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THE UTILIZATION OF GLYCOGEN AND ACCUMULATION OF SOME INTERMEDIATES DURING ANAEROBIOSIS IN *MYTILUS EDULIS* L.

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Abstract—1. Glycogen degradation in the mussel under anaerobic conditions was measured at two temperatures. Glycogen decrease at 6·6°C was about 3 mg and at 20°C about 6 mg/24 hr per mussel. A Pasteur effect was observed.

2. The decrease of glycogen was almost entirely restricted to muscles, including the foot and hepatopancreas.

3. During anaerobiosis there was a significant increase in succinate and alanine, whereas almost no increase in L-lactate was found. There was some accumulation of D-lactate. Alanine and succinate were formed in almost equimolar amounts and accounted for about 50 per cent of the degraded glycogen.

INTRODUCTION

IT HAS BEEN shown that large amounts of polysaccharides are accumulated in bivalves. Values of glycogen ranging from 10 to 35 per cent of dry weight are common, with extremes lying around 60 per cent (Favretto, 1968; Walne, 1970; de Zwaan & Zandee, 1971). The occurrence of most of the enzymes and intermediates of glycolysis and Krebs' cycle have been established for many bivalve species (Usuki & Okamura, 1956; Bennett & Nakada, 1968; Hammen, 1969; Engel & Neat, 1970; de Zwaan, 1971a, b). It is thus obvious that glycogen can be regarded as an energy store. It is well known that intertidal bivalves can withstand anaerobic conditions for long periods of time, depending upon temperature (Theede *et al.*, 1969). One way to derive energy under anaerobic conditions is by degrading carbohydrates by way of the Embden–Meyerhof scheme. Evidence of glycogen consumption during anaerobiosis was given as early as 1936 by Glaister & Kerly from a study on the retractor muscle in the foot of *Mytilus edulis*. Their observation was remarkable in that lactate production was irregular and small.

This paper summarizes a study about the role of glycogen during anaerobiosis and the effect of glycogen degradation on the physiological levels of D- and L-lactate, alanine and succinate.

MATERIALS AND METHODS

Selection of the samples

For all experiments (I, II and III) mussels were collected from a natural bed in the Waddenzee. Only animals of 5·5 ± 0·2 cm in length were selected. In experiment II they

were also selected on a total weight basis of between 14 and 15 g. In experiment I the mussels were used immediately after they were taken from the sea. The mussels from experiments II and III were kept in aerated natural sea water in the laboratory for 2 and 7 days respectively before being used.

Experiment I (*November* 1968)

The mussels were divided at random into thirteen groups of fifteen animals. The soft part of each mussel was dissolved individually in 30% KOH; the first group after being left lying dry for 2 hr, the second group after 6 hr and subsequent groups at 6 hr intervals up to a total of 72 hr. Each mussel was placed in an open plastic bag which was used to collect any sea water pressed out from the mussel whilst it was lying dry. The sea water expressed from each mussel was added to its solution of soft parts. After saponification (see below), the dry weight of each animal was calculated by taking 20% volume from each sample and drying desiccator to constant weight. The mussels were left lying dry at an average temperature of 6·6°C (min. 3·7, max. 7·9).

Experiment II (*mid-April* 1969)

Five groups of fifteen mussels were chosen at random. Group A was the control group being analysed immediately after dividing the groups. Group B was kept in 1400 ml of well-aerated sea water. Group C was also in 1400 ml of sea water, previously boiled to remove oxygen and through which was bubbled a constant stream of nitrogen. Groups D and E were left lying dry in constant streams of air and nitrogen respectively. Each of the four groups was placed in a desiccator in a water-bath at 20°C for 48 hr. Groups D and E were then placed in sufficient sea water for 15 min to enable the mussels to open their shells so that the surrounding sea water could be easily collected for examination of L-lactate without damaging the animals.

All mussels were dissected into the following five parts: hepatopancreas, all muscles including the foot, gills, mantle and residue. The surrounding sea water from the animals of the five groups was added to their residues. Each individual part was dissolved in 30% KOH and these solutions were examined for glycogen and L-lactate content. An L-lactate examination was also conducted on each of the 1400 ml of sea-water which held groups B and C during the experiment.

From the above experiments the samples in 30% KOH were saponified by keeping them at 50°C for 2 hr. They were neutralized with 5 N HCl and trichloroacetic acid was added in a to a final concentration of 3% for protein precipitation and then centrifuged at 10,000 *g* for 20 min. After filtration the supernatant was used for the determination of glycogen and L-lactate.

