

## THE FATE OF [<sup>14</sup>C]GLUCOSE DURING COLD-HARDENING IN *EUROSTA SOLIDAGINIS* (FITCH)

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(Received 18 November 1985; revised and accepted 7 May 1986)

**Abstract**—Glucose catabolism in overwintering larvae *Eurosta solidaginis* was examined to determine the relative contributions of glycolysis and the pentose phosphate pathway to polyol synthesis at different temperatures. Rates of <sup>14</sup>CO<sub>2</sub> evolution were determined after injection of [<sup>14</sup>C]1-glucose, [<sup>14</sup>C]6-glucose, and [<sup>14</sup>C]3,4-glucose. In addition incorporation of label from each isotope into sorbitol and glycerol was monitored. The respirometric studies showed a relative increase in pentose phosphate activity between 10 and 5°C. Similar results were obtained from the changes of radioactivities incorporated into glycerol, although the activation of the pentose phosphate pathway was low. The conversion of [<sup>14</sup>C]glucose to glycerol was highest at 10°C, suggesting that maximum glycerol synthesis may occur at this temperature. Radioactivity appeared in the sorbitol fraction of larvae incubated at temperatures below 5°C. Late autumn larvae converted more [<sup>14</sup>C]glucose than did early autumn larvae.

**Key Word Index:** Cold hardiness, overwintering, antifreeze production, polyols

### INTRODUCTION

The gall fly, *Eurosta solidaginis* overwinters as third instar larvae in goldenrod galls and displays freezing tolerance. The third instar survives temperatures below -40°C in winter. The larvae accumulate two cryoprotectant polyols, sorbitol and glycerol (Morrissey and Baust, 1976). These two polyols show distinct patterns of accumulation. Glycerol is synthesized at warm temperatures and sorbitol is produced at low temperatures with cessation of glycerol synthesis (Morrissey and Baust, 1976; Storey *et al.*, 1981). A temperature dependent metabolic switch occurs between 10 and 5°C (Baust and Lee, 1982; Rojas *et al.*, 1983) for glycerol and sorbitol synthesis.

Glycogen is the major precursor of glycerol and sorbitol in *E. solidaginis* larvae (Storey *et al.*, 1981) as in other insects (Wyatt, 1967). Two routes of glycerol and sorbitol synthesis from glycogen in this species have been proposed by Storey (1981). Phosphofructokinase activity of *E. solidaginis* is strongly inhibited by low temperature *in vitro*, suggesting that low temperature may block glycolysis and trigger the metabolic switch to divert carbon flow from glycerol synthesis to sorbitol synthesis (Storey, 1982). In addition, glycerol synthesis via the pentose phosphate pathway may be possible. The pentose phosphate pathway is activated during sorbitol and glycerol synthesis in diapausing eggs of *Bombyx mori* (Kageyama, 1976) and during cold-induced glycerol synthesis in *Protophormia terranova* (Wood and Nordin, 1980).

The purpose of this study was to measure the metabolism of glucose and the activities of glycolysis and the pentose phosphate pathway at different temperatures by using specifically labeled [<sup>14</sup>C]glucose in overwintering *E. solidaginis* larvae.

### MATERIALS AND METHODS

Galls of the goldenrod plant (*Solidago canadensis*) were collected monthly from September to December from coastal Texas. Third instar larvae of the gall fly, *Eurosta solidaginis* were removed from the galls, placed on moist filter paper and maintained at 15°C for 7 days. A control group was held at 15°C for the duration of the experiment. The remainder of the larvae were transferred to 10°C for 5 days. At the end of 5 days the rest were transferred to 5°C and the same procedure was followed. This acclimation scheme of lowering the larvae 5°C every 5 days was followed until -10°C was attained.

[<sup>14</sup>C]1-glucose (57.1 mCi/mmol), [<sup>14</sup>C]3,4-glucose (10.3 mCi/mmol), and [<sup>14</sup>C]6-glucose (56.1 mCi/mmol) were purchased from New England Nuclear.

