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STUDIES ON THE MECHANISM OF PHOSPHATE ACCUMULATION BY SEA URCHIN EMBRYOS

by

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INTRODUCTION

The problem of phosphate entry into cells has been the subject of many studies with radioactive phosphorus during the past fifteen years. These studies, when compared with similar experiments with monovalent cations or chloride, present more difficulty in interpretation due to the fact that the greater part of the intracellular phosphate is in the form of organic compounds. These compounds often have a high turnover rate with respect to phosphorus. This usually exceeds the rate of phosphate transfer across the membrane (Hevesy, 1948).

In general two hypotheses have been proposed, either implicitly or explicitly, to explain the passage of phosphate into cells. Earlier papers explained phosphate uptake on the basis of simple diffusion (Furchgott and Shorr, 1943, Kalckar, Dahlinger, and Mehler, 1944, Krogh, 1946). This theory requires that the intracellular inorganic phosphate be derived directly from extracellular inorganic phosphate. The alternative to this is a passage brought about by the formation of an organic phosphate compound on or in the cell surface, and the transfer of this compound across the cell membrane. This would presumably involve enzyme action and imply that the intracellular inorganic phosphate was derived from the breakdown of an organic phosphate compound and not from the extracellular inorganic phosphate (Sacks, 1948). The latter hypothesis seems to have been supported in recent studies, particularly those involving cell suspensions. Included in these studies would be the work of Kamen and Spiegelman (1948), Mickerson and Mullins (1948), Sacks (1951), and Gourley (1952a).

The cells and tissues that have been most frequently studied are striated and cardiac muscle, liver, erythrocytes, yeast, and marine invertebrate eggs. Low temperature, anaerobiosis, and metabolic inhibitors such as cyanide, azide, and substituted phenols have been used as tools.

Much of the work that has been done with intact animals or tissue slices has led to contradictory conclusions. The experimental tissues, largely cardiac and striated muscle, contain appreciable amounts of extracellular inorganic phosphate which must either be removed or accounted for. Otherwise determinations of intracellular inorganic phosphate will be in error and lead to difficulties in interpreting the data.

Sacks and Aitchuler (1942), in a study of striated and cardiac muscle, concluded that phosphate did not diffuse across the cell membrane, but that the formation of an organic phosphate compound on the cell surface was necessary for the passage of phosphate into the cell. P^{32} was injected subcutaneously and at various times after the injection the tissues were removed and frozen. The extracellular inorganic phosphate was calculated by assuming that the chloride space coincided with the extracellular space for phosphate, and that the extracellular inorganic phosphate was in equilibrium with the plasma inorganic phosphate. This calculated value was subtracted from the total inorganic phosphate to give the intracellular inorganic phosphate. This method has been criticized since wide variations were obtained in similar experiments. The major evidence supporting an enzymatically controlled transport was

obtained from the cardiac muscle experiments. At one, two, and four hours after the injection of P^{32}, the intracellular inorganic phosphate had a lower specific activity then the plasma inorganic phosphate. At twenty-four hours, the specific activity was highest in the intracellular inorganic phosphate. Phosphocreatine and adenosinetriphosphate had intermediate activities at all times. If diffusion were operative, this specific activity gradient at twenty-four hours would require diffusion against a concentration gradient which is unlikely. This same relationship has been found in two-hour liver experiments (Secks, 1948).

Further experiments (Secks, 1951), which followed the time course of the relative specific activity of liver phosphate compounds, showed that ATP-ADP and glucose-1-PO_4 had higher turnover rates than other organic phosphate compounds. According to the criteria of Zilversmit, Entenman, and Fishler (1943), the plasma inorganic phosphate fulfilled the requirements of a precursor substance for both ATP-ADP and glucose-1-PO_4. This would imply that phosphate entered the cell via ATP or glucose-1-PO_4 formation. These criteria require that the specific activity of the precursor substance be higher than that of the product before the latter reaches a maximum. Furthermore, when the specific activity of the product reaches a maximum value, it must be equal to the specific activity of the precursor.

In experiments using cardiac muscle slices, Furchgott and Shorr (1943) concluded that radioactive phosphate entered mainly by means of simple diffusion. Extracellular inorganic phosphate was removed by

washing the slices with phosphate-free Ringer's solution at 2°C. Therefore direct measurements of intracellular inorganic phosphate were possible. In no case did the specific activity of the intracellular inorganic phosphate rise above the specific activity of the organic phosphate compounds, as was the case in the experiments of Sacks and Altschuler (1942). In thirty minutes at 37.5°C the specific activities of intracellular inorganic phosphate, creatine phosphate, and the terminal phosphate of ATP were equal. It was concluded that intracellular inorganic phosphate was the source of the two organic phosphate compounds. The most direct evidence in favor of a diffusion theory is that in experiments at 2°C, where metabolism was at a low level, the intracellular inorganic phosphate had a much higher relative specific activity than any organic phosphate compound. However the total penetration of P^{32} into the cells at 37.5°C was 5-10 times as rapid as at 2°C, and it was suggested that under normal conditions, a process other than simple diffusion might also be operating. It may be noted, however, that according to Danielli's theory of activated diffusion, a high temperature coefficient, of the order of 2 or more, does not necessarily imply that a chemical process is operative. It may merely indicate that the cell membrane represents a high potential energy barrier, and consequently the resistance to diffusion is high (Danielli, 1952).

Kalckar, Dehlinger, and Mehler (1944) gave support to the diffusion theory in a series of experiments with rabbit striated muscle. Radioactive phosphate was injected intravenously and 15 to 20 minutes later one

leg was perfused with 1 to 1.5 liters of phosphate-free Ringer's solution to remove the extracellular phosphate. The other leg was not perfused, and, therefore, it acted as a control on the adequacy of the perfusion. The specific activities of the phosphocreatine and ATP were always lower than that of the inorganic phosphate. It is of interest, however, that the specific activity of hexose-mono-phosphate was extremely variable. In one three hour experiment it was considerably higher than that of the inorganic phosphate. Therefore, the authors do not exclude the possibility that incorporation of phosphate into polysaccharide molecules is a process whereby phosphate may enter the cell.

In general, experiments with cell suspensions have given more conclusive information on phosphate entry. They have tended to support the theory of an enzymatically controlled transport mechanism. Early experiments of Hevesy, Linderstrøm-Lang, and Nielsen (1937) showed that at low temperatures or in the absence of substrate there was little phosphate uptake by yeast. These observations were confirmed by Lawrence, Erf, and Tuttle (1941) and Mullins (1942). Further evidence indicating that phosphate uptake by yeast is an active process, closely associated with metabolism, is derived from the fact that uptake depends on the pH of the medium. Experiments carried out by Malm, cited in Hevesy (1947), show that uptake was maximum at pH 4 to 5 and non-existent at pH 7. The pH of the cells was not changed during the course of the experiment. It was concluded that a pH dependent reaction at the cell surface was important for phosphate entry into the cell. The possible nature of such a surface reaction was suggested in experiments with

riboflavin (Nickerson and Mullins, 1948). In yeast utilizing glucose P^{32} exchange was markedly increased by adding riboflavin to the medium. It was suggested that phosphate enters by combining with a complexing compound on the cell surface and that riboflavin may be such a compound.

Another approach to the problem of phosphate uptake has made use of metabolic inhibitors (Kamen and Spiegelman, 1948). The rationale behind the use of these agents is as follows. If phosphate enters by diffusion, agents affecting phosphorylations within the cell would not have any effect.[1] However, if phosphate entry is due to some esterification reaction, these agents might inhibit the exchange between extracellular and intracellular ortho-phosphate. In experiments with glucose-fermenting yeast, azide, arsenate and monoiodoacetic acid all inhibited P^{32} uptake. These results were, therefore, consistent with the theory that esterification is important in phosphate penetration.

The same conclusion was also reached with an entirely different type of experiment by the same workers. The esterification theory requires the presence of a phosphate acceptor, and it is not unreasonable to assume that this acceptor is produced directly or indirectly as a result of carbohydrate metabolism. Therefore, if yeast fermented in a phosphate-free medium, one might expect that this acceptor would accumulate. On subsequent addition of phosphate, an increased uptake might be observed. This indeed proved to be the case. After yeast had fermented in a phosphate-free medium, phosphate was added and the rate of uptake was

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1. An exception would occur if phosphorylations within the cell were affecting diffusion by maintaining a favorable inorganic phosphate gradient.

2 to 3 times greater than usual.

If an esterification reaction is involved in phosphate entry into yeast, it is possible that enzymes would be present on the cell surface. It is interesting that Rothstein and Meier (1948) have reported an ATP-ase on the cell surface of yeast. ADP, triose-PO_4, glycero-PO_4, and phenyl-PO_4 are also hydrolyzed, thereby suggesting that several enzymes may be present.

Experiments, comparable to those with yeast, have been carried out with erythrocyte suspensions with similar results. The temperature coefficient for phosphate entry into human erythrocytes is one that could be characteristic of a chemical process (Eisenman, Ott, Smith and Winkler, 1940). However, the reservations introduced by Danielli (1952) must be considered. At 7°C no phosphate entry was observed, in contrast to the marked uptake at 38°C. In experiments designed to follow the time course of the relative specific activities of plasma inorganic phosphate and intracellular phosphate compounds, ATP was found to be the precursor of the intracellular inorganic phosphate (Gourley, 1952a). Again the criteria for a precursor substance were those of Zilversmit, Entenman, and Fishler (1943). It was therefore suggested that phosphate enters by formation of ATP. This suggestion received further confirmation in experiments with metabolic inhibitors (Gourley, 1952b). Iodoacetic acid and sodium fluoride were known to reduce phosphate uptake to one fifth of the normal value. It was, therefore, anticipated that, if uptake were via ATP formation, these agents would reduce the specific activity of ATP. Iodoacetic acid and sodium fluoride in concentrations which inhibited all

but a fraction of the normal P^{32} uptake, also reduced by 90% the P^{32} turnover in the labile phosphate of ATP.

Enzymes have also been found on the cell surface of erythrocytes. Clarkson and Maizels (1952) report the presence of an apyrase on the cell surface of human erythrocytes. These experiments were carried out with "ghosts" isolated from hemolysates. Other studies (Ernst and Komola, 1952) have shown that an enzyme releasing three phosphates from ATP, two phosphates from ATP, and two ortho-phosphates from each mole of inorganic pyro-phosphate, is part of the isolated cell membrane of mammalian erythrocytes.

A third type of cell suspension that has been widely used is one involving marine invertebrate eggs, notably echinoderm eggs. Additional importance has been attached to this work because of interest in events associated with fertilization and early cleavage stages in these eggs. The study by Brooks (1943) on P^{32} entry into echinoderm eggs established that these eggs were permeable to phosphate. Subsequent studies have confirmed this, and have been mainly concerned with differences between the unfertilized and fertilized eggs, changes at fertilization and, as with other cells and tissues, the mechanism of transfer across the cell membrane. This information is summarized in Table 1.

