Marshall, G. R., and Merrifield, R. B. (1965), *Biochemistry 4*, 2394.
Medzihradszky-Schweiger, H. (1962), *Acta Chim. Acad. Sci. Hung. 34*, 213.
Meienhofer, J. (1962), *Chimia 16*, 385.
Merrifield, R. B. (1963), *J. Am. Chem. Soc. 85*, 2149.
Merrifield, R. B. (1964), *J. Am. Chem. Soc. 86*, 304.
Merrifield, R. B. (1967), *Recent Progr. Hormone Res. 23*, 451.
Ondetti, M. A., Narayanan, V., von Saltza, M., Sheehan, J. T., Sabo, E. F., and Bodanszky, M. (1968a), *J. Am. Chem. Soc. 90*, 4711.
Ondetti, M. A., Sheehan, J. T., and Bodanszky, M. (1968b), *in* Pharmacology of Hormonal Polypeptides and Proteins, New York, N. Y., Plenum, p 18.
Schröder, E., and Klieger, E. (1964), *Ann. 673*, 208.
Schwyzer, R., Iselin, B., Kappeler, H., Riniker, B., Rittel W., and Zuber, H. (1963), *Helv. Chim. Acta, 46*, 1975.
Shalitin, Y., and Bernhard, S. A. (1966), *J. Am. Chem. Soc. 88*, 4711.
Sondheimer, E., and Holley, R. W. (1954), *J. Am. Chem. Soc. 76*, 2467.
Swallow, D. L., and Abraham, E. P. (1958), *Biochem. J. 70*, 364.
Werum, L. N., Gordon, L. T., Thornburg, W. (1960), *J. Chromatog. 3*, 125.
Wieland, Th., and Mohr, H. (1956), *Ann. 599*, 222.
Woodward, R. B., Olofson, R. A., and Mayer, H. (1961), *J. Am. Chem. Soc. 83*, 1010.

An Examination of the Polymerization Behavior of *Jasus lalandii* Haemocyanin and Its Relation to the Allosteric Binding of Oxygen*

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ABSTRACT: *Jasus lalandii* haemocyanin in solution is shown by ultracentrifuge studies to undergo a pH-dependent polymerization. In acetate buffer of pH 5.5 and ionic strength of 0.1, the protein exists essentially as a form of mol wt 455,000, characterized by a weight-average sedimentation coefficient of 15 S; but as the pH is increased this unit slowly dissociates to a series of lower polymers in equilibrium. Plots of the amount of oxygen bound to holohaemocyanin *vs.* the partial pressure of unbound oxygen (binding curves) are sigmoidal, the extent of sigmoidality varying with pH in the range 5.5–8.9. It is shown that results obtained on the polymerization behavior of both holo- and apohaemocyanins are consistent with the postulate that polymerization of the protein is the basis of these observed allosteric binding effects. The regeneration of holohaemocyanin by the combination of apoprotein with added cuprous chloride is shown to be inhibited by silver ions. It, therefore, appears that these ions bind at the oxygen binding site. While cupric and magnesium ions do not inhibit the regeneration of haemocyanin, their addition to haemocyanin solutions at alkaline pH values results in a shift of the polymerization equilibria in favor of the formation of the 15S species. Inhibition studies performed with mixtures of these divalent metal ions and silver ions indicate that haemocyanin in the presence of divalent metal ions is capable of existing in various forms, all of the same size, which exhibit a differential capacity toward the binding of silver ions. These observations are employed to interpret oxygen binding curves obtained with the haemocyanin in the presence of cupric and magnesium ions in terms of the coexistence of various isomeric forms of the protein. Results obtained with *Jasus* serum further suggest that the allosteric binding of oxygen to haemocyanin, operating *in vivo*, is affected by the metal ion content of the serum.

Pantin and Hogben (1925) showed that oxygen binding curves obtained with *Palinurus* haemocyanin were sigmoidal and similar curves have been obtained with haemocyanins from other sources (Redfield, 1934; Wolvekamp, 1949; Redmond, 1955). Recently, considerable attention has been given to the physical basis of sigmoidal binding curves, because they manifest an allosteric effect important in metabolic control (Wyman, 1964; Atkinson, 1966; Stadtman, 1966; Changeux *et al.*, 1968; Gerhart and Schachman, 1968; Changeux and Rubin, 1968). It has been shown that multiple binding of ligand to equivalent sites on isomeric and/or polymeric forms of protein acceptor molecules leads to sigmoidal binding curves, provided the phenomena of binding and self-interaction of acceptor are competitive (Nichol *et al.*, 1967). Koshland *et al.* (1966) interpreted the sigmoi-

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dal binding curves obtained with the oxygen–mammalian haemoglobin system in terms of isomeric forms of the protein. Benesch *et al.* (1965, 1966) proposed a detailed mechanism of interconversion between the isomeric forms, which included dissociation and hybridization steps. On the other hand, rapidly attained equilibria between polymeric forms of lamprey eel haemoglobin and preferential binding of oxygen to the monomer appear to be the basis of allosteric effects observed with this system (Briehl, 1963). Although the same form of binding equation, derived on a thermodynamic basis, describes binding of a ligand to an acceptor undergoing self-interaction, the nature of the latter reaction (an isomerization, polymerization, or reaction of the type A + B $\rightleftharpoons$ C) appears to vary with different systems.

A common property exhibited by all haemocyanins, so far examined in this respect, is their ability to exist in solution in a variety of polymeric forms, the extent of polymerization depending upon environmental factors, such as pH and ionic strength (Brohult, 1940). Several workers have commented upon the possible relation between sigmoidal binding curves observed with different types of haemocyanin and the known polymerization reactions of the proteins (*e.g.*, Larimer and Riggs, 1964; Johnston *et al.*, 1967). The present study attempts to examine the validity of the postulated relationship for the particular system, oxygen–*Jasus lalandii* haemocyanin. First, the polymerization behavior of the protein is characterized in a variety of experimental environments. Secondly, the relative effects of certain metal ions on the extent of polymerization and on the degree of regeneration of the haemocyanin from its apoprotein (Ghiretti, 1956) are examined. Thirdly, the effects of metal ions on the above factors are correlated with their effect on oxygen binding curves obtained with the protein.

Experimental Section

Protein Preparations. Blood was collected from the ventral sinus of *J. lalandii* and allowed to clot. The clot was frozen, allowed to thaw, and the serum was collected by filtration. The serum was then dialyzed exhaustively against water, after which the haemocyanin was crystallized by dialysis against buffer of pH 4.6, ionic strength 0.015 (0.005 M sodium acetate, 0.005 M acetic acid, and 0.01 M sodium chloride). The water used in all experiments was glass distilled and then passed through a Chelex 100 ion-exchange resin. The crystals were dissolved in a 1% (w/v) sodium bicarbonate solution and recrystallized by reiterating the above dialysis procedure. The light absorption spectrum of the oxygenated protein was measured with a Cary Model 14 spectrophotometer and exhibited absorption maxima at 280, 338, and 550 mμ. The results are typical of those obtained with most other haemocyanins (Ghiretti, 1962). In general, after three recrystallizations the sample was characterized by an extinction ratio, E_{280}/E_{338}, of 5.4 and a constant copper content of 0.176% (w/w). Analysis for copper was performed by the method of Klotz and Klotz (1955), employing 2,2′-biquinolyl. Samples of haemocyanin not used immediately were stored under toluene at 4°; the E_{280}/E_{338} ratio remained unchanged after 3-months storage.

