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SHORT COMMUNICATION

HAEMOLYMPH BUFFERING IN THE LOCUST *SCHISTOCERCA GREGARIA*

BY JON F. HARRISON, CALVIN J. H. WONG AND JOHN E. PHILLIPS
Department of Zoology, University of British Columbia, Vancouver BC, V6T 2A9 Canada

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The biochemical basis of buffering in the haemolymph of insects has received little attention, and the chemical complexity of insect haemolymph (reviewed by Mullins, 1985) suggests that the relative importance of various compounds to buffering may be quite different from that in other arthropods or vertebrates. Moreover, the role of bicarbonate as a buffer in insect haemolymph is difficult to evaluate because precautions to prevent carbon dioxide loss during sampling from insect haemolymph have generally been ignored, and titration techniques previously used to measure haemolymph buffer value allowed inadequate time for equilibration of the bicarbonate buffer system. In this study, we describe the biochemical basis of haemolymph buffering in the migratory locust *Schistocerca gregaria*.

Recent texts of insect physiology (Mullins, 1985; Woodring, 1985) emphasize early observations of a U-shaped haemolymph buffer curve over the range of pH 3 to pH 10, with minimum values near the normal extracellular pH (Craig and Clark, 1938; Babers, 1941; Pepper *et al.* 1941; Hastings and Pepper, 1943; Levenbrook, 1950*b*). The elevated buffer values at extreme pH may be attributed primarily to buffering by the ammonium and carboxyl groups of amino acids, which commonly occur at high concentrations in insect haemolymph. Levenbrook proposed that the U-shaped buffer curve of insect blood might be advantageous in the minimization of pH changes due to carbon dioxide accumulation during activity (Levenbrook, 1950*b*). However, in the insects that have been examined recently, pH excursions during activity are less than 1 unit (Downer and Mathews, 1977; Harrison *et al.* 1990), and only compounds which buffer in this physiological pH range of 6.3–7.3 will contribute to acid–base regulation in living insects.

The biochemical nature of buffers in insect haemolymph in a physiological pH range has only been determined for *Gastrophilus intestinalis*. This larval parasite of horse intestines is unusual among insects in that it is routinely exposed to P_{CO_2} values of 66.7 kPa (Levenbrook, 1950*b*). During titration with acid, the haemolymph buffers are bicarbonate (58%), proteins (30%), succinate (6%) and inorganic phosphate (5%, Levenbrook, 1950*b*). Other potential buffers in insect

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haemolymph include organic phosphates, citrate, malate, fumarate, α-ketoglutarate and histidine (Wyatt, 1961; Florkin and Jeuniaux, 1974). Quantification of the contribution of proteins to haemolymph buffering requires measurement of the specific buffer value of insect haemolymph proteins. Protein concentration, and probably composition, varies in the haemolymph of female locusts in correlation with the ovarian cycle (Hill, 1962). We estimated a pooled specific buffer value of *S. gregaria* haemolymph proteins by measuring the haemolymph buffer value of individual locusts differing in the protein concentration of their haemolymph.

Locusts were maintained at the University of British Columbia as previously described (Thomson *et al.* 1988). All animals were adult females, 3–4 weeks past the final moult. Individuals were fed synchronously on lettuce *ad libitum* for 2 h at 35 °C on the day before haemolymph sampling, and then were maintained individually with access to cotton soaked with distilled water for 14–18 h at 21 °C.

Haemolymph was sampled and analyzed for pH as previously described (15 μl sample, Harrison, 1988). Total carbon dioxide (C_{CO_2}, mmol l^{-1}) was measured with a 10 μl sample from the same individual by gas chromatography as described by Boutilier *et al.* (1985). The C_{CO_2} was measured with cell-containing haemolymph; however, haematocrits were less than 1 %. Samples were then centrifuged to remove cells and prevent clotting, as previously described (Harrison, 1988), and frozen. Attempts to control clotting were only partially successful with these techniques. Therefore, haemolymph buffer value was also measured using haemolymph collected directly onto ice (without delays due to measurement of acid–base parameters) and then centrifuged to remove cells. The concentration of protein *in vivo* (before clotting) was measured by injecting 10 μl haemolymph samples directly into 90 μl of 72 % trichloroacetic acid (TCA).

Haemolymph nonbicarbonate buffer values ($\Delta[HCO_3^-]\Delta pH^{-1}$, mequiv l^{-1} pH unit^{-1}) at 21 °C were calculated by measuring the change in pH of 15 μl drops equilibrated with different water-saturated gas mixtures (2 %, 5.2 % and 8 % CO_2, balance N_2). The pH range during these trials was 7.3–6.6. $[HCO_3^-]$ was calculated using CO_2 solubility coefficients and pK_{app} values for locust haemolymph (Harrison, 1988). Tonometered haemolymph also contained approximately 1 mg l^{-1} each of iodoacetamide (to inhibit clotting) and NaF (Harrison, 1988). Carbonic anhydrase (25 Wilbur–Anderson units ml^{-1}, 10 μg protein ml^{-1}) was added immediately before tonometry. Equilibration time was 30–45 min; each drop was exposed to ascending, followed by descending, values of P_{CO_2}. Small changes in pH at a given P_{CO_2} over time were assumed to be due to linear drift. As previous studies had indicated that plots of $[HCO_3^-]$ *vs* pH were linear between pH 7.1 and pH 6.5 (Harrison, 1988), buffer values were calculated using linear regression analysis.

