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THE USE OF SEDIMENTATION COEFFICIENTS TO DISTINGUISH BETWEEN MODELS FOR PROTEIN OLIGOMERS

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The sedimentation coefficients of proteins are dependent on their sizes, shapes and densities and on the density and viscosity of the solvent. However, when the sedimentation coefficients of an oligomeric protein and its protomer are measured under the same experimental conditions, the ratio of the two coefficients depends only on the protomer shape and the mode of aggregation. This property, which we shall call the sedimentation ratio, therefore provides a way of distinguishing between models for oligomeric proteins. To allow examination of the behaviour of the sedimentation ratio, sedimentation coefficients are calculated for a comprehensive range of protomer shapes and modes of aggregation in hexameric systems using equations derived by Kirkwood. As illustrations of the method the resulting sedimentation ratios are compared with experimental values for insulin and arthropod hemocyanin, which eliminates many of the possible structures for these proteins. When experimental estimates of degree of hydration and molecular dimensions are also considered, all but a group of virtually identical structures are eliminated for the insulin hexamer and a single most likely structure remains for arthropod hemocyanin. The insulin structure is in good agreement with that determined by X-ray crystallography while the hemocyanin hexameric structure is a hexagonal prism formed by the cyclic aggregation of prolate ellipsoids of axial ratio about 2.5 : 1.

1. Introduction

Theoretical methods for predicting the hydrodynamic properties of structures of complex shape have been available for some years [1,2] and have been used to test proposed models for aggregated structures by comparing an experimentally measured quantity, usually the sedimentation coefficient, with that calculated for a given model. Systems to which this approach has been applied include protein oligomers [3–5] polysomes [6] and aggregates of small molecules [7,8]. Some reports, [5,6,9] have included tables relating the sedimentation coefficients of oligomer and protomer for a selected number of arrangements of the protomers to form the oligomer. While a structure with the required sedimentation coefficient is often found, the confidence which can be placed in it may be questioned because only a limited number of models is considered and because the protomers comprising the aggregated form are almost invariably assumed to be spherical.

In this paper we explore the behaviour of the calculated sedimentation coefficients of aggregated structures formed from identical subunits, taking into account a much more comprehensive range of protomer shapes and modes of aggregation than examined previously. The development here is made in terms of the formation of protein hexamers but the procedure is applicable to aggregates formed by the accretion of any (relatively small) number of subunits.

2. Theory

2.1. Definitions

The terms oligomer, protomer, monomer, subunit, heterologous, and isologous are applied in the sense defined by Monod et al. [10]. In describing the arrangement of protomers in an oligomer the terms cyclic and spherical symmetry are employed rather than the point group notation. For the case of cyclic symmetry the protomers lie in a plane about an n-fold rotation axis while in an oligomer with spherical symmetry the pro-

tomers occupy equivalent positions on the surface of a sphere; the former case thus has cyclic point group symmetry while the latter includes the dihedral and cubic point groups. It is assumed that no change in conformation or volume occurs in the protomers when they aggregate to form the oligomer.

2.2. Protomer shape and mode of aggregation

Association of protomers to form a specific closed structure requires that they take up spatially equivalent positions in the oligomer which consequently has cyclic symmetry in the case of heterologous association, and either cyclic or spherical symmetry for isologous or mixed isologous–heterologous binding. The structural alternatives for hexamers composed of spherical protomers may be represented by the cyclic and spherical arrangements shown in fig. 1A. However, consideration of only spherical protomers is a rather severe restriction, and while treatment of all subunit shapes is clearly not possible, we have chosen to compromise by using the set of alternatives shown in fig. 1B. These protomers, which for the purpose of calculation are composed of a suitable number and arrangement of identical spheres, correspond roughly to oblate and prolate ellipsoids of revolution and are labelled O and P, respectively. A number following the letter O or P gives the axial ratio of the corresponding ellipsoid. The possible hexameric structures which may be formed from protomers of the shapes shown in fig. 1B are illustrated for the case of the P_3 protomer in fig. 2A. The forma-