Apohaemocyanin was prepared by reacting recrystallized haemocyanin in 0.1 M diethyl barbiturate buffer of pH 8.5 with 0.1 M sodium cyanide. The cyanide was added until the solution was colorless and the pH was maintained at a value just less than 9.0 by the addition of 0.2 M acetic acid. When the addition of cyanide was completed, the pH was lowered to 7.0 by the cautious addition of more acetic acid. The excess reagents and the copper cyanide complex were removed by dialysis against two changes of 0.05 M phosphate buffer of pH 7.0 and finally against two changes of distilled water. The entire procedure was performed at 1°. It was found that even after the addition of excess sodium cyanide (>100 moles of cyanide/mole of initial copper), ~7% (w/w) of the initial copper bound was retained. Ghiretti-Magaldi and Nardi (1963) and Lontie *et al.* (1965) have reported similar findings, with the residual copper ranging from 7 to 15% (w/w). The absorption spectrum of apohaemocyanin proved to be identical with that of deoxyhaemocyanin and exhibited only one absorption maximum at 280 mμ in the range 250–600 mμ.

Regeneration of the haemocyanin was effected in the following way. The apoprotein was dissolved in 0.1 M phosphate buffer (pH 7.0) and deoxygenated by bubbling with nitrogen. Cuprous chloride was then added as a saturated solution. The solution was allowed to stand for 1 hr at 0°, and the excess copper was oxidized to the cupric form with oxygen and complexed with an excess of EDTA. After exhaustive dialysis against water, the holoprotein was precipitated by dialysis against the acetate buffer of pH 4.6, ionic strength 0.015. The regenerated form was characterized by the same values of the E_{280}/E_{338} ratio and copper content as the original holoprotein.

Analysis of Serum. A freeze-dried sample of *J. lalandii* serum was arced in a Hilger medium quartz spectrograph. The result revealed that sodium, magnesium, and calcium were the only ions present in a large amount. Separate samples of sera from different crayfish were used to determine quantitatively the magnesium and calcium ion concentrations, employing an atomic absorption spectrophotometer (Tectron Model AAS). Each sample contained 1×10^{-2} M magnesium ions and 1.2×10^{-2} M calcium ions. Analysis of a recrystallized sample of holohaemocyanin by the same technique revealed only trace amounts of magnesium ions ($\sim 4 \times 10^{-6}$ M).

Ultracentrifugation. Sedimentation velocity experiments were performed in a Spinco E ultracentrifuge at 50,740 rpm and 20°. Prior to sedimentation, all samples were dialyzed against the appropriate buffer for 3 days in the cold and allowed to equilibrate with the buffer at 20° for at least a further 14 hr. In experiments where metal ions were added to dialyzed solutions prior to sedimentation velocity analysis, a further 2-hr standing was allowed after the addition of metal ions. The schlieren patterns obtained were measured with a two-coordinate comparator (Nikon Model 6C), fitted with a projection screen and accurate to 2×10^{-4} cm. The resulting data were used to calculate the rate of movement of the square root of the second moment of schlieren

peaks, and hence the weight-average sedimentation coefficients, s, pertaining to 20° in the buffer employed (Goldberg, 1953). Areas under peaks were found by trapezoidal integration, corrected for radial dilution (Svedberg and Pedersen, 1940), and related to protein concentration by assuming a specific refractive increment of 1.8×10^{-3} dl g^{-1} (Perlmann and Longsworth, 1948). In some instances, the data from the plates were also used to perform a boundary analysis by the method of Fujita (1956), described in detail by Baldwin (1957). The method permits not only a test for homogeneity in terms of molecular size; but also an estimation of the apparent diffusion coefficient.

The Archibald method (Archibald, 1947) was used to evaluate the weight-average molecular weight of haemocyanin in the environments specified in the text. The selected value of the angular velocity was 7445 rpm and the experiments were conducted at 20°, a temperature controlled with the RTIC unit. Four photographs were taken within the 60-min duration of the experiment and values of the observed refractive index gradient at the meniscus, $(dn/dx)_m$, were employed to calculate the required ratio, $(dn/dx)_m/x_m c_m \omega^2$, for each exposure (where x_m is the distance from the center of rotation, c_m is the concentration at the meniscus in appropriate refractometric units, and ω is the angular velocity). Evaluation of c_m from eq 9 of Klainer and Kegeles (1955) requires the refractometric determination of the initial concentration. This was obtained by accelerating the rotor to 50,740 rpm after the initial photographs had been taken and performing a trapezoidal integration on the peak observed in sedimentation velocity (Nichol and Roy, 1965). A value of 0.73 for the partial specific volume of the protein was assumed in all molecular weight calculations.

Oxygen Binding Curves. The method used to obtain oxygen binding data was essentially that described by Pantin and Hogben (1925). Extinction values due to oxyhaemocyanin were obtained at different air pressures, measured with a mercury manometer. At each pressure, the protein solution was gently shaken in a constant-temperature water bath at 20° to accelerate the attainment of equilibrium, judged by the constancy of the observed extinction. The parameter, per cent oxygen bound, was obtained by dividing the extinction observed at a particular pressure by that at atmospheric pressure and multiplying the ratio by 100. The procedure ensures that the maximum value of the parameter at atmospheric pressure is identical (100%) for all concentrations of protein investigated and assumes that the measured extinction is directly proportional to the amount of oxygen bound at all pressures. Plots of the per cent oxygen bound *vs.* the corresponding partial oxygen pressure measured by the manometer are termed binding curves.

Results

Ultracentrifuge Data Relating to the Polymerization. Oxyhaemocyanin was dissolved in borate buffer (pH 8.9), approximate ionic strength 0.1 (0.01 M sodium tetraborate, 0.01 M boric acid, and 0.07 M sodium chloride), and subjected to sedimentation velocity analysis. The result is shown in Figure 1a, which reveals a major peak, characterized by a sedimentation coefficient of ~5 S and a small amount of ~15S material. Dialysis of aliquots of the haemocyanin solution against the following buffers, all of ionic strength 0.1, was employed to lower the pH of the original solution: pH 8.7 (0.014 M sodium tetraborate, 0.036 M boric acid, and 0.06 M sodium chloride), pH 8.0 (0.02 M sodium diethylbarbiturate, 0.01 M diethylbarbituric acid, and 0.08 M sodium chloride), and pH 5.5 (0.07 M sodium acetate, 0.01 M acetic acid, and 0.03 M sodium chloride). Sedimentation velocity results obtained with each of these solutions are shown in Figure 1b–d. It is clear that the relative proportion of the faster sedimenting material (15 S) increases progressively as the pH is decreased. The effect was shown by sedimentation velocity analysis to be reversible on increasing the pH value from 5.5 to 8.9 and then decreasing the value again to pH 5.5. The same result was obtained with three different samples of oxyhaemocyanin. It could also be noted from Figure 1a–c that the refractive index gradient falls to zero between the 5S and 15S peaks. This suggests that the equilibrium reaction between the species constituting the two peaks is slowly attained in comparison with the difference in migration rates of the individual species (Longsworth and MacInnes, 1942; Nichol *et al.*, 1964). The hypothesis is supported by the data summarized in Figure 2, which shows the time dependence of the increase of the relative area of the 5S peak, following the rapid adjustment of the pH value from 5.5 to 8.7 of an 0.7% haemocyanin solution.