#### *Injection of isotopes*

At each acclimation temperature (10, 5, 0 and -10°C) a group of larvae was injected with [<sup>14</sup>C]glucose after the second day of acclimation. Those held at 15°C were injected 5 days after acclimation. All larvae were injected once. Only larvae in the acclimation group to be used for respirometric data and analyzed for <sup>14</sup>C-incorporation into polyols were injected with [<sup>14</sup>C]glucose the remainder were transferred to the next lower temperature. Larvae were first anaesthetized in small vials containing cotton moistened with ethyl ether. After anaesthesia, 1 µl of [<sup>14</sup>C]glucose containing 0.3 µg glucose dissolved in a ethanol-water (9:1) solution was injected into the abdomen with a glass capillary attached to a 10 or 25 µl microsyringe. The point of injection was ligated with a cotton thread to prevent bleeding.

#### *Measurement of label distribution*

For measurement of <sup>14</sup>CO<sub>2</sub> production, larvae were transferred to a 5 ml syringe as a container. The CO<sub>2</sub> trap

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apparatus and methods were similar to those previously described (Tsumuki and Kanehisa, 1980). Evolved  $\text{CO}_2$  gas was trapped in an ethanolamine solution. The larvae were then weighed 12 hr (15, 10, 5, and  $0^\circ\text{C}$ ) or 24 hr ( $-10^\circ\text{C}$ ) after injection and kept at  $-45^\circ\text{C}$  until analyzed. The  $\text{CO}_2$  recovered as  $^{14}\text{CO}_2$  is expressed as percentage of the total activity in the 1  $\mu\text{l}$  injection of isotope [ $^{14}\text{CO}_2$  activity (%)].

For assay of label incorporation into polyols larvae were homogenized in 5 ml of 80% ethanol using a teflon glass homogenizer. The extraction procedures are as follows (Table 1): the supernatant from the ethanol extraction was used for polyol and carbohydrate analysis following further removal of lipids by partition against chloroform-methanol (2:1). The aqueous phase was evaporated to dryness and resuspended in water prior to high pressure liquid chromatography (HPLC) analysis using both ion-exchange and amine modified silica columns (Baust *et al.*, 1983). The fractions were collected at 0.3 min with a microfractionator. The organic phase of the chloroform-methanol extraction was used for determination of radioactivity incorporation into lipids.

The precipitate from the 80% ethanol extract was used for glycogen and protein analysis. Five milliliters of 5% trichloroacetic acid (TCA) was added to the precipitate and heated for 10 min at  $100^\circ\text{C}$ , chilled and centrifuged. The supernatants were combined with the resultant supernatants from a double wash of the precipitate with 5% TCA. These

combined supernatants were then analyzed for  $^{14}\text{C}$ -incorporation into glycogen. The precipitates were used for  $^{14}\text{C}$ -incorporation into protein by digestion of the precipitate in 1 M hyamine methanol solution at  $50^\circ\text{C}$ .

The radioactivities in the  $\text{CO}_2$ , 80% ethanol, chloroform-methanol, 5% TCA and the residues were assayed in 10 ml of scintillation solution (PPO 4.2 g, POPOP 0.0525 g, Triton X-100 333 ml, and toluene 667 ml) and the activities in fractions separated by HPLC in 5 ml of scintillation solution. The radioactivities in all fractions were assayed with a Scintillation Counter (Beckman LS-230).

## RESULTS

The isotope content of various fractions 12 or 24 hr after injection of specifically labeled glucose is expressed as a percentage of the total radioactivity recovered. Table 2 lists a typical recovery pattern for the 12 hr  $10^\circ\text{C}$  experimental group.

