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THE INFLUX OF ORTHOPHOSPHATE INTO SQUID GIANT AXONS

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SUMMARY

1. The influx of ^{32}P, applied externally as orthophosphate, into the axoplasm of squid giant axons has been studied.
2. An average orthophosphate influx of 20·9 f-mole/cm^2.sec is obtained if the ^{32}P found in the axoplasm is assumed to be indicative of orthophosphate which has crossed the axolemma.
3. The influx does not show very much dependence on external orthophosphate concentration in the range 0·02–0·5 mM.
4. The influx is reduced by cyanide, 2,4-dinitrophenol, ouabain and by the absence of external potassium.
5. The ^{32}P appears to arrive in the axoplasm as orthophosphate.
6. It is concluded that there is an inward movement of orthophosphate into the axons which is mediated by an active transport process and that this may have some connexion with the active transport of sodium and potassium.

INTRODUCTION

The measurement of the entry of radioactively labelled orthophosphate into cells is often complicated by a tendency for much of the activity to become incorporated into a phosphate fraction which is situated at the cell membrane and which may be extracellular (e.g. Causey & Harris, 1951). This difficulty can be overcome to some extent by investigation of the uptake of labelled phosphate entering the cell into intracellular compounds like ATP (e.g. Fleckenstein, Janke & Davies, 1956). This approach is, however, limited by two factors. First, it must be assumed that the incorporation of labelled orthophosphate into ATP is fast compared with the rate at which it enters the cell. Secondly, it is not possible to study the effect of metabolic inhibitors on the phosphate entry, since the inhibitors may also reduce the rate at which orthophosphate is incorporated into intracellular compounds like ATP.

These uncertainties can be overcome if the squid giant axon is used for

the study of phosphate uptake. If an axon is exposed for a time to isotopically labelled orthophosphate and then briefly washed in an inactive medium, it is possible to extrude the axoplasm containing the labelled phosphate which has penetrated and to remove it from the axolemma and various cells and structures outside the axon. Baker, Hodgkin & Shaw (1962) have shown that the axolemma remains largely intact after careful extrusion of the axoplasm and it seems very likely that the radioactive phosphate found in the extruded axoplasm has penetrated across the axolemma, any phosphate which has not penetrated being left behind with the axolemma and its surroundings.

In the work described in this paper, this method has been used in the study of various aspects of phosphate entry into squid giant axons, including the effects of inhibitors on the entry. A preliminary account of some of this work has been presented to the Physiological Society (Caldwell & Lowe, 1966).

METHODS

Material. Pairs of the hindmost giant axon were dissected from specimens of the squid *Loligo forbesi*. The axons were used uncleaned, but normally about half the axon surface was comparatively free of other tissues. The ^{32}P used was supplied as orthophosphate by the UKAEA Radiochemical Centre, Amersham (Code No. PBS. 1).

General experimental procedure. The axons were normally bathed in an artificial sea water having the following composition (mM): NaCl, 460; $MgCl_2$, 55; $CaCl_2$, 11; KCl, 10·4; $NaHCO_3$, 2·5. The orthophosphate concentration in the artificial sea water was usually 0·1 mM, but this was sometimes varied. Normally one axon of a pair was used as a control and immersed in artificial sea water containing 0·1 mM [^{32}P]orthophosphate, while the other was exposed to a particular set of experimental conditions in another artificial sea water containing [^{32}P]orthophosphate. After a given time, the two axons were removed and washed several times in non-active artificial sea water. The axon diameter was measured with a microscope with a micrometer eyepiece, and a centimetre of the axon, about one centimetre away from the proximal end, was cleaned of adhering tissue. After a final wash the axon was cut in the cleaned region and the axoplasm was extruded onto a weighed planchet, great care being taken to allow only a small part of the cleaned part of the axon to come into contact with the planchet. About 1 cm of the axoplasm situated at the distal end of the axon was left unextruded. The weight of axoplasm extruded was then recorded, the axoplasm dispersed with a small quantity of distilled water and finally dried. The radioactivity in each sample of axoplasm was measured with an end-window Geiger tube. The radioactivity in a standard volume of artificial sea water was also measured. The phosphate influx in f-mole/cm^2.sec (i.e. 10^{-15} mole/cm^2.sec) was then calculated from the relation