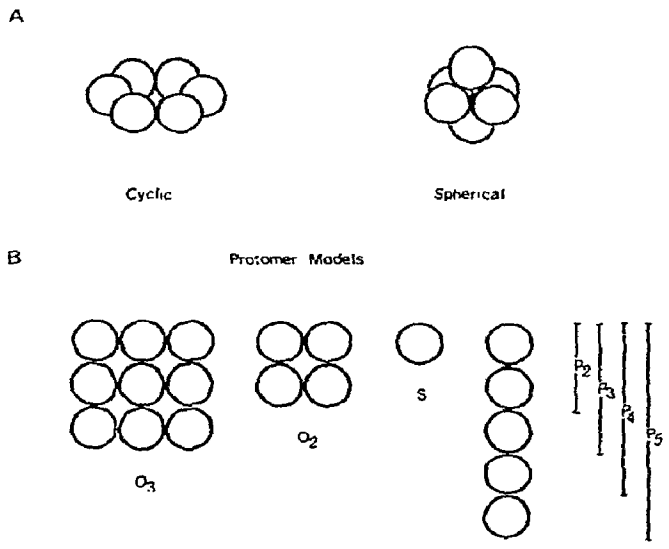

Fig. 1. Basic models used for the calculation of frictional coefficients for hexameric structures. A: Arrangements of spherical protomers in closed hexamers. B: The range of protomer models used in the calculation, showing how oblate and prolate ellipsoids were approximated by assemblages of identical spheres (see text).

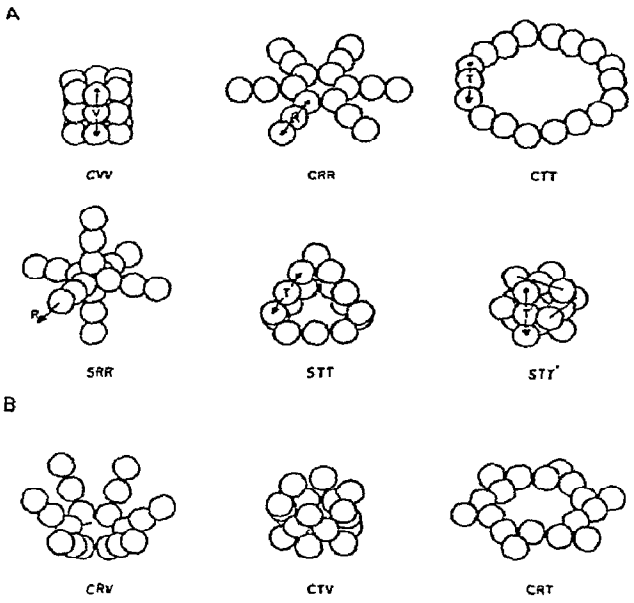

Fig. 2. The hexameric forms for which frictional coefficients were calculated. The structures illustrated are those formed from P_3 protomers but the calculations were made for all the types of protomer shown in fig. 1B. Three letters, of which the first denotes whether it is derived from the cyclic, C, or spherical, S, arrangement of spheres shown in fig. 1A, serve to identify each structure. The remaining letters denote in which axial direction the original sphere was considered to be stretched (or compressed) in order to form a particular protomer. The directions of these axes are shown on the figure as V, vertical, R, radial, and T, tangential. Thus for the cyclic structures the vertical axis is parallel to the six-fold axis, the radial axis intersects it at right angles, and the tangential axis is tangential to two adjacent subunits. For the spherical structures the radial axis passes through the centres of two opposite subunits in the octahedral arrangement shown in fig. 1A, and is normal to a tangential plane in which all axis directions are equivalent because of spherical symmetry. Two tangential axes have been employed in the calculations and are indicated in the figure as T' and T. A: The six basic arrangements employed in the calculations. B: Three intermediate structures for which calculations were made for the purposes of illustration, with CRV representing a transition from CRR to CVV; CTV from CVV to CTT, and CRT from CRR to CTT.

tion of these structures may be visualized as a process of symmetrical stretching (for prolate ellipsoids) or compressing (for oblate ellipsoids) to the required extent, of the spherical subunits shown in fig. 1A, along trangential, radial and vertical axes for the cyclic model, and along tangential and radial axes for the spherical case. The resulting cylindrical or disc-shaped protomers are then approximated by the appropriate assemblage of spheres from the set shown in fig. 1B. The directions of the axes referred to, and the system of nomenclature adopted for the resulting oligomeric structures, is explained in the caption to fig. 2A. We believe that this group of structures provides a reasonably comprehensive set of alternatives for hexamers, but in order to test this proposition the set of intermediate structures illustrated in fig. 2B have also been included for consideration. These structures represent forms between some of those given in fig. 2A, with CTV for example, being the result of a rotation around axis R from structure CTT halfway to structure CVV. It is noted, however, that the models considered for both protomers and hexamers still provide no more than reference points on a continuum of possible structures.

