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GLYCOGEN CONTENT AND RELEASE OF GLUCOSE FROM RED BLOOD CELLS OF THE SIPUNCULAN WORM *THEMISTE DYSCRITA*

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SUMMARY

The coelomic red blood cells, or haemocytes, of the sipunculan worm *Themiste dyscrita* (Fisher, 1952) were found to contain high concentrations of glycogen: the haemocytes accounted for approximately 56 % of the total glycogen in the organism. Haemocytes, incubated in a glucose-free medium *in vitro*, released D-glucose into the medium. At 10°C and at a physiological haematocrit, the concentration in the initially glucose-free medium reached physiological plasma levels of D-glucose ($0{\cdot}27\,\mathrm{mmol\,l^{-1}}$) in about 3 h. The cells continued to release D-glucose and the medium concentration reached about $0{\cdot}65\,\mathrm{mmol\,l^{-1}}$ after 24 h. Cells resuspended in plasma also released D-glucose to produce medium concentrations that also appeared to exceed physiological levels. Kinetic analysis of membrane transport of D-glucose suggested that membrane transfer was not the rate-limiting step in this release. Mammalian insulin, epinephrine and glucagon were not effective in influencing D-glucose release. No reduction in the medium concentration of D-glucose could be detected when haemocytes were incubated in $2\,\mathrm{mmol\,l^{-1}}$ D-glucose in plasma or sea water. The results suggest that the haemocyte is involved in the glucose regulation of the organism, although the regulatory mechanism(s) remains to be elucidated. Furthermore, the results suggest that, in addition to its function in oxygen transport, the red blood cell is the principal storage tissue for glycogen in this organism.

INTRODUCTION

The haemerythrin-containing coelomic red blood cells, or haemocytes, of sipunculids are involved in respiratory physiology of these organisms (Edmonds, 1957, 1970; Manwell, 1960; Mangum & Kondon, 1975; Pörtner, Heisler & Grieshaber, 1985). Haemocytes from *Themiste dyscrita* possess a stereospecific transport mechanism which facilitates uptake of monosaccharides, and the rate of D-glucose uptake by this mechanism exceeds that by simple diffusion by several orders of magnitude (Ingermann, Hall, Bissonnette & Terwilliger, 1985). This rapid uptake is not a phylogenetically ubiquitous property of red blood cells (Jacquez, 1984; Ingermann, Bissonnette & Hall, 1986). The physiological significance of rapid D-glucose transfer

Key words: Sipuncula, haemocyte, blood cell, glycogen.

across the membrane of a respiratory cell is not clear. Conceivably, rapid rates of transfer may support high rates of cellular metabolism or may allow the cell to store carbohydrates.

Based on electron microscopy, Terwilliger, Terwilliger & Schabtach (1985) reported that glycogen-like structures exist in the cytoplasm of the coelomic haemocytes of *T. dyscrita*. Coelomocytes of a polychaete contain glycogen (in addition to haemoglobin) and are thought to function as storage sites for chemical energy for the organism as a whole (Dales, 1964). Blood lymphocytes and granular amoebocytes of some ascidians also appear to play a role in energy storage (Freeman, 1964; Fujimoto & Watanabe, 1976). Red blood cells of some invertebrates, in addition to those of *T. dyscrita*, appear to contain glycogen (Ochi, 1969, 1970), but whether this serves as an energy reserve exclusively for the cell, or whether it is a reserve for the other tissues of the organism is not clear. Consequently, we have examined the glycogen content of the haemocytes of *Themiste dyscrita* and their ability to release D-glucose under hypoglycaemic conditions. Although *T. dyscrita* contains both vascular and coelomic haemocytes (Terwilliger *et al.* 1985), this report deals exclusively with the coelomic haemocytes and the plasma of the coelomic fluid.

MATERIALS AND METHODS

Specimens of *Themiste dyscrita* were collected in intertidal regions of Cape Arago on the Pacific coast of Oregon during summer months and were maintained in 40 l of aerated sea water at 4–5 °C.