### Production of $^{14}\text{CO}_2$

The larvae injected with glucose labeled in different positions were monitored for periods up to 12 hr at 15, 10, 5, 0 and  $-10^\circ\text{C}$ .  $^{14}\text{CO}_2$  from C-1 labeled sugar occurs primarily as a result of the

Table 1. Flow diagram of extraction procedure for determination of isotope distribution in various fractions (lipid, protein and polyol) obtained from *E. solidaginis* third instar larvae

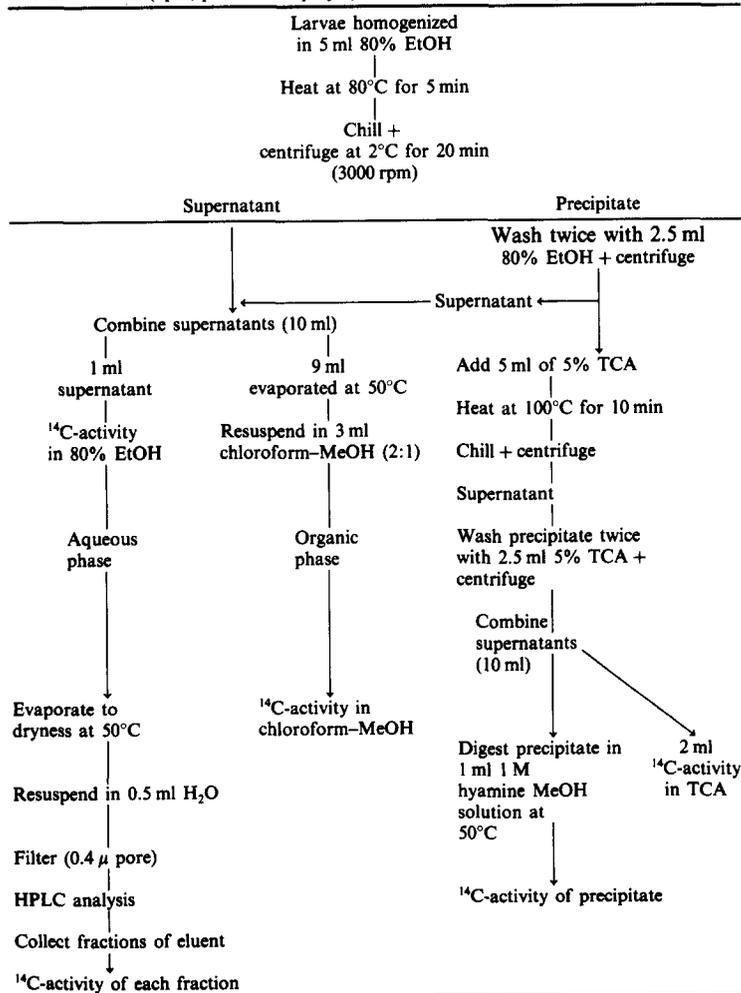


Table 2. Distribution of isotope in each fraction of the larvae 12 hr after injection of specifically labeled glucose at 10°C

	[ <sup>14</sup> C]1-glucose	[ <sup>14</sup> C]3,4-glucose	[ <sup>14</sup> C]6-glucose
80% ethanol (%)	84.4 ± 1.8	74.2 ± 2.2	91.7 ± 0.9
5% TCA (%)	3.8 ± 0.3	5.0 ± 0.9	3.2 ± 0.4
Precipitate (%)	4.1 ± 0.4	3.7 ± 0.2	2.5 ± 0.1
Liberated CO <sub>2</sub> (%)	7.7 ± 0.8	17.3 ± 1.6	2.6 ± 0.4
Total (%)	100.0	100.0	100.0