2.3. Calculations

The translational frictional coefficient, f, of a collection of n identical spherical subunits each with a frictional coefficient, ξ, is given by [1]

$$f = \frac{n\xi}{1 + \frac{\xi}{6\pi n\eta} \sum_{l=1}^{n} \sum_{\substack{s=1 \\ l \neq s}}^{n} \langle R_{ls}^{-1} \rangle} \tag{1}$$

where η is the solvent viscosity, R_{ls} is the distance between the centres of subunits l and s and the angular brackets denote an average over all internal degrees of freedom of the assembly. No averaging is needed in the present work which is concerned only with rigid protomers; the angular brackets are therefore omitted from subsequent equations. If a protomer and oligomer contain respectively n_p and n_o identical frictional subunits their respective frictional coefficients $f(p)$ and $f(o)$ are given by

$$f(p) = \frac{n_p\xi}{1 + \frac{\xi}{6\pi n_p\eta} \sum_{l=1}^{n_p} \sum_{\substack{s=1 \\ l \neq s}}^{n_p} R_{ls}^{-1}} \tag{2a}$$

$$f(o) = \frac{n_o\xi}{1 + \frac{\xi}{6\pi n_o\eta} \sum_{l=1}^{n_o} \sum_{\substack{s=1 \\ l \neq s}}^{n_o} R_{ls}^{-1}} \tag{2b}$$

and it follows that the corresponding sedimentation coefficients are related by the expression

$$\frac{s(o)}{s(p)} = \left(1 + \frac{\xi}{6\pi n_o\eta} \sum_{l=1}^{n_o} \sum_{\substack{s=1 \\ l \neq s}}^{n_o} R_{ls}^{-1}\right) \times \left(1 + \frac{\xi}{6\pi n_p\eta} \sum_{l=1}^{n_p} \sum_{\substack{s=1 \\ l \neq s}}^{n_p} R_{ls}^{-1}\right)^{-1} . \tag{3}$$

For structures which are built up from spherical subunits of radius a, $\xi = 6\pi\eta a$ and eq. (3) reduces to

$$\frac{s(o)}{s(p)} = \left(1 + \frac{1}{n_o} \sum_{l=1}^{n_o} \sum_{\substack{s=1 \\ l \neq s}}^{n_o} (R'_{ls})^{-1}\right) \times \left(1 + \frac{1}{n_p} \sum_{l=1}^{n_p} \sum_{\substack{s=1 \\ l \neq s}}^{n_p} (R'_{ls})^{-1}\right)^{-1} , \tag{4}$$

where R'_{ls} is expressed in units of the radius, a, of the frictional subunit. Thus the ratio $s(o)/s(p)$, which we will call the sedimentation ratio, may be calculated independent of the solvent viscosity, the protein and the solvent density and the absolute size of protomer and oligomer: it depends solely on the shape of the protomer, represented by

$$\sum_{l=1}^{n_p} \sum_{\substack{s=1 \\ l \neq s}}^{n_p} (R'_{ls})^{-1},$$

and the mode of aggregation, represented by

$$\sum_{l=1}^{n_o} \sum_{\substack{s=1 \\ l \neq s}}^{n_o} (R'_{ls})^{-1}.$$

The magnitudes of the R'_{ls} values depend on the geometry of the structure under consideration while the number of such distances which appear in the summation is equal to $n(n-1)$ where n is the number of spherical frictional subunits from which the structure is built up. Thus for example, calculation of $s(o)$ for the hexamer composed of O_3 protomers (fig. 1B) involves 2862 intersubunit distances. The sedimentation coefficients were therefore obtained with the aid of a PDP 8/I computer which was also programmed to produce diagrammatic representations of the oligomeric structures as a check on the input data. The resulting sedimentation ratios are plotted in fig. 3.

The experimental value of the sedimentation ratio (corrected for concentration dependence) may be compared with the theoretical alternatives as expressed in fig. 3 to yield, in general, a number of combinations of protomer shape and mode of aggregation which fit the experimental hydrodynamic data. Some of the models may be eliminated if the axial ratio of the protomer, treated as an ellipsoid of revolution, is known. This information may be available for example from viscosity measurements, or from the degree of hydration of the protein together with the value of f/f_o for the protomer [11]. Protomers have been treated as assemblages of identical spheres approximating ellipsoids of revolution in this work and therefore to maintain consistency of approach, a contour diagram of protomer axial ratio versus degree of hydration was calculated for a range of values of f/f_o for such protomers. The resulting curves differed so slightly from those originally calculated by Oncley [11] for true ellipsoids of

