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DEMONSTRATION OF THE FORMATION OF 1,3-DIPHOSPHOGLYCERIC ACID AS AN INTERMEDIATE IN THE HIGH-ENERGY PHOSPHATE METABOLISM DURING REINITIATION OF DEVELOPMENT IN *ARTEMIA*

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Extensive labelling of the glycolytic intermediate 2,3-diphosphoglycerate by $^{32}PO_4^{3-}$ during the early periods of development in *Artemia* is reported. At 30 min of activation this is the major labelled compound. The mobilization of inorganic phosphate through glycolysis leading to the formation of 1,3-diphosphoglycerate results in the formation of a high-energy phosphate donor. The label from this compound could be chased to high-energy phosphates (adenine derivatives). The location and subsequent high degree of labelling of 2,3-diphosphoglycerate in the yolk platelets further demonstrate the important role played by this organelle in the metabolic events accompanying the breakdown of dormancy in *Artemia*.

Introduction

The breakdown of dormancy of the cryptobiotic embryos of *Artemia* is accomplished in response to a simple trigger such as a decrease in salinity of the environment [1]. The study of the nature of the early macromolecular and biochemical changes has been difficult on account of the extreme impermeability of the cysts to precursors [2]. We have demonstrated that carrier-free $^{32}PO_4^{3-}$ can be taken up by the cysts after sodium hypochlorite treatment [3]. This enabled us to study the turnover of preformed mRNA species during early development [4].

The energy requirements of the developing cysts are not clearly understood. The cysts are rich in storage materials like trehalose and glycerol [5–7]. Careful analysis of the changes in their levels accompanying the initiation of development has shown that trehalose is rapidly metabolised to glycerol while the glycerol level remains constant [8]. Apparently, there is a compartmentation of the glycerol in the developing embryos as it is required both for the preservation of the osmotic conditions [2] and for glycolytic reactions.

Using $^{32}PO_4^{3-}$-labelled cysts in the present studies, we have analysed the pattern of labelling of high-energy phosphate intermediates during early development. An unusual observation is reported here concerning the formation of 1,3-diphosphoglycerate as the major high-energy phosphate derivative during early periods of activation.

Materials and Methods

Materials

Uniformly labelled [^{32}P]orthophosphate (carrier-free) was purchased from Bhabha Atomic Research Centre, Bombay, India. *Artemia* cysts (Parthenogenetic strain) were collected from the salt pans at Tuticorin, South India. DEAE-cellulose, NADH, glyceraldehyde-3-phosphate dehydrogenase, ATP, AMP, ADP, GMP and CTP were from Sigma Chemicals, U.S.A.

Methods

Actiation. Cysts treated with 20% hypochlorite for 7 min were activated in 1% saline with a 40 W light source [21] and aeration at 30°C. For labelling experiments [^{32}P]orthophosphate (carrier-free) was provided, while maintaining the same incubation conditions.

Analysis of acid-soluble nucleotides. Cysts (500 mg) incubated in 5 ml of 1% saline with 250 μCi $^{32}PO_4^{3-}$ following activation were washed with 0.1 M phosphate buffer, pH 7, and homogenised in 7 ml of 2.5 M perchloric acid and left in ice for 30 min. The homogenate was centrifuged at 10000 rpm for 10 min in an RC 5B Sorvall centrifuge. The supernatant was neutralised with KOH solution and spun again at 10000 rpm for 10 min. The acid-soluble materials were analysed in DEAE columns [20].

Neutralised acid-soluble material (100 $A_{260\,nm}$) was loaded on a DEAE-cellulose (capacity 0.89 mequiv./g) column (1 × 20 cm) which was previously activated and equilibrated with 0.002 M NH_4HCO_3 (pH 8.6). The column was precalibrated with ATP, AMP, ADP, CTP and GMP. Elution of nucleotides was carried out by using a linear salt gradient from 0.002 (700 ml) to 0.25 M NH_4HCO_3, pH 8.6 (700 ml), at a flow rate of 1 ml/min. Fractions (10 ml) were collected in a 2112 Redirac LKB fraction collector. The fractions were monitored for their absorbance at 260 nm in a DU 2 Beckman spectrophotometer and radioactivity was measured in a Beckman LS 100 C scintillation counter. Individual peaks were further identified by spectral analysis.