6-phosphogluconate dehydrogenase reaction of the pentose phosphate pathway. <sup>14</sup>CO<sub>2</sub> produced from C-3,4 labeled glucose comes from the pyruvate dehydrogenase reaction, whereas C-6 labeled sugar yields <sup>14</sup>CO<sub>2</sub> almost exclusively from TCA cycle reactions, as does [<sup>14</sup>C]1-glucose which is oxidized glycolytically. Conversion of specifically labeled [<sup>14</sup>C]glucose into <sup>14</sup>CO<sub>2</sub> at different temperatures is shown in Figs 1, 2,

and 3 and is expressed as the percentage of total radioactivity injected. The radioactivities in CO<sub>2</sub> increased linearly with time at all temperatures tested, although the activities were greatly affected by temperature. C-1, C-3,4, and C-6 refer to the <sup>14</sup>CO<sub>2</sub> produced from [<sup>14</sup>C]1-glucose, [<sup>14</sup>C]3,4-glucose, and [<sup>14</sup>C]6-glucose, respectively. At 15 and 10°C the rate of CO<sub>2</sub> production from [<sup>14</sup>C]3,4-glucose was greater

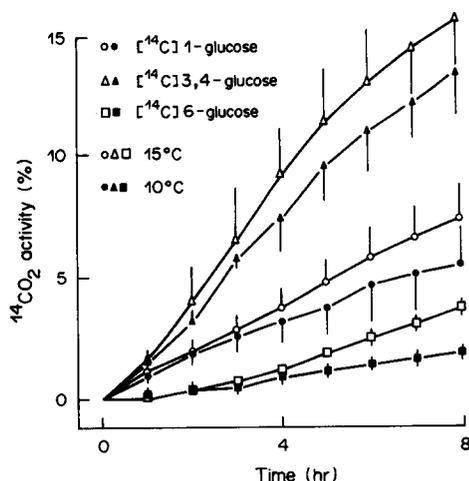


Fig. 1. Incorporation of label into CO<sub>2</sub> evolved by gall fly larvae injected with specifically labeled glucose at 15 and 10°C. Values (±SEM) are expressed as the percentage of radioactivity incorporated into CO<sub>2</sub> to the total radioactivity of the injected isotope (% <sup>14</sup>CO<sub>2</sub> activity).

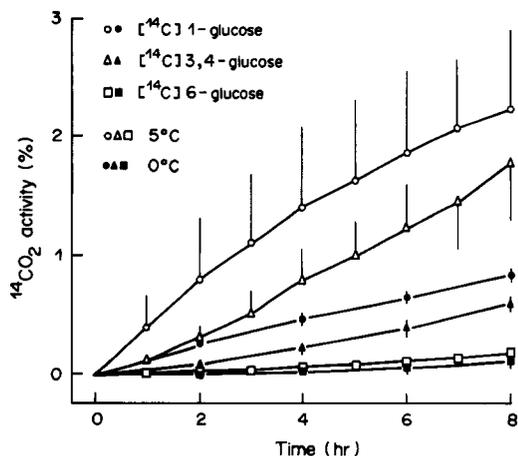


Fig. 2. Incorporation of label into CO<sub>2</sub> evolved by gall fly larvae injected with specifically labeled glucose at 5 and 0°C. Values (±SEM) are expressed as the percentage of radioactivity incorporated into CO<sub>2</sub> to the total radioactivity of the injected isotope (% <sup>14</sup>CO<sub>2</sub> activity).

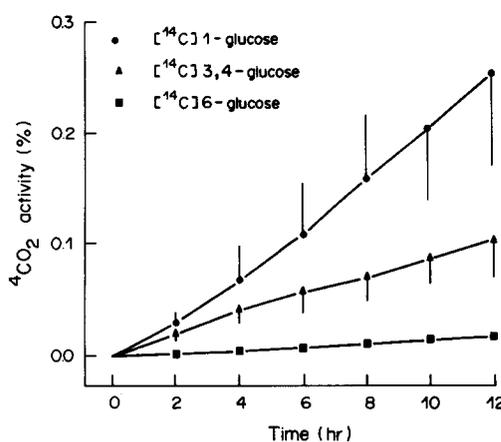


Fig. 3. Incorporation of label into CO<sub>2</sub> evolved by gall fly larvae injected with specifically labeled glucose at -10°C. Values (±SEM) are expressed as the percentage of radioactivity incorporated into CO<sub>2</sub> to the total activity of the injected isotope (% <sup>14</sup>CO<sub>2</sub> activity).