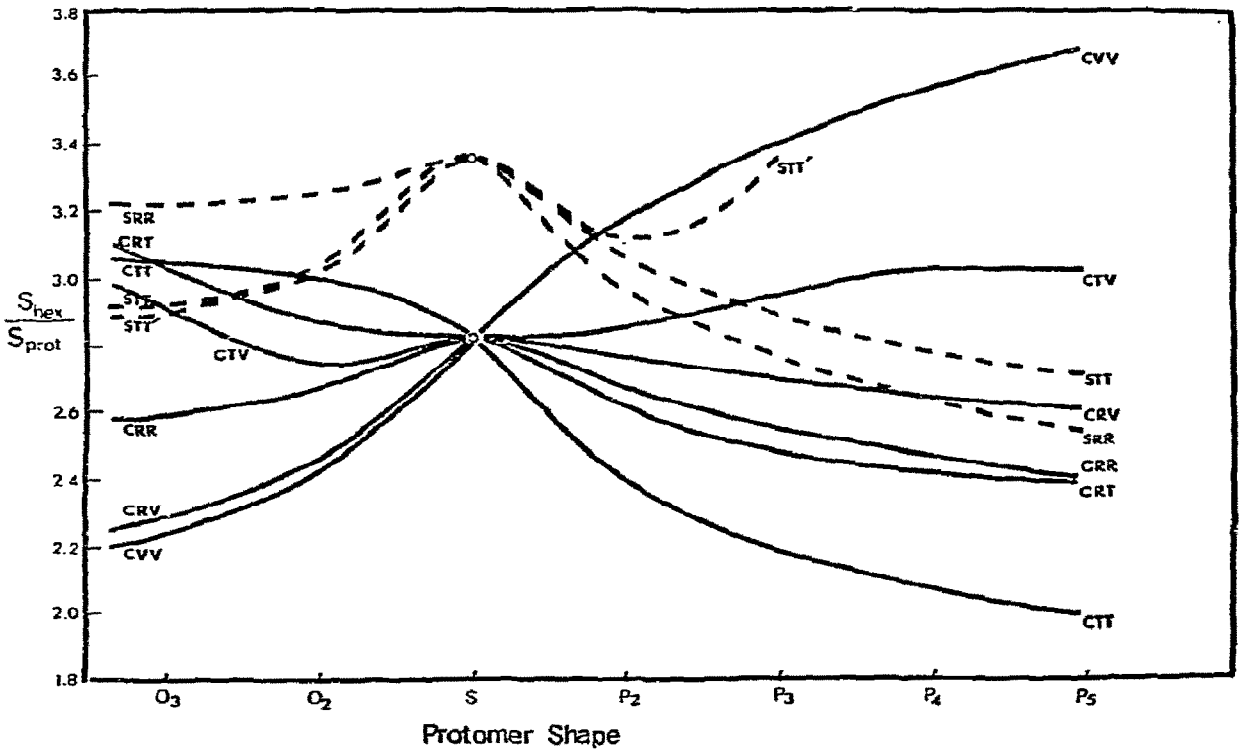

Fig. 3. A plot of the calculated sedimentation ratio versus protomer shape for each of the nine ways of arranging the protomers shown in fig. 2. The dashed lines are for structures with spherical symmetry, the solid lines for those with cyclic symmetry. It is not possible to form the structure STT′ when the axial ratio exceeds 3 : 1. The figure shows that aggregates with spherical symmetry are generally, but not invariably, more compact (i.e. sediment faster) than those with cyclic symmetry. In structures with spherical symmetry, but not in those with cyclic, the aggregate formed from spherical protomers is the most compact with respect to its hydrodynamic behaviour.

Table 1
Dimensions of hexameric structures a)

	Protomer type						
	O3	O2	S	P2	P3	P4	P5
Number of spheres	54	24	6	12	18	24	30
	6.241	5.262	3.722	2.954	2.581	2.345	2.177
CVV	6.082	5.052	3.390	2.690	2.350	2.135	1.982
	0.596	0.782	1.241	1.969	2.580	3.126	3.628
	4.015	3.699	3.722	4.924	6.022	7.034	7.981
CRR	3.856	3.699	3.390	4.396	5.330	6.197	7.009
	1.789	1.563	1.241	0.985	0.860	0.782	0.726
	4.175	3.908	3.722	4.659	5.791	6.825	7.787
CTT	3.696	3.489	3.390	4.659	5.561	6.406	7.204
	1.789	1.563	1.241	0.985	0.860	0.782	0.726
	3.579	3.126	2.481	2.954	3.441	3.908	4.353
STT	3.579	3.126	2.481	2.975	3.344	3.715	4.077
	4.814	4.098	2.995	2.593	2.967	3.334	3.688
	2.633	2.669	2.481	3.362	4.154	4.879	5.556
SRR	2.633	2.669	2.481	3.362	4.154	4.879	5.556
	2.633	2.669	2.995	4.347	5.518	6.576	7.556
	3.579	3.126	2.481	2.954	2.581		
STT'	3.579	3.126	2.481	2.954	2.581		
	4.814	4.098	2.995	2.954	2.581		
	4.350	4.346	3.722	3.770	3.293	3.545	3.804
CTV	4.443	4.259	3.390	3.396	2.967	3.653	4.280
	1.440	1.334	1.241	1.681	2.077	2.440	2.778
	5.476	4.656	3.722	4.347	5.014	5.661	6.281
CRV	5.542	4.804	3.390	3.896	4.457	5.007	5.537
	1.440	1.334	1.241	1.681	2.077	2.440	2.778
	3.579	3.699	3.722	4.660	5.162	6.043	6.530
CRT	3.696	3.699	3.390	4.660	5.329	6.406	7.009
	1.789	1.563	1.241	0.985	0.860	0.782	0.726

