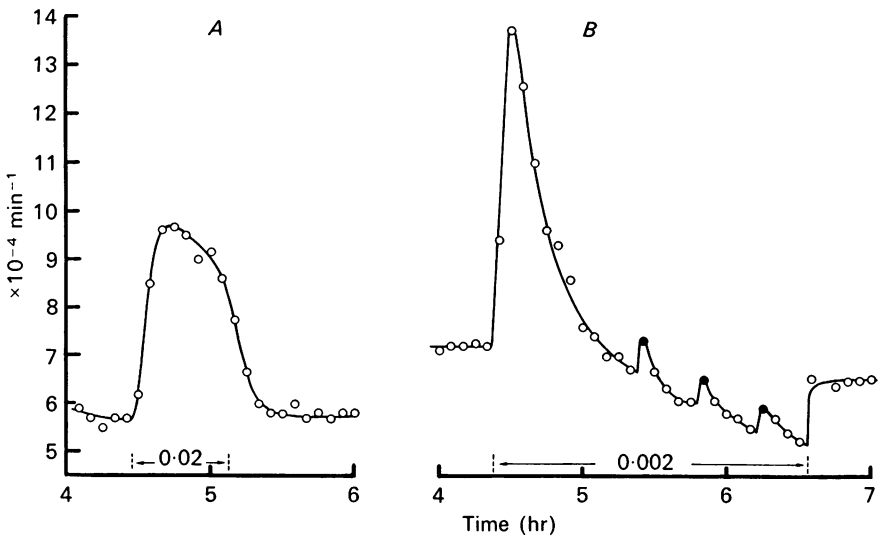

Fig. 2. Effect of changing phosphate concentration on phosphate efflux in garfish olfactory nerve. The ordinate indicates the fractional loss of radiophosphate in the resting preparation (open circles) and during the stimulation at 0·5/sec for 2·5 min (filled circles). The abscissa is the time after beginning of washing. The phosphate concentration of the washing fluid was 0·2 mM unless otherwise indicated. Temperature: *A*, 23·2 °C; *B*, 22·7 °C.

this increase was transient and was followed by a slow fall (Fig. 2), so that after some time the efflux in these low phosphate solutions was almost equal to, or even lower than (Fig. 2) the efflux in 0·2 mM-phosphate. In this latter case, i.e. when the efflux was lower than in 0·2 mM-phosphate, addition of phosphate *increased* the efflux (Fig. 2*B*). This increase resembles the trans-effects seen in rabbit vagus (Ferrero *et al.* (1976) and in vesicles from rat renal brush border membranes (Hoffman, Thees & Kinne, 1976).

The results from measurements in near steady-state conditions, summarized in Table 2, show that the resting efflux was largest in 0·2 mM-phosphate, and smaller at both 2 and 0·02 mM-phosphate.

In sodium-free solution a decrease in phosphate from 0·2 to 0·02 mM also caused a transient increase in efflux. Raising the phosphate from 0·2 to 2 mM decreased the efflux; but when the nerve was then restored to 0·2 mM-phosphate, the efflux reached a value that was much larger than before the addition of the 2 mM-phosphate solution (two experiments).

TABLE 2. Effect of changing the external phosphate concentration both on the normalized phosphate efflux at rest (K_r) and on the corresponding extra efflux with stimulation (K_s) in garfish olfactory nerve

Phosphate (mM)	2·0		0·2		0·02		0·002	
	K_r	K_s	K_r	K_s	K_r	K_s	K_r	K_s
	0·69	0·73	1·00	1·00	0·82	0·88	0·80	0·60
	0·91	1·17	1·00	1·00	0·52	0·91	—	—
	0·61	0·29	1·00	1·00	—	—	0·41	0·60
	—	—	1·00	1·00	0·78	0·90	—	—
Mean values	0·74	0·73	1·00	1·00	0·71	0·90	0·61	0·60