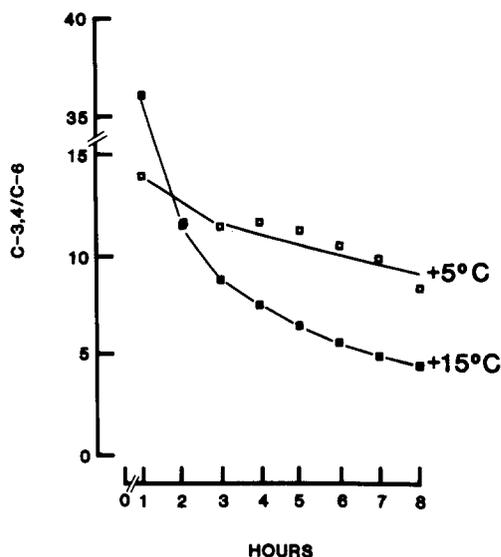


Fig. 4. Ratios of <sup>14</sup>CO<sub>2</sub> from [<sup>14</sup>C]3,4-glucose vs <sup>14</sup>CO<sub>2</sub> from [<sup>14</sup>C]6-glucose at 15 and 5°C.

Table 3. The ratio of per cent label recovered as  $^{14}\text{CO}_2$  from injected  $[^{14}\text{C}]1$ -glucose to that from injected  $[^{14}\text{C}]6$ -glucose

	[C-1/C-6]				vs 15°C*
	2 hr	4 hr	6 hr	8 hr	
15°C	5.6	3.0	2.4	2.0	1
10°C	4.8	3.6	3.3	2.9	0.69
5°C	31.3	20.6	15.5	10.9	0.18
0°C	15.2	10.3	7.7	6.1	0.33
-10°C	13.9	15.1	16.2	15.8	0.13

\*The value was calculated from

$$\frac{\text{C-1/C-6 at 15°C}}{\text{C-1/C-6 at different temperature for 8 hr}}$$

Table 4. The ratio of percent of administered  $^{14}\text{C}$  in glycerol following  $[^{14}\text{C}]1$ -glucose and  $[^{14}\text{C}]6$ -glucose injection for 8 hr

Temperature		$^{14}\text{C-1}$ (%)	$^{14}\text{C-6}$ (%)	$^{14}\text{C-6/C-1}$ (%)
15°C	Early autumn	2.54	3.17	1.25
	Late autumn	1.30	1.85	1.42
10°C	Early autumn	5.04	6.30	1.25
	Late autumn	1.47	2.03	1.37
5°C	Early autumn	0.93	1.55	1.67
	Late autumn	0.41	0.80	1.95
0°C	Early autumn	0.34	0.64	1.87
	Late autumn	0.33	0.78	2.36

than that from C-1 and C-6, whereas at 5, 0, and -10°C the rate for C-1 was greater than that for C-3,4 and C-6.  $Q_{10}$  was calculated 8 hr after injection. The  $Q_{10}$  of  $\text{CO}_2$  evolved at 15°C is 3.28 for C-1 and 17.65 for C-6; 10°C vs 0°C is 6.33 for C-1 and 13.43 for C-6; 0°C vs -10°C is 2.18 for C-1 and 5.48 for C-6.

Figure 4 shows that the ratio of C-3,4/C-6 is higher at 5 than 15°C. These data can be used to compare the C-3,4 to C-6 ratio between 15 and 5°C at various times (Wood and Nordin, 1980). The  $(\text{C-3,4/C-6 at } 15^\circ\text{C})/(\text{C-3,4/C-6 at } 5^\circ\text{C})$  was calculated for the 8 hr interval and a value of 0.53 was obtained. A lower ratio arises when there is a larger number in the denominator of the above expression, reflecting a situation in which the rate of glycolysis relative to the TCA cycle is greater at 5 than 15°C.

