CELL INTERFEROMETRY AND THE SPECIFIC REFRACTION INCREMENT OF CRYSTALLINE PROTEINS

I. β-LACTOGLOBULIN

H. G. DAVIES

Medical Research Council, Biophysics Research Unit, King's College, London (Great Britain)

(Received June 26th, 1958)

SUMMARY

The refractive indices of orthorhombic and monoclinic crystals of β-lactoglobulin have been measured by microscope interferometry and found to be 1.4536 ± 0.0015 and 1.4765 ± 0.0015 respectively, these values being for a particular orientation of the light electric vector relative to the crystal axes. Combining these values with data[4] on the dry weight of protein per unit volume of crystals, the specific refraction increments of orthorhombic and monoclinic crystals are calculated to be 0.00196 ± 0.00003 and 0.00191 ± 0.00002 respectively. The values are in fairly good agreement with that of 0.00182 found[8] for dilute solutions of β-lactoglobulin. This conclusion is not altered when the birefringence of the crystals, which has also been measured, is taken into account. Both types of crystal approximate in behaviour to uniaxial crystals with birefringence values of 0.00086 ± 0.00003 and about 0.0036 for orthorhombic and monoclinic crystals respectively.

INTRODUCTION

This investigation into the optical properties of protein crystals arose mainly from an interest in investigating the physical basis of cell interferometry. This is a method[1] for measuring the so-called dry mass of biological cells by means of the interferometer microscope, and it is being used in the study of single cells in much the same way that the balance is used in ordinary analytical chemistry. The subject has recently been reviewed[2]. Cell interferometry is based on the empirical observation that the specific refraction increments (α) of most chemical substances found in cells are very nearly the same and approximately independent of concentration. The accuracy of the method therefore depends on the extent to which α may vary. α is defined as the increase in refractive index of the solution per unit increase in concentration, the latter being expressed in g/100 ml of solution. Proteins constitute the largest fraction of the dry weight of most animal cells, so that the accuracy depends mainly on the behaviour of the proteins. Their specific refraction increments lie between 0.0018 and 0.0019, when they are non-conjugated. When conjugated the values of α depend somewhat on the nature of the substance to which the protein is linked, but usually

References p. 232.

α does not differ appreciably from the above values. However, specific refraction increments of protein are usually measured in dilute solutions, less than 10 g/100 ml, and the values of concentration of dry substance per unit volume in biological material may often considerably exceed this value. Protein crystals in which concentrations may be as high as about 75 g/100 ml have therefore been used here to test the relationship between refractive index and concentration over a wider range than has previously been investigated.

The measurements have been made on β-lactoglobulins, in two crystalline forms, βA and βAB rectangular orthorhombic crystals and βB lozenge-shaped monoclinic crystals. The terms βA and βB refer to the two varieties of protein isolated from cow's milk, which differ slightly in their mobilities upon paper electrophoresis (ASCHAFFENBURG AND DREWRY[3]), βAB refers to mixtures of the two proteins. GREEN, NORTH AND ASCHAFFENBURG[4] conclude from their X-ray studies that these proteins are almost identical, probably differing in composition by only a few amino acids. In order to obtain the specific refraction increments of the crystals it is necessary to know both the refractive index and the concentration of dry substance per unit volume. This latter value can be obtained from the data on density and fractional weight of water in the crystal given by GREEN *et al.*[4]. Hence it is necessary to measure only the refractive indices of the crystals. Since the value of α may be expected to vary somewhat with the direction of the light electric vector relative to the crystal axes, the birefringence has also been measured.

METHODS

The refractive indices of the protein crystals were determined by a method introduced by AMBROSE[5] and by KLUG AND WALKER[6]. The specimen was mounted in its mother liquor between slide and coverslip, separated conveniently by a ring of vaseline. As the coverslip was pressed down, a series of readings of optical path difference (*o.p.d.*) relative to the mother liquor was made through the crystal (Δc) and an air-bubble (Δa) which had been introduced nearby. As the air bubble was deformed so that its thickness approached that of the crystal, the value of the ratio $\Delta c/\Delta a$ approached a maximum which was not affected by a slight deformation of the crystal. The refractive index (n_c) of the crystal is given by $n_m + (n_m - 1)\,\Delta c/\Delta a$, n_m being the refractive index of the mother liquor. The measurements of *o.p.d.* were made in a HUXLEY[7]-type interferometer microscope of numerical aperture 0.20 by measuring the displacement of the interference bands. In this microscope the object is illuminated by two plane polarized wave-trains in which the vibration directions are at right angles. It is therefore to be expected that the value obtained for *o.p.d.* will depend on the orientation of the crystal.

