

## GAS TRANSPORT IN THE HAEMOLYMPH OF ARACHNIDS

### I. OXYGEN TRANSPORT AND THE PHYSIOLOGICAL ROLE OF HAEMOCYANIN

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Accepted 23 November 1993

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#### Summary

Oxygen equilibrium curves and the relationships between the partial pressure of CO<sub>2</sub> and pH were determined for the haemolymph of the arachnids *Eurypelma californicum*, *Pandinus imperator* and *Cupiennius salei*. A new type of experimental apparatus was constructed, tested and used to make these measurements on small undiluted cell-free haemolymph samples. Most of its components were made in our workshop and were inexpensive. The apparatus proved to be very reliable as demonstrated by control (oxygen concentration) experiments. In previous reports on O<sub>2</sub> equilibrium curves of *E. californicum* haemocyanin, Tris–HCl buffer was used to set the pH, whereas in our experiments, pH was adjusted by altering the partial pressure of CO<sub>2</sub>. The O<sub>2</sub> concentration measurements demonstrate an increase in oxygen affinity of *E. californicum* haemocyanin when using Tris–HCl buffer. The position and shape of the O<sub>2</sub> equilibrium curves of *E. californicum* and *P. imperator* haemocyanin show a distinct dependency on pH. Oxygen affinity is lower in *E. californicum* and cooperativity changes with pH in *E. californicum*, but not in *P. imperator*. Oxygen transport in the haemolymph of *E. californicum* during rest, activity and recovery was calculated on the basis of the O<sub>2</sub> equilibrium curves of undiluted haemolymph. Apart from oxygen transport, there are indications that haemocyanin is also involved in other physiological processes. For example, it may function as a storage protein.

#### Introduction

Arachnids (spiders, scorpions) are a large group in the animal kingdom, comprising at least 65 000 species. In spite of their great numbers and ecological importance, studies of their physiology are not very numerous. Many arachnids use lung-like respiratory organs

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Key words: arachnids, oxygen transport, haemocyanin, *Eurypelma californicum*, *Pandinus imperator*, *Cupiennius salei*.

(book lungs) for gas exchange between their haemolymph and the ambient air, with the haemolymph acting as a gas transport medium between the book lungs and the tissues. Essential components of such a gas transport system are respiratory pigments (haemocyanin) and a well-developed circulatory system.

Our studies of the environmental physiology and metabolism of arachnids focused on the tarantula *E. californicum* and the scorpion *P. imperator*, with some comparative studies on the ctenid spider *C. salei*. In previous reports, we have described the function and performance of the book lungs (Paul *et al.* 1987, 1989a; Paul and Fincke, 1989; Fincke and Paul, 1989) and interactions between the circulation and the specific hydraulic locomotory system of spiders (Paul *et al.* 1989b). We now communicate studies on (i) O<sub>2</sub> equilibrium curves of whole blood (haemolymph), haemolymph oxygen-transport and the physiological role of haemocyanin in *E. californicum* (this paper) and (ii) carbon-dioxide-related properties of the arachnid haemolymph (CO<sub>2</sub> transport, acid–base status) as well as results on haemolymph ions (Paul *et al.* 1994). We also describe a novel experimental apparatus used to measure gas transport properties of whole blood.

Haemocyanin, the respiratory pigment in the haemolymph of many arthropods and molluscs, has received much physiological attention in recent years (e.g. Mangum, 1980, 1983, 1990; McMahon, 1986). There are numerous studies on structure-related properties of spider haemocyanin (e.g. Markl *et al.* 1981; Linzen *et al.* 1985; Decker and Sterner, 1990). Oxygen equilibrium curves of diluted, dialysed, purified or modified haemocyanin of *E. californicum* (Loewe, 1978; Savel-Niemann *et al.* 1988; Decker and Sterner, 1990) or *P. imperator* (Decker, 1990) have revealed the molecular mechanisms of cooperative oxygen binding and its allosteric modulation by ions.

To assess the physiological role of haemocyanin in oxygen-transport processes, it is necessary, however, to study the O<sub>2</sub> equilibrium curves of whole blood, because the presence of effectors (Truchot, 1980; Mangum, 1983; Bridges and Morris, 1986; Morris and Bridges, 1986) or other factors may influence the position and shape of the curves. Apart from one study (Bridges, 1988) on *Lasiadora erythrocythara* haemocyanin, there are no data published about O<sub>2</sub> equilibrium curves of whole blood in spiders.

Especially for the tarantula *E. californicum*, the lack of appropriate studies is regrettable, because data are available on haemolymph oxygen-transport, including a detailed study of haemolymph P<sub>O<sub>2</sub></sub> and pH (Angersbach, 1978). Oxygen transport in the haemolymph of *E. californicum* can be quantified using these data and the O<sub>2</sub> equilibrium curves of whole blood presented in the present study. We discuss the significance of haemocyanin for physiological processes other than oxygen transport.

## Materials and methods

### *Animals*

For these studies, the haemolymph of the North American tarantula *Eurypelma californicum* (Theraphosidae; determined according to Comstock, 1965), the African scorpion *Pandinus imperator* C. L. Koch (Scorpionidae) and the Latin American spider *Cupiennius salei* Keyserling (Ctenidae) was used. *E. californicum* was obtained from Carolina Biological Supply Co., Burlington, NC, USA. *P. imperator* was supplied by

P. Hoch, Waldkirch im Breisgau, Germany. *C. salei* was bred according to Melchers (1963). The tarantulas and emperor scorpions were kept in 40 cm×40 cm×15 cm plastic containers with peat dust for the former and gravel for the latter. A piece of moistened carpeting and a broken pot were provided as shelter for the scorpions. The ctenid spiders were kept in glass jars filled with moistened peat dust. The animals were fed weekly with newborn mice, crickets or flies, and water was given *ad libitum*. The animal room was maintained at 25 °C.

#### *Haemolymph sampling*

Before haemolymph sampling, the animals were kept in small (20 cm×10 cm×10 cm) plastic boxes (equipped with air-holes and a piece of moistened carpeting) placed into a 251 portable cooling box (Super Fridge; Coleman, Germany) for at least 1 day. A thermostat (ITR1; Eberle, Germany) was used to stabilize the temperature in the box to 25±0.5 °C. The portable box was kept in a quiet dark room.

Haemolymph was usually sampled in the morning. One of the small plastic boxes was rapidly taken out and opened. The animal was held in place by pressing down the prosoma and tail (scorpions) using one or two large paintbrushes. The dorsal opisthosomal cuticle (in spiders), or the dorsal membrane between two segments (in scorpions), and the pericardium were punctured using a 0.9 mm (o.d.) cannula connected to a syringe. The haemolymph which flowed out was aspirated into a Pasteur pipette. The whole sampling procedure took, at most, 15 s. To obtain haemolymph samples of 'post-active individuals', the spiders or scorpions were rapidly taken out of their plastic boxes and put into a larger plastic container. Burst activity was then provoked by touching the animals with a plastic rod. After 3 min of intense exercise, the animals were given 10 min to recover before haemolymph was sampled. The haemolymph samples were put into plastic vessels (1 ml; Eppendorf, Germany) and centrifuged (6000 g; 4 °C) for 6 min to remove haemocytes. The supernatant was used for photometric measurements, determinations of O<sub>2</sub> and CO<sub>2</sub> equilibrium curves and measurements of ions or metabolites. The whole procedure that followed haemolymph sampling (centrifugation, filling the measuring chambers) took about 10 min, after which the experiment began immediately.

#### *Experimental apparatus*

A novel apparatus was constructed, tested and used to determine O<sub>2</sub> equilibrium curves and *P*<sub>CO<sub>2</sub></sub>/*pH* relationships of undiluted haemolymph (or blood) (Fig. 1). Its main components were (i) two gas-mixing pumps (Wösthoff, Bochum, Germany), which were connected in parallel, (ii) a measuring chamber used for the determination of O<sub>2</sub> equilibrium curves and (iii) a chamber for measuring haemolymph *pH*. Many of the components were made in our workshop and were relatively inexpensive. The apparatus proved to be very reliable.

