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# ALLOSTERIC MODULATION OF HAEMOCYANIN OXYGEN-AFFINITY BY L-LACTATE AND URATE IN THE LOBSTER *HOMARUS VULGARIS*

## I. SPECIFIC AND ADDITIVE EFFECTS ON HAEMOCYANIN OXYGEN-AFFINITY

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### Summary

The specific effects of L-lactate and urate on oxygen binding by the haemocyanin of the lobster *Homarus vulgaris* were investigated. Increasing concentrations of L-lactate were found to increase haemocyanin oxygen-affinity. The relationship between the oxygen affinity ($\log P_{50}$) and [L-lactate] expressed as $(\Delta\log P_{50})(\Delta\log[\text{L-lactate}])^{-1}$ was $-0.11$ at L-lactate concentrations between 0.3 and 11 mmol $l^{-1}$ and pH 7.99±0.03. Urate, likewise, had a potentiating effect on haemocyanin oxygen-affinity: $(\Delta\log P_{50})(\Delta\log[\text{urate}])^{-1}$ was $-0.18$ at urate concentrations between 0 and 0.93 mmol $l^{-1}$ and pH 7.99±0.03. Cooperativity, expressed as $n_{50}$, was reduced by the presence of both modulators.

The influence of the simultaneous presence of both factors on haemocyanin oxygen-affinity was also investigated. The effects of L-lactate and urate on haemocyanin oxygen-affinity were found to be additive. The possible physiological role of these modulators is discussed.

### Introduction

The oxygen affinity of many arthropod haemocyanins can be modulated by allosteric effectors. It has been known for some time that inorganic ions, such as $H^+$, $Ca^{2+}$, $Mg^{2+}$ and $Cl^-$, affect haemocyanin oxygen-affinity (for a review, see Ellerton *et al.* 1983). The organic modulator that was first demonstrated to increase the oxygen affinity of *Carcinus maenas* and *Cancer pagurus* haemocyanin was L-lactate (Truchot, 1980). This effect of L-lactate on haemocyanin oxygen-affinity has been confirmed for many other species of crustaceans (see table in Bridges and Morris, 1986). Similarly, urate and other purine bases have a potentiating effect on haemocyanin oxygen-affinity (Morris *et al.* 1985, 1986*a*).

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Key words: lobster haemocyanin, oxygen equilibrium curve, L-lactate, urate, cooperativity, *Homarus vulgaris*.

Dopamine has also been demonstrated to increase the oxygen affinity of *Cancer magister* haemocyanin *in vitro* (Morris and McMahon, 1989*a*,*b*).

In contrast to their well-documented effect on oxygen affinity, there is still some uncertainty whether allosteric effectors also influence cooperativity of oxygen binding to haemocyanin. A higher cooperativity was measured in the presence of $Ca^{2+}$ for *Limulus polyphemus* and *Callinectes sapidus* (Brouwer *et al.* 1983), but L-lactate was reported to decrease cooperativity in *Cancer magister* (Graham *et al.* 1983) and *Callinectes sapidus* (Johnson *et al.* 1984; Bridges and Morris, 1986). Other authors have observed no measurable influence on cooperativity in *Callinectes sapidus* and *Homarus vulgaris* (Booth *et al.* 1982; Taylor and Whiteley, 1989). Urate has been demonstrated to have no effect on the cooperativity of oxygen binding in *Austropotamobius pallipes* (Morris *et al.* 1985) and *Astacus leptodactylus* (Bridges, 1990).

These previous studies were carried out *in vitro* for each modulator separately, leaving all other factors unchanged. Therefore, less information is available concerning the interactions of two or more allosteric modulators. The experimental approach for investigations on simultaneous effects of several effectors includes variation of the concentrations of all components studied. This was done by Morris *et al.* (1986*b*, 1987) for L-lactate and $Ca^{2+}$ in *Austropotamobius pallipes*; they found mutual agonistic effects of both ions (Morris *et al.* 1986*b*). Various combinations of L-lactate and urate concentrations present in the haemolymph of *Austropotamobius pallipes* were able to enhance haemocyanin oxygen-affinity (Morris *et al.* 1986*a*; Morris and Bridges, 1986); however, the effects were not additive.

In the present study, the effect of L-lactate and urate on lobster haemocyanin oxygen-affinity is first considered for each modulator individually and then the simultaneous effect of L-lactate and urate is examined. The results are discussed in the light of our present knowledge of modulation of haemocyanin oxygen-affinity.

## Materials and methods

### *Animal supply and haemolymph sampling*

Five European lobsters (*Homarus vulgaris* Milne-Edwards) were purchased in Roscoff, Brittany (France). The animals were transported to Düsseldorf and held there at the Institut für Zoologie, Lehrstuhl für Tierphysiologie, in a recirculating artificial seawater aquarium at 15±1°C, pH 8.0±0.1 and a salinity of 38±1‰. Sea salt was obtained from Wiegandt (Krefeld, Germany). Animals were fed regularly with squid muscle tissue and exposed to a 12 h:12 h light:dark regime.

Haemolymph samples were taken by piercing the arthrodial membrane at the base of the walking legs and drawing a haemolymph sample into a syringe. A 5 ml sample was taken every 4 weeks from each individual and samples were pooled. To avoid clotting, the haemolymph was filtered through gauze and cellular material was removed by centrifugation at 8000 *g* (Hermle ZK 400, Kontron, Munich, Germany) at 4°C for 10 min. To remove endogenous effectors, the

haemolymph was dialysed three times against Ringer's solution whose volume was 40 times the haemolymph volume. The Ringer's solution was made up as follows: 600 mmol $l^{-1}$ NaCl, 10 mmol $l^{-1}$ $CaSO_4$, 10 mmol $l^{-1}$ $MgSO_4$, 5 mmol $l^{-1}$ KCl, 5 mmol $l^{-1}$ $NaHCO_3$, pH 7.99±0.03. This reflected the concentrations of inorganic ions in the artificial sea water and those measured in whole haemolymph. Bacterial growth was prevented by adding 0.02 % $NaN_3$ to the Ringer's solution. The haemocyanin solutions were stored at 4 °C for a maximum of 4 weeks.

### *Concentrations of protein and haemocyanin*

The concentrations of protein and haemocyanin in the haemolymph were determined by spectrophotometric analysis at wavelengths between 250 and 450 nm (Uvikon 810, Kontron, Munich, Germany) according to the method of Nickerson and van Holde (1971). The maximum absorption of haemocyanin was measured at 334 nm. Haemocyanin and protein concentrations were calculated using the extinction coefficients reported for *Homarus americanus* ($\epsilon_{334}=2.69^{1\,\%}_{1\,cm}$, $\epsilon_{280}=14.3^{1\,\%}_{1\,cm}$; Nickerson and van Holde, 1971).

