CHANGES IN LEVELS OF TRIPHOSPHOPYRIDINE NUCLEOTIDE IN MARINE EGGS SUBSEQUENT TO FERTILIZATION

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SUMMARY

1. The steady-state levels of oxidized and reduced TPN have been measured in two species of marine eggs, *Arbacia punctulata* and *Spisula solidissima*, before and after fertilization.

2. Within 1 to 3 h after fertilization a 3 to 7 fold increase in concentrations of TPNH was observed, but no significant change in concentrations of oxidized TPN.

3. The net synthesis of TPN is discussed in relation to the predominant role of the hexose monophosphate shunt in glucose utilization by these eggs.

INTRODUCTION

It has been shown that during the early stages of cleavage of eggs of *Arbacia punctulata* utilization of glucose is predominantly via the hexose monophosphate shunt[1,2]. As development proceeds, the glycolytic pathway becomes quantitatively more important. However the amount of glucose oxidized via pathways requiring TPN increases many fold as development continues. The activities of two enzymes concerned with oxidation of glucose-6-phosphate, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, are considerably greater than the actual amount of glucose metabolized through the hexose monophosphate shunt[3].

To determine some of the factors controlling the rate of carbohydrate utilization, the steady state concentrations of TPN and TPNH were determined in the unfertilized and fertilized eggs of *Arbacia* as well as in the eggs of the surf-clam *Spisula Solidissima*. An increase of 3 to 6 fold in the levels of TPNH subsequent to fertilization has been regularly observed.

MATERIALS

TPN, TPNH, DPN, and DPNH were obtained from the Sigma Chemical Company. Alcohol dehydrogenase from yeast, 2 times crystallized was obtained from the

Abbreviations: TPN, oxidized triphosphopyridine nucleotide; TPNH, reduced triphospohopyridine nucleotide; DPN, oxidized diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

Sigma Chemical Company. Isocitric dehydrogenase was prepared from an acetone powder of pig heart by a modification of the method of MOYLE AND DIXON[7]. Glutathione reductase was prepared according to CIOTTI AND KAPLAN[6].

METHODS

The experiments were carried out at Woods Hole during the summers of 1958 and 1959. Eggs of *Arbacia punctulata* were obtained and handled by methods previously described[4]. *Spisula* eggs were handled according to the methods of ALLEN[5], although much larger numbers of eggs than he used were required for these experiments. *Arbacia* eggs were assayed during 3 stages of development at 20–25°, unfertilized, 1 and 3 h after fertilization and 24 h later when the eggs had reached the pluteus stage. *Spisula* eggs were assayed before and 1 to 3 h after fertilization at 20–25°.

At each stage of development extracts for assay were prepared as follows:

Oxidized pyridine nucleotides

After determination of the volume of eggs by centrifugation, a suspension in filtered sea water was made. Of this suspension 2.0 ml was homogenized for 1 min with 0.2 ml of 50 % trichloracetic acid, using a motor driven teflon or glass homogenizer. Following centrifugation, 1.0 ml of the supernatant was added to 1.0 ml of phosphate buffer at 0.1 *M*, pH 7.4 and was brought to pH 7.4 with NaOH. This solution was assayed for TPN according to CIOTTI AND KAPLAN by measuring the decrease in fluorescence induced by methyl-ethyl-ketone with and without TPN-specific isocitric dehydrogenase[6]. TPN was also assayed by a modification of the method of LOWRY, measuring the increase in the native fluorescence of TPNH in the presence and absence of TPN isocitric dehydrogenase[8]. DPN was assayed by the methyl-ethyl-ketone procedure on the same acid extracts, using yeast alcohol dehydrogenase, Tris buffer, pH 10.1, and ethanol 0.5 *M*. In the assay of the oxidized and reduced pyridine nucleotides the Farrand Model A Photofluorometer was used with Corning No. 5860 as primary filter and combining Corning Nos. 4308, 5562 and 3387 as secondary filters. Concentrations of TPN and DPN standards used in the fluorometric assays were previously determined by absorption at 340 mμ after reduction with isocitrate and isocitric dehydrogenase and ethanol and alcohol dehydrogenase respectively.

Reduced pyridine nucleotides

Of a suspension in filtered sea water of a known volume of eggs, 2.0 ml was added to an homogenizing tube containing 25 mg of Na_2CO_3 and mixed by shaking. The tube was heated in a water bath at 100° for 30 sec, homogenized mechanically for 60 sec, and returned to the 100° bath for an additional 60 sec. The homogenate was chilled on ice and centrifuged at 4°. To 1.0 ml of the supernatant was added 1.0 ml of phosphate buffer 0.1 *M* pH 7.4 and the solution cautiously brought to pH 7.4 by the stepwise addition of 0.4 *N* HCl. TPNH was assayed on this solution by the methyl-ethyl-ketone procedure by measuring the increase in fluorescence in the presence of oxidized glutathione and glutathione reductase partially purified from peas[6]. By controlling the concentration of the enzyme, temperature and time of incubation there was no oxidation of DPNH in this assay. DPNH was assayed

using methyl-ethyl-ketone by measuring the increase in fluorescence in the presence of $8.3 \cdot 10^{-3}$ *M* acetaldehyde and yeast alcohol dehydrogenase at pH 7.4. The concentrations of TPNH and DPNH standard used in the fluorometric assay were determined by measuring the decrease in optical density at 340 mμ with oxidized glutathione and glutathione reductase and acetaldehyde and alcohol dehydrogenase respectively.

