Refractometry of Living Cells

Part I. Basic Principles

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With one plate (fig. 1)

SUMMARY

The principles underlying a new method of refractometry of living cells are discussed. The method was evolved from the chance observation that the amoebocytes of the blood of the earthworm, examined in their own blood, appeared bright instead of dark by positive phase-contrast microscopy. This was shown to be due to the presence of dissolved haemoglobin which raised the refractive index of the medium above that of the cytoplasm. In order to determine the refractive index of the latter it was only necessary to dilute the blood until the cytoplasm became virtually invisible. Non-pigmented proteins and other high molecular weight substances have now been substituted for haemoglobin.

The nature of the initial observations suggested that if the cell could be regarded to a first approximation as being composed entirely of proteins, the cytoplasmic protein concentration could be equated to the protein concentration of the immersion medium which made the cell appear with minimum contrast. This would only be true if equal concentrations of different proteins in solution had the same refractive index. The nature of refractive index and its relationship to density are discussed and it is shown that for nearly all unconjugated proteins so far investigated the specific refraction increments (i.e. the increase in refractive index per 1 per cent. increase in concentration) have almost the same values ($\cdot 00185 \pm 2$ per cent.). The effects of many factors such as pH, salts, temperature, wavelength, concentration, and nature of the solvent are discussed. Since living cells contain substances other than proteins the specific refraction increments of protein derivatives, lipides, carbohydrates, and salts are considered and it is shown that the presence of moderate amounts of such substances is unlikely to affect the refractive index of cells to any great extent. It is suggested that the mean specific refraction increment of protoplasm should be taken as $\cdot 0018$ and that this value can be used in order to calculate the solid and water content of protoplasm from values of refractive index.

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INTRODUCTION

VERY little work has been carried out in the past on the measurement of the refractive index of living cells. This is surprising in view of the numerous investigations of other physical properties of protoplasm such as viscosity, elasticity, density, absorption of light of different wavelengths, &c. Even in the case of the few values which have been obtained no attempt appears to have been made to interpret the results, and the latter were usually quoted as bare figures for the refractive index which seemed to have little meaning in themselves. With the advent of phase-contrast microscopy, interest in refractometry revived somewhat, as it was soon observed that the appearance of many objects varies greatly with the refractive index of the mounting medium. Investigations on fixed and dehydrated tissue sections showed that the refractive index of such materials was usually about 1·53–1·54 (Crossmon, 1949). This value was determined by examining the specimen by phase-contrast microscopy, using mounting media of different refractive indices. When the refractive index of the medium matched that of the tissue, the latter became virtually invisible. This technique is essentially the well-known immersion method which has long been used in mineralogy and crystallography. It did not appear possible to use immersion methods for measurements on living as opposed to dehydrated cells, because most of the common immersion media were incompatible with viability. Some biologists apparently imagined that the values obtained for tissue sections could be applied to living protoplasm without realizing that the considerable hydration of the latter must inevitably lower its refractive index. This was perhaps another reason why the search for suitable immersion media was not pursued more energetically, as aqueous media with a refractive index exceeding 1·50 are comparatively uncommon.

The development of interference microscopy, which in some cases can be used for the quantitative determination of optical path difference, at first sight seemed to offer a solution to the problem, but since the optical path difference depends on the *product* of refractive index and thickness, the refractive index itself cannot be measured without some method of determining the thickness of living cells. This determination is subject to very considerable errors.

The method which we have developed depends on a purely chance observa-

tion which not only indicated how the problem might be solved but suggested a simple practical interpretation. Both the method and the interpretation may seem obvious now, but they were not so at the time.

The initial observation was made at the beginning of 1952 during an examination of various types of amoebocytes with the phase-contrast microscope (Barer and Ross, 1952). The purpose of this investigation was in no way concerned with the measurement of refractive index, but was an attempt to find suitable material for studying the action of ultra-violet light on living cells. It was thought that the free-living amoebocytes in earthworm blood might provide a readily available source of suitable material. Blood was therefore drawn from the region of the hearts by means of a fine pipette and examined by phase-contrast microscopy. At first sight the appearance of many of the amoebocytes was disappointing as the contrast seemed to be unexpectedly low. We then observed to our surprise that the cytoplasm of many of the cells appeared *bright* instead of dark when a positive phase-contrast objective was used (fig. 1). Under the conditions of the experiment the only reasonable explanation seemed to be that the refractive index of the cytoplasm was less than that of the surrounding fluid. This was astonishing to us, as it was assumed at first that the refractive index of the latter was roughly the same as that of water. On further consideration, however, it was realized that unlike mammalian blood, earthworm blood contains *dissolved* haemoglobin, and that if the latter were present in sufficient concentration it might conceivably raise the refractive index of the medium above that of the amoebocyte cytoplasm. Simple experiments showed that this explanation was correct. On running in isotonic saline solution beneath the coverslip, the cytoplasm gradually faded, disappeared, and then became progressively darker, until after several changes of saline solution it remained constant in appearance and showed the dark contrast normally associated with other types of amoebocyte when viewed by positive phase-contrast. It was therefore clear that this method could be used to determine the refractive index of the cytoplasm of these cells; all one had to do in principle was to dilute the blood with a suitable saline solution until the cytoplasm, as far as one could judge, disappeared by phase-contrast microscopy. At the same time a possible interpretation of the meaning of refractive index suggested itself. If the cell could be regarded in a first approximation as being composed entirely of protein, could it be assumed that at the disappearance point the concentration of protein in the immersion medium was equal to that within the cytoplasm? Further experiments and a detailed search of the literature showed that this was in fact a reasonable assumption. Aqueous solutions of all proteins so far investigated have very nearly the same refractive indices for the same concentration. Moreover, other substances likely to be present in protoplasm, particularly lipides and carbohydrates, would for reasons discussed below not be expected greatly to affect this basic conclusion. In fact *the refractive index of a living cell can be taken as a measure of the total organic solids present.* The evidence for this statement will be discussed in detail later. It was also

apparent that if the concentration of solids could be determined, that of water could be obtained by simple subtraction. In other words, a comparatively simple measurement under the microscope could yield quantitative information concerning two very important cellular constituents.