a) The three numbers for each structure when multiplied by the cube root in Å of the volume of a particular protein protomer give the dimensions in Å of the hexamer in three mutually perpendicular directions. One of the calculated dimensions is the maximum value for the structure in question.

revolution that his fig. 1 may be used to obtain axial ratios for use in conjunction with fig. 3 without significant error. Further differentiation between model structures which satisfy the $s(o)/s(p)$ data may be made on the basis of a comparison of the dimensions of the various models with those of the oligomer under study. Calculated dimensions for each of the oligomers considered are given in table 1. These values are appropriate to oligomers composed of protomers of unit volume (one cubic Ångstrom). To obtain absolute dimensions for a given oligomer they must be multiplied by the cube root in Ångstrom units of the

protomer volume. The latter can be calculated from the molecular weight and partial specific volume of the protomer. The dimensions so obtained can then be compared with those determined independently, for example from measurements of electron micrographs. Of course if electron micrographs of sufficiently high resolution can be obtained it should be possible to postulate a limited number of structures directly; the present treatment then provides a means of checking which of the structures so derived are compatible with the sedimentation behaviour of the protein.

3. Examples

3.1. Insulin

It should be noted that the treatment given in this paper applies to oligomers in which the protomers occupy equivalent positions and is therefore not always appropriate to hexamers which have been formed via stable intermediates, that is dimers or trimers. The formation of the insulin hexamer is an example of an aggregation proceeding through dimerization to give a structure in which both the dimers and the protomers are equivalently arranged [15] and is therefore amenable to the present analysis. Since the crystal structure is known in detail consideration of the insulin hexamer provides a direct test for the method.

The best values available for $s(p)$ and $s(o)$ for the insulin protomer and hexamer are 1.2 ± 0.07 S [12] and 3.12 ± 0.03 S [13], respectively. Both sedimentation coefficients are values at infinite dilution, and when the stated experimental error is taken into account, yield a range of values for $s(o)/s(p)$ of 2.43–2.79. Application of the measured hydration of 0.25 g of water/g of protein [14] and of the frictional ratio, f/f_o = 1.12 [15] shows that the insulin protomer may be represented as either an oblate or prolate ellipsoid of revolution of axial ratio about 1.5 : 1 [11]. Reference to figs. 3 and 4 shows that these values of the sedimentation and axial ratio indicate that the insulin hexamer is a cyclic structure composed of either oblate ellipsoids of axial ratio 1.5 : 1 arranged in the fashions denoted CRR or CVV, or prolate ellipsoids of the same axial ratio with the structures CRR or CTT, or combinations of these forms. The four major alternatives are shown diagramatically in fig. 5.