Further examination was made of the 15S material, existing alone in acetate buffer (pH 5.5) and ionic strength 0.1 (Figure 1d), by employing the Archibald procedure to evaluate the weight-average molecular weight. Values obtained using different protein concentrations, shown in parentheses, were 465,000 (0.68%), 430,000 (0.53%), and 470,000 (0.26%). In each case, values of $(dn/dx)_m/x_m c_m \omega^2$ were independent of time, showing that the 15S material was essentially homogeneous with respect to molecular size. Accordingly, the molecular weight of this form of haemocyanin was taken as the arithmetic average of the above values, 455,000. The value is in excellent agreement with that of 450,000 reported by Joubert (1954) on the basis of light-scattering measurements on *Jasus* haemocyanin at pH 5.5, ionic strength 0.1. Estimates of the apparent diffusion coefficient (and hence apparent molecular weight) of the 15S species sedimenting in the presence of 5S material were obtained by analyses of the boundary shapes (Fujita, 1956; Baldwin, 1957) of the faster sedimenting peaks shown in Figure 1b,c. In each case, the calculated apparent diffusion coefficients proved to be time independent, suggesting that only one type of species constituted the 15S peak. For the solutions of pH 8.7 and 8.0, the apparent molecular weight values obtained were 500,000 and 480,000, respectively. It should be stressed that these values are approximate, for any modification of the boundary form induced by the operation of a Johnston–Ogston effect (1946) has been ignored. Moreover, it was assumed that the dependence of the sedimentation coefficient of the 15S peak upon *total* protein concentration was appro-

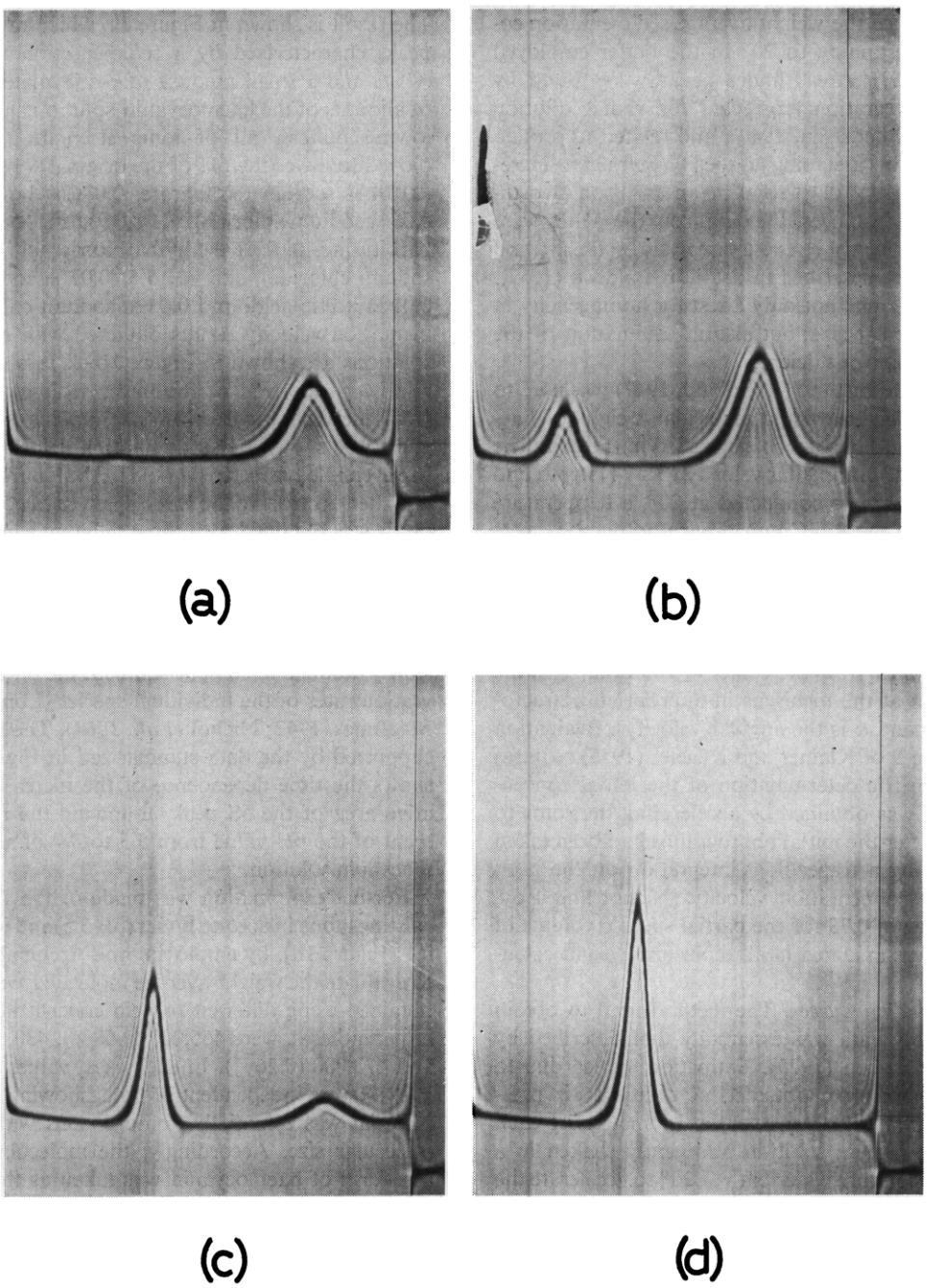

FIGURE 1: Sedimentation velocity patterns of *J. lalandii* holohaemocyanin in buffers of ionic strength 0.1 at 20°. The angular velocity was 50,740 rpm and the bar angle 70°. Sedimentation is from right to left. Protein concentration in parentheses: (a) pH 8.9 (0.43%), (b) pH 8.7 (0.82%), (c) pH 8.0 (0.63%), and (d) pH 5.5 (0.70%).

priate to the reiterative calculation (Baldwin, 1957). The latter dependence was obtained by sedimenting five different concentrations of protein (c, g/100 ml) at both pH values and was described for both cases by the linear relation, $\bar{s} = 15.6(1 - 0.09c)$S.

The nature of the slow-sedimenting peak evident in Figure 1a–c was also subjected to further examination, by determining values of $\bar{s}$ at a series of proteins concentrations. The results, relevant to pH values of 8.9 (●), 8.7 (x), and 8.0 (○), are presented in Figure 3, where the abscissa axis refers to the concentration of the material sedimenting alone as the slower peak. It is clear that at corresponding concentrations, values of $\bar{s}$ progressively increase as the pH is decreased. Moreover, at pH 8.0 (○) the curve exhibits an initial positive slope, which is characteristic of systems comprising a series of polymers in equilibrium (*e.g.*, Schwert, 1949; Nichol *et al.*, 1964). Thus, it is possible that at pH 8.9, where almost all 15S material has dissociated (Figure 1a), the slower sedimenting peak corresponds *essentially* to monomer, characterized by a linear concentration dependence, $\bar{s} = 5.2(1 - 0.15c)$ S. As the pH is decreased, reassociation to 15S material proceeds together with an increase in relative amounts of lower polymers (higher

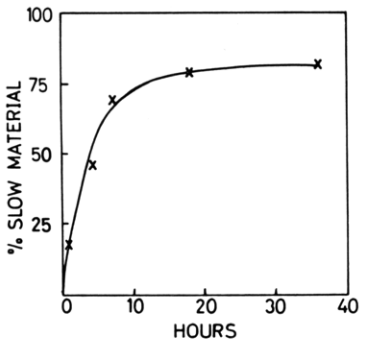

FIGURE 2: The time dependence of the dissociation of *J. lalandii* holohaemocyanin in borate buffer (pH 8.7) at 20°. The total protein concentration was 0.7% and the extent of dissociation is shown as the amount of slow-sedimenting material expressed as a per cent of the total.