The pH of the haemolymph drop was measured with a glass microelectrode constructed with a procedure modified from Harrison and Walker (1977). The pH-sensitive tips of these electrodes averaged 500 μm in length. Tips were blown to a bulb with a diameter of approximately 50 μm. Electrodes were backfilled with a pH 7.0 phosphate buffer. The reference electrode used was a PE-10 KCl–agar

bridge (3 mol l^{-1} KCl, 3 % agar) in series with a calomel electrode. The potential difference between the reference and pH electrodes was measured with a high input impedance (2×10^{-14} Ω) Keithley model 616 electrometer. Electrodes were calibrated against Radiometer precision buffers (S1500, S1510). Electrodes were rejected if EMF slopes were not in the range 50–60 mV pH $unit^{-1}$ or 90 % response times were greater than 10 s. The pH and reference electrodes were inserted into the drop through a port in the tonometry vessel, so the pH of the haemolymph was measured during continuous equilibration with the reference gas. Electrodes were calibrated against the Radiometer pH buffers before and after each haemolymph pH measurement. All measurements were conducted at room temperature (21 °C, range 20.2–21.7 °C).

The concentration of protein in individual haemolymph samples was measured using a Biuret reagent with bovine serum albumin as a standard (Layne, 1957). Inorganic phosphate concentrations were measured with a modified Fiske–Subbarow method (Chen *et al.* 1956).

A large sample (>1 ml) of *S. gregaria* haemolymph was also collected by pooling haemolymph from approximately 30 individual females as described above. This sample was analyzed for buffer value, protein, total and inorganic phosphorus and succinate. Succinate was assayed as this compound was an important buffer in *G. intestinalis*, and its concentration in *S. gregaria* haemolymph was unknown. Succinate was measured using an enzymatic assay with a Boehringer Mannheim kit (176281). Total phosphate was analyzed using a procedure modified from Lowry *et al.* (1954) with glucose-6-phosphate as a standard, and organic phosphate concentrations were calculated from the difference between total and inorganic phosphate. The buffer values (β, mequiv l^{-1} pH $unit^{-1}$) for various compounds were calculated using pK values appropriate for the ionic strength of locust haemolymph at 21 °C, using data compiled by Robinson and Stokes (1959). As the specific organic phosphate compounds in locust haemolymph have not been identified, and the pK values of organic phosphate compounds range from 6.4 to 6.8 (Robinson and Stokes, 1959), we assumed a pK for organic phosphates of 6.6.

The nonbicarbonate buffer value of individual haemolymph samples increased with increasing protein concentration (Fig. 1). The specific buffer value for protein was estimated from the slope of the regression as 0.182 mequiv l^{-1} pH $unit^{-1}$ mg^{-1} (S.E. of the slope=0.053). The nonbicarbonate buffer values of individual haemolymph samples were not correlated with inorganic phosphate levels (Pearson's product moment correlation coefficient=−0.03). Using the specific buffer value for protein estimated from Fig. 1, protein and total phosphates accounted for approximately 90 % of the nonbicarbonate buffer value in the pooled haemolymph sample (Table 1). These results indicate that unmeasured organic compounds are unlikely to contribute more than 1–2 mequiv l^{-1} pH $unit^{-1}$ in buffer value in the haemolymph of *S. gregaria* (10–20 % of the total buffer value).

Under *in vivo* conditions (Table 2), protein is estimated to account for 66 %, total phosphates 20 %, and citrate and histidine 3 %, of the nonbicarbonate buffer

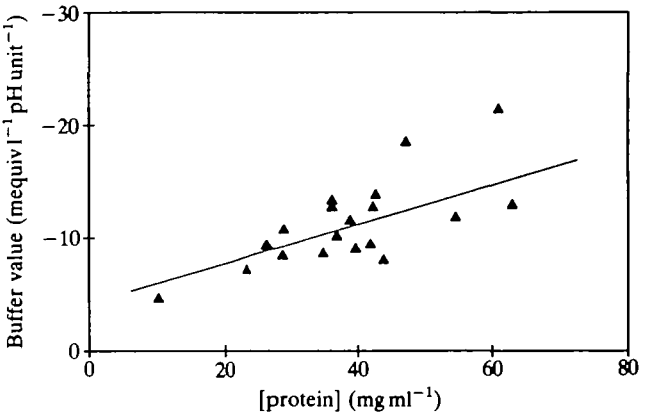

Fig. 1. Scatterplot of haemolymph buffer values (β, mequiv l^{-1} pH unit^{-1}) as a function of the concentration of protein (mg ml^{-1}) in individual *Schistocerca gregaria* females. β=4.0+0.182[protein] (r^2=0.39, P<0.01).