The equivalence of the flow rates (0.51 min<sup>-1</sup>) of the two gas-mixing pumps was checked. One pump mixed O<sub>2</sub> and N<sub>2</sub>, and the other one mixed CO<sub>2</sub> and N<sub>2</sub>. The medical-quality gases (Linde, Germany) were humidified to ensure a high degree of saturation with water vapour. All connections were made using polyvinylchloride tubes. About

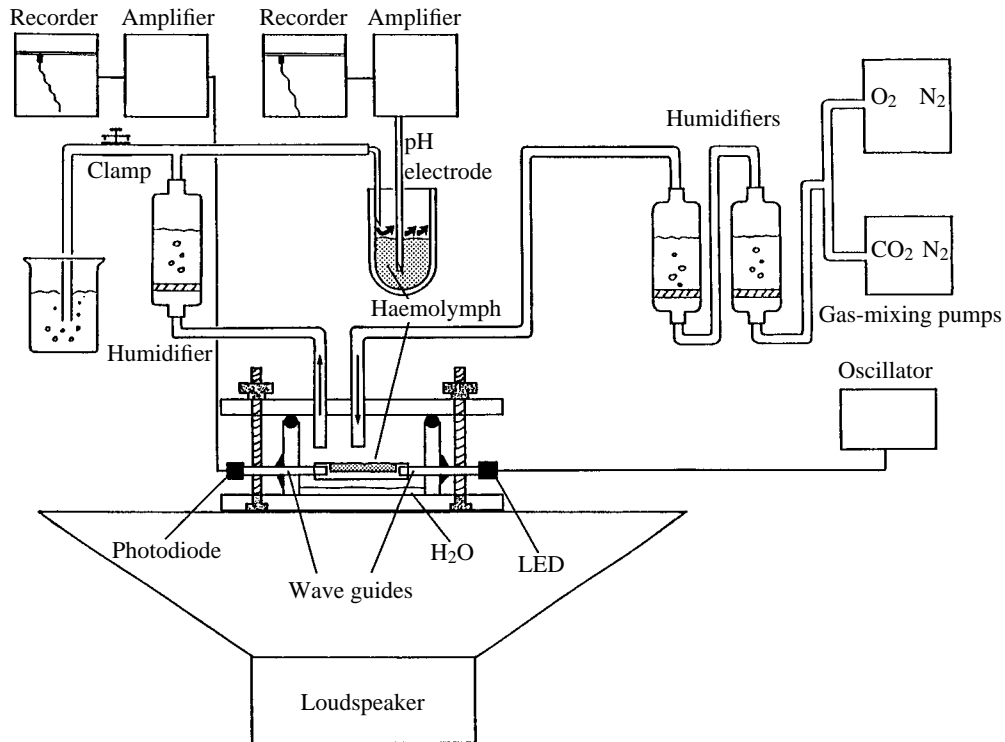


Fig. 1. Experimental apparatus for the measurement of oxygen equilibrium curves and  $P_{\text{CO}_2}$ /pH relationships (see text for details). LED, light-emitting diode.

30  $\mu\text{l}$  of cell-free haemolymph was placed into a Perspex tube that was open at the top (a cuvette) inside a Perspex equilibrium chamber. The chamber was hermetically sealed (using screws and an O ring) and, to increase the saturation with water vapour further, its bottom was covered with water. To speed up equilibration with the gas mixtures, the chamber was glued to the membrane of a loudspeaker driven by a function generator. (Preliminary tests using a stirrer revealed haemocyanin denaturation.) The frequency (approximately 80 Hz) and amplitude of a square-wave signal were adjusted in preliminary equilibration tests. Small lengths of wave guide (2 mm o.d., Crofon; Du Pont, USA), polished at the endings, were fixed on both sides of the cuvette. A light-emitting diode (LED, ESPY 5501; Stanley, Japan) and a photodiode (OSD 1-5, Centronic, UK) were connected to the respective wave guides. The maximum emission wavelength of the LED ( $\lambda_{\text{max}}$  570 nm) was the same as the maximum absorbance wavelength of haemocyanin in the visible range. Special electronics (lock-in detection; Horowitz and Hill, 1990) were built to reduce noise in the photodiode signal. The resulting signal, which corresponded to light transmission, was passed to a recorder. Calculations were performed later to obtain the normalized absorbance (percentage haemocyanin saturation).

After leaving the equilibrium chamber, the gas mixtures passed through another

humidifier and then over a second sample of haemolymph (80–100  $\mu$ l) in a glass vessel (sealed with Parafilm) where the pH was measured using an electrode (SA 4 combination glass pH electrode, o.d. 0.9 mm; WPI, USA). The amplified signal was passed to another recorder. To avoid breaking this fragile electrode, vibration (to improve equilibration speed) was not applied. A (moderate) turbulence in the haemolymph was generated by altering the adjustment of a clamp, which controlled a second outflow. It was not possible to use this method for the optical measurements described above. The whole apparatus, including gas bottles and pumps, was in a room whose temperature was stabilized to  $25 \pm 1$  °C using a thermostat (ITR1; Eberle, Germany) and a fan heater.

#### *Checking the apparatus*

The electronic circuit was designed to have a linear relationship between light intensity ( $I$ ) and signal over the whole working range. To test its performance, we checked the linearity between 100% transmission ( $I=I_0$ ; the cuvette was filled with water) and 1% transmission (the range used in our experiments), using either (i) ten different concentrations of a blue water-based paint or (ii) five neutral density filters (Zeiss, Germany).

The function of the pH electrode was tested by repeating the determination of the  $P_{CO_2}$ /pH relationship (in the haemolymph of resting *E. californicum*) using another type of electrode (N6000A; Schott, FRG). This second electrode was much larger, and about 1 ml of (pooled) haemolymph was needed for these experiments. The two electrodes gave similar results ( $N=3$ ).

The measured  $O_2$  equilibrium curves were checked by determinations of total oxygen concentration (see below).

#### *Additional photometric measurements*

Before and after each experiment ( $CO_2$  and  $O_2$  equilibration), the absorbance ( $A$ ) of small haemolymph samples (10  $\mu$ l; diluted 1:100 in spider Ringer's solution; according to Schartau and Leidescher, 1983) was measured using a Hitachi spectrophotometer. These measurements, which show, for example, changes in haemolymph protein concentration ( $C_{HP}$ ), were used during testing of the apparatus to determine the specific arrangement of humidifiers. Later they were used to evaluate the individual  $C_{HP}$  values (Paul and Efinger, 1990) and to check for any evaporation (or dilution) of the haemolymph in the Perspex tube or in the glass vessel. Experiments in which there was a difference of more than 1% in the absorbance at 280 nm (before and after the equilibration experiments) were rejected. Similarly, experiments were rejected if a deviation of the  $A_{280}/A_{250}$  ratio signalled that denaturation of proteins had occurred (denaturation is reflected by a relative increase of scattering at shorter wavelengths compared with that at longer wavelengths).

#### *Measurement of $P_{CO_2}$ /pH relationships*

The pH electrode was calibrated before and after each  $CO_2$  equilibration experiment using standard buffer solutions (Merck, Germany). During the measurements, one gas-mixing pump was switched to 10%  $O_2$ :90%  $N_2$ , and the other was successively switched

to different CO<sub>2</sub>:N<sub>2</sub> ratios. Equilibration was ascertained using a recorder. It took approximately 1.5 h to measure one curve. The electrode was then cleaned for 1 h in a solution of 5 % pepsin in 0.1 mol l<sup>-1</sup> HCl and stored in 3 mol l<sup>-1</sup> KCl.

#### *Measurement of oxygen equilibrium curves*

Oxygen equilibrium curves were measured at different pH values. The preceding determination of the  $P_{\text{CO}_2}$ /pH relationship was fitted with a linear regression ( $\log P_{\text{CO}_2}$  versus pH) to give the correct setting for the CO<sub>2</sub>/N<sub>2</sub> mixing pump. Because the pump can only be set in integer steps, a small (negligible) deviation of the respective pH sometimes occurred ( $\Delta\text{pH} < 0.025$ ). (In some experiments, a higher precision was necessary. So a gas bottle with a 5 % CO<sub>2</sub>:95 % N<sub>2</sub> mixture was used instead of pure CO<sub>2</sub>.) The measurements started from the deoxygenated state of the haemolymph, and the fully oxygenated state was reached in steps. Finally, measurements in the absence of O<sub>2</sub> were repeated. Equilibration was ascertained using a recorder with 0 % transmission set to the baseline. Any drift was corrected for by using the transmission values at the beginning and end of an experiment (no O<sub>2</sub>). It took approximately 2 h to measure one curve.

