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MODELS FOR THE ANALYTICAL ULTRACENTRIFUGE BEHAVIOR OF *HELIX POMATIA* α-HEMOCYANIN

I. EXPERIMENTAL TESTING OF PREVIOUS MODELS

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The 'microheterogeneity model' (R.J. Siezen and R. van Driel, Biochim. Biophys. Acta 295 (1973) 131) and the 'incompetent whole molecule model' (G. Kegeles, Arch. Biochem. Biophys. 180 (1977) 530) for the dissociation of *Helix pomatia* α-hemocyanin whole molecules to half molecules were tested experimentally, using ultracentrifugation and stopped-flow dilution analysis. Results of differential sedimentation experiments followed by stopped flow analysis of separated fractions of 60 S and 100 S molecules were not entirely as predicted by the incompetent model, the agreement depending on the pH and ionic strength of analysis. A considerable amount of stopped-flow dilution response could be attributed to material sedimenting between 60 and 100 S. This material appears to be the main equilibrating fraction, and its amount is considerably larger than that predicted by the microheterogeneity model. Increased hydrostatic pressure was found to enhance this fraction, whereas fixation or low ionic strength reduced or eliminated this fraction. Nonequilibrium components of 30, 50 and 80 S were detected and partially purified by differential sedimentation.

1. Introduction

The sedimentation behavior of α-hemocyanin of *Helix pomatia*, the Roman orchard snail, has usually been described by assuming that the intact whole molecules (100 S) are not in equilibrium with their dissociated species, halves (60 S) and tenths (20 S). The α-hemocyanin fraction constitutes about 75–80% of total hemocyanin, and is characterized by its ability to dissociate into half molecules in acetate buffer (pH 5.7) in the presence of 1 M NaCl [1].

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The sedimentation behavior of α-hemocyanin under a variety of conditions has been described in several publications [2–5]. Siezen and Van Driel [3] demonstrated unequivocally the presence of ionic charge microheterogeneity and its correlation with fractionation by differential sedimentation, through reversible boundary spreading in electrophoresis. They also demonstrated that, even though pure samples contained only whole molecules and their dissociation products, moving boundaries of whole and half molecules invariably separated in analytical ultracentrifugation, which should not occur, according to Gilbert [6], as long as reequilibration is rapid. Siezen and Van Driel also showed that when the pH is changed, a shift in molecular

weight as detected by turbidity takes place, in a time range too fast to follow in hand-mixing experiments. Similar rapid molecular weight changes resulting from hand-mixing changes of ionic strength were reported [5]. An estimate based on calculations corresponding to the case of lobster hemocyanin, but having broader generality, indicates that partial separation in ultracentrifugation caused by slow reaction kinetics would not be expected in a time range much smaller than 60 s [7].

In addition, Siezen and Van Driel demonstrated that isolated fractions corresponding to portions of the ultracentrifuge patterns, when reexamined, reproduced only those portions of these patterns. They remained stable over long periods of time, instead of reequilibrating and reproducing the original ultracentrifuge behavior of the original unfractioned samples. One further puzzling phenomenon described by several authors [2–5] is the presence of a raised baseline between the 60 S and 100 S peaks in the moving boundary pattern. The problem became even more perplexing when it was demonstrated [2,3] that the ratio of wholes to halves (and tenths) remained virtually independent of concentration as judged by sedimentation analysis using Schlieren optics and light absorption in the total protein concentration range from 0.09 to 24 mg/ml, if the material in the elevated baseline region between peaks was counted as whole molecules [3]. All of these pieces of information, except for the rapid kinetics displayed upon shifts of ionic strength or pH, would serve to classify α-hemocyanin as a 'nonequilibrium' system. Two models have been put forward to explain this unusual sedimentation behavior, the 'microheterogeneity model' [3] and the 'incompetent whole molecule model' [8]. In the present paper several aspects and predictions of these models are tested experimentally, using sedimentation and stopped-flow dilution analysis. In addition, the origin of the elevated baseline between 60 S and 100 S peaks is analyzed in more detail, e.g., by changes in pressure, ionic strength and fixation. The following paper [9] describes computer simulations of sedimentation and stopped-flow patterns based on a new model which incorporates the results of this work.

2. Theory

2.1. Microheterogeneity model

In order to explain the rapid kinetics produced by a change in buffer system, while no changes at

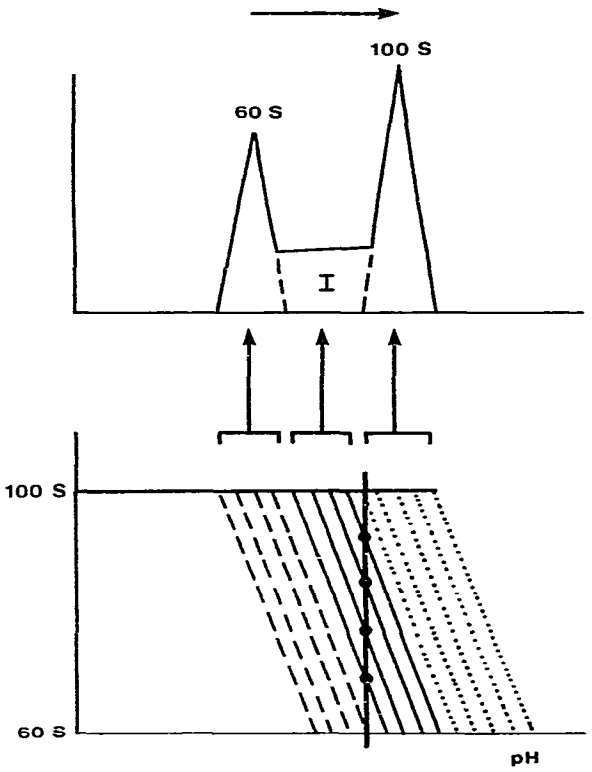

Fig. 1. Schematic representation of the Siezen and Van Driel microheterogeneity model, adapted from ref. 15. The lower illustration represents a 'stability diagram' with a number of hemocyanin components with slightly different 100 S–60 S dissociation constants, i.e., different dissociation pH values, represented by diagonal titration curves. Above is shown an idealized sedimentation pattern, with sedimentation from left to right. At the pH indicated by the vertical solid line only those components with the solid titration curves are in equilibrium. All components contributing to the 60 S peak are represented by the dashed titration curves entirely to the left (lower pH) of the vertical solid line. All components of the 100 S peak are represented by dotted 'titration curves' to the right (higher pH) of the vertical line. Equilibrating components only contribute to the intermediate region I.