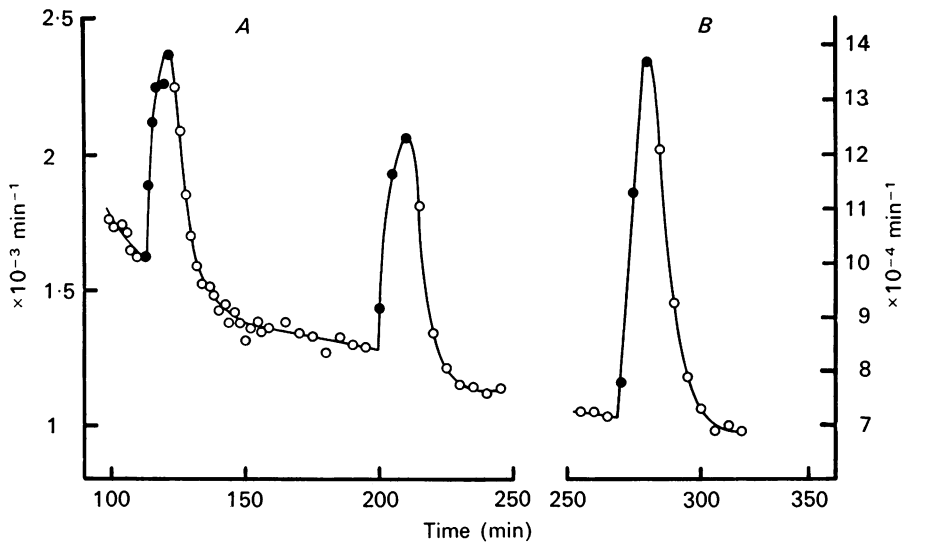

Fig. 3. Effect of stimulation of phosphate efflux in garfish olfactory nerve. The ordinate indicates the fractional loss of radiophosphate in the resting preparation (open circles), and during stimulation at 0·5/sec for 11 and 13 min (filled circles). The abscissa is the time after beginning of washing. Temperature: *A*, 18·5 °C; *B*, 19·2 °C.

Efflux of phosphate in stimulated nerve

Electrical stimulation led to a prompt increase in phosphate efflux. Fig. 3, for example, shows how the efflux increased during, and immediately after, stimulation at 0·5 shocks/sec. In ten different nerves stimulated for 2·5 to 14 min at 0·5 shocks/sec, the mean fractional loss per impulse was $12{\cdot}00 \pm 1{\cdot}33 \times 10^{-6}$. The fractional loss per impulse did not seem to depend much on the duration of stimulation; for a

stimulation period of 2·5 min it was $10{\cdot}3 \pm 0{\cdot}8 \times 10^{-6}$ impulse^{-1} ($n = 7$), for 4–5 min $8{\cdot}9 \pm 1{\cdot}0 \times 10^{-6}$ impulse^{-1} ($n = 4$), and for 11–14 min $14{\cdot}3 \pm 2{\cdot}2 \times 10^{-6}$ impulse^{-1} ($n = 12$).

With stimulation at 1/sec for 50 or 100 sec the fractional loss was $23{\cdot}09 \pm 4{\cdot}22 \times 10^{-6}$ impulse^{-1} ($n = 4$); with 5/sec (for 10 or 40 sec) it was $10{\cdot}51 \pm 3{\cdot}79 \times 10^{-6}$ impulse^{-1} ($n = 3$). Thus, the frequency of 0·5/sec was probably too low and the frequency of 5/sec too high for a maximal effect.

At the end of stimulation at 0·5/sec or 1/sec (1–13 min, $n = 5$) the increased efflux returned to the resting level with a mean time constant of $4{\cdot}4 \pm 0{\cdot}4$ min (2 or 5 min sampling periods).

Often the resting efflux after stimulation remained at a lower level than before activity.