To determine the relative contribution of the HMS, C-1/C-6 ratios were calculated (Table 3). The ratio rose considerably between 10 and 5°C, showing that relative pentose phosphate activity is activated at 5°C. The  $(\text{C-1/C-6 at } 15^\circ\text{C})/(\text{C-1/C-6 at } 5^\circ\text{C})$  for 8 hr interval gave a value of 0.18. This value indicates that the HMS increases relative to glycolysis at 5°C when compared to 15°C. Again, these data show that the contribution of the pentose phosphate pathway increases at low temperature relative to glycolysis.

#### Incorporation of $^{14}\text{C}$ into polyols

Figure 5 shows the incorporation of radioactivity into glycerol at different temperatures 12 hr after injection with  $[^{14}\text{C}]1$ -glucose and  $[^{14}\text{C}]6$ -glucose, respectively. Radioactivity incorporated into glycerol decreased abruptly at temperatures between 10 and 5°C. The activities in glycerol were higher in the larvae collected in early autumn than late autumn. Furthermore, these data show that glycerol synthesis is greatly reduced below 5°C. The radioactivity in glycerol from  $[^{14}\text{C}]6$ -glucose was higher than that from  $[^{14}\text{C}]1$ -glucose. The ratio of C-6/C-1 was calculated, where C-1 is the incorporation rate of label into glycerol from  $[^{14}\text{C}]1$ -glucose and C-6 is from  $[^{14}\text{C}]6$ -glucose (Table 4). These values indicate that the activity of the pentose phosphate pathway increases relative to glycolysis at temperatures between 10 and 5°C when glycerol synthesis is curtailed.

Sorbitol was not detected at either 15 or 10°C and the radioactivity in sorbitol fractions could not be detected at these temperatures. Figure 6 shows the radioactivity in sorbitol from  $[^{14}\text{C}]1$ -glucose and  $[^{14}\text{C}]6$ -glucose at 5, 0 and -10°C. Incorporation increased with decreasing temperatures between 5 and 0°C and then decreased at -10°C. The activity in sorbitol was higher in larvae collected in late autumn than in early autumn. In early autumn larvae  $[^{14}\text{C}]6$ -glucose incorporation was the same as  $[^{14}\text{C}]1$ -glucose incorporation, indicating that the contribution of the pentose phosphate pathway is minimal. It is most likely that sorbitol is produced directly from glucose by a single enzyme reaction which would explain the C-6/C-1 ratio of unity.

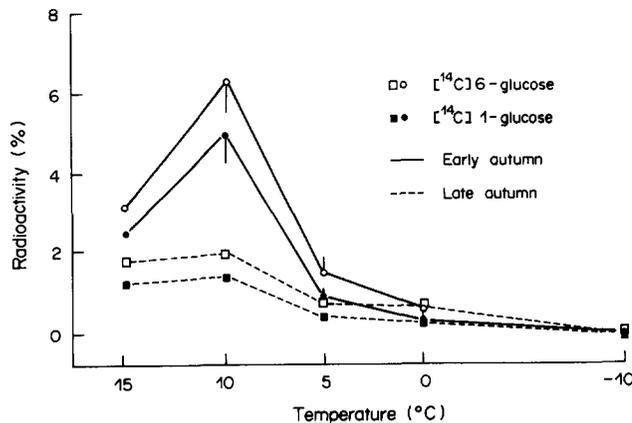


Fig. 5. Incorporation of isotope from specifically labeled glucose into glycerol in gall fly larvae at different temperatures 12 hr after injection. Values ( $\pm$  SEM) are expressed as the percentage of radioactivity incorporated into glycerol to the total radioactivity of the injected isotope (% radioactivity).

