

ELECTRON MICROSCOPE STUDIES ON THE HAEMOGLOBIN MOLECULES

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ABSTRACT

Haemoglobin molecules isolated from normal human subjects have been directly micrographed under the electron microscope following in general Hall's technique. The average height (h) and the widths along (w_{11}) and perpendicular (w_{\perp}) to the shadow direction of the molecules have been measured as 56.5 ± 6.6 A, 122.7 ± 15 A, and 120.9 ± 20 A, respectively. The exaggeration in the molecular widths due to the deposition of metal cap ranges between 60 to 70 A. The probable resolution of the substructure of the molecule, *e.g.*, presence of "holes" and dimples, in the present electron microscopic evidence has been discussed. The electron microscopic results on the size of the individual haemoglobin molecules are in satisfactory agreement with the recent x-ray diffraction model of Perutz and his associates for horse haemoglobin.

The haemoglobin molecule, the carrier of oxygen in blood, has been studied extensively by different authors. Its molecular weight is known very accurately (mol. wt. = 66,700) and the molecular structure has been determined from the x-ray diffraction studies of Bragg, Perutz, and their associates (1). According to these authors, the haemoglobin molecule is ellipsoidal in shape and has dimensions of $53 \times 53 \times 71$ A in the hydrated condition and $45 \times 45 \times 65$ A in the dry condition. Small angle x-ray scattering data are, however, not in very good agreement with the above model. Fournet (2) obtained a radius of gyration, $R = 23$ A, for horse haemoglobin. The radius of gyration corresponding to the x-ray diffraction model of dry haemoglobin molecule is 20 A and the axial ratio is 1.4. X-ray scattering data of Ritland, Kaesberg, and Beeman (3) and Rothwell (4) give an axial ratio of 1.5. The scattering curve of Rothwell (4) corresponds to an ellipsoidal molecule of dimensions $56 \times 56 \times 84$ A. Very recently Perutz *et al.* (5) considered this problem again and obtained a revised model of the molecule. In this

model, the molecule looks like an ellipsoid of dimensions $64 \times 55 \times 50$ A but has a dimple on the surface in the central region and a hole passing right through the molecule. In the present work, attempts have been made to micrograph these molecules directly under the electron microscope, measure the dimensions, and find their agreement with the indirect physicochemical data.

MATERIALS AND METHODS

Oxalated red blood cells, collected by venipuncture from normal human subjects, were washed with cold isotonic saline four times and then hemolysed with twice their volume of distilled water and 0.4 volume of toluene. The hemolysed cells were spun down at 1,000 *g* for 30 minutes and the clear haemoglobin solution was pipetted off. For further clarification, the haemoglobin solution thus obtained was centrifuged at 10,000 *g* for 15 minutes.

The specimens for electron microscopic studies were prepared in general according to Hall's method (6). The purified human haemoglobin solution so obtained was dissolved in a buffer containing 0.05 M ammonium carbonate and 0.1 M ammonium ace-