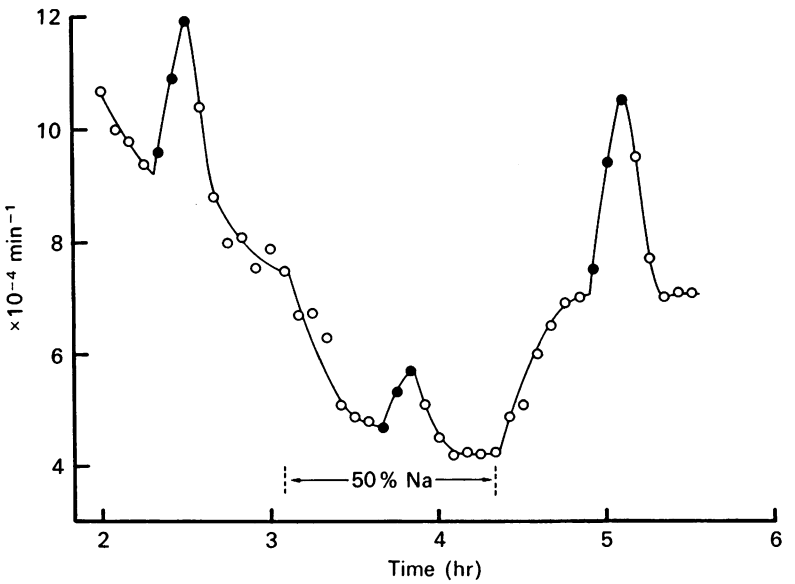

Fig. 4. Effect of changing the sodium concentration on phosphate efflux at rest and during stimulation. The ordinate indicates fractional loss of radiophosphate (open circles) and during stimulation at 0·5/sec for 13 min (filled circles). The abscissa is the time after beginning of washing. Sodium concentration of washing fluid was 120 mM unless otherwise indicated. Temperature: 18·8 °C.

Effect of external sodium

When the sodium concentration in the perfusing fluid was reduced, the fractional loss per impulse decreased (Fig. 4). The effects, obtained at different sodium concentrations, are summarized in Table 1. The observation that in sodium-free solution, when the action potential was absent, stimulation did not increase the efflux demonstrates that the stimulating current *per se* did not affect the phosphate efflux: the effluent of the part under the stimulating electrodes was thus effectively separated from the remaining effluent.

When the preparation was restored to normal sodium-containing medium the fractional loss was larger (158 ± 45 %, five experiments) than before the application of the low sodium solution.

In one experiment, in which sodium was replaced by lithium, the preparation became rapidly inexcitable and the effect of stimulation could not be tested.

Effect of external phosphate

The effects of stimulation at different external phosphate concentrations are summarized in Table 2. These measurements, taken when a quasi-steady resting efflux was reached, show that the stimulated efflux did not much depend on the external phosphate concentration. This agrees with the finding that the steady state resting efflux was also fairly independent of the external phosphate concentration.

Phosphate content of nerves

In five nerves in which the total water soluble phosphate was measured at the end of the experiment a mean value of $0{\cdot}0174 \pm 0{\cdot}0040$ mole.kg wet^{-1} was found. This agrees quite well with measurements in garfish olfactory nerves of McDougal & Osborn (1976) from which a total aqueous intracellular phosphate content of 0·0135 mole.kg wet^{-1} can be calculated. The lower values of these authors may be due to their using phosphate-free solutions for incubation; however, in two of their experiments in which *in vivo* determinations for ATP and inorganic phosphate were made, these values were not much different from the *in vitro* values of these compounds.

Rabbit experiments

Resting efflux

The mean resting rate constant of phosphate efflux in solutions with 154 mM-sodium and 0·2 mM-phosphate was $4{\cdot}91 \pm 0{\cdot}55 \times 10^{-4}$ min^{-1}. This value was obtained by taking the mean of 6 individual samples and using twelve such means from four different nerves.

Lowering the sodium concentration decreased the efflux, which recovered when the nerve was restored to normal sodium concentration. The effects of changing the sodium concentration at room temperature were thus similar to those found at 37 °C (see Anner *et al.* 1976; Ferrero *et al.* 1976).