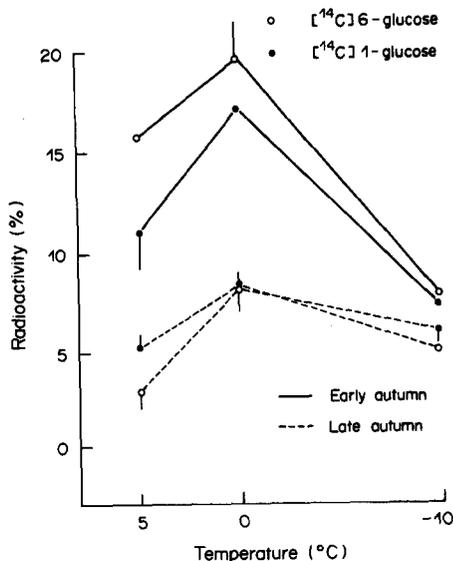


Fig. 6. Incorporation of isotope from specifically labeled glucose into sorbitol in gall fly larvae at different temperatures 12 hr after injection. Values ( $\pm$ SEM) are expressed as the percentage of radioactivity incorporated into sorbitol to the total radioactivity of the isotope injection (% radioactivity).

DISCUSSION

Gall fly larvae produce sorbitol and glycerol in their tissues as antifreeze/cryoprotectant agents (Morrissey and Baust, 1976). Glycerol synthesis occurs at warm temperatures (Baust and Lee, 1982; Rojas *et al.*, 1983). The radioactivities incorporated into glycerol from [<sup>14</sup>C]glucose decreased abruptly at temperatures between 10 and 5°C, and below 5°C the activities were lowered to trace levels. The rate of conversion of [<sup>14</sup>C]glucose to glycerol at 10°C was the highest of all temperatures tested, showing that maximum glycerol production may occur at 10°C (Fig. 5).

Sorbitol synthesis in this species is initiated at low temperatures concomitant with the cessation of glycerol production and ceases at -10°C (Storey *et al.*, 1981). Similar results were found in the larvae incubated at temperatures below 5°C. The highest activities were found in both early and late autumn larvae incubated at 0°C showing that maximum sorbitol production occurs at 0°C as Baust (1983) and Rojas *et al.* (1983) have reported.

The possible carbon flow routes from glycogen to glycerol and sorbitol are glycolysis and the pentose phosphate pathway. The pathways of carbohydrate metabolism can be estimated using specifically labeled glucose (Wood *et al.*, 1963). Pentose phosphate and glycolysis contributions were calculated from the ratio of <sup>14</sup>CO<sub>2</sub> yields using the equation of Silva *et al.* (1958):

$$\text{pentose phosphate pathway (\%)} = \frac{C-1 - C-6}{C-1 - C-6 + C-3,4} \times 100.$$

From these data it appears that the relative rate of glycolysis activity decreased while the relative pentose phosphate activity rate increased at temperatures

between 10 and 5°C. However, caution must be used in calculating relative pathway contributions in glucose catabolism when utilizing respirometric data (Katz and Wood, 1960, 1963; Cheldelin *et al.*, 1962). Indeed, a major problem in analyzing respirometric data in cold stressed *E. solidaginis* larvae arises from incomplete oxidation of triose phosphates. Storey and Storey (1983) have shown that in *E. solidaginis* at low temperatures pyruvate kinase is inhibited. This would decrease the amount of <sup>14</sup>CO<sub>2</sub> from [<sup>14</sup>C]6-glucose. As a result <sup>14</sup>CO<sub>2</sub> from [<sup>14</sup>C]1-glucose would increase relative to that from [<sup>14</sup>C]6-glucose, spuriously making the contribution of the pentose phosphate pathway appear greater at lower temperatures. Specific yields as described by Katz and Wood (1963) were used to partially overcome this problem. Specific yields express the <sup>14</sup>CO<sub>2</sub> data in terms of utilized glucose. Specific yields showed a slight decrease in the percentage of the absolute pentose phosphate pathway activity with decreasing temperatures. The ratio of C-1/C-6 using specific yields shows an increase in pentose phosphate pathway activity relative to glycolysis between 10 and 5°C (1.7-fold increase).