all seem to be effected by large concentration changes or enormous displacements from equilibrium (i.e., isolation of purified fractions), Konings et al. [2] and Siezen and Van Driel [3] developed a model which may be illustrated by fig. 1. A given type of molecule with a single equilibrium constant, represented by a single 'titration curve' of sedimentation coefficient versus pH is either virtually all in the form of whole molecules or virtually all in the form of half molecules at one given pH value such as represented by the vertical solid line. Only a small amount of material is in equilibrium at any pH. This picture then explains why halves and wholes are resolved in the sedimentation diagram, why isolated fractions do not reequilibrate over long periods of time, and also why, by shifting ionic strength, the relative population of wholes and halves can be shifted suddenly, as the titration curves of many individual homogeneous subspecies are crossed. In addition, this picture would support the apparent finding that the ratio of wholes to halves, as judged by sedimentation analysis, is virtually independent of total concentration. It was clear that in stopped-flow experiments, we should expect large shifts of molecular weight by changing pH or ionic strength, which occurred as expected [3,5]. It would also be predicted from the evidence reviewed above, and the model illustrated in fig. 1, that in contrast to the reequilibrating system in lobster hemocyanin [10–12], there would be no change in molecular weight on simple dilution of α-hemocyanin in a constant buffer system. Instead, there were decreases in light scattering completed in the time range of 1–2 s, comparable to those initiated by a 100 atm jump in pressure [13]. The motivation for the model illustrated in fig. 1 was to explain why the extent of dissociation is independent of concentration. The direct experimental evidence from dilution experiments [13], particularly at pH 5.7 in 0.4 M NaCl, proved that the extent of dissociation is in fact quite dependent upon concentration.

2.2. *Incompetent whole molecule model*

The simplest alternative model suggested by Kegeles [8] was that the 100 S species are unreactive or incompetent whole molecules, and that the 'half molecules' peak seen in the sedimentation patterns represents, in fact, a typical Gilbert [6] rapidly reacting monomer-dimer system which cannot resolve in moving boundary sedimentation. In this model, relative peak areas of 100 S (incompetent wholes) and 60 S (wholes-halves reaction mixture) are also independent of protein concentration, and isolated fractions should also appear not to reequilibrate when resubjected to ultracentrifugation. The stopped-flow dilution response was attributed to rapid reequilibration within the 60 S peak reaction mixture. The incompetent model fitted the equilibrium light scattering data of Engelborghs and Lontie [5] at pH 5.7 in the presence of 0.4 M NaCl very well, and led to an estimate of 35% incompetent whole molecules and a formation constant of wholes from halves of 0.176 l/g. The model is also quantitatively consistent with kinetic studies on the 60 S–100 S system by pressure-jump and concentration-jump methods [13]. In these procedures it is necessary to know what fraction of the material is reactive, before one can measure the recombination rate constant from halves to wholes. If only a tiny amount of material could react, the recombination rate constant estimate would be proportionately increased, leading to an equilibrium formation constant very much larger than that estimated [8] from the equilibrium light scattering data of Engelborghs and Lontie [5]. When the estimate [8] of 35% nonreactive material was used [13], the formation constant derived from the kinetic measurements, 0.154 l/g, was in very satisfactory agreement with the value of 0.176 l/g derived on the basis of equilibrium measurements [8]. There was one initially recognized flaw in the incompetent whole molecule model: the raised baseline between 60 S and 100 S peaks in the sedimentation diagrams was not explained. It was already suggested that the incompetent whole molecule – single reactive species model was the simplest one possible, and might need extension [8,13].

Both models have been reviewed and compared in detail by Kegeles and Cann [14] and Van Holde and Miller [15].

3. Experimental

3.1. Protein solution

H. Pomatia α-hemocyanin solutions were prepared in buffers of various pH and ionic strength as described previously [3,4]. The main buffers used were 0.1 *I* Tris buffer, pH 7.76 (0.05 M Tris-HCl, 0.05 M NaCl), 0.45 *I* Tris buffer, pH 7.0 (0.05 M Tris-HCl, 0.4 M NaCl), and 0.5 *I* acetate, pH 5.7 (0.1 M sodium acetate, 0.4 M NaCl).

3.2. Analytical ultracentrifugation

Sedimentation velocity experiments were conducted at 20–25°C as before [2–4], using Beckman Spinco model E ultracentrifuges equipped with Schlieren optics; 12-mm pathlength cells were used unless otherwise specified. Sedimentation coefficients were determined from the Schlieren peak maximum and corrected to standard conditions of viscosity and density of water at 20°C ($s_{20,w}$). Theoretical $s_{20,w}$ values were calculated from $s^0_{20,w}$ values of 102, 64 and 19 S for whole, half and tenth molecules, respectively, and their linear concentration dependence [2], according to the equation

$$s_{20,w} = s^0_{20,w}(1 - kc) \quad (1)$$

where c is protein concentration (mg/ml) and k a constant. In theoretical calculations of the concentration dependence of $s_{20,w}$ values in mixtures, account was taken of the fact that half molecules sediment in the presence of tenths, and whole molecules sediment in the presence of both halves and tenths.

Weight percentages of each peak were determined from their areas, corrected for radial dilution. When the baseline was elevated between peaks of whole and half molecules the intermediate area was estimated separately, assuming symmetrical shape for both adjoining peaks (fig. 1).

In studies of pressure effects, approx. 0.35 ml of Beckman silicone oil 6377 ($\rho_{20} = 0.97$) was layered over about 0.35 ml protein solution ($\rho_{20} = 1.003$). The pressure at the cell bottom was calculated from

$$\Delta P = \omega^2 \rho (r_b^2 - r_m^2)/2 \quad (2)$$

where ω is angular velocity, ρ density and r_m and r_b distances from the center of rotation to meniscus and cell bottom, respectively.

3.3. Differential sedimentation

Series of differential sedimentation experiments were performed in a 50Ti or an SW50L rotor in a Beckman type L2 or L2-65 preparative centrifuge at 20°C. The fractions were collected with a rack and pinion-driven syringe and needle, which reached to within 0.65 mm of the tube bottom. The rotor was spun for a time just sufficient to centrifuge to the bottom of the tube most '100 S whole molecules'. The supernatant was carefully removed from above this level, and the pellet material was then resuspended in fresh buffer of the original composition. A small sample of this pellet solution was examined in the analytical ultracentrifuge to assess the progress of the purification of 100 S material, and the whole process was repeated for up to 20 cycles. A few of the early supernatants were also examined in the analytical ultracentrifuge; if required, these supernatants were reconcentrated by pelleting at 226 000 × *g* for 4 h.