#### *Measurement of oxygen concentration*

A TC 500 Tucker cell (Strathkelvin Instruments, Glasgow, UK) was used to determine total O<sub>2</sub> concentration of haemolymph samples (all experiments at 25 °C), according to the method of Tucker (1967) as modified by Bridges *et al.* (1979).

#### *Measurement of gas-equilibrated haemolymph samples*

To check the results obtained by the spectrophotometric method, O<sub>2</sub> equilibrium curves of approximately 1 ml of pooled undiluted haemolymph of resting *E. californicum* (pH adjusted by the  $P_{\text{CO}_2}$ ) were measured using the Tucker cell. Equilibration with the different gas mixtures (permanently monitored by spectrophotometry) took place in a Perspex vessel. Further checks included photometric and pH measurements before and after each experiment. After reaching equilibrium, 20  $\mu\text{l}$  of haemolymph was transferred to the Tucker cell using a gas-tight Hamilton syringe. For each equilibration step, at least three measurements of total oxygen concentration ( $C_{\text{O}_2}$ ) were made. Before computing the percentage saturation curves, the physically dissolved O<sub>2</sub> was calculated and subtracted:  $\text{O}_2 (\mu\text{l})/\text{haemolymph volume } (\mu\text{l}) = \alpha' \times P_{\text{O}_2}/760 \text{ mmHg}$  (where  $\alpha' = 0.0276$ ; Angersbach, 1978). (To check the O<sub>2</sub> solubility  $\alpha'$ , its value was also measured at 25 °C.)

Further experiments were carried out with dialysed haemolymph. We started with a stock of pooled undiluted haemolymph and used the Tucker cell and three different Perspex vessels. We simultaneously measured oxygen equilibrium curves of whole haemolymph (stored at 10 °C for 1–2 days) and of haemolymph dialysed at 10 °C for 1–2 days against either (i) spider Ringer's solution (which contains a weak phosphate buffer) or (ii) 0.1 mol l<sup>-1</sup> Tris-HCl (with 5 mmol l<sup>-1</sup> CaCl<sub>2</sub> and 5 mmol l<sup>-1</sup> MgCl<sub>2</sub>). The total Cl<sup>-</sup> concentration in Tris buffer solution was calculated to be approximately 86 mmol l<sup>-1</sup>. The pH of the buffer solutions (pH 7.5) was adjusted using HCl and NaOH, and the gas mixtures used for the equilibration of dialysed haemolymph were free of CO<sub>2</sub>. Checks included photometric and pH measurements (samples with a deviation of more

than  $\pm 0.05$  pH were rejected). The O<sub>2</sub> solubility of spider Ringer's solution ( $\alpha''$ ) was assumed to be equal to that of haemolymph ( $\alpha'$ ). The O<sub>2</sub> solubility of 0.1 mol l<sup>-1</sup> Tris-HCl ( $\alpha'''$ ) was measured to be 0.0264 at 25 °C.

#### *Measurement of in vivo haemolymph samples*

Oxygen concentration was determined in arterial (from the pericardium) and venous (from the ventral lacuna, coxa or the opisthosomal lateral sacs) haemolymph samples. We used either gas-tight Hamilton syringes or pulled-glass (made using a puller) microcapillaries which filled with haemolymph because of capillary action. Sampling time was, at most, 15 s. We tried to cannulate the animals using metal, glass or plastic cannulae, but these experiments failed because of rapid coagulation that could not be prevented.

#### *Measurement of arterial P<sub>O<sub>2</sub></sub>*

To check published haemolymph P<sub>O<sub>2</sub></sub> measurements in *E. californicum* (Angersbach, 1978), the P<sub>O<sub>2</sub></sub> in the pericardium was continuously measured using an intravascular P<sub>O<sub>2</sub></sub> sensor (Continucath 1000; Biomedical Sensors, Kansas City, USA). The dorsal opisthosomal cuticle (and pericardium) was punctured at the posterior end of the heart and, after calibration, about 10 mm of the flexible sensor (o.d. 0.55 mm) was introduced towards the anterior. The remaining part of the sensor was covered with a close-fitting metal cannula (sealed at the top with wax-colophonium). Wax-colophonium was also used to form an air-tight connection between the cannula and the cuticle. This type of preparation causes some stress to the animals, and the response of the sensor was not very fast, but these studies were carried out to check, rather than to re-evaluate completely, previous determinations of P<sub>O<sub>2</sub></sub> values and the time courses of P<sub>O<sub>2</sub></sub> change.

Some additional studies were carried out to investigate whether haemocyanin participates in physiological processes other than oxygen transport.

#### *Protein extraction for polyacrylamide gel electrophoresis and immunoelectrophoresis*

Cuticle of the opisthosoma of *E. californicum* was separated from adhering tissues by simply peeling it off the tissues lying below, washing it in Ca<sup>2+</sup>, Mg<sup>2+</sup> Ringer (both 10 mmol l<sup>-1</sup>; other ions according to Schartau and Leidescher, 1983) and drying it with filter paper. Weighed quantities of exuviae of *E. californicum* (immediately cooled after moulting and used within 3 weeks) or cuticle were mixed with an identical amount of sand and crushed in a mortar. The mixture was suspended in Ca<sup>2+</sup>, Mg<sup>2+</sup> Ringer, stirred for at least 24 h (4 °C) and centrifuged. The protein concentrations in the supernatant and in the pellet were determined according to Lowry *et al.* (1951). For polyacrylamide gel electrophoresis (PAGE), the proteins in the supernatant were denatured using sodium dodecyl sulphate (SDS) and  $\beta$ -mercaptoethanol. For immunoelectrophoresis, they were dissociated by dialysing overnight (4 °C) against 0.05 mol l<sup>-1</sup> glycine/NaOH buffer, pH 9.6. The dialysed cuticle solutions and cell-free diluted (Ca<sup>2+</sup>, Mg<sup>2+</sup> Ringer; protein concentration 1 mg ml<sup>-1</sup>) and dialysed *E. californicum* haemolymph were used.

#### *Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis was performed in slab gels using the system of

Laemmli (1970) for SDS-denatured subunits. The acrylamide concentration ranged from 3.7% to 20%, pH 8.8.

#### *Immunoelectrophoresis*

Crossed immunoelectrophoresis of dissociated proteins was performed according to Weeke (1973). Rabbit antiserum was raised against dissociated purified *E. californicum* haemocyanin as described elsewhere (Lamy *et al.* 1979). To allow a comparison between the immunoelectrophoresis patterns of the dissociated exuvial and cuticular proteins and the pattern of dissociated haemocyanin (cf. Lamy *et al.* 1979), the run time in the first dimension was shortened.

### Results

#### *Oxygen equilibrium curves*

Our measurements were highly repeatable. Determinations of O<sub>2</sub> equilibrium curves of samples from one haemolymph pool were congruent in a graphical presentation and are therefore not shown. Only if the haemolymph sample was left in the equilibrium chamber for more than a few hours was there a distinct shift of the curves towards higher affinity and smaller cooperativity, probably because of proteolysis (Strych *et al.* 1983).

The affinity of the haemolymph of resting *E. californicum* for O<sub>2</sub> is strongly pH-dependent (Bohr effect), especially above pH 7.5; the equilibrium curves at pH 7.25 and 7 are more similar to each other (Fig. 2, see also Fig. 6). The shift in the curves at different

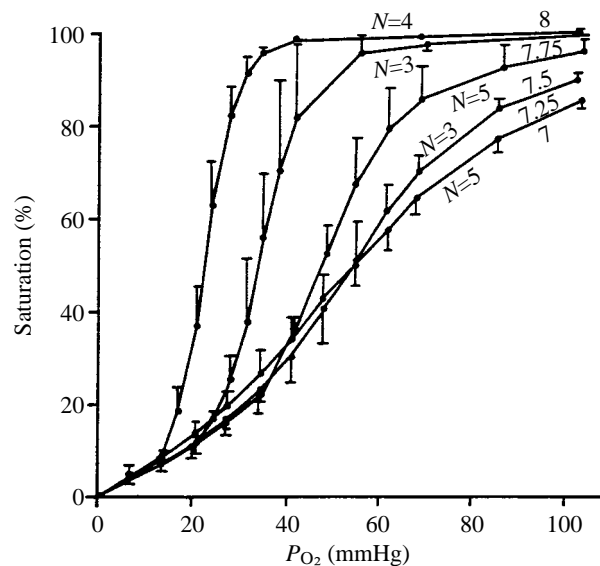


Fig. 2. Mean oxygen equilibrium curves of undiluted haemolymph of resting *Eurypelma californicum* at different pH values (7, 7.25, 7.5, 7.75 and 8). Values are mean  $\pm$  S.D. Numbers of individuals tested ( $N$ ) are shown.