Subcellular distribution of nucleotides. Following activation to the desired time, the cysts were gound and homogenised in buffer containing 10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl. The homogenate was centrifuged at 1000 × *g* for 10 min and the pellet which contained both nuclei and yolk platelets [12] were washed with the same buffer, and the yolk platelets were purified by sucrose gradient (30–70%) centrifugation. The yolk platelets attained 90% purity by repeated passing through the gradient. The degree of nuclear contamination was determined microscopically by staining with 0.1% methyl green. The nucleus-free cytoplasmic fraction was spun at 30000 × *g* for 45 min in a Sorvall RC 5B centrifuge to obtain the particulate and cytosolic fractions. From the subcellular fractions acid-soluble nucleotides were extracted and analysed on DEAE-cellulose columns.

Quantitative estimation of diphosphoglycerate. The level of diphosphoglycerate is estimated by following the degree of oxidation of NADH to NAD which is catalysed by glyceraldehyde-3-phosphate dehydrogenase (GAP) and is the reverse of the reaction [22]:

$$GAP + \text{phosphate} + NAD^+ \rightleftharpoons \text{diphosphoglycerate} + NADH + H^+$$

Results

Incorporation of $^{32}PO_4^{3-}$ into the acid-soluble and -insoluble materials of developing embryos of Artemia

The uptake of $^{32}PO_4^{3-}$ into the cysts upon activation increased steadily (Table I). The initial level of label in the acid-soluble material was very high (83%), which declined with a concomitant increase in the acid-insoluble material until the pre-nauplius stage. It may be mentioned that incorporation of $^{32}PO_4^{3-}$ into acid-insoluble material could be detected as early as 30 min of activation.

TABLE I

INCORPORATION OF $^{32}PO_4^{3-}$ INTO ACID-SOLUBLE AND ACID-INSOLUBLE MATERIALS OF THE DEVELOPING EMBRYOS OF *ARTEMIA*

Batches of cysts (500 mg) were activated in the presence of 250 μCi $^{32}PO_4^{3-}$. After the given period of activation they were homogenized in 2.5 M perchloric acid and the incorporation data analysed.

Time of activation	Total incorporation (cpm) (× 10^6)	% cpm in	
		Acid-soluble material	Acid-insoluble material
10 min	8.6	83.2	16.8
30 min	6.6	71.0	29.0
60 min	17.6	68.0	32.0
120 min	33.6	64.0	36.0
3 h	45.8	61.0	39.0
8 h	67.8	57.0	43.0
12 h	68.9	51.2	48.8
18 h	72.6	48.5	51.5

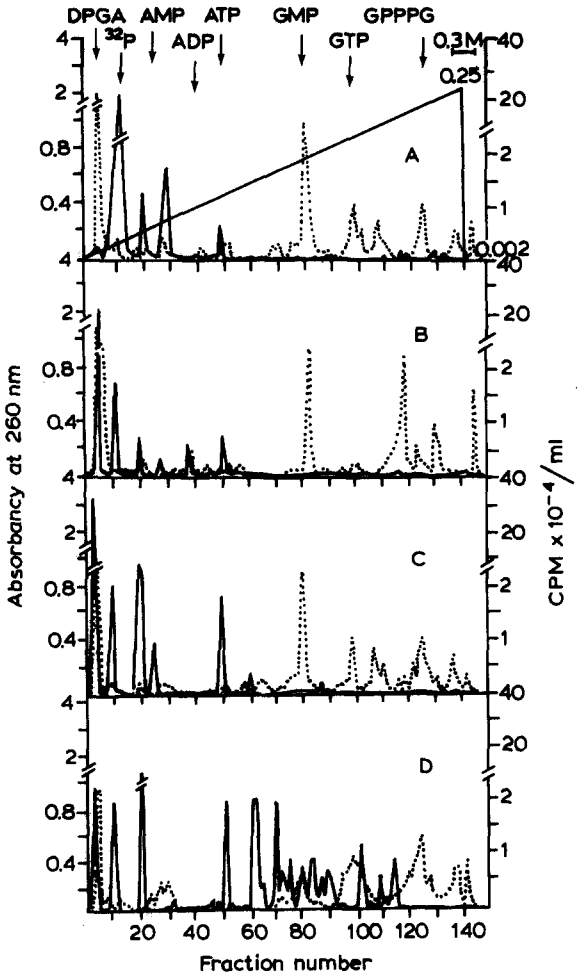

Fig. 1. DEAE-cellulose chromatographic profiles of acid-soluble materials of cysts activated for 10 (A), 30 (B), 60 (C) and 120 min (D). (———) cpm/ml, (- - - - -) absorbance at 260 nm.

