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Metabolic Shifts in Carbohydrate Metabolism during Embryonic Development of Non-Diapause Eggs of the Silkworm, *Bombyx mori*

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In order to clarify the pathways of carbohydrate metabolism during embryonic development in *Bombyx mori* eggs, changes in oxygen consumption, free sugar content, glycolytic intermediates and selected enzyme activities were monitored during the nine days of embryonic development. Two distinct phases of metabolism were found - a phase of differentiation (phase I), from day 1 to day 4, and then a phase of organogenesis (phase II), from day 5 to day 9. A gradual degradation of glycogen during phase I resulted in the production of trehalose, fructose and sorbitol. In this phase, phosphofructokinase was inhibited due to a sharp fall in levels of its activator, fructose-2,6-bisphosphate. Glycolytic flux leads to lactate formation during phase I. During phase II, activity of the mitochondrial enzyme, NAD-isocitrate dehydrogenase increased eight-fold, and oxygen consumption rose sharply by six-fold. These changes correlated with increased TCA cycle activity and the activated biosynthesis of mitochondria in phase II. Sharp increases in phosphofructokinase (eight-fold) and pyruvate kinase (18-fold) activities were observed during phase II. The increased glycolytic flux correlated with decreases in substrates (glycogen, trehalose, sorbitol and fructose), and the increased carbon flux was funneled into the TCA cycle as indicated by increased oxygen consumption and decreased lactate levels between days 3 and 6. The intense activation of glycolysis apparently overwhelmed mitochondrial capacity from days 6 to 9, as indicated by the marked increase in glycolytic intermediates including phosphoenolpyruvate, dihydroxyacetone phosphate and pyruvate, and the production of lactate even in the presence of high oxygen uptake. Levels of auxiliary enzymes changed during development; NAD-sorbitol dehydrogenase activity increased during phase I and trehalase activity increased during phase II. Glycerol-3-phosphatase activity decreased slightly and fructose-1,6-bisphosphatase-1 increased slightly over the nine days of development. In summary, phase I (differentiation) is fueled by glycogen and characterized by low glycolytic flux under largely anaerobic conditions, whereas phase II (embryogenesis) is characterized by increased glycolytic flux under aerobic conditions fueled by glycogen, in addition to the mixture of sugar substrates.

Key words: *Bombyx mori*, embryogenesis, phosphofructokinase, pyruvate kinase, fructose-2,6-diphosphate

INTRODUCTION

Carbohydrate metabolism in insects during embryonic development and embryonic diapause has been studied extensively from the viewpoint of cold hardiness and energy supply (Storey and Storey, 1991). Many researchers have reported fluctuations in free sugars (Furusawa and Yang, 1987), intermediary metabolites and activities of the rate-limiting enzymes (Kageyama and Ohnishi, 1971; Yaginuma and Yamashita, 1979) during embryonic diapause and development of *Bombyx* eggs. Various metabolic events have been characterized during embryonic development of non-diapause silkworm eggs incubated at 25°C. The eggs contain high levels of glycogen until about 92 h after oviposition, but then the amount of glycogen declines markedly until hatching (Yamashita, 1965), corresponding to the appearance of glycogen phosphorylase *a* activity (Yamashita *et al*., 1975). On day 2, sorbitol levels increase but then decrease rapidly on day 3 (Furusawa and Yang, 1987). The disappearance of sorbitol correlates with a sharp increase in activity of NAD-sorbitol dehydrogenase (NAD-SDH) (Yaginuma and Yamashita, 1979; Yaginuma *et al*., 1990b). Trehalose levels increase after days 2 or 3, but then decrease as hatching approaches, and trehalase activity increases around day 6 (Yamashita, 1965).

Although most of these studies have focussed on identification of the rate-limiting enzymes in the metabolic pathway of each sugar, there is fragmentary knowledge on the regulatory mechanisms of these enzyme activities. For example, glycogen phosphorylase activation is reported to be triggered by anaerobic conditions in diapause eggs as well as non-diapause eggs (Yamashita *et al*., 1975). The appearance of NAD-SDH activity at the termination of diapause (Yaginuma and Yamashita, 1979) results from an increase in enzyme amount due to activation of the NAD-SDH gene by low temperature (Niimi and Yaginuma, 1992; Niimi *et al*., 1992, 1993). However, the low level of phosphofructokinase (PFK) activity during the early stages of developing eggs (Kageyama and Ohnishi, 1971; Suzuki and Miya, 1975) appears to result from enzyme inactivation via protein phosphorylation during the early period of embryogenesis, whereas subsequent dephosphorylation causes PFK activity to appear late in

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embryonic development (Furusawa *et al*., 1999).