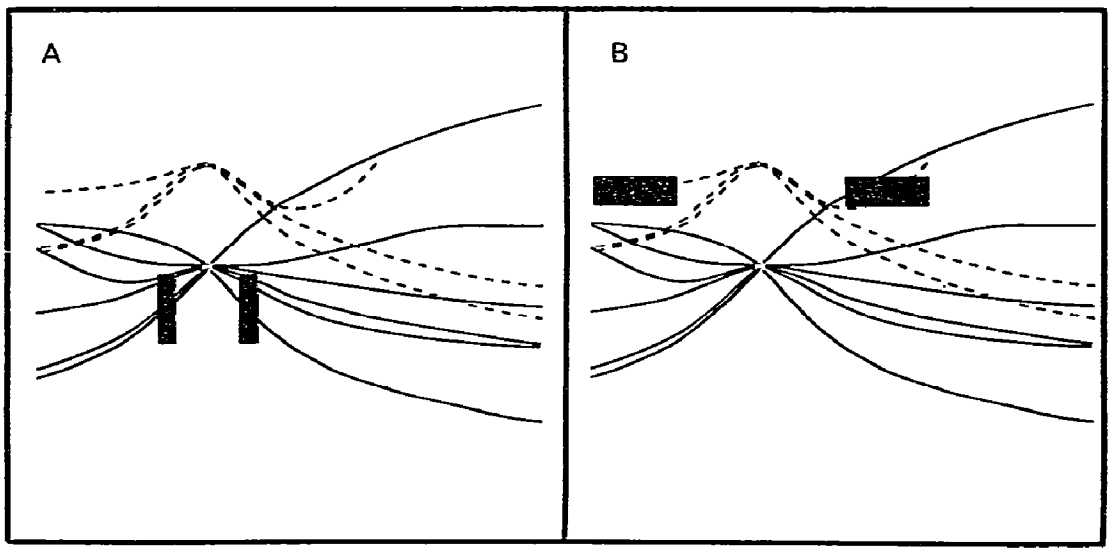

Fig. 4. Hexameric structures consistent with the sedimentation behaviour of A, insulin; B, arthropod hemocyanin. The shaded areas represent the regions of fig. 3. encompassed by the experimental sedimentation coefficients and axial ratios of insulin and hemocyanin, respectively.

The dimensions derived from table 1 for the insulin hexamer structures using a value of 0.73 ml/g for the partial specific volume and 5775 for the molecular weight of the protomer [16] are indicated on fig. 5 together with the structure of the insulin hexamer deduced from X-ray analysis [15]. Although the available hydrodynamic data do not allow selection of a unique structure, the four alternatives are closely related, and the approach may therefore be judged successful in that it suggests that the insulin hexamer is roughly an oblate ellipsoid of dimensions 20–30 Å × 60–80 Å and is formed by cyclic aggregation of the protomers, a description quite consistent with the structures derived by crystallographic analysis.

3.2. *Arthropod hemocyanin*

There seems little doubt that the arthropod hemocyanin aggregate usually referred to as the "16 S" form is a hexamer [17,18] of the species usually referred to as the "5 S" form, and is therefore an appropriate oligomer for study in the present context. If the ratio $s(o)/s(p)$ is formed as 16 S/5 S it has the value 3.20, and while these sedimentation coefficients are only approximate "rounded" values, it appears that the value of the ratio is likely to be close to 3.2 for most arthropod species. For example, the sedimentation coefficients ($S^{\circ}_{20,w}$) measured for the freshwater crayfish, *Cherax destructor*, were 5.3 S and 17.5 S respectively