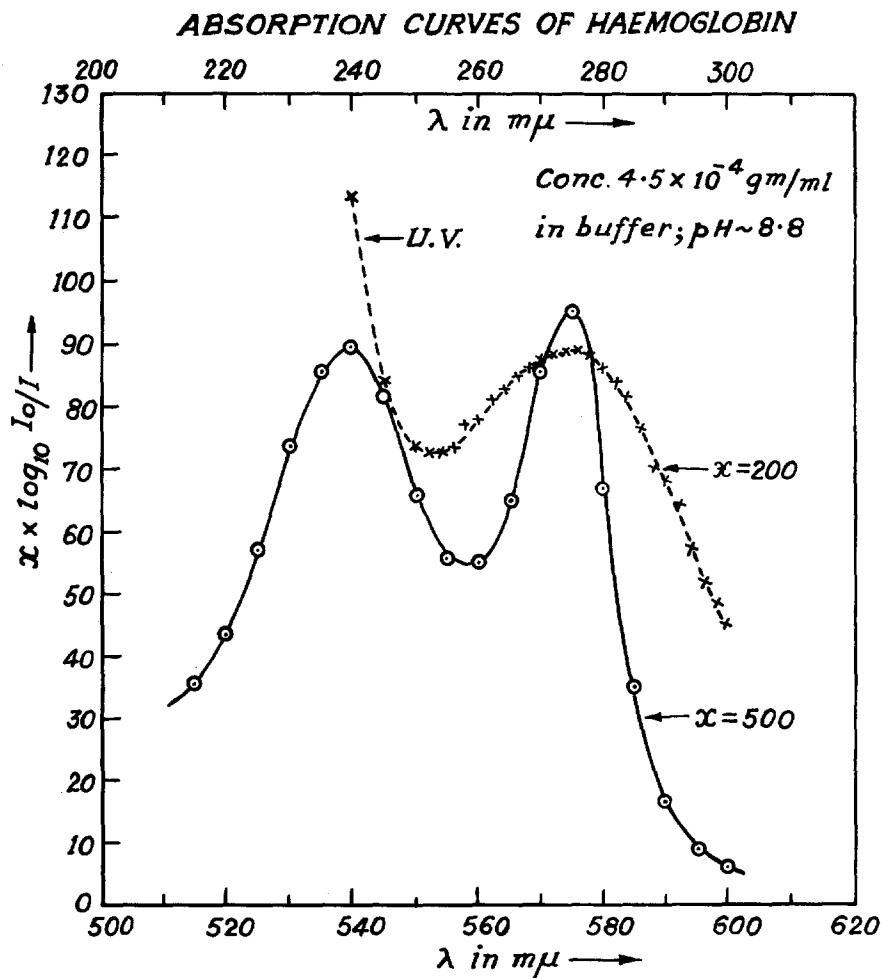


FIGURE 1

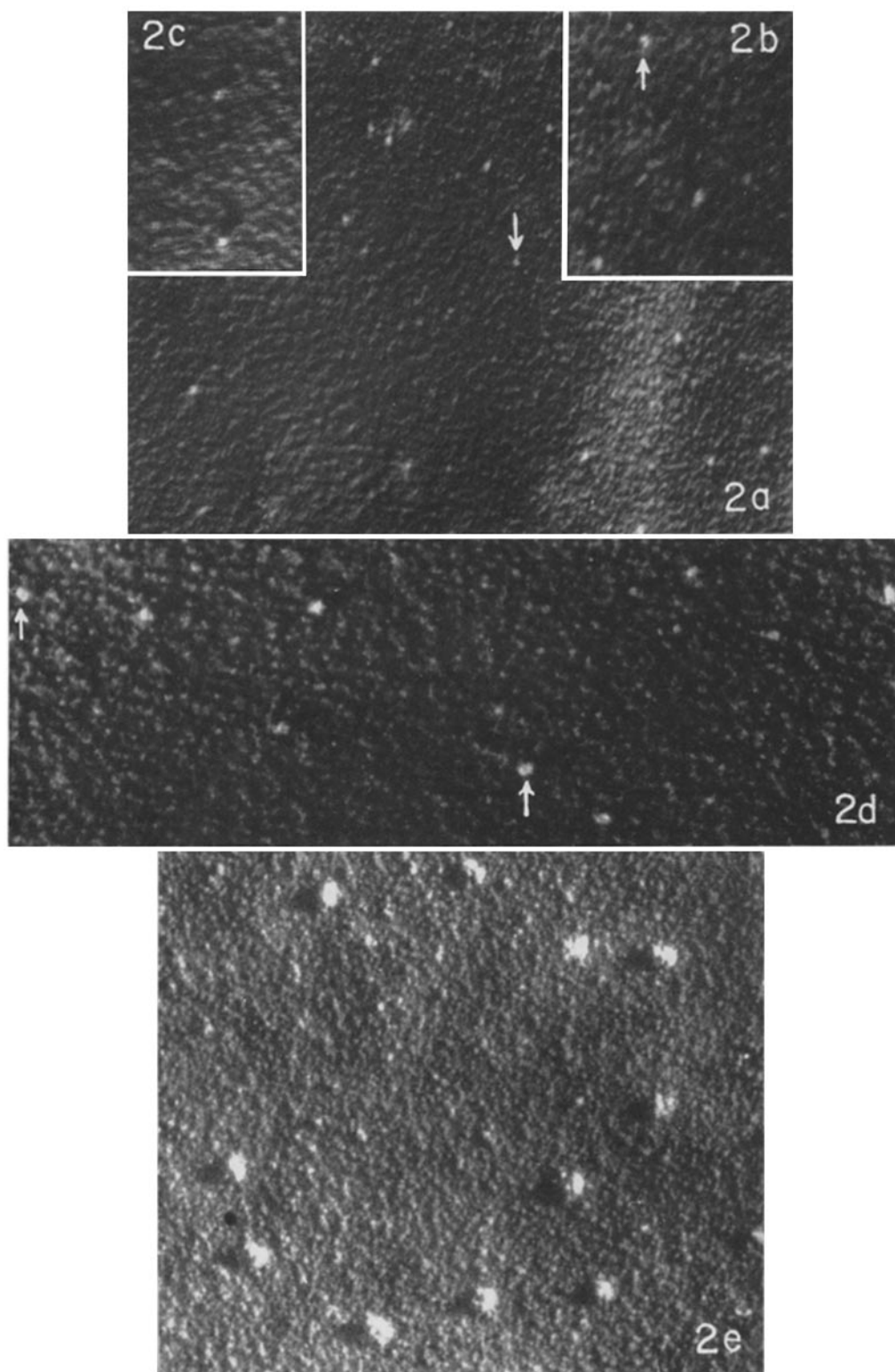
The optical absorption curves of the haemoglobin solution in buffer.