P^{32} uptake by unfertilized eggs is negligible. An exchange is thought to take place between the external and intracellular phosphate. However this does not result in an increase in the internal concentration of phosphate (Chambers and White, 1949, 1954; Brooks and Chambers, 1954). Lindberg (1948, 1950) considered that the surface of unfertilized eggs

Table 1

Species	P^{32} Uptake of Unfertilized Eggs	P^{32} Uptake of Fertilized Eggs	Length of Lag Period	Time of Maximum Uptake	Temperature Effect	Inhibitors	Incorporation into Phosphate Compounds	Remarks	Source
Dendraster excentricus		61.8% increase in P^{31} from fertilization to gastrulation						swimming embryos can get sufficient P^{31} from sea water	Needham and Needham (1930)
Arbacia punctulata, Asterias forbesii		some penetration, not constant							Brooks (1943)
Arbacia punctulata	40 x the unfertilized eggs, constant for 4 hours	7-10 minutes	Fert.-uptake at 10°C is one seventh that at 23°C	Fert.-1.6×10^{-5}M 4,6-dinitro-o-cresol diminished uptake by factor of 6			activity of acid soluble compounds 10 x that of acid insoluble	P^{32} uptake closely associated with cellular activity	Abelson (1947)
Arbacia punctulata							Fert.-97% of activity in acid soluble compounds		Abelson (1948)

Table 1 (Continued)

Species	P^{32} Uptake of Unfertilized Eggs	P^{32} Uptake of Fertilized Eggs	Length of Lag Period	Time of Maximum Uptake	Temperature Effect	Inhibitors	Incorporation into Phosphate Compounds	Remarks	Source
Strongylocentrotus purpuratus, S. franciscanus	slow and constant for hours	130-160 x the unfertilized eggs, a 0.1% increase in total P/hour	6 min.	60 min. after fertilization, constant during 1st 3 cleavages		10^{-4}M NaCN decreased penetration three-fold		rate remains constant irrespective of cleavage and mitotic cycles	Brooks and Chambers (1948)
Lytechinus pictus		P^{32} accumulates after fertilization					Fert. and Unfert.-activity mainly in acid soluble fraction		Chambers, Whiteley, Chambers, and Brooks (1948)
Arbacia			0°C inhibited uptake		Fert.-5×10^{-5}M DNP, 10^{-2} malononitrile, and 10^{-4}M uranyl nitrate inhibit uptake				Villee, Lowens, Gordon, Leonard, and Rich (1948)
Psammechinus miliaris	impermeable, rapid surface metabolism coupled with transphosphorylation of ATP	permeable, rapid surface metabolism coupled with transphosphorylation of ATP						only a fraction of ATP and ortho-PO_4 stores of unfertilized eggs involved in P turnover, therefore, it is assumed that these are on surface	Lindberg (1949)

Table 1 (Continued)

Species	P^{32} Uptake of Unfertilized Eggs	P^{32} Uptake of Fertilized Eggs	Length of Lag Period	Time of Maximum Uptake	Temperature Effect	Inhibitors	Incorporation into Phosphate Compounds	Remarks	Source
Arbacia						Fert.-dunnic acid inhibits uptake		cleavage also inhibited	Marsbak & Herting (1948)
Strongylocentrotus purpuratus	exchange	accumulation					90-95% of P^{32} is in TCA extracts, 86% of this is in ATP		Chambers & White (1949)
Lytechinus pictus			12-15 minutes					Unfert.-light half has 1-2.4 x as much activity as the heavy half. Fert.-heavy half has 2 x as much activity as light half	Whiteley (1949)
Paracentrotus lividus						Max. amount of labelled ATP formed in 30 min. in U.Fert., ascending curve for ATP labelling in Fert.			Lindberg (1950)

Table 1 (Continued)

Species	P^{32} Uptake of Unfertilized Eggs	P^{32} Uptake of Fertilized Eggs	Length of Lag Period	Time of Maximum Uptake	Temperature Effect	Inhibitors	Incorporation into Phosphate Compounds	Remarks	Source
Arbacia					$Q_{10} = 2$	Fert.-eserine, pilocarpine, & atropine inhibit uptake in certain conc.		Q_{10} indicates an enzyme controlled reaction	Villee & Villee (1952)
Strongylocentrotus purpuratus		Rate of uptake increases up to 35 hrs. or mid-gastrulation, then decreases		35 hrs. or mid-gastrulation					Bolst (1952)
Strongylocentrotus purpuratus, S. franciscanus, Lytechinus pictus	exchange, internal conc. of P remains constant	85-132% unfert. S. purp., 113 x Unfert. S. fran., uptake represents an accumulation	15-20 min.	S. purp.-50-60 min., S. fran.-80-90 min.				jelly, fert. membrane or hyaline layer do not affect, uptake is constant despite decrease of P^{32} in medium, P^{31} is removed at same rate as P^{32}	Brooks and Chambers (1954)
Strongylocentrotus purpuratus, S. franciscanus, Lytechinus pictus	exchange	an accumulation against a conc. gradient, rate of uptake is constant if medium contains over 15-20 Mg P/l.	0-30 min.	60 min., constant for 1st 430 min. after fert.		Unfert.-95.7-95.9% activity in TCA extracts. Fert.-96.3-96.4% activity in TCA extracts, majority of the activity is in easily hydrolyzable P cpds.		P^{32} not removed from medium, may even increase-Unfert. P^{31} & P^{32} removed, removal rate = P^{32} uptake rate--Fert.	Chambers and White (1954)

metabolized phosphate, but that there was no penetration of phosphate into the interior of the egg. The small amount of P^{32} incorporated into the unfertilized egg is rapidly converted into ATP. The amount of labelled ATP reaches a maximum at the end of 30 minutes. No larger amount is formed after this time and an exchange equilibrium is established with the orthophosphate in the eggs and in the medium. Since the labelled ATP represents only a fraction of the ATP stores of the eggs, it is assumed that only the surface ATP is involved in this labelling. Consequently, Lindberg concluded that the unfertilized egg is impermeable to P^{32}, although a rapid surface turnover of P^{32} is carried on.

The fertilized egg presents a radically different picture. Immediately following fertilization there is no change in P^{32} uptake, but within 6-30 minutes there is a noticeable increase in uptake. The rate of uptake usually reaches a maximum by 60 minutes and remains constant for up to seven hours after fertilization. These initial events have been described by Abelson (1947), Brooks and Chambers (1948, 1954), Whiteley (1949), and Chambers and White (1954). In a study extending throughout the larval life, however, Bolst (1952) found the rate of penetration continued to increase up to mid-gastrulation (35 hours) and then subsequently decreased. The maximum uptake rate of the fertilized eggs is up to 160 times as great as the uptake in unfertilized eggs (Brooks and Chambers, 1948). Slight changes in rate associated with cleavage or mitotic cycles have been reported by Zeuthen (1951), but these changes have not been found by Brooks and Chambers (1948), or Whiteley (1949). This penetration of phosphate represents an accumulation against a concentration gradient which may be

as high as a thousandfold (Chambers and White, 1954). However, this figure is thought to be too large by the authors. The rate is not affected by the accompanying decrease in P^{31} and P^{32} concentrations in the suspension medium (Brooks and Chambers, 1954). However, uptake of P^{32} can be inhibited by P^{31}, if the concentration of the latter is sufficiently high (Lindberg, 1950). Evidence that this uptake represents a real penetration is the fact that only 2 to 5% of the P^{32} activity is removed by continuous washing with sea water (Brooks and Chambers, 1948).

Radioactive phosphate is largely incorporated into the acid soluble phosphate compounds (Abelson, 1947, 1948; Chambers, Whiteley, Chambers, and Brooks, 1948; Chambers and White, 1949, 1954). This is true both in the fertilized and unfertilized eggs. Among the acid soluble components, the easily hydrolyzable phosphate compounds have the highest activity (Abelson, 1948; Lindberg, 1948, 1950; Chambers and White, 1949, 1954). Lindberg's work (1948, 1950) on ATP turnover in fertilized eggs has shown that, in contrast to the unfertilized condition described previously, the fertilized egg is permeable to P^{32} and that there is an ascending curve for ATP labelling. The specific activity of this fraction eventually greatly exceeds that of the ortho-phosphate in the eggs. If P^{32} is removed from the medium, equilibrium between the labelled ATP and the ortho-phosphate is not reached until the swimming blastula stage.

There is also a difference between the unfertilized and fertilized eggs in regard to the cytological distribution of P^{32}. In experiments with the eggs of Lytechinus pictus, centrifuged into light and heavy halves, Chambers, Whiteley, Chambers and Brooks (1948) and Whiteley (1949) found

that in unfertilized eggs, the light half had 1 to 2.4 times as much activity as the heavy half. Upon fertilization this situation was reversed, and the heavy half had twice the activity of the light half.

Low temperature markedly inhibits uptake by fertilized eggs. Abelson (1947) reported that the uptake at 10°C was one seventh of that at 23°C. A Q_{10} of approximately 2 has been calculated by Villee and Villee (1952). They interpreted this result as characteristic of an enzymatically controlled transport.

Experiments with metabolic inhibitors also suggest that a process other than diffusion is in operation in the fertilized egg. 1.6×10^{-5}M 4,6 dinitro-o-cresol diminishes uptake by a factor of six (Abelson, 1947). It is not directly stated when the inhibitor was added, but it seems likely that it was added at or near fertilization. Increased penetration also results when eggs are incubated with 10^{-4}M sodium cyanide (Brooks and Chambers, 1948). Villee, Loosma, Gordon, Leonard, and Rich (1948) in studies designed to observe P^{32} incorporation into nucleoproteins, found that dinitrophenol, malononitrile, and uranyl nitrate significantly inhibited uptake by the entire egg. This finding was inferred from the fact that the P^{32} incorporated into the acid soluble phosphate fraction was low. Dinitrophenol was most effective in this regard. In similar experiments, pilocarpine, eserine, and atropine decreased uptake (Villee and Villee, 1952).

The experiments on sea urchin eggs just reviewed have led to the view that, while the unfertilized egg is characterized by a phosphate uptake that does not represent penetration into the interior of the egg

and involves a simple exchange between the phosphate of the medium and the inorganic phosphate of the egg, the developing embryo has an enzymatic mechanism that predominantly controls the penetration and consequent accumulation of phosphate. In the present study the nature of this enzymatic mechanism has been examined, the time of its establishment and a partial relation to the events of fertilization determined, its localization as a surface system has been established with reasonable certainty, and some information concerning the relation of the energy metabolism of the egg to the mechanism has been obtained. The experiments have largely involved an analysis of the effect of various metabolic effectors (2,4-DNP, cyanide, arsenate, ATP, ADP, AMP, and temperature) on the rate of uptake of P^{32} in fertilized eggs.

