Effect of Anaerobiosis on Glycerol Formation in Larvae of the Rice Stem Borer, *Chilo suppressalis* WALKER¹

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Effect of anaerobiosis on glycerol formation was investigated in developing and diapausing larvae of the rice stem borer, *Chilo suppressalis* WALKER. Glycerol accumulation was stimulated in both the pre-diapausing and diapausing larvae exposed to hypoxia, especially to 20% nitrogen-air mixed gas and the accumulation was gradually reduced with increasing nitrogen rates. On the other hand, glycerol formation could not be detected in developing larvae incubated anaerobically. This fact was also confirmed by experiments using ¹⁴C-glucose and ¹⁴C-glycerol.

Lactic acid content was increased in developing larvae exposed to nitrogen gas, but was not increased so much in diapausing larvae. Pyruvic acid content decreased with increase in lactic acid content.

These metabolisms were greatly dependent on the activities of α -glycerophosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase.

Anaerobic metabolism changed according to physiological state of larvae and environmental temperatures.

INTRODUCTION

The accumulation of glycerol as well as sorbitol is closely associated with a conspicuous decline of oxygen uptake in diapausing silkworm eggs, while the polyol decreases progressively with increase of oxygen uptake (CHINO, 1958). These changes in respiratory rates are associated with those in the cytochrome system. CHINO (1958, 1960), WYATT and MEYER (1959) and MEYER (1977, 1978) suggested that the reduction in the cytochrome system leads to accumulation of reduced diphosphopyridine nucleotide (NADH), which is utilized for glycerol formation.

Even if glycerol was not observed in developing insects, it was accumulated in some insects when they were transferred under anoxia (WHILHELM et al., 1961; cf. CHEFURKA, 1965; SØMME, 1966, 1967; KAGEYAMA and OHNISHI, 1973; ZACHARIASSEN and PASCHE, 1976; MEYER, 1977, 1978).

On the other hand, in rice stem borer larvae glycerol concentration in haemolymph increased with increasing activity of α glycerophosphate dehydrogenase (α -GP Dase) in fat body during pre-diapausing to diapausing stage (TSUMUKI and KANEHISA, in preparation). But the respiratory rate of diapausing larvae of the rice stem borer was

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not reduced so much as that of diapausing silkworm eggs as described by CHINO (1958) (TSUMUKI and KANEHISA, 1978). However, anaerobiosis probably plays an important role in glycerol production in the rice stem borer.

This study was carried out to investigate the difference of anaerobic effect on formation of glycerol and other components according to the physiological state of rice stem borer larvae and environmental temperatures.

MATERIALS AND METHODS

Experimental animals. Larvae of the rice stem borer were collected at Okayama Agricultural Experiment Station in Okayama Prefecture. The hibernating larvae were taken from the rice stems. Developing larvae were reared on rice seedlings as described previously (TSUMUKI and KANEHISA, 1978). Only mature larvae were used.

Chemicals. ^{14}C -(U)-glucose (250 mCi/m mole) and ^{14}C -(U)-glycerol (46 mCi/m mole) were purchased from the Radiochemical Centre (Amersham, England). Fructose-1,6-diphosphate (F-1,6-P), nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide reduced form (NADH), glucose-6-phosphate (G-6-P) and aldolase were purchased from Sigma Chem. Co., (St. Louis, Missouri). All other chemicals were of reagent grade.

Method for anaerobiosis. A flask (volume; 100 ml) was employed for the exchange of gas. The top of a flask was fitted with a double-bored rubber stopper. A glass pipe was inserted nearly to the bottom of the flask and another one protruded at the surface of the rubber stopper. The junction between rubber and glass was sealed with vaseline to prevent gas leakage. Larvae were put into the flasks and the air in the flasks was replaced with 20, 50, 80 and 100% nitrogen-air mixed gasses. Anaestization was performed by passing over 2 l of each mixed gas. The 100% nitrogen gas in the flask was obtained by passing it directly from the nitrogen cylinder for several min. After gas exchange, the interception of the gas in the flask from the open air was done with pinchcocks pinching rubber tubes joined at the tops of the glass pipes and the flasks were incubated at 25°C, 15°C and 4°C. The gas in the flask was renewed every day.

Determination of glycerol and sugars. Glycerol, sugars and glycogen contents were determined by the method of TSUMUKI and KANEHISA (1978).