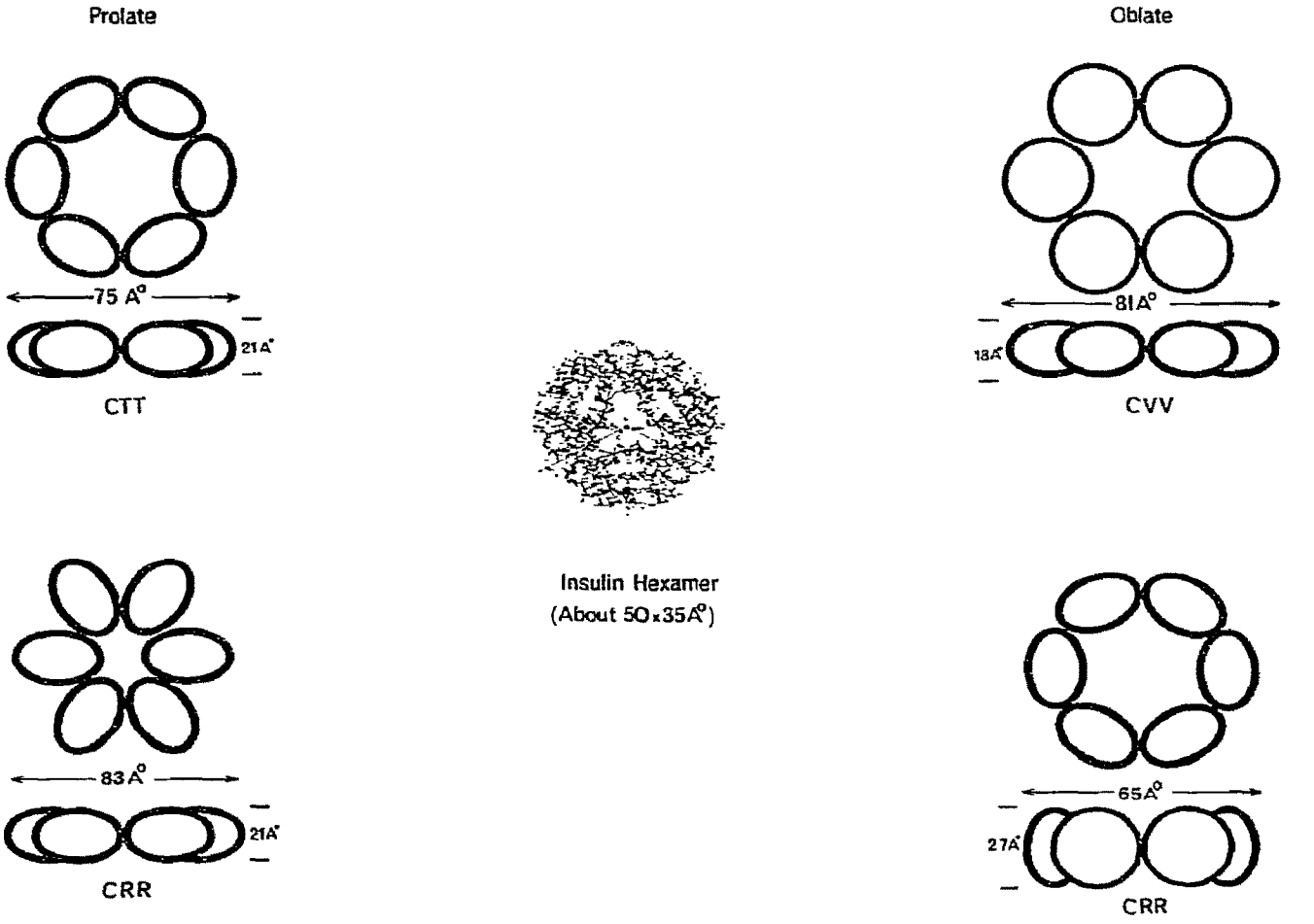

Fig. 5. The possible arrangements of the protomers in the insulin hexamer as deduced from the hydrodynamic analysis. The structure obtained by X-ray crystallography [15] is included for comparison by kind permission of the authors and Academic Press. It is reproduced to the same scale as CRR oblate.

[19] giving $s(o)/s(p) = 3.30$ and for the Dungeness crab *Cancer magister* [20], 5.7 S and 18.0 S, giving $s(o)/s(p) = 3.16$. Accordingly a range of values from 3.15–3.30 is taken to be representative of the sedimentation ratio for arthropod hemocyanins. The hydration of hemocyanin is taken to lie in the range 0.15 g water/g protein [21] – 0.30 g water/g protein, and the frictional ratio f/f_0 as 1.17, the value calculated from the measured sedimentation coefficient of the *Cherax destructor* hemocyanin protomer for which all relevant data were available. With these values it is found that the sedimentation properties of the arthropod hemocyanin protomer may be described in terms of prolate or oblate ellipsoids of revolution of axial ratio 2 : 1 – 3 : 1. This information, together with the previously quoted range of values for $s(o)/s(p)$ and in conjunction with fig. 3, indicates (see fig. 4) that possible modes of aggregation for the hemocyanin hexamer are, for protomers which are oblate ellispoids, the structure denoted SRR, and for protomers which are prolate ellipsoids, the structures denoted CVV and STT'. These structures are shown in diagrammatic form in fig. 6 and their dimensions (calculated from table 1 with a value of $\bar{v} = 0.73$ ml/g and $M_{protomer} = 75000$ [17]) are given in table 2. The hydrodynamic data do not allow of a choice between these three possibilities, but they may be used in combination with electron micrographs to select a structure which is consistent with the results obtained by the two techniques. Electron micrographs of arthropod hemocyanin hexamers do not show good resolution, the ambiguity of interpretation being illustrated by the fact that five different models [22–26] for the structure have been proposed. Nevertheless, there is agreement that in the electron microscope these hexamers present profiles which are hexagons, squares, and rectangles of dimensions about 80–120 Å [17]. The only hydrodynamic structure consistent with this description is that denoted CVV in table 2, that is, a hexagonal prism about 124 Å across and 102 Å deep, formed by the cyclic aggregation of six prolate ellipsoids of axial ratio 2.5 : 1.

Table 2
Structures derived for arthropod hemocyanin hexamers

Oligomer structure	Protomer shape	Appearance and dimensions	
SRR	$O_{2.5}$	Square to circular	119 × 119 Å
		Hexagon	124 Å across
CVV	$P_{2.5}$	Rectangle	124 × 102 Å
		Squarer rectangle	113 × 102 Å
STT'	$P_{2.5}$	Square to circular	124 × 124 Å

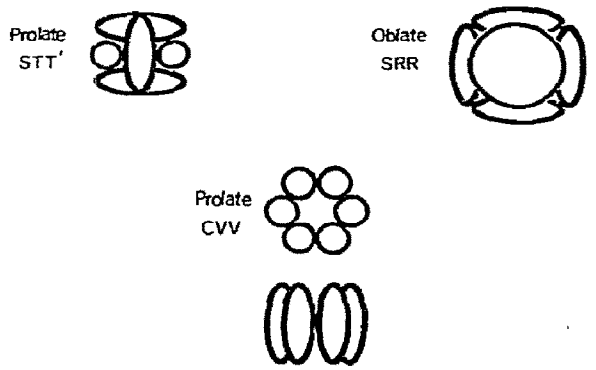

Fig. 6. The possible arrangements of the protomers in the arthropod hemocyanin hexamer as deduced from the hydrodynamic analysis. The structures shown are those formed from protomers of axial ratio 2.5 : 1. That denoted prolate CVV is believed to be the best approximation to the actual protein (see text).