$$\text{Influx (in f-mole/cm}^2\text{.sec)} = \frac{M_s C_a d\rho}{4 C_s W t} \times 10^{15},$$

where M_s is the number of moles of phosphorus in a given volume of the artificial sea water and C_s is the number of counts/unit time found for this volume. C_a is the number of counts/unit time for the axoplasm sample, W is its weight (g) and t is the

time (sec) for which the axon was immersed in the radioactive artificial sea water. d is the axon diameter (cm) and ρ is the density of the axoplasm which was assumed to have a value of 1·0.

RESULTS

The size of the orthophosphate influx

A total of thirty measurements of the influx of labelled orthophosphate into unpoisoned axons bathed in artificial sea water containing 0·1 mM orthophosphate was carried out. The pH of these sea waters was in the range 6·9–8·2 and the temperature in the range 17–21° C. A mean value of 20·9 f-mole/cm^2.sec (S.D. = 9·6) was obtained for the influx.

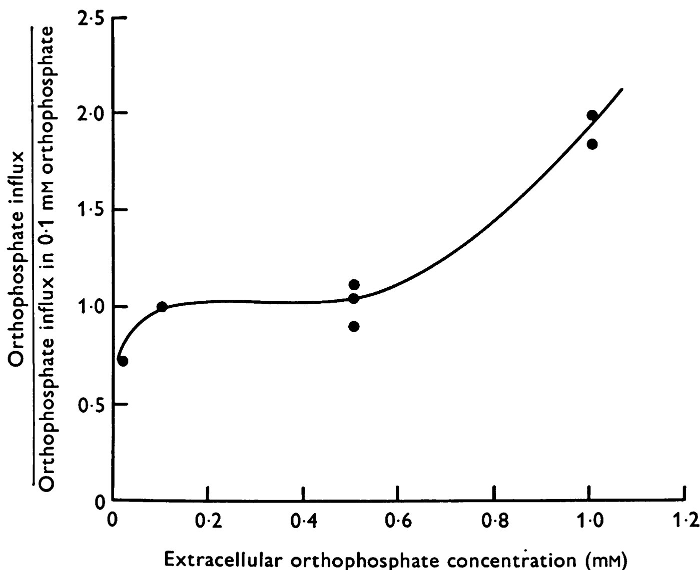

Fig. 1. Variation of the orthophosphate influx into squid giant axons with extracellular orthophosphate concentration. The influx into the control axon of each pair of axons used was measured in artificial sea water containing 0·1 mM orthophosphate. The influx into the other axon of each pair was measured at a different extracellular orthophosphate concentration, the value obtained being expressed in terms of that obtained for the control axon. Temperature = 18–19° C. pH = 7·3–8·2.

Three preliminary experiments were carried out. In the first the orthophosphate influxes in artificial sea waters of pH 6·8 and 7·9 were found to be roughly the same while in the second the presence of 1 mM choline in the artificial sea water was found to have little effect on the influx. In the third experiment electrical stimulation at 50 shocks/sec increased the influx by a factor of only 1·2.

The effect of external orthophosphate concentration on the orthophosphate influx

The results of experiments in which one axon of each pair was bathed in artificial sea water containing 0·1 mM orthophosphate and the other in artificial sea water containing a different orthophosphate concentration are summarized in Fig. 1. There appears to be little variation in the absolute size of the influx in the concentration range 0·02–0·5 mM, but it tends to increase when the external concentration of orthophosphate is raised to 1 mM.

The effects of metabolic inhibitors on the orthophosphate influx

The effects of the metabolic inhibitors cyanide and 2,4-dinitrophenol on the orthophosphate influx are summarized in Table 1. In each case the inhibitor was added to the artificial sea water used for one axon of each pair. Most of the experiments were done with artificial sea water containing 0·1 mM orthophosphate but in two experiments this concentration was 0·5 mM. The pH of the artificial sea waters was low (6·7–7·2) in the experiments in which 2,4-dinitrophenol was used, since this inhibitor has been found to have greater effects on phosphorylation and sodium transport in squid axons in this pH range (Caldwell, 1960; Caldwell, Hodgkin, Keynes & Shaw, 1960*a*, *b*). The results given in Table 1 show that in each experiment the orthophosphate influx was greatly reduced in the axon exposed to cyanide or dinitrophenol.