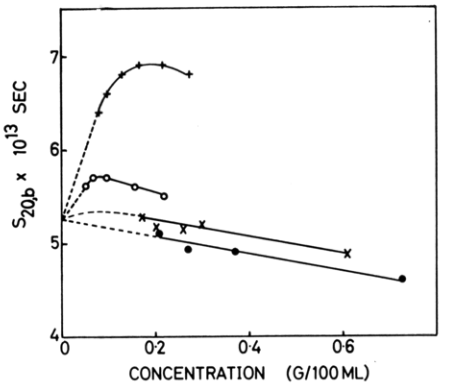

FIGURE 3: The concentration dependence of the weight-average sedmentation coefficient, $\bar{s}$, of the slow-sedimenting species present in solutions of haemocyanin. Solid curves refer to holohaemocyanin in buffers of pH values 8.9 (●), 8.7 (×), and 8.0 (○), and to apohaemocyanin at pH 8.0 (+). The broken lines represent attempts to extrapolate the data to infinite dilution.

than the monomer), which are associated with the slower sedimenting peak. The results of attempted extrapolations of data shown in Figure 3 (---) indicate that the sedimentation coefficient of monomer is approximately 5.2 S. Since the slower sedimenting peaks evident in Figure 1 are possibly reaction boundaries (Longsworth, 1959; Gilbert, 1959), no attempt was made to analyze their shape. In an attempt to evaluate the molecular weight of monomer, haemocyanin solutions in sodium carbonate–bicarbonate buffer (pH 11.0) and ionic strength 0.1 were examined by the Archibald procedure. Values of 86,000 and 90,000 were obtained for solutions of concentration 0.7 and 0.35%, respectively. In the chosen environment of pH 11.0 sedimentation velocity analysis revealed a symmetrical peak of 4S and no 15S material.

A 0.6% solution of apohaemocyanin in diethyl barbiturate buffer (pH 8.0) and ionic strength 0.1 was subjected to sedimentation velocity analysis and the result is shown in Figure 4. The concentration dependence of $\bar{s}$, referring to the faster sedimenting peak, was found to be described by the same relation given previously for the corresponding peak observed with holohaemocyanin samples. Application of the method of Fujita, showed that the 15S material was homogeneous and had mol wt 440,000. The dependence of $\bar{s}$ of the slower sedimenting peak on its concentration is given in Figure 3 (+), from which it is clear that the initial slope, $d\bar{s}/dc$, is positive. Accordingly, the boundary must be regarded as a reaction boundary, representing gradients in refractive index of various polymeric forms. The broken line in Figure 3 represents an attempt to extrapolate the data to infinite dilution and suggests that in common with holohaemocyanin, the monomeric form of apoprotein is characterized by a sedimentation coefficient of ~5.2 S.

Metal Ion Binding and Sedimentation Studies. The effect on the sedimentation pattern of the addition of 1.5×10^{-2} M magnesium chloride to a 0.7% solution of holohaemocyanin in borate buffer (pH 8.7) is shown in Figure 5a. Comparison of this figure with Figure 1b, obtained under the same conditions in the absence of added magnesium ions, shows that the ions promote formation of the 455,000 unit. (The effect was not due to the increase in ionic strength caused by the addition of magnesium chloride; for in a separate experiment it was shown that the extent of association was not affected by increasing the ionic strength to 0.5 with sodium chloride.) Results similar to those shown in Figure 5a were also obtained with apohaemocyanin in the presence of 1.5×10^{-2} M $MgCl_2$. The effect of the addition of divalent copper ions (cupric acetate) on the extent of association of apohaemocyanin is illustrated in Figure 5b–d. Figure 5b refers to a 0.7% solution of apohaemocyanin in 0.025 M phosphate buffer (pH 7.0). The experiment provides an additional observation of the effect of

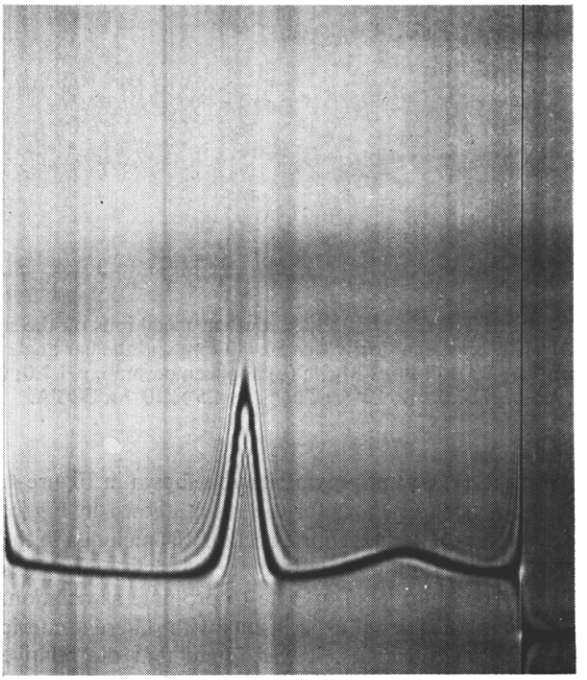

FIGURE 4: Sedimentation velocity pattern of *J. lalandii* apohaemocyanin (0.6%) in diethyl barbiturate buffer (pH 8.0) and ionic strength 0.1 at 20°. The angular velocity was 50,740 rpm and the bar angle was 70°. Sedimentation is from right to left.