Having established the feasibility of the method, a search was carried out for more suitable immersion media than haemoglobin solutions. Although haemoglobin is not difficult to prepare, and large quantities are readily available, its deep colour may be a disadvantage and, so far as is known, sufficiently pure material is not commercially obtainable. A colourless commercially available protein was obviously preferable, and we were fortunate in finding at once a material which proved to be suitable. This was Armour's bovine plasma albumin, fraction V, which is sold in the form of a dry powder. Crystalline bovine plasma albumin is also produced by the same firm, but does not show any special advantages and is much more expensive. Many other substances have been investigated as possible substitutes for plasma albumin, and although one or two appear to be promising for certain purposes, none has been so universally successful. Even plasma albumin, however, suffers from certain disadvantages, which will be mentioned later, and it is hoped that a more suitable substance will eventually be discovered.

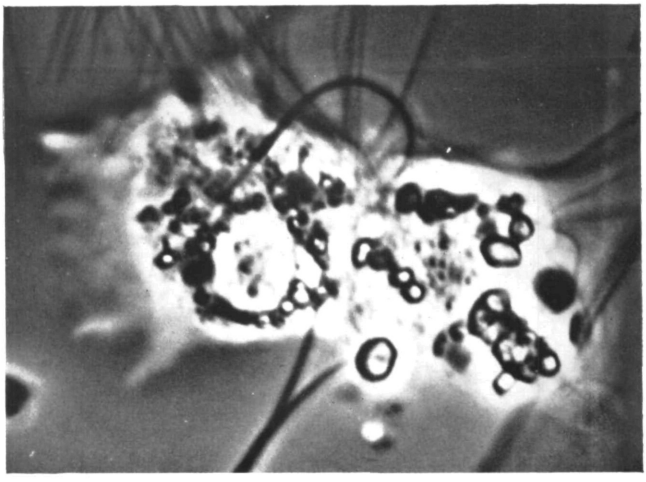
As the technique and interpretation of the results are quite new, we have felt that a detailed consideration of the practical methods and the various difficulties associated with them, together with a discussion of the underlying fundamental principles, would be useful to workers in many fields in which the method is applicable. This paper is therefore concerned with general principles rather than with detailed results. The latter will be described elsewhere in relation to the various problems investigated. Some indication of the results will, however, be given in order to compare them with the figures for solid and water concentration obtained by other methods (see also Barer, Ross, and Tkaczyk, 1953).

The length of this communication makes it necessary to publish it in separate parts. The first part is concerned with fundamental principles. Later parts will deal with the properties of the immersion medium, technical methods and precautions, interpretation of results in terms of solid and water content of cells, estimation of errors, and comparison with other methods.

Remarks on the Theory of Refraction

Many aspects of the theory of refraction of electromagnetic waves are well understood, and the subject has been investigated mathematically both by

Fig. 1 (plate). Positive phase-contrast photographs of a living earthworm amoebocyte in an isotonic solution containing 16 per cent. bovine plasma albumin. This cell was originally observed in the animal's own blood, and appeared faintly reversed in contrast. The blood was then replaced by the protein solution and the cytoplasm has become strongly reversed. The reversal is seen in both photomicrographs. These pictures are from a series showing the cell in active motion and apparently in good health. Note how the disposition of the inclusion-bodies changes during movement. The fibrous structures around the cell are spermatozoa with which the preparation became contaminated.



10 μ

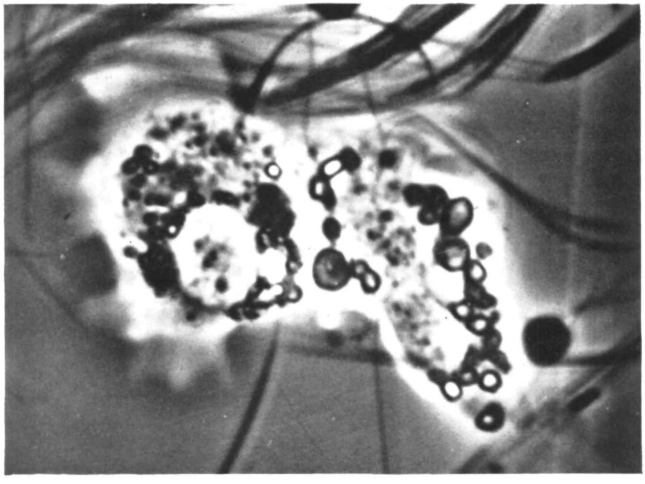


FIG. 1

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classical Electromagnetic Theory and by Quantum Theory. The results are essentially identical. Unfortunately the theory is rather complex and it is difficult to give a satisfactory explanation based on simple physical concepts alone. The remarks which follow must not be regarded as a rigorous treatment of the subject, but may be helpful in indicating in an approximate manner the sort of factors which are responsible for the refractile properties of different substances.

According to classical theory an electromagnetic wave falling on an atom or molecule may set the whole molecule and its electron shells into vibration. In the case of ultra-violet and visible light it is mainly the outer electrons which are made to vibrate. These do so with the same frequency as the incident wave, but in general with a different phase. The vibrating electrons emit secondary light waves. The secondary waves interfere destructively with each other in a lateral direction, but in the original forward direction they do not disappear but combine with the primary wave, altering its phase and in this way changing the wave velocity. The ratio of the velocity of light *in vacuo* to its velocity through a transparent substance is defined as the refractive index n. The phase of the forced vibrations which the electrons undergo depends on the frequency of the incident light wave, so that the refractive index will also depend on this frequency and hence on the wavelength.

