J. exp. Biol. (1978), **74**, 37–45
With 1 figure
Printed in Great Britain

THE ROLE OF THE THERMAL HYSTERESIS FACTOR IN *TENEBRIO MOLITOR* LARVAE

JEAN L. PATTERSON AND JOHN G. DUMAN
Biology Department, University of Notre Dame, Notre Dame, Indiana 46556 *U.S.A.*

(*Received* 25 *July* 1977)

SUMMARY

The haemolymph of larvae of *Tenebrio molitor* contains a factor which produces a thermal hysteresis (a difference between the freezing and melting points) of approximately 0·75 °C. When larvae were acclimated to low temperatures or short photoperiod the thermal hysteresis increased more than twofold. Coincident with the increase in thermal hysteresis the supercooling points and lower lethal temperatures of the larvae were depressed. Therefore, the thermal-hysteresis-producing factor seems to function as an antifreeze.

The factor may also act as an adaptation to prevent desiccation. Thermal hysteresis increased almost three-fold in larvae acclimated to low relative humidity. Also, larvae with high levels of the thermal hysteresis factor survived low relative humidities much better than did larvae with lower levels.

INTRODUCTION

Ramsay (1964) found a thermal hysteresis (a difference between the freezing and melting points) in haemolymph and in fluid from the perirectal space of larvae of the mealworm, *Tenebrio molitor*. Grimstone, Mullinger & Ramsay (1968) later reported that this thermal hysteresis was due to a protein of molecular weight 10000–12000 daltons. The function of the thermal-hysteresis-producing protein was not speculated upon in either study; however, Wall (1971) has suggested that the proteins may play a role in the water reabsorption mechanism of the rectal complex.

Recently, a protein that produces a thermal hysteresis has been reported in the haemolymph of overwintering larvae of the darkling beetle, *Meracantha contracta* (Duman, 1977*a*, *b*). This protein enhances low-temperature survival by depressing the freezing and supercooling points of the larval haemolymph. Protein and glycoprotein antifreezes, which produce a thermal hysteresis similar to that of these insect larvae, have been found in several species of cold water marine teleost fishes (DeVries & Wohlschlag, 1969; DeVries, 1971; Duman & DeVries, 1975, 1976; Raymond, Lin & DeVries, 1975). The fish antifreezes function to depress the freezing point of the body fluids of the fish below that of sea water and thereby prevent freezing. The degree of difference between freezing and melting points (thermal hysteresis) increases with increasing antifreeze concentration in aqueous solutions of purified protein or glycoprotein antifreeze. Theede, Schneppenheim & Beress

(1976) isolated a thermal-hysteresis-producing glycoprotein, which also appears to function in low-temperature survival, from the intertidal mollusc, *Mytilus edulis*. Thus, thermal-hysteresis-producing solutes are known to occur in three widely divergent groups of animals – teleost fishes, insects and molluscs. Except in the case of the *Tenebrio* larvae these thermal-hysteresis-producing solutes have been demonstrated to function to enhance survival of the animal at subzero temperature. The purpose of this study was to ascertain the function of the thermal-hysteresis-producing factor in *Tenebrio* and to investigate the environmental cues which effect the production of this factor.

MATERIALS AND METHODS

Tenebrio cultures were originally purchased from Carolina Supply and thereafter maintained in the laboratory on wheat bran at 20 °C and 90% relative humidity, in a 12L/12D photoperiod.

Haemolymph samples were collected by puncturing the cuticle of the larvae with a 23-gauge needle in the dorsal midline. Three to six μl of haemolymph were then collected in one end of a 10 μl glass capillary tube by capillary action. The opposite end of the capillary was sealed in a flame. Care was taken not to heat the haemolymph. The sample was then centrifuged briefly to force the haemolymph into the sealed end of the capillary. Finally, the capillary was sealed with mineral oil, allowing an air space to remain between the oil and the haemolymph. If the melting and freezing points could not be run immediately, the samples were frozen for later analysis.

The freezing and melting points of the haemolymph samples were determined according to the following technique, which is a modification of the method of Ramsay & Brown (1955). A small seed crystal was spray-frozen in the sample with Cryokwik and the capillary placed in a refrigerated (alcohol) bath with a viewing port through which the sample could be observed with a microscope. The temperature of the bath was finely controlled ($\pm$0·02 °C). The temperature of the bath was raised 0·05 °C/5 min until the crystal disappeared. The temperature at which the crystal disappeared was taken as the melting point of the sample. Another crystal was then spray-frozen in the capillary and the temperature lowered 0·1 °C/2·5 min until the crystal began to grow. This temperature was taken as the freezing point of the sample.