tate to a concentration of 4.5×10^{-4} gm./ml. and sprayed almost instantaneously at room temperature on the freshly cleaved surface of mica with the help of a throat sprayer. The preparation was then shadowed with Pt (25 mm. of 0.2 mm. Pt-wire) at

an estimated shadow-to-height ratio of 10:1. The shadowed samples were then coated with SiO film (1 mg. at 10 cm.) at normal incidence. This was subsequently backed with a collodion film, stripped from the mica surface, floated on water, and picked

FIGURE 2

- a. Electron micrograph of the haemoglobin molecules. Note fine structure at arrow. $\times 60,000$.
- b. Enlarged version of the part of Fig. 2a containing the arrowed particle. $\times 100,000$.
- c. Micrograph of two haemoglobin molecules from a different field, resolving the fine structure. $\times 100,000$.
- d. Micrograph of the haemoglobin molecules showing dark central region (at arrow). $\times 100,000$.
- e. Micrograph showing the agglomerated molecules. $\times 100,000$.



up on specimen grids. The collodion was then dissolved from above by adding drops of amyl acetate. The preparation thus consisted of shadowed haemoglobin molecules on a very thin SiO backing layer. This was micrographed with the help of a Siemens Elmiskop I at an electronic magnification of $20,000\times$ in through-focus series. Measurements were made of the shadow lengths of the particles from the optically enlarged prints and the molecular heights obtained from the known shadow geometry.

Optical absorption data for the above solution were also obtained with the help of Zeiss spectrophotometer model PMQ II. Quartz cells (5 mm.) were used for the ultraviolet region and all measurements were taken with a fixed slit width.

RESULTS AND DISCUSSION

After we had sent a preliminary report of this investigation (7), we recorded several more micrographs and have carefully revised the data. Fig. 1 shows the optical absorption curves of the haemoglobin solution in the said buffer with the

prominent characteristic peaks (Langstroth, 1950). A representative picture of the electron micrographs of the individual molecules of haemoglobin is reproduced in Fig. 2a. In order to have an estimation of the molecular dimensions, the molecular heights were calculated from the measurements of the shadow lengths. The shadow-to-height ratio was calibrated with the help of TMV particles and is obtained as 9:1 compared to the estimated ratio of 10:1. Fig. 3 gives the distribution in the molecular heights as obtained from the present electron microscopic evidence, the number average height being 56.5 ± 6.6 A. The widths of the particles were also measured both along (w_{\parallel}) and perpendicular (w_{\perp}) to the shadow direction. The distribution in the measured values of w_{\parallel} and w_{\perp} are shown in Fig. 4a and b respectively. The number average values of w_{\parallel} and w_{\perp} are 122.7 ± 15 A and 120.9 ± 20 A, respectively. The diameters of the theoretical spheres equivalent to the models of the molecules deduced by different authors on the