METHODS AND MATERIALS

The basic experimental approach used in all the experiments to be described, was to measure continuously with a Geiger counter the accumulation of P^{32} by sea urchin eggs which were being perfused with sea water containing P^{32}. In some experiments, the perfusion fluid included various metabolic effectors. The perfusion chamber (Fig. 1) was an improved version of that used by Chambers and Whiteley (Whiteley, 1949), and Chambers, White and Zeuthen (Zeuthen, 1951). It consisted of a circular lucite chamber with two compartments. Water from a constant temperature water bath was pumped through the upper compartment to regulate the temperature of the bottom compartment which contained the eggs. The two chambers were separated by a No. 1 cover slip. The inlet tube for the bottom chamber was made of 1 mm. capillary tubing, which diverted the incoming water against the center of the upper cover slip, while the outlet tube was of 3 mm. glass tubing. The flow characteristics of the chamber were tested by perfusing a dye through it. The dye spread quickly, and fairly homogeneously to all parts of the chamber. The rate of flow through the egg compartment, which had a volume of 1.7 c.c., was regulated by a piece of capillary tubing attached to the outlet tube outside of the chamber. The flow, of from 3 to 4 ml./min., was sufficient to change the sea water in the compartment approximately twice a minute. The eggs were introduced into the chamber through a 3 mm. opening drilled in the side. The opening could be closed with a lucite plug. The lower surface of the bottom compartment was also a No. 1 cover slip.

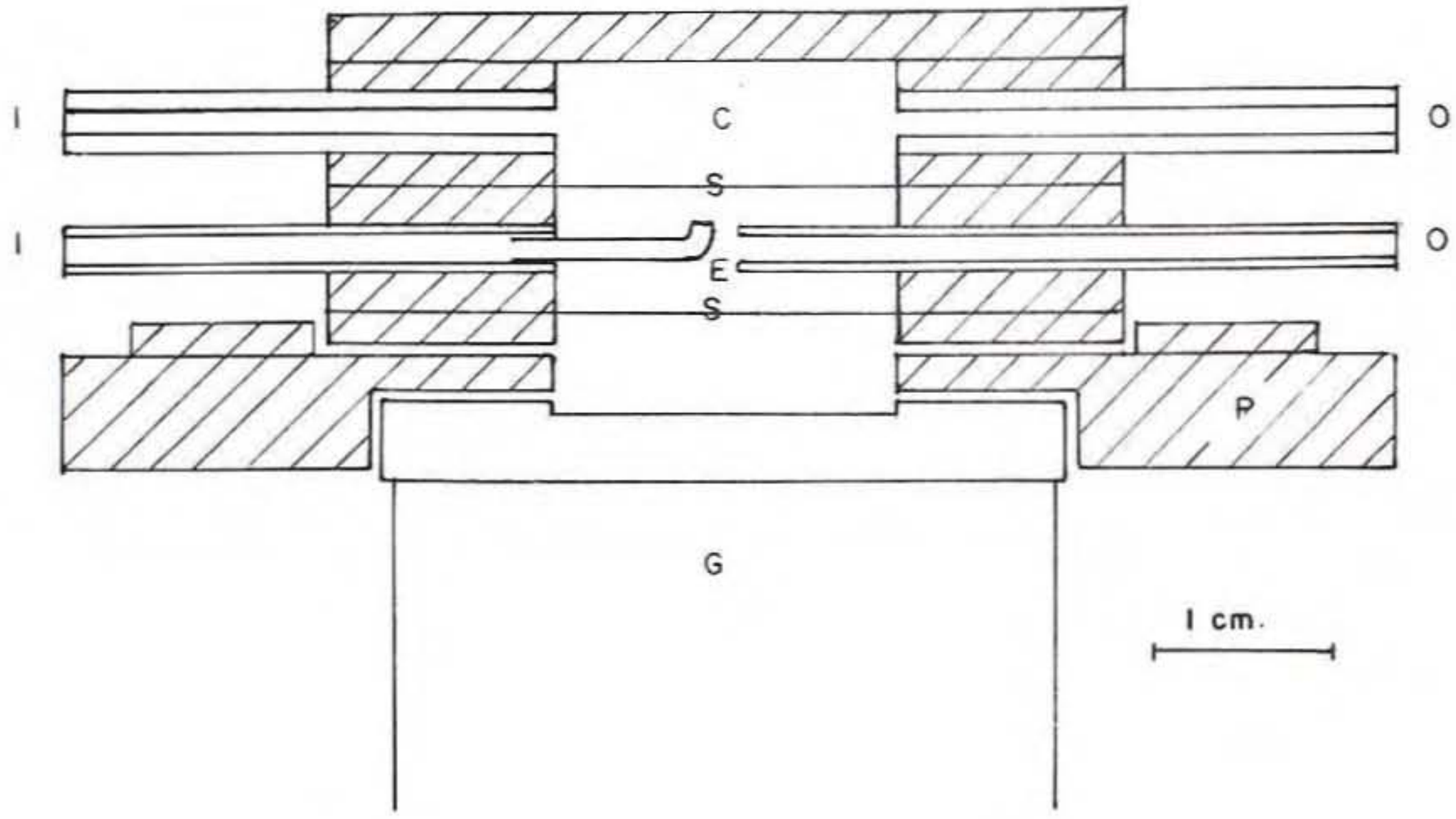

Figure 1. Perfusion Chamber.
c, cooling chamber; e, egg chamber; g, end-window Geiger-Mueller tube; i, inlet tube; o, outlet tube; p, platform; s, No. 1 cover slip.

Prior to entry into the egg chamber, the sea water was cooled by passage through coiled glass tubing, in a constant temperature water bath. In most experiments, the temperature of the bath, and therefore in the chamber, was 18.0°C, however, for several experiments conducted at 8.0°C, the temperature of the water bath was lowered. Before each of these low temperature experiments, it was established with a thermocouple inserted into the chamber, that the temperature of the water flowing through the egg chamber was 8.0°C.

The chamber was placed on a lucite platform which in turn rested on the top of the end-window Geiger-Mueller tube. Consequently the geometrical relationship between the tube and the chamber was always the same.

At the beginning of each experiment, the eggs were perfused with sea water for fifteen minutes. During this time a background count for the experiments was obtained. Sea water containing P^{32} was then turned on and the activity in the chamber reached a new level with the unfertilized eggs. Fifty to sixty minutes after the start of the experiment, the eggs were fertilized. This was accomplished by injecting 0.1 to 0.2 cc of a 10% sperm suspension in P^{32} sea water into the inlet tube with a hypodermic syringe. Fertilization and subsequent development of the eggs were observed with a Zeiss Opton stereoscopic microscope placed above the lucite chamber. At the end of each experiment, the eggs were removed from the chamber, washed, and allowed to develop further. This served as a check on their normality, or on their recovery from the effects of any reagent that was being tested. After each experiment, the chamber was rinsed alternately with concentrated HCl and NaOH and perfused with tap

water and sea water in order to remove P^{32} absorbed on the inner surfaces of the bottom compartment. Any radioactivity left in the chamber was accounted for by measuring the activity of the empty chamber just before each experiment.

The eggs used were those of the sea urchin Strongylocentrotus purpuratus (Stimpson), collected at San Juan Island and at Neah Bay, Washington. The eggs from a single animal were used in each experiment. Shedding was induced either by injection of 4.2% KCl (Tyler, 1949), or by an electric shock of 30 volts, applied intermittently for several minutes. Eggs were shed into filtered sea water and washed until 95 to 100% fertilization was obtained. If fertilization was abnormal, the eggs were not used. In the majority of experiments, the eggs were used within 1 to 3 hours after shedding. Sperm were collected in dry Syracuse watch glasses, and suspensions were made up just before use.

An 0.5% egg suspension was prepared and 1 ml. aliquots of this suspension were counted in a Sedgewick-Rafter counting chamber. The required number of milliliters were taken from the suspension in order to secure 15,000 eggs. These were collected by light centrifugation, excess sea water was removed, and the eggs were transferred to the experimental chamber. During transfer and distribution of the eggs in the chamber, it is estimated that from 15 to 20% of the eggs were lost, so that in each experiment approximately 12,000 to 13,000 eggs were used. This number of eggs was sufficient to cover the bottom of the chamber in a single layer. Eggs from the 0.5% suspension were also taken for controls. They were diluted and cultured at 18.0°C during the course of the

experiment, and consequently could be compared with the experimental eggs which were at the same temperature.

All perfusion solutions were made up in filtered sea water obtained from the vicinity of Friday Harbor or from Puget Sound. P^{32} was obtained from the Abbott Laboratories as sodium phosphate in 0.9% NaCl. P^{32} sea water solutions were made up to have an activity of 0.005 μc/ml. In all but two experiments, the amount of carrier phosphate added was considerably less than the 31 to 62 M μg/l. normally found in sea water in this area. The additional phosphate in these two experiments had no adverse effect on the eggs. One solution of P^{32} sea water was prepared for each experiment, and from this, P^{32} solutions containing various test substances were made. These included disodium-ATP and monosodium-ADP which were obtained from the Pabst Brewing Company, and adenosine-5-phosphoric acid which was a product of Schwarz Laboratories, Inc. All solutions were adjusted to pH 8.

A Tracerlab 64 Scaler was used in all experiments. This was checked before and after each experiment against a Tracerlab calibrated standard of 1.47×10^{-5} mc. Several Geiger-Mueller end-window tubes were used during the course of the experiments. The thickness of the mica windows ranged from 2.3 to 3.1 mg./cm^2. In the majority of experiments, corrections were made for the differences in sensitivity resulting from these differences in window thickness.

In some experiments, eggs were activated parthenogenetically by the double treatment method of Loeb (Just, 1939). The butyric acid and hypertonic sea water solutions were injected by means of a syringe into

the egg chamber containing the unfertilized eggs. At the end of the treatment, the eggs were perfused with sea water, followed by P^{32} sea water as in the other experiments.

RESUME

Experiments with unfertilized eggs confirm previous observations that there is practically no P^{32} accumulation at this time. A few minutes after the P^{32} sea water first enters the egg chamber, the activity in the chamber reaches a level which remains relatively constant for hours. Such an experiment is indicated in Fig. 2. During the course of several hours the activity may rise slightly, showing a small accumulation over the level in the sea water; however, this is probably mainly due to absorption of P^{32} on the surfaces of the chamber. This is indicated by the fact that when P^{32} flows through an empty chamber, there is also a slight increase in activity during the course of three hours. The curves obtained with unfertilized eggs and with an empty chamber are very similar. This similarity is shown in Fig. 2, from which it is concluded that no accumulation of P^{32} occurs in the unfertilized eggs. This conclusion is strengthened by the results of several experiments in which it was found that the level of activity in the chamber is not affected by varying the number of unfertilized eggs from 5,000 to 20,000.