Plasma and haemocytes for D-glucose and glycogen analyses were obtained within 2 days of animal collection and were prepared by centrifuging whole blood for 2 min in a Fisher microcentrifuge. Plasma was stored frozen until analysis. Cell pellets were resuspended in sea water (which had been passed through a 0·22 μm Millipore filter), passed through a plug of glass wool, centrifuged 1–4 times at 100 ***g*** for 10 min, resuspended in sea water, recentrifuged in the microcentrifuge, and the final cell pellet was frozen until analysed for glycogen. After removal of the blood, the animal carcase was rinsed several times in ice-cold sea water, blotted dry, weighed, frozen within 3 min, and stored until analysed for glycogen.

For physiological studies of the cells, haemocytes were collected and pooled from 3–7 animals into Millipore-filtered sea water containing antibiotic (0·1 % Distrycillin AS, Squibb, Princeton, NJ) and 50 mmol l^{-1} Tris titrated to pH 8·0 at 10°C with HCl (SW). This haemocyte preparation was then filtered through glass wool and centrifuged to yield a relatively homogeneous cell preparation as previously described (Ingermann *et al.* 1985). For studies of D-glucose release and uptake by haemocytes in plasma, the final, washed cell pellet was resuspended so that the final suspension fluid consisted of 75–85 % plasma. The plasma had previously been collected from 10 animals and stored frozen prior to use. To assess whether the apparent release of D-glucose from the haemocytes was due to cell lysis and subsequent hydrolysis of glycogen, a 1·5 % haematocrit suspension was frozen and thawed twice, then incubated at 10°C. There was no discernible D-glucose release

into the medium after 12 h. The influence of several hormones on D-glucose release and uptake was examined. Glucagon and epinephrine were obtained from Sigma Chemical Company (St Louis, MO); porcine insulin was obtained from Lilly Research Laboratories (Indianapolis, IN).

D-Glucose analyses were conducted with an assay kit (no. 510) from Sigma Chemical Company. The most concentrated standard glycogen solution used to generate the standard curve for glycogen analysis (see below) was analysed with the glucose assay (without amyloglucosidase) and no D-glucose was discernible. This indicated that the presence of glycogen would not give a false D-glucose value either by directly interfering with the assay or by liberating D-glucose *via* hydrolysis during the assay. Although glucose appears to be the primary sugar in sipunculid plasma, galactose, mannose, fucose, ribose and maltose are also present (Gonse, 1957; Sundara Rajulu, 1970). Therefore, a 2·0 $mmol\,l^{-1}$ solution of each of these sugars was assayed with the glucose analysis procedure. Less than 5 % of any of these sugars appeared as D-glucose, showing that the enzyme assay was specific for D-glucose.

To determine the amount of glycogen in the haemocytes, an equal volume of 2 % (v/v) Triton X-100 was added to a frozen and thawed pellet of packed haemocytes. This mixture was then diluted 1:1 with 0·06 $mol\,l^{-1}$ HCl. After thorough mixing, 50–75 μl samples were pipetted onto 2 cm squares of Whatman no. 2 filter paper. These squares were then washed in 67 % ethanol, dried, resuspended in acetate buffer and digested with amyloglucosidase (Sigma Chemical Company product no. A 3514) as described by Chan & Exton (1976). To determine body glycogen levels, the animal carcase was minced with scissors in 0·03 $mol\,l^{-1}$ HCl, homogenized with a Brinkmann homogenizer 15 times, each for 15 s, and then a sample was ground with a glass plunger until visually homogeneous. All homogenization procedures were conducted at 0°C. Samples of 60 μl were pipetted onto filter paper squares and treated as above. Analysis of the enzymatic digest for D-glucose was conducted as described above for the plasma samples using the assay kit. Glycogen concentration was determined from a standard curve derived using bovine liver glycogen (Sigma Chemical Company product no. G 0885) treated in the same way as the haemocyte samples. Addition of glycogen to a haemocyte suspension and to minced bodies prior to homogenization showed glycogen recoveries of 99 and 104 %, respectively. A sample of body homogenate was analysed for free D-glucose; none could be detected, indicating that glycogen was not appreciably hydrolysed to D-glucose as a result of the homogenization procedure.