### *Aggregation state of the haemocyanin*

The aggregation state of haemocyanin was determined by gel filtration using a fast protein liquid chromatography (FPLC) system equipped with a Superose-6 column (Pharmacia, Freiburg, Germany). About 2 mg of protein was used for this purpose and eluted with 0.1 ml $min^{-1}$ of Ringer's solution (see above). The haemolymph proteins were monitored spectrophotometrically at 280 nm. The different aggregation states of haemocyanin were identified by their relative molecular mass using the values reported by Markl *et al.* (1979) for dodecamers and hexamers of this species. Ovalbumin, aldolase, catalase, ferritin and thyreoglobulin were used for calibration.

### *Concentration of L-lactate and urate*

L-Lactate concentrations were measured using the method of Gutmann and Wahlefeld (1974), modified by addition of EDTA to the assay (Engel and Jones, 1978). Urate concentration was determined using the HPLC technique according to Wynants *et al.* (1987). Proteins were removed from the samples by extraction with perchloric acid (0.6 mol $l^{-1}$), coagulated proteins being sedimented by centrifugation at 8000 *g* at 4 °C for 10 min (Biofuge A, Heraeus Christ, Osterode, Germany). A 20 $\mu$l sample of the supernatant was used for chromatography without preceding neutralisation.

### *Oxygen equilibrium curves*

Oxygen equilibrium curves were determined spectrophotometrically using a diffusion chamber (Sick and Gersonde, 1969; modified by Bridges *et al.* 1979). The $O_2$ tensions in a 10 $\mu$l sample were varied using gas-mixing pumps (303a/f Wösthoff, Bochum, Germany). The pH was altered by changing the $CO_2$ tension from 0.1 to 0.8 kPa. pH values were measured in a corresponding sample

equilibrated in a BMS II tonometer (Radiometer, Copenhagen, Denmark) with the same gas mixtures. The pH was determined at the half-saturation point ($P_{50}$) of each equilibrium curve. The oxygen-dependent changes in haemocyanin absorption were measured at a wavelength of 365 nm using a spectrophotometer (Eppendorf 1101M, Hamburg, Germany). The experimental equipment was thermostatted at 15°C. The $P_{50}$ and $n_{50}$ values were calculated employing regression analysis of the data between 25 and 75 % saturation according to the Hill equation (Hill, 1910).

Oxygen binding experiments were carried out with native haemolymph and dialysed haemocyanin solutions. To obtain different L-lactate and urate concentrations in the haemocyanin samples, 200 $\mu$l samples of the dialysed solutions were centrifuged at 100 000 *g* for 1 h (Airfuge, Beckman, CA, USA). 100 $\mu$l of the supernatant was replaced by Ringer's solutions containing defined amounts of L-lactate and/or urate; the haemocyanin pellet was redissolved (replacement technique according to Bridges *et al.* 1984). The exact L-lactate and urate concentrations of the resulting haemocyanin solutions were measured as described above. The following results are based on 42 oxygen equilibrium curves.

Unless otherwise stated, values given are means±S.D. The data were analysed according to Student's *t*-test. When covariance analysis was carried out, the *P* values are given in the text.

## Results

The concentration of haemocyanin in native *Homarus vulgaris* haemolymph was 37.8±10.3 mg ml$^{-1}$, and total protein concentration was 39.6±10.7 mg ml$^{-1}$ ($N$=21). In these measurements, individual haemolymph samples of four animals were taken at different times. L-Lactate concentrations averaged 0.5±0.2 mmol l$^{-1}$ ($N$=7) and those of urate 0.08±0.02 mmol l$^{-1}$ ($N$=7) in undisturbed control animals. In the native haemolymph more than 96 % of the haemocyanin molecules were dodecamers and hexamers; at least 80 % were dodecamers. The concentration of monomers was negligible. Only solutions with more than 80 % dodecameric haemocyanin were used for the experiments.

Oxygen binding curves constructed in the presence of different L-lactate concentrations at pH 7.99±0.02 ($P_{CO_2}$=0.1 kPa, 0.8 mmHg) are shown in Fig. 1. The $P_{50}$ in the dialysed haemolymph (0.3 mmol l$^{-1}$ L-lactate) was 0.89 kPa (6.7 mmHg). Increasing the concentration of L-lactate to 1.5 mmol l$^{-1}$ resulted in a left shift of the curve, the $P_{50}$ being 0.79 kPa (5.9 mmHg). Half-saturation was further lowered to 0.65 kPa (4.9 mmHg) by raising the L-lactate concentration to 4.5 mmol l$^{-1}$. Augmentation of the L-lactate concentration to 11 mmol l$^{-1}$ only resulted in a small further increase in the oxygen affinity, the $P_{50}$ reaching 0.63 kPa (4.7 mmHg). The decrease in $P_{50}$ as an exponential function of the L-lactate concentration is illustrated in Fig. 2. At low L-lactate concentrations, up to 4.5 mmol l$^{-1}$, $P_{50}$ is proportional to the added L-lactate concentration. Raising the