In contrast with the unfertilized eggs, fertilized eggs accumulate P^{32}, so that the activity in the chamber greatly exceeds that of the P^{32} sea water. An experiment that typifies the course of this uptake is given in Fig. 3. Two other experiments are similar in all essential respects. This accumulation commences after a short lag period, which is quite variable, ranging from 7 to 30 minutes in 24 experiments, with the average at 18. The maximum rate of uptake is not established until

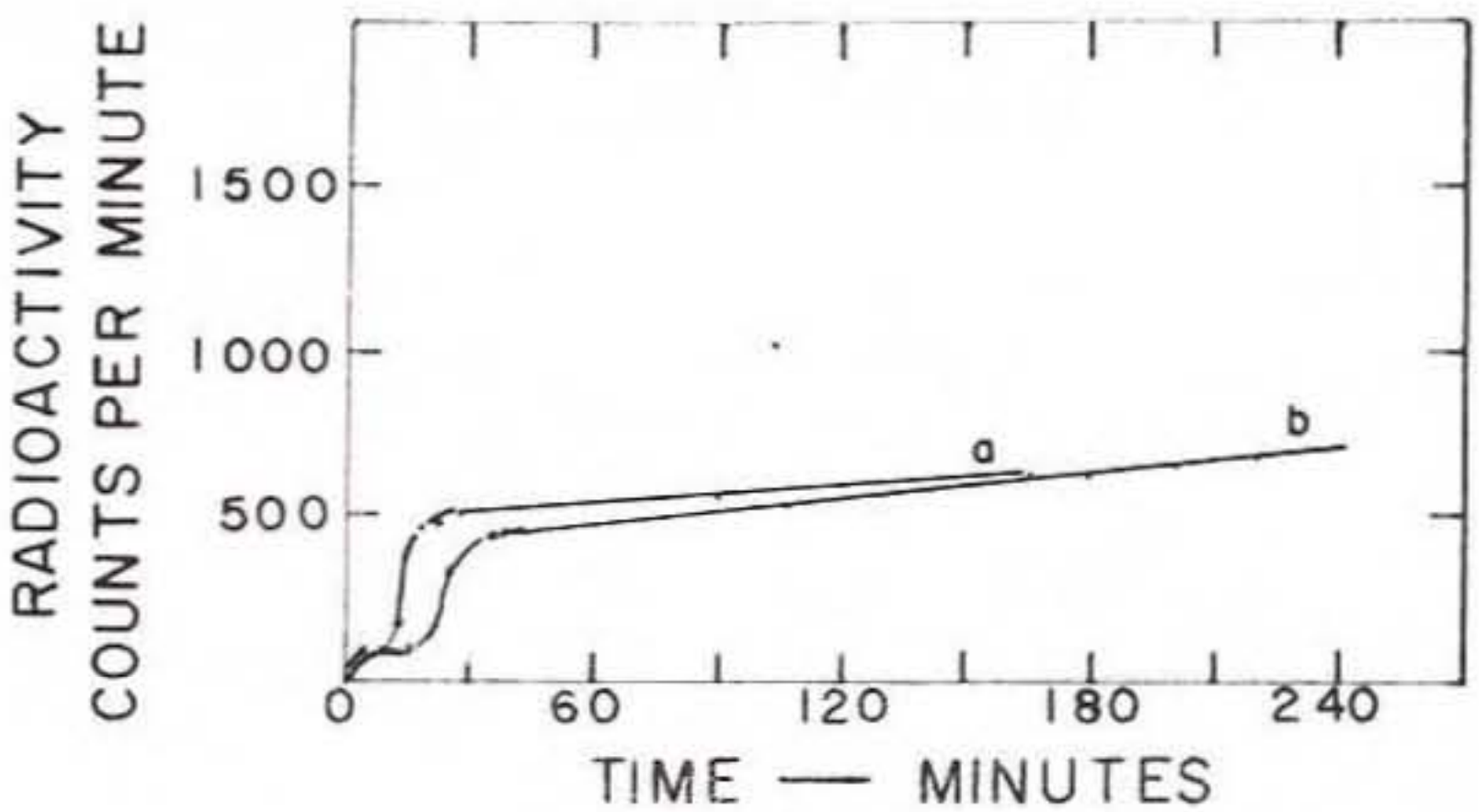

Figure 2. Uptake of P^{32} by unfertilized eggs of Strongylocentrotus purpuratus (Stimpson) and absorption of P^{32} by the perfusion chamber.
The initial low level of activity represents the background count.
a, unfertilized eggs.
b, empty chamber.

approximately 40 minutes after fertilization. This again is variable, ranging from 22 to 60 minutes in 14 experiments. While an explanation of these variabilities is not at hand, there seems to be no correlation between the length of the lag period and the length of time the eggs have been out of the ovary, which varied from 1 to 5.75 hours in 24 comparable experiments. It is probable that the cause of the variability resides in inherent differences in the gametes of different animals. Once the maximum rate of uptake is established, it remains constant. This constant rate of uptake was observed in experiments that continued for 5 hours after fertilization.

It seemed important to determine to what extent the characteristic uptake pattern of the fertilized eggs was dependent on the penetration of the sperm, and to what degree it was inherent in the potentialities of the eggs. To answer this question, eggs were activated parthenogenetically and the uptake of P^{32} followed. As is shown in Fig. 3, such eggs exhibit a P^{32} accumulation comparable to that of fertilized eggs. Uptake was initiated by both a single treatment with butyric acid and a double treatment of butyric acid and hypertonic sea water. The rate of P^{32} uptake in these experiments was approximately 3/4 of the fertilized egg rate. This difference was probably due to the activation of only 60-70% of the eggs in the chamber, as measured by membrane elevation. Moreover none of the activated eggs cleaved normally. It seems probable that under optimum conditions of activation, with activation approaching 100%, the uptake would more nearly approach that of the fertilized eggs. The onset of phosphate accumulation is also preceded by a lag period.

These results are not surprising for other activities attributed to the fertilized egg have been demonstrated in artificially activated eggs. Respiration in artificially activated eggs is comparable to that in sperm fertilized eggs (Warburg, 1910). This has been confirmed by Keltch and Clowes for Arbacia (1947). The respiration in artificially activated eggs can also be stimulated by substituted phenols (Keltch, Walters, and Clowes, 1947). In their experiments no more than 45% recognizable cell divisions occurred. They concluded that O_2 consumption was correlated with changes in the egg associated with formation of the fertilization membrane and was not dependent on subsequent cell division. This same relationship may be true for P^{32} uptake.

These results, confirming the existence of phosphate accumulation in the fertilized eggs, suggest that an enzymatically controlled transport is established in the activated eggs and that the sperm is not essential for this establishment. Among the criteria frequently used to determine if an enzymatically controlled transport is operative is that the transport should be affected by various enzyme effectors (Rosenberg and Wilbrandt, 1952; Danielli, 1952). 2,4-dinitrophenol (DNP) is a metabolic inhibitor known to interfere especially with aerobic phosphorus metabolism with the result that it uncouples phosphorylations from oxidations (Loomis and Lipmann, 1948).

It would seem probable that if P^{32} uptake in sea urchin eggs is an enzymatically controlled process, DNP would be an inhibitor. Inhibition has been reported by Abelson (1947) for 4,6-nitrocresol, another substituted phenol. However, in some unpublished experiments, Whiteley (1949) had

observed that when DNP was added some time after fertilization, there was no inhibition of phosphate uptake. Further investigation of this point has shown that the time when DNP is applied has a direct bearing on its effect on P^{32} uptake. In these experiments DNP in a concentration of 10^{-4} M in sea water was introduced into the perfusion chamber at various times before and after fertilization, and the effect produced on the rate of P^{32} uptake was measured. Since this concentration will reversibly inhibit cleavage, it is assumed that it interferes with the generation or utilization of high energy phosphate (Clowes, Kelch, Strittmatter, and Walters, 1950).

When DNP is applied before fertilization or any time within the first thirty minutes following fertilization, there is a marked inhibition of P^{32} accumulation, as may be seen in Fig. 4. After thirty minutes, the effect of DNP decreases until, when it is added at sixty minutes after fertilization, it has no effect on P^{32} accumulation. When the rate of P^{32} uptake after fertilization is plotted against the time after fertilization when DNP is applied, it can be seen that the inhibition gradually decreases between 30 and 60 minutes (Fig. 5). The maximum inhibition by DNP is associated with the first 30 minutes following fertilization, a time when virtually no P^{32} uptake has been established. The degree of inhibition at 40 minutes is variable. This variability may be correlated with the length of the lag period and the onset of the maximum uptake rate: if the latter is not established until after the application of DNP at 40 minutes, the inhibition seems to be appreciable;