Similarly the effects of changing the external phosphate concentration resembled those found at 37 °C (see Ferrero *et al.* 1976): increasing the phosphate concentration from 0·2 to 2 mM increased the efflux by a factor of 1·23 (mean of three nerves), lowering the phosphate to 0·02 lowered the normalized efflux to 0·92 ($n = 2$), and with 0·002 it was 0·49 ($n = 1$).

Effect of activity

Stimulation of the nerve produced an increased loss of radiophosphate. The mean extra fractional loss per impulse was $0{\cdot}87 \pm 0{\cdot}11 \times 10^{-6}$. This value is based on 12 measurements in four nerves, stimulated with 150 shocks at 30/sec or 1/sec and bathed in solutions with 154 mM-sodium and 0·2 mM-phosphate. The extra fractional loss per impulse with stimulation at 30/sec was $0{\cdot}98 \pm 0{\cdot}22 \times 10^{-6}$ ($n = 9$); with 1/sec it was $0{\cdot}50 \pm 0{\cdot}14 \times 10^{-6}$ ($n = 3$).

The effect of stimulation was smaller than in the garfish. It was therefore more difficult to measure the time course of the recovery towards the resting efflux. In all experiments recovery was complete within 5 min, so that the time constant was probably around 2 min.

Lowering the sodium to 60 mM decreased the extra loss by 80% (one experiment). Changing the external phosphate concentration to 2 mM increased the extra loss by a factor of $1{\cdot}98 \pm 0{\cdot}83$ ($n = 3$), lowering the phosphate to 0·02 mM lowered the normalized loss to 0·73 ($n = 2$).

DISCUSSION

Garfish

Phosphate turnover and labelling

During incubation in radiophosphate solution the water soluble phosphates of the garfish olfactory nerve are almost certainly equally labelled. The mean efflux rate constant for phosphate is $9{\cdot}82 \times 10^{-4}$ min^{-1}, which corresponds to an efflux of 0·0169 mmole.kg wet^{-1}.min^{-1} and, in steady-state conditions, to an efflux of the same size. The turnover of the phosphate can be estimated from the potassium fluxes in garfish nerves. The rate constant of potassium efflux is 0·0108 min^{-1} (Ritchie & Straub, 1975) so that, with an intracellular potassium content of 73·7 m-mole.kg wet^{-1}, the potassium efflux becomes 0·80 m-mole.kg wet^{-1}.min^{-1}. Taking the potassium loss to be equal to the potassium uptake, and to be equal to the sodium extrusion, and taking a ratio of one ATP molecule used for every three sodium ions extruded, one calculates the phosphate turnover to be at least 0·265 m-mole.kg wet^{-1}.min^{-1}. The phosphate turnover is thus more than 16 times faster than the influx or efflux of phosphate, and a 2 hr period of incubation is certainly sufficient for equal labelling.

Effect of stimulation

The mean extra fractional loss of 12×10^{-6} impulse^{-1} corresponds to an extra phosphate efflux per impulse of $2{\cdot}06 \times 10^{-4}$ m-mole.kg wet^{-1}, i.e. an efflux of 3·2 f-mole.cm^{-2}, which is about 70 times smaller than the potassium efflux associated with an action potential.

Chromatography of the efflux has shown that in resting vagus nerve the radiophosphate is almost entirely found in the inorganic fraction (Anner *et al.* 1976). During activity the inorganic phosphate in the nerve increases (Baker, 1965; Chmouliovsky *et al.* 1969; McDougal & Osborn, 1976), and ATP and CrP are lowered: there is thus no reason to assume that the extra efflux during activity is not mostly released from the inorganic pool.

This conclusion is supported by observations in myelinated nerve, where Silinsky & Hubbard (1973) found release of ATP at the terminals but not along the nerve proper.