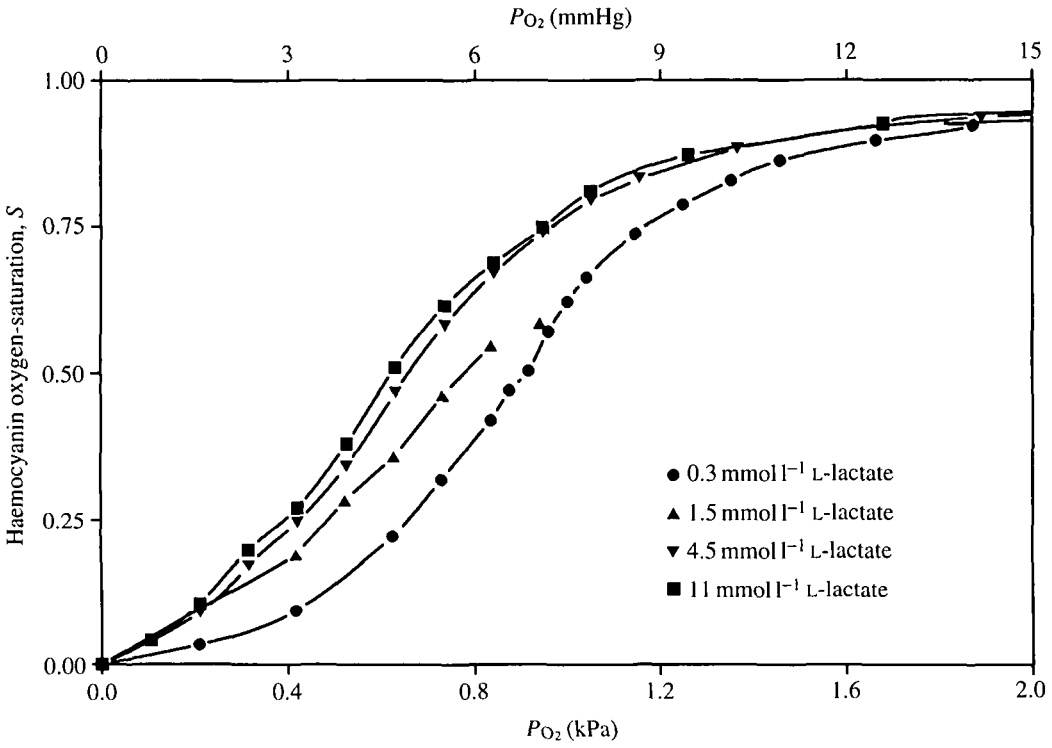


Fig. 1. Effect of L-lactate concentration on haemocyanin oxygen-affinity. The saturation of haemocyanin with oxygen ($S$) is shown at different oxygen tensions ($P_{O_2}$). Oxygen tensions are given in mmHg and kPa. The oxygen binding curves are shifted to the left by increasing L-lactate concentrations, indicating a higher oxygen affinity. Different L-lactate concentrations were achieved with the replacement technique (Bridges *et al.* 1984). $T$=15°C, pH 7.99±0.03.

L-lactate concentration beyond 4.5 mmol $l^{-1}$ resulted only in a small increase in oxygen affinity.

A quantitative estimate of the L-lactate effect can be obtained from the quotient $(\Delta\log P_{50})(\Delta\log[\text{L-lactate}])^{-1}$, which was −0.11 at pH 7.99±0.03 (Table 1B). Whole haemolymph showed a higher oxygen affinity than did dialysed haemolymph at the same L-lactate concentration (Fig. 2): $\Delta P_{50}$ was 0.16 kPa (1.2 mmHg).

The Bohr effect can be estimated by the ratio $(\Delta\log P_{50})(\Delta\text{pH})^{-1}$, which is derived from the regression lines given in Table 1A for the L-lactate concentrations examined. For L-lactate concentrations between 0.3 mmol $l^{-1}$ and 4.5 mmol $l^{-1}$ no significant change in the Bohr coefficient was observed. At 11 mmol $l^{-1}$ L-lactate, however, the slope of the regression line $(\Delta\log P_{50})(\Delta\text{pH})^{-1}$ was significantly lowered ($P$<0.01).

Similar investigations were carried out with haemocyanin solutions containing different urate concentrations (Fig. 3) at constant pH. The oxygen affinity of

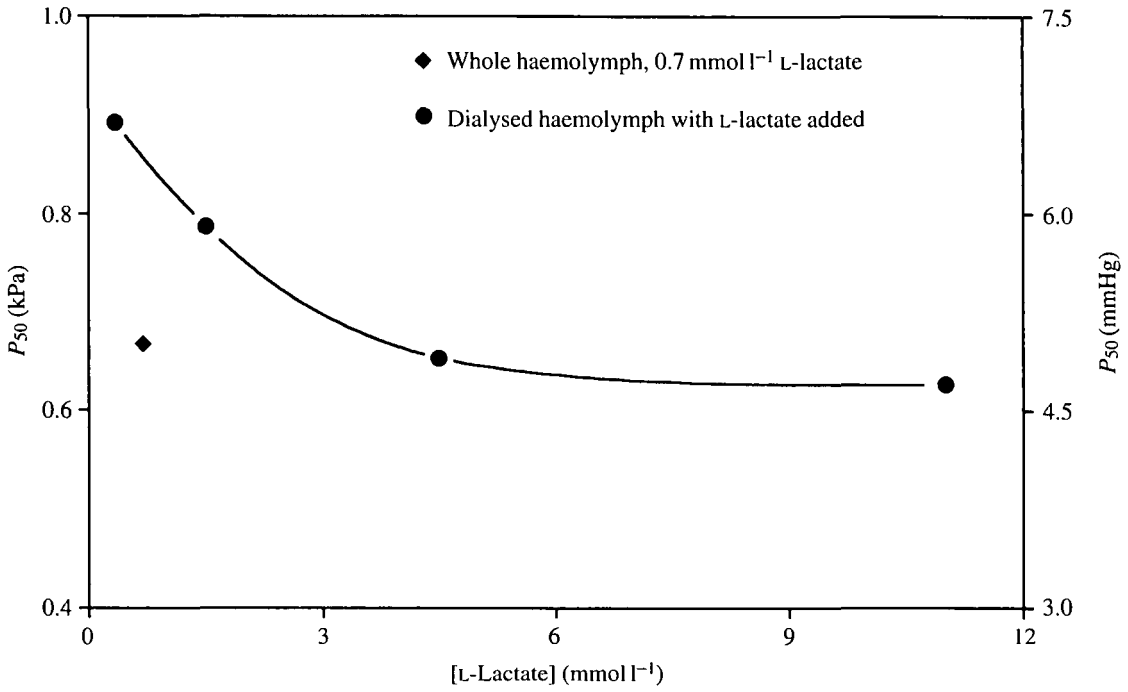


Fig. 2. Effect of L-lactate concentration on haemocyanin oxygen-affinity. The half-saturation points ($P_{50}$, given in mmHg and kPa) are calculated from oxygen binding curves (Fig. 1) at pH 7.99±0.03. $P_{50}$ values are shown in relation to the L-lactate concentrations added to dialysed haemolymph. As [L-lactate] is increased, $P_{50}$ decreases exponentially. The $P_{50}$ of the whole haemolymph containing 0.7 mmol l$^{-1}$ L-lactate is also indicated (◆). $T$=15°C.

haemocyanin was raised by increasing urate concentrations. The $P_{50}$ in the dialysed solution was 0.89 kPa (6.7 mmHg), with 0.17 mmol l$^{-1}$ urate it decreased to 0.72 kPa (5.4 mmHg), with 0.56 mmol l$^{-1}$ urate to 0.53 kPa (4.0 mmHg). A further increase in the urate concentration to 0.93 mmol l$^{-1}$ only led to a slight decrease in $P_{50}$ to 0.52 kPa (3.9 mmHg). As for L-lactate, $P_{50}$ is an exponential function of the urate concentration (Fig. 4). The measured urate effect at pH 7.99±0.03 according to the quotient $(\Delta\log P_{50})(\Delta\log[\text{urate}])^{-1}$ was −0.18 (Table 1D). Whole haemolymph showed a higher oxygen affinity than did dialysed haemolymph with the same urate concentrations (Fig. 4): $\Delta P_{50}$=0.10 kPa (0.8 mmHg).

The dependence of the oxygen affinity on pH was calculated for a constant urate concentration by linear regression, as in the case of L-lactate, giving the Bohr coefficients listed in Table 1C. Urate concentrations exceeding 0.56 mmol l$^{-1}$ led to a significant decrease in the Bohr coefficient ($P$<0.01; Table 1C).