The present paper provides a comprehensive analysis of changes in the levels of glycolytic intermediates, polyols, and related enzymes over the full nine-day course of development in non-diapause eggs. The data show that the eggs pass through at least three distinct metabolic stages with respect to carbohydrate metabolism, and the enzymes PFK and pyruvate kinase (PK) are identified as key loci involved in the conversion from anaerobic metabolism to aerobic metabolism in the middle of embryonic development.

MATERIALS AND METHODS

Eggs

The eggs were obtained from moths of a polyvoltine strain, N_4. Mated female moths were allowed to lay eggs within a single period of 3 h. The eggs were then pooled and incubated at 25°C and 75% relative humidity under dark conditions. Under these conditions, eggs underwent blastokinesis on day 4, head pigmentation on day 8, and hatched into larvae on day 9.

Chemicals

All chemicals were purchased from Sigma Chemical Co. or Wako Pure Chemical Industries Ltd.

Measurement of O_2 uptake

Respiration in silkworm eggs was measured from day 0 through larval hatching using a Taiyo O_2UP Tester (Taiyo-kogyo Co. Ltd.). Briefly, 1 g of eggs was placed in a cylindrical container (30 mm $\phi \times$ 30 mm), which was closed except for a side opening into a glass capillary tube. Filter paper that was soaked in 1 N KOH was placed beneath an operculum under the cover of the container. The whole container was submerged in a tank of water at 25°C. As the eggs consumed O_2 and released CO_2, the CO_2 was absorbed into the filter paper, and the water was drawn through the capillary tube. Gradations along the side of the tube allowed for determination of the reduction in gas volume in the system, which was proportional to O_2 consumption.

Assay of glycogen content

Eggs were homogenized in ice-cold 80% ethanol (1:5 v/v). The homogenate was centrifuged, and the precipitate was dissolved in 30% (w/v) KOH and boiled for 1 h. Glycogen was precipitated by the addition of 99% cold ethanol and then redissolved completely in distilled water. Glycogen content was determined by the method of Dubois *et al*. (1956).

Assay of free sugars

To estimate free sugars (sorbitol, glycerol, trehalose, glucose and fructose), eggs were ground in 99% ethanol (1:5 w/v) containing 0.5 mg/ml pentaerythritol as an internal standard, and centrifuged at 12,000 $\times$ g for 20 min at 4°C. The supernatant was passed through an ion-exchange column (IR-120 and IR-4B, ORGANO; 12 mm $\phi \times$ 50 mm) by centrifugation at 12,000 $\times$ g for 20 min. The eluate (20 µl) was fractionated through a Shim-Pac SCR-101C column (9.9 mm $\phi \times$ 30.0 cm: Shimadzu) at 72°C with water as the mobile phase and an elution rate of 0.8 ml/ml. The peaks were detected with a refractive index detector (RID-10A, Shimadzu). This resulted in the following retention times: 7.2 min for trehalose, 8.7 min for glucose, 10.9 min for fructose, 12.1 min for pentaerythritol, 13.1 min for glycerol, and 17.1 min for sorbitol. Sugar contents were calculated by comparison with internal standards.

Sample preparation and metabolite assays

Egg samples were ground (1:5 w/v) in ice cold 6% perchloric acid solution containing 1 mM EDTA. The homogenate was centrifuged at 12,000 $\times$ g for 20 min at 4°C to remove precipitated protein. The extracts were neutralized by the addition of a solution containing 15 N KOH, 0.4 M KCl and 0.3 M Tris (hydroxymethylaminomethane) and then centrifuged again to remove precipitated $KClO_4$. Aliquots of neutralized extract were used for the assay of glycolytic intermediates.

Metabolites, other than sorbitol, glycerol, trehalose, glucose and fructose, were assayed enzymatically using a UV-visible recording spectrophotometer (UV-1600, Shimadzu) and the assays of Lowry and Passonneau (1972). Fructose-2,6-bisphosphate ($F2,6P_2$) content was assayed separately after alkaline extraction of eggs (1:5 w/v) in 50 mM NaOH according to the method of van Schaftingen (1984).

Sample preparation for enzyme assay

Eggs were homogenized (1:5 w/v) in ice-cold 20 mM imidazole-HCl buffer (pH 7.2) containing 10 mM 2-mercaptoethanol, 50 mM NaF, 5 mM EDTA, 5 mM EGTA (homogenization buffer) and a few crystals (about 0.1 mM) of phenylmethylsulphonyl fluoride (a protease inhibitor) using a Ultra Turax homogenizer. Homogenates were centrifuged at 12,000 $\times$ g for 20 min at 4°C, and the supernatant was removed and desalted by centrifugation through a Sephadex G-25 column (1 cm $\phi \times$ 6 cm) pre-equilibrated in the homogenization buffer. Samples for GPase activity determination were homogenized as above but were not subjected to centrifugation or applied to the spin column. Instead, the homogenate was allowed to settle for about 30 min on ice and was then used as the source of enzyme. Samples for the determination of treha-

lase activity were homogenized (1:5 w/v) in 0.1 M acetate buffer (pH 5.6). One unit of enzyme activity was defined as the amount of enzyme that converted 1 μmol of substrate per min at 25°C. Protein was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as the standard.