The supercooling point (temperature at which spontaneous nucleation occurs when the temperature of the insect is lowered below its freezing point) of *Tenebrio* larvae was determined with a thermoelectric technique. A thermistor was attached to a YSI Model 42SC telethermometer and a recorder. The larvae were placed in a flask in a refrigerated bath and the temperature was lowered 0·4 °C/min. The supercooling point of the insect was easily determined as the temperature at which a rapid increase in the body temperature occurred due to the release of the heat of fusion as the body fluids froze (Salt, 1966). Larvae were starved for 24 h prior to supercooling point determination.

Lower lethal temperatures were determined by holding larvae (n = 8–10) in a flask in an alcohol bath for 24 h at a constant temperature. At the end of this period the larvae were removed from the cold chamber and held at room temperature for

24 h. The insects were considered to be dead if they failed to respond to a lighted match. Prior to lower lethal temperature determinations the larvae were starved for 24 h.

The time to 50% mortality was determined at a relative humidity of 15% at 22 °C. Larvae were placed in individual vials in a desiccator containing Drierite desiccant. No food was supplied. The larvae were monitored for a darkening of the cuticle which indicated death.

Glycerol concentrations in the haemolymph were determined using Calbiochem glycerol reagents. The enzymic technique is based on the method of Eggstein & Kreutz (1966) as modified by Bucolo & David (1973).

Acclimation experiments were conducted either in Precision Scientific Model 805 incubators or in Controlled Environments Inc. environmental chambers. In the cold acclimation experiments larvae were gradually taken from 20 to 5 °C over the course of 7 days. Relative humidity was controlled by equilibrating the atmosphere over the larvae with distilled water (90% R.H.) or with various saturated salt solutions (Winston & Bates, 1960; Ahearn & Hadley, 1969). Saturated aqueous solutions of NaCl and NaI were used to attain humidities of 75% and 38% respectively. A group of larvae were acclimated to 15% R.H. in a desiccator containing Drierite after they had been acclimated to 38% R.H. for 21 days.

The water content was determined by comparing the wet and dry weights of the larvae. Dry weights were measured after the larvae had been dried for 48 h at 105 °C in a drying oven.

RESULTS

When the freezing and melting points of a normal aqueous solution are measured using the technique described above the two points can easily be seen to be nearly identical (within 0·02 °C) as theory predicts. As the bath temperature is slowly lowered from the melting temperature the seed crystal grows immediately. But, when the temperature of a sample of *Tenebrio* haemolymph is slowly lowered from the melting-point temperature the seed crystal does not immediately begin to grow. In some cases the temperature must be lowered 2–3 °C below the melting point before crystal growth occurs. When the freezing point is reached the crystal generally grows as long monocline spears which rapidly spread throughout the entire sample. This freezing behaviour is quite similar to that of aqueous solutions which contain the protein or glycoprotein antifreezes of teleost fishes. It appears likely that the *Tenebrio* thermal-hysteresis-producing factor is basically similar to the teleost antifreezes. Therefore it is reasonable to assume that the amount of thermal hysteresis in a sample of *Tenebrio* haemolymph is proportional to the concentration of the thermal-hysteresis-producing factor, as is the case with the teleost antifreezes (DeVries, 1971; Lin, Duman & DeVries, 1972; Duman & DeVries, 1976).

Larvae maintained under summer environmental conditions of 20 °C and a long photoperiod (16L/8D) had a thermal hysteresis of 0·75 °C in the haemolymph (Table 1(1)). Acclimation of larvae to 20 °C but to a short photoperiod resulted in a more than twofold increase in thermal hysteresis (Table 1(2)). Likewise, acclimation of larvae to 5 °C in combination with either a short or a long photoperiod resulted in a significant increase (F prob. $< 0{\cdot}05$) in thermal hysteresis (Table 1 (3, 4)).

Table 1. *The effects of acclimation temperature and photoperiod on the melting point, freezing point and thermal hysteresis in the haemolymph of* Tenebrio *larvae*

The relative humidity of the larvae was maintained at 90%. Numbers in parentheses indicate sample size. Values are means ± standard deviation. See text for details.

