J. exp. Biol. **138**, 535–539 (1988)
Printed in Great Britain © *The Company of Biologists Limited 1988*

SHORT COMMUNICATION

EFFECTS OF FREEZING ON THE FUNCTION AND ASSOCIATION STATE OF CRUSTACEAN HAEMOCYANINS

BY S. MORRIS

Department of Biological Sciences, University of Calgary, 2500 University Drive NW, Calgary, Alberta, Canada T2N 1N4

Accepted 14 April 1988

The addition of protease inhibitors and antibiotics provides a valuable means of stabilizing proteins in solution but this is less desirable if the physiological properties of a biological fluid, such as haemolymph, are to be accurately evaluated. Freezing haemolymph has proved to be a convenient way both of transporting samples from remote sites and of storage for longer periods. Although many investigators have suspected and intimated that freezing may affect haemocyanin function there are few data describing exactly what these effects may be. Mangum (1983*a*) provided some data for *Squilla empusa* haemocyanin indicating that freezing did not alter the P_{50} affinity but did reduce the cooperativity of O_2 binding. Similar results were reported for *Palaemon elegans* (Bridges *et al.* 1984; Morris *et al.* 1985). These reports that cooperativity may be reduced by freezing indicate that structural changes occur that may lead to changes in affinity, despite the P_{50} remaining unchanged.

The haemocyanins from two macruran and two brachyuran species were investigated; *Homarus vulgaris* (Selbie) and *Cancer pagurus* (L.) from Europe, together with *Hemigrapsus nudus* (Dana) and *Cambarus diogenes* (Girard) from North America.

The effect of freezing on the affinity and cooperativity of oxygen binding was determined by the construction of oxygen equilibrium curves for fresh, native haemolymph and for the same haemolymph subjected to a known number of freezing cycles. The freezing protocol consisted of flash freezing (−80°C) the haemolymph in small samples which were kept frozen for 30 min before thawing at room temperature. Any required sample was withdrawn and the freezing cycle repeated appropriately. Oxygen equilibrium curves were constructed at 15°C as described by Bridges *et al.* (1984) or Morris *et al.* (1988). Both methods are spectrophotometric and directly comparable. Oxygen affinity was analysed as the P_{50} in dependence of pH and the cooperativity was calculated for the same equilibrium curves (Fig. 1). Using a fiducial limit of $P = 0{\cdot}05$, covariance analysis showed no significant change in $\Delta \log P_{50}/\Delta pH$ with respect to freezing. None of the four haemocyanins exhibited significant dependence of cooperativity (n_{50}) on

Key words: haemocyanin, freezing, oxygen affinity, association state.