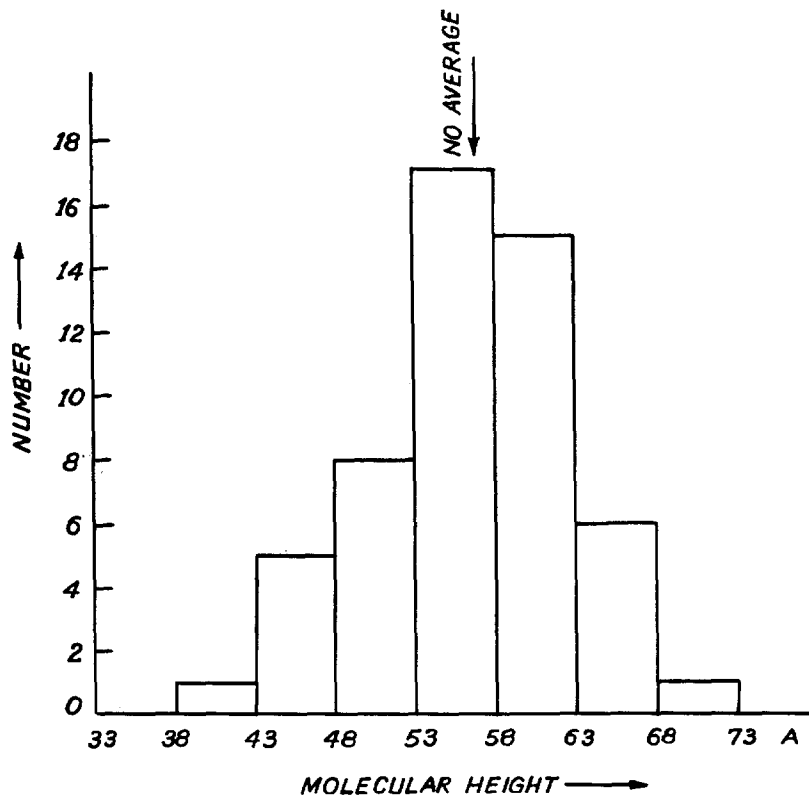


FIGURE 3

Histogram showing the distribution in the molecular heights as obtained from the shadow lengths.