Kinetics of D-glucose uptake by haemocytes was measured as described by Ingermann *et al.* (1985). D-[^{14}C]glucose was obtained from New England Nuclear (Boston, MA) and was dried and resuspended in SW immediately prior to initiation of the experiments.

RESULTS

Although the total [glycogen] in the haemocytes and carcase varied appreciably among the individuals examined, the fraction of glycogen in the haemocytes relative

to that of the whole organism was much less variable (Table 1). The haemocytes contained 56 ± 11 % ($N = 10$) of the total body reserves of glycogen, excluding any glycogen in the non-haemocyte portion of the coelomic fluid. In contrast, the haemocytes constituted only about 18 ± 4 % ($N = 10$) of the total wet tissue mass of the organism, excluding non-haemocyte coelomic fluid contents.

Haemocytes that had been washed in SW and resuspended at 25 % haematocrit in SW released D-glucose into the medium (Fig. 1). [Haematocrit for several freshly caught animals was found to be 28 ± 11 % ($N = 7$).] After an incubation of 24 h at 10°C, the D-glucose concentration in the medium had risen to 0·65 ± 0·03 mmol l^{-1} ($N = 3$), but haemocyte glycogen showed no discernible decline. Release of D-glucose was insensitive to the presence of 0·1 μmol l^{-1} insulin, glucagon or epinephrine. The haematocrit had changed by less than 0·5 % (Hct units) after 12 h and by 4 % after 24 h incubation. Haemocytes incubated in 80 % plasma also released D-glucose to 0·63 and 0·61 mmol l^{-1} in the medium after 9 h (two experiments); the concentration at time zero was 0·25 mmol l^{-1}. Haemocytes suspended in SW or plasma containing 2·0 mmol l^{-1} D-glucose showed no discernible net uptake of glucose, as there was no change in the medium concentration during the 12-h incubation. Insulin and glucagon did not appear to have an influence on D-glucose uptake under such hyperglycaemic conditions in SW.

Table 1. *Plasma concentrations of* D-*glucose and haemocyte and body glycogen in* Themiste dyscrita

Animal no.	Total mass	Plasma [glucose] (mmol l^{-1})	Haemocyte [glycogen] (mg ml^{-1})*	Total haemocyte [glycogen] (mg)	Body [glycogen] (mg g^{-1})†	Total body [glycogen] (mg)†	Total [glycogen]: haemocyte / body + haemocyte (%)
1	3·51	0·593	61·2	14·2	5·13	9·2	61
2	4·22	0·028	0·6	0·3	0·30	0·6	33
3	4·78	0·379	62·6	22·2	5·93	12·8	63
4	5·93	0·299	17·8	13·0	4·47	14·2	48
5	7·59	0·242	50·8	30·8	5·47	19·9	61
6	7·72	0·298	67·0	41·5	6·48	22·0	65
7	7·98	0·230	31·0	33·1	3·79	16·3	67
8	8·24	0·463	57·4	24·7	5·79	23·4	51
9	9·18	0·268	56·2	39·5	4·19	18·0	69
10	9·43	0·180	20·4	12·3	3·82	14·3	46
Mean (all data):‡		0·273	41·8		4·91		56
±S.D.		±0·147	±22·9		±1·81		±11
		($N = 21$)[a]	($N = 16$)[b]		($N = 12$)[b]		($N = 10$)

* ml: packed cell volume.
† Body: animal less coelomic fluid contents.
‡ All data: includes data in addition to those from the 10 animals above.
[a] Each N is the mean of 2–3 determinations per animal.
[b] Each N is the mean of 3–5 determinations per animal.