Oxygen binding curves were then constructed for dialysed haemocyanin solutions containing different concentrations of L-lactate and urate at the same

Table 1. *Summary of regression analysis of the data obtained for varying* L*-lactate and urate concentrations at different pH values*

| [L-Lactate] (mmol $l^{-1}$) | Equation of regression line | $r$ | $N$ |
|---|---|---|---|
| A Bohr effect in the presence of L-lactate | | | |
| 0.3 | $\log P_{50}=-1.24pH+10.74$ | −0.97 | 6 |
| 1.5 | $\log P_{50}=-1.22pH+10.55$ | −0.99 | 3 |
| 4.5 | $\log P_{50}=-0.98pH+8.49$ | −0.98 | 4 |
| 11.0 | $\log P_{50}=-0.83pH+7.30$** | −0.99 | 4 |
| B L-Lactate effect at pH 7.99±0.03 calculated from Fig. 1 | | | |
| | $\log P_{50}=-0.11\log$[L-lactate]$-0.1$ | −0.98 | 4 |
| [Urate] (mmol $l^{-1}$) | Equation of regression line | $r$ | $N$ |
| C Bohr effect in the presence of urate | | | |
| 0 | $\log P_{50}=-1.24pH+10.74$ | −0.97 | 6 |
| 0.29 | $\log P_{50}=-1.17pH+9.99$ | −0.99 | 3 |
| 0.56 | $\log P_{50}=-0.83pH+7.24$** | −0.99 | 3 |
| 0.93 | $\log P_{50}=-0.85pH+7.39$* | −0.96 | 3 |
| D Urate effect at pH 7.98±0.03 calculated from Fig. 3 | | | |
| | $\log P_{50}=-0.18\log$[urate]$+0.3$ | −0.98 | 6 |

For the calculation of regression data $P_{50}$ values were used in kPa.

The Bohr effect was analysed in the range of pH from 7.5 to 8.0.

$r$ is the correlation coefficient, $N$ is the number of data points used for the regression lines.

The slope of each regression line was compared to the value for dialysed haemolymph containing 0.3 mmol $l^{-1}$ L-lactate and 0 mmol $l^{-1}$ urate.

The asterisks indicate the results of covariance analyses of the slopes (* $P<0.05$; ** $P<0.01$).

time (Fig. 5). At any given concentration of one effector, the oxygen affinity could still be enhanced by increasing the concentration of the other effector. The lowest half-saturation value of 0.35 kPa (2.6 mmHg) was achieved at the highest L-lactate (11 mmol $l^{-1}$) and urate (0.39 mmol $l^{-1}$) concentrations studied.

To check whether these experimentally determined $P_{50}$ values agree with the hypothesis that the effects of L-lactate and urate are additive, half-saturation values were calculated from the equations given in Table 1B and Table 1D for the combinations of L-lactate and urate concentrations studied. For this calculation the shift in oxygen affinity of the dialysed haemolymph expected for the investigated L-lactate concentration was added to the expected decrease in $P_{50}$ for the studied urate concentration, giving a predicted value of half-saturation ($P_{50(calc)}$) which could be compared to the experimental data ($P_{50(det)}$) of Fig. 5. These differences between the expected and measured values, $P_{50(calc)}-P_{50(det)}$, are given in Table 2. The deviation between the calculated and the determined values averaged −0.02±0.06 kPa (−0.15±0.45 mmHg). Statistical analysis re-

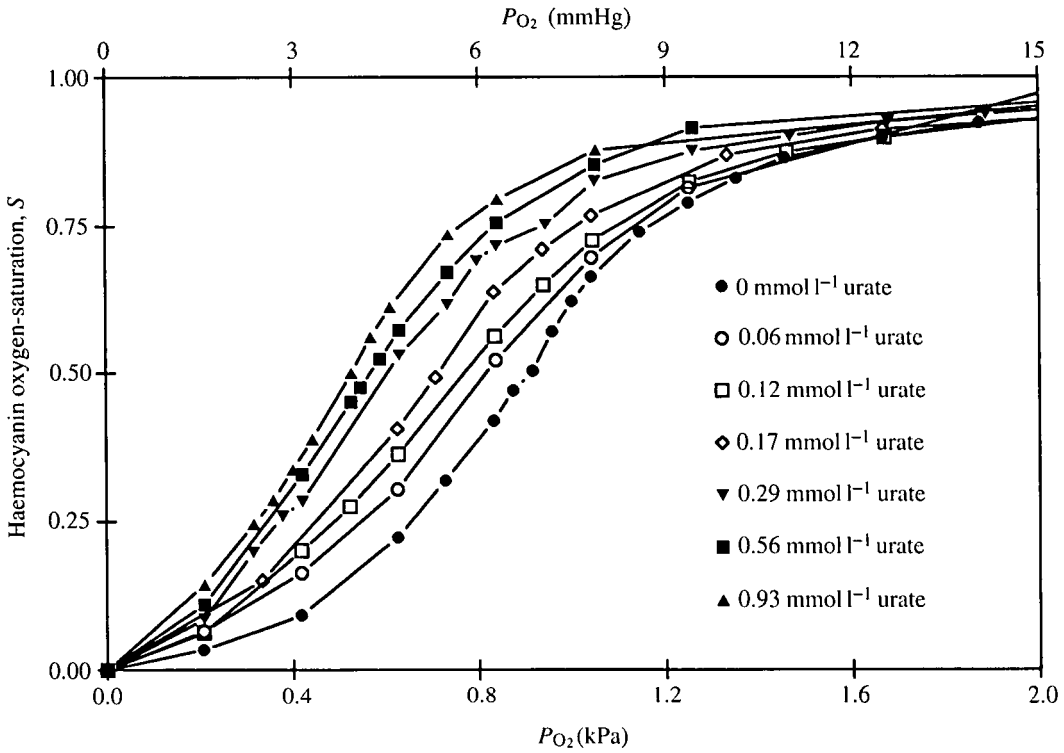


Fig. 3. Effect of urate concentration on haemocyanin oxygen-affinity. The oxygen saturation ($S$) of haemocyanin was measured at different oxygen tensions. Oxygen binding curves were constructed for different urate concentrations, which were achieved with the replacement technique (Bridges *et al.* 1984). The half-saturation point is shifted to lower oxygen tensions in the presence of increasing urate concentrations. $T$=15°C, pH 7.99±0.03.

vealed that the $P_{50}$ values of Fig. 5 did not differ significantly from the calculated values based on the hypothesis that the effects of both modulators are additive.