	Acclimation conditions				
	T (°C)	Photoperiod (L/D)	Melting point (°C)	Freezing point (°C)	Melting point minus freezing point (°C)
(1) (7)	20	16/8	−1·24 ± 0·12	−2·20 ± 0·59	0·75 ± 0·12
(2) (10)	20	6/18	−1·08 ± 0·08	−2·75 ± 0·68	1·67 ± 0·70
(3) (8)	5	18/6	−1·30 ± 0·19	−2·77 ± 0·42	1·47 ± 0·26
(4) (8)	5	6/18	−1·38 ± 0·28	−2·80 ± 0·36	1·35 ± 0·38

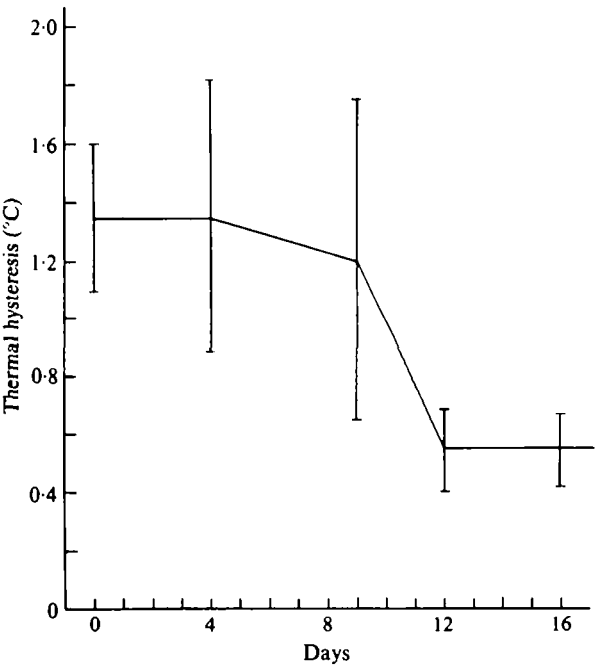

Fig. 1. The effect of acclimation to 20 °C and a long photoperiod (16L/8D) on the thermal hysteresis in the haemolymph of *Tenebrio* larvae which had previously been acclimated to 5 °C and an 8L/16D photoperiod. Each point represents the mean of 3–4 individuals ± standard deviation.

The freezing points and thermal hysteresis of groups 2, 3 and 4 were all significantly different from those of group 1, but groups 2, 3 and 4 were not significantly different from one another.

When larvae which had previously been acclimated for 2 months to low temperature (5 °C) and a short photoperiod (6L/18D) were acclimated to 20 °C and a long photoperiod (16L/8D) the thermal hysteresis in the haemolymph decreased. The time course for this loss of thermal hysteresis is shown in Fig. 1. Some individuals lost the thermal hysteresis more quickly than others. This is indicated by the increase in variability after the warm acclimation was initiated.

Table 2. *The effect of acclimation to various relative humidities at 22 °C and a 12L/12D photoperiod on the melting point, freezing point and thermal hysteresis of the haemolymph of* Tenebrio *larvae. Numbers in parentheses indicate sample size. Values are mean ± standard deviation*

	Relative humidity (%)	Melting point (°C)	Freezing point (°C)	Melting point minus freezing point (°C)
(1) (8)	90	−1·00 ± 0·09	−1·81 ± 0·27	0·81 ± 0·30
(2) (8)	75	−1·10 ± 0·12	−2·30 ± 0·57	1·21 ± 0·65
(3) (7)	38	−0·92 ± 0·12	−3·01 ± 0·59	2·09 ± 0·56
(4) (7)	15	−1·07 ± 0·13	−3·21 ± 0·63	2·14 ± 0·61

Table 3. *The effects of acclimation temperature, photoperiod and humidity on the supercooling point and lower lethal temperature of* Tenebrio *larvae*

Acclimation to low temperature (2), short photoperiod (3) or low humidity (4) is known to increase thermal hysteresis of the haemolymph. These same acclimations also significantly depressed both the supercooling points and lower lethal temperatures of the larvae. Numbers in parentheses indicate the sample size for the supercooling point determinations. Supercooling point values indicate mean ± standard deviation. See text for details.