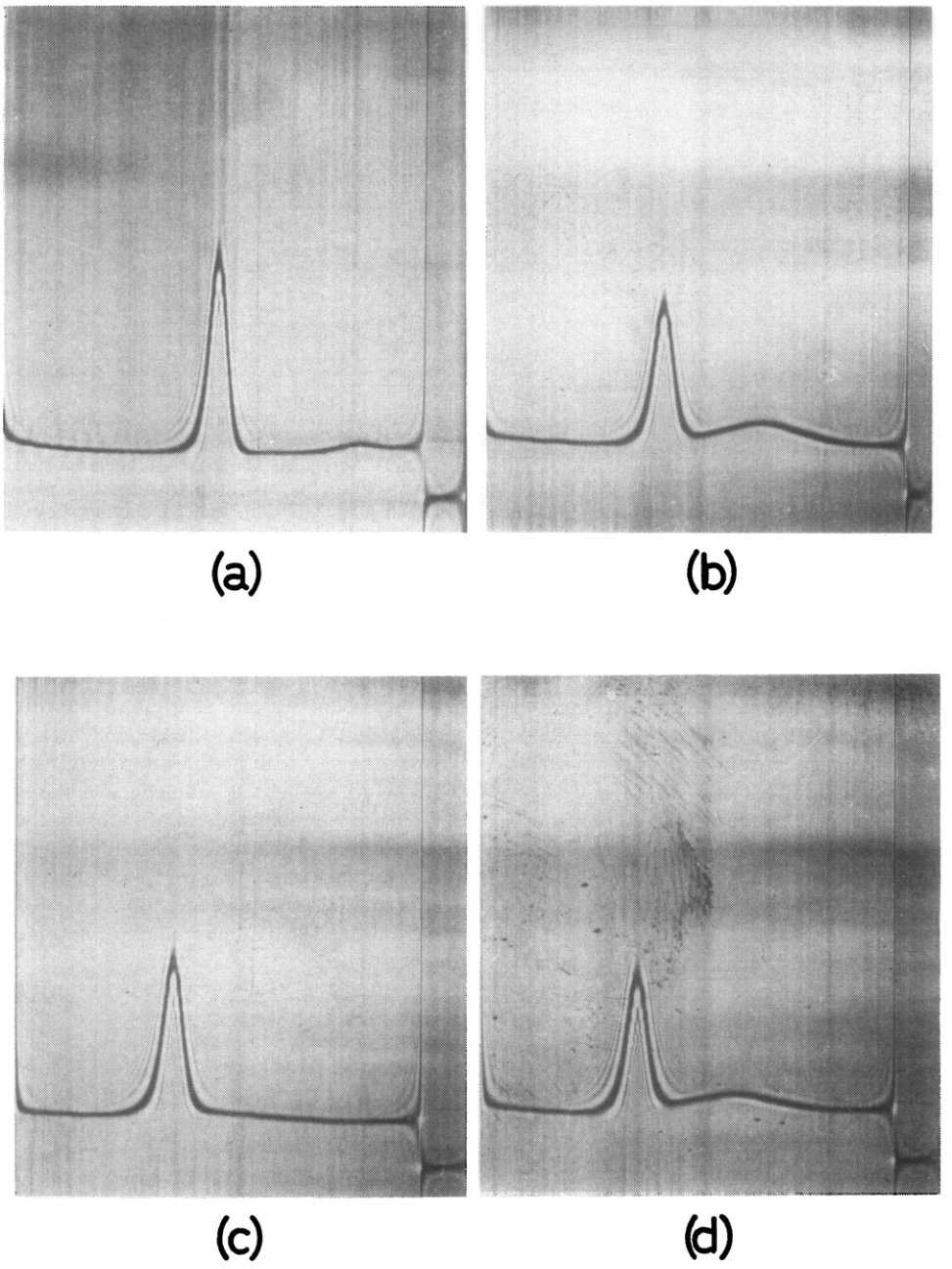

FIGURE 5: The effects of added divalent metal ions on the sedimentation velocity patterns of *J. lalandii* haemocyanin (0.7%) at 20°. The angular velocity was 50,740 rpm and the bar angle was 70°. Sedimentation is from right to left. (a) Holohaemocyanin, pH 8.7, 1.5×10^{-2} M Mg^{2+}; (b) apohaemocyanin, pH 7.0; (c) apohaemocyanin, pH 7.0, 6×10^{-4} M Cu^{2+}; and (d) apohaemocyanin, pH 7.0, 6×10^{-4} M Cu^{2+} plus 4.5×10^{-3} M EDTA.

pH on the extent of polymerization shown in Figure 4. It also acts as a control for the result shown in Figure 5c, where 6×10^{-4} M cupric acetate was included in the specified environment. It is observed that cupric ions (like magnesium ions) operate to promote association. Indeed, in additional experiments with different cupric acetate concentrations, it was found that the relative amounts of slow- and fast-sedimenting material could be controlled. Figure 5d shows that the addition of 4.5×10^{-3} M EDTA after the addition of 6×10^{-4} M cupric acetate reverses the effect caused by the latter reagent. The addition of EDTA of the same concentration to solutions of holohaemocyanin at pH values of 5.5, 8.0, 8.7, and 8.9 was without effect. Sedimentation patterns were similar to those shown in Figure 1. In contrast to the effects of the divalent cations on the extent of polymerization, it was found that the addition of 1.5×10^{-4} M silver acetate 2 hr prior to sedimentation velocity analysis had no effect on sedimentation patterns obtained with either apo- or holohaemocyanin at pH 7.0. However, introduction of 6×10^{-4} M cupric ions following the addition of the silver acetate resulted in one peak, sedimenting with an s of 15 S.

The effects were studied of the presence of magnesium,

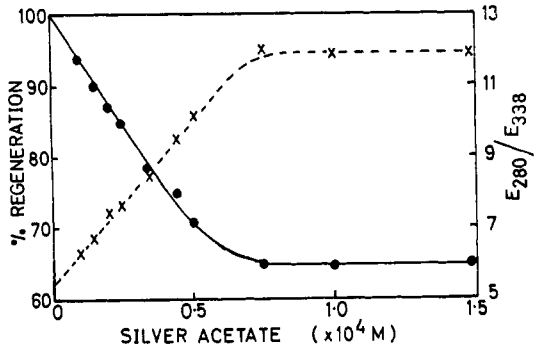

FIGURE 6: The effect of added silver ions on the regeneration of *J. lalandii* haemocyanin. The solid line averages data pertaining to the extent of the reaction measured as per cent regeneration (left-hand scale). The broken line averages data measured as the ratio E_{280}/E_{338} (right-hand scale).

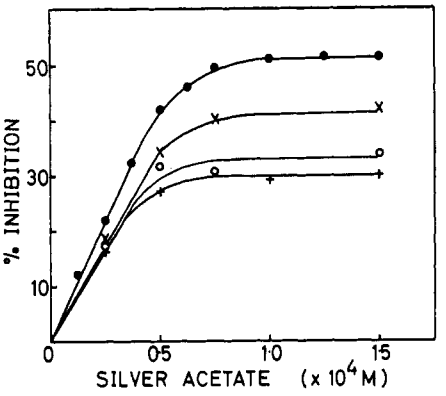

FIGURE 7: The effect of added silver ions on the per cent inhibition of regeneration of *J. lalandii* haemocyanin (0.7%) (pH 7.0) in the presence of cupric acetate of the following concentrations: 6×10^{-4} M (+), 4.5×10^{-4} M (○), 3×10^{-4} M (×), and 0 M (●).

cupric, and silver ions on the regeneration of haemocyanin. In these experiments, the metal ions were added to the protein prior to the addition of excess cuprous chloride and, as described in the Experimental Section, the regenerated protein was isolated free of metal ions not bound at the oxygen binding site. In this connection, it could be noted that EDTA suffices to complex any bound cupric ions (Figure 5d). The extinction ratio, E_{280}/E_{338}, found with the regenerated protein provides a measure of the copper bound at the oxygen binding site. Moreover, copper analyses were used to determine the per cent regeneration, defined as the ratio of the amount of copper bound in the presence of metal ions (Mg^{2+}, Cu^{2+}, or Ag^{+}) to that bound in their absence, multiplied by 100 (% inhibition = 100 − % regeneration). It was found in a separate series of experiments, conducted at pH 7.0, that magnesium chloride ($0–3 \times 10^{-2}$ M) and cupric acetate ($0–6 \times 10^{-4}$ M) did not affect regeneration, *i.e.*, 0% inhibition was found. The results of regeneration studies in the presence of silver ions are shown in Figure 6, in which the final concentration of silver ions (silver acetate) is shown as a function of the E_{280}/E_{338} ratio (broken line) and per cent regeneration (solid line). As the concentration of silver ions increases, the E_{280}/E_{338} ratio increases steadily due to a reduction in the extinction at 338 mμ. This reflects a decreasing copper content at the oxygen binding site. The ratio reaches a constant plateau value commencing at a point corresponding to a silver ion concentration of 7.5×10^{-5} M. Approximately the same concentration corresponds to the onset of the plateau region evident in the per cent regeneration curve. The reasonable inference may be drawn that silver ions bind at or near the binding site of cuprous ions (and hence of oxygen). It was also found that mercurous chloride inhibited the regeneration of apohaemocyanin to the same extent as silver acetate; but that methylmercuric iodide and *p*-mercuribenzoate did not inhibit regeneration.