Experiment III

Thirty mussels were divided at random into two groups of fifteen animals. The first group was analysed immediately after dividing the groups, the second group after keeping dry for 48 hr at room temperature. The mussels were cooled in ice before removing them from their shells. The soft parts were cut into small pieces with scissors, homogenized in a Potter–Elvehjem homogenizer with an equal volume of cooled 6% (v/v) perchloric acid and centrifuged at 13,000 *g*. The supernatant was neutralized with a solution of 5 N KOH and cooled in a refrigerator for a few hours. The $KClO_4$ formed was removed by centrifugation and the clear supernatant was used for determination of D-lactate and alanine. For the determination of succinate the neutralized supernatant was passed through a strong anion exchange column containing Dowex 1 × 2 in the OH^- form. The column was eluted by 100 ml twice-distilled water and 30 ml of 0·5 M NaCl. The second fraction was used for the determination of succinate.

Determination of glycogen

The glycogen was precipitated by adding 2 vol. of 96% ethanol with Na_2SO_4 as a co-precipitant (van Handel, 1965), placed in boiling water for 2 min, cooled in an ice-bath for 1 hr, and then centrifugated at 10,000 *g* for 20 min. The sediment was then incubated for 20 min at 90°C with a fresh solution of 0·15% anthrone in diluted sulfuric acid ($d = 1{\cdot}75$), and measured spectrophotometrically at 620 nm.

Determination of L- *and* D-*lactate, alanine and succinate*

These compounds were determined by spectrophotometric methods described by Hohorst (1957), Pfleiderer (1965) and Kmetec (1966) respectively. For the determination of D-lactate, D-lactate dehydrogenase (Boehringer & Soehne) was used. For L-lactate 88–90 per cent of the total L-lactate could be measured by the given procedure. (This was checked by control samples of known lactate content.)

Statistical procedure

Differences between groups in the results were examined by the Wilcoxon two-sample test of significance (de Jonge, 1963). Differences are significant when

$$S_0 \leqslant S_1(\alpha, n) \text{ or } S_0 \geqslant S_r(\alpha, n).$$

RESULTS AND DISCUSSION

Table 1 and Fig. 1 gave the results of experiment I. Although there is no significant difference between the various groups in average glycogen and L-lactate percentages, Fig. 1 shows a clear tendency for a glycogen decrease. The glycogen decrease per 24 hr was about 1·5 per cent, corresponding to $740 \times 10^{-2} \times 29 \times 1{\cdot}5 \times 10^{-2} = 3$ mg glycogen/mussel (average dry wt. 740 mg, average

TABLE 1—THE AVERAGE VALUES OF GLYCOGEN AND L-LACTATE AS PERCENTAGES OF DRY WEIGHT DURING INCREASING NUMBER OF HOURS LEFT LYING DRY (EXPERIMENT I)

Hours	Glycogen / Dry weight × 100	L-Lactate / Dry weight × 100	No. of mussels*
2	31 ± 6	0·009 ± 0·004	15
6	34 ± 7	0·009 ± 0·004	15
12	28 ± 4	0·012 ± 0·005	14
18	31 ± 5	0·011 ± 0·003	15
24	32 ± 9	0·009 ± 0·003	15
30	27 ± 6	0·010 ± 0·004	13
36	28 ± 4	0·013 ± 0·006	15
42	30 ± 8	0·010 ± 0·003	15
48	28 ± 6	0·012 ± 0·005	13
54	31 ± 5	0·013 ± 0·004	15
60	27 ± 5	0·011 ± 0·003	12
66	28 ± 4	0·013 ± 0·007	14
72	26 ± 7	0·014 ± 0·006	12

*Where the number is less than fifteen, the animals died during the experiment and were discarded.

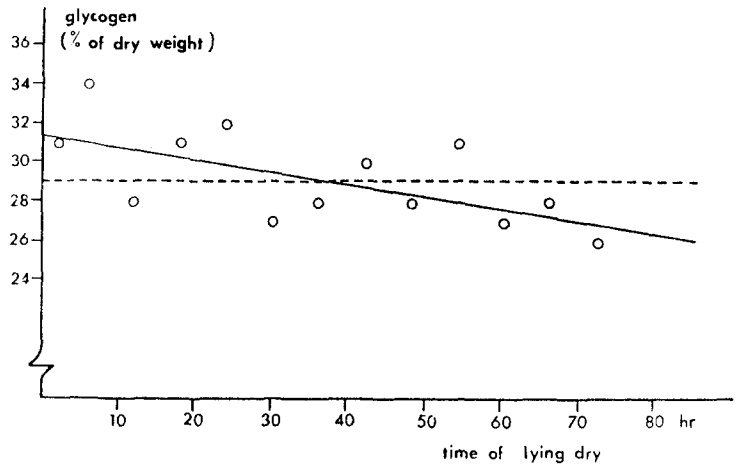

FIG. 1. The correlation between the percentage of glycogen and the time left lying dry at an average temperature of 6·6°C. The dotted line gives the average glycogen percentage (29 per cent) of the thirteen groups analysed.

glycogen percentage 29 per cent from the dry wt.). From 3 mg glycogen $2 \times 3 \times 10^3 \times 180^{-1} = 30$ μmole L-lactate can be formed, whereas an average L-lactate content of about 1 μmole/mussel was found. From Table 1 it is clear that there is almost no increase in L-lactate content when mussels were left lying dry for 24 hr. This means that the degraded glycogen is not converted into L-lactate.