Kinetics of incorporation of $^{32}PO_4^{3-}$ into different acid-soluble materials during activation

We analysed the labelled phosphorylated compounds of cysts activated for different periods. During the first 10 min of activation the majority of the label (93.3%) was present as inorganic phosphate (Fig. 1A). Soon after (30 min) the label was shifted to another fraction which was eluted well before $^{32}PO_4^{3-}$ (Fig. 1B) at a salt concentration of 0.003 M NH_4HCO_3. At this period of activation this fraction had the highest amount of label (75%). Upon further activation (60 min), labelling of adenine nucleotides such as AMP, ADP and ATP increased (Fig. 1C). At 120 min of activation the labelling in fraction 3 decreased further, with increased labelling of other adenine nucleotides (Fig. 1D). The kinetics of labelling of the various acid-soluble materials in developing embryos up to 18 h are given in Table II.

Characterization of the materials of the highly labelled fraction

Lyophilised fraction 3 containing the highly labelled materials obtained from cysts activated for 30 min was used to characterise the components. The labelled materials were not retained in a charcoal column, indicating that they were not nucleotides. The major labelled compound, containing more than 90% label, was identified as 2,3-diphosphoglyceric acid initially by paper chromatography [9] by comparison with 2,3-diphosphoglycerate isolated from pig blood erythrocytes [10] and staining for glycerate.

TABLE II

KINETICS OF INCORPORATION OF $^{32}PO_4^{3-}$ INTO DIFFERENT ACID-SOLUBLE MATERIALS DURING ACTIVATION

Cysts were continuously labelled with $^{32}PO_4^{3-}$ to the desired time. The acid-soluble materials were fractionated on DEAE-cellulose columns and individual fractions were monitored for their absorbance at 260 nm and radioactivity. % label = $\frac{\text{cpm in fraction}}{\text{total cpm input}} \times 100$. n.d., not detectable.

Time of activation	% label in						
	P_i	PP_i	AMP	ADP	ATP	GpppG/GppppG	Fraction 3
10 min	93.0	2.0	n.d.	0.3	0.4	n.d.	0.4
30 min	10.5	1.8	1.0	1.3	0.8	0.1	75.0
60 min	4.8	2.2	27.0	0.4	3.2	1.4	61.0
3 h	2.8	3.9	24.3	1.1	2.4	10.9	38.0
8 h	2.7	6.4	13.3	2.4	6.8	7.8	27.0
18 h	3.9	5.1	9.6	7.4	12.6	4.5	3.8

TABLE III

LEVELS OF 2,3-DIPHOSPHOGLYCERATE IN DEVELOPING EMBRYOS OF *ARTEMIA*

Fraction 3, derived from the samples activated to the desired time, was estimated quantitatively by an assay system involving glyceraldehyde-3-phosphate dehydrogenase and NADH. The level of diphosphoglycerate is expressed as nmol NADH oxidised/250 mg cysts.

Time of activation (min)	Level of diphosphoglycerate
0	450
30	17400
60	6600
120	7650
240	2400

A small amount of label was also present in the materials that co-migrated with 1,3-diphosphoglycerate and serine. Further identification of 2,3-diphosphoglycerate and the quantitative estimation of its level in developing embryos was made with an assay system involving glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) and NADH [11]. The level of diphosphoglycerate in the developing embryos is listed in Table III. The other major compounds present were unlabelled NADH and CTP. NADH was identified by an assay system involving malic dehydrogenase (EC 1.1.1.37), oxaloacetate and CTP by spectral analysis.

Correlation between labelling of 2,3-diphosphoglycerate and adenine nucleotides

Cysts labelled with $^{32}PO_4^{3-}$ for 30 min were washed with an excess of phosphate buffer and were further activated in the presence of unlabelled phosphate in 1% saline for another 30 min and the distribution of labelling in the acid soluble materials was analysed. As can be seen from Table IV there was a considerable decline in the level of labelling in 2,3-diphosphoglycerate accompanied by an increase in labelling of ADP and ATP over and above the normal level observed at 60 min of activation. Moreover, we found that AMP, ADP and ATP were labelled at all their phosphate positions. When cysts were activated in the presence of unlabelled phosphate for a period of 30 min and then with $^{32}PO_4^{3-}$ until 60 min, most of the label

TABLE IV

CORRELATION BETWEEN LABELLING OF 2,3-DIPHOSPHOGLYCERATE AND ADENINE NUCLEOTIDES

Two batches of cysts (500 mg) were labelled for 30 min with $^{32}PO_4^{3-}$ (250 μCi), one batch was homogenised in 2.5 M perchloric acid and acid-soluble materials were analysed on a DEAE-cellulose column. The other batch was washed with unlabelled phosphate incubated for another 30 min in the presence of unlabelled phosphate in 1% saline. n.d., not detectable. 2,3-DPG, 2,3-diphosphoglycerate.