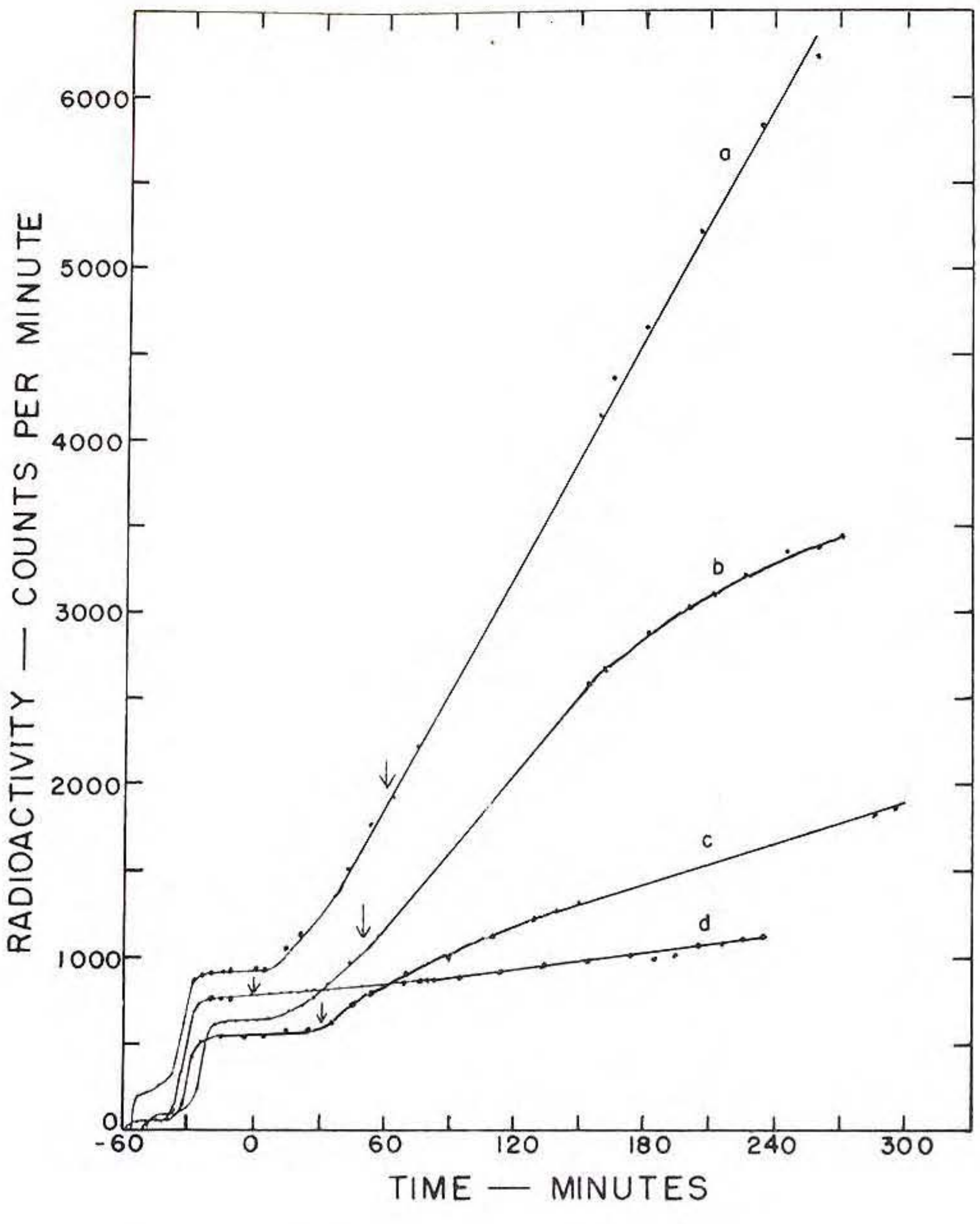

Figure 4. Effect of 10^{-4}M DNP on P^{32} uptake.
The initial low level of activity represents the background count. Fertilization was at zero time. Arrows indicate the addition of DNP to the perfusion chamber.
a, DNP added at 60 minutes.
b, DNP added at 50 minutes.
c, DNP added at 30 minutes.
d, DNP added at fertilization.

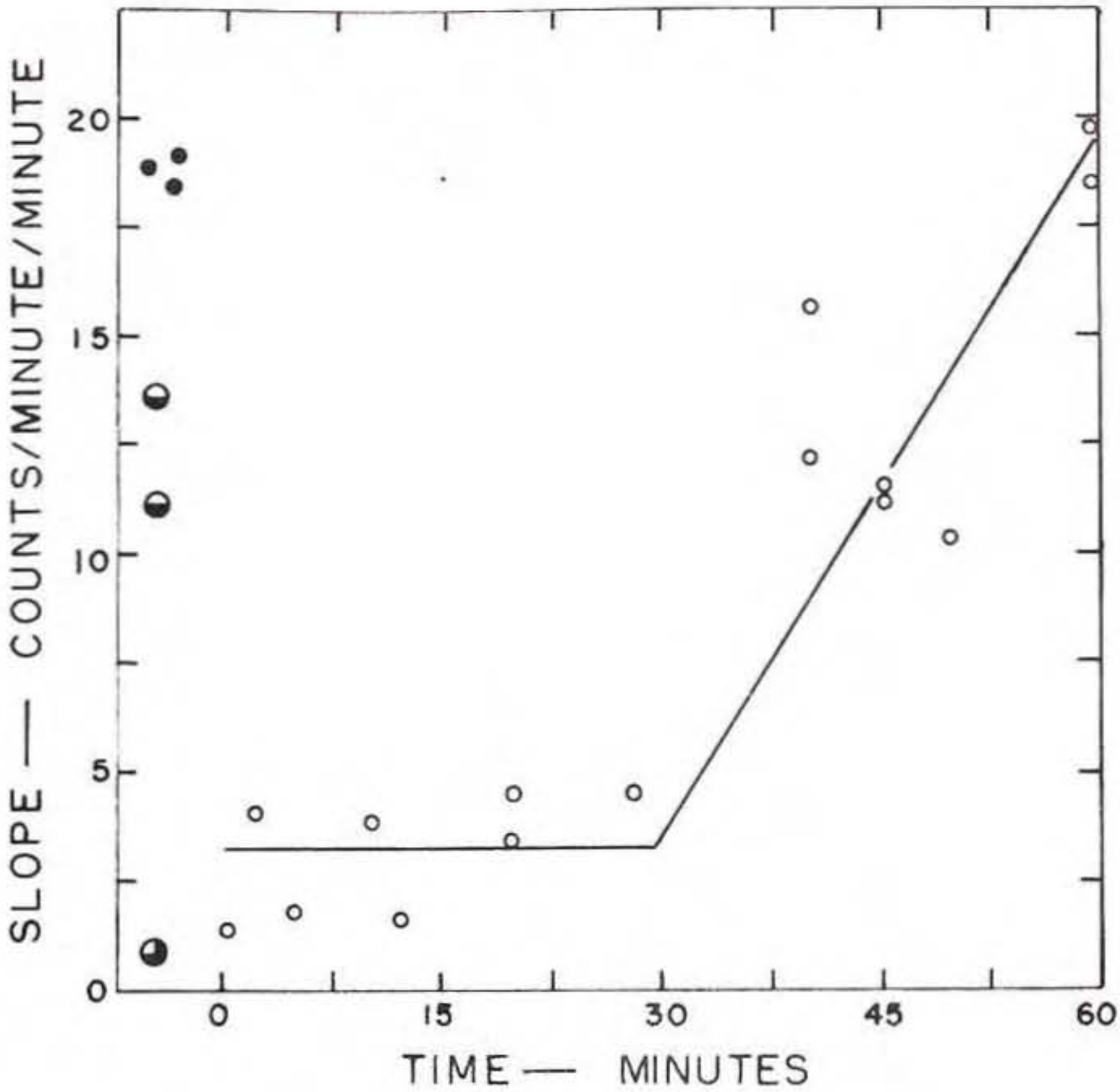

Figure 5. Effect of 10^{-4} M DNP on the rate of accumulation of P^{32} when the DNP is added at various times after fertilization.
ordinate - average slope from fertilization to the end of the experiment.
abscissa - time after fertilization when the DNP was added.
○ - fertilized eggs and DNP.
● - fertilized eggs.
◒ - artificially activated eggs.
◕ - unfertilized eggs.

if the maximum rate of uptake has been established by 40 minutes, the inhibition is considerably reduced. Experiments continued for three hours after the application of DNP at 50 minutes showed no signs of decreased uptake. It appears that once P^{32} uptake has been firmly established, as at 40 minutes after fertilization, DNP has no effect on this accumulation.

It therefore seems that fertilized eggs are sensitive to DNP, as far as P^{32} uptake is concerned, only when the DNP is introduced during the first 30-45 minutes following fertilization. The mode of action of DNP is generally thought to be to the uncoupling of phosphorylation from oxidation. The idea stems from the observations of Loomis and Lipmann (1948), who found that DNP stimulated respiration in rabbit kidney homogenates at the same time that phosphate uptake from the medium was prevented. Further work on the mechanism of action indicates that DNP either inhibits the production of high-energy phosphate by the tricarboxylic acid cycle or subsequently breaks it down. The proposed site of action seems to be related to high-energy phosphate production associated with electron transport from substrate to O_2 (Lardy and Wellman, 1953). Consequently it has been observed that many energy requiring processes, such as adaptive enzyme formation in Escherichia coli, yeast growth, and phosphate uptake by yeast, are inhibited by DNP. This information has been summarized recently by Simon (1953).

2,4-DNP and other substituted phenols inhibit cell division in the eggs of Arbacia at the same time that respiration is stimulated. The effects of these agents on sea urchin eggs have been studied in an

extensive series of experiments by Clowes, Krahl, and coworkers which have been reviewed by Clowes (1951). For a number of substituted phenols, the concentration giving the maximum respiratory stimulation causes a 50% inhibition of cell cleavage. The cleavage inhibition is reversible upon removal of the substituted phenol. Experiments with cell-free particulate systems from Arbacia (Clowes, Keltch, Strittmatter, and Walters, 1950), capable of exhibiting oxidative phosphorylation, show that DNP inhibits phosphorylation and stimulates respiration. It is interesting that the concentration of a substituted phenol that will inhibit cleavage is the same as the concentration that will inhibit phosphorylation in a cell-free system. The conclusions drawn from this similarity in the effective concentrations, is that DNP inhibits cleavage by interfering with high-energy phosphate production. Here again is an energy-requiring process that is inhibited by DNP.

This effect of DNP on oxidative phosphorylations, and, therefore, cleavage, would not seem to be limited by any time factor as evidenced by the inhibition of cell division at all times. The time-limited sensitivity shown in P^{32} uptake is, therefore, surprising. The inhibition during the first 30 to 45 minutes could be attributed to an interference by DNP with aerobic phosphorus metabolism and therefore with energy requiring processes, but it would be most unlikely that such interference would cease after 40 minutes or so. An hypothesis that would explain this limited inhibition is that there is a synthesis of a phosphate entry mechanism in the egg surface during the first 30 to 40 minutes following fertilization. The energy requirements for the establishment of this

system might be greater than those necessary for its maintenance and operation. Therefore, when DNP is applied during the first 30 or 40 minutes it might inhibit the establishment of this system, but when added at a later time, would not affect its maintenance and operation. This suggestion will be discussed.

It seemed desirable in evaluating this hypothesis to work with other inhibitors whose action would be entirely different from that of DNP. It was also advantageous to use a non-penetrating agent, as an important criterion for determining if a phosphate entry mechanism were located on the cell surface would be interference by a non-penetrating inhibitor (Rosenberg and Wilbrandt, 1952). Experiments were therefore undertaken with arsenate, which is a competitive inhibitor of phosphate because of its structural similarity. Kamen and Spiegelman (1948) have demonstrated that arsenate inhibits P^{32} uptake in yeast. This inhibition was dependent on the relative concentrations of phosphate and arsenate as would be required by a competitive inhibitor. It had been tentatively concluded by Yeas (1950) that arsenate does not penetrate the eggs of the echinoid Lytechinus pictus. This conclusion was based on experiments that showed that arsenate had no effect on respiration or on cleavage. It was assumed that if it had penetrated, there would have been an interference with oxidative phosphorylation, and consequently respiration and cleavage would have been affected. Therefore any inhibition of P^{32} uptake in eggs by arsenate would presumably be due to an interference with some surface reaction.