Experiment II was performed in April when the glycogen content of the mussel is at its lowest (de Zwaan & Zandee, 1971). A temperature of 20°C was chosen, because this is a normal temperature for mussels uncovered by the tide in summer. The differences in glycogen content were, where necessary, compared for significance by the Wilcoxon two-sample test. Compared to control group A, there is a significant decrease of glycogen in groups C, D and E (Table 2a and 2b). This decrease is almost entirely restricted to two fractions, namely muscles, including the foot, and the hepatopancreas. For gills, mantle and residue no significant difference between the control group and other groups could be measured. Also no significant difference in changes in glycogen in all fractions was found between groups D and E. Oxygen uptake during exposure by means of diffusion seems unlikely. This is in agreement with observations for many bivalves that an oxygen debt which occurs at shell closure is repaid under aerobic conditions (Mitchell, 1912; Collip, 1921; van Dam, 1935; Schlieper & Kowalski, 1959).

The glycogen used during 48 hr in groups C, D and E is about 25 per cent of the total glycogen stored in the digestive gland and muscles including the foot. The average values for groups C, D and E of the total glycogen consumed are respectively 14·5 (5·5–23·5), 11 (1·5–20·5) and 12 (3–21) mg/mussel (Tables 2a and 2b). As in experiment I, the content of L-lactate does not increase noticeably.

TABLE 2a—AVERAGE VALUES OF WET WEIGHTS, GLYCOGEN AND L-LACTATE IN VARIOUS PARTS OF THE MUSSELS FROM GROUPS A, B, C, D AND E*

	Group	Hepato-pancreas	Muscles	Gills	Mantle	Residue
Wet weight (g)	A	0·61 ± 0·05	1·54 ± 0·07	0·57 ± 0·05	1·18 ± 0·07	1·60 ± 0·16
	B	0·58 ± 0·05	1·51 ± 0·07	0·46 ± 0·06	1·13 ± 0·07	1·51 ± 0·14
	C	0·60 ± 0·06	1·51 ± 0·09	0·54 ± 0·07	1·11 ± 0·08	1·45 ± 0·13
	D	0·59 ± 0·05	1·56 ± 0·09	0·49 ± 0·04	1·12 ± 0·08	1·58 ± 0·13
	E	0·60 ± 0·06	1·52 ± 0·07	0·51 ± 0·06	1·09 ± 0·06	1·52 ± 0·16
Glycogen (mg/g wet wt.)	A	31	22	17	14	14
	B	30	23	14	18	15
	C	23	15	14	17	15
	D	24	17	16	16	13
	E	25	16	14	15	13
L-Lactate (μmole/g wet wt.)	A	0·07	0·24	0·08	0·04	0·06
	B	0·09	0·22	0·11	0·06	0·08
	C	0·06	0·20	0·07	0·03	0·16
	D	0·08	0·22	0·12	0·07	0·21
	E	0·09	0·18	0·10	0·05	0·17

*Group A was the control group, being analysed immediately after dividing the groups; groups B and C were kept in sea water through which was bubbled a constant stream of air and nitrogen respectively; and groups D and E were left lying dry in a constant stream of air and nitrogen respectively. (Experiment II, experimentation period 48 hr, temperature 20°C.)

TABLE 2b—WILCOXON TWO-SAMPLE TEST OF SOME OF THE FIGURES PRESENTED IN TABLE 2a (EXPERIMENT II)

Organ	Compared groups	Wilcoxon value of S_0*	Difference (mg)	Confidence interval for $\alpha = 0{\cdot}05$
Hepatopancreas	A–B	221	—	–
	A–C	293	8	2–14
	A–D	279	7	0–14
	A–E	280	6	0–12
Muscles	A–B	242	—	–
	A–C	297	7	3–11
	A–D	288	5	1–9
	A–E	295	6	2–10

*Critical values for S_0 ($n_1 = n_2 = 15$; $\alpha = 0{\cdot}05$) 184–281; for S_0 ($n_1 = n_2 = 15$; $\alpha = 0{\cdot}10$) 191–274.