3.4. Stopped-flow dilution

All original protein solutions were extensively dialyzed against buffer prepared with freshly boiled water, to avoid gas bubbles. 90° scattering measurements were monitored in a Durrum stopped-flow apparatus at 436 nm. For the purposes of qualitative observation, greater dilutions were made, if needed for generation of sufficient amplitude, than had been used in small perturbation measurements [13]. Control experiments were made on original solution, diluted to the initial identical concentration of fractions being studied.

3.5. Electron microscopy and fixation

Negative staining and electron microscopy were conducted as before [16]. In fixation experiments protein solutions were mixed 1:1 (v/v) with

buffered solutions of uranyl oxalate, phosphotungstate or glutaraldehyde (0.5% final concentration) just prior to ultracentrifugation. The measured pH change after mixing was less than 0.05.

4. Results and discussion

4.1. Protein concentration effects

It was predicted [8] that if the incompetent whole molecule model holds, the sedimentation coefficient of the nominal 60 S reaction boundary peak should increase with increasing protein concentration, finally approaching that of the hypothesized incompetent whole molecules at sufficiently high concentration. How high that concentration needs to be to observe this effect depends on the value of the formation constant for whole molecules, which must be known before predictions can be made. An early attempt (M.S. Tai and G. Kegeles, unpublished results) to make this test at pH 5.7 in 0.1 sodium acetate/0.4 M NaCl buffer at protein concentrations of 5.4 and 56.6 mg/ml showed that, as predicted, the two peaks had still not merged at 56.6 mg/ml, but that the sedimentation coefficient of the slower peak at this high concentration was indeed considerably higher than might have been predicted for a non-reacting 60 S species. However, hydrodynamic concentration effects are so large (as much as 50%) at the higher concentration that the experiments were felt to be inconclusive. Van Holde and Miller [15] emphasized that this might be a simple way to distinguish between the two models.

Extensive data for sedimentation were available [3] in Tris buffer at pH 7.76, where there are no corresponding data available for the formation constant of whole molecules. Table 1 presents the $s_{20,w}$ values calculated from the previously published sedimentation experiments, ranging from 0.75 to 24 mg/ml, in 0.1 I Tris buffer (pH 7.76) at 20°C [3]. Under those conditions a virtually constant composition with weight fractions of approx. 0.58 for wholes, 0.29 for halves and 0.13 for tenths was found (average of table 1 values in ref. 3, excluding the 24 mg/ml values).

Theoretical sedimentation coefficients were calculated for both models using this weight composition (table 1). For the microheterogeneity model a linear decrease of $s_{20,w}$ with protein concentration for each peak in the mixture was assumed, as found for the purified components [2]. The experimental sedimentation coefficients of the peaks which are assumed to represent whole, half and tenth molecules are seen to decrease regularly with protein concentration, in excellent agreement with the theoretical values calculated for the microheterogeneity model. The only deviation occurs for the fastest peak at the highest concentration,

Table 1

Concentration dependence of experimental and theoretical sedimentation coefficients of α-hemocyanin whole, half and tenth molecules

Conditions: Tris buffer (pH 7.76), ionic strength 0.1. Experimental details in ref. 3.

c (mg/ml)	Experimental $s_{20,w}$			Theoretical $s_{20,w}$ *					
	Tenths	Halves	Wholes	Microheterogeneity model			Incompetent model, $K_{halves\text{-}wholes}$ (l/g)		
				Tenths	Halves	Wholes	0.00176	0.176	17.6
24	18.0	53.2	73.1	17.7	51.7	62.8	52.2	67.9	83.3
12	18.8	58.6	85.7	18.3	57.9	82.4	58.1	69.5	89.9
6	17.6	59.1	91.7	18.7	60.9	92.2	61.1	68.6	92.1
3	19.1	62.0	97.1	18.8	62.5	97.1	62.6	67.0	91.7
1.5	19.3	62.5	98.9	18.9	63.2	99.6	63.3	65.8	89.7
0.75	20.2	62.9	100.3	19.0	63.6	100.8	63.6	65.0	86.5

* Calculated using $s^0_{20,w}$ values of 19, 64 and 102 S and their protein concentration dependence as given by ref. 2. In the reaction boundary model a weight fraction of 0.29 was assumed to be in halves-wholes equilibrium.

where eq. 1 presumably no longer applies.

For the reaction boundary model a weight average sedimentation coefficient ($\bar{s}_{20,w}$) was calculated for the halves-wholes boundary, due allowance being made for protein concentration dependence of individual $s_{20,w}$ values. The weight fraction of half molecules, α, was calculated from the law of mass action

$$K = (1-\alpha)/(1-f_d)c\alpha^2 \quad (3)$$

in which f_d is the weight fraction of molecules not participating in the equilibrium, and K the formation or association constant in l/g of whole from half molecules. As both the fastest peak (incompetent whole molecules) and the slowest peak (tenth molecules) should not participate in the equilibrium, a value of 0.71 was used for f_d.

Our calculations in the penultimate column in table 1 used the K value of 0.176 l/g evaluated by Kegeles [8] from the equilibrium light-scattering data obtained in 0.5 I acetate buffer (pH 5.7) [5]. Unfortunately, the sedimentation experiments were performed in a different buffer, 0.1 I Tris buffer (pH 7.76) [3], in which the association and dissociation rate constants and hence the equilibrium constant K are presumably quite different than at pH 5.7 (see section 4.5). Therefore, theoretical values of $s_{20,w}$ were also calculated using K values two orders of magnitude lower (0.00176 l/g) and higher (17.6 l/g), as tabulated in table 1. The calculations show that for $K = 0.176$ l/g the theoretical $s_{20,w}$ values for the reaction boundary increase to nearly 70 S, and hence are not in agreement with the experimental $s_{20,w}$ values. On the other hand, an excellent agreement is found with a 100-fold lower value for K. For this K value, the weight fraction of half molecules in the reaction boundary is 0.99 at the highest concentration studied. In other words, the two models have become virtually identical.

That the Schlieren peaks for whole and half molecules approach each other at high concentration can be adequately explained by the protein concentration dependence of $s_{20,w}$ values, since in moving-boundary sedimentation whole molecules always migrate in a higher total protein concentration than half molecules.