Table 1. *Concentrations and estimated buffer value (β, mequiv l^{-1} pH unit^{-1}) of buffer compounds in a pooled sample of* Schistocerca gregaria *haemolymph with a nonbicarbonate buffer value of 10.2 mequiv l^{-1} pH unit^{-1}*

	Concentration	β
Protein (mg ml^{-1})	29.0	5.3
Organic phosphate (mmol l^{-1})	4.7	2.5
Inorganic phosphate (mmol l^{-1})	2.7	1.6
Succinate (mmol l^{-1})	0.2	0.03

value (Table 3). Possibly the remaining 10% of the buffer value is due to unmeasured organic acids. However, given the variance in the individual data, and the uncertainties in assigning values of pK, it is also possible that all buffer compounds of physiological significance have been measured.

While protein is the dominant nonbicarbonate buffer in this system, protein is quantitatively less important than in vertebrates or crustaceans (Truchot, 1976; Heisler, 1986). The variation in protein concentration only explains a portion of the variance in nonbicarbonate buffer value among individuals (Fig. 1). The estimated specific buffer value of haemolymph protein (0.182 mequiv l^{-1} pH unit^{-1} mg^{-1}) is high relative to that measured for human haemoglobin (0.152, Davenport, 1974) or crab haemocyanin (0.139, Truchot, 1976); however, confidence intervals for the slope of the regression line (Fig. 1) include these values. The specific buffer value for *S. gregaria* haemolymph is also high relative to that measured for *G. intestinalis* larvae (0.096 mequiv l^{-1} pH unit^{-1} mg^{-1}, Levenbrook, 1950*b*). However, it is possible that the calculated specific buffer value for *Gastrophilus* haemolymph protein may be a low estimate because (1) equilibration

Table 2. *Acid–base status, nonbicarbonate buffer value (β, mequiv l^{-1} pH unit^{-1}) and concentrations of inorganic phosphate and protein in* Schistocerca gregaria *haemolymph at 21 °C*

	Mean	S.D.
pH	7.31	0.351
C_{CO_2} (mmol l^{-1})	9.4	1.55
P_{CO_2} (kPa)	2.19	0.34
[HCO_3^-] (mmol l^{-1})	8.7	1.46
Protein (mg ml^{-1})	54.8	3.51
Inorganic phosphate (mmol l^{-1})	3.5	1.18
β	15.8	

N=8 for each, except for nonbicarbonate buffer value, which was measured with a pooled sample (protein concentration of pooled sample=52 mg ml^{-1}).

Table 3. *Estimated components of buffering in the haemolymph of quiescent* Schistocerca gregaria *at 21 °C, pH 7.31 and constant* P_{CO_2}

	β (mequiv l^{-1} pH unit^{-1})
Bicarbonate	20.0
Protein	10.0
Inorganic phosphate	1.6
Organic phosphate	1.5
Citrate	0.4
Histidine	0.1
Total	33.6

The contribution of protein was estimated from Fig. 1 and the average *in vivo* protein concentration.

The buffer value of bicarbonate was calculated as 2.303 times bicarbonate concentration (Heisler, 1986).

Concentrations of citrate and histidine in the haemolymph of *S. gregaria* were taken from the literature (Baumeister *et al.* 1981; Chamberlin and Phillips, 1982).

Measured compounds accounted for approximately 90 % of the nonbicarbonate buffer value of 15.2 mequiv l^{-1} pH unit^{-1}

is difficult to obtain during titration of solutions high in bicarbonate and (2) protein precipitation may have occurred during the acid titration.

Only nonbicarbonate buffers are of physiological importance when locusts are exposed to environmental hypercapnia, or during the respiratory acidosis associated with activity (Harrison, 1989*b*; Harrison *et al.* 1991). However, when haemolymph P_{CO_2} is maintained constant by the locust, as occurs across most temperature changes and after acid injection (Harrison, 1988, 1989*a*; Wong *et al.* 1989), the bicarbonate buffer system accounts for approximately 57 % of the total buffer value of the haemolymph (Table 3). In *G. intestinalis*, Levenbrook calculated a very similar bicarbonate buffer value of 19 mequiv l^{-1} pH unit^{-1}

based on differences between the titration curves of dialyzed and undialyzed haemolymph. However, the theoretical value for bicarbonate buffering at the haemolymph concentrations in *G. intestinalis* of 50 mmol l^{-1} (Levenbrook, 1950*a*) is over five times the measured value, strongly suggesting that equilibration was not attained during the *G. intestinalis* titration.

We have two conclusions. (1) As in vertebrates, the major buffer systems of physiological importance in locusts (and probably most insects) during non-respiratory acid–base disturbances are bicarbonate and protein. (2) The much-quoted U-shaped buffer curve of insect haemolymph is physiologically irrelevant. The intraspecific variation in haemolymph composition and pH of insects suggests that the quantitative importance of various haemolymph buffer compounds must show considerable variation. For example, organic acids seem likely to be substantially more important to buffering in lepidopteran larvae, as succinate, citrate and organic phosphate levels may each exceed 30 mmol l^{-1} and haemolymph pH values are between 6.4 and 6.9 (Wyatt, 1961).

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