As shown in Figure 6, a concentration of 10^{-4}M sodium arsenate

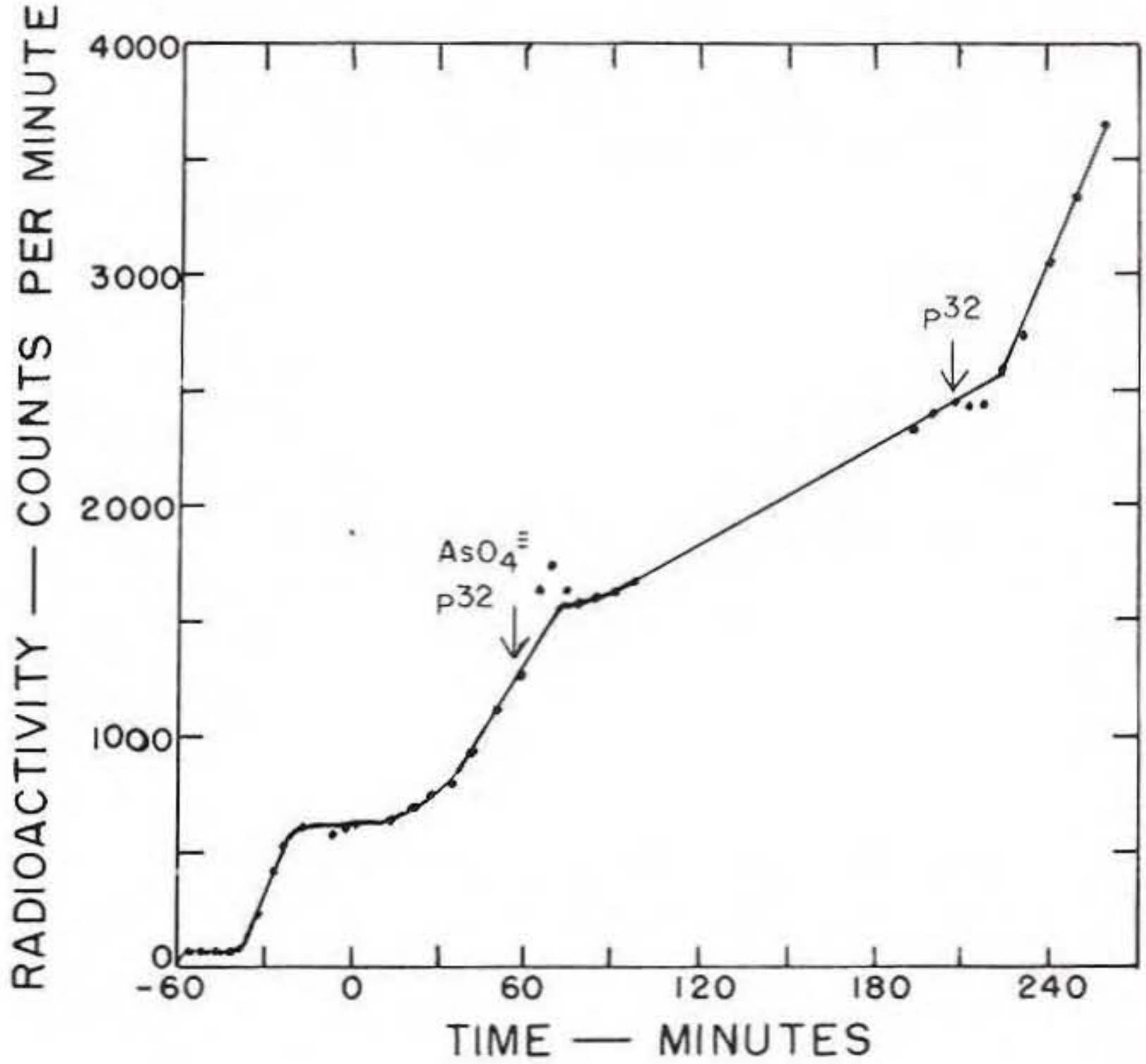

Figure 6. Effect of 10^{-4} M arsenate on P^{32} uptake. The initial low level of activity represents the background count. Fertilization was at zero time. Arrows indicate a change in the perfusion solution. The new solution reached the chamber 2 to 5 minutes later.

markedly inhibits P^{32} uptake. Unlike DNP, this inhibition occurs whenever arsenate is applied from fertilization to 3½ hours after fertilization, and is readily reversible upon removal (Fig. 6, Table 2). A decrease in the rate of P^{32} accumulation sets in within 1 to 5 minutes after the arsenate has first entered the chamber. Recovery from this inhibition takes place within a comparable period of time after the arsenate has been removed from the egg chamber. When arsenate is applied at fertilization, it does not appear to have any effect on the length of the lag period, and a limited rate of uptake commences at the end of this period.

When both DNP and arsenate are applied at 60 minutes following fertilization, a decrease in uptake occurs. Subsequent removal of only the arsenate results in an increased rate of P^{32} accumulation (Table 2). This would be expected since at 60 minutes after fertilization, 10^{-4}M DNP alone seems to be without any effect on uptake. In view of the evidence that Ycas has presented that arsenate does not penetrate the sea urchin egg, nor inhibit its respiration, its interference with phosphate uptake is interpreted as meaning that it is inhibiting a surface reaction, and this reaction is probably not directly linked to respiratory metabolism.

Krizat and Rumstrom (1951) and Bennett (1953) have reported that ATP will partially reverse the cleavage block due to DNP. In the light of these results it was thought that ATP might overcome the inhibitory effect of DNP on P^{32} uptake when applied during the lag period. To the contrary, however, when the eggs were perfused with ATP alone in a

Table 2

AsO_4 Experiments			AsO_4-DNP Experiments			ATP, ATP-ADP Experiments		
Time Added Min. After Fert.	Perfusion Solution	Slope-Counts/ Min/Min	Time Added Min. After Fert.	Perfusion Solution	Slope-Counts/ Min/Min	Time Added Mins. After Fert.	Perfusion Solution	Slope-Counts/ Min/Min
0	AsO_4, P^{32} sea water	6.0	0	P^{32} sea water	14.6	0	P^{32} sea water	16.7
89	P^{32} sea water	25.0	56.0	AsO_4, DNP, P^{32} sea water	4.5	65	ATP, P^{32} sea water	1.3
144	AsO_4, P^{32} sea water	7.0	101.0	DNP, P^{32} sea water	13.9	95	P^{32} sea water	20.0
						197	ATP, P^{32}	2.4
0	P^{32} sea water	20.3	0	P^{32} sea water	12.0	0	P^{32} sea water	21.2
55	AsO_4, P^{32} sea water	6.9	59.0	AsO_4, DNP, P^{32} sea water	3.0	67	ADP, P^{32} sea water	9.3
205	P^{32} sea water	28.0	131.0	DNP, P^{32} sea water	9.3	181	P^{32} sea water	32.3
			0	P^{32} sea water	12.9	240	ADP, P^{32} sea water	31.0
			57.0	AsO_4, DNP, P^{32} sea water	4.7	296	ADP, P^{32} sea water	3.4
			105.0	DNP, P^{32} sea water	10.2			
			295.0	P^{32} sea water	9.5			

concentration of 10^{-4}M, P^{32} uptake was very markedly inhibited (Fig. 7, Table 2). This inhibition, while more complete, was comparable to that obtained with 10^{-4}M sodium arsenate in that it is not limited to any particular time following fertilization, its effects can be readily reversed upon removal, and both inhibition and recovery occur within 1 to 5 minutes. The almost immediate effect of ATP, once it has entered the egg chamber, suggests that it may be acting on the surface of the egg. In this respect its action is again similar to that of arsenate. The possible significance of these similarities will be discussed.

Inhibition of approximately the same degree is obtained with 10^{-4}M ADP, but not with 10^{-4}M AMP (Fig. 8, Table 2). The latter has no effect on uptake.

In view of the probability that a chemical reaction is involved in phosphate entry, it was anticipated that low temperatures would result in considerable inhibition of uptake. Two experiments were carried out in which the temperature was initially 8.0°C, followed by an increase to 18.0°C in the middle of the experiment. At 8.0°C there was a comparatively low rate of P^{32} uptake, which was readily stimulated when the temperature was raised to 18.0°C (Fig. 9). From these experiments a Q_{10} of 2 and 2.3 was calculated, comparable to the value of 2 obtained by Villee and Villee (1952) with Arbacia.

Several experiments were carried out with cyanide in an attempt to investigate the relationship between respiration and P^{32} uptake. Cyanide is known to suppress respiration in sea urchin eggs up to 80% because of