RESULTS

The values of $\Delta c/\Delta a$ obtained for βA and βAB orthorhombic are similar with an average of 0.360 ± 0.0005 (Table I). In this series of measurements the vibration directions in the microscope were always set parallel to an edge in the rectangular crystals. Since the setting was at random part of the variation encountered in $\Delta c/\Delta a$ may be due to the birefringence. The average value of $\Delta c/\Delta a$ for the βB monoclinic

References p. 232.

crystals was 0.420 ± 0.005 (Tables II). In this series the orientation of the crystal was chosen so that its birefringince was small as shown from the measurements in the polarizing microscope. The mother liquors were salt-free and the refractive indices indicated a protein concentration of 0.7% and 1.8% for orthorhombic and monoclinic crystals, respectively. These values are based on the specific refraction increment value of 0.001822 for dilute solutions of β-lactoglobulin (HALWER, NUTTING AND BRICE[8]). The refractive indices calculated from these values are 1.4536 ± 0.0015 and 1.4765 ± 0.0015 for orthorhombic and monoclinic crystals, respectively.

TABLE I

No. of crystal	*Type*	*Δc*	*Δc/Δa*
1	βA orthorhombic	5.4	0.36
2	βA orthorhombic	2.8	0.365
3	βA orthorhombic	8.1	0.34
4	βA orthorhombic	5.6	0.37
5	βAB orthorhombic	2.3	0.38
6	βAB orthorhombic	2.0	0.35
7	βAB orthorhombic	3.3	0.34
8	βAB orthorhombic	2.5	0.37
			Av. 0.360 ± 0.005

TABLE II

No. of crystal	*Type*	*Δc*	*Δc/Δa*
1	βB monoclinic	2.0	0.41
2	βB monoclinic	3.0	0.435
3	βB monoclinic	2.0	0.42
4	βB monoclinic	7.1	0.415
5	βB monoclinic	5.0	0.415
6	βB monoclinic	4.0	0.42
			Av. 0.420 ± 0.005

The crystals are birefringent, which property may be considered to arise in part from an intrinsic birefringence of the protein molecule and partly from form birefringence. The retardation (τ) was measured with the polarizing microscope, employing a Berek compensator. Next, the optical path difference (Δc) of the same crystal was measured in the interferometer microscope. Since the refractive index (n_c) is known from previous data, this enables crystal thickness to be simply computed. The birefringence (δn) is given by $\tau\,(n_c - n_m)/\Delta c$. It will be noted that the value obtained for δn will depend on the way Δc is measured and on the value chosen for n_c. This fact can of course be taken into account; in these experiments the resulting uncertainty in δn is negligible. The optical properties of the orthorhombic crystals (βA) were similar to those those of a uniaxial crystal, with two of the refractive indices equal and a birefringence of 0.00086 ± 0.00003, a value obtained from readings on seven crystals. The optical properties of the monoclinic crystals also approximated to those of a uniaxial crystal with two refractive indices approximately equal and a

References p. 232.

birefringence of about 0.0036. The birefringence of the monoclinic crystals is thus about four times larger than that of the orthorhombic crystals.

The values for concentration of dry substance per unit volume of crystal were calculated to be 61.6 and 75.2 g/100 ml for the orthorhombic and monoclinic crystals, respectively. By combining these values with the data on refractive indices, the values of specific refraction increment for orthorhombic and monoclinic crystals are found to be 0.00196 ± 0.00003 and 0.00191 ± 0.00002 respectively. These values were computed on the assumption that the refractive index of the fluid phase of the crystal is the same as that of water. The values do not differ appreciably if the refractive index of the fluid inside the crystal is taken to be that of the mother liquor. In this respect proteins crystallized in salt solution would be less suitable for the measurement of a, since there would be some uncertainty as to the extent to which the water in the crystal is free to dissolve salt. Due to the birefringence the value of a will vary depending on the direction of the electric vector and the resulting effect can be most simply summarized as follows. In the orthorhombic crystals the spread in the values of a calculated from the birefringence values obtained by the polarizing microscope will be within the limits of error obtained by measurement in the interferometer microscope. Hence the effect of birefringence may be neglected. In the monoclinic crystals, because of the birefringence, there are two different refractive indices which, taking into account the sign of birefringenece, are about 1.4765 and 1.4765— 0.0036, *i.e.* 1.4739. These correspond to values of a which are 0.00191 and 0.00186. It is concluded that the specific refraction increments of the β-lactoglobulins in the crystalline state are in fairly good agreement with the value of 0.001822 obtained[8] in dilute solution.