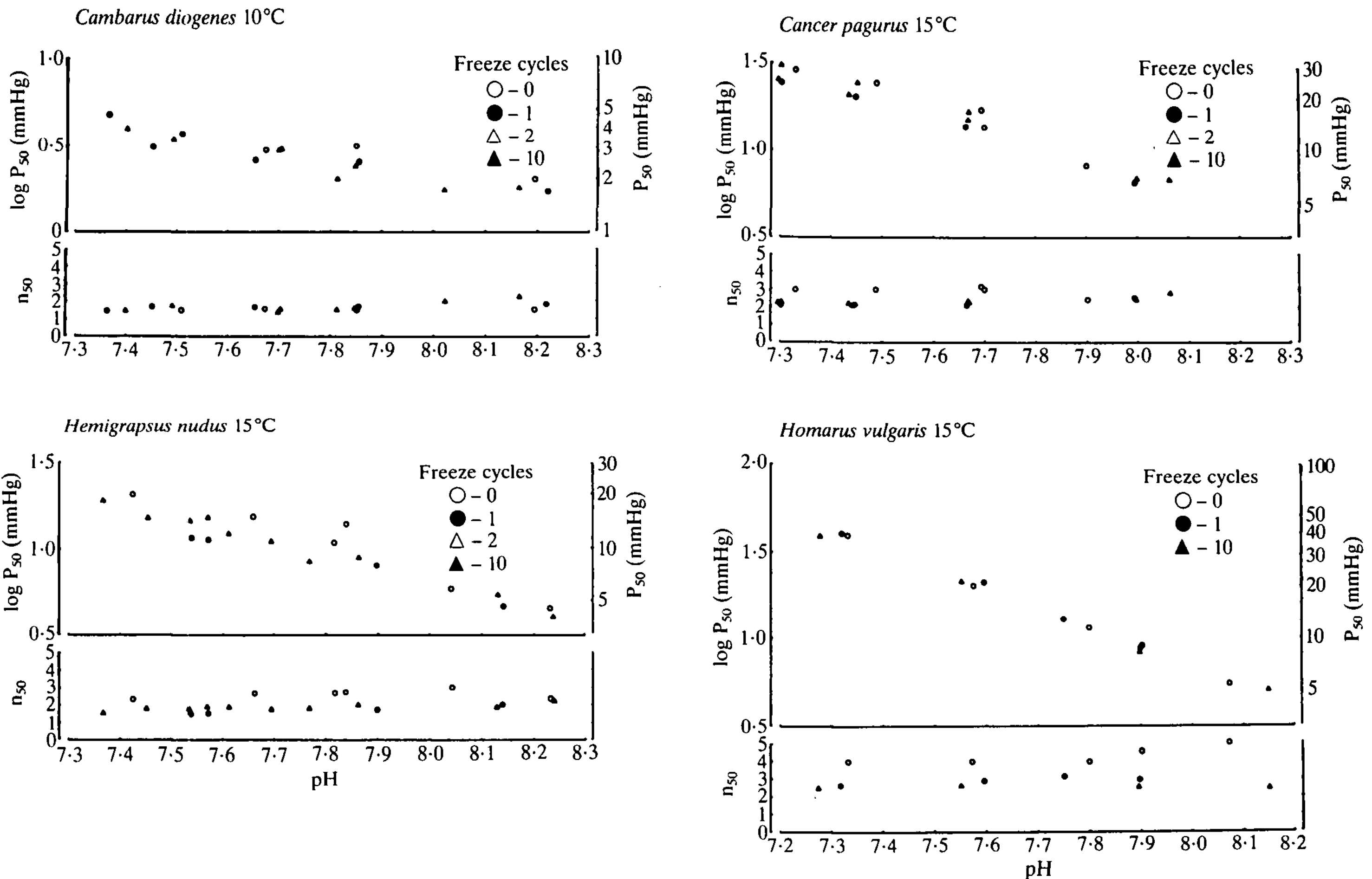

Fig. 1. The dependence of oxygen affinity, as $\log P_{50}$, and cooperativity (n_{50}) on pH. The number of freezing cycles is indicated in each case (0, 1, 2 or 10).

pH. Subjecting the n_{50} data to Student's *t*-test showed, however, that freezing one or more times significantly reduced cooperativity. Importantly, the most significant effect was after freezing once only. Subsequent freeze cycles elicited a relatively small decrease in the value of n_{50}. A reduction in cooperativity with no change in P_{50} describes an increase in the value of P_{75} and a corresponding decrease in the P_{25} value. Affinity changes at oxygen tensions away from the P_{50} are then more significant. The magnitude of the effect of freezing was directly related to the initial value of n_{50}, being large in *H. vulgaris* but small in *C. diogenes*.

Changes in the association state of non-frozen haemocyanin and material frozen a known number of times were investigated using fast protein liquid chromatography (FPLC, Pharmacia) with the appropriate columns (Fig. 2). Calibration markers were ferritin, aldolase, ovalbumin and chymotrypsinogen A. The fresh haemolymph was withdrawn from the animal, passed through a Millipore filter to remove particulate material and applied to the column within 30 min. Frozen haemolymph was prepared as described previously.