Refractive index and density

We can see in a general way that the refractive index of a substance is related to its density. A light wave passing through an empty space will encounter no atoms, and it will proceed with unchanged velocity. If the same space is now partially filled with atoms or molecules, the incident wave will interact with these as described above, so that its velocity will be reduced. It is reasonable to assume that since the reduction in velocity is the result of the interaction between the light and atoms in its path, the refractive index will depend on the number of the latter per unit length, or in other words on the density. The difference in optical path due to the presence of matter depends on $n-1$, so that the quantity $\frac{n-1}{d}$, where d is the density, might be expected to be approximately constant. This was in fact suggested and verified experimentally by Gladstone and Dale (1858, 1863). For a given wavelength they found that $\frac{n-1}{d}$ was almost independent of temperature for many liquids. This empirical relationship was given a sounder theoretical basis by H. A. Lorentz (1880) and L. V. Lorenz (1880), who calculated independently from the electromagnetic theory of light that the quantity $\frac{n^2-1}{n^2+2}\cdot\frac{1}{d}$ should be a constant. This expression can be written in the form $\frac{n-1}{d}\left(\frac{n+1}{n^2+2}\right)$, which is

simply the Gladstone–Dale expression multiplied by the term in brackets. The latter only varies slowly with n. Thus it falls from ·62 when $n = 1{\cdot}3$, to ·57 when $n = 1{\cdot}6$, a range of n covering most common liquids. Hence the Lorentz–Lorenz relationship approximates to the simpler Gladstone–Dale formula. In fact both formulae express the behaviour of many substances quite well and the quantity R defined as

$$R = (n-1).\frac{M}{d}$$

or

$$R = \frac{n^2-1}{n^2+2}.\frac{M}{d},$$

which is known as the molecular refractivity, is a characteristic property of a given molecule (M = molecular weight). The Lorentz–Lorenz formula is usually preferred to the Gladstone–Dale one because it is assumed to have a sounder theoretical foundation, but it must be remembered that both are approximations, and, indeed, in a few cases it has been shown that the Gladstone–Dale expression fits the experimental facts rather better. One must beware therefore of ascribing a greater validity to the Lorentz–Lorenz relationship than it really possesses. The assumptions on which it rests have been critically examined by Böttcher (1952), Oster (1949), and others. Another relationship based on theoretical considerations which attempt to eliminate some of the difficulties inherent in the Lorentz–Lorenz theory is that of Onsager (1936). His formula is

$$\frac{(n^2-1)(2n^2+1)}{9n^2}.\frac{1}{d} = \text{const.}$$

This can be written in the form

$$\frac{n-1}{d}\left[\frac{(n+1)(2n^2+1)}{9n^2}\right] = \text{const.}$$

This more complicated formula again approximates to the simple Gladstone–Dale relationship, and, indeed, the quantity in square brackets changes extremely slowly with n. Thus it varies only from ·662 to ·694 as n varies from 1·3 to 1·6. Onsager's formula can thus be regarded as the theoretical justification of the Gladstone–Dale relationship, and it has been shown to fit many experimental facts very well (Oster, 1949).

One of the assumptions on which the Lorentz–Lorenz relationship is based is interesting in that it gives us a further physical insight into the nature of refraction. This assumption is that the molecules of a substance are conducting spheres. According to theory it turns out that if n is measured at very long (strictly infinite) wavelengths, the molecular refractivity is nothing more than the actual volume of these spherical molecules in one mol of the substance. This is, of course, only a comparatively crude approximation for real sub-

stances, but it is nevertheless a useful concept. It suggests, for example, that if we imagine a vessel containing given numbers of atoms of different kinds the refractivity of the contents of the vessel will be independent of the way in which these atoms are combined, assuming that the volume they occupy will not be very much changed in different combinations. Also, if we changed one sort of atom for another having about the same volume, there would again be little change in refractivity. If only a small proportion of the atoms present were changed for others of a very different size, the refractivity would again not be greatly affected. These predictions are with certain important exceptions roughly borne out in practice. It has been shown that the molecular refraction is an additive property. By measuring the refractive indices of a large number of substances it has been possible to deduce atomic refractivities for different types of atoms and in this way to predict the molecular refractivity of many substances. In some cases the agreement between theory and experiment is remarkably good. Experience has shown, however, that atomic refractivities do in some cases depend on the way in which an atom is bound to its neighbours. Thus the atomic refractivity of oxygen in a carbonyl group is nearly 50 per cent. greater than that of oxygen in a hydroxyl group. The presence of double and treble bonds raises the molecular refractivity, and the influence of conjugated double bonds is particularly strong in this respect. Many substances which are visibly pigmented contain groups of this type, and show so-called 'anomalous' dispersion or refraction in the region of an absorption band (see Wood, 1934; Ditchburn, 1952). In regions remote from absorption bands the refractive index changes comparatively slowly with wavelength.

These principles lead to certain important conclusions when applied to organic substances, especially those of high molecular weight. Since most fairly large organic molecules are composed mainly of carbon, hydrogen, nitrogen, and oxygen, one might not expect very great differences in refractile properties provided that not many unsaturated groups are present. This is roughly true, and gross departures only occur when atoms such as sulphur or iodine, for example, are introduced into a molecule. In the case of extremely large molecules such as the proteins even quite big differences in the amino-acid composition would be unlikely to affect the refractive index significantly. The unpigmented proteins show no specific absorption bands in the visible region, so that anomalous refraction is only to be expected in the ultra-violet (at about 280 mμ) where the absorption due to the aromatic amino acids becomes manifest. In the visible region, therefore, all proteins would be expected to have roughly the same refractive indexes.

The concept of specific refraction increment

If we consider the Gladstone–Dale relationship in the form $\frac{n-1}{d} = \text{constant}$, it is possible to derive a formula connecting refractive index and concentration. If the density of a solution can be taken as varying linearly with

the concentration of dissolved substance, the Gladstone–Dale formula can be written

$$n = K+\alpha C,$$

where n is the refractive index of the solution whose concentration is C, and K and α are constants. By writing $C = 0$, we see that $K = n_m$, the refractive index of the pure solvent. Hence

$$n-n_m = \alpha C.$$

This formula has been found to hold experimentally for a large number of different substances, *provided that the concentration C is measured in terms of weight per unit volume*, i.e. grams of solute per 100 ml. of solution. α then becomes the increase in refractive index of the solution for every 1 per cent. increase in concentration, and is known as the *specific refraction increment*.