In the previous section it was shown that the phosphate influx did not change very much over a wide range of extracellular orthophosphate concentrations. This means that an increase in the amount of inactive orthophosphate in the external medium, resulting in a decrease in the specific activity of the orthophosphate, would lower the amount of labelled orthophosphate entering the axons. Metabolic inhibitors might therefore decrease the entry of labelled orthophosphate by causing a leakage of unlabelled orthophosphate from the axons so that the specific activity in the medium was reduced, rather than by affecting the orthophosphate uptake mechanism. Some measurements were carried out to check the amount of orthophosphate leaking from the axons during exposure to metabolic inhibitors. This was found to be much too small to account for the decreases observed in the influx of labelled orthophosphate, particularly if the ganglion had been removed from the axons, as was the case in most of the experiments.

The effects of ouabain on the orthophosphate influx

The glycoside ouabain is well known as an inhibitor of active transport mechanisms, in particular those involved in the transport of sodium and

potassium (see, for example, Glynn, 1964). Its effects on the orthophosphate influx into the axons were examined and the results are summarized in Table 2. These results show that the presence of 10^{-4} M ouabain in the

TABLE 1. The effect of metabolic inhibitors on the influx of orthophosphate into squid giant axons

Extracellular ortho-phosphate concentration	Inhibitor	Influxes (f-mole/ cm².sec) Control	In inhibitor	Influx in inhibitor / Control influx
0·1 mM	2 mM cyanide	5·0	1·5	0·33
		20·4	2·9	0·14
		4·1	2·8	0·68
		47·6	6·7	0·14
		19·4	3·6	0·19
		8·7	3·7	0·43
		36·1	6·4	0·18
	0·4 mM dinitrophenol	22·4	8·0	0·36
0·5 mM	2 mM cyanide	29·8	14·7	0·49
	0·4 mM dinitrophenol	30·7	21·7	0·71
	Mean	22·4	7·2	0·36

Temperature = 18–21° C. The extracellular pH was 7·5–8·0 in the experiments in which cyanide was used and 6·7–7·2 in the experiments in which 2,4-dinitrophenol was used.

TABLE 2. The effect of ouabain on the influx of orthophosphate into squid giant axons

	Influxes (f-mole/cm².sec) Control	In artificial sea water containing 10^{-4} M ouabain	Influx in ouabain / Control influx
	7·5	7·9	1·05
	21·7	9·5	0·44
	22·6	11·0	0·49
	21·5	7·5	0·35
	28·7	8·7	0·30
	9·7	4·4	0·45
Mean	18·6	8·2	0·51

Extracellular orthophosphate concentration = 0·1 mM. Temperature = 18–21° C. Extracellular pH = 7·9–8·1.

artificial sea water brings about a decrease in that influx, this decrease having a high degree of significance ($P < 0{\cdot}01$) even if the single experiment in which no change was observed is taken into account. It should be noted

that the metabolic condition of the control axon in the experiment in which no change was observed may have been less good than usual since the absolute value of the orthophosphate influx was lower than normal.

The effects of the removal of potassium from the artificial sea water on the orthophosphate influx

The removal of potassium from the artificial sea water used as a bathing medium reduces the efflux of sodium from squid giant axons (Hodgkin & Keynes, 1955*a*; Caldwell *et al.* 1960*a*, *b*). The effects of potassium removal on the orthophosphate influx were investigated and the results are summarized in Table 3. These results show that potassium removal causes some reduction of the phosphate influx, but that this reduction is less marked than that brought about by metabolic inhibitors and ouabain.