Determination of lactic acid and pyruvic acid. Larvae were weighed and homogenized with 10 ml of 10% trichloroacetic acid. After centrifugation at 3,000 r.p.m. for 10 min, pyruvic acid and lactic acid in the supernatant were determined by the method of SHIMIZU (1950) and BARKER and SUMMERSON (1941), respectively.

Enzyme assays. G-6-P dehydrogenase (G-6-P Dase), α -GP Dase, glyceraldehyde-3-phosphate dehydrogenase (GA-3-P Dase) and lactate dehydrogenase (lactate Dase) activities were determined by slight modification of the methods of CHINO (1960), and KAGEYAMA and OHNISHI (1971) (TSUMUKI and KANEHISA, in preparation). That is, G-6-P Dase activity was measured by the production rate of NADPH and GA-3-P Dase, α -GP Dase and lactate Dase activities were measured by the production or expenditure rates of NADH from NAD.

^{14}C -glucose and ^{14}C -glycerol metabolism under anaerobic condition. The developing

larvae were kept in nitrogen gas for one day at temperatures of 25°C, 15°C and 4°C. Each 1 μ l of ^{14}C -glucose or ^{14}C -glycerol was injected into haemocoel through a leg of the abdomen. The leg was tied fast with cotton thread. Then the larvae were transferred into incubating glass containers at 25°C, 15°C and 4°C, and nitrogen gas passed continuously from the nitrogen cylinder through the container. $^{14}\text{CO}_2$ gas that evolved was trapped by the method of TSUMUKI and KANEHISA (1980). After haemolymph was collected, the remaining tissues were homogenized in 10 ml of 80% ethanol 2 days after injection of ^{14}C -substance. Sugars and glycerol in both haemocoel and the remaining tissues were extracted with 80% ethanol and separated by paper chromatography (TSUMUKI and KANEHISA, 1978). Radioactivity incorporated into sugars and glycerol was measured by a liquid scintillation counter using paper strip method (TSUMUKI and KANEHISA, 1980).

RESULTS

Effect of anaerobiosis on glycerol concentration in haemolymph

The effect of anaerobiosis on glycerol formation in haemolymph of pre-diapausing larvae was examined (Table 1). Pre-diapausing larvae exposed to 100% nitrogen gas failed to exhibit a substantial increase of glycerol concentration in haemolymph at 15°C and glycerol was rather decreased at 25°C, whereas anaerobiosis had a marked effect on elevation of glycerol at 4°C. When pre-diapausing larvae were exposed to 20% nitrogen gas at 15°C, glycerol concentration in haemolymph was largely increased and the increase was gradually reduced with increasing nitrogen rates (Table 2). The effect of anaerobiosis on glycerol formation in haemolymph of developing larvae was examined with the elapse of time at various temperatures. No glycerol synthesis could be detected in developing larvae exposed to these different air-nitrogen mixture ratio gasses at 25°C, 15°C and 4°C. Sorbitol could not be detected in developing and diapausing larvae exposed to hypoxia.

Table 1. EFFECT OF ANAEROBIOSIS ON GLYCEROL CONCENTRATION IN HAEMOLYMPH OF PRE-DIAPAUSING LARVAE OF THE RICE STEM BORER AT VARIOUS TEMPERATURES FOR 3 DAYS

	Glycerol concentration ($\mu\text{g}/10 \mu\text{l}$ haemolymph)		
	25°C	15°C	4°C
Air	73.8	89.6	109.9
N ₂	63.6	89.3	123.7

Table 2. EFFECT OF ANAEROBIOSIS ON GLYCEROL CONCENTRATION IN HAEMOLYMPH OF PRE-DIAPAUSING LARVAE OF THE RICE STEM BORER AT 15°C FOR 3 DAYS

N ₂ /Air	Glycerol concentration ($\mu\text{g}/10 \mu\text{l}$ haemolymph)			
	0%	20%	50%	80%
	91.3	186.7	161.2	136.4

Effect of anaerobiosis on total sugars and glycogen contents

The contents of total sugars and glycogen in developing and diapausing larvae exposed to 100% nitrogen gas for 3 days are shown in Table 3. No significant difference in total sugar contents were found between aerobic and anaerobic larvae. On the other hand, glycogen content in developing larvae under anaerobic condition was somewhat lower than that under aerobic condition, whereas in diapausing larvae no significant differences were found between aerobic and anaerobic insects.