Coupled enzyme assays

All enzyme activities were measured at 25°C using a UV-visible recording spectrophotometer. Controls for non-specific activity were run for all assays and any blank value was subtracted to yield final activity values. Optimal conditions for maximal activities were determined to be:

Glycogen phosphorylase (GPase) (total $a + b$): 48 mM potassium phosphate buffer (pH 7.0), 9.7 mM $MgCl_2$, 0.24 mM EDTA, 2.0 mg/ml glycogen, 10 μM glucose-1,6-bisphosphate, 0.2 mM $NADP^+$, 0.5 IU phosphoglucomutase, 0.2 IU glucose-6-phosphate dehydrogenase, 1.6 mM AMP. The active form of the enzyme (*a*) was measured in the presence of 5 mM caffeine instead of AMP.

Phosphofructokinase (PFK): 20 mM imidazole-HCl buffer (pH 7.2), 5 mM fructose-6-phosphate, 2 mM ATP, 0.15 mM NADH, 5 mM KCl, 5 mM $MgCl_2$, 0.5 IU aldolase, 0.5 IU triosephosphate isomerase, 2 IU α-glycerophosphate dehydrogenase.

Pyruvate kinase (PK): 20 mM imidazole-HCl buffer (pH 7.2), 6 mM phosphoenolpyruvate (PEP), 2 mM ADP, 0.15 mM NADH, 5 mM KCl, 5 mM $MgCl_2$, 2 IU lactate dehydrogenase.

Fructose-1,6-bisphosphatase (FBPase): 20 mM imidazole-HCl buffer (pH 7.2), 0.05 mM fructose-1,6-bisphoshate, 5 mM $MgCl_2$, 0.2 mM $NADP^+$, 1 IU phosphoglucose isomerase, 1 IU glucose-6-phosphate dehydrogenase.

6-Phosphogluconate dehydrogenase (6PGDH): 20 mM imidazole-HCl buffer (pH 7.2), 1 mM 6-phosphogluconate, 0.2 mM $NADP^+$.

Polyol dehydrogenase, with glucose as the substrate (PDH gluc): 20 mM imidazole-HCl buffer (pH 7.2), 0.52 M D-glucose, 0.1 mM NADPH.

NAD-isocitrate dehydrogenase (NAD-IDH): 20 mM Tris-HCl buffer (pH 7.2), 5 mM DL-isocitrate, 0.2 mM NAD^+, 5 mM $MgCl_2$.

NAD-sorbitol dehydrogenase (NAD-SDH): 0.1 M Tris-HCl buffer (pH 8.8), 0.2 M sorbitol, 0.15 mM NAD^+, 20 mM $MgSO_4$. The enzyme activity was monitored by following NADH/NADPH oxidation or reduction at 340 nm using a UV-visible recording spectrophotometer.

Trehalase: 0.1 M acetate buffer (pH 5.6), 0.2 M trehalose. The reaction mixture was incubated at 25°C for 30 min. To estimate the content of glucose produced, 200 μl of the solution was mixed with 3 ml of Glucose B-Test Wako (GOD method) (Wako Pure Chem. Ind. Ltd) according the instructions of the supplier.

Glycerol-3-phosphatase (G3Pase): 20 mM imidazole-HCl buffer (pH 7.2), 10 mM $MgCl_2$, 10 mM D,L-glycerol-3-phosphate. The content of released P*i* was measured by the method of Bartlett *et al*. (1970).

RESULTS

O_2 uptake and changes in NAD-isocitrate dehydrogenase during embryogenesis of non-diapause eggs

Oxygen uptake by non-diapause eggs held at 25°C was low for the first day after oviposition but then began to increase gradually, doubling by day 5 (Fig. 1). Between days 5 and 6, O_2 consumption rose sharply and continued to increase until day 9 (hatching) when respiration was five-fold higher than on day 0. The activity of the mitochondrial tricarboxylic acid cycle enzyme, NAD-isocitrate dehydrogenase (NAD-IDH), showed a similar pattern to O_2 consumption over the course of embryogenesis. Activities were under $0.3\ U \times 10^{-3}$/mg protein (low) over the first four days, began to rise on day 5, and then increased to $3.8\ U \times 10^{-3}$/mg protein by day 8 (between days 6 and 8). Data for both oxygen uptake and NAD-IDH activities document two phases of embryonic life, a period of low metabolic rate over the early days followed by high aerobic metabolic rate in late embryonic development from day 6 onwards.