The question sometimes arises whether, given the value of α, we can calculate by extrapolation the refractive index of the pure dry solute. It is important to observe that this cannot be done simply by substituting $C = 100$ in the formula, as might appear at first sight. C is the number of grams dissolved in 100 ml. of solution and 1 gram of a substance may occupy more or less than 1 c.c., depending on its density. In the case of proteins in solution, for example, the density can be taken as approximately 1·33, so that 133 grams of protein would occupy 100 ml. of 'solid solution'. On this basis the refractive index of dried protein should be given by $n = n_m+133\alpha$. Taking n_m as the refractive index of water, namely, 1·333, and α as ·00185 (in water), the refractive index of pure protein should be approximately 1·58. This is in quite good agreement with many of the published figures for the refractive indices of protein crystals, though exact comparison is difficult because such crystals are often strongly birefringent and the refractive index varies with the axis of measurement. The refractive index of pure proteins can also be calculated by extrapolation, by using the Lorentz–Lorenz relationship. This has been done by Putzuys and Brosteaux (1936), who found that the refractive index of most proteins was approximately 1·60. This is only a little higher than the value given by the Gladstone–Dale formula and it is not in fact in any better agreement with the rather scanty published results. It is rather remarkable that both formulae give figures which are higher than the refractive indexes of dried protein fibres and gels, which are generally stated to have a refractive index of about 1·53 to 1·54. The exact reason for this discrepancy is not known. It may be that the so-called 'dry' gels and fibres still contain a considerable amount of water, though one might expect the same to hold in protein crystals. Another possible factor is that we have considered the density of proteins to be the same in the dissolved state as in the solid state. This may not be the case, and the density may differ in dried gels or crystals. These are difficult theoretical problems, but it is clear that values for refractive indices of solids obtained by extrapolation must be regarded with some caution. The same applies to the reverse procedure of attempting to derive the

value of α from a knowledge of the refractive index of a solid. This is illustrated in a note published by Davies and Wilkins (1952). The value of α measured for dilute solutions of tobacco mosaic virus is ·0017, whereas that calculated from the refractive index of the dry solid (1·534) is ·0015. The discrepancy in the case of inorganic salts is even greater. Thus for aqueous solutions of sodium chloride $\alpha = ·00163$, whereas the value calculated from the properties of the solid is only ·00097. The corresponding figures for calcium chloride are ·0021 and ·00075. In view of these facts it is evident that the calculation of α from the properties of the solid is highly dangerous, and it is essential to rely only on results which have been determined experimentally for solutions.

The refraction increment of protein solutions

Refractometry was suggested as a method for estimating protein concentration as early as 1903 by Reiss. He was particularly concerned with the estimation of blood serum proteins, and obtained values of α of ·00183 for serum albumin and ·00224 and ·00230 for two different pseudoglobulin fractions. The following decade saw a great expansion of work on the subject, due to the influence of T. B. Robertson, who investigated the refractile properties of many proteins and used refractometry for measuring the concentration of various protein constituents of the blood. A summary of this work and references to the considerable early literature on the problem will be found in Robertson's book on the physical chemistry of proteins (1918). Robertson's work was detailed and careful and he undoubtedly laid the foundations for future exact work in this field. He was, however, hampered by the comparative crudity of methods for separating and purifying proteins which were available in his day, so that many of his samples were probably not very pure by modern standards. Nevertheless, the values he obtained for the refraction increments of several proteins are quite close to those obtained by modern workers. In addition he investigated many basic problems such as the effects of concentration, temperature, and pH.

Modern work on the subject may be said to have been instituted by Adair and Robinson in 1930. They introduced two main advances, namely, the use of more highly purified samples and the application of the Donnan equilibrium theory to calculate the salt concentration in solutions of proteins purified by dialysis. This is particularly important in the case of those proteins which are only soluble in the presence of salts, for the Donnan effect results in an unequal distribution of ions between the protein and its solvent. In such cases Adair and his collaborators have distinguished between α, the refraction increment in water, and α', the refraction increment in the presence of a Donnan distribution of ions. In general, α and α' differ by only a very small amount. In their work on horse serum albumin, Adair and Robinson used material which had been recrystallized several times and dialysed for varying periods. They found that between 14·7° and 20·1° C. and over the concentration range 2·9–8·47 per cent. α was essentially constant, the extreme values being ·001816 and ·001852, with a mean of ·0018295.

In the next twenty years steady improvements were made in the separation and purification of proteins, particularly by means of ultracentrifugation and electrophoresis. A knowledge of α is important for quantitative work by these methods, as it is also for the determination of molecular weights by light-scattering measurements in which the value of α^2 is used. These methods have thus stimulated interest in work on refractometry of proteins and allied substances.

It will be convenient at this stage to tabulate some of the main results obtained by different workers and to discuss the influence of various factors. In a few cases details such as pH, concentration range, temperature, and wavelength used are given, but unfortunately such facts are not always stated in published work, and slight discrepancies in results may be due to diverse experimental conditions. Another important cause of differences is that there is considerable variation in the method of estimating the protein concentration. Some authors have used dry weight measurements, others have used weight of crystalline protein, and many have estimated the nitrogen concentration by Kjeldahl's method. In the latter, in order to convert the nitrogen content to protein concentration, it is necessary to use a conversion factor which varies slightly for different proteins and whose value is not very precisely known even for a given protein. When, however, due allowance is made for these various factors, it is found that the results for a given protein obtained by different authors show very good agreement. For example, some of the values obtained by Halwer and others (1951) differ from those of Perlmann and Longsworth (1949) by nearly 2 per cent., even when differences of temperature and wavelength are taken into account. The former workers estimated concentration by drying samples to a constant weight, whereas the latter used Kjeldahl nitrogen determinations.