In all assays analyses were performed in duplicate.

RESULTS

In both *Spisula* and *Arbacia* eggs, whether fertilized or unfertilized, the level of TPN was low. In *Spisula* concentrations were 11 and 5 mμmoles/ml of packed cells in unfertilized and fertilized eggs respectively; in *Arbacia* levels were 11 and 14 mμmoles/ml of packed cells in unfertilized and fertilized eggs, respectively. In all instances these values were at the lower limit of sensitivity of the method used. Recovery of TPN added at the instant of homogenization was 100 %.

The values for TPNH levels are summarized in Table I. In *Spisula* eggs recovery of TPNH added at the instant of homogenization was 95–100 %. In *Arbacia* eggs recovery of TPNH was low, from 33–56 %, and the use of an internal standard was required. A known amount of TPNH was added to a duplicate suspension of eggs and the values for *Arbacia* were corrected for the recovery of the added TPNH. In *Arbacia* TPNH levels increased from a mean of 19 mμmoles/ml of cells in the unfertilized eggs to 59 mμmoles in eggs 3 h after fertilization. In each instance in which the fertilized eggs of one batch were compared to the unfertilized cells of the same batch the increase in TPNH was 3 to 7 fold. In Expt. 2 with *Spisula* eggs (Table I) the level of TPNH in the fertilized eggs was low compared to the other experiments. On this day, however, for undetermined reasons, only about 15 % of the eggs underwent cleavage and lost their nuclei despite the appearance of fertilization membranes in over 90 %. In the other experiments with *Spisula* eggs a complete assay of pyridine nucleotide was performed. These values are given in Table II. The relative proportion of TPNH increased dramatically after fertilization from 5 to 30 % of the total pyridine

TABLE I

TPNH CONCENTRATION IN *Spisula* AND *Arbacia* EGGS, EXPRESSED IN MμMOLES/ML PACKED CELLS

Expt.	*Species*	*Minutes after fertilization*			
		Zero	*60–80*	*150–170*	*1440*
1	*Spisula*	23	—	71	—
2	*Spisula*	9	—	12*	—
3	*Spisula*	6	19	41	—
4	*Spisula*	9	—	65	—
1	*Arbacia*	13	—	—	90
2	*Arbacia*	—	—	—	60
3	*Arbacia*	7	57	—	57
4	*Arbacia*	22	61	—	—
5	*Arbacia*	32	—	—	61

* Only 15 % of the cells had undergone cleavage.

nucleotides. Of interest is the low level of DPNH and the slight fall in the DPN concentration consistent with the previous observations of JANDORF AND KRAHL in *Arbacia*[9].

TABLE II

TOTAL PYRIDINE NUCLEOTIDE OF *Spisula* EGGS, EXPRESSED IN MμMOLES /ML PACKED CELLS

Nucleotide	*Minutes after fertilization*	
	Zero	*150*
DPN	163	140
DPNH	5	11
TPN	11	5
TPNH	9	65

DISCUSSION

The results of these experiments are consistent with the observation that the predominant pathway of glucose utilization in fertilized and unfertilized marine eggs is via the hexose monophosphate shunt. Although the glycolytic pathway becomes more important as development proceeds the absolute amount of glucose metabolized via the hexosemonophosphate shunt increases strikingly after fertilization occurs. The increase in total triphosphopyridine nucleotide (TPN+TPNH) indicates net synthesis from DPN, not merely increase in TPNH at the expense of TPN. Preliminary experiments have demonstrated the presence of DPN kinase in homogenates of fertilized and unfertilized *Arbacia* eggs using a fluorescence assay[10]. The activity of this enzyme, difficult to assay in crude extracts because of the presence of inhibitors[11], was about the same in either stage of development.

It is of interest that the pattern of pyridine nucleotide levels observed in the present studies in fertilized marine eggs is similar to that reported by GLOCK AND MCLEAN in the guinea pig ovary[12]. In the mammary gland, another tissue in which the hexose monophosphate shunt is highly active, the steady-state concentrations of TPNH also increases out of proportion to DPNH as lactation proceeds[13]. In the latter tissue, however, levels of oxidized DPN also increase parallel to those of TPNH.

The observed increases in steady-state concentrations of TPNH in a rapidly dividing cell such as the fertilized egg are consistent with current concepts concerning the role of this pyridine nucleotide in synthetic processes[14]. Investigation into the mechanisms relating the changes in pyridine nucleotide levels to the increase in activity of the hexose monophosphate shunt is in progress.

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