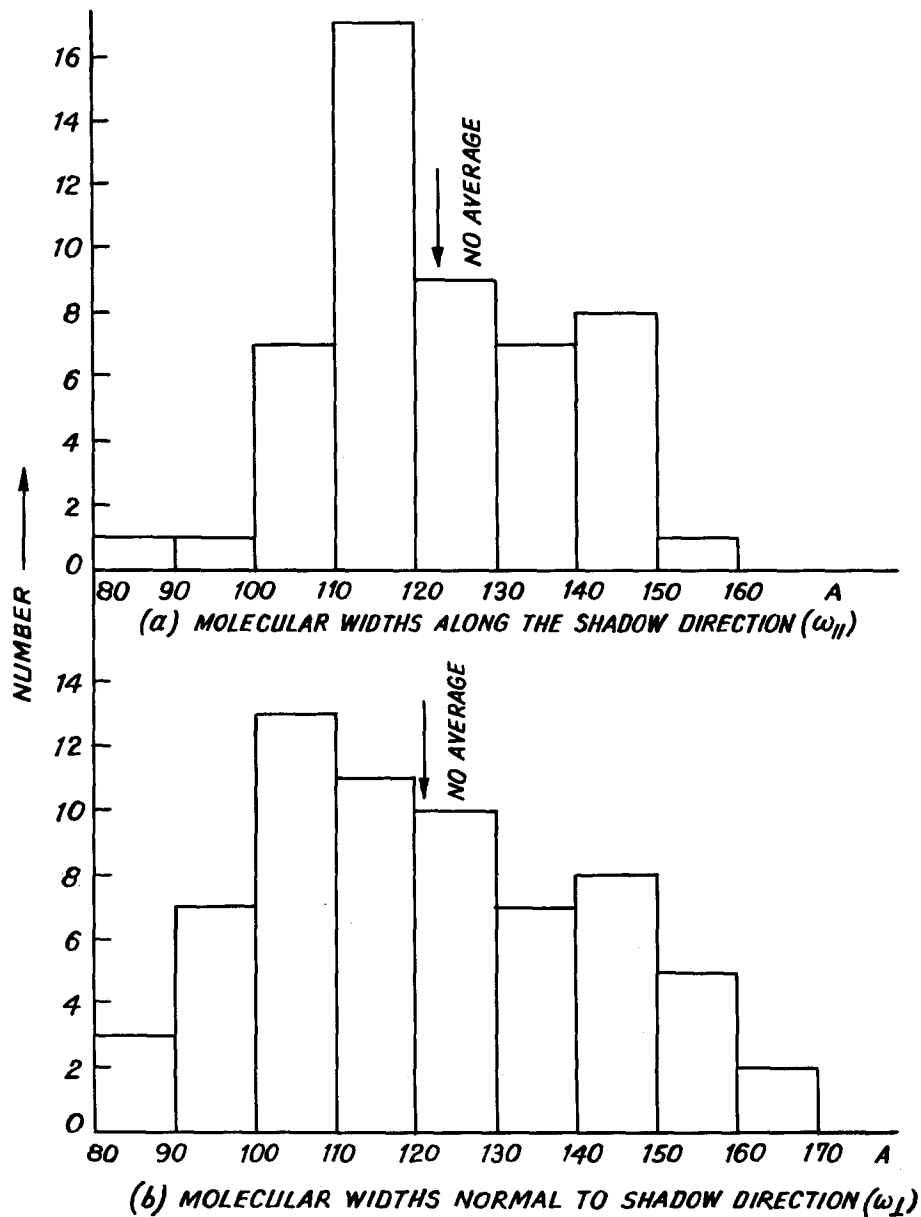


FIGURE 4

Histogram showing the distribution in the molecular widths (a) along and (b) perpendicular to the shadow direction.

basis of the indirect physicochemical methods have been calculated and given in Table I. This will allow a fair comparison to be made of their results with the present electron microscopic evidence. It can be seen from the table that the average value of the molecular height (h) is satis-

factorily close to the diameter of the sphere equivalent to the Perutz model. On the other hand the measured values of the molecular widths (w_{\parallel} and w_{\perp}) are 60 to 70 A larger than the molecular heights (h). This exaggeration in the width is most likely due to the accumulation of the

TABLE I
Size of the Haemoglobin Molecules Obtained by Different Authors

	Electron Microscope Data of the Present Authors*			Diameter of the equivalent sphere for the indirect physicochemical models		
	No. Average	Wt. Average	Peak frequency	Perutz <i>et al.</i> (5)	Rothwell (4)	Fournet (2)
	<i>A</i>	<i>A</i>	<i>A</i>	<i>A</i>	<i>A</i>	<i>A</i>
<i>h</i>	56.5	56.8	55.5			
w_{\perp}	120.9	124.1	105	56	64	59.7
w_{11}	122.7	118.1	115			
$w_{\perp} - h$	64.4	67.3	49.5			
$w_{11} - h$	66.2	61.3	59.5			

* For symbols see the text.

shadowing metal on the molecular surface. Hall (9) has also recently found in the case of a number of globular proteins an exactly equal deformation in the molecular widths. He has also measured the width of the metal cap forming on the ends of the TMV particles lying parallel to the shadow direction and has consistently obtained a similar value. In the light of these considerations it is fairly reasonable to assume that the measured values of h , w_{11} , and w_{\perp} are in satisfactory agreement with one another so as to represent the diam-

eter of the assumed spherical haemoglobin molecule. The deposition of a metal cap on the molecular surface is very undesirable and makes it difficult to have any idea of the slight asymmetry, if present, in the molecular dimensions. It is to be noted, however, that the electron micrographs show slightly different shapes of the molecules. Although practically nothing can be concluded from this, yet it is likely that the molecules are not exactly spherical. The different shapes are probably due to the different relative orientations of the slightly asymmetrical molecules with respect to the shadow geometry. Had the molecules been spherical, the deformed shapes would always be alike.

Some of the particles have been found to exhibit a fine structure (arrow shown in Fig. 2a). This observation may have two possible interpretations: (a) it may be due to a genuine substructure of the molecule, or (b) this apparent fine structure is due to the presence of two molecules very close to each other. As regards the arguments in favour of the first possibility, let us recall the dimple present on the molecular surface in the model put forward by Perutz *et al.* (5). If such a molecule is shadowed at a very low angle, as in the present case, in the direction shown in Fig. 5a, it is evident that the dip region will not receive any metal. Consequently, when such a shadowed molecule is micrographed, that region will be much more transparent to the electrons than the two sides, and hence the image will bear a narrow, central dark line as shown in Fig. 5b. As the shadow direction deviates more and more from the above, the probability of resolving this dip in the final image