It is evident from Figure 6 that, even in the presence of $>7.5 \times 10^{-5}$ M Ag^{+}, a maximum value of 36% inhibition was observed. With other samples of apoprotein, the per cent inhibition was found to range between 30 and 50%; but in all cases a plateau value was reached independent of silver ion concentration. If equilibria existed between bound and unbound Ag^{+} and all apoprotein were available for binding, the occurrence of such plateaux would be difficult to explain. An alternative postulate is that less than ~50% of apoprotein is available for the binding reaction involving Ag^{+}. In this connection, it is noteworthy that the sample showing 36% inhibition in Figure 6 contained 34% slower sedimenting material. Moreover, per cent inhibition by Ag^{+} was shown to decrease as the pH was lowered from 8.0 to 5.5. Values for the per cent inhibition were 45% (pH 8.0), 36% (pH 7.0), 10% (pH 6.0), and 0% (pH 5.5). Over the same pH range 100% regeneration of apohaemocyanin with cuprous ions was achieved in the absence of Ag^{+}. As we have seen in Figure 1, the relative amounts of slower sedimenting material also decreased as the pH was lowered over this range until at pH 5.5 (where 0% inhibition by Ag^{+} was observed) the 455,000 unit predominates (Figure 1d). The tentative conclusion is drawn that Ag^{+} binds less readily (if at all) to the 455,000 unit; but that no such differentiation exists with regard to the binding of cuprous ions. If this were the case, it might be expected that at pH 7.0, the per cent inhibition by Ag^{+} would decrease as the relative amount of faster sedimenting material increased. The latter could be achieved by adding various amounts of Cu^{2+} or Mg^{2+} to the apoprotein, prior to the addition of silver ions. The results of an experiment of this design are shown in Figure 7, from which it is clear that per cent inhibition (in plateaux regions) does decrease as the initial cupric content increases. On the other hand, the plateau value of per cent inhibition proved to be independent of magnesium ion concentration ($0–3 \times 10^{-2}$ M), when magnesium chloride was added to the apoprotein at pH 7.0, prior to the addition of Ag^{+}.

Oxygen Binding Curves. Typical oxygen binding curves found at 20° with holohaemocyanin (2.5%) dissolved in buffer of ionic strength 0.1 and pH values 8.0 (---), 6.5 (●), and 5.5 (-·-·-) are shown in Figure 8a. In each case, the curves are sigmoidal in that the slopes of the tangents to the curves at low oxygen pressures increase with oxygen pressure. In addition, it is seen that the curves approach differently the same maximum limiting value of

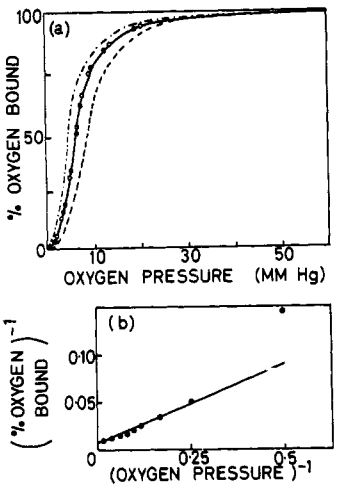

FIGURE 8: Oxygen binding curves of *J. lalandii* holohaemocyanin (2.5%) at 20° as a function of pH. (a) A direct plot of data: (-·-·-) pH 5.5, (- - -) pH 8.0, and (——) pH 6.5 with (●) no EDTA and (○) 1.5×10^{-2} M EDTA. (b) A double-reciprocal plot of data obtained at pH 8.9.

the ordinate. It was found experimentally that the amount of oxygen bound by each sample was the same at atmospheric pressure, regardless of the experimental environment. The addition of 1.5×10^{-2} M EDTA to the protein prior to oxygen binding studies had no effect on the binding curve obtained at pH 6.5 (○). Similar data obtained with holohaemocyanin at pH 8.9 (also in the absence of added metal ions) is plotted in double-reciprocal form in Figure 8b. The points appear to describe a curve concave to the abscissa, which suggests that the corresponding binding curve may exhibit slight sigmoidality (Nichol *et al.*, 1967). It could be noted, however, that points obtained at large values of reciprocal oxygen pressure are subject to the greatest experimental error.

The effect of divalent metal ions on the binding curves obtained at 20° is illustrated in Figure 9. When 6×10^{-4} M cupric ions were added to a 2.5% holohaemocyanin solution at pH 6.5, the resultant curve (—) appears to be of the form of a rectangular hyperbola. A double-reciprocal plot of the data (not shown) is concave to the abscissa axis, but the departure from linearity is even less than shown in Figure 8b. On the other hand, additions of 1.5×10^{-2} M magnesium ions to 2.5% solutions of protein at pH values of 8.0, 6.5, and 5.5 result in binding curves (all described by - - - in Figure 9), which are clearly sigmoidal. Comparison of the latter curve with those shown in Figure 8a, relevant to the same pH values, shows that the introduction of magnesium ions under these conditions results in a decrease in the amount of oxygen bound at all pressures, below atmospheric. Finally, the binding curve obtained with *J. lalandii* serum (-·- - of Figure 9) is clearly more sigmoidal than any obtained with mixtures of recrystallized haemocyanin and divalent metal ions.

Discussion

The basic hypothesis which emerges from studies on the polymerization behavior of *Jasus lalandii* haemocyanin is that at alkaline pH values the protein dissociates in a relatively slow reaction from a homogeneous 15S pentamer or hexamer ($n = 5$ or 6) of mol wt 455,000 to form monomer (90,000), which coexists in equilibrium with dimer and perhaps trimer and tetramer. The hypothesis will now be discussed in the light of all available evidence. The results of Archibald experiments at pH 5.5 and boundary analyses of the 15S peak evident at pH 8.0 and 8.7 (Figure 1) serve to demonstrate that an essentially homogeneous unit of mol wt 455,000 exists in each environment studied. The estimation that this unit is a pentamer rests on the determination of the monomer molecular weight (90,000) found at pH 11.0, where no 15S material was evident in sedimentation velocity patterns. However, an alternative value of $n = 6$ is indicated from the copper content of 0.176% (w/w) on the basis that each base-mole of haemocyanin contains two copper atoms associated with one oxygen binding site (Redfield *et al.*, 1928a,b; Guillemet and Gosselin, 1932; Rawlinson, 1940). Certainly, the value of $n = 6$ cannot be excluded on the basis of the Archibald data, since no account was taken in molecular weight determinations of a possible pH dependence of partial specific volume of the protein (Van Holde and Cohen, 1964).

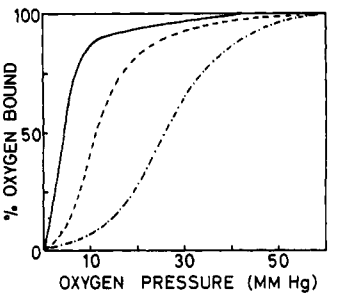

FIGURE 9: The effect of metal ions on the oxygen binding curves of *J. lalandii* holohaemocyanin at 20°. (——) 2.5% holohaemocyanin, pH 6.5, 6×10^{-4} M Cu^{2+}. (- - -) 2.5% holohaemocyanin, pH values of 5.5, 6.5, and 8.0, 1.5×10^{-2} M Mg^{2+}. (-·-·-·-) *Jasus* serum, pH 7.2.