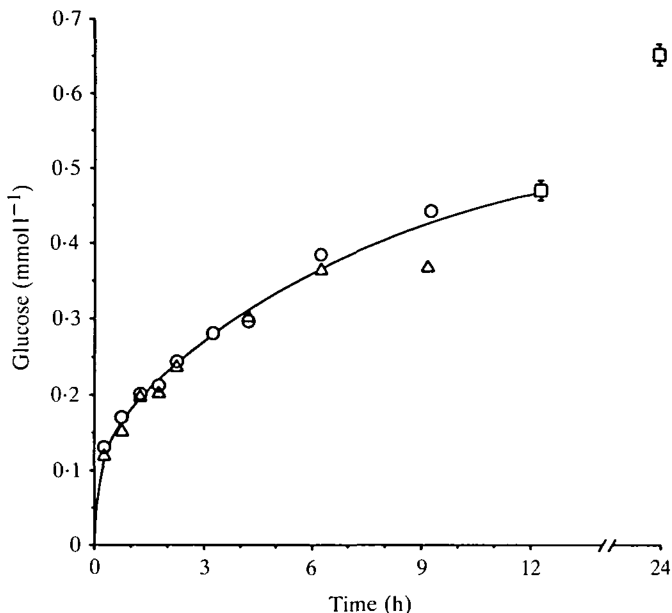

Fig. 1. The release of D-glucose from the haemocytes as a function of time. The haematocrit was 25 % and the incubation was conducted at 10°C. Different symbols represent different trials. Data at 12 h are mean ± S.D., $N = 5$; at 24 h, $N = 3$. The line is fitted to the data by eye.

The kinetics of uptake by the haemocytes in SW showed that D-glucose was taken up with a K_m of 0·3 mmol l^{-1} and a V_{max} of 5·7 μmol l cell vol.$^{-1}$ s^{-1} (Fig. 2).

DISCUSSION

The coelomic haemocytes of *Themiste dyscrita* have a specific membrane transporter for monosaccharides (Ingermann *et al.* 1985). These cells also contain glycogen-like structures in the cytoplasm as seen by electron microscopy (Terwilliger *et al.* 1985). Taken together, these observations suggest that the haemocytes may function as a carbohydrate storage site for the organism. Consequently, we tested for the presence of glycogen in the haemocytes as well as in the carcase and for the ability of the haemocytes to release D-glucose into a glucose-free medium.

The haemocytes contained about 35 mg glycogen g^{-1} wet tissue. This is comparable to the amount of glycogen in the livers of fed rats reported by Carroll, Longley & Roe (1956) to be 43 ± 13 mg g^{-1} wet tissue (as determined by trichloroacetic acid extraction of the tissue). (By comparison, they reported that the livers of fasted rats contain 0·6 ± 0·5 mg glycogen g^{-1} wet tissue.) Thus, the haemocytes, which constituted less than 20 % of the total wet mass of the organism, contained most (about 56 %) of the total glycogen in the organism. Furthermore, these cells contained glycogen levels which were comparable to those of livers from fed rats.

Plasma levels of D-glucose were measured in organisms within 48 h of animal capture (Table 1). The mean value was 0·27 mmol l^{-1} and is taken to be a

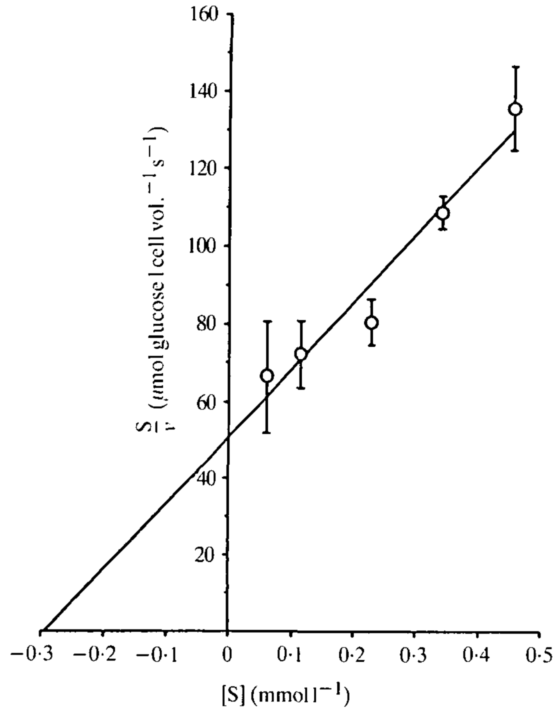

Fig. 2. Substrate dependence of D-glucose uptake. Data are expressed according to the Hanes–Woolf equation,

$$S/v = S/V_{max} + K_m/V_{max},$$

where S is the glucose concentration of the medium and v is the uptake rate calculated from the measured first-order rate constant (see Ingermann, Hall, Bissonnette & Terwilliger, 1985). Data are means ± S.D., $N = 6$, $r = 0{\cdot}92$ for regression. K_m and V_{max} are 0·29 mmol l^{-1} and 5·7 μmol l cell vol.$^{-1}$ s^{-1}, respectively. (The 95 % confidence intervals are 0·15–0·47 mmol l^{-1} and 4·9–6·8 μmol l^{-1} s^{-1}, respectively, calculated as described by Snedecor & Cochran, 1967.)