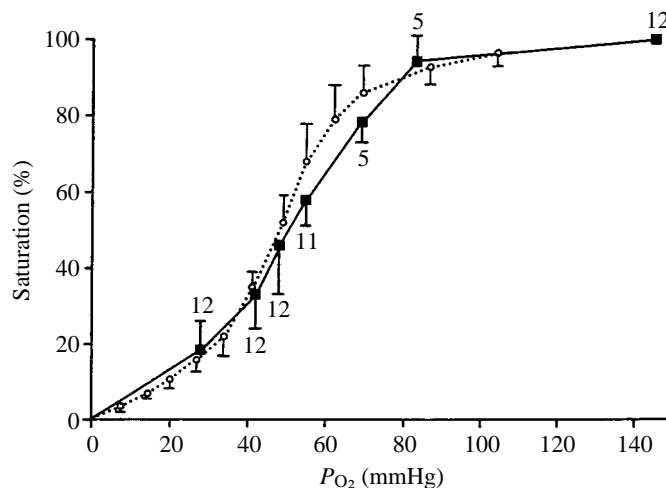


Fig. 3. Mean oxygen equilibrium curves of undiluted haemolymph of resting *Eurypelma californicum* (pH 7.5 adjusted by the  $P_{CO_2}$ ) measured either with the spectrophotometric method (open circles; dotted line;  $N=5$ ) or using a Tucker cell to determine total  $CO_2$  (filled squares; solid line). Values are mean  $\pm$  S.D. Numbers of individuals tested are shown beside the points.

pH values is much more distinct at higher O<sub>2</sub> saturations; this means that the Bohr effect could play no role at venous  $P_{O_2}$  values, which are low (see Discussion). The variance should mainly be due to individual differences between the haemolymph samples, because deviations from the stated pH value were small ( $\pm 0.025$ ; see Materials and methods).

Because we found lower affinities compared with previous results (which were measured using a fluorescence technique; see below), we checked our spectrophotometrically determined ( $\lambda_{max}=570$  nm) O<sub>2</sub> equilibrium curves of resting *E. californicum* by applying a non-optical method (Tucker cell). The agreement between the results of the two techniques was satisfactory (Fig. 3).

Previous measurements of O<sub>2</sub> equilibrium curves of *E. californicum* haemocyanin have been made using a fluorimetric/polarographic method ( $\lambda_{excitation}=294$  nm,  $\lambda_{emission}=348$  nm) and highly diluted samples (protein concentrations of less than  $0.2$  mg ml<sup>-1</sup>). Tris-HCl buffer ( $0.1$  mol l<sup>-1</sup>) was used to set and stabilize pH. These studies (e.g. Loewe, 1978; Decker and Sterner, 1990) reported higher O<sub>2</sub> affinities. To study the reason for these differences, we determined O<sub>2</sub> equilibrium curves (using the Tucker cell) of cell-free whole blood and of haemolymph dialysed against either spider Ringer's solution or Tris-HCl buffer (Fig. 4). The O<sub>2</sub> equilibrium curves of whole blood and haemolymph dialysed against spider Ringer's solution were almost congruent with the curve determined spectrophotometrically, but the curve for haemolymph dialysed against Tris-HCl buffer showed a distinct shift to higher affinities.

Oxygen equilibrium curves of undiluted haemolymph of resting *P. imperator* were determined at different pH values (Fig. 5). The Bohr effect was again more distinct at

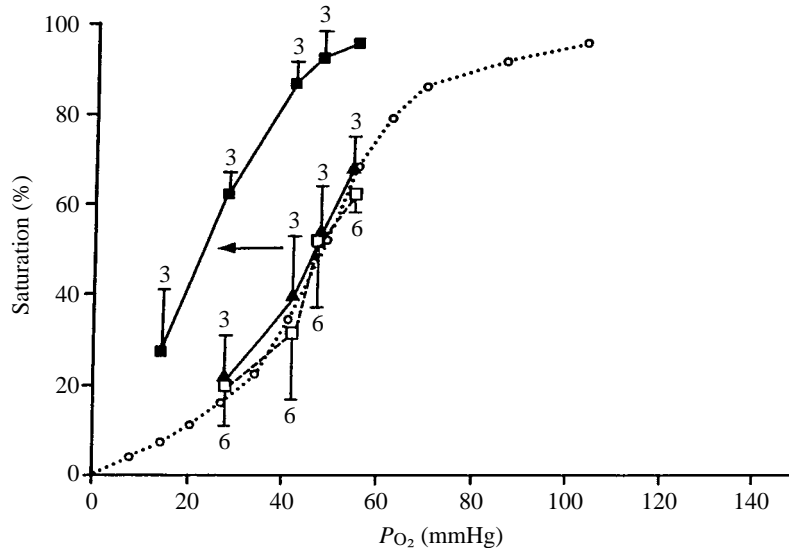


Fig. 4. Mean oxygen equilibrium curves of undiluted haemolymph of resting *Eurypelma californicum* (pH 7.5) measured either (i) using the spectrophotometric method (open circles; dotted line) or using a Tucker cell with the haemolymph (ii) not dialysed (open squares; dashed line), (iii) dialysed against spider Ringer's solution (filled triangles; solid line) or (iv) dialysed against  $0.1 \text{ mol l}^{-1}$  Tris-HCl (filled squares; solid line). The Tucker cell measurements were carried out only at intermediate (near the  $P_{50}$ ) and high  $P_{O_2}$  values. The pH was adjusted either by the  $P_{CO_2}$  (i, ii) or using HCl/NaOH (iii, iv). Dialysis against Tris-HCl caused a distinct shift of the curve to the left (arrow). Values are mean  $\pm$  S.D. Numbers of individuals tested are shown beside the points.

higher pH values, but no differences were found between the curves at pH 7 and 7.25, so these curves were pooled.

Oxygen affinity (expressed as  $\log P_{50}$ ) is clearly lower in *E. californicum* than in *P. imperator* haemolymph (Fig. 6A). The Bohr coefficient ( $\Delta \log P_{50} / \Delta \text{pH}$ ) depends on pH. In *E. californicum* it is  $-0.01$  (pH 7–7.25),  $-0.21$  (pH 7.25–7.5),  $-0.6$  (pH 7.5–7.75) or  $-0.7$  (pH 7.75–8). In *P. imperator* it is  $-0.02$  (pH 7–7.25),  $-0.23$  (pH 7.25–7.5) or  $-0.52$  (pH 7.5–7.75).

Cooperativity (expressed as  $n_{50}$ ) depends on pH in *E. californicum* (Fig. 6B) and ranges from 2.4 (pH 7) to 6.3 (pH 7.75). In *P. imperator*, it is more or less constant as pH changes ( $n_{50} \approx 4$ ).

The  $O_2$  equilibrium curve (at pH 7.5) of *C. salei* haemolymph (Fig. 7) shows a lower affinity ( $\log P_{50} = 1.69$ ) and cooperativity ( $n_{50} = 2.8$ ) than the corresponding curves for *E. californicum* and *P. imperator*.

#### Oxygen transport in the haemolymph of *Eurypelma californicum*

The different utilization of haemocyanin as an  $O_2$  carrier in *E. californicum* haemolymph of animals at rest and during early recovery (a few minutes after locomotor activity) was evaluated using data from Angersbach (1978) on mean haemolymph  $P_{O_2}$

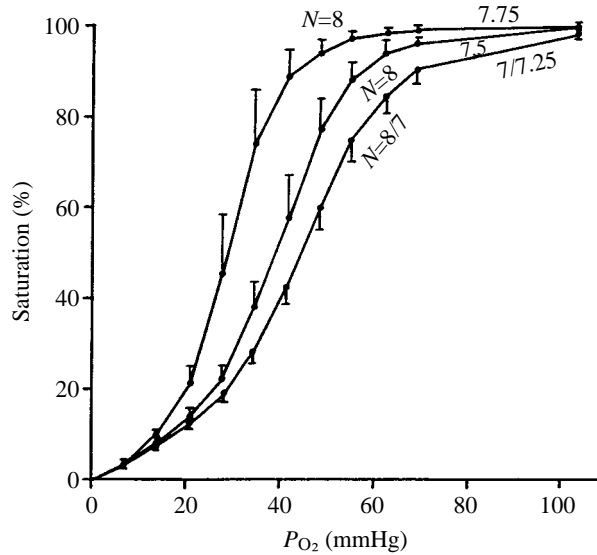


Fig. 5. Mean oxygen equilibrium curves of undiluted haemolymph of resting *Pandinus imperator* at different pH values (7, 7.25, 7.5 and 7.75). Values for pH7 and 7.25 were averaged. Values are mean  $\pm$  s.d. Numbers of individuals tested are shown beside the curves.