Activation conditions	% label in					
	2,3-DPG	P_i	PP_i	AMP	ADP	ATP
Control 30 min activation with $^{32}PO_4^{3-}$)	76.0	15.0	3.6	1.6	n.d.	1.0
30 min activation with $^{32}PO_4^{3-}$ + 30 min chase	48.4	5.8	6.2	17.0	7.0	9.5

appeared in fraction 3, indicating that the 2,3-diphosphoglycerate was the principal labelled derivative of $^{32}PO_4^{3-}$.

Labelling of 2,3-diphosphoglycerate in the subcellular fraction

In order to localise the site of formation of labelled 2,3-diphosphoglycerate we labelled the

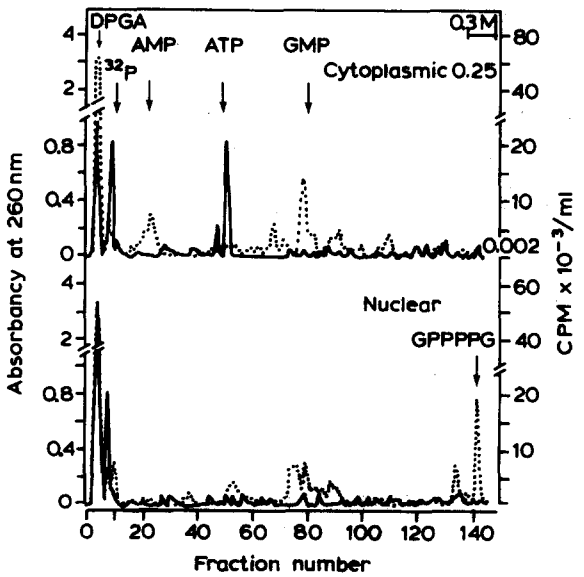

Fig. 2. DEAE-cellulose profile of acid-soluble materials of subcellular fractions from embryos activated for 30 min. (———) cpm/ml, (- - - - -) absorbance at 260 nm.

cysts with $^{32}PO_4^{3-}$ for 30 min and the nuclear and cytoplasmic fractions were analysed for labelling of the acid-soluble components. As shown in Fig. 2, the majority of labelled 2,3-diphosphoglycerate was present in the nuclear fraction containing yolk platelets (73%). When we further purified the yolk platelets which are present in abundance in *Artemia* embryos [12] from the nuclear fraction by sucrose gradient centrifugation, labelled 2,3-diphosphoglycerate was found to be predominant in the yolk platelets (86%).

Discussion

In our earlier studies, we demonstrated that $^{32}PO_4^{3-}$ can be incorporated into the poly(A) region of the preformed RNA [4]. Although we had demonstrated an increase in the levels of the enzyme polyadenylate polymerase along with a high turnover of poly(A) tails, formation of labelled ATP from $^{32}PO_4^{3-}$ was unclear. The present studies have brought forth an interesting mode of utilisation of inorganic phosphate in the formation of high-energy intermediates. The initial step would obviously be the formation of the high-energy phosphate donor 1,3-diphosphoglycerate from glyceraldehyde 3-phosphate and $^{32}PO_4^{3-}$ through glyceraldehyde-3-phosphate dehydrogenase. The presence of high levels of NADH in the nuclear fraction containing yolk platelets suggests the possibility of this conversion. On account of the highly unstable nature of 1,3-diphosphoglycerate [14], we were unable to demonstrate its labelling. The demonstration of a considerable amount of labelling in the glycolytic intermediate 2,3-diphosphoglycerate (75% of the total acid-soluble labelled material at 30 min of activation) suggests that glycolysis plays a major role in the utilisation of inorganic phosphate. However, only the donation of $^{32}PO_4^{3-}$ from 1,3-diphosphoglycerate can account for the high labelling of the adenine nucleotides, shown in the chase experiments (Table IV).

The presence of adenosine kinase, nucleoside and nucleotide phosphokinase [15] in the developing embryos suggests the stepwise synthesis of adenine nucleotides. The formation of 2,3-diphosphoglycerate from 1,3-diphosphoglycerate is well documented in erythrocytes [10]. The dormant cysts of *Artemia* are characterised by the presence of a high quantity of yolk platelets which sediment along with nuclei [12]. The presence of trehalose, diguanosine polyphosphates and the enzymes that utilise purines for DNA synthesis during development after pre-emergence [4] indicates that these yolk platelets play an important role as storage organelles. Our studies have indicated that they may also play a vital role in early energy metabolism.

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