representative, physiological concentration. Wilber (1948) reported a plasma D-glucose concentration of 0·26 mmol l^{-1} for *Phascolopsis gouldii* (formerly named *Goldfingia* and *Phascolosoma*; phylum Sipuncula) (Stephen & Edmonds, 1972).

To examine whether haemocyte glycogen could be mobilized and made available to the rest of the organism, haemocytes were incubated in a glucose-free medium and the release of D-glucose to the medium was measured. D-Glucose was released into this medium (Fig. 1). At 10°C and at a haematocrit of 25 %, the D-glucose concentration in the experimental medium rose from 0 mmol l^{-1} to the physiological concentration (0·27 mmol l^{-1}) within about 3 h, or about 25 % of a tidal cycle. The D-glucose concentration then continued to rise and reached 0·65 mmol l^{-1} by the end of the 24-h incubation. The glycogen content of haemocytes after 24 h incubation was not measurably diminished. This is consistent with calculations of the amount of glycogen hydrolysis necessary to account for the observed increase in the concentration of D-glucose in the medium.

The *T. dyscrita* haemocyte contains a D-glucose transporter which is functionally similar to the human transporter (Ingermann *et al.* 1985), and the human transporter appears to be directionally symmetrical with respect to function (Carruthers & Melchior, 1985). It is therefore likely that kinetics of influx is comparable to those of efflux in these haemocytes. Assuming that the intracellular D-glucose concentration is equal to that of the medium at 24 h, the rate of accumulation of D-glucose in the suspension medium between 15 min and 135 min (Fig. 1) represents approximately 2 % of the maximum rate of membrane transport of D-glucose. Therefore, membrane transport does not seem to be the rate-limiting step in the release of D-glucose from the haemocytes. It is more likely that the conversion of glycogen to glucose is rate-limiting.

To determine whether some plasma factor limits the release of D-glucose from haemocytes, haemocytes were incubated in about 80 % plasma, 20 % SW. Under these conditions, they released D-glucose into media of concentrations slightly above those previously seen in the plasma-free media. Thus, nothing in the frozen and thawed plasma restricted D-glucose release from the haemocytes; in fact, exposure to plasma appeared to stimulate D-glucose release slightly. Haemocytes incubated in media containing 2 mmol l^{-1} D-glucose did not appear to accumulate glucose as no change could be detected in the medium concentration. To examine further the control of D-glucose release or uptake, haemocytes in either 2 mmol l^{-1} or glucose-free media were exposed to mammalian insulin, glucagon or epinephrine. There was no discernible effect of any hormonal treatment.

The coelomic haemocytes of *T. dyscrita* contain high levels of glycogen. It appears that D-glucose from this glycogen can be rapidly (within 3 h) mobilized and released to achieve physiological concentrations in the coelomic fluid. Since glycogen is an important source of chemical energy in the sipunculid *Sipunculus nudus* under conditions of environmental hypoxia (such as those which may exist during the tidal cycle) (Pörtner *et al.* 1984), the ability of the haemocyte to store and release carbohydrate may be very important in the environmental physiology of the whole organism. The high concentration of glycogen stored in the haemocytes and their ability to liberate D-glucose to the blood plasma makes these cells functionally comparable to mammalian hepatocytes. Despite the similarities, however, we found no mechanisms that limit the release of D-glucose from the haemocytes. Nonetheless, the results of this study indicate that the red blood cell of this sipunculid is an important tissue for carbohydrate storage and probably functions in glucose regulation as well as in the respiratory physiology of the organism.

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