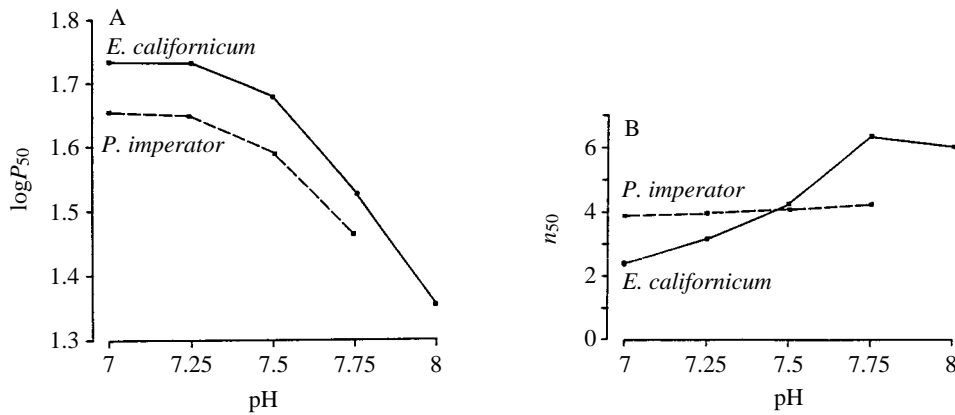


Fig. 6. (A) Plot of haemolymph oxygen-affinity ( $\log P_{50}$ ) versus pH for *Eurypelma californicum* and *Pandinus imperator*. (B) Plot of cooperativity ( $n_{50}$ ) versus pH for *Eurypelma californicum* and *Pandinus imperator*. The  $P_{50}$  and  $n_{50}$  values were calculated from the data shown in Figs 2 and 5.

and pH. Because haemolymph pH varies, different O<sub>2</sub> equilibrium curves were used: at rest, we used that determined at pH 7.5 (for arterial and venous haemolymph) and during recovery we used those determined at pH 7.25 (arterial) and pH 7 (venous). At rest, arterial ( $P_{aO_2}$ ) and venous ( $P_{vO_2}$ ) oxygen partial pressure values are very low and the arterio-venous percentage saturation difference is only approximately 8% (Fig. 8A).

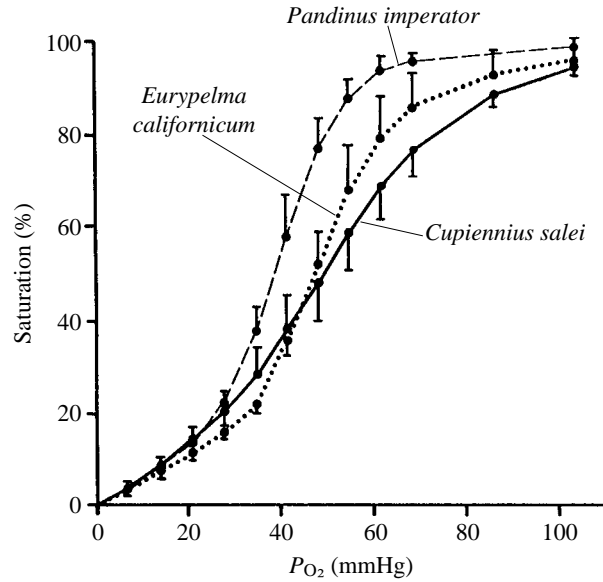


Fig. 7. Mean oxygen equilibrium curve of *Cupiennius salei* haemolymph (solid line) at pH 7.5 compared with the corresponding curves for haemolymph from *Eurypelma californicum* (dotted line) and *Pandinus imperator* (dashed line). Values are mean  $\pm$  s.d.; haemolymph from three individuals was tested.

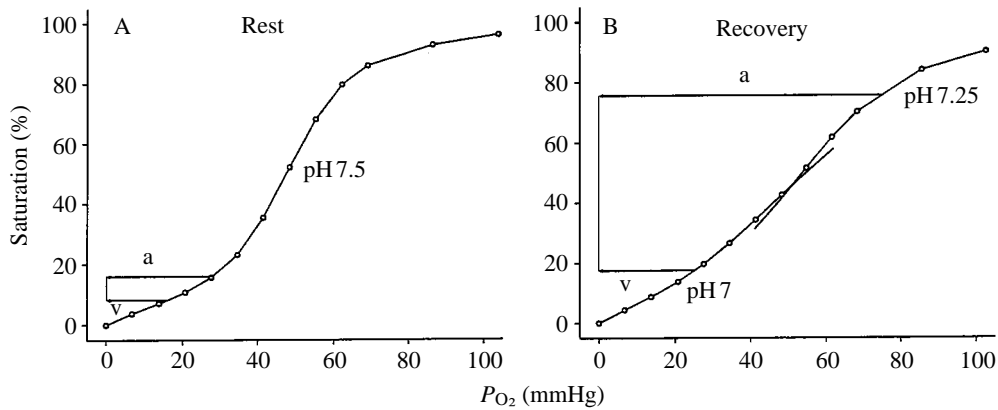


Fig. 8. Arterio-venous (a-v) percentage saturation difference of *Eurypelma californicum* haemolymph at rest (A;  $pH_a \approx pH_v$ ;  $O_2$  equilibrium curve at pH 7.5) and during recovery from activity (B;  $pH_a \gg pH_v$ ; upper and lower parts of the  $O_2$  equilibrium curves at pH 7.25 and pH 7). The increase in the a-v percentage saturation difference after exercise is due to an elevation of arterial  $P_{O_2}$ . During recovery, the partial pressure gradients from the haemolymph to the tissues are also higher (see text for details).

During recovery,  $P_{aO_2}$  rises markedly, which increases the a-v percentage saturation difference to almost 60% (Fig. 8B).