The reversibility of the 15S → 5S transition induced by changing the pH was demonstrated with several different samples of holohaemocyanin. In addition, the data summarized in Figure 2 show that reequilibration (in the direction of dissociation) at a particular pH value proceeds relatively slowly. From Figure 1a–c it is clear that 5S material may exist alone without noticeable *reassociation* to the 15S material within the duration of the sedimentation velocity experiments (~1 hr). Even in prolonged experiments of 3-hr duration at low angular velocity the refractive index gradient between the 5S and 15S peaks remained at zero. In addition to the relation between 5S and 15S material, it is proposed that the 5S peak is a reaction boundary, representing gradients in refractive index of various polymeric forms in equilibrium. The postulate is based on the observation that the initial slopes, ds/dc, of curves ○ and x of Figure 3 are positive. It also provides a direct explanation of the effect of increasing the pH on the curves shown in Figure 3 for holohaemocyanin, especially as such an increase results in dissociation of the 455,000

unit (Figure 1). Attempts at curve fitting the data summarized in Figure 3 (○) for holohaemocyanin at pH 8.0, according to eq 2, 3, and 5 of Nichol *et al.* (1964), led to the following result for the model of best fit. Monomeric and dimeric forms of holohaemocyanin constitute the 5S reaction boundary, the apparent association constant increasing systematically from 0.2 to 0.4 l. per g with increasing concentration of 5S material. This observed concentration dependence may be due to the coexistence of small amounts of other polymers (trimer and tetramer) in equilibrium with monomer and dimer. A more detailed characterization of the 5S system would involve the evaluation of successive equilibrium constants, obtainable only from precise weight-average molecular weight data over a wide concentration range (Steiner, 1952; Rao and Kegeles, 1958). Such data, relating only to the 5S system, are unavailable at pH 8.0 since 15S material also exists in this environment. The postulate that the 5S peak is a reaction boundary is of interest, since it suggests that classification of subunits of haemocyanin using sedimentation coefficients alone is an unwise practice (Eriksson-Quensel and Svedberg, 1936; Schramm and Berger, 1952). Also, it provides an explanation of the observation from Figure 1a–c that the 5S peak is asymmetric on the leading edge and shows no tendency to resolve into separate peaks. Numerical examples, calculated on the basis of equations presented by Gilbert (1959) employing parameters relevant to this study, indicate that the behavior could be attributed to the operation of rapidly established equilibria between monomer and polymers (lower than the pentamer).

The possibility will now be examined that the polymerization reactions of holohaemocyanin, discussed above, are involved in the allosteric effects observed in Figure 8, which presents oxygen binding curves of holohaemocyanin in the absence of added metal ions. The addition of 1.5×10^{-2} M EDTA to the protein had no effect on the binding curve obtained at pH 6.5 (*cf.* ● and ○ of Figure 8). Likewise, the reagent had no effect on the sedimentation velocity patterns obtained with holohaemocyanin in the pH range 5.5–8.9. It appears unlikely, therefore, that the allosteric effects could be attributed to an effect induced by divalent metal ion contaminants. In this connection, only $\sim 4 \times 10^{-6}$ M Mg^{2+} was detected in the purified holohaemocyanin samples. At pH 8.9, the data in Figures 1a and 3 (●) show that oxygenated haemocyanin exists essentially in the form of monomer; but that relatively small amounts of higher polymers (including the 455,000 unit) may also coexist. These observations are consistent with the finding that the oxygen binding curve found at pH 8.9 deviates little in form from that of a rectangular hyperbola (Figure 8b). The results are essentially described by eq 9 of Nichol *et al.* (1967), with $p = 1$, where p is the number of oxygen binding sites per 90,000 unit. As the pH is decreased from 8.9 to 8.0, the relative amounts of higher polymers increase. The increase is not only in the amount of 455,000 unit (Figure 1), but also in the amounts of dimer and (perhaps) trimer and tetramer (Figure 3). A decrease in pH from 8.9 to 8.0 also results in the binding curve becoming more sigmoidal (Figure 8a (---)). It appears unlikely that an equilibrium between 5S and 15S species can be invoked to explain these findings, since each experiment leading to a sigmoidal binding curve (Figure 8) was completed in 1 hr and yet reequilibration (either association or dissociation) between these species is evidently a slow process (Figures 1a–c and 2). The same objection does not apply, however, to the polymerization reactions between species constituting the 5S *reaction* boundary; for, as we have seen, these equilibria may be rapidly established. This in no way implies that the 455,000 unit is incapable of binding oxygen. Indeed at pH 5.5, where this form predominates, 100% regeneration of apoprotein was achieved. Thus, each curve in Figure 8 must be regarded as the sum of a curve due to oxygen binding to the 455,000 unit (a rectangular hyperbola, if all sites are equivalent except for a statistical effect) and a sigmoidal curve due to oxygen binding to lower polymeric forms. This would result in sigmoidality becoming more pronounced as the latter effect predominated, *i.e.*, as the relative amount of 455,000 unit decreased with an increase of pH. The prediction is consistent with the findings in Figure 8a where values of the slopes of tangents to the curves at low oxygen pressure increase less rapidly the higher the pH value.

It is also of interest to note that comparison of + and ○ of Figure 3 shows that the concentration dependence of s values, pertaining to the 5S peak and measured at pH 8.0 and ionic strength 0.1, is different for oxygenated holohaemocyanin and deoxygenated apohaemocyanin. The difference indicates that monomer is favored in the oxygenated form. Indeed, it is evident on comparison of Figures 1c and 4 that the slower moving boundary in the sample of apoprotein (Figure 4) is more diffuse and approaches the faster sedimenting boundary, which from boundary analysis results appears to represent the 455,000 unit. Although no information is available on rates of reequilibration between polymeric forms of apohaemocyanin, it is clear that the *relative amounts* of lower polymeric forms appears to depend upon the amount of oxygen bound. This is in accord with the findings of Nichol *et al.* (1967) that binding of ligand to polymeric forms of acceptor will produce an allosteric effect only if the phenomena of polymerization and binding are competitive.

Whereas allosteric effects observed with recrystallized holohaemocyanin in the absence of metal ions find a rational explanation (albeit qualitative) on the basis of polymerization reactions, it appears that a different interpretation must be given results obtained in the presence of divalent metal ions (Figure 9). The addition of 1.5×10^{-2} M Mg^{2+} or 6×10^{-4} M Cu^{2+} is associated with essentially comple e conversion of the haemocyanin into material, characterized by the same s value of 15 S observed with the 455,000 unit of holohaemocyanin in the absence of metal ions (Figures 1 and 5). Since neither divalent metal ion inhibits the regeneration of apohaemocyanin (*i.e.*, 100% regeneration was achieved in their presence), it is clear that neither ion acts at the oxygen binding site. At first sight, therefore, it appears that in the presence of Mg^{2+} or Cu^{2+} the binding curve might be a rectangular hyperbola, describing the multiple bind-

ing of oxygen to equivalent sites on a single form (455,000) of holohaemocyanin. The solid curve in Figure 9 obtained in the presence of 6×10^{-4} M Cu^{2+} approaches this form; but it is clear from --- of Figure 9 that the addition of 1.5×10^{-2} M Mg^{2+} results in a markedly sigmoidal binding curve. A difference in the effects of added Mg^{2+} and Cu^{2+} is also observed in the results pertaining to the inhibition of regeneration of apohaemocyanin (Figures 6 and 7). In all experiments employing Ag^{+} alone, it was found that a plateau value of per cent inhibition was attained. In Figure 6 the value was 36%, but with other samples of haemocyanin studied under identical experimental conditions the value ranged from 30 to 50%. This variation possibly finds explanation in the varying amounts of residual cuprous ion retained in the apohaemocyanin (*cf.* Experimental Section), but also may reflect differences in the relative concentrations of genetic variants of the protein in the samples studied. It was found, however, with all samples studied that the plateau values of per cent inhibition by Ag^{+} depended upon the concentration of added Cu^{2+} (Figure 7) but not on the concentration of added Mg^{2+} ($0–3 \times 10^{-2}$ M). These results, together with those in Figure 9, strongly suggest that the 15S form of haemocyanin induced by the addition of Cu^{2+} differs from that induced by Mg^{2+} in their abilities to bind Ag^{+} and oxygen at the oxygen binding site.