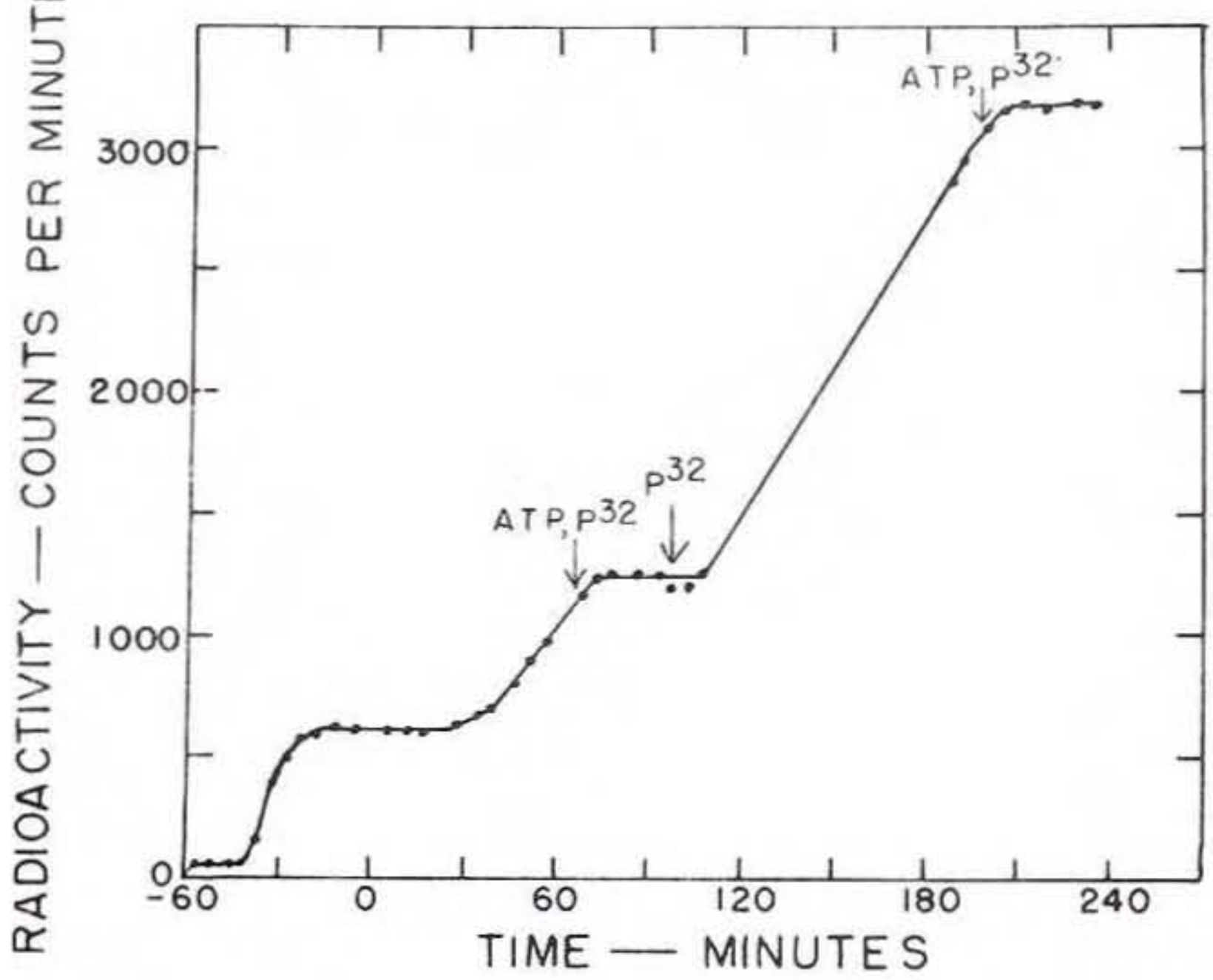

Figure 7. Effect of 10^{-4} M ATP on P^{32} uptake. The initial low level of activity represents the background count. Fertilization was at zero time. Arrows indicate a change in the perfusion solution. The new solution reached the chamber 2 to 5 minutes later.

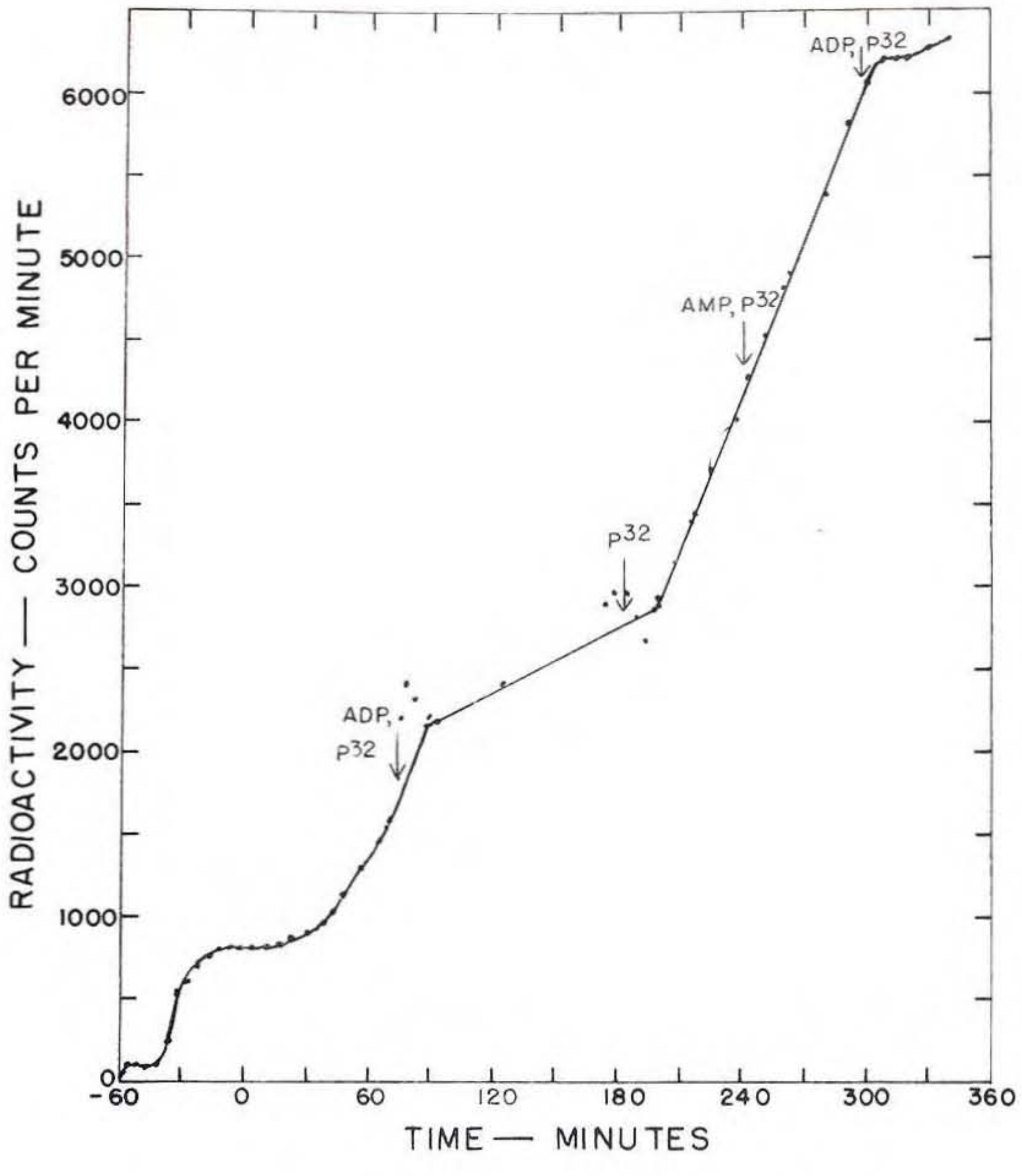

Figure 8. Effect of 10^{-4} M ADP and 10^{-4} M AMP on P^{32} uptake. The initial low level of activity represents the background count. Fertilization was at zero time. Arrows indicate a change in the perfusion solution. The new solution reached the chamber 2 to 5 minutes later.

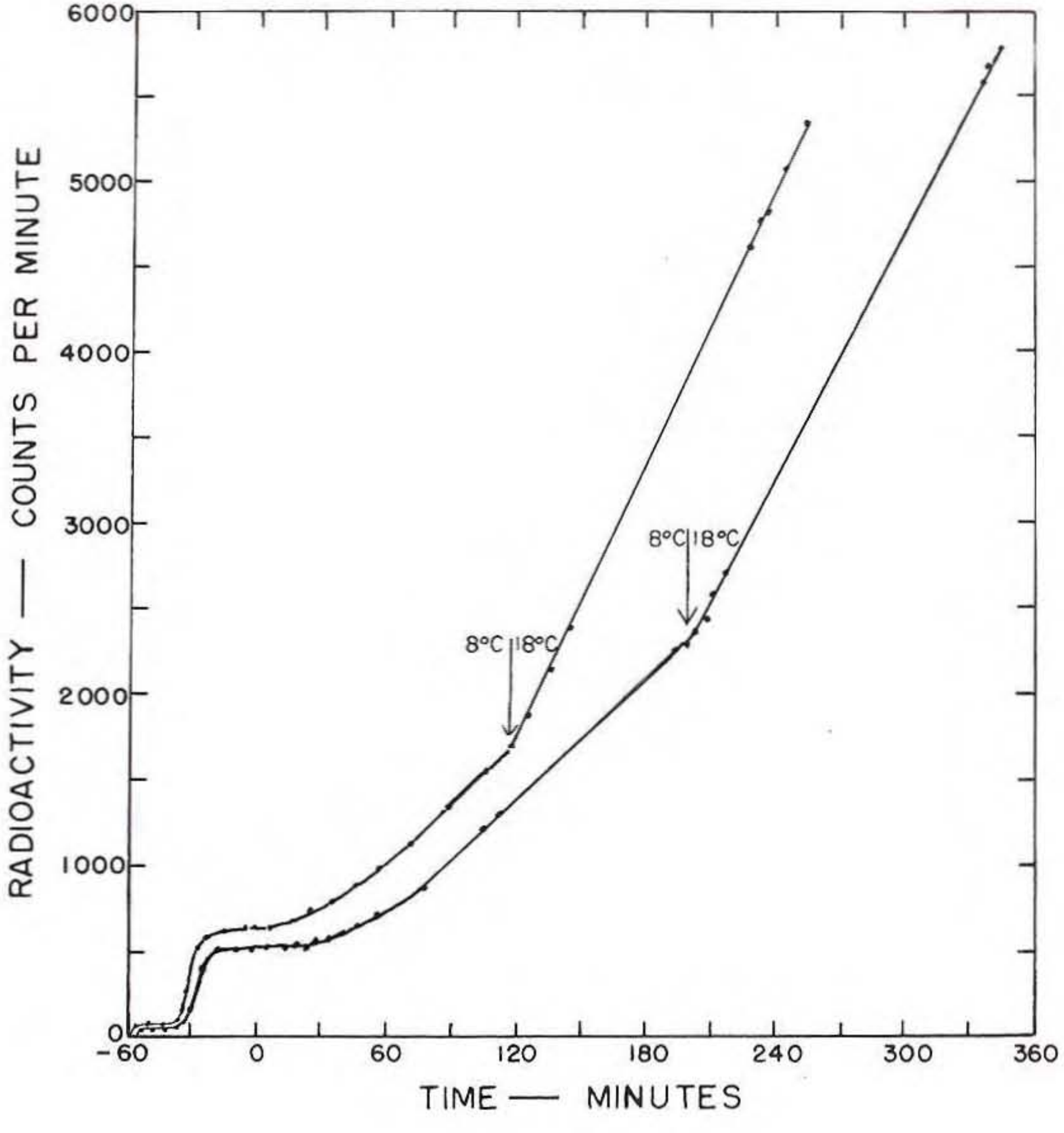

Figure 9. Effect of temperature on P^{32} uptake.
The initial low level of activity represents the background count. Fertilization was at zero time. Arrows indicate a change in the temperature of the perfusion solution.

its action on cytochrome oxidase (Keilin, Hartree, Rothschild, and Glover, 1941). A concentration of 10^{-3}M sodium cyanide in sea water was added at various times from fertilization to 60 minutes post-fertilization, and the effects on P^{32} uptake were followed. This is a concentration that will decrease respiration and inhibit normal development. It also causes some cytolysis (Robbie, 1946, 1948). The results were inconclusive, as the degree of inhibition was variable and was not clearly related to the time when the cyanide was added. In all cases, cell division was blocked, but the subsequent development of the eggs after removal from the chamber was variable and was never completely normal. A possible relation between the degree of inhibition and a secondary effect of the cyanide is revealed, however, by close examination of the data. In those experiments exhibiting the least inhibition of P^{32} uptake, recovery upon the removal from the cyanide was more nearly complete than in those experiments showing greatly reduced uptake. If this relation is borne out in future experiments, the explanation of the cause of the inhibition of uptake might be more closely associated with the cytolysis caused by the cyanide than by its effects on respiration, and the variability might lie more in the sensitivity of the structure of the cöplasm than in the sensitivity of its cytochrome system to cyanide. It seems very probable, in view of the previous experiments, that any agent causing cytolysis, particularly on the surface of the egg, would inhibit P^{32} uptake. It would be interesting to test the effect on P^{32} uptake of agents causing a mild surface cytolysis and in comparison with anaerobiosis. The results obtained might indicate whether cyanide is effecting P^{32} uptake by some surface action or by its effect on respiration, or by both means.