A similar calculation can be carried out for the oxygen affinity of native haemolymph containing 0.7 mmol $l^{-1}$ L-lactate and 0.08 mmol $l^{-1}$ urate, showing a $P_{50}$ value of 0.67 kPa (5.0 mmHg) at pH 7.99. Dialysis raised the $P_{50}$ to 0.89 kPa (6.7 mmHg). Assuming their effects to be additive, the addition of 0.7 mmol $l^{-1}$ L-lactate and 0.08 mmol $l^{-1}$ urate to the dialysed haemolymph should result in a $P_{50}$ of 0.71 kPa (5.3 mmHg). This value differs by 0.04 kPa (0.3 mmHg) from the $P_{50}$ of native haemolymph. Thus, the combined effect of L-lactate and urate accounts for 82.4 % of the shift in oxygen affinity.

Oxygen binds to the haemocyanin of *Homarus vulgaris* in a cooperative way. The $n_{50}$ values derived by analysis of the binding data in the Hill equation are summarized in Table 3. The maximal cooperativity is achieved in dialysed haemolymph ($n_{50}$=4.1). Addition of either L-lactate or urate or the presence of

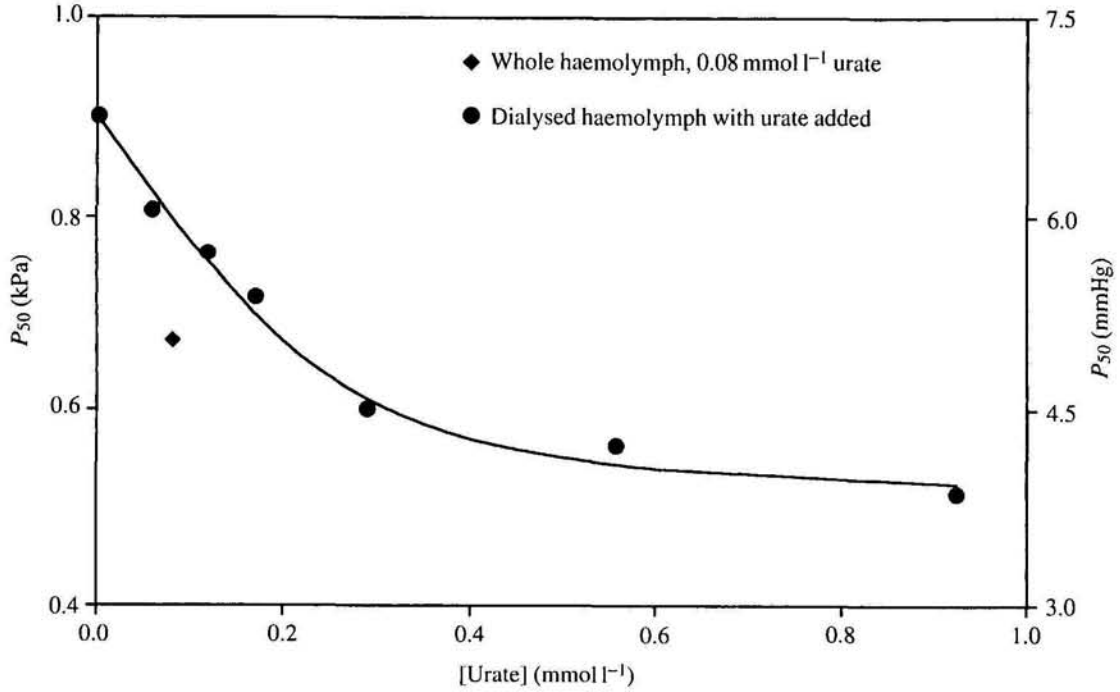


Fig. 4. Effect of urate concentration on haemocyanin oxygen-affinity. $P_{50}$ values (in mmHg and kPa) were estimated according to the urate concentrations in the haemolymph. The half-saturation point of whole haemolymph containing 0.08 mmol $l^{-1}$ urate (◆) is not part of the curve. The higher affinity indicates the presence of other potentiating factors. $T$=15°C, pH 7.99±0.03.

Table 2. *Effect of* L-*lactate amd urate concentrations on haemocyanin oxygen-affinity*

| [L-Lactate] (mmol $l^{-1}$) | [Urate] (mmol $l^{-1}$) | | | | |
|---|---|---|---|---|---|
| | 0.06 | 0.12 | 0.17 | 0.29 | 0.39 |
| 0.5 | ND | 0.02 | ND | 0.01 | ND |
| 1.5 | 0.10 | 0.01 | −0.11 | −0.13 | ND |
| 2.5 | ND | 0.01 | ND | −0.06 | −0.05 |
| 4.5 | 0.07 | −0.07 | −0.09 | ND | 0.01 |
| 9.3 | 0.02 | −0.06 | ND | ND | −0.04 |
| 11.0 | ND | −0.01 | 0.00 | ND | −0.03 |

Experimentally determined $P_{50}$ values from Fig. 5 are compared to expected values calculated from equations B and D in Table 1, assuming the effects of L-lactate and urate to be additive.

$P_{50}$ values are given in kPa (0.133 kPa=1 mmHg) for each combination of L-lactate and urate studied, the difference $P_{50\ (calc)}-P_{50\ (det)}$ is given in the table.

ND indicates that no value was determined for this combination of effector concentrations.

Table 3. *Effect of L-lactate and urate on cooperativity*

A Dependency of cooperativity on L-lactate concentration in the absence of urate

| | [L-Lactate] ($mmol\,l^{-1}$) | | | |
|---|---|---|---|---|
| | 0.3 | 1.5 | 4.5 | 11.0 |
| $n_{50}$ | 4.1 | 2.3 | 2.9 | 2.6 |

B Dependency of cooperativity on urate concentration with a constant L-lactate concentration of $0.3\,mmol\,l^{-1}$

| | [Urate] ($mmol\,l^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 0.06 | 0.12 | 0.17 | 0.29 | 0.56 | 0.93 |
| $n_{50}$ | 4.1 | 3.3 | 2.7 | 3.1 | 2.4 | 2.3 | 2.6 |

C Dependency of cooperativity ($n_{50}$) on the L-lactate and urate concentrations with both modulators present in the solution

| [L-Lactate] ($mmol\,l^{-1}$) | [Urate] ($mmol\,l^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 0.06 | 0.12 | 0.17 | 0.29 | 0.39 |
| 0.3 | 4.1 | 3.3 | 2.7 | 3.1 | 2.4 | ND |
| 0.5 | ND | ND | 3.7 | ND | 3.4 | ND |
| 1.5 | 2.3 | 4.0 | 2.7 | 4.0 | 2.7 | ND |
| 2.5 | ND | ND | 3.0 | ND | 3.0 | 3.1 |
| 4.5 | 2.9 | 2.4 | 2.9 | 2.0 | ND | 2.4 |
| 9.3 | ND | 2.5 | 2.7 | 2.7 | ND | 1.8 |
| 11.0 | 2.6 | ND | 2.9 | ND | ND | 1.7 |

ND indicates that no value was determined for this combination of effector concentrations.
The listed $n_{50}$ values were determined by fitting the data of the oxygen binding curves to the Hill equation between 25 % and 75 % saturation.
In all experiments the pH averaged 7.99±0.03.

both factors reduces cooperativity. The smallest $n_{50}$ value (1.7) was measured in the presence of $0.39\,mmol\,l^{-1}$ urate and $11\,mmol\,l^{-1}$ L-lactate.