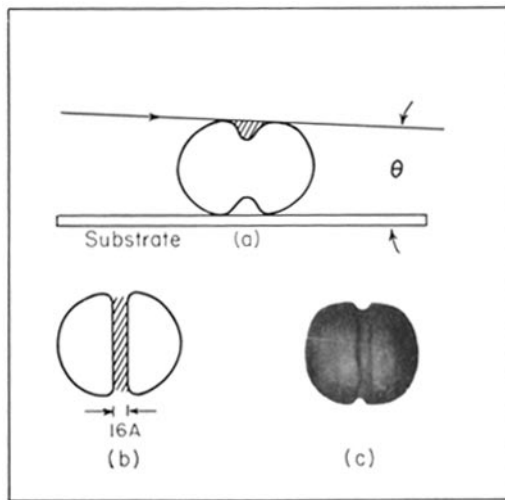


FIGURE 5

a. Schematic diagram of the shadow geometry that will resolve the fine structure. b. Schematic diagram of the molecule in the micrograph resolving the fine structure. c. A possible model of the haemoglobin molecule (not in scale).

becomes smaller and smaller. Fig. 5c shows a possible model (not in scale) of the molecule as evidenced by the electron micrographs. Considering all possible orientations of this molecule on the substrate, the probability of the particular orientation relative to the shadow geometry at which the said fine structure is resolvable becomes extremely small. This can possibly explain why relatively few such particles (about 5 in 50) have been observed in the micrographs. Fig. 2b shows an enlarged version ($\times 100,000$) of the part of Fig. 2a containing the arrowed particle. From this enlarged picture the width of the region at the centre of the particle was measured carefully under a microscope provided with a micrometer eyepiece and was found to be between 10 to 16 Å. This is clearly above the resolution limit of the electron microscope (Siemens Elmiskop I) used (10). Fig. 2c shows two other particles obtained from a different field but exhibiting an almost similar fine structure. As regards the second possibility, a similar fine structure can be expected for two molecules placed very close to each other in an identical shadow geometry. But since the distributions of the molecules in the fields concerned are very sparse, such close associations of two molecules and particularly along the shadow direction in every case are highly unlikely. Moreover, in such cases the shape of such particles would appear more elongated along the shadow direction than perpendicular to it. Since the measured widths of such particles both along and perpendicular to the shadow direction (130 to 150 Å) fall reasonably within the respective distribution curves, it is more likely that this evidence reveals a genuine substructure of the haemoglobin molecule in conformity with the Perutz model.

In addition to the above, the Perutz model postulates a hole passing right through the centre of the molecule. The presence of the hole can be expected to be revealed in the electron micrograph in the form of a dark central spot only in the case of the particular orientation of the molecule in which the axis of the hole coincides more or less with the beam direction. Fig. 2d shows at least two molecules (arrow) exhibiting a comparatively dark central region. Unfortunately, it is very difficult to come to any conclusion from the above evidence, especially because the measured diameter of the dark central region (~ 25 Å) seems to be larger than the dimension of the hole in the

Perutz model. In this figure the molecular widths (w_{\perp} and w_{\parallel}) are also slightly larger (~ 180 Å) than the normal. These probably indicate a loosening in the molecular structure starting from the centre as a preliminary to fragmentation, occurring in the course of specimen preparation. However, the statistical significance of this evidence is difficult to judge as the total number of their incidences, as obtained in the present work, is small (3 in 50).

Some evidence of agglomeration of the molecules in the rather densely populated fields was also observed in the present investigation. Fig. 2e shows a beautiful example of such agglomeration. From the measurement of the shadow lengths it is found that the heights of the agglomerated particles are almost the same and on the average equal to that of a single molecule (~ 56 Å), that is, the agglomeration taking place in a monomolecular layer. This is, however, not the general picture of the agglomeration of the molecules.

In the present work it has thus been possible to demonstrate the individual haemoglobin molecules having dimensions in reasonably good agreement with the recent x-ray diffraction data (5). The observed distribution is most likely due to some real differences in the molecular dimensions and in this respect the electron microscopy has distinct advantages over the indirect physicochemical methods. Unfortunately, the deposition of the metal cap on the molecular surface is rather disturbing but nevertheless we believe, along with Hall (9), that, with due considerations to this, the present electron microscopic technique can give significant information on the molecular dimensions of the globular proteins.

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