In this study three possible isomeric forms of the 15S material seem relevant: Cu^{2+}-induced isomer, Mg^{2+}-induced isomer, and preexisting isomer (the 455,000 unit formed as in Figure 1d in the absence of added metal ions). The latter isomer appears not to bind Ag^{+}, since 0% inhibition by Ag^{+} of the regeneration was found at pH 5.5 where the 455,000 unit predominates (Figure 1d) and at pH 7 the value of per cent inhibition (36%) corresponded approximately to the per cent slow-sedimenting material (34%). With regard to the Cu^{2+}-induced isomer, it is seen in Figure 7 that the per cent inhibition decreases (*i.e.*, less Ag^{+} is bound) as the Cu^{2+} concentration increases (*i.e.*, as the proportion of 455,000 unit increases). However, with the highest cupric content (6.0×10^{-4} M), where essentially only the 455,000 unit exists (Figure 5c), a value of 27% inhibition was observed in the plateau region of Figure 7, suggesting that Cu^{2+}-induced isomer may bind Ag^{+} in contrast to the behavior of the preexisting isomer. It would also follow that Mg^{2+}-induced isomer is the most efficient with regard to Ag^{+} binding, since addition of Mg^{2+} does not affect per cent inhibition by Ag^{+}, but does promote association (Figure 5a). These conclusions are tentative; but it is of interest that the indicated order of efficiency with regard Ag^{+} binding at the oxygen binding site (Mg^{2+}-induced isomer > Cu^{2+}- induced isomer > preexisting isomer) parallel the order of magnitudes of intrinsic binding constants (describing the binding of oxygen to the isomers) predicted on the basis of results shown in Figure 9. Thus, it follows from eq 7 and 10 of Nichol *et al.* (1967) (with $n = 1$ and $p = q$) that sigmoidality of binding curves becomes more pronounced as the values of intrinsic binding constants diverge in magnitude. If these values were close for Cu^{2+}-induced and preexisting isomers the curve (—) in Figure 9 would find a rational explanation. Also, curve --- of Figure 9, which is pronouncedly sigmoidal, suggests that the oxygen binding capacities of Mg^{2+}-induced and preexisting isomers are quite different.

Curve -·-·- of Figure 9 shows that the most pronounced allosteric binding effect was obtained with serum. The serum composition (pH 7.2, 4–8% haemocyanin, 1×10^{-2} M Mg^{2+}, and 1.2×10^{-2} M Ca^{2+}) is conducive to the formation of isomeric forms of mol wt 455,000. In this connection, Brohult (1940) has shown that the addition of calcium ions to *Helix* haemocyanin induces polymerization. The interesting point arises that the allosteric binding of oxygen to haemocyanin, operating *in vivo*, may be governed by the metal ion content in the serum, which determines the relative amounts of isomeric forms induced by the metal ions. The effect on binding curves of varying the relative amounts of isomeric forms of acceptor has been illustrated for a simple isomerizing system in Figure 1a of Monod *et al.* (1965).

In conclusion, it appears that the allosteric effects observed with *J. lalandii* haemocyanin have their physical basis in both polymerization and isomerization reactions of the protein. It is of interest that the same thermodynamic approach suffices to describe both situations in macroscopic terms and that a binding equation may be written to include both effects (Nichol *et al.*, 1967). It is hoped that the present work may provide a basis for the evaluation of the physical parameters included in the binding equation; for then a quantitative interpretation of the sigmoidal binding curves would be possible.

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References

Archibald, W. J. (1947), *J. Phys. Chem. 51*, 1204.
Atkinson, D. E. (1966), *Ann. Rev. Biochem. 35*, 85.
Baldwin, R. L. (1957), *Biochem. J. 65*, 503.
Benesch, R., Benesch, R. E., and Tyuma, I. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1268.
Benesch, R. E., Benesch, R., and MacDuff, G. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 535.
Briehl, R. W. (1963), *J. Biol. Chem. 238*, 2361.
Brohult, S. (1940), *Nova Acta Regiae Soc. Sci. Upsaliensis 12*, 1.
Changeux, J.-P., Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7*, 531.
Changeux, J.-P., and Rubin, M. M. (1968), *Biochemistry 7*, 553.
Eriksson-Quensel, I., and Svedberg, T. (1936), *Biol. Bull. 71*, 498.
Fujita, H. (1956), *J. Chem. Phys. 24*, 1084.
Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7*, 538.
Ghiretti, F. (1956), *Arch. Biochem. Biophys. 63*, 165.
Ghiretti, F. (1962), *in* Oxygenases, Hayaishi, O., Ed., New York, N. Y., Academic, p 517.
Ghiretti-Magaldi, A., and Nardi, G. (1963), *in* Protides

of the Biological Fluids, Vol. II, Peeters, H., Ed., p 507.
Gilbert, G. A. (1959), *Proc. Roy. Soc. (London) A250*, 377.
Goldberg, R. J. (1953), *J. Phys. Chem. 57*, 194.
Guillemet, R., and Gosselin, G. (1932), *Compt. Rend. Soc. Biol. 111*, 733.
Johnston, J. P., and Ogston, A. G. (1946), *Trans. Faraday Soc. 42*, 789.
Johnston, W., James, T. W., and Barber, A. A. (1967), *Comp. Biochem. Physiol. 22*, 261.
Joubert, F. J. (1954), *Biochim. Biophys. Acta 14*, 127.
Klainer, S. M., and Kegeles, G. (1955), *J. Phys. Chem. 59*, 952.
Klotz, I. M., and Klotz, T. A. (1955), *Science 121*, 477.
Koshland, D. E., Némethy, G., and Filmer, D. (1966), *Biochemistry 5*, 365.
Larimer, J. L., and Riggs, A. F. (1964), *Comp. Biochem. Physiol. 13*, 35.
Longsworth, L. G. (1959), *in* Electrophoresis, Bier, M., Ed., New York, N. Y., Academic, p 91.
Longsworth, L. G., and MacInnes, D. A. (1942), *J. Gen. Physiol. 25*, 507.
Lontie, R., Blaton, V., Albert, M., and Peeters, B. (1965), *Arch. Intern. Physiol. 73*, 150.
Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.
Nichol, L. W., Bethune, J. L., Kegeles, G., and Hess, E. L. (1964), *Proteins 2*, 305.
Nichol, L. W., Jackson, W. J. H., and Winzor, D. J. (1967), *Biochemistry 6*, 2449.
Nichol, L. W., and Roy, A. B. (1965), *Biochemistry 4*, 386.
Pantin, C. F. A., and Hogben, L. T. (1925), *J. Marine Biol. Assoc. 13*, 970.
Perlmann, G. E., and Longsworth, L. G. (1948), *J. Am. Chem. Soc. 70*, 2719.
Rao, M. S. N., and Kegeles, G. (1958), *J. Am. Chem. Soc. 80*, 5724.
Rawlinson, W. A. (1940), *Australian J. Exptl. Biol. Med. Sci. 18*, 131.
Redfield, A. C. (1934), *Cambridge Phil. Soc. Biol. Rev. Proc. 9*, 175.
Redfield, A. C., Coolidge, T., and Montgomery, H. (1928a), *J. Biol. Chem. 76*, 197.
Redfield, A. C., Coolidge, T., and Shotts, M. A. (1928b), *J. Biol. Chem. 76*, 185.
Redmond, J. R. (1955), *J. Cellular Comp. Physiol. 46*, 209.
Schramm, G., and Berger, G. (1952), *Z. Naturforsch. 7b*, 284.
Schwert, G. W. (1949), *J. Biol. Chem. 179*, 655.
Stadtman, E. R. (1966), *Advan. Enzymol. 28*, 41.
Steiner, R. F. (1952), *Arch. Biochem. Biophys. 39*, 333.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, Oxford, Oxford University.
Van Holde, K. E., and Cohen, L. B. (1964), *Biochemistry 3*, 1803.
Wyman, J. (1964), *Advan. Protein Chem. 19*, 223.
Wolvekamp, H. P. (1949), *in* Haemoglobin, Roughton, F. J. W., and Kendrew, J. C., Ed., London, Butterworths Scientific Publications, p 301.