If we ignore the figure for total human serum, which contains substances besides proteins, the lowest value for α in table 1 is ·00181. The highest value measured with green or yellow light and at approximately room temperature is ·00188. Taking a mean of ·001845, the extremes differ from this by only ± 2 per cent. Considering the diversity of materials and methods used by various workers this degree of agreement is remarkable, and one might almost say exceptional, in biological work. Table 1, however, only refers to unconjugated soluble proteins.

Pigmented proteins

A number of figures for the refraction increments of haemocyanins and haemoglobins are available. These have been collected in table 2. It should be noted that the values given by Redfield (1934) are for haemocyanins dissolved in water, whereas the figures of Adair and his collaborators refer to haemocyanins dialysed against phosphate buffers at pH 7·5. Adair's results therefore are for α' rather than for α. The same applies to his values for haemoglobins, with the exception of Stoddard and Adair's figure for human haemoglobin. The value obtained by Howard (1920) for haemoglobin is

considerably less than that found by Stoddard and Adair (1923). This may perhaps be accounted for by the fact that the latter workers based their concentration measurements on weight of oven-dried protein, whereas Howard used the weight of crystals, which contain water. On the whole the values for

TABLE I

Specific refraction increment (α) *of unconjugated proteins*

Protein	*C*	*pH*	*T*	λ	α	*Reference*
Bovine serum albumin	3·7–10·1	5·05	·5°	5780	·001901	a
,, ,,	3·7–10·1	5·05	20°	5780	·00187	a
,, ,,		5·2	25°	4360	·001924	b
,, ,,		5·2	25°	5460	·001854	b
Horse serum albumin	2·9–8·5		14–20°	white	·001830	c
,, ,,		4·8		4360	·001912	b
,, ,,		4·8		5460	·001844	b
Human total serum				white	·00179	d
,, ,, albumin				white	·00181	d
,, crystalline albumin				white	·00181	d
,, serum albumin	1·78–7·7	4·85	·5°	5780	·001887	a
,, ,,	1·78–7·7	4·85	20°	5780	·001862	a
,, pooled plasma	3–6		25°	5893	·001833	e
,, crystalline albumin	5·1–14·6	4·4–7·7	25°	5893	·001860	e
Egg albumin					·001813	f
,,	·74–6·6	4·5–5·8			·001854	g
,, (denatured)	·65–6·6	6·9–7·4			·001844	g
,,	1·6–6·45	4·95	·5°	5780	·001876	a
,,		4·8	25°	4360	·001883	b
,,		4·8	25°	5460	·001820	b
Human γ globulin			25°	5893	·00188	e
,, ,,			·5°	5780	·001875	a
Horse serum globulin	3·3–17			white	·00186	c
Human α_2 globulin				5893	·00183	e
,, β_1 globulin				5893	·00185	e
,, euglobulin					·00183	d
,, pseudoglobulin					·00181	d
Lactoglobulin	2			4360	·001892	h
,,	2			5460	·001818	h
β lactoglobulin		5·2		4360	·001890	b
,,				5460	·001822	b
Human fibrinogen			25°	5893	·00188	e

The concentration range *C* is given in terms of grams of protein per 100 ml. of solution. Temperature *T* in degrees centigrade. Wavelength λ in Ångström units.

Key to references: a, Perlmann and Longsworth, 1948; b, Halwer and others, 1951; c, Adair and Robinson, 1930; d, Adair, 1952; e, Armstrong and others, 1947; f, Taylor and others, 1932; g, Barker, 1934; h, Pedersen, 1936.

proteins containing pigment groups are higher than those for unpigmented proteins as is to be expected on theoretical grounds. Redfield's value of ·002 for the specific refraction increment of Homarus haemocyanin is probably the highest fairly reliable figure reported for aqueous protein solutions. It can be stated with reasonable certainty, therefore, that the refraction increments of nearly all known proteins will lie within the range of ·00170–·00200.

TABLE 2

Specific refraction increment of pigmented proteins

Protein	*C*	*pH*	*T*	*γ*	*α*	*References*
Haemoglobin, Ox, CO		7·4		white	·001930	i
,, ,, ,,		5·6		white	·001935	j
,, ,, ,,		7·7		white	·001988	j
,, sheep, CO				white	·001945	k
,, human	·04–8·17			white	·001942	l
Haemocyanin *Helix*		7·5		white	·00179	m
,, *Octopus*		7·5		white	·00184	m
,, ,,	1·1				·00197	n
,, *Carcinus*		7·5		white	·00187	m
,, *Homarus*	11·85		20°		·002	o
,, *Limulus*	4·9–7·3		15–23°		·00198	o
,, *Busycon*	1–11		20–24°		·00197	o

Headings as in table 1.

Key to references. i, Adair and others, 1946; j, Roche and others, 1932; k, Adair and Adair, 1934; l, Stoddard and Adair, 1923; m, Roche and others, 1935; n, Quagliariello, 1920; o, Redfield, 1934.

Effect of pH

Except at their isoelectric points proteins normally exist as salts. Since in such cases salt formation consists in the replacement of a relatively small number of hydrogen or hydroxyl groups by, for example, sodium or chloride ions, it would seem inherently unlikely that a change of pH would have very much effect on the refractive index of large molecules such as proteins. This has been borne out experimentally. Adair and Robinson (1930) prepared solutions of sodium albuminate by dialysing against buffers at pH 7·4. Under these conditions there is an unequal distribution of ions due to the Donnan effect, so that α' is measured. The mean value found for α' for blood albumin from various sources was ·00177. The value of α calculated according to the Donnan equations was ·00180, in reasonably good agreement with the experimental result of ·00183 for crystalline isoelectric albumin. Adair and Robinson point out that the difference between α and α' depends on the concentrations of hydrogen ions, salts, and the protein. They quote one instance in which the difference between α and α' was negligible. In the case of horse serum globulin, which is insoluble in pure water, α' was determined at both pH 5·35 and pH 7·4. The values obtained were ·001815 and ·00181 respectively. α was calculated to be ·001857 and ·00187 respectively, so that the influence of pH was very small.