In view of the fact that the assumption of 100-fold lower value for K at pH 7.76 than that estimated [8] for pH 5.7 does correctly predict the concentration dependence for the sedimentation coefficient of the 60 S peak taken as a reaction boundary in the incompetent whole molecule model, it is likely that there is at pH 7.76 a considerable fraction of material with an extremely small formation constant. Such a model was tried [8] and found not to fit the experimental light-scattering data of Engelborghs and Lontie [5] at pH 5.7.

4.2. *Pressure effects*

The elevated baseline between Schlieren peaks of 100 S and 60 S molecules has been ascribed to a continuous dissociation of 100 S molecules due to increasing hydrostatic pressure [2]. This explana-

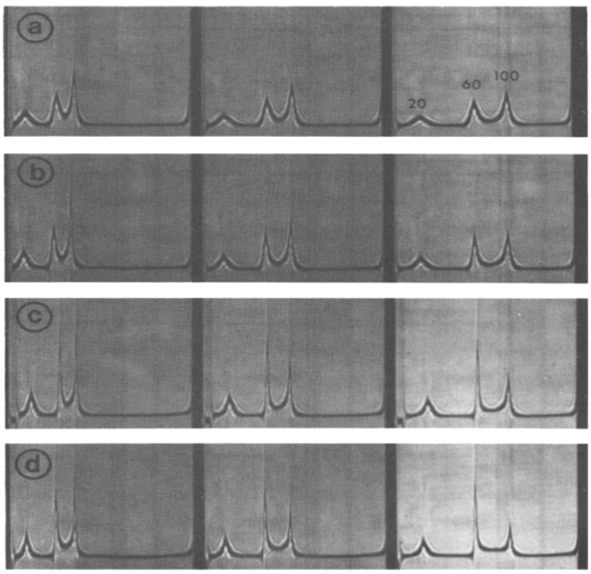

Fig. 2. Effects of hydrostatic pressure by varying rotor speeds on Schlieren velocity sedimentation pattern of α-hemocyanin (16.8 mg/ml) in 0.1 Tris buffer (pH 7.76) at 20°C; 3 mm cell. Rotor speeds and pressure increase (ΔP) from meniscus to bottom were: (a) 20410 rpm, 40 atm; (b) 29500 rpm, 85 atm; (c) 42040 rpm, 166 atm; (d) 59780 rpm, 345 atm. Sedimentation is from left to right. The indicated sedimentation coefficients (in S) serve only to identify the peaks of tenths (20 S), halves (60 S) and wholes (100 S), and are not the measured values.

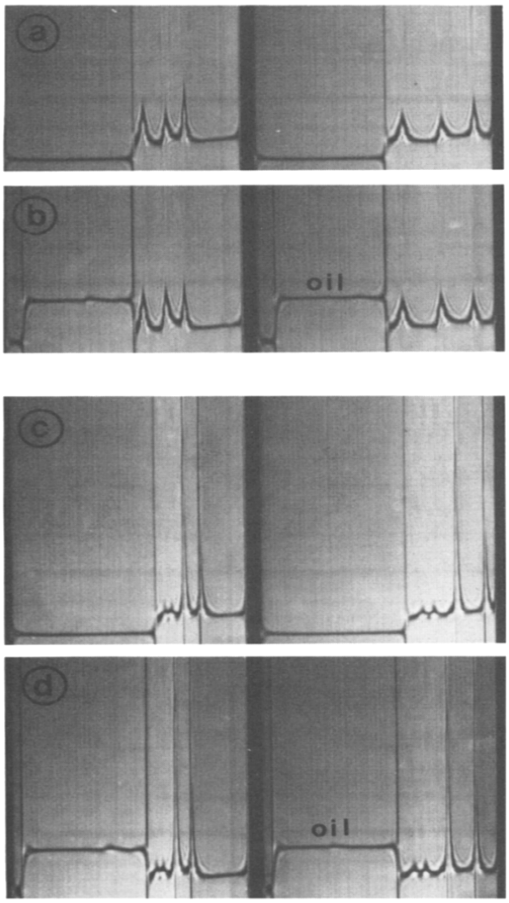

Fig. 3. Effect of hydrostatic pressure by overlayering with oil on α-hemocyanin in: (a,b) 0.1 *I* Tris buffer (pH 7.8), protein concentration 3 mg/ml, and (c,d) 0.005 *I* Tris buffer (pH 7.6), protein concentration 5.3 mg/ml. All runs at 37020 rpm, 20°C. ΔP values: (a) 65 atm, (b) 125 atm = 59 atm (oil)+66 atm (protein), (c) 55 atm, (d) 128 atm = 67 atm (oil)+61 atm (protein).

tion is not completely satisfactory, however, as indicated by experiments performed in 0.1 *I* Tris buffer (pH 7.76) over a larger pressure range than examined previously (fig. 2).

Four rotor speeds were chosen such that the pressure approximately doubled between speeds, i.e., $\Delta P = 40$, 85, 166 and 345 atm between meniscus and cell bottom, and pictures were taken at equivalent distances of migration. At any speed (and at any protein concentration as in table 1) the amount of intermediate material extrapolates to about 15% of total protein at zero time of centrifugation. This suggests that this fraction of α-hemocyanin is in a rapid whole-half equilibrium at atmospheric pressure.

During every run, the intermediate area increases at the expense of the 100 S peak to maximally 30% of total protein at the highest pressure. Hence, dissociation due to pressure accounts for only part of the material between 60 S and 100 S peaks. The observed increase of about 15% during sedimentation correlates reasonably well with the 8% decrease of weight-average molecular weight (= 16% dissociation of wholes to halves) measured by light scattering at 200 atm [2].

Alternatively, one can keep rotor speed constant and increase hydrostatic pressure by layering oil over the protein solution, as shown in fig. 3. A 59 atm pressure increase (from 66 to 125 atm at r_b) led to an approx. 10% shift in the relative amounts of 60 S and 100 S components (fig. 3a and b) in 0.1 *I* Tris buffer (pH 7.8). Smaller pressure dissociation effects have been observed in 0.5 *I* acetate buffer (pH 5.7) [5].

4.3. *Suppression of baseline elevation*

A number of conditions have been found under which baseline elevation between the Schlieren peaks for whole and half molecules of α-hemo-

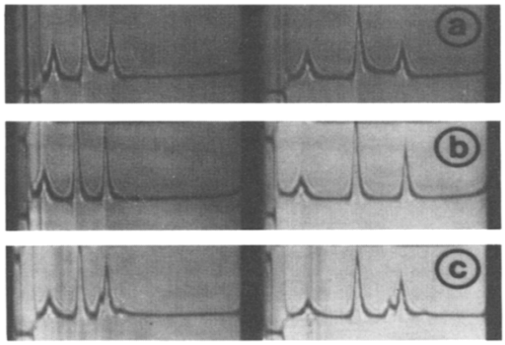

Fig. 4. Effect of fixation on sedimentation patterns of α-hemocyanin in 0.1 *I* Tris buffer (pH 7.8). Protein concentration, 3.5 mg/ml; 37020 rpm, 20°C. (a) Original solution, (b)+0.5% uranyl oxalate, (c)+0.5% glutaraldehyde.