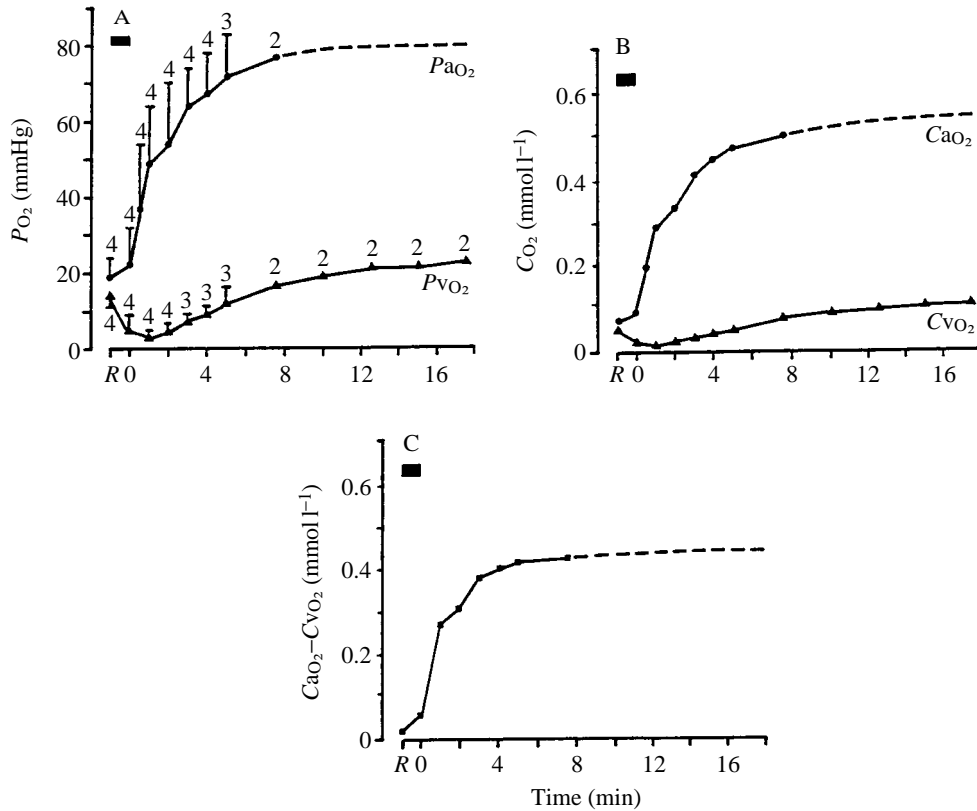


Fig. 9. Oxygen transport in the haemolymph of *Eurypelma californicum*. (A) Arterial (a) and venous (v)  $P_{O_2}$  at rest (R), during 1 min of burst activity (black bar) and during recovery. Values are mean  $\pm$  s.d. Numbers of individuals tested are shown beside the points. The dashed part of the line is an extrapolation. Data from Angersbach (1978 and unpublished results). (B) Time courses of  $CaO_2$  (a) and  $CvO_2$  (v) changes calculated from  $P_{O_2}$  (A) and pH time courses (from Angersbach, 1978 and unpublished results) and  $O_2$  equilibrium curves of undiluted haemolymph taking physically dissolved  $O_2$  into account (haemocyanin concentration 45 mg ml<sup>-1</sup>). (C) Time course of arterio-venous  $CO_2$  difference ( $CaO_2 - CvO_2$ ) calculated from the  $CO_2$  time courses (B), showing the changes in  $O_2$  transport in *E. californicum* haemolymph.

Dynamic changes of oxygen transport in *E. californicum* haemolymph during the transition from rest to recovery were assessed by calculating the arterial ( $CaO_2$ ) and venous ( $CvO_2$ ) oxygen concentrations, as well as the arterio-venous  $CO_2$  difference ( $CaO_2 - CvO_2$ ) (Fig. 9). The calculations were based on exercise-related  $P_{O_2}$  and pH time courses measured by D. Angersbach (Angersbach, 1978 and unpublished results).

To check these calculations, we used the Tucker cell to measure the  $CO_2$  of haemolymph sampled from animals at rest and at different times during recovery. With this approach, however, we could find no significant differences between  $CO_2$  at rest and during recovery (see Discussion). At rest,  $CaO_2$  was  $0.5 \pm 0.1$  mmol l<sup>-1</sup> ( $N=10$  animals) and  $CvO_2$  was  $0.33 \pm 0.1$  mmol l<sup>-1</sup> ( $N=8$  animals). These data correspond to a  $P_{aO_2}$  of

approximately 66 mmHg and to a  $P_{\text{vO}_2}$  of approximately 49 mmHg (1 mmHg=0.1333 kPa) (Bergner, 1991; calculated using data from Fig. 3). The tarantulas were not active or even alert, for we monitored the heart rate before haemolymph sampling.

To check the published data on haemolymph  $P_{\text{O}_2}$  in *E. californicum* (Angersbach, 1978), we used an intravascular  $P_{\text{O}_2}$  sensor to determine the pericardial  $P_{\text{aO}_2}$  in three individuals. Considering the methodological problems mentioned above, we found reasonably similar time courses and absolute values (e.g.  $P_{\text{aO}_2}$  values of approximately 40 mmHg at rest and 70 mmHg during early recovery).

#### *The physiological role of haemocyanin in Eurypelma californicum*

There are indications that haemocyanin (at least in *E. californicum*) not only functions as an  $\text{O}_2$  carrier but is also involved in other physiological processes (see Discussion). To investigate the possibility that haemocyanin could play a role as a storage protein and a cuticular structural component, we extracted and studied cuticular and exuvial proteins from *E. californicum* using PAGE and crossed immunoelectrophoresis ( $N=3$  experiments on different individuals or exuviae). Using  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  Ringer, extracted protein mass was, at most, 8% of the cuticle wet mass and 3% of the exuvial dry mass. (Quantities extracted using sodium hydroxide were much higher, but this procedure caused protein degradation that was detected by PAGE.) The cuticle extraction showed, in addition to a small amount of haemocyanin subunits (perhaps due to haemolymph impurities), several protein fractions with relative molecular masses between  $14 \times 10^3$  and more than  $20 \times 10^3$  (Fig. 10A). Extractions from the exuviae resulted in only the latter protein bands. The crossed immunoelectrophoresis patterns (Fig. 10B) showed an immunoreaction of both the extracted cuticular and exuvial proteins against antibodies to *E. californicum* haemocyanin (see Discussion). Compared with the haemocyanin subunits, the migration of the cuticular and exuvial proteins was much faster because of their lower relative molecular masses. This also shows that the immunoreaction is not due to haemolymph (haemocyanin) impurities.

## Discussion

### *Oxygen equilibrium curves*

We have used a novel experimental apparatus to determine  $\text{O}_2$  equilibrium curves and  $P_{\text{CO}_2}$ /pH relationships of cell-free undiluted haemolymph of resting *E. californicum*, *P. imperator* and *C. salei*. Oxygen affinity in *E. californicum* and *P. imperator*, and cooperativity in *E. californicum*, depend strongly on pH.

Our measures of  $\text{O}_2$  affinity differed from previous results on *E. californicum* haemocyanin (see below), apparently because of the different methods used to set and stabilize pH. In previous experiments Tris-HCl buffer was used, whereas in the present measurements pH was adjusted by altering the  $P_{\text{CO}_2}$ , which is equivalent to the physiological condition. The spectrophotometric results on undiluted haemolymph presented here are almost identical to  $\text{O}_2$  equilibrium curves of undiluted haemolymph or haemolymph dialysed against spider Ringer's solution, as determined by  $\text{O}_2$  concentration measurements. Haemolymph dialysed against Tris-HCl buffer shows a

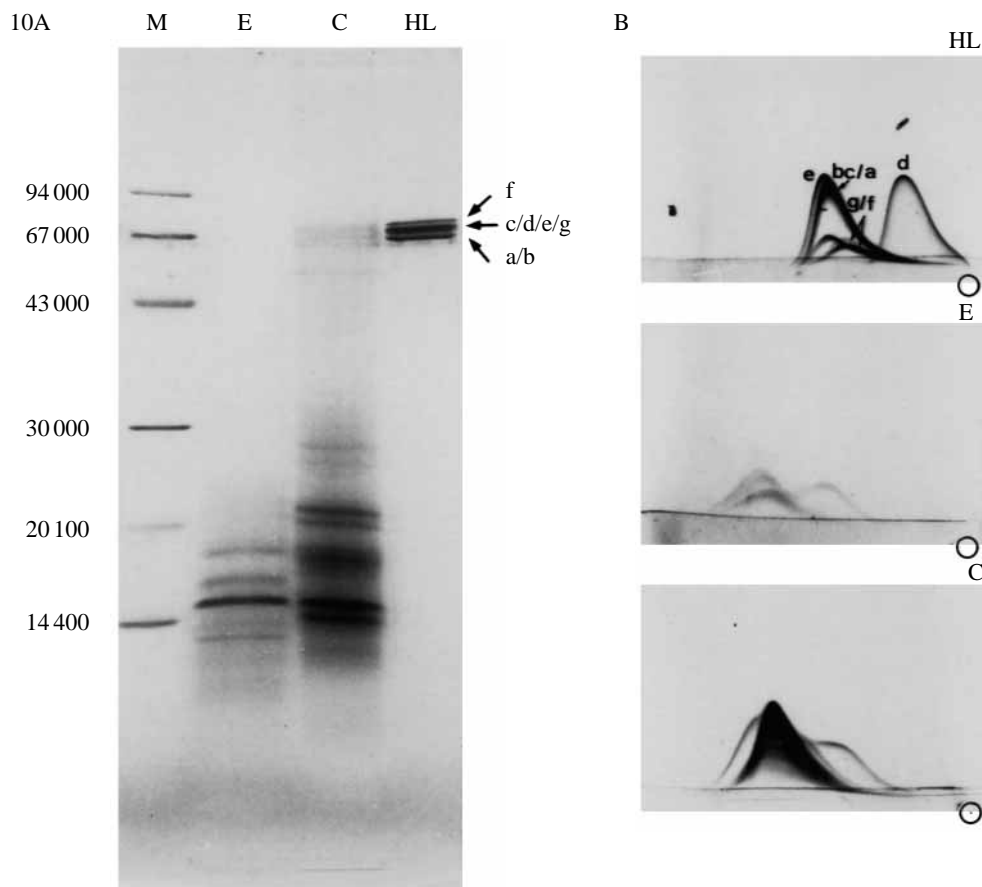


Fig. 10. (A) PAGE patterns of dissociated denatured (SDS) proteins of the haemolymph (HL; lower case letters designate the individual subunits), cuticle (C) and exuviae (E) of *Eurypelma californicum* at pH 8.8. The relative molecular masses of several standard proteins (M) are shown on the left. An acrylamide gradient using the system of Laemmli (1970) was employed. The anode was at the bottom. (B) Crossed immunoelectrophoresis patterns of dissociated haemolymph proteins (HL; lower case letters designate the individual subunits), exuvial proteins (E) and cuticular proteins (C) of *E. californicum*, tested against antiserum raised against dissociated purified *E. californicum* haemocyanin. In the horizontal direction, the anode is on the left. The points of sample application are indicated by the open circles.