The effect of pH was also investigated by Perlmann and Longsworth (1948) in two different ways. In the first method they determined the refraction increment of salt-free isoelectric proteins to which small amounts of sodium hydroxide had been added. For egg albumin the refraction increment increased from ·001877 at pH 4·95 to ·001909 at pH 10·5. For bovine serum albumin the value increased from ·001902 at pH 5·02 to ·001979 at pH 10·72. (All measurements were carried out at ·5° C.) They concluded that the specific refraction increment α_H at any pH was related to the specific refraction increment at the isoelectric point α, by the formula

$$\alpha_H = \alpha(1+ae),$$

where e is the net charge on the protein and a is a constant whose value was determined experimentally to be approximately 45. The second method used was to change the pH by dialysis against buffers, taking into account the Donnan equilibrium. When sodium phosphate buffers were used, the results obtained agreed very well with those for the isoelectric solutions treated with caustic soda. With diethyl barbiturate buffers, on the other hand, the results were slightly higher, a tentative explanation being that the diethyl barbiturate ion was bound by the protein.

Although the investigations of Adair and Robinson and of Perlmann and Longsworth on the effect of pH are the most detailed and reliable so far available, it should be mentioned that other workers have frequently noted in passing that the effect of pH was either small or negligible (Robertson, 1918; Barker, 1934; Armstrong and others, 1947).

Effect of salts

Perlmann and Longsworth carried out measurements on egg albumin, bovine serum albumin, and human serum albumin, equilibrated by dialysis against various strengths of sodium chloride solutions. According to their results there is a slight rise in α for bovine and human serum albumin as the salt concentration is raised. The effects are small, and in the case of bovine serum albumin the maximum increase obtained was about 2 per cent. Egg albumin, on the other hand, appeared to be unaffected by salt concentration. Halwer and others were unable to confirm these results and found that α was not affected by salt concentration.

Effect of temperature

Most workers were unable to observe any significant change of α with temperature, possibly because they did not work over a sufficient range. Perlmann and Longsworth have published curves showing the variation of α with temperature between 0·5° and 25° C. for bovine and human serum albumin, egg albumin, and β lactoglobulin. In these cases there was a slight increase amounting to about 0·5 per cent. as the temperature fell from 25° to 5° C., with a slightly steeper rise towards 0°. In the case of human albumin α only

rose from ·001854 at 25° to ·001887 at ·5° C. Human γ globulin showed a negligible variation of α with temperature. Kenchington (1954) has also found that the value of α for gelatin is virtually independent of temperature.

Effect of wavelength

Most measurements of α are carried out with either green or yellow light from mercury or sodium lamps with appropriate filters. Some workers, notably Adair, have used white light, in which case the wavelength of measurement probably corresponds to the maximum spectral sensitivity of the eye in

TABLE 3

Variation of specific refraction increments (α) *with wavelength*

λ ($m\mu$)	*Serum albumin*	*Serum globulin*	*Lactoglobulin*
366	·00198	·00202	·001963
436	·00190	·00195	·001892
546	·00185	·00187	·001818
579	·00183	·00186	·001810
589	·00183	·00186	
656	·00179	·00182	

The figures for serum albumin and serum globulin are by Pedersen and Andersson, quoted by McFarlane (1935). The figures for lactoglobulin are by Pedersen (1936).

the green region. The refractive indexes of proteins rise considerably as the wavelength is shortened, and even over the visible spectrum there is an appreciable change in α. The widest range of wavelength so far used for the determination of α is from 366 to 656 $m\mu$, as employed by Pedersen and Andersson (quoted by McFarlane, 1935). Their results are given in table 3. Perlmann and Longsworth also carried out measurements over a narrower spectral range and found that their results as well as those of Pedersen and Andersson could be expressed by a formula of the type

$$\alpha_\lambda = \alpha_0\left(A+\frac{B}{\lambda^2}\right),$$

which is similar to the well-known Cauchy dispersion formula (see Wood, 1934; also Putzuys and Brosteaux, 1936). Since the effect of wavelength on α is quite considerable, it is generally advisable to work in the green or yellow regions of the spectrum or with white light sources in which the blue components are naturally weak or have been filtered out. The combination of the spectral sensitivity of the eye and the spectral emission of ordinary tungsten filament lamps ensures that for visual work with such sources the intensity of the blue rays is negligible as compared with that of the green and yellow rays.

No detailed work appears to have been carried out on the variation of α with wavelength in the case of pigmented proteins such as haemoglobin and haemocyanin. Since it is known that anomalous dispersion occurs in the region of absorption bands, one would expect α to show variations in such regions. The dispersion of refractive index of haemocyanin was in fact investigated by

Putzuys and Brosteaux (1936), but no anomalies were observed. This is not altogether surprising, because in order to detect strong anomalous dispersion the absorption band should be very intense (i.e. the molecular extinction coefficient should be high) and narrow. Haemocyanin has a rather broad absorption maximum in the yellow region, so that sharp changes of refractive index would not be expected. Perhaps the most important factor, however, is that the chromophore group that is conjugated to the protein is small as compared with the latter, so that the molecular extinction coefficient can never become very great. Possibly if the work of Putzuys and Brosteaux on haemocyanin were repeated with narrower wavelength intervals and perhaps a more sensitive method of measuring refractive index, such as an interferometer, slight anomalous dispersion might be detected. The same arguments apply in the case of haemoglobin. The two visible bands in the yellow and green regions of the spectrum are far too weak for any but very slight anomalies of refractive index or refraction increment to be expected. The haem pigments have, on the other hand, a very intense Soret band at about 415 to 420 mμ. The effective molecular extinction is of course greatly reduced by conjugation with protein, but even so one might expect to find some anomalies of refraction. So far as is known no measurements of refractive index of haemoglobin have been carried out in this region, but indirect evidence for the existence of anomalous dispersion has been obtained (Barer, 1952 and unpublished observations). As already noted, the specific refraction increments of pigmented proteins tend to be distinctly higher than those of unpigmented ones. In the case of the latter, anomalous dispersion would only be expected to occur in the ultra-violet at a wavelength of about 280 mμ. No figures are available for the refractive indexes of proteins in this region of the spectrum, but the work of Pedersen and Andersson already quoted shows a fairly considerable rise in α even at 366 mμ.