Effect of anaerobiosis on lactic acid and pyruvic acid contents

The contents of lactic acid and pyruvic acid in whole bodies of developing and diapausing larvae exposed to 100% nitrogen gas for 3 days are shown in Tables 4 and 5. In developing larvae, lactic acid content in anaerobic insects rose greatly in comparison with that in aerobic insects, whereas pyruvic acid content varied with temperature.

Table 3. EFFECT OF ANAEROBIOSIS ON CARBOHYDRATE CONTENTS IN WHOLE BODY OF RICE STEM BORER LARVAE AT VARIOUS TEMPERATURES FOR 3 DAYS

Stage	Temperature	Carbohydrate content ($\mu\text{g}/100\text{ mg whole body}$)			
		Total sugars		Glycogen	
		N ₂	Air	N ₂	Air
Development	25°C	8.2	8.1	14.9	20.9
	15°C	9.5	10.0	16.9	20.4
	4°C	11.0	11.7	23.3	23.5
Diapause	25°C	13.6	13.4	34.5	32.3
	15°C	14.4	14.4	34.2	33.5
	4°C	16.6	15.3	27.1	26.5

Table 4. EFFECT OF ANAEROBIOSIS ON LACTIC ACID CONTENT IN WHOLE BODY OF RICE STEM BORER LARVAE AT VARIOUS TEMPERATURES FOR 3 DAYS

		Lactic acid content ($\mu\text{g}/100\text{ mg whole body}$)		
		25°C	15°C	4°C
Development	Air	1.4	3.8	5.7
	N ₂	352.9	145.3	29.0
Diapause	Air	1.0	1.5	3.1
	N ₂	8.2	14.2	17.8

Table 5. EFFECT OF ANAEROBIOSIS ON PYRUVIC ACID CONTENT IN WHOLE BODY OF RICE STEM BORER LARVAE AT VARIOUS TEMPERATURES FOR 3 DAYS

		Pyruvic acid content ($\mu\text{g}/100\text{ mg whole body}$)		
		25°C	15°C	4°C
Development	Air	46.0	23.2	20.4
	N ₂	13.9	21.8	26.9
Diapause	Air	9.8	11.5	11.0
	N ₂	7.0	12.1	13.1

Table 6. EFFECT OF ANAEROBIOSIS ON ENZYME ACTIVITIES IN WHOLE BODY OF RICE STEM BORER LARVAE FOR ONE DAY AT 25°C

		Enzyme activity (m μ mole/mg protein/min)			
		α -GP Dase	GA-3-P Dase	G-6-P Dase	Lactate Dase
Development	Air	75.3	123.5	58.3	190.5
	N ₂	103.5	60.9	72.8	176.8
Diapause	Air	378.4	94.2	201.6	70.3
	N ₂	339.4	20.9	144.6	61.5

Table 7. EFFECT OF ANAEROBIOSIS ON ¹⁴C-GLYCEROL AND ¹⁴C-GLUCOSE METABOLISMS IN THE DEVELOPING LARVAE OF THE RICE STEM BORER AT VARIOUS TEMPERATURES ^a

	Percent of ¹⁴ C-activity					
	¹⁴ C-glucose injection			¹⁴ C-glycerol injection		
	25°C	15°C	4°C	25°C	15°C	4°C
¹⁴ C-glycerol in haemolymph	0.4	0.6	0.5	1.7	2.6	14.4
¹⁴ C-glycerol in tissues	0.7	1.5	2.4	4.1	4.4	41.5
¹⁴ CO ₂	29.0	14.2	2.0	23.0	10.4	2.1

^a The larvae were kept for 2 days at various temperatures.

However, the content of lactic acid was higher than that of pyruvic acid in anaerobic larvae. Furthermore, anaerobiosis was not so effective on lactic acid and pyruvic acid contents in diapausing larvae as those in developing larvae.

Effect of anaerobiosis on enzyme activities

Table 6 shows changes of α -GP Dase, GA-3-P Dase, G-6-P Dase and lactate Dase activities in developing and diapausing larvae exposed to 100% nitrogen gas for one day. In developing larvae exposed to 100% nitrogen gas, α -GP activity increased slightly and GA-3-P Dase activity decreased. Although α -GP Dase activity of developing larvae in nitrogen gas increased slightly, α -GP Dase activity was considerably lower than that of diapausing larvae. Nitrogen gas shows a negative effect on the activities of four enzymes in diapausing larvae.