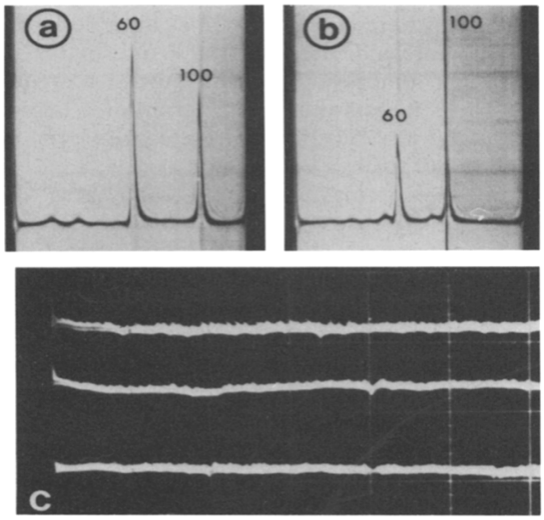

Fig. 5. Effect of low ionic strength on sedimentation patterns and stopped-flow dilution patterns of α-hemocyanin: (a) sedimentation in 0.01 *I* Tris buffer (pH 7.65), protein concentration 6 mg/ml; (b) sedimentation in 0.002 *I* Tris buffer (pH 7.24), protein concentration 5 mg/ml, 37020 rpm, 20°C; (c) replicate stopped-flow dilution patterns in 0.01 *I* Tris buffer (pH 7.65) at 25.5°C; 5 mg/ml solutions were diluted to 5/7 of original concentration; oscilloscope sweep time 5 s/division.

cyanin is either suppressed or completely eliminated.

4.3.1. Fixation

Addition of some fixing agents commonly used in electron microscopy has a variety of effects on the 60 S–100 S system. Uranyl oxalate eliminates the elevated baseline altogether, shifting the small fraction of protein that is initially in rapid equilibrium completely to whole molecules (fig. 4a and b). The opposite is true for phosphotungstate which shifts all intermediate material and the 100 S material to the 60 S peak (not shown). Glutaraldehyde eliminates the smooth baseline elevation, but produces two new minor peaks of 87 S and 145 S (fig. 4c). The latter is due to three half molecules joined together, whereas the former may represent two incorrectly joined half molecules, i.e., not aligned as a perfect cylinder. No elevated baseline occurs after cross-linking with dimethyl suberimidate [16].

4.3.2. Low ionic strength

As ionic strength is reduced below 0.1 the dissociation of whole into half molecules occurs at

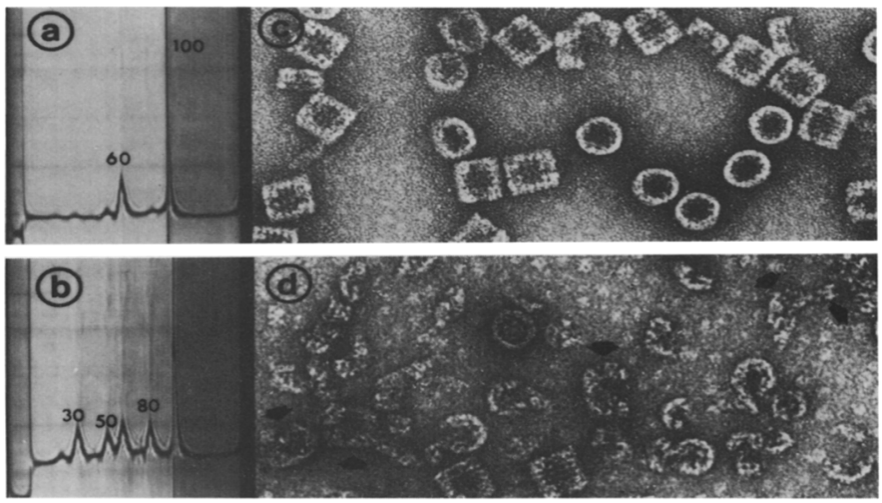

Fig. 6. Differential sedimentation enrichment of intermediate components of α-hemocyanin in 0.005 *I* Tris buffer (pH 7.4). (a) Sedimentation pattern of original solution. Protein concentration, 5 mg/ml; 37020 rpm, 20°C. (b) Sedimentation pattern of reconcentrated supernatant after partial pelleting of 100 S molecules at 145000 × g for 60 min; 30 mm cell, 37020 rpm, 20°C. Sedimentation coefficients of enriched intermediates as indicated. (c) Electron micrograph of negatively stained original mixture of whole and half molecules; magnification, × 200000 (d) Electron micrograph of supernatant; arrows indicate large irregular molecules, presumably 80 S molecules; same magnification.

progressively lower pH in the alkaline range [4]. Simultaneously, the elevation of the baseline between 60 S and 100 S peaks diminishes, and it is virtually absent below 0.01 *I*. Examples of sedimentation patterns in 0.01 *I* Tris buffer (pH 7.65) and 0.002 *I* Tris buffer (pH 7.24) are shown in fig. 5. It is of additional interest that in 0.01 *I* Tris buffer (pH 7.65) there is essentially no reactivity in stopped-flow dilution experiments, as shown in fig. 5c.

Hence, the protein fraction that is in equilibrium at any pH appears to be much smaller at low ionic strength. In pressure studies under such conditions no shift occurred in the relative amounts of 60 S and 100 S peaks, nor did any new baseline elevation arise as a result of increasing pressure, either during a run, at different speeds or after overlayering with oil (fig. 3c and d).

A previously published statement that no elevated baseline occurs in borate buffers at alkaline pH is incorrect [2], as the sedimentation pattern was found to be independent of buffer type [4]. As that statement referred to experiments performed in low ionic strength borate buffers, the absence of baseline elevation is consistent with our present results, and no compensating effects of pressure on protein partial specific volume versus borate p*K* are required to explain this phenomenon. Light-scattering studies of α-hemocyanin at 200 atm showed no pressure-induced dissociation in low ionic strength borate buffers which again is consistent with the present results [2].