4. Discussion

The mode of aggregation of insulin protomers to form the hexamer, as derived from the sedimentation analysis, is in good agreement with results from X-ray crystallography, and it might be noted that this implies that the structure of the insulin hexamer in solution is the same as that in the wet crystal. It is evident, though, that the dimensions of the hydrodynamic model most nearly resembling the X-ray structure (CRR, oblate) differ from it by about 30%. The differences tend to compensate for each other, the hydrodynamic structure being larger than the X-ray structure in one direction and smaller in the other. The most likely source of this discrepancy resides in the approximation of protomer shapes by groups of spheres or ellipsoids of revolution. These are necessarily crude representations of those of real proteins as is illustrated by the arrangement and shape of the six protomers in the insulin hexamer in the crystallographic studies [15]. A second restriction is revealed by reference to fig. 3 which shows that, for insulin, the

intermediate structures oblate CTV and CRV, and prolate CRV and CRT, would also fulfill the hydrodynamic requirements. The sedimentation characteristics of these particular intermediates were calculated and plotted on fig. 4 to illustrate how such structures could be included in the analysis if required. They were not discussed in connection with the insulin hexamer for which the structures shown in fig. 3, representing extremes, were sufficient. Their possible inclusion, however, serves to re-emphasize that fig. 3 is somewhat artificial, in the sense that it shows the sedimentation ratios of only certain selected structures, those thought to give a practically useful coverage of the possibilities. The blank spaces on the diagram also represent possible structures, each point in the field corresponding to some average of those structures actually plotted. These reservations, which are even more severe in treatments based only on spherical protomers and a restricted set of models, do not imply that the hydrodynamic approach is not useful. Indeed, inspection of fig. 4 shows that a great number of possibilities can be eliminated rather simply by the analysis presented here.

As noted earlier, the models which have been proposed for the quaternary structure of the "16 S" component of arthropod hemocyanin have been based on the appearance of electron micrographs. These are not easy to interpret and the difficulty has been increased by uncertainty in the number of protomers comprising the structure. Thus the models have consisted of twelve units in a truncated tetrahedron [22] twelve units in a hexagonal prism [23], six units in a trigonal antiprism [24], eight units in a cube [25], and a modification of the first model mentioned, with the twelve subunits arranged in pairs in a distorted octahedron [26]. With the knowledge that the "16 S" component is a hexamer [20,27] some of these models obviously become inappropriate. The most likely structure from the present hydrodynamic treatment is a hexagonal prism formed from six protomers of axial ratio about 2.5 : 1. This is very similar to that proposed by di Giamberardino and de Haën [23] for if the twelve subunits in their model are considered in pairs they approximate prolate ellipsoids of axial ratio about 3 : 1 and are arranged cyclically in a hexagonal prism. As in the case of insulin, the dimensions calculated for the hemocyanin structures with ellipsoidal protomers seem to be slightly at variance with those derived from electron micrographs, presumably for the reason advanced earlier. We conclude that on the basis of present knowledge the best approximation to the quarternary structure of arthropod hemocyanin hexamer in solution is the hexagonal prism diagramatically represented as CVV in fig. 5, which is consistent with both the electron microscope and sedimentation velocity results.

It is evident from the foregoing discussion that a fairly good idea of the mode of aggregation of a given oligomer can be obtained from hydrodynamic studies alone, but that results from a combination of techniques are generally required to specify a structure which can confidently be regarded as a good representation of the actual oligomer in solution. The same conclusion is reached by Haschemeyer [28] in his thorough discussion of the problems associated with the application of electron microscopy to the determination of the arrangement of subunits in an aggregated structure. We hope that the treatment of sedimentation results proposed here will be of assistance in this context and recommend that where at all possible such combined studies should be carried out.

Acknowledgement

We would like to thank Academic Press for permission to reproduce the diagram of the crystal structure of the insulin hexamer which appears in fig. 5. We are specially grateful to one of the authors, Dr. G. Dodson, for supplying us with a negative of the diagram for use in this paper.

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