Cause of increased efflux

The extra fractional loss during activity could be caused either by the increase in intracellular sodium or the increase in intracellular phosphate, or by both. Consider the increase in intracellular sodium first. The loss of intracellular potassium during activity in garfish olfactory nerve is at least 0·015 m-mole.kg wet^{-1}.impulse^{-1} (see Ritchie & Straub, 1975); it is probably accompanied by an approximately equal increase in sodium. This can be compared to the value by which the phosphate efflux is increased after activity. The increased phosphate efflux disappears almost expo-

nentially (with a time constant of about 4·4 min), and it is measured by summing the increase in efflux over about 15–20 min, which corresponds to about four time constants. If the total extra fractional loss per impulse is Q, it follows that $Q = Y_0\tau/(1-e^{-4})$ where Y_0 is the initial increase of the rate constant (min^{-1}). With $\tau = 4{\cdot}4$ min, $Q = 12 \times 10^{-6}$ $impulse^{-1}$, Y_0 becomes $2{\cdot}7 \times 10^{-6}$ min^{-1}. From Table 1 the sodium-dependent resting efflux is $0{\cdot}40 \times 9{\cdot}82 \times 10^{-4}$ min^{-1}, and it is driven by an intracellular sodium concentration of 12·7 m-mole.kg wet^{-1} (pike olfactory nerve, Howarth *et al.* 1975). If the sodium-dependent phosphate efflux were directly proportional to the internal sodium concentration, the increase in internal sodium after one impulse (0·015 m-mole/kg wet) would cause an initial increase in phosphate efflux (Y_0) of $0{\cdot}46 \times 10^{-6}$ min^{-1} which is 7 times smaller than the observed effect. Even if some error was made in the estimation of the change in internal sodium after activity, or if the relation between internal sodium and phosphate efflux is not linear, it would still be difficult to account for the increase of the phosphate efflux by the change in internal sodium.

The increased efflux of inorganic phosphate thus seems unlikely to result only from the raised intracellular sodium concentration. The alternative is that it results from the increase in the intracellular inorganic phosphate concentration. McDougal & Osborn (1976) found after 120 sec stimulation at 1/sec a 15% increase in inorganic phosphate, i.e. the increase per impulse was 0·125%. This would produce a value for Y_0 of $1{\cdot}2 \times 10^{-6}$ min^{-1}, which is of the same order as the value observed experimentally.

Although the increased intracellular concentration of inorganic phosphate thus seems adequate to account for the extra efflux of orthophosphate with stimulation, it might still be possible that there might be a contribution from the increased membrane potential during the post-tetanic hyperpolarization that follows activity. This possibility, however, can be excluded. Using plausible assumptions for the equilibrium potential for the inorganic phosphate, we were unable to account by post-tetanic hyperpolarization for more than about 3% of the observed efflux, unless an increase in the permeability to inorganic phosphate occurred, a possibility for which we have no independent evidence.

Rabbit

In rabbit vagus too, the increase in inorganic phosphate could account for the extra phosphate loss associated with activity.

The extra fractional release of phosphate is $0{\cdot}98 \times 10^{-6}$ $impulse^{-1}$ which, with a time constant of 2 min gives a value for Y_0 of $0{\cdot}49 \times 10^{-6}$ min^{-1}. From the values of Keynes & Ritchie (1965) for potassium release during activity, the intracellular potassium and sodium concentrations given by these authors, and a sodium-dependent resting efflux of 70% (Ferrero *et al.* 1976), the increase in internal sodium after one impulse can be calculated to account for an increase in phosphate efflux of $0{\cdot}134 \times 10^{-6}$ min^{-1}, i.e. for about 27% of the observed effect (again on the assumption that the efflux is proportional to the sodium concentration).

The effect of internal phosphate can be estimated from the results of Chmouliovsky *et al.* (1969): after 750 impulses the inorganic phosphate increased from 1·6 to 2·7 mmole.kg wet^{-1} which, on the basis of direct proportionality, would increase

the phosphate efflux by $0{\cdot}53 \times 10^{-6}$ min^{-1}, sufficient to account for the observed effect.

For the post-tetanic hyperpolarization the effect is again small, only about 1% of the observed loss could be accounted for by the post-tetanic hyperpolarization.

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