DISCUSSION

The above data may be compared with that of F. T. JONES[9], who has measured the refractive indices and fractional water content by weight in wet and air-dried crystals of lysozyme chloride. From the experimentally determined value for the water content the dry weight per unit volume can be computed, provided that an assumption is made as to partial specific volume of protein. Assuming the usual value of 0.74 ml/g for the partial specific volume of protein in solution (inverse of density, 1.35 g/ml), the concentrations of dry substance per unit volume of wet and air-dried crystals are 64 and 119 g/100 ml, respectively. That this assumption is reasonably in the case of the wet protein is shown by the fact that when it is used together with the data on fractional water content of the β-lactoglobulins the values obtained for the densities of the wet crystals are within a few per cent of the experimentally determined values. The values calculated for the specific refraction increments of wet and air-dried crystal are 0.00250 and 0.00187 respectively. The value of a for the air-dried crystal will be only about 4% higher if the value of 0.79 (inverse of density 1.27) is taken for the partial specific volume of protein. This may be the correct value to assume for the partial specific volume if reliance can be placed on the data of CHICK AND MARTIN[10] (see however COHN AND EDSALL[11]). Neither are the values of a appreciably altered if assumptions are varied regarding the influence of salt in the mother liquor on the refractive index and water content of the crystal. We may therefore conclude that while the data on air-dried protein indicate a similar factor of proportionality between refractive index and concentration as in dilute solutions,

References p. 232.

the data on the wet crystal are not in agreement. It may be noted that the technique used by JONES in measuring the refractive index of the crystals is open to criticism in that crystals wiped free of mother liquor were measured in oily media which might penetrate the crystal to some extent. Neither were the measurements of water content made on a single crystal as is usual but on a mass of small crystals from which the mother liquor may not have been completely removed by filter paper. This would result in too high a value of a for the wet crystal.

These experiments on β-lactoglobulin suggest that, apart from small changes due to the alignment of protein molecules resulting in birefringence, the proportionality factor between refractive index and concentration is very similar in the crystalline state and in dilute solution. Protein molecules, therefore, do not behave in a manner similar to electrolytes, *e.g.* salts and amino acids, in which refractive index varies non-linearly with concentration in solutions, and changes very markedly upon crystallization. Apparently, protein molecules behave in the manner of non-electrolytes, for example sucrose, in which the factor relating refractive index and concentration is the same in solutions as it is in the water-free crystalline state. However this data cannot be taken to exclude the possibility of some alteration in the specific refraction increment of protein upon denaturation during cellular fixation, as discussed in detail elsewhere[2].

ACKNOWLEDGEMENTS

I wish to thank Professor J. T. RANDALL, F.R.S., for his encouragement and for providing facilities for the work. I am most grateful to Dr. R. ASCHAFFENBURG for providing me with the crystals of β-lactoglobulin and to Dr. P. M. B. WALKER for permitting met to use his mechanical device for controlling the pressure of coverslip onto air bubble. I also wish to thank Dr. M. H. F. WILKINS and Dr. A. KLUG for helpful discussion.

REFERENCES

1 H. G. DAVIES AND M. H. F. WILKINS, *Nature*, 169 (1952) 541.
2 H. G. DAVIES, in J. F. DANIELLI, *General Cytochemical Methods*, Academic Press, New York, 1958.
3 R. ASCHAFFENBURG AND J. DREWRY, *Nature*, 180 (1957) 376.
4 D. W. GREEN, A. C. T. NORTH AND R. ASCHAFFENBURG, *Biochim. Biophys. Acta*, 21 (1956) 583.
5 E. J. AMBROSE, private communication.
6 A. KLUG AND P. M. B. WALKER, private communication.
7 A. F. HUXLEY, *J. Physiol.*, 117 (1952) 52.
8 M. HALWER, G. C. NUTTING AND B. A. BRICE, *J. Am. Chem. Soc.*, 73 (1951) 2786.
9 F. T. JONES, *J. Am. Chem. Soc.*, 68 (1946) 854.
10 H. CHICK AND J. MARTIN, *Biochem. J.*, 7 (1913) 92.
11 E. J. COHN AND J. T. EDSALL, *Proteins, Amino Acids and Peptides*, Reinhold, New York, 1943, p. 378.