Effect of concentration

Most work on specific refraction increment has been carried out on protein solutions of low concentration. In view of the fact that high concentrations of proteins may be encountered in living cells, it is especially important in relation to the present work to find out over what range of concentration of protein α remains constant. Nearly all the earlier workers stated that the refractive index of a protein solution varied linearly with concentration over the range studied, though full details were rarely given. Adair and Robinson (1930) found no significant change in α for horse serum albumin betwen 2·9 and 8·5 per cent. Perlmann and Longsworth (1949) found only small and irregular variations for bovine plasma albumin between 3·8 and 10·1 per cent. Armstrong and others obtained very constant results for crystallized human albumin between 5·1 and 14·6 per cent. The highest concentration range so far investigated appears to be up to 17 per cent., in the case of haemoglobin (Howard 1920). Stoddard and Adair (1923) also found that α was constant for human haemoglobin up to 8 per cent.

Since in our work we have frequently used protein concentrations exceeding 30 per cent. it was felt necessary to investigate the variation of α with concentration over a much wider range than hitherto. The problem is not as simple as one might at first suppose. One could in principle measure the refractive index of a concentrated protein solution and then determine its concentration by drying a known volume or by nitrogen estimation on a known volume. Unfortunately, with increasing concentrations, particularly those exceeding 30 per cent. or so, the bovine serum albumin preparations become increasingly viscous and difficult to handle. For accuracy, therefore, rather large volumes would be required, necessitating the use of several grams of protein for each reading. Since commercial bovine plasma albumin is moderately expensive, the cost of such an investigation becomes an important factor. Another obvious approach would be to weigh out a definite amount of dried protein and make it up to a given concentration by addition of water. This, however, is quite impracticable because even with moderately concentrated solutions some of the protein, despite all reasonable precautions, becomes caked into a hard mass which tends to adhere to the container or stirring rod, making it virtually impossible to remove all the protein from the latter and to measure the volume of the solution accurately. For these reasons an alternative method of estimating protein concentration was sought. All proteins containing the aromatic amino acids tyrosine, tryptophane, and phenylalanine show characteristic absorption bands in the ultra-violet at about 280 mμ (Beaven and Holiday, 1952). For a given protein the extinction coefficient for the peak in this region can be measured and hence the concentration of protein present in a given solution can be determined. This method has the advantage of requiring very little protein. In the case of bovine plasma albumin a 1 cm. thickness of a ·2 per cent. solution gives an optical density of approximately 1·3. If the Beer–Lambert laws concerning the proportionality between concentration of solute, length of optical path, and optical density were obeyed, there would be no difficulty in diluting the unknown sample by a known amount, measuring its optical density for a given thickness, and thus deducing the concentration. It is well known that concentrated solutions of proteins show slight opalescence and possibly other effects, such as polymerization or association, which result in some departure from these laws. In order to avoid this, therefore, our solutions were diluted accurately by varying amounts in order to obtain approximately the *same* final optical density at 278 mμ. The final concentration was chosen so as to make the optical density about 1·0 for a 1 cm. path-length. This corresponds to a concentration of about ·2 per cent. At this dilution the turbidity of the solution is negligible and the Beer–Lambert laws can be assumed to hold for a moderate range of concentrations around this value.

The experimental procedure was as follows. Concentrated solutions of bovine plasma albumin were made in distilled water (the absorption of which was carefully checked at 278 mμ and found to be negligible). The solutions were allowed to stand for at least several hours, and preferably overnight, and

were stirred carefully at intervals in order to ensure homogeneity. Several samples were taken from different regions of each solution and their refractive indices measured. These precautions were essential in order to obtain consistent results. In the early stages of this work the results were rather variable because it was not realized that 'stratification' occurs in concentrated protein solutions, so that there is a gradient of concentration from the surface to the bottom of the containing vessel. Refractive indices were measured on a Bellingham and Stanley Abbe-type refractometer at 20° C., with monochromatic yellow mercury light (λ 579 mμ). Readings were also taken with a hand sugar refractometer in order to compare the accuracy of the two instruments. The protein concentration was calculated roughly from the formula $C = \frac{n - 1{\cdot}3330}{\alpha}$, taking ·00185 for α. A small volume of the solution was measured in an accurately graduated volumetric pipette and diluted with distilled water to a final concentration of approximately ·2 per cent. in a 250 ml. volumetric flask. Great care is necessary in this part of the procedure, particularly when dealing with concentrated solutions. The high viscosity of the latter makes it extremely difficult to suck them up into a narrow calibre pipette. It is important not to overshoot the mark, for if the viscous solution is deposited on the walls of the pipette it will take a very long time to fall to the desired level. All solution on the outside of the lower end of the pipette must be carefully removed by repeated wiping with plugs of cotton wool soaked in distilled water. Finally, one of the most potent sources of error was found to be the incomplete emptying of the pipette. Ordinary washing through with a few changes of water is quite inadequate as the film of viscous protein adheres firmly to the walls, and between 50 and 100 rinses were necessary before the concentration reached a final stable value. The diluted solution was mixed thoroughly and several samples were selected for measuring the light absorption at 278 mμ. This wavelength was chosen because a preliminary measurement of the absorption spectrum of bovine plasma albumin, fraction V, showed that it was the position of the absorption maximum. Measurements of absorption were carried out with a special Hilger Uvispek ultra-violet spectro-photometer with a non-standard extra-large quartz prism. The slit width used corresponded to a band width of about ·5 mμ. Silica absorption cells 1 cm. thick were used.