	Acclimation conditions			Supercooling point (°C)	Lower lethal temperature (°C)
	T (°C)	Photoperiod (L/D)	Relative humidity (%)		
(1) (7)	20	16/8	90	−7·7 ± 2·6	−7·0
(2) (8)	5	6/18	90	−14·9 ± 2·1	−13·0
(3) (10)	20	6/18	90	−13·6 ± 2·7	−13·0
(4) (8)	20	12/12	15	−12·80 ± 4·3	−11·5

Acclimation of *Tenebrio* to low humidity, even at 20 °C and long photoperiod, resulted in an increase in haemolymph thermal hysteresis (Table 2). In fact, the thermal hysteresis of larvae acclimated to 15% R.H. was nearly three times that of larvae held at 90% R.H. The freezing points and thermal hysteresis of groups 1 and 2 were significantly different (F prob. < 0·001) from those of groups 3 and 4. But group 1 was not significantly different from group 2 nor was group 3 different from group 4 at any significantly different probability level. The water content of larvae acclimated to the various humidities listed in Table 2 remained constant.

As Table 3 indicates, any of the acclimation treatments (low temperature, short photoperiod or low relative humidity) which induced increased thermal hysteresis, also significantly (F prob. < 0·001) lowered the supercooling points of the larvae below those of larvae held at 20 °C, long photoperiod and high relative humidity. In addition, those groups (2, 3, 4) with the lowered supercooling points also had depressed lower lethal temperatures. Because *Tenebrio* larvae are not freeze-tolerant the supercooling points and lower lethal temperatures should be nearly identical for a particular group. The glycerol concentration of cold-acclimated *Tenebrio* was not significantly different from that of warm-acclimated larvae. Haemolymph glycerol levels in both groups were < 10 mg %.

Larvae which had been acclimated to conditions which do not induce production of the thermal-hysteresis-producing factor (i.e. high temperature, long photoperiod

Table 4. *The effect of acclimation temperature, photoperiod and humidity on the survival of* Tenebrio *larvae at* 15% *relative humidity*

Acclimation to short photoperiod (2), low humidity (3) or low temperature (4) is known to increase thermal hysteresis in the haemolymph. These same acclimation conditions greatly extended the time to 50% mortality of the larvae at 15% relative humidity. The acclimation time for each group was 30 days, except group 3 which had an acclimation time of 7 days.

	Acclimation conditions			
	T (°C)	Photoperiod (L/D)	Relative humidity (%)	Time to 50% mortality (days)
(1)	20	16/8	90	8
(2)	20	8/16	90	30+
(3)	20	16/8	38	30+
(4)	5	16/8	90	33

and high relative humidity – Table 1) had a time to 50% mortality of 8 days when placed in a desiccator at 15% R.H. and 22 °C (Table 4(1)). However, when larvae were acclimated to any of the environmental factors which increase the levels of the thermal-hysteresis-producing factor (low temperature, short photoperiod or low relative humidity) the ability of the larvae to survive the low relative humidity of the desiccator was greatly enhanced (Table 4(2, 3, 4)). Tests in which the larvae did not die within 30 days were generally terminated after this time.

DISCUSSION

All of the thermal hysteresis factors which have been isolated to date have been shown to be either glycoproteins or proteins (DeVries, 1971; Lin *et al.* 1972; Raymond *et al.* 1975; Duman & DeVries, 1976). The *Tenebrio* thermal hysteresis factor was partially purified and identified as a protein by Grimstone *et al.* (1968). Because the thermal hysteresis of aqueous solutions of purified glycoprotein or protein antifreeze varies directly with antifreeze concentration (DeVries, 1971; Lin *et al.* 1972; Duman & DeVries, 1976), it is reasonable to assume that increases in thermal hysteresis in the haemolymph of *Tenebrio* larvae result from increases in concentration of an antifreeze protein.

The original studies on the thermal-hysteresis-producing protein in *Tenebrio* larvae failed to determine a function for the protein (Ramsay, 1964; Grimstone *et al.* 1968) and relegated the protein to the status of a 'biological curiosity'. Evidence presented in this study indicates that the factor is an adaptation to low temperature. Acclimation of larvae to environmental conditions normally associated with the onset of winter (low temperatures or short photoperiods), resulted in increased hysteresis in the larval haemolymph. In addition, when larvae which had high levels of thermal hysteresis (induced by low-temperature acclimation) were warm-acclimated, the thermal hysteresis decreased noticeably. This strongly suggests that the thermal hysteresis factor is an adaptation to low temperature. More specifically, the factor appears to depress the supercooling point and therefore the lower lethal temperature of the frost-susceptible larvae. Acclimation of larvae to any of the three environmental conditions known to increase thermal hysteresis (low temperature, short photoperiod