marked increase in O<sub>2</sub> affinity (Fig. 4). Chloride concentration was different in the haemolymph samples dialysed against spider Ringer's solution (about 220 mmol l<sup>-1</sup>) or Tris-HCl buffer (about 86 mmol l<sup>-1</sup>), as were, to some extent, cation concentrations (5 mmol l<sup>-1</sup> Ca<sup>2+</sup>, 5 mmol l<sup>-1</sup> Mg<sup>2+</sup> versus 4.2 mmol l<sup>-1</sup> Ca<sup>2+</sup>, 0.4 mmol l<sup>-1</sup> Mg<sup>2+</sup>), but we think that the increase in affinity is caused by Tris. The main point of these measurements, however, was to check the spectrophotometric results and to look for reasons for the differences from previous data. In subsequent experiments, Tris-HCl buffer increased oxygen affinity of *E. californicum* haemocyanin measured using a

continuous fluorimetric/polarographic method (Bardehle, 1991). The higher O<sub>2</sub> affinity of *P. imperator* haemocyanin (as previously reported by Decker, 1990) compared with the data presented in this study may also be due to the Tris-HCl buffer used in the previous study.

Our values of O<sub>2</sub> affinity ( $\log P_{50}$ ) at pH 7, 7.5 and 8 (1.73, 1.68 and 1.35, respectively, at 25 °C) differ consistently by approximately 0.25 units from previous data on *E. californicum* haemocyanin: 1.49, 1.3 and 0.96 (20 °C; Decker and Sterner, 1990); 1.5, 1.4 and 1.1 (25 °C; Loewe, 1978). The O<sub>2</sub> equilibrium curves of whole blood are shifted to the right by approximately 23 mmHg (pH 7) to 10 mmHg (pH 8). The physiological consequences of this are discussed below.

In *E. californicum*, the Bohr effect is pH-dependent with coefficients ( $\Delta \log P_{50} / \Delta \text{pH}$ ) of  $-0.21$  (pH 7.25–7.5) and  $-0.7$  (pH 7.75–8). Decker and Sterner (1990) reported values (caused by differences in affinity) of  $-0.38$  (pH 7–7.6) and  $-1.04$  (pH 7.7–8). Cooperativity ( $n_{50}$ ) is also pH-dependent (at pH 7, 7.5 and 8), having values of 2.4, 4.3 and 6, respectively. Decker and Sterner (1990) give  $n_{50}$  values of 2.2, 3 and 6, respectively.

Our data on *P. imperator* haemocyanin O<sub>2</sub>-affinity [ $\log P_{50}$ : 1.65 (pH 7), 1.59 (pH 7.5) and 1.46 (pH 7.75), at 25 °C] differ by approximately 0.6 from Decker's (1990) values: 1.1 (pH 7), 0.98 (pH 7.4) and 0.78 (pH 7.75) (20 °C). Our value for the Bohr coefficient is  $-0.52$  (pH 7.5–7.75), whereas Decker (1990) reported  $-1.3$  at pH 7.8. Cooperativity is independent of pH, with an  $n_{50}$  value of approximately 4. Decker (1990) gives an  $n_{50}$  value of approximately 6.

At pH 7.5, *C. salei* haemolymph shows a  $\log P_{50}$  of 1.69 and an  $n_{50}$  value of 2.8. Loewe and Linzen (1975) measured an O<sub>2</sub> affinity of 1.46 and an  $n_{50}$  value of 5.2.

In the whole blood of the Middle American tarantula *Lasiodora erythrocythara*, Bridges (1988) determined O<sub>2</sub> affinities of 1.6, 1.36 and 1 and cooperativity values of 2.2, 3.4 and 3.5 (at pH 7.17, 7.64 and 8.06, respectively, 25 °C). He measured a Bohr coefficient of  $-0.71$  (pH 7.3–8.1). Bridges showed that there was no specific effect of CO<sub>2</sub> on oxygen affinity of *Lasiodora* haemolymph.

For *E. californicum* haemocyanin (haemolymph) also, no specific influence of CO<sub>2</sub> on the O<sub>2</sub> equilibrium curves can be demonstrated: O<sub>2</sub> equilibrium curves of undiluted haemolymph (where pH is adjusted by the  $P_{\text{CO}_2}$ ) are almost identical to curves of haemolymph dialysed against spider Ringer's solution (where pH is set by HCl and NaOH). This result (Fig. 4) also shows that, at least in resting individuals, the position and shape of the O<sub>2</sub> equilibrium curves of *E. californicum* are not influenced by dialysable effectors with a low molecular mass.

In view of the above discussion, we may now consider the O<sub>2</sub> equilibrium curves determined for cell-free undiluted *E. californicum* haemolymph to be valid for the *in vivo* condition. This allows a discussion of oxygen transport in this species.

#### *Oxygen transport in the haemolymph of Eurypelma californicum*

*E. californicum* and *P. imperator* have a very low oxygen consumption at rest (Paul *et al.* 1989a). Accordingly, the utilization of carbohydrate and lipid stores in the body is reduced; i.e. these animals have a strategy to save energy (Paul, 1990). Fast locomotion



(to catch prey or to defend themselves) is mainly based on the degradation of phosphagen and carbohydrate stores, with D-lactate as the final product, probably because these pathways yield more energy (ATP) per unit time than does aerobic metabolism (McGilvery, 1975). After locomotion, a long-lasting recovery period with an increased aerobic metabolism takes place, during which the phosphagen and carbohydrate stores of the muscles are replaced (Werner, 1991; Eschrich, 1991). Oxygen uptake, carbon dioxide release and heart rate increase markedly after locomotion (Paul *et al.* 1989a; Paul and Fincke, 1989).

There are differences in the utilization of the haemocyanin oxygen-transport system of *E. californicum* at rest and during early recovery (Fig. 8). At rest, mean  $P_{aO_2}$  is very low (approximately 28 mmHg) and mean  $P_{vO_2}$  is 16 mmHg. Mean pH values in the haemolymph are between 7.49 (pHa) and 7.45 (pHv). Accordingly, the arterio-venous percentage saturation difference is approximately 8% at rest. The large 122 mmHg difference between  $P_{aO_2}$  and ambient  $P_{O_2}$  (which is approximately 150 mmHg) is due to high diffusion resistances along the airways of the book lungs. At rest, the spiracles (the entrance slits of the book lungs) are almost closed, causing a marked drop in O<sub>2</sub> partial pressure (see Paul, 1992, for details). Because oxygen partial pressures are very low in *E. californicum* tissues (1–10 mmHg; Angersbach, 1975), the partial pressure gradients from the haemolymph to the tissues are at most 27 mmHg.

During the final phase of locomotor activity, the spiracles start to open and, during early recovery, they are fully opened (Fincke and Paul, 1989). The reduction of diffusion resistance in the book lungs brings ambient  $P_{O_2}$  and haemolymph  $P_{O_2}$  closer and, accordingly,  $P_{aO_2}$  rises to a mean value of 74 mmHg in *E. californicum*. With some delay,  $P_{vO_2}$  also rises to a mean value of approximately 25 mmHg. During fast locomotion, the prosomal muscles utilize their glycogen stores with D-lactate as the final metabolite (Werner *et al.* 1989). After locomotion, D-lactate accumulates in the haemolymph (Paul and Storz, 1987), and pHa and pHv decrease. pHa drops by, at most, 0.25 units, pHv by 0.5 units. This difference between pHa and pHv is due to buffering by increased CO<sub>2</sub> release from the book lungs during early recovery (Paul and Fincke, 1989). The arterio-venous percentage saturation difference is almost 60% and the haemocyanin oxygen-transport system is utilized more heavily during recovery. The oxygen partial pressure gradients from the haemolymph to the tissues also markedly increase to values of more than 70 mmHg, which cause much higher oxygen fluxes to the tissues.