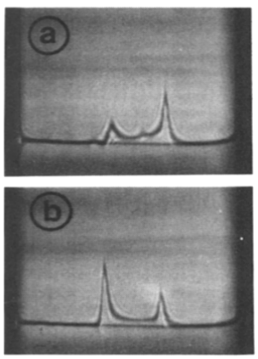

Fig. 7. Sedimentation pattern of α-hemocyanin (4.2 mg/ml) in 0.3 *I* Tris buffer, 37020 rpm, 20°C. (a) pH 7.0, (b) pH 7.4.

It is noteworthy that sedimentation patterns of some other molluscan hemocyanins such as *Murex trunculus* [17], *Busycon canaliculatum* [18], *Buccinum undatum*, *Neptunea antiqua* [19] and *Pila leopoldvillensis* [20] do not show the elevated baseline between 60 S and 100 S peaks, even at ionic strength of 0.05–0.1. No explanation is available to explain this difference between species.

4.4. Intermediate nonequilibrium components

Additional small Schlieren peaks are often seen between the three main peaks of tenth, half and whole molecules, such as those in fig. 5b. These intermediate components occur mainly between pH 7.0 and 7.5, and are most pronounced at low ionic strength.

Their relative amounts can be dramatically enhanced by differential sedimentation, as illustrated in fig. 6 in 0.005 *I* Tris buffer (pH 7.4). The profile in fig. 6b was obtained for the supernatant after most whole molecules in the original solution (fig. 6a) were pelleted to the bottom of the tube. This pattern is stable, in that no reequilibration takes place over several days, suggesting that the various intermediate components are not in equilibrium with each other, or with tenth, half and whole molecules.

The three main extra peaks of about 30, 50 and 80 S presumably correspond to assemblies of two-, four- and eight-tenth molecules. Electron microscopy shows an increased number of circle segments corresponding to two-tenth and four-tenth molecules (fig. 6c and d), which resemble those seen after cross-linking and dissociation of half molecules [16], but also a number of large irregular structures (arrows) which must be the 80 S molecules. These structures appear to be incorrectly assembled.

Although we have now demonstrated that stable nonequilibrium components of size between half and whole molecules do exist, their concentrations are normally very low, and they certainly do not contribute to baseline elevation effects between 60 S and 100 S peaks as observed above pH 7.5. That these are two distinct phenomena is best illustrated at ionic strength 0.3 where dissociation of whole molecules is initiated at lower pH [4]. At

pH 7.4 only a smooth elevated baseline is observed (fig. 7b), but upon lowering pH to 7.0 additional 50 S and 80 S peaks appear, the latter superimposed on the elevated baseline (fig. 7a).

4.5. Differential sedimentation and stopped-flow analysis

In this section mixtures of 60 S and 100 S material are subjected to series of differential sedimentation runs, in a attempt to isolate material responsible only for 60 S sedimentation, and material responsible only for 100 S sedimentation behavior, and to subject these fractions not only to resedimentation analysis, but also to quantitative stopped-flow light-scattering dilution analysis. If the incompetent whole molecule model [8] is rigorously followed, '100 S material' should show no reactivity on stopped-flow dilution and all the reactivity should reside in '60 S material'.

Separate series were performed under three different conditions of pH and ionic strength corresponding to those used most in previous relevant literature [3,5,13].

4.5.1. 0.5 I acetate buffer (pH 5.7)

One cascade of successive pelletings and resuspensions and resedimentations was pursued through 20 cycles. A comparison of the analytical sedimentation patterns obtained on material from the 20th pellet and from an equally diluted aliquot of the original solution is shown in fig. 8. This shows that while a nonreactant 50 S peak has been removed, there is still a residue of material sedimenting near 60 S. The relative amount of this material remained virtually constant during the last few fractionations; its carrying over may be the result of excessive time of centrifugation. Almost complete removal of 100 S material with the aid of the Yphantis-Waugh separation cell [21] produced a fraction which did not reequilibrate to 100 S. Also, prolonged storage of fractions such as shown in fig. 8 produced no further regeneration of 60 S material, confirming that there is no slow reequilibration between 60 S and 100 S materials.

Samples of the 20th pellet and the combined 2nd and 3rd supernatant were compared with control samples of original solution in light-scattering stopped-flow dilution with pH 5.7 buffer. It is seen in fig. 9 that the original mixture shows a larger scattering amplitude than does either the 20th pellet material or the supernatant. However, if only the 60 S material were responsible for dissociation on dilution, as hypothesized [8], then the dilution response for the supernatant should be more than that of the original solution. In addition, the dilution response for the pellet material containing primarily incompetent 100 S material (fig. 8) would be expected to be far less than shown in fig. 9.

The conclusion to be derived from these experiments is that the reactivity on dilution at pH 5.7 does not appear to be attributable entirely to species associated with the slower moving boundary in sedimentation, as postulated [8]. While this finding does not agree with the simplest incompetent whole molecule model which was developed by one of us [8] at pH 5.7 as a result of the measurements of Engelborghs and Lontie [5] at that pH, it does not appear to contradict directly the results of any published separation attempts under such buffer conditions.

4.5.2. 0.45 I Tris buffer (pH 7.0)

The original solution has only two Schlieren peaks, which travel at roughly 60 S and 100 S, connected by an elevated baseline (fig. 10a). The

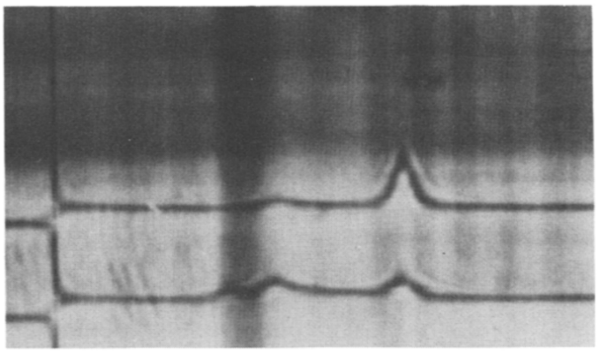

Fig. 8. Differential sedimentation of α-hemocyanin in 0.5 *I* acetate buffer (pH 5.7). Bottom: sedimentation pattern of original unfractionated control. Top: sedimentation pattern of 20th pellet solution. Protein concentration of both, 1.75 mg/ml; 29500 rpm, room temperature.