Haemocyanin is normally present in a number of association states in the haemolymph but the relative proportions vary both between species and with changes in the haemolymph microenvironment (reviewed by Mangum, 1983*b*). It was clear in all cases that a single freeze and thaw had a significant effect on haemocyanin association, ranging from merely noticeable in *C. pagurus* to dramatic in *H. vulgaris*. In all four species the pattern was for the fully associated dodecameric haemocyanin (approx. M_r 830 000) to decline; and for smaller hexameric (M_r 415 000) haemocyanin and monomers (M_r 70 000) to increase (Fig. 2). Further freezing exacerbated the effect in all cases.

Reduced temperatures (>0 °C) leading to a progressive dissociation of the haemocyanin was suggested as a possible cause of changes in the affinity of haemocyanin from *Callianassa californiensis* (Miller & Van Holde, 1974), although in re-evaluating these data Miller & Van Holde (1981) suggest that uncontrolled temperature changes may make these data suspect. Similar significant cold-induced decreases in cooperativity have also been associated with marked oxygen affinity changes in the haemocyanin of the two holarctic crabs *Hyas coarctatus* and *H. araneus*, where the changes may have some physiological significance (S. Morris & C. R. Bridges, in preparation). These reductions in cooperativity differ from those reported in the present study in the important respect of being reversible. The effect of freezing on crustacean haemocyanins appears to be irreversible.

In conclusion, the present study demonstrates three facts. First, if the native properties of the haemocyanin are at issue the effect of freezing must be carefully determined in each case. Second, the dependence of cooperative oxygen binding on the higher association states of haemocyanin was apparent in all four species. Third, since some intertidal species occasionally experience extracellular freezing or near freezing, the data presented here may indicate that haemocyanin is irreversibly damaged, requiring synthesis of new pigment.