## Discussion

### *Concentrations of L-lactate and urate in the haemolymph*

The *in vivo* concentrations of L-lactate shown in the present study are within the range reported by other authors for *Homarus vulgaris* (Phillips *et al.* 1977; Bridges and Brand, 1980; Bouchet and Truchot, 1985). Little information is available concerning *in vivo* concentrations of urate in the haemolymph of this species. The average value of $0.08\pm0.02\,mmol\,l^{-1}$ urate determined in this study is low in comparison with $0.31\,mmol\,l^{-1}$ measured by Morris and Bridges (1986) but agrees

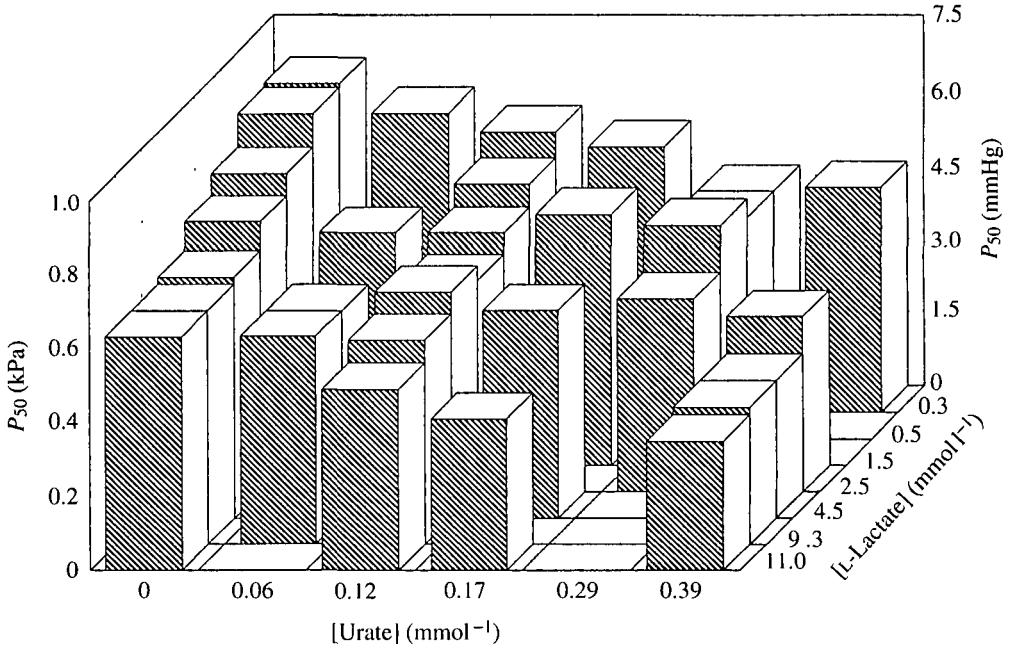


Fig. 5. Interaction of L-lactate and urate. The half-saturation values ($P_{50}$, given in mmHg and kPa) for several L-lactate and urate concentrations are depicted. At a given concentration of L-lactate, the oxygen affinity can still be enhanced by increasing the concentration of urate and *vice versa*. The lowest half-saturation value of 0.35 kPa is achieved at the highest L-lactate and urate concentrations studied. Bars are omitted for the concentration combinations that were not investigated. Note that only the $P_{50}$ axes are to scale. $T$=15°C, pH 7.99±0.03.

well with data for *Astacus leptodactylus* (Czytrich *et al.* 1987; Bridges, 1990), *Carcinus maenas* (Lallier *et al.* 1987) and *Penaeus japonicus* (Lallier and Truchot, 1989*b*).

### *Effect of* L-*lactate*

The effect of L-lactate in increasing the oxygen affinity of crustacean haemocyanin has been established for many species (for a review see Bridges and Morris, 1986). In the present study, the potentiating effect of L-lactate on the oxygen affinity of lobster haemocyanin $(\Delta\log P_{50})(\Delta\log[\text{L-lactate}])^{-1}$ was −0.11 at pH 7.99±0.03. This value is similar to that cited for *Homarus vulgaris* (−0.11 at pH 7.8, −0.19 at pH 7.4) by Bridges *et al.* (1984), whereas Bouchet and Truchot (1985) measured a higher value (−0.16 at pH 7.9) and Taylor and Whiteley (1989) measured a value of −0.18. The influence of L-lactate calculated by different authors implies a moderate potentiating effect on oxygen affinity in *Homarus vulgaris* compared to its effect in other species. The highest reported value of $(\Delta\log P_{50})(\Delta\log[\text{L-lactate}])^{-1}$ is −0.56 at pH 7.8 and 15°C for *Palaemon elegans*

(Bridges *et al.* 1984). The L-lactate effect may, however, be absent in a number of crustaceans (Bridges, 1988).

*Effect of urate*

The calculated value for $(\Delta\log P_{50})(\Delta\log[\text{urate}])^{-1}$ of $-0.18$ at pH $7.99\pm0.03$ and 15°C agrees well with the value of $-0.16$ at pH 7.8 and 15°C calculated from the data of Morris and Bridges (1986) for *Homarus vulgaris*. In other species, slightly higher values are reported, indicating a more pronounced effect of urate on oxygen affinity. In *Carcinus maenas* it amounts to $-0.23$ at pH 7.6 (Lallier and Truchot, 1989*a*) and in *Austropotamobius pallipes* to $-0.39$ at pH 7.8 (Morris *et al.* 1985), both at 15°C. A value of $-0.48$ is reported by Bridges (1990) for *Astacus leptodactylus* at pH 7.8 and 15°C. Spicer and McMahon (1991) report values of $-0.38$ and $-0.23$, respectively, for the amphipods *Apohyale pugettensis* and *Megalorchestia californiana* at 10°C. In *Penaeus japonicus*, however, urate causes only a small increase in haemocyanin oxygen-affinity, $(\Delta\log P_{50})(\Delta\log[\text{urate}])^{-1}$ being $-0.03$ at pH 7.6 and 25°C (Lallier and Truchot, 1989*b*).