or low relative humidity) also lowered the supercooling point and the lower lethal temperature of the larvae. Glycerol was present in concentrations of less than 10 mg % in the haemolymph and could not have been responsible for this effect. Therefore the thermal-hysteresis-producing factor seems to function as an antifreeze to depress the freezing and supercooling points of the larvae in winter. It should be kept in mind that *Tenebrio molitor* originally came from Central Europe (Metcalf & Flint, 1951) where it certainly encountered subzero temperatures, and that, in the United States, populations of *Tenebrio* are found in stored grain or grain products in regions where low temperatures are common in winter.

The antifreeze function of the thermal-hysteresis-producing factor does not account for the 'low' levels of the factor in larvae reared under summer temperature and photoperiod conditions where at a high relative humidity the larvae had 0·75 °C thermal hysteresis in the haemolymph. If the *Tenebrio* thermal-hysteresis-producing factor has activity comparable to that of the glycoprotein or protein antifreezes of teleost fishes then the antifreeze concentration of the haemolymph of these *Tenebrio* should be from 12 to 15 mg/ml. This is a very high antifreeze concentration to maintain in the haemolymph over periods when it is not needed. The alternative is that the thermal hysteresis factor may serve another function in addition to that of an antifreeze.

The cryptonephridial rectal complex of *Tenebrio* is an extremely efficient water reabsorption system (Ramsay, 1964; Grimstone *et al.* 1968) which even has the ability to absorb water from an unsaturated atmosphere (Dunbar & Winston, 1975). Studies have shown that a thermal-hysteresis-producing factor was concentrated in the perirectal space (Ramsay, 1964; Grimstone *et al.* 1968), which appears to be enticing preliminary evidence that this protein might be involved in the water reabsorption mechanism. But these studies were unable to correlate the protein with water reabsorption, or any other function. Oschman & Wall (1969) have observed a flocculent material in the intercellular spaces of the rectal pads of dehydrated *Periplaneta*, and an analogous material is present in the rectal papillae of *Calliphora* (Berridge & Gupta, 1967). Wall (1971) has suggested that these and other similar macromolecular solutes may play a role in the concentration mechanism of the rectal complex.

Acclimation of larvae to a low relative humidity induced production of the thermal-hysteresis-producing antifreeze. Climatological data on the South Bend, Indiana region collected by the National Oceanic and Atmospheric Administration shows that there is no tendency toward lower relative humidities during the autumn and winter. Therefore low relative humidity could not be used by the larvae as a cue to the approach of winter. However, the increased production of the thermal hysteresis factor in low humidity indicates that the factor might be a desiccation-resistance adaptation and may somehow be involved in the water reabsorption mechanism of the rectal complex. This function could also account for the presence of low levels of the factor in larvae held under summer conditions of temperature and photoperiod and 90% R.H. Better, but still preliminary, evidence that the factor might be involved in resistance to desiccation is provided by the extended survival in the desiccator of larvae with high antifreeze levels. This was true whether the high antifreeze concentration was induced by acclimation of the larvae

to low temperature, short photoperiod or low humidity. Gradual acclimation of larvae to low humidity would be expected to induce adaptive physiological mechanisms which would enhance survival in the desiccator. But there is no *a priori* reason why acclimation to low temperature or short photoperiod should induce these mechanisms. However, these factors should and do commonly induce adaptations to low temperature, including production of the antifreeze. This indicates that the increased levels of the thermal hysteresis factor may serve the dual functions of both depressing the supercooling point and therefore the lower lethal temperature of the larvae, and also of inhibiting dehydration by perhaps contributing to the water reabsorption mechanism of the cryptonephridial rectal complex in an as yet unknown manner.

This work was funded by N.I.H. Biomedical Sciences Support Grants FR/RR-07033-9 and FR/RR-07033-10 by a Grant in Aid of Research from Sigma Xi and by National Science Foundation Grant No. PCM77-03475.

REFERENCES

AHEARN, G. A. & HADLEY, N. F. (1969). The effects of temperature and humidity on water loss in two desert Tenebrionid beetles, *Eleodes aramata* and *Cryptoglossa verrucosa*. *Comp. Biochem. Physiol.* **30**, 739–749.