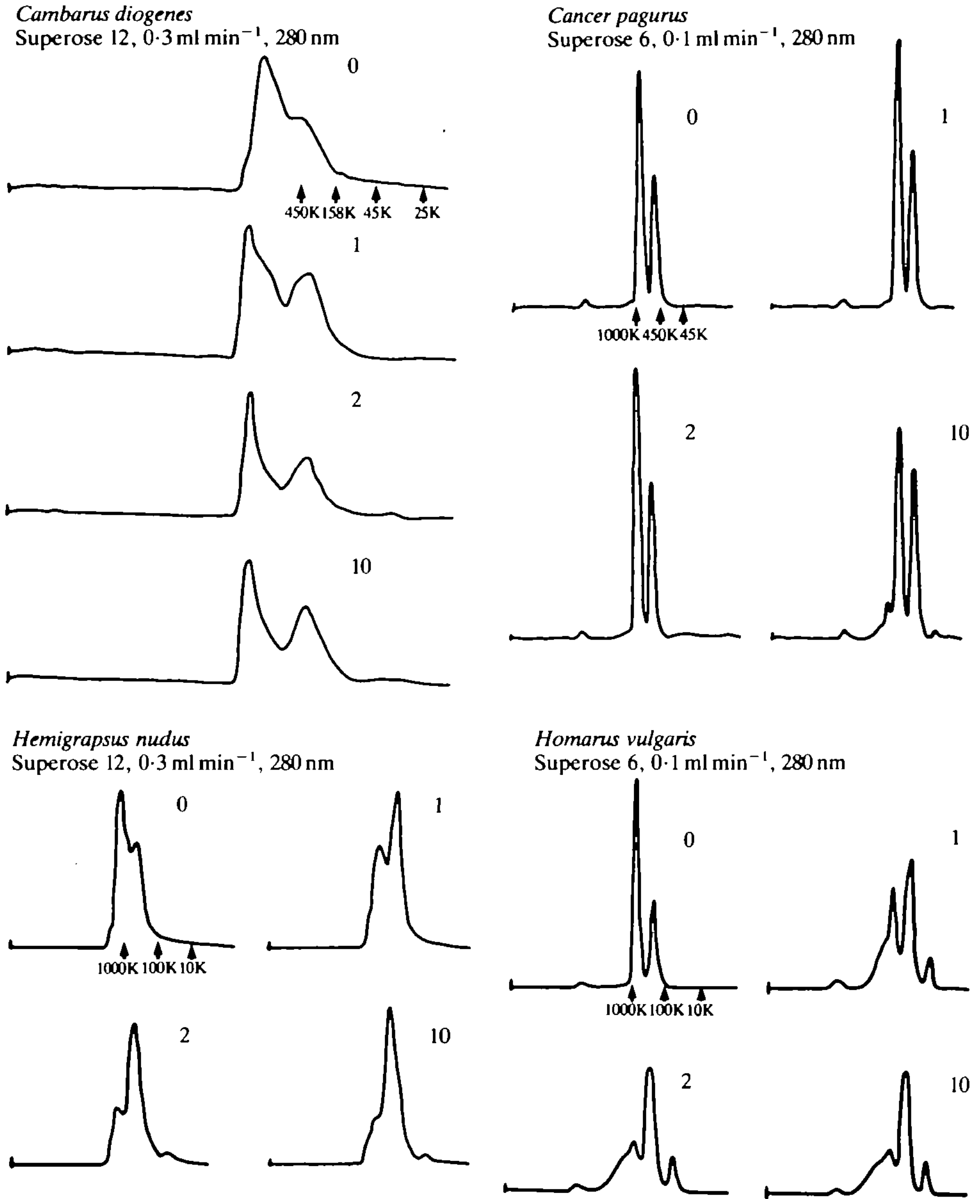

Fig. 2. PFLC chromatograms for the four crustacean haemocyanins, non-frozen and frozen 1, 2 and 10 times. Either a Superose 6 or a Superose 12 column was used and the eluant was a physiological saline based on measured haemolymph ion values. Flow rates are indicated in each case. M_r markers are given as kilo Daltons (K).

I should like to thank Professor M. K. Grieshaber and Dr C. R. Bridges for facilities at the Institut für Zoologie IV, Universität Düsseldorf, FRG and also Dr B. R. McMahon at Calgary. Thanks are due also to A. Koop and G. Hogan for assistance with the FPLC in Düsseldorf and Calgary, respectively. Support for this

work was in the form of a Royal Society (London) European Exchange Fellowship, a NATO Overseas Fellowship awarded by the NERC (UK) and an independent Research Grant from the Alberta Heritage Foundation for Medical Research to SM.

References

BRIDGES, C. R., MORRIS, S. & GRIESHABER, M. K. (1984). Modulation of haemocyanin oxygen affinity in the intertidal prawn *Palaemon elegans* (Rathke). *Respir. Physiol.* **57**, 187–200.

MANGUM, C. P. (1983*a*). On the distribution of lactate sensitivity among the haemocyanins. *Mar. biol. Lett.* **4**, 139–149.

MANGUM, C. P. (1983*b*). Oxygen transport in the blood. In *Biology of the Crustacea*, vol. 5, *Internal Anatomy and Physiological Regulation* (ed. L. H. Mantel), pp. 373–429. New York, London: Academic Press.

MILLER, K. I. & VAN HOLDE, K. E. (1974). Oxygen binding by *Callianassa californiensis* haemocyanin. *Biochemistry, N.Y.* **13**, 1668–1674.

MILLER, K. I. & VAN HOLDE, K. E. (1981). The effect of environmental variables on the structure and function of haemocyanin from *Callianassa californiensis*. *J. comp. Physiol.* **143**, 253–260.

MORRIS, S., GREENAWAY, P. & MCMAHON, B. R. (1988). Oxygen and carbon dioxide transport by the haemocyanin of an amphibious crab, *Holthuisana transversa*. *J. comp. Physiol.* **157**, 873–882.

MORRIS, S., TAYLOR, A. C., BRIDGES, C. R. & GRIESHABER, M. K. (1985). Respiratory properties of the haemolymph of the intertidal prawn; *Palaemon elegans* (Rathke). *J. exp. Zool.* **233**, 175–186.