The activity of the pentose phosphate pathway was also estimated using radioactivity in glycerol and sorbitol by the methods of Wood *et al.* (1963) (Figs 7 and 8). The equation is as follows:

$$\text{pentose phosphate pathway (\%)} = \frac{C-6/C-1 - 1}{C-6/C-1 + 2} \times 100$$

where C-6 and C-1 are incorporation of label into glycerol or sorbitol from [<sup>14</sup>C]6-glucose and [<sup>14</sup>C]1-glucose, respectively. The pentose phosphate activity calculated from the different radioactivities in glycerol converted from [<sup>14</sup>C]1-glucose and [<sup>14</sup>C]6-glucose shows an increase of over 2-fold between 10 and 5°C. The activity changes coincided with those calculated from different respirometric data, although the activation was low compared with that from respirometric data. The pentose phosphate activity calculated from the different radioactivity in sorbitol show that activity decreased with decreasing temperature. It is most likely that sorbitol is being

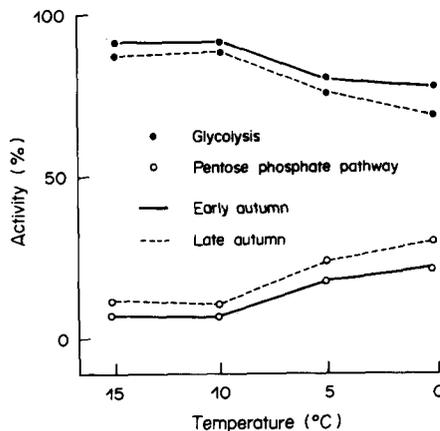


Fig. 7. Change in glycolytic and pentose phosphate activity calculated from the radioactivities incorporated into glycerol.

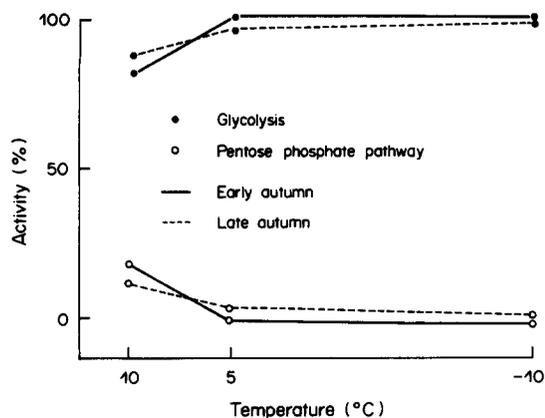


Fig. 8. Change in glycolytic and pentose phosphate activity calculated from the radioactivities incorporated into sorbitol.

produced directly from glucose by a single enzyme reaction which would explain the C-6/C-1 ratio of unity. Support for this comes from Storey and Storey (1981), who have shown that there is a parallel increase in glucose and sorbitol. However, NADPH is required for this reaction and some of the NADPH may be obtained from the relative increase in pentose phosphate pathway activity observed with lower temperatures.

Finally, the early autumn larvae have a greater ability to synthesize glycerol than in late autumn (Fig. 5). However, for sorbitol late autumn larvae have a greater ability for synthesis (Fig. 6). In outdoor northern U.S.A. populations, Morrissey and Baust (1976) showed that glycerol accumulation began during early autumn and sorbitol accumulated later in autumn coinciding with first frost exposure. This accumulation pattern may now be explained in part by changes in the cellular synthesizing machinery.

**Acknowledgements**—This study was supported by NSF research grant PCM81-10327 and the University of Houston, University Park Coastal Center. We wish to thank John Nordin for his comments on this manuscript.

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