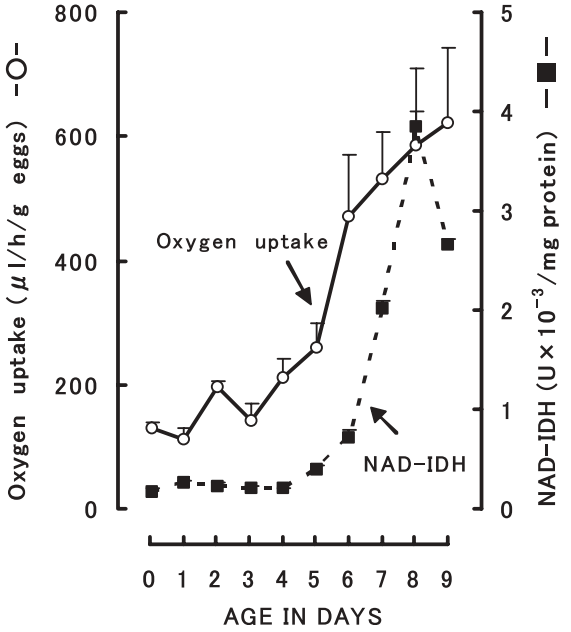

Fig. 1. Changes in oxygen uptake (μl/h/g eggs, —○—) and NAD-IDH activity (units × 10^{-3}/mg protein, —■—) in non-diapause eggs of *B. mori* held at 25°C after oviposition. Data are the means ± SEM of n = 3 independent experiments.

Changes in carbohydrates and enzyme activities during embryonic development

Figs. 2 and 3 show changes in selected carbohydrates, sugar phosphates and enzymes over the course of embryonic development in non-diapause eggs at 25°C. Glycogen content was high in eggs at oviposition and remained high at 63-66 μmoles/g eggs over the first three days (Fig. 2a). Over this same time, the total amount of glycogen phosphorylase (GPase) remained low, and the percentage of GPase present as the active *a* form (GPase *a*) remained between 40-50%. However, from day 3 onward, total GPase activity increased, rising about six-fold by day 9, and the percentage of enzyme present in the active form rose to about 70%. The consumption of egg glycogen, which fell steadily to a final value of about 15 μmoles/g eggs by day 9, was inversely correlated with the increase in GPase activity.

Fig. 3a showes changes in the concentrations of the hexose phosphate intermediates of glycolysis and sugar products (glucose, fructose, sorbitol and trehalose) over the nine days of embryonic development. Glucose-1-phosphate (G1P) was undetectable just after oviposition but rose to a peak of 0.38 μmoles/g eggs on day 3. Thereafter, levels fell to less than 0.1 μmoles/g eggs until hatching (Fig. 2b). The content of glucose-6-P (G6P) was ~0.5 μmoles/g eggs just after oviposition, but it increased thereafter and peaked at 1.5 μmoles/g on day 3 (Fig. 2b). Similarly, fructose-6-P (F6P) and fructose-1,6-bisphosphate ($F1,6P_2$) were low over the first two days after oviposition (F6P at 0.05-0.2 μmoles/g eggs and $F1,6P_2$ undetectable) but rose on day 4, with F6P reaching 0.3-0.35 μmoles/g eggs and $F1,6P_2$ rising to ~0.08 μmoles/g. F6P fell to 0.12 μmoles/g eggs and $F1,6P_2$ rose to 0.22 μmoles/g eggs on day 8, suggesting an increase in glycolytic rate at that time (Fig. 2b).

Eggs showed an early accumulation of sorbitol, rising from about 1.5 μmoles/g eggs in newly laid eggs to ~5 μmoles/g by day 2 (Fig. 2b). Sorbitol levels dropped again on day 3, and this was inversely correlated with a sharp rise in fructose on days 3 and 4 (Fig. 3a). Fructose is the product of sorbitol catabolism *via* NAD-sorbitol dehydrogenase (NAD-SDH). Both sorbitol and fructose accumulated again on day 8 but decreased on day 9. The rise in hexose phosphate levels on day 3 correlated with the beginning of the increase in GPase activity and glycogen consumption, and with the initiation of trehalose synthesis. Trehalose peaked at ~10 μmoles/g eggs on day 5 and thereafter declined. Glucose stayed below ~2 μmoles from day 0 to 5, but levels rose on day 6 (correlated with trehalose consumption), and thereafter declined towards hatching (Fig. 3a).