Also, L-lactate was not detected in the sea water containing groups B and C and the sea water surrounding groups D and E.

The results of experiments I and II indicate that mussels survive exposure by producing energy anaerobically from the breakdown of glycogen. In experiment II a Pasteur effect could be observed because more glycogen was broken down under anaerobic conditions (groups C, D and E) than under aerobic conditions (group B). Anaerobic degradation of glycogen in vertebrates is possible by the Embden–Meyerhof scheme in which NAD is reduced by the breakdown of glycogen or glucose and reoxidized by the reducing of pyruvate to L-lactate. The results with the sea mussel indicate that the Embden–Meyerhof scheme does not operate the same as in vertebrates. This is in agreement with the observation of many authors that succinate rather than lactate is the major fermentation product of a number of micro-organisms, intestinal helminths and bivalves which depend on anaerobic metabolism for their supply of energy (Lynch & Calvin, 1952; Saz & Beuding, 1966; Simpson & Awapara, 1966; Hammen, 1969). Stokes & Awapara (1968) incubated under nitrogen the mantle of *Rangia cuneata*, a brackish water bivalve, with randomly labeled ^{14}C-glucose. They found that succinate and alanine in equimolar amounts constituted the major portion of the end-products. In their scheme for glucose degradation the reduction of pyruvate to lactate for the re-oxidation of glycolytic NADH is replaced by two other steps, namely oxalacetate → malate, and fumarate → succinate. For this reason only half of the glucose molecule is transformed into succinate, while using both moles of NADH produced per molecule of glucose degraded. The second half undergoes reactions not involving oxidoreductions, namely transamination into alanine. According to this scheme the NADH/NAD redox pair is kept in the same state necessary to maintain glycolysis. As there was no significant L-lactate production during anaerobiosis in *M. edulis* normal concentrations of alanine and succinate were determined as well as those which had been influenced by 48 hr anaerobiosis. This was also done for D-lactate, because the enzymatic test for lactate used in experiment II was specific to the L-isomer. The results are summarized in Table 3. All metabolites underwent a significant increase, but the increase in D-lactate is relatively unimportant compared to succinate and alanine. Succinate and alanine are formed in an almost equimolar amount, resembling the findings of Stokes & Awapara (1968) for *R. cuneata*.

The two acids examined (plus D-lactate) together give an accumulation of 635 μmole/gram wet tissue in 48 hr. This is about 60 μmole for the total animal (average weight = 9·6 g), being equivalent to 30 μmole or 5·4 mg glucose. This is equivalent to about 6 mg glycogen (mg glycogen = mg glucose × 1·111 according to Morris, 1948). From experiment II it became clear that about 10–15 mg glycogen was used during 48 hr of exposure. Therefore alanine and succinate (plus D-lactate) account for about 50 per cent of the degraded glycogen. The presence of D-lactate is remarkable. Michejda *et al.* (1969) found a D-lactate dehydrogenase in *Helix pomatia*, while the same enzyme was present in *Xiphosura*, a moon snail and the bivalve *Mercenaria mercenaria* (Massaro, 1970).

TABLE 3—THE EFFECT OF BEING LEFT FOR 48 hr LYING DRY (GROUP II*) ON THE CONCENTRATION OF SOME ORGANIC ACIDS IN THE SEA MUSSEL

	Wet wt. (g)		D-Lactate		Succinate		Alanine	
Mussel	I	II	I	II	I	II	I	II
1	10·2	8·8	8	13	21	229	560	780
2	9·7	8·4	19	40	20	350	918	1182
3	9·3	8·5	9	42	17	301	315	1074
4	10·6	10·6	2	69	14	475	673	960
5	9·7	9·5	13	52	23	102	416	764
6	11·0	9·7	2	36	10	365	752	1190
7	8·6	9·5	4	16	13	405	674	1271
8	10·3	9·0	12	20	25	294	385	854
9	8·8	10·7	52	1	13	319	1120	713
10	10·6	9·4	11	70	15	237	411	784
11	10·7	8·9	9	61	41	250	490	645
12	8·6	8·6	6	39	20	372	662	792
13	7·4	8·1	5	37	36	305	542	1222
14	9·7	8·3	10	46	51	187	463	961
15	9·0	9·0	6	64	40	215	411	574
$\bar{x}$	9·6	9·1	11	44	24	294	586	918
II − I			33		270		332	
S_0†							325	

*Group I was the control group being analysed immediately after dividing the groups. The values express 10^{-2} μmole/g wet wt. (experiment III).

†Wilcoxon two-sample test: for $n_1 = n_2 = 15$ and $\alpha = 0{\cdot}05$ the difference between I and II is significant when $184 \geqslant S_0 \geqslant 281$.

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