*Bohr effect*

For the lobster, haemocyanin oxygen-affinity showed a pronounced dependence on pH, with a Bohr coefficient of $\psi=-1.20$. This value corresponds well with the results of other authors for this species ($\psi=-1.00$, Taylor and Whiteley, 1989; $\psi=-1.17$, Bouchet and Truchot, 1985). Increasing the concentrations of either L-lactate or urate slightly decreases the Bohr coefficient at high modulator concentrations (Table 1A,C). It is not clear, however, whether the Bohr effect changes as a function of the effector concentration. This question requires a more detailed investigation. In *Ocypode saratan* the Bohr effect is dependent on L-lactate concentration (Morris and Bridges, 1985), whereas in *Carcinus maenas* and *Callinectes sapidus* the Bohr effect is independent of the L-lactate concentration (Truchot, 1980; Johnson *et al.* 1984). Previous studies on the urate effect did not show a dependency of the Bohr effect on urate concentration (Morris *et al.* 1985; Lallier *et al.* 1987; Lallier and Truchot, 1989*a*; Bridges, 1990).

*Interaction of* L-*lactate and urate*

The use of dialysed haemolymph with added L-lactate and urate guarantees constant concentrations of other factors which may have influenced haemocyanin oxygen-affinity. Observed changes in oxygen affinity must thus result from the combined effects of L-lactate and urate. Comparison of predicted and measured $P_{50}$ values for various combinations of L-lactate and urate concentrations showed that the values determined were slightly higher than the expected values by 5.2 % (Fig. 5, Table 2). Since the statistical analysis revealed no significant difference between calculated and experimental data, this deviation may be due to experimental errors. Therefore, in the concentration range investigated, the

effects of L-lactate and urate on oxygen affinity of dialysed haemolymph of *Homarus vulgaris* are additive.

The decrease in oxygen affinity brought about by dialysis amounted to 0.22 kPa (1.7 mmHg). The effect on $P_{50}$ due to the native concentrations of both effectors, 0.7 mmol $l^{-1}$ L-lactate and 0.08 mmol $l^{-1}$ urate, is calculated to be 0.19 kPa (1.4 mmHg). Several reasons for the difference of 0.04 kPa (0.3 mmHg) between the experimental and calculated values must be considered. It is possible that low molecular weight factors present in the whole haemolymph other than urate and L-lactate are removed by dialysis. Morris *et al.* (1986*a*) have clearly shown that other purines such as hypoxanthine, adenine and inosine increase oxygen affinity in *Austropotamobius pallipes*. Likewise, dopamine has been shown to increase haemocyanin oxygen-affinity *in vitro* in *Cancer magister* (Morris and McMahon, 1989*a*,*b*).

At L-lactate and urate concentrations exceeding the values measured in undisturbed animals, the oxygen affinity of haemocyanin can be raised *in vitro* beyond the affinity determined in whole haemolymph. At the highest modulator concentrations, a half-saturation value of 0.35 kPa (2.6 mmHg) was achieved, which is 0.32 kPa (2.4 mmHg) lower than the $P_{50}$ determined in whole haemolymph. Even at high L-lactate concentrations, the addition of urate further increases haemocyanin oxygen-affinity and *vice versa*. Yet the increasing effect of L-lactate and urate present in the haemolymph on haemocyanin oxygen-affinity must be limited, since the effect of each single modulator is progressively reduced at high concentrations (Figs 2, 4, 5).

Some representative curves were analysed in more detail using modified Scatchard plots (Edsall *et al.* 1954; Imai, 1973; Savel-Niemann *et al.* 1988; Morris and Bridges, 1989). For this purpose, the oxygen binding to haemocyanin can be described by the quotient $Q$, which is given by the following equation:

$$Q = \frac{S}{(1-S)P} = \frac{K_1 P + 2K_1K_2 P^2 + \ldots + 12K_1K_2\ldots K_{12} P^{11}}{12 + 11K_1 P + 10K_1K_2 P^2 + \ldots + K_1K_2\ldots K_{11} P^{11}},$$

with $S$ being the fractional saturation of haemocyanin and $P$ being the oxygen tension. $K_1$–$K_{12}$ are the intrinsic oxygen affinity constants for each oxygen binding step to haemocyanin. As the oxygen tension reaches zero or infinity the saturation becomes $S=0$ or $S=1$, respectively. For these cases, the limiting values $limQ$ are $K_1$ and $K_{12}$, respectively. According to the MWC model for haemoglobin (Monod *et al.* 1965), the deoxyhaemocyanin is in the low-affinity (T) state, corresponding to $K_1$. As a result of oxygenation, the conformation of haemocyanin changes to the high-affinity (R) state, corresponding to $K_{12}$. In a plot of $\log Q$ *vs* $S$ (Fig. 6A,B) the values of $K_{T,ass}$ and $K_{R,ass}$ can be estimated with regard to the interpolated data at minimal and maximal saturation (Table 4). Although this method cannot provide exact values for all affinity constants, it is possible to estimate the effect of urate and L-lactate on $K_{T,ass}$ and $K_{R,ass}$. While the changes in $K_{R,ass}$ are small and not clearly correlated with the effector concentration, the effect on $K_{T,ass}$ is apparent (Table 4). The affinity of haemocyanin in the T state is increased in the presence of