Changes in the activities of four enzymes (trehalase, 6PGDH, NAD-SDH and PDHgluc) during embryonic development are shown in Fig. 3b. Trehalase activity was under 18×10^{-3} U/mg protein from day 0 to day 5 and then rose continuously until hatching, rising from an initial ~9 $\times 10^{-3}$ U/mg protein to over 250×10^{-3} U/mg protein by day 9 (Fig. 3b). NAD-SDH activity was low (~0.2×10^{-3} U/mg protein) over the first two days of development but then rose sharply on day 3 to reach a peak on day 4 at 3.5

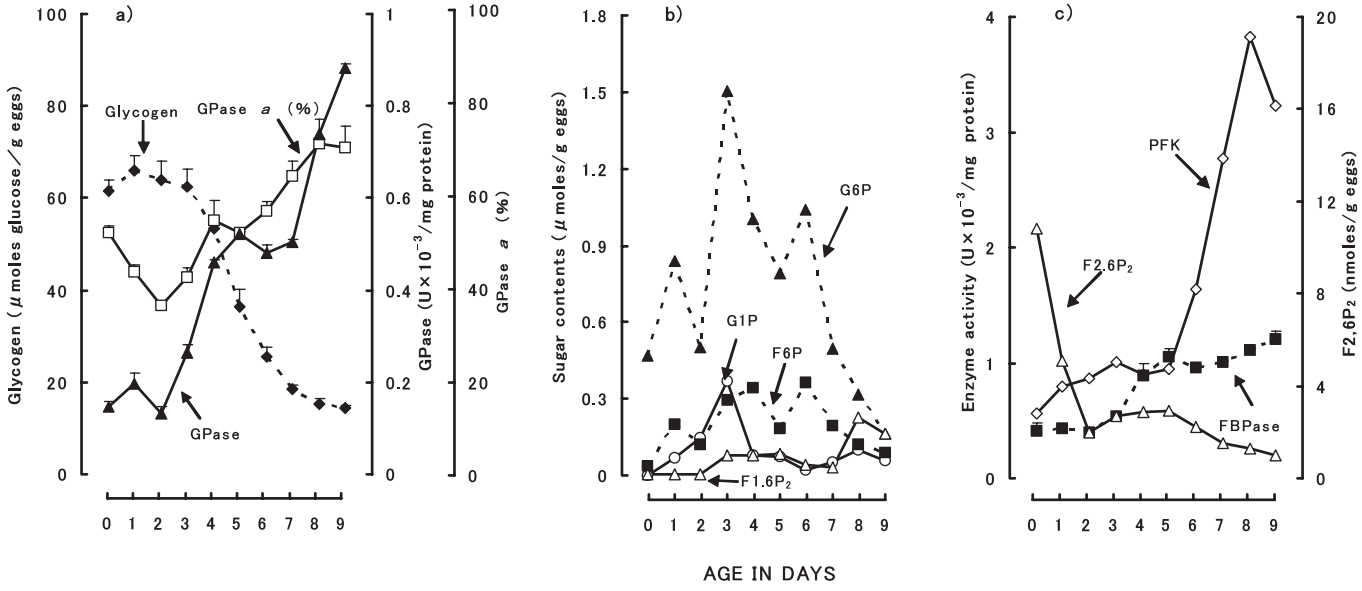

Fig. 2. Changes in the leveles of glycogen, sugars, hexose phosphate intermediates of glycolysis and in non-diapause eggs of *B. mori* held at 25°C after oviposition. Metabolite levels are given in μmoles/g eggs. Enzyme activities are expressed as units $\times 10^{-3}$/mg protein whereas glycogen phosphorylase *a* is shown as a percentage of the total activity. Data are the means ± SEM of n = 3 independent experiments. Symbols are: (a) glycogen (—◆—, left axis), Gpase total activity (—▲—, right axis), percent Gpase in the active *a* form (—□—, far right axis); (b) G1P (—○—), G6P (—▲—), F6P (—■—), $F1,6P_2$ (—△—); (c) activities of PFK (—◇—, left axis), FBPase (—■—, left axis), $F2,6P_2$ (—△—, right axis).

$\times 10^{-3}$ U/mg protein. Activity then fell to ~0.6×10^{-3} U/mg protein by day 6, but then rose again to an intermediate value (2×10^{-3} U/mg protein) that was maintained until hatching. Activities of two enzymes involved in sorbitol synthesis, polyol dehydrogenase (PDHgluc; assayed with glucose as the substrate) and 6-phosphogluconate dehydrogenase (6PGDH; one of two pentose phosphate cycle enzymes that produces the NADPH reducing power for the PDHgluc reaction) were assessed. As shown in Fig. 3c, PDHgluc fluctuated between 0.3 and 0.7×10^{-3} U/mg protein. 6PGDH was highest (2.7×10^{-3} U/mg protein) over the first two days of development, when it correlated with the synthesis of sorbitol. It then decreased to 1.9×10^{-3} U/mg protein on day3 and increased again to 2.9×10^{-3} U/mg protein.