TABLE 3. The effect of extracellular potassium concentration on the influx of orthophosphate into squid giant axons

Influxes (f-mole/cm^2.sec)

	Control (10 mM-K$^+$)	In artificial sea water containing 0 mM-K$^+$	$\frac{\text{Influx in 0 mM-K}^+}{\text{Influx in 10 mM-K}^+}$
	19·7	15·4	0·78
	21·4	12·4	0·58
	29·0	20·7	0·71
	21·3	15·6	0·73
	19·9	15·9	0·80
Mean	22·3	16·0	0·72

Extracellular orthophosphate concentration = 0·1 mM. Temperature = 19° C. Extracellular pH = 8·0.

The incorporation of externally applied [^{32}P]orthophosphate into the phosphate fractions in squid axons

One question which is raised by the results which have been presented is whether the ^{32}P applied on the outside of the axons as orthophosphate arrives in the axoplasm as orthophosphate or in the form of some other organic phosphate. To try and answer this question some experiments were carried out in which single axons were immersed for 10 min in artificial sea water containing 0·02 mM orthophosphate of very high specific activity. After three brief washes in ice-cold artificial sea water the axoplasm was extruded into a cold solution of 5 % trichloroacetic acid (w/v) and the resulting extract subjected to paper chromatography at 0° C in a solvent of trichloroacetic acid (30 ml. 20 % aqueous trichloroacetic acid solution) and ethanol (200 ml. 96 % ethanol). The counts and the phos-

phorus in each fraction were determined by methods which have been described previously (Caldwell, 1953, 1960; Caldwell, Hodgkin, Keynes & Shaw, 1964).

The specific activities calculated for the orthophosphate, ATP and arginine phosphate from the data obtained in these experiments are summarized in Table 4. In each experiment the specific activity of the orthophosphate fraction was found to be appreciably higher than that of the arginine phosphate and that estimated for the γ-phosphorus atom of ATP. This indicates that externally applied orthophosphate passing across the axolemma arrives in the axoplasm as orthophosphate and not as ATP or arginine phosphate. Two further experiments were done in which axons were immersed in artificial sea water containing [^{32}P]orthophosphate for periods of 1·5 and 4 hr. The specific activity of all the ATP phosphorus in the axoplasm from these axons was close to that of the orthophosphate, presumably because there had been sufficient time for equilibration (cp. Caldwell *et al.* 1964).

TABLE 4. The incorporation of externally applied [^{32}P]orthophosphate into the intracellular phosphorus of squid giant axons

Mean value obtained for % of axoplasmic phosphorus (orthophosphate + ATP + arginine phosphate) present as		Specific activity			
		Expt. 1	Expt. 2	Expt. 3	Expt. 4
Orthophosphate	16·5	3·1	2·4	2·1	2·6
ATP	42·2	0·4	0·3	0·3	0·4
γ phosphorus of ATP (ATP/3)	14·1	1·2	1·0	0·9	1·3
Arginine phosphate	41·4	0·9	1·1	1·4	0·9

Extracellular orthophosphate concentration = 0·02 mM. Extracellular potassium was absent in Expt. 4. Temperature = 18–19° C. The mean values of the % phosphorus in each fraction are those obtained for the four experiments. The specific activities were calculated as (Counts in fraction/total counts as orthophosphate + ATP + arginine phosphate)/(Phosphorus in fraction/total phosphorus in axoplasm as orthophosphate + ATP + arginine phosphate). ^{32}P incorporation was allowed to proceed for 10 min.

DISCUSSION

The results obtained in this work provide evidence that the movement of orthophosphate into squid giant axons involves an active transport process linked to metabolism. Although this might have been anticipated from the fact that orthophosphate has to enter the axons against a potential gradient and probably against a concentration gradient, the existence of such a process has not always been suggested by other work which has been done on phosphate entry into cells. Gerlach, Deuticke & Duhm (1964), for example, found that iodoacetate, fluoride and arsenate, which are

inhibitors of glycolysis, had little effect on labelled orthophosphate uptake by red cells and they concluded that an active transport process was not operating. On the other hand 2,4-dinitrophenol has been found to suppress the uptake of labelled orthophosphate into developing echinoderm eggs if applied within 30 min of fertilization (Litchfield & Whiteley, 1959), although it has little effect if applied at a later stage.