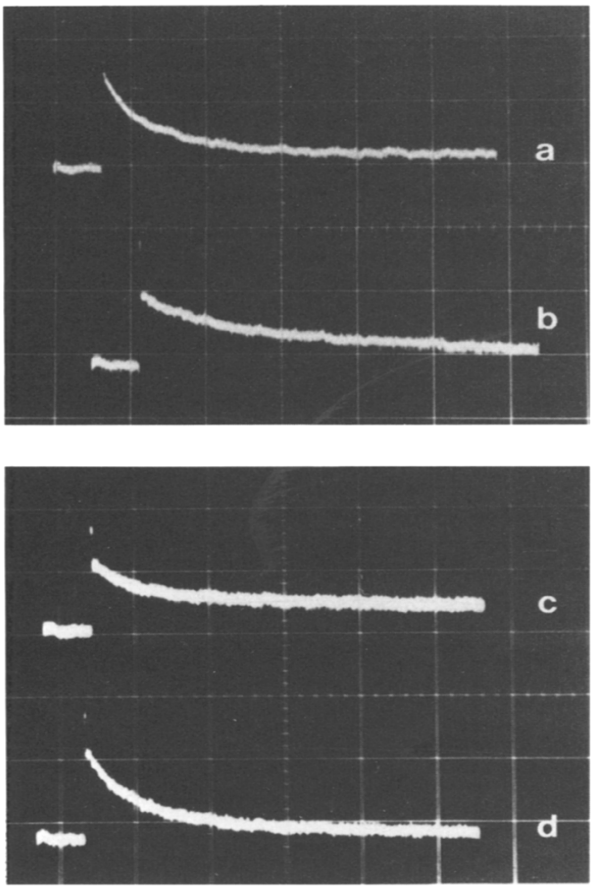

Fig. 9. Differential sedimentation of α-hemocyanin in 0.5 *I* acetate buffer (pH 5.7). Stopped-flow dilution patterns (sweep time 6.83 s/division, 25°C) of: (a) original unfractionated control, (b) 20th pellet solution; (c) combined 2nd and 3rd supernatant; and (d) original mixture. Concentration changes were (a,b) 3.5 to 1.75 mg/ml, (c,d) 2.3 to 1.15 mg/ml.

ultracentrifuge patterns of 20th pellet material and 3rd supernatant each show only a single peak, travelling near 100 S and 60 S, respectively, indicating that complete separation has been achieved (fig. 10b and c).

A comparison of the stopped-flow dilution of the 20th pellet material, whose sedimentation pattern is shown in fig. 10c, is made with that of original solution at the same concentration in fig. 11a and b. It is seen that there is essentially no residual reactivity in this 100 S material, in agreement with the incompetent whole molecule model [8]. On the other hand, the supernatant fraction which contains only 60 S material shows less dilution reactivity than the original sample (fig. 11c and d), implicating some intermediate material sedimenting between 60 S as a contributor to the observed reactivity on dilution of the original material. In this respect, a hypothesis of only one homogeneous reactive species is too simple [8].

4.5.3. 0.1 I Tris buffer (pH 7.76)

Under these conditions some tenth (20 S) molecules are present in addition to 60 S and 100 S molecules (fig. 12a). As demonstrated previously [3], isolation of 20 S material by differential sedimentation is relatively easy: the top half of the first supernatant is shown in fig. 12b. The 20 S material shows no relaxation amplitude in stopped-flow dilution. While this material could conceivably reassociate on being reconcentrated, it did not need to be considered as a reactive species

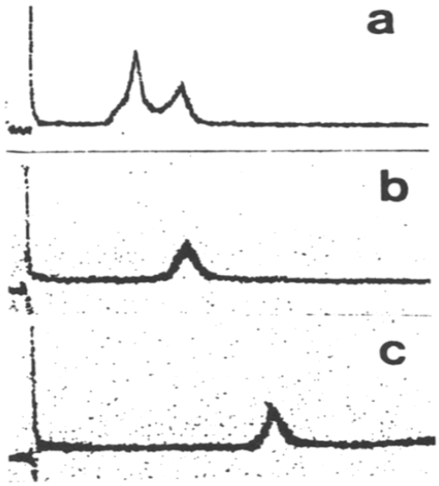

Fig. 10. Differential sedimentation of α-hemocyanin in 0.45 *I* Tris buffer (pH 7.0). Sedimentation patterns at 29500 rpm of: (a) original unfractionated solution, 17.4 mg/ml, 3 mm cell, after 10 min sedimentation; (b) 3rd supernatant, 1.62 mg/ml, after 8 min sedimentation; (c) 20th pellet, 1.85 mg/ml, after 12 min sedimentation.

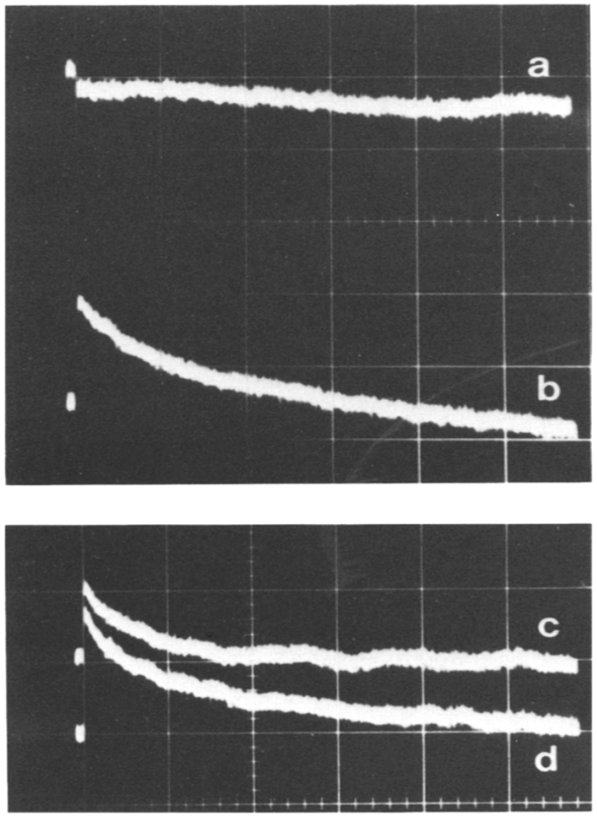

Fig. 11. Differential sedimentation of α-hemocyanin in 0.45 *I* Tris buffer (pH 7.0). Stopped-flow dilution patterns (sweep time 13.7 s/division) of: (a) 20th pellet solution, (b) original unfractionated solution, (c) 3rd supernatant, (d) original solution. Concentration changes were (a,b) 1.85 to 1.32 mg/ml, (c,d) 1.62 to 1.15 mg/ml.

for the purposes of the dilution experiments conducted.