Oxygen transport in the haemolymph has to adapt to changes in oxygen uptake at the book lungs, and both must follow the oxygen requirements of the tissues. Therefore, a rapid change in the utilization of the haemocyanin oxygen-transport system takes place in *E. californicum* at the transition from rest to recovery (Fig. 9). At rest, both  $P_{aO_2}$  and  $P_{vO_2}$  are very low. During early recovery,  $P_{aO_2}$  rises fast (Fig. 9A), corresponding to an opening of the spiracles and an increase in O<sub>2</sub> uptake (Paul *et al.* 1989a; Fincke and Paul, 1989).  $P_{vO_2}$  (in the prosoma) decreases during exercise, which is probably because prosomal perfusion stops (Paul *et al.* 1989b). With some delay (probably due to circulation time),  $P_{vO_2}$  starts to rise, but more slowly than  $P_{aO_2}$ . The CO<sub>2</sub> time courses (Fig. 9B) were calculated from  $P_{O_2}$  and pH time courses (measured by D. Angersbach, 1978 and unpublished results) using oxygen equilibrium curves of whole blood at

different pH values. Although the pH time courses and equilibrium curves are non-linear functions, the correlation between  $P_{O_2}$  and  $C_{O_2}$  time courses in arterial and venous blood is highly linear ( $r$  is approximately 0.99). The crucial variable for oxygen transport in the haemolymph is the arterio-venous  $C_{O_2}$  difference (Fig. 9C), which shows a marked increase during early recovery, mainly because of the increase in  $P_{aO_2}$ . This is when the haemocyanin oxygen-transport system is most heavily utilized. Additionally, heart rate increases and becomes maximal during early recovery, which further improves oxygen transport from the book lungs to the tissues.

We tried to check the  $C_{O_2}$  calculations by measuring  $C_{O_2}$  directly, but we found no significant differences between rest and recovery values. Two different factors may negatively influence a haemolymph gas analysis of fresh samples in *E. californicum* (see also Paul *et al.* 1994). It is possible (i) that the animals started to open their spiracles during sampling, which would have caused an increase in  $P_{aO_2}$  (the circulation time between the book lungs and the pericardium is very short); or (ii) that  $O_2$  from the surroundings entered the samples, as indicated by the high  $P_{vO_2}$ , although great care was taken to avoid this. The latter problem is probably related to the low  $O_2$  capacity of *E. californicum* haemolymph.

#### *The physiological role of haemocyanin in Eurypelma californicum*

Respiratory pigment concentration is a further important variable that affects oxygen transport in blood. In *E. californicum*, as well as in *P. imperator*, we found amazingly high individual variations in haemolymph protein concentration ( $C_{HP}$ ). In both species, individual  $C_{HP}$  values are between approximately 5 and 100 mg ml<sup>-1</sup> (Paul and Efinger, 1990). We found particularly high values in the scorpions (see van Aardt, 1991). The haemocyanin concentration follows the  $C_{HP}$  variability (because haemocyanin is the main haemolymph protein, constituting approximately 80% of total  $C_{HP}$  in *E. californicum*; Markl *et al.* 1976), as deduced from a comparison of absorbance measurements at 340 and 280 nm. The behaviour or activity patterns of individuals with different  $C_{HP}$  values did not show any striking differences. To study the physiological consequences of  $C_{HP}$  (and  $C_{Hc}$ ) differences, we determined, in individual tarantulas, the relationships between  $C_{HP}$  and different physiological variables (e.g. basal  $O_2$  uptake, minimal and maximal heart rate,  $P_{50}$  of the  $O_2$  equilibrium curves). Surprisingly, we only found weak correlations (Paul and Efinger, 1990):  $r=0.43$  for oxygen uptake,  $r=-0.31$  and  $-0.46$  for minimal and maximal heart rate, respectively, and  $r=0.36$  for the  $P_{50}$  values (at pH 7). The duration of recovery, deduced from heart rate time courses after exhausting exercise, did not correlate with  $C_{HP}$  (at maximum activity, tarantulas exhaust very rapidly, within a few minutes). From these results, we may conclude that different haemocyanin concentrations in individual tarantulas are not compensated for by an adaptation of a single physiological variable, but by the sum of different adaptational changes. One variable that we were not able to measure, the arterio-venous percentage saturation difference, may be the most important. It seems likely that individuals with a low  $C_{HP}$  value utilize their haemocyanin oxygen-transport system much more heavily than do those with a high  $C_{HP}$  value (up to 100 mg ml<sup>-1</sup>). In individuals (at least of *E. californicum*) with a 'normal'  $C_{HP}$  value (approximately 45 mg ml<sup>-1</sup>), haemocyanin

plays its most important role as an oxygen carrier during the recovery phase. Aerobic metabolism increases markedly during this phase to provide sufficient energy to restore phosphagen and carbohydrate concentrations in the muscles to resting levels (Paul, 1991). In individuals with a low  $C_{HP}$ , the haemocyanin oxygen-transport system may be utilized more intensely at rest.

Apart from the physiological consequences of the variability in  $C_{HP}$ , there are further questions about its origin and meaning. We found close relationships between food and water supply and  $C_{HP}$  in *E. californicum* (Paul and Efinger, 1990). Individuals that were well supplied with food showed a marked increase in  $C_{HP}$ . Starvation resulted in a decrease in  $C_{HP}$ , but deprivation of both water and food caused an increase in  $C_{HP}$  due to dehydration. (Theoretically, deprivation of water alone should cause an even larger increase in  $C_{HP}$ .) Giving the animals water after a period of food and water deprivation showed that the dehydration is reversible:  $C_{HP}$  reached a value a little below the initial one within a few days.

Why do  $C_{HP}$  and haemocyanin concentrations increase to such an extent that, even during recovery, the haemocyanin oxygen-transport system is not fully utilized? The direct dependence of  $C_{HP}$  on food supply suggests that the haemolymph proteins, particularly the dominant haemocyanin fraction, may additionally serve as a store for amino acids or peptides. In this context, it is interesting to note that there is a very high degree of similarity (27%) between the amino acid sequences of arthropod haemocyanins and that of arylphorin, a larval serum and storage protein (LSP) from *Manduca sexta* (Fujii *et al.* 1989; Willott *et al.* 1989). Markl and Winter (1989) were able to show that monoclonal antibodies against *E. californicum* haemocyanin cross-react with calliphorin, an LSP from *Calliphora vicina*. The LSPs of insects are storage proteins (stores of amino acids), which play a central role during metamorphosis (Munn *et al.* 1971; Scheller, 1983, 1987; König *et al.* 1986). During the final larval stadium of holometabolous insects, the larval serum proteins (or larval storage proteins) accumulate in the haemolymph and reach very high concentrations (up to 95 mg ml<sup>-1</sup>). During metamorphosis, they enter the cuticle and appear to be utilized for cuticle sclerotization (König *et al.* 1986). We observed changes in  $C_{HP}$  during moulting in *E. californicum* (Paul and Efinger, 1990). In the days and weeks before and after ecdysis, the animals stop eating and lose body mass (Stewart and Martin, 1982). During this time (mainly after ecdysis), we found a slow decrease in  $C_{HP}$ . This may be related to the increased water uptake, but an additional use of haemolymph protein in the post-moulting phase is possible.

To test the possibility that haemocyanin plays a role in the moulting processes, we extracted cuticular and exuvial proteins from *E. californicum* and studied them by PAGE and crossed immunoelectrophoresis. The relatively small amount of extracted proteins (8% from the cuticle, 3% from the exuviae) is due to the gentle treatment with Ca<sup>2+</sup>, Mg<sup>2+</sup> Ringer. (NaOH extraction resulted in much higher quantities, but the proteins were degraded.) Most extracted proteins had relative molecular masses between 14 × 10<sup>3</sup> and more than 20 × 10<sup>3</sup>, and they showed a positive immunoreaction against antibodies to *E. californicum* haemocyanin subunits. An explanation for these findings could be that fragments of haemocyanin molecules are utilized as structural components for cuticle construction. However, conclusive evidence must include the proof of haemocyanin

transport from the haemolymph to the cuticle and of its incorporation there (König *et al.* 1986).

We thank Chris Bridges, Michael Colmorgen and Bettina Zeis for reading the manuscript critically. Supported by the Deutsche Forschungsgemeinschaft (Pa 308/1-2, Pa 308/1-3).

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