Janke, Fleckenstein, Marmier & Koenig (1966) have found that the incorporation of externally applied [^{32}P]orthophosphate into the ATP and creatine phosphate of frog rectus muscle is reduced by 2,4-dinitrophenol. Since studies with [^{18}O]H_2O indicated that the rate of incorporation of labelled intracellular orthophosphate into the ATP was increased rather than reduced by the dinitrophenol, Janke *et al.* (1966) argued that the reduced incorporation of externally applied [^{32}P]orthophosphate was due to an inhibition of the process involved in the uptake of this phosphate into the fibres. However, they considered that inhibition arose from the increased transmembrane orthophosphate gradient brought about by the dinitrophenol and they did not take into account the possibility that it could arise from an inhibition of a phosphate transport mechanism. If their interpretation were accepted, the increased orthophosphate gradient in dinitrophenol would presumably slow down orthophosphate uptake by a single-file effect analogous to that discussed by Hodgkin & Keynes (1955*b*) for potassium ions.

In view of these considerations the evidence which has been put forward in this paper for an active uptake of orthophosphate into squid axons should perhaps be examined more closely. The clearest indication that an active transport system of some sort is involved is provided by the inhibitory effect of ouabain on the orthophosphate influx, ouabain being a powerful inhibitor of active transport systems. Ouabain does not increase the orthophosphate gradient across the axolemma since it does not increase the orthophosphate concentration in the axoplasm of squid axons (Caldwell, 1960; Baker & Shaw, 1965) and has in fact been found to decrease the orthophosphate concentration in *Maia* nerve (Baker, 1963). In this case the orthophosphate influx is inhibited by a known inhibitor of active transport and the complication of an increased orthophosphate gradient across the axolemma arising from the action of the inhibitor is absent. The small reduction in orthophosphate influx observed on removal of the external potassium suggests that the active process involved in this influx may be linked in some way with the sodium pump mechanism, which is also slowed down by the removal of the external potassium. The decrease in orthophosphate influx on removal of external potassium is unlikely to be due to an increase in the orthophosphate gradient across the axolemma since Baker (1963) has shown that the orthophosphate content of *Maia*

nerve is reduced in the absence of external potassium. It must, however, be admitted that it cannot be argued conclusively that the decreases in orthophosphate influx found in the presence of cyanide and 2,4-dinitrophenol are due to an inhibition of an uptake mechanism rather than to an increase in the orthophosphate gradient since both these inhibitors cause an increase in the axoplasmic orthophosphate concentration (Caldwell, 1960). Also there is some indication in the results given in Table 1 that the effects of cyanide and dinitrophenol may be less marked if the external orthophosphate concentration is 0·5 instead of 0·1 mM. The size of the orthophosphate gradient across the axolemma may therefore modify the effect of metabolic inhibitors on the orthophosphate influx into squid axons and this aspect of phosphate uptake would seem to require further investigation.

It seems possible in the light of this and other work that orthophosphate might enter into cells in two different ways, the first of which operates at extracellular orthophosphate concentrations below about 1 mM. This mode of uptake, which would be that mainly studied in the present work, would involve an active transport mechanism linked to metabolism which was saturated with orthophosphate outside the cell so that the uptake was comparatively insensitive to changes in extracellular orthophosphate concentration. It would be directly affected by ouabain and would be affected by cyanide and 2,4-dinitrophenol as a result of interference with oxidative phosphorylation and the synthesis of ATP. Orthophosphate transport by this means might also be affected by the orthophosphate concentration gradient across the axolemma. The second type of entry could operate at extracellular orthophosphate concentrations above 1 mM and could be a passive entry through the same channels used by the phosphate efflux. This influx, like the efflux (P. C. Caldwell & P. F. Baker, unpublished), would not be affected by metabolic inhibitors. Gerlach *et al.* (1964) studied the orthophosphate influx into erythrocytes at extracellular orthophosphate concentrations of 1 mM and above and this influx would be expected to be of the second, passive type. An indication that this is so is perhaps given by the failure of Gerlach *et al.* (1964) to find effects of metabolic inhibitors on orthophosphate influx under their conditions.

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