While the differential sedimentation separations were not carried through as many cycles, the purification of nonreactive whole molecules was succeeding during these cycles (fig. 12d, table 2 and ref. 3). The bottom half of the first supernatant shows an enhancement of intermediate material relative to 60 S and 100 S peaks (fig. 12c and table 2). This suggests that it may be possible to enrich the solution even further with material that is in rapid equilibrium by careful choice of sedimentation conditions, alternately removing 100 S molecules by pelleting and 60 S molecules from the upper supernatant.

In fig. 13 and 14 a comparison is shown of stopped-flow dilution of the original mixture, 4th pellet and total second supernatant. It is seen that there is a greatly reduced reactivity for the pellet on dilution, in agreement with the incompetent whole molecule model (fig. 13). The reaction response indicates the removal from this fraction of some species having the greatest rates of dilution

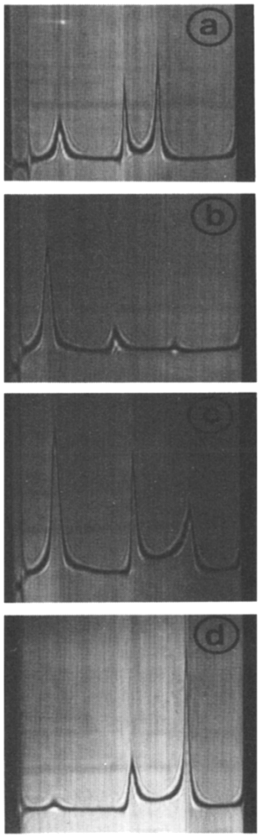

Fig. 12. Differential sedimentation of α-hemocyanin in 0.1 *I* Tris buffer (pH 7.76). Sedimentation patterns (at 37020 rpm, 20°C) of: (a) original unfractionated solution, 25 mg/ml, 3 mm cell; (b) upper half of 1st supernatant; (c) lower half of 1st supernatant; and (d) 2nd pellet solution.

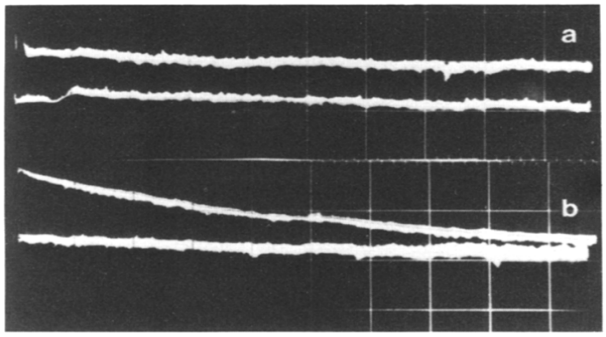

Fig. 13. Differential sedimentation of α-hemocyanin in 0.1 *I* Tris buffer (pH 7.76). Stopped-flow patterns (sweep time 15 s/division) of: (a) duplicate dilutions of 4th pellet solution, diluted from 1.48 mg/ml to 1.07 mg/ml; and (b) two successive scans from dilution of original solution, from 1.48 mg/ml to 1.07 mg/ml.

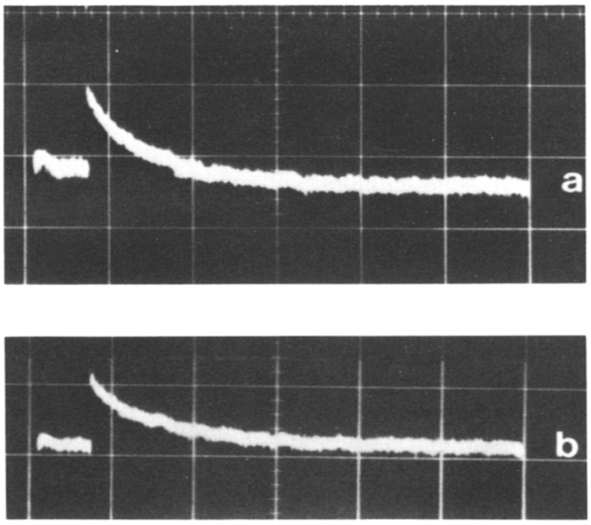

Fig. 14. Differential sedimentation of α-hemocyanin in 0.1 *I* Tris buffer (pH 7.76). Stopped-flow patterns (sweep time 68.3 s/division) of: (a) original unfractionated solution, diluted from 4.65 mg/ml to 2.33 mg/ml; and (b) total 2nd supernatant, diluted from 4.0 to 2.0 mg/ml.

reaction in the original material, but the continued presence of more slowly reacting species. Again, the slower response of the supernatant material, relative to the original mixture, suggests the partial loss of reactive species intermediate between 60 S and 100 S in sedimentation velocity (fig. 14).

5. General discussion

A more general description of α-hemocyanin association-dissociation is required now, which takes the present results into consideration. The microheterogeneity model as originally presented [3] needs to be modified so that it can predict the ability of whole-half molecule mixtures to react on dilution. In addition, experimental work in this paper indicates that, at least at pH 5.7 and 7.76, more extensive microheterogeneity of reactive

Table 2

Differential sedimentation of α-hemocyanin.

Conditions: Tris buffer (pH 7.76), ionic strength 0.1.

	Weight composition (%)				
	Tenths (T)	Halves (H)	Intermediate species (I)	Wholes (W)	$\frac{I}{H+I+W}$ *
Original mixture	23	21	17	39	0.22
1st supernatant (top half)	85	10	3	2	0.20
1st supernatant (bottom half)	39	19	24	18	0.39
2nd pellet	2	19	29	50	0.30
4th pellet	0	20	18	62	0.18
6th pellet	0	16	15	69	0.15

* Fraction of intermediate material relative to 60 S and 100 S peaks.

half-whole molecule systems is necessary than in the single reactive species model presented before [8].

Both models as previously published need to be extended to account for apparent reactivity in the region between 60 S and 100 S peaks. At each pH studied, some reactivity in stopped-flow dilution appears to be associated with material having sedimentation coefficients between 60 S and 100 S. At pH 5.7, the observed relaxation speeds of the overall dissociation processes are relatively fast, compared to those at neutral and alkaline pH values. Our pressure studies also indicate that at least 15% of the protein is in rapid 60 S–100 S equilibrium, even in the absence of applied pressure, in buffers of sufficiently high ionic strength. This fraction can be 'frozen' by either lowering the ionic strength or addition of fixation agents.

One mechanism, which is based on a whole series of rapidly equilibrating half molecule-whole molecule interactions with a large range of equilibrium constants, is proposed in the accompanying paper [9].

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