BERRIDGE, M. J. & GUPTA, B. L. (1967). Fine structural changes in relation to ion and water transport in the rectal papillae of the blowfly, *Calliphora*. *J. Cell. Sci.* **2**, 89–112.

BUCOLO, G. & DAVID, H. (1973). Quantitative determination of serum triglycerides by the use of enzymes. *Clin. Chem.* **19**, 476–482.

DEVRIES, A. L. (1971). Glycoproteins as biological antifreeze agents in Antarctic fishes. *Science, N.Y.* **172**, 1152–1155.

DEVRIES, A. L. & WOHLSCHLAG, D. E. (1969). Freezing resistance in some Antarctic fishes. *Science, N.Y.* **163**, 1073–1075.

DUMAN, J. G. & DEVRIES, A. J. (1974). Freezing resistance in winter flounder *Pseudopleuronectes americanus*. *Nature, Lond.* **247**, 237–238.

DUMAN, J. G. & DEVRIES, A. L. (1974). The effects of temperature and photoperiod on antifreeze production in cold water fishes. *J. exp. Zool.* **190**, 89–98.

DUMAN, J. G. & DEVRIES, A. L. (1975). The role of macromolecular antifreezes in cold water fishes. *Comp. Biochem. Physiol.* **52**A, 193–199.

DUMAN, J. G. & DEVRIES, A. L. (1976). Isolation characterization, and physical properties of protein antifreezes from the winter flounder, *Pseudopleuronectes americanus*. *Comp. Biochem. Physiol.* **54**B, 375–380.

DUMAN, J. G. (1977*a*). The role of macromolecular antifreeze in the darkling beetle, *Meracantha contracta*. *J. comp. physiol.* **115**, 279–286.

DUMAN, J. G. (1977*b*). Variations in macromolecular antifreeze levels in larvae of the darkling beetle, *Mercantha contracta*. *J. exp. Zool.* **201**, 85–92.

DUNBAR, B. S. & WINSTON, P. W. (1975). The site of active uptake of atmospheric water in larvae of *Tenebrio molitor*. *J. Insect Physiol.* **21**, 495–500.

EGGSTEIN, M. & KREUTZ, F. H. (1966). A new method for the determination of neutral fats in blood serum and tissues. I. Principle, performance and discussion of method. *Klin. Wschr.* **44**, 262–267.

GRIMSTONE, A. V., MULLINGER, A. M. & RAMSAY, J. A. (1968). Further studies on the rectal complex of the mealworm *Tenebrio molitor* (Coleoptera, Tenebrionidae). *Phil. Trans. R. Soc.* B **253**, 343–382.

LIN, Y., DUMAN, J. G. & DEVRIES, A. L. (1972). Studies on the structure and activity of low molecular weight glycoproteins from an Antarctic fish. *Biochem. Biophys. Res. Comm.* **46**, 87–92.

METCALF, C. L. & FLINT, W. P. (1951). *Destructive and Useful Insects*. McGraw-Hill Book Company.

OSCHMAN, J. L. & WALL, B. J. (1969). The structure of the rectal pads of *Periplaneta americana* with regard to fluid transport. *J. Morph.* **127**, 475–510.

RAMSAY, J. A. & BROWN, R. A. (1955). Simplified apparatus and procedure for freezing point determinations upon small volumes of fluid. *J. Scient. Instrum.* **32**, 372–375.

RAMSAY, J. A. (1964). The rectal complex of the mealworm *Tenebrio molitor* L. (Coleoptera, Tenebrionidae). *Phil. Trans. R. Soc.* B **248**, 279–314.

RAYMOND, J. A., LIN, Y. & DEVRIES, A. L. (1975). Glycoprotein and protein antifreezes in two Alaskan fishes. *J. exp. Zool.* **193**, 125–130.

SALT, R. W. (1966). Factors influencing nucleation in supercooled insects. *Can. Ent.* **95**, 1190–1202.
THEEDE, H., SCHNEPPENHEIM, R. & BERESS, L. (1976). Frostschutz-Glykoproteine bei *Mytilus edulis*? *Marine Biology* **36**, 183–189.
WALL, B. J. (1971). Local osmotic gradients in the rectal pads of an insect. *Fed. Proc. Fedn. Am. Socs exp. Biol.* **80**, 42–48.
WINSTON, P. W. & BATES, D. H. (1960). Saturated solutions for the control of humidity in biological research. *Ecology* **41**, 232–237.