Effect of anaerobiosis on ¹⁴C-glucose and ¹⁴C-glycerol metabolisms

Little incorporation of ¹⁴C-glucose into glycerol was found in haemolymph and tissues of developing larvae exposed to 100% nitrogen gas (Table 7). ¹⁴C-glycerol was rapidly metabolized by the larvae under anaerobic condition as ¹⁴C-glucose. From these results, glycerol formation is unlikely to be found in developing larvae under anaerobic condition.

DISCUSSION

The glycerol and sorbitol derived from glycogen were closely associated with a conspicuous decline of oxygen uptake in diapausing silkworm eggs (CHINO, 1958;

YAGINUMA and YAMASHITA, 1978). When non-diapausing eggs of the silkworm were incubated under anaerobic condition their glycogen content decreased almost at the same rate as that of the diapausing eggs (OKADA, 1971). Further, these polyols were produced in the non-diapausing eggs of the silkworm under anaerobic condition (KAGEYAMA and OHNISHI, 1973). These phenomena were also observed in other insects irrespective of diapause or non-diapause (WHILHELM et al., 1961; *cf.* CHEFURKA, 1965; SØMME, 1966, 1967; HESLOP et al., 1963; ZACHARIASEN and PASCHE, 1976; MEYER, 1977, 1978). In the rice stem borer glycerol formation was not found in developing larvae exposed to hypoxia as shown in cynthia pupae (WHILHELM et al., 1961). However, in pre-diapausing and diapausing larvae of the rice stem borer exposed to hypoxia, glycerol formation was stimulated. In particular, the formation rate of glycerol was at the highest level in the larvae exposed to 20% nitrogen-air mixed gas, 100% nitrogen gas had no effect on glycerol formation at 15°C. These results are confirmed by the observations that oxygen uptake decreased slightly during pre-diapausing to diapausing stage when glycerol increased in haemolymph (TSUMUKI and KANEHISA, 1978).

Although anaerobiosis failed to stimulate glycerol formation in developing larvae, lactic acid content increased as shown in other insects (*cf.* CHEFURKA, 1965). That is to say, metabolites from glycogen were not glycerol but lactic acid and pyruvic acid in developing larvae under anaerobic condition, whereas in diapausing larvae the main metabolites were glycerol. In developing larvae, the content of pyruvic acid was smaller under anaerobic condition than under aerobic condition. Pyruvic acid occupies a central position in several metabolic sequences such as lactic acid and alanine and the level may vary with the elapse of time when the insects are exposed to anoxia as shown by CHEFURKA (1965). Since GA-3-P Dase activity was reduced to half and lactate Dase activity was slightly inhibited in anaerobic larvae, pyruvic acid may be decreased. However, metabolism of pyruvic acid in larvae exposed to anoxia needs to be investigated further.

Glycerol was produced presumably by the reduction and hydrolysis of triose phosphate (CHINO, 1960) and triose phosphates produced by the way of pentose phosphate pathway and glycolysis (KAGEYAMA, 1976) are metabolized by α -GP Dase or GA-3-P Dase. Serious differences in α GP Dase activity between developing and diapausing eggs of the silkworm were not observed by CHINO (1960) and KAGEYAMA and OHNISHI (1971). However, in the rice stem borer α GP Dase was extremely active and GA-3-P Dase was of low activity in diapausing larvae in comparison with the corresponding enzyme activities in developing larvae (TSUMUKI and KANEHISA, in preparation). Although glycerol production from glycogen may be regulated by phosphorylase activity in diapausing larvae (TSUMUKI and KANEHISA, 1979a), the change of glycerol content was coincident with that of α -GP Dase activity. In this study, the lowered α -GP Dase activity in diapausing larvae exposed to 100% nitrogen gas resulted in decrease of glycerol production. α -GP Dase activity in developing larvae exposed to nitrogen increased slightly, but the activity was much lower than that in diapausing larvae and the activity of lactate Dase. Consequently, glycerol formation may not be observed in the developing larvae under anaerobic condition. Thus the glycerol formation may be regulated by other factors such as low temperature and hormones (TSUMUKI and KANEHISA, 1979b) rather than anaerobiosis.

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