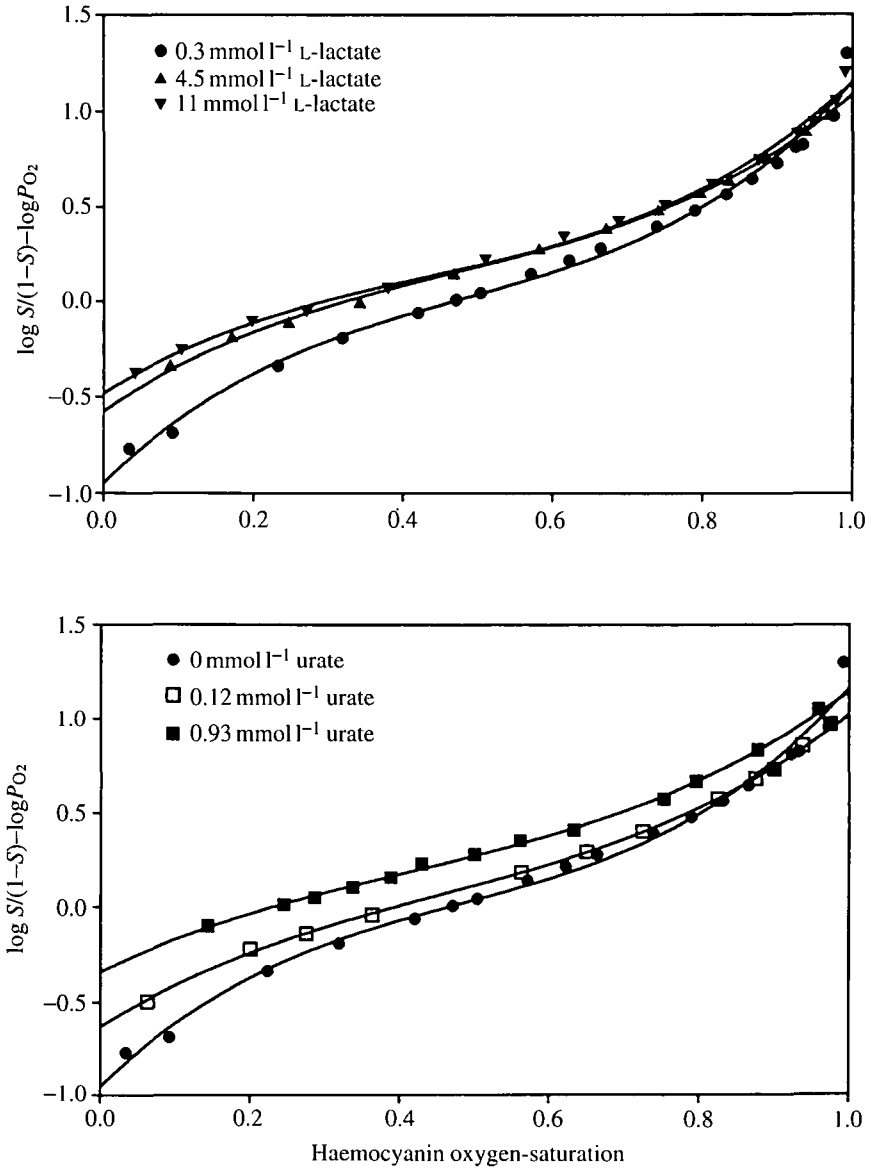


Fig. 6. Modified Scatchard plots at various L-lactate (A) and urate concentrations (B) used for the graphical determination of $K_{T,ass}$ and $K_{R,ass}$ for the binding of oxygen to haemocyanin. The data were calculated from the interpolated curves to $S=0$ and $S=1$. The derived values are given in Table 4. $T=15$°C, pH 7.99±0.03, $P_{O_2}$ is given in kPa.

both effectors. This must be concluded from the raised $K_{T,ass}$ values. So the potentiating effect of L-lactate and urate on oxygen affinity can be interpreted as a change in affinity of the T state or as a shift in the T/R equilibrium, or both (Morris and Bridges, 1989).

Table 4. *Affinity constants* $K_{T,ass}$ *and* $K_{R,ass}$ *derived from modified Scatchard plots (Fig. 6) measured at pH 7.99±0.03*

| | $K_{T,ass}$ | $K_{R,ass}$ |
|---|---|---|
| [L-Lactate] (mmol $l^{-1}$) | | |
| 0.3 | $1.1\times10^{-4}$ | $2.0\times10^{-2}$ |
| 4.5 | $3.1\times10^{-4}$ | $1.0\times10^{-2}$ |
| 11.0 | $3.1\times10^{-4}$ | $1.6\times10^{-2}$ |
| [Urate] (mmol $l^{-1}$) | | |
| 0 | $1.1\times10^{-4}$ | $2.0\times10^{-2}$ |
| 0.12 | $2.2\times10^{-4}$ | $1.1\times10^{-2}$ |
| 0.93 | $4.4\times10^{-4}$ | $1.4\times10^{-2}$ |

$K_{T,ass}$ and $K_{R,ass}$ are given in $kPa^{-1}$.

### *Cooperativity*

In the presence of L-lactate and urate, the $n_{50}$ values decrease markedly at the same time as the oxygen affinity increases. Morris and Bridges (1989) suggest that this is the case when $K_{T,ass}$ is affected to a greater extent than $K_{R,ass}$ by the studied modulators. This is true in the present investigation. Cooperativity has been explained by the MWC model (Monod *et al.* 1965) as a symmetrical conformational change of the entire protein, with the binding of the first ligand facilitating the binding of further ligand molecules. According to this model, the maximal cooperativity is determined by the number of binding sites for a ligand on the protein molecule. So, in the case of the dodecameric haemocyanin, the maximal $n_{50}$ possible for oxygen binding is 12. The highest value achieved here, however, was 4.1, which is far below the maximum. Moreover, the presence of both L-lactate and urate in a haemocyanin solution decreased cooperativity to a greater extent than each effector was able to do alone (Table 3). These results suggest that the binding of one ligand does not affect the entire haemocyanin molecule, but only a limited environment adjacent to the ligand binding site. This asymmetry is not compatible with the MWC model. For this reason, several authors have extended this model for large protein molecules composed of many subunits; the nesting model (Decker *et al.* 1986) and the model of interacting cooperative units (Brouwer and Serigstad, 1989) are perhaps better suited to explain the binding properties and cooperativity characteristics of haemocyanin.

### *Physiological importance of L-lactate and urate effects*

While the potentiating effects of L-lactate and urate on haemocyanin oxygen-affinity have been confirmed by several *in vitro* investigations, little information is available on the influence of these factors on the animal's physiology. To serve as true modulators *in vivo*, their concentration in the haemolymph must vary with different physiological conditions.

Exercise increases L-lactate concentrations in the haemolymph, as was shown

for *Callinectes sapidus* (Booth *et al.* 1982) and for *Cancer magister* (Graham *et al.* 1983). L-Lactate concentrations are also reported to increase in hypoxic situations in *Homarus vulgaris* at the critical $P_{O_2}$ (Bridges and Brand, 1980; Bouchet and Truchot, 1985; Taylor and Whiteley, 1989). With this increase in L-lactate concentration, a concomitant reduction occurs in $P_{50}$. Hypoxia also leads to elevated urate concentrations in *Astacus leptodactylus* (Czytrich *et al.* 1987), *Carcinus maenas* (Lallier *et al.* 1987) and *Penaeus japonicus* (Lallier, 1988) because the urate concentration in the haemolymph is dependent upon the available oxygen, since the urate oxidising enzyme (uricase) requires molecular oxygen (Mahler, 1963). In contrast to L-lactate formation, urate metabolism is limited by moderate hypoxia, resulting in an accumulation of urate in the haemolymph. L-Lactate, which is the anaerobic end product in Crustacea (Chang and O'Connor, 1983; Gäde and Grieshaber, 1986), accumulates during severe hypoxic situations (Bouchet and Truchot, 1985) and exercise (Booth *et al.* 1982; Graham *et al.* 1983), when the energy metabolism is switched to anaerobic pathways. Thus, a shortage of oxygen leads to an initial increase in urate concentration followed by an increase in L-lactate concentration as hypoxia becomes more severe. Both factors, in turn, elevate the oxygen affinity of haemocyanin. This feedback may increase the ability to take up oxygen in the gills and enhance the amount of oxygen transported in the haemolymph.

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