Fig. 4 shows changes in the concentrations of triose phosphate intermediates of glycolysis, triose products (glycerol and lactate) and enzymes involved in their synthesis. As shown in Fig. 4a, both dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP) were present at substantial levels (0.09-0.15 μmoles/g eggs) in newly laid eggs on day 0 (Fig. 4a). However, lev-

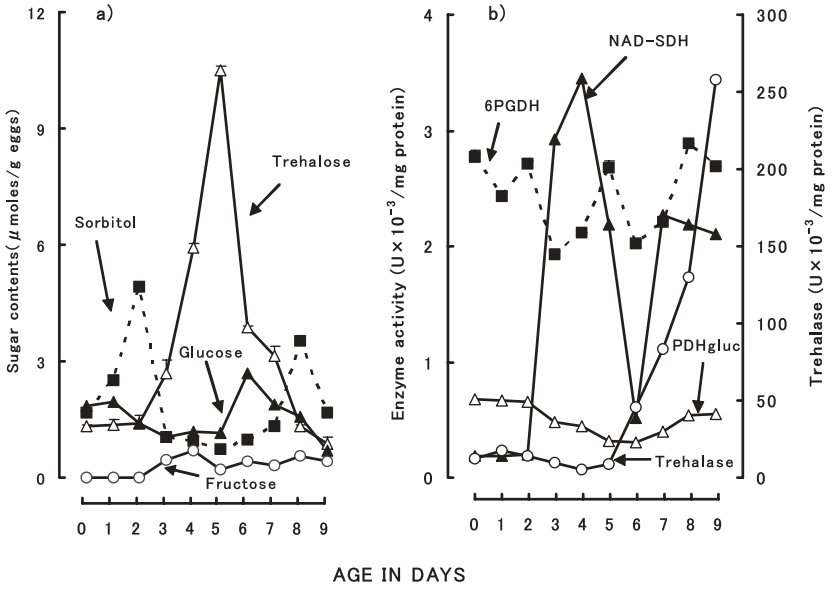

Fig. 3. Changes in the leveles of sugars and activities of related enzymes. Other information as in Fig. 2. (a) sorbitol (—■—), trehalose (—△—), glucose (—▲—), fructose (—○—); (b) activities of 6PGDH (—■—, left axis), NAD-SDH (—▲—), left axis), PDHgluc (—△—, left axis), trehalase (—○—, right axis).

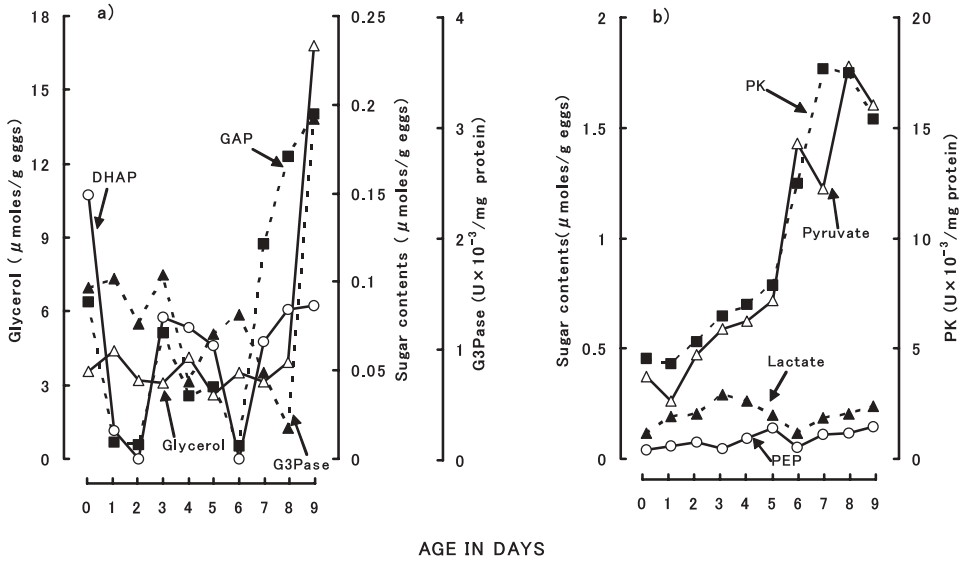

Fig. 4. Changes in the levels of triose phosphate intermediates of glycolysis, glycerol and lactate in non-diapause eggs of *B. mori* held at 25℃ after oviposition. Other information as in Fig. 2. (a) glycerol (—△—, left axis), DHAP (—○—, right axis), GAP (—■—, right axis), activities of G3Pase (—▲—, far right axis); (b) PEP (—○—, left axis), lactate (—▲—, right axis), pyruvate (—△—, left axis), activities of PK (—■—, far right axis).

els of both dropped dramatically to < 0.01 μmoles/g eggs on days 1 and 2 before rising again to intermediate levels on days 3-5. Both then showed a second sharp decrease to < 0.01 μmoles/g eggs on day 6 and then rose again over the final three days of embryonic development. Glycerol content of the eggs was maintained between 2 and 4 μmoles/g until day 8, but increased rapidly to 16 μmoles/g just before hatching (Fig. 4a). Pyruvate showed a similar pattern, low through days 0-5 and then rising sharply by about two-fold on day 6 and remaining high until hatching (Fig. 4b). PEP content was relatively constant over the first four days of development and then levels increased over the subsequent days as hatching approached (Fig. 4b). The end-product of anaerobic glycolysis, lactate, showed two peaks of accumulation. Lactate rose from ~1.2 μmoles/g eggs on day 0 to 3 μmoles/g on day 3, then decreased again to ~1.2 μmoles/g on day 6 before rising over the following days to reach 2.5 μmoles/g at the time of hatching (Fig. 4b).