If the measured optical density of a diluted solution is d and the number of times it has been diluted as compared with the original concentration is s, then the concentration of the original solution is proportional to $s.d$. (It should be noted that under the conditions of the experiment this is true whether or not the Beer–Lambert laws hold.) If this quantity is plotted against the refractive index of the concentrated solution, the variation of refraction increment with concentration is found. In order to convert $s.d$ to actual concentration, a knowledge of $E_{1\,\text{cm.}}^{1\%}$ (i.e. the optical density of a 1 per cent. solution in a 1 cm. path) is required. The main purpose of this investigation was to determine the slope of the refractive index / concentration curve and not to

measure the absolute value of α accurately. $E_{1\,\text{cm.}}^{1\%}$ was not determined experimentally, therefore, but the value of 6·6 found by Cohn and others (1947) was used. The concentration of protein was thus taken as $\frac{s.d}{6{\cdot}6}$ and these values are plotted against the refractive index n in fig. 2. It will be seen that within the limits of experimental error the curve is a straight line. The accuracy is least at high concentrations because of the various difficulties associated with the viscosity of such solutions. Nevertheless, the departure from linearity

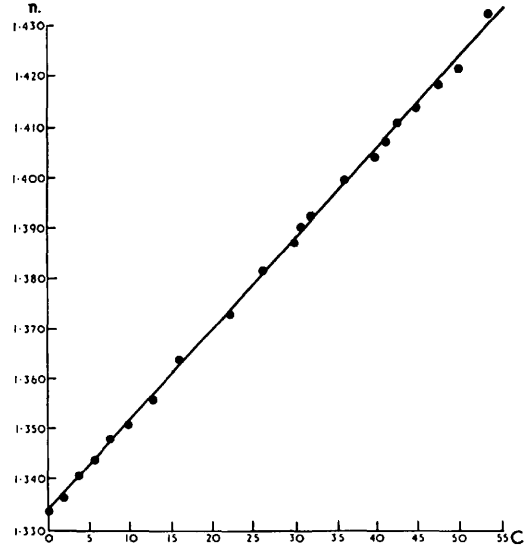

FIG. 2. Variation of refractive index (n) of bovine plasma albumin, fraction V, with concentration (C). From Barer and Tkaczyk (1954).

is negligible at concentrations up to nearly 50 per cent. and is small even at 55 per cent., the practical limit of solubility. The value of α, the slope of the line, was found to be ·00182. This is in quite good agreement with other reported values in table 1, but no special accuracy is claimed because of the uncertainty in the value of $E_{1\,\text{cm.}}^{1\%}$. The main point of the result is that α is constant over a very wide range of concentration, so that it is possible to deduce concentrations with considerable accuracy from measurements of refractive index.

(Since this manuscript was prepared a personal communication has been received from Mr. A. W. Kenchington concerning work he has carried out on the refraction increment of gelatin. In order to use high concentrations it was necessary to work at a temperature of 34·5° C. It was found that the refractive index varied linearly with concentration up to the highest concentration used

(just over 30 per cent.). The refraction increment was found to be ·0018. This is about the same as that found for other proteins, which is of some interest in view of the fact that gelatin contains almost no tryptophane or tyrosine and comparatively little phenylalanine. Gelatins from very different collagenous sources were found to have the same refraction increment, and chemical treatment such as processing with lime or with acid did not affect the refractive index, nor did heating at 100° C. for 1 hour in a sealed tube. The specific refraction increment of collagen has been measured recently by M'Ewen and Pratt (1953). They obtained values between ·00189 and ·00192 at 5,780 Å. These values are a little higher than those for gelatin and most other unpigmented proteins, but collagen is insoluble in water and has to be dissolved in dilute acids.)

Effect of medium

Putzuys and Brosteaux (1936) and Hand (1935) have pointed out that the value of α may vary with the nature of the solvent. This is fairly obvious because in the limiting case, when the 'solution' consists of pure solute only, the final refractive index attained will be that of the latter and will be independent of the solvent. Thus, if we imagine a solute whose refractive index in the solid state is 1·6, the refraction increment will be greater in a solvent of refractive index 1·33 than in one of refractive index 1·50. Differences of this sort are unlikely to be of any importance in the case of constituents of living cells which can be regarded as being dissolved in a very dilute salt solution, but they must be taken into account in interpreting values for refraction increment obtained with substances which are only soluble in non-aqueous media or in strong salt solutions.

The refraction increment of certain cellular constituents

Protein derivatives and constituents

Denatured protein. Barker (1934) measured the refraction increment for native and heat-denatured egg albumin solutions, using first a dipping refractometer accurate to six decimal places, and then an interferometer reading to eight places. The change in α was extremely small. A value of ·001854 was found for the native albumin, and values for the heat-denatured albumin were ·001844 and ·001833, measured with the refractometer and interferometer respectively. The change was thus approximately 1 per cent. The pH of the native solution varied between 4·6 and 5·8, that of the denatured material between 6·9 and 7·4, so that it is possible that part of the variation in α may be due to a change in pH. At all events the result is interesting in that it shows how little the refractive index is affected by quite drastic treatment.

Protein digests. As long ago as 1912 Robertson, using a refractometer accurate to ·000078, found no detectable change in refractive index during the complete hydrolysis of a sodium caseinate solution by trypsin. We have ourselves been unable to find any change in the refractive index of a bovine plasma albumin solution subjected to digestion by pepsin.

Peptones. In order to obtain some information about the behaviour of mixtures of protein derivatives, we have measured the refractive indices of peptone solutions in the concentration range 0–50 per cent. Commercial peptone preparations differ considerably in their composition and in the amount of insoluble matter which they contain. Evans's bacteriological peptone, which is completely soluble in concentrations of 50 per cent. w/v, was used. This