Chapter II

The evidence, taken as a whole, suggests the view that the rapid penetration of phosphate into sea urchin embryos is an enzymatically controlled transport. The reaction has a temperature coefficient of 2 to 2.3, which is compatible with this possibility though not, by itself, conclusive (Danielli, 1952). It is inhibited by arsenate, a competitive analogue of phosphate. Certain phosphate compounds, TP and ATP, but not AMP, inhibit the reaction. Furthermore, other investigators have found that the uptake is independent of the external phosphate concentration over a wide range, and have calculated that phosphate can penetrate against a steep concentration gradient (Brooks and Chambers, 1954; Chambers and White, 1954).

That the enzymatic mechanism is surface located seems most probable from the results of the arsenate and adenosine phosphate experiments. The bases for this conclusion from the effects of arsenate and of cyanide have been discussed earlier. The almost immediate effect brought about by the addition of ATP and ADP, and the unlikelihood that they would inhibit by any internal action also support this conclusion.

The specific enzymatic reaction by which phosphate enters the embryo is not definitely elucidated by these experiments, but certain possibilities are suggested by the arsenate experiments. Arsenate is known to be a competitive analogue of phosphate, and therefore will substitute for it in enzymatic reactions. The resulting arsenate compound is usually unstable and is hydrolyzed instantly. Hence, the term arsenolysis has been applied to the action of arsenate (Doudoroff, Barker, and Hassid, 1947). Although

arsenate would be expected to compete with phosphate in any reaction, Crane and Lipmann (1953) report that P^{32} incorporation into ATP is particularly sensitive. Phosphate incorporation into ATP has been the proposed method for phosphate entry into erythrocytes (Gourley, 1952) and liver cells (Sacks, 1951). If the same mechanism were operative in the fertilized eggs, this inhibition by arsenate would be expected, although if some other phosphate compound were involved, arsenate should also inhibit.

The unexpected inhibition by ATP and ADP may also be interpreted as favoring this mechanism. All phosphate compounds do not inhibit phosphate entry as evidenced by adenylic acid, and therefore the inhibition by ATP and ADP may be specific. This, however, cannot be definitely stated until a wide variety of phosphate compounds have been tested. If ADP and ATP are unique among phosphate compounds in inhibiting P^{32} uptake, it suggests that the incorporation of P^{32} into ATP may be important in phosphate penetration. If some further reaction, after ATP formation, were necessary for phosphate entry, the added ATP might be expected to compete with the newly formed ATP containing P^{32} and thereby effectively inhibit P^{32} uptake. It is known that added P^{31} will prevent appreciable uptake of P^{32}, presumably by dilution (Lindberg, 1950). Added ATP would seem to be acting in the same way as the added P^{31}. Formation of ATP as a means of phosphate entry into sea urchin eggs receives support from Lindberg's work on Paracentrotus lividus (1950) which has been mentioned previously.

The time of establishment of the mechanism is within the first 40 to 50 minutes, varying with eggs from different urchins. Whether its appearance

as a functional system is during the lag period of 7 to 30 minutes, or whether the subsequent period of increasing activity represents the time of its establishment is not answered by these experiments. The experiments with parthenogenesis show clearly that the establishment of the mechanism is not dependent on the sperm, nor on the existence of a cleavage mechanism, since such activation did not lead to cleavage.

There remains to be considered the relation between the egg's metabolism and the transport mechanism. The present experiments with DNP show that the inhibition of formation of high-energy phosphate by aerobic oxidations during the first 30 minutes after fertilization prevents very markedly the later uptake of phosphate. However, it appears that there is enough energy for this process at 60 minutes after fertilization despite the presence of DNP. At this same time, DNP is presumably having its effect on oxidative phosphorylation since cleavage is blocked.

Two interpretations of these results present themselves. According to one, the initial period may be sensitive to DNP because aerobic phosphate bond energy is needed for the synthesis of the enzymes of the transport mechanism as is the case with adaptive enzyme synthesis (Monod, 1944; Reiner, 1946; Spiegelman, 1947), or perhaps for the spatial rearrangement of the preformed system. The later period may be insensitive to DNP because the maintenance and operation of the mechanism requires quantitatively smaller amounts of aerobic phosphate bond energy.

An alternative to the idea that the energy requirements for the establishment of such a transport mechanism may be quantitatively larger than those for its operation and functioning, has been suggested by a paper

by Sielevitz and Potter (1953). It may be that the energy source for the establishment of a phosphate entry mechanism may be different from that for its maintenance and operation. In experiments with rat liver mitochondria, they concluded that the ATP generated within the mitochondria diffused out and mingled very slowly with that generated by glycolysis outside of the mitochondria. Consequently, there may be a separation in the functions of the ATP formed in these two locations. Synthetic reactions within the mitochondria would preferentially utilize the ATP generated locally, while an energy requiring reaction outside of the mitochondria would be served by ATP produced by glycolysis externally. It may be that the establishment of a phosphate transport mechanism involves enzyme synthesis which is favored by ATP formed within the mitochondria. DNP could inhibit such a process by its action on the tricarboxylic acid cycle, as the enzymes for this cycle are associated with the mitochondria. Once the mechanism is established it might be maintained by high-energy phosphate resulting from glycolysis. DNP presumably does not inhibit the formation of high-energy phosphate by this means, although this has not been fully investigated (Simon, 1953). However, Clowes and Keltch (1951) were unable to inhibit phosphorylations associated with glycolysis by means of dinitrocresol. These experiments were with the soluble fraction from rat liver and brain homogenates. According to these interpretations, inhibitors known to interfere with the production of ATP by glycolysis should interfere with the operation of the established phosphate entry mechanism but not with its formation. Such inhibitors would include azide and monoiodoacetic acid. It is

suggestive that these are known to inhibit uptake by yeast (Kamen and Spiegelman, 1948).

According to the results of Holst (1952) the rate of penetration of phosphate increases rapidly for 35 hours in the embryos of Strongylocentrotus purpuratus. This is in accord with the present findings that the transport system is surface located because during the development to the gastrula, the number of cells, and therefore the surface area of the embryos, increases through cleavage, and it is reasonable to suppose that the newly formed surface would possess the transport mechanism.

SUMMARY

1. The accumulation of phosphate by the eggs and embryos of the sea urchin, Strongylocentrotus purpuratus (Stimpson) was analyzed by determining the action of various metabolic effectors on the uptake of P^{32} from sea water flowing at a constant rate over the eggs in a special perfusion chamber.

2. The rate of uptake of P^{32} by unfertilized eggs is nearly zero. The rate for the first 7 to 30 minutes after fertilization (lag phase) is also nearly zero, but increases rapidly during the next 20 to 30 minutes (augmentative phase), and becomes maximal 22 to 60 minutes after fertilization (accumulation phase) at a level many times that of the unfertilized eggs.

3. Artificial parthenogenesis, by either the single or double treatment, results in the same pattern of uptake as does fertilization, even in the absence of cleavage.

4. Phosphate accumulation is markedly inhibited by 10^{-4}M 2,4-dinitrophenol if this agent is added during the lag phase, moderately inhibited if added during the augmentative phase, but is unaffected if added during the accumulation phase.

5. 10^{-4}M arsenate markedly inhibits P^{32} uptake at all times after fertilization.

6. 10^{-4}M ATP almost completely inhibits P^{32} uptake at all times after fertilization; 10^{-5}M ATP markedly inhibits uptake during the accumulation phase, while 10^{-4}M ATP does not affect uptake at all when added during the accumulation phase.

7. Inhibition in the presence of cyanide was variable and may be partially due to a surface cytolysis by this agent.

8. P^{32} uptake has a temperature coefficient of 2 to 2.3 during the accumulation phase.

9. The evidence resulting from the use of these effectors indicates that P^{32} uptake in sea urchin embryos is enzymatically controlled and that the enzymatic mechanism is located on the surface. It is suggested that formation of ATP may be involved in phosphate entry. The period immediately following fertilization is believed to be a time when the uptake mechanism is being established. This process appears to be dependent on phosphate bond energy, the production of which is DNP sensitive. During the accumulation phase it is suggested that the energy requirements for the operation and maintenance of this mechanism are quantitatively much smaller, or are satisfied by phosphate bond energy the production of which is DNP insensitive. Possible reasons for this difference in sensitivity are discussed.

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P^{32} uptake

Suggestions for further expts — from Judy Littlefield

1. S. faec — time to max uptake rate = 80-90'
   vs 50-60 for S. f., therefore DNP resistant period should be longer

2. Does IP pick up = egg due to DNP
   eg.- P released can't go on to P acceptors

3. Time course / relative spec. act. of P cpds. to get precursor of intracell. IP.
   Also IP = rad. is precursor of what P-cpd = egg? Use criteria of fractionat.

4. Test theory that glycolytic P furnishes energy for uptake by P^{32} uptake and estab.. by use of inhibitors known to interfere = steps in glycolytic cycle known to lead to prod. of ~~HE~~ ~P. NaN_3, MIA, F?, malononitrile.

5. Test effects of Analog. + some agent {but. acid??} causing acidosis cytologic to see whether CN effects might be due to both resp. effect + cytol.

6. Test effects of other P-cpds. to see if ATP-ADP uptake is specific.

7. Long term exp. (a) to check Bolet's method — rate (b) see if any direct correl.

with increased = surface area for cleavage.

8. Maybe not active transport but have "active patches" = surface which are selective for PO_4 + will allow rapid diffusion (Daniellí — Proc. Roy Soc. 142:153 (1954) — Energy required to maintain these patches (also probably for their formation) but no stoichiometric relation between energy required & no. of moles. passing across membrane — no conc. gradients built up as in true active transport e.g. — term facilitated diffusion vs. selective active transport

The conc. of PO_4 would seem very important to deter. as Chambers + White 1954 question their high values + suggest increased uptake but fact due to decrease in IP conc. due to form. of PO_4 esters at fast +:. favorable gradient.

3/

9. F - Dont abort Richardes exp! -

eg. separate endoplasm from cortex by

bursting egg = C_2SW — ends → nosedevel. ; cortex → devel.

?!

if could get enough of both Ca & P^{32} uptake by both. Maybe only cortices would take it up.