Changes in the levels of $F2,6P_2$, an important activator of phosphofructokinase (PFK), are shown in Fig. 2c. Levels were high on day 0 just after oviposition (~10 nmoles/g eggs) but fell to a low of ~2.5 nmoles/g on day 2 before rising to a second peak at 3 nmoles/g on days 5-6 and decreasing to 1.0 nmoles/g eggs on day 9.

The activities of two important glycolysis enzymes, PFK and pyruvate kinase (PK), were low, but gradually increased over the first five days of embryonic development (PFK to 0.5-1 $\times 10^{-3}$ U/mg protein and PK to 5-8 $\times 10^{-3}$ U/mg protein) (Figs. 2c, 4b). However, activities of both rose sharply during days 6-8 with an approximate four-fold increase in PFK and 2.25-fold increase in PK activity compared with their values on day 5 (Figs. 2c, 4b). The activity of fructose-1,6-bisphosphatase (FBPase) was about 0.4-0.5 $\times 10^{-3}$ U/mg protein over the first few days but then rose gradually to about 1.2 $\times 10^{-3}$ U/mg protein by hatching (Fig. 2c). The activity of glycerol-3-phosphatase (G3Pase), which is the final enzyme in the production of glycerol, decreased gradually from 1.5 $\times 10^{-3}$ U/mg protein on day 0 to 0.2 $\times 10^{-3}$ U/mg protein on day 8. However, its activity rose again suddenly to about 3 $\times 10^{-3}$ U/mg protein just before hatching (Fig. 4a), which correlated with the accumulation of glycerol just before hatching.

DISCUSSION

Embryonic development of the silkworm requires approximately 10 days at 25°C and can be divided into two parts: the differentiation period and the period of organogenesis (Ohtsuki, 1979). Differentiation begins at oviposition, continues through day 4 (embryonic stage 20) and is followed by organogenesis from day 4 until hatching. These two stages also show distinct differences in energy metabolism. Fig. 1 and 2c, as well as the data reported by Yaginuma and Yamashita (1999), illustrate that the initial few days (phase I) are characterized by low oxygen consumption and very low activities of PFK and NAD-IDH, a rate-limiting enzyme of the tricarboxylic acid cycle. Hence, aerobic ATP production by mitochondria appears to be very low during the first four days of the differentiation period. This is further supported by an accumulation of lactate that peaks on day 3 (Fig. 4b), and indicates that anaerobic glycolysis is involved in ATP production during phase I. Subsequently, however, both oxygen uptake and NAD-IDH activity rose rapidly (and lactate fell), which indicates that the second stage of development is supported by strong aerobic metabolism.

The data show shifting patterns of carbohydrate metabolism during the embryonic development of non-diapause eggs. In terms of the carbohydrate substrate (glycogen) and sugar products (sorbitol, glucose, fructose, trehalose and glycerol), at least three phases can be identified: (1) an initial temporary accumulation of sorbitol and (2) synthesis of trehalose, both in phase I, followed by (3) glycolysis and trehalose degradation accompanied by elevated activities of PFK, PK and trehalase in phase II. The first phase is an accumulation of sorbitol over the first two days of embryonic life. This occurs despite low GPase activity. However, increased levels of G1P, G6P and F6P on days 1 and 2 suggest that glycogenolysis rates are low at this time of sorbitol production. Thus, the 3 μmoles/g increase in sorbitol by day 2 greatly exceeds the approximately 0.08 μmoles/g increase in the glycolytic end product, lactate, over the same time. The premise that glycolytic carbon flows primarily into sorbitol over the first two days of embryogenesis is further supported by the extremely low PFK activity at this time and by changes in the concentrations of glycolytic intermediates, which strongly indicate a glycolytic block at the PFK locus. Thus, between day 0 and day 1, levels of G6P and F6P (the PFK substrate) rise, whereas levels of $F1,6P_2$ (the PFK product) remain undetectable and levels of the triose phosphates (DHAP and GAP) produced from $F1,6P_2$ by the aldolase reaction are strongly suppressed. This substrate build-up during product suppression is indicative of PFK inhibition and is further supported by the significant decline in $F2,6P_2$ levels, a potent activator of PFK, over the first two days of embryogenesis (Fig. 2c). With PFK blocked, carbohydrate catabolism for energy production can only go forward by shunting carbon through the reactions of the pentose phosphate cycle and then feeding one of the products of the cycle, GAP, back into glycolysis at the triose phosphate level of the pathway. This route of carbohydrate catabolism has also been described in other insects (Storey, 1982) where it is

a route of anaerobic metabolism and is specifically utilized as a mechanism for producing high concentrations of polyhydric alcohols (glycerol and sorbitol) for cryoprotection in cold-hardy species (Storey and Storey, 1990, 1991).

Two possible uses for this anaerobic metabolism during early embryogenesis in *B. mori* eggs have been identified. In the first scenario, this anaerobic carbohydrate catabolic route suits the particular cellular circumstances of early embryogenesis. Alternatively, this route can rapidly synthesize protectants (e.g. sorbitol or glycerol) if non-diapause eggs encounter unfavorable environmental stresses (e.g. cold or desiccation). Indeed, Furusawa *et al*. (1989, 1992) found that non-diapause eggs exposed to cold-stressed (5°C) on day 1 after oviposition, rapidly accumulate sorbitol and glycerol. Furthermore, diapause eggs use this route to accumulate extremely high concentrations of polyols, which enable the eggs to withstand long-term exposure to cold (Furusawa *et al*., 1982; Yamashita *et al*., 1988).

When non-diapause eggs develop in a favorable environment (such as at 25°C), this initial route of anaerobic metabolism is abandoned by the third day of development in order to allow metabolic rate and growth to accelerate. Our data supported this premise as both oxygen and glycogen consumption increased substantially between days 3 and 4 (Figs. 1, 2a). The induction of NAD-SDH on day 3 quickly catabolizes sorbitol, resulting in a temporary peak in fructose, the product of the NAD-SDH reaction (Fig. 3). The catabolism of sorbitol at this time may also contribute to the rising ATP needs of the developing embryo because the NADH, which is produced by the SDH reaction, can be funneled into the mitochondrial electron transport system to drive ATP synthesis (Yaginuma *et al*., 1990a). The accumulation of trehalose during days 3-5 is the second major event in carbohydrate metabolism. After reaching a peak on day 5, trehalose is consumed following the appearance of the enzyme trehalase on day 6. An increase in the concentration of the trehalase product, glucose on day 6 is also consistent with trehalase action. The changes in trehalose may be explained as follows. At this time in development, the process of organogenesis is well underway and multiple tissues are differentiating, each with their own requirements for metabolic fuels. Trehalose is the sugar used for intertissue transport of carbohydrate fuel in insects and is typically made from glycogen reserves in the fat body and exported into the hemolymph for uptake and use by other tissues (Wyatt, 1967). Trehalose would undoubtedly have the same function in pharate larvae once organs have differentiated and a circulatory system has been established. Trehalose is provided from a central depot (the developing fat body), and the induction of trehalase activity in different tissues allows each developing organ to then begin to regulate its individual uptake and use of the sugar. Net trehalose levels in the eggs on days 6-9 would, therefore, represent the balance between trehalose synthesis from glycogen and trehalose catabolism as a tissue fuel. The final level of trehalose would thus be quite low just prior to hatching due to the depletion of glycogen reserves by the end of development.

On day 6 of embryogenesis, another phase of metabolism appears to begin (phase II). Oxygen consumption greatly increases, elevated NAD-IDH activity indicates a proliferation of mitochondria, activities of glycolytic enzymes (PFK and PK) increase sharply, and trehalase activity is induced. All of these enzyme changes are consistent with enhanced metabolic activity in the growing embryo and activities of all these enzymes continued to increase until hatching. The increase in enzyme activities during this time may result from one or more regulatory mechanisms. For example, PFK activation may involve at least two mechanisms: covalent modification and allosteric activation. Previous studies have shown that PFK is phosphorylated (inactivated) during the differentiation period and then dephosphorylated and activated beginning around day 5 (Furusawa *et al*., 1999). Levels of $F2,6P_2$ also increase just before the rise in PFK activity - $F2,6P_2$ is a potent allosteric activator of PFK that is known to facilitate the use of carbohydrate reserves for anabolic purposes (Pilkis *et al*., 1987; Hue and Rider, 1987). These two factors (dephosphorylation and $F2,6P_2$ increase) will elevate PFK activity, and together with the concurrent activation of GPase *a*, will result in increased glycolytic flux. Indeed, evidence that the normal pathway of glycolysis begins to function in the second half of embryogenesis comes from the appearance of $F1,6P_2$ on day 3, and increased flux is indicated by the rising levels of $F1,6P_2$ and triose phosphate intermediates on days 3-5. However, the molecular mechanism that causes the increase in PK activity during phase II may be reversible phosphorylation, similar to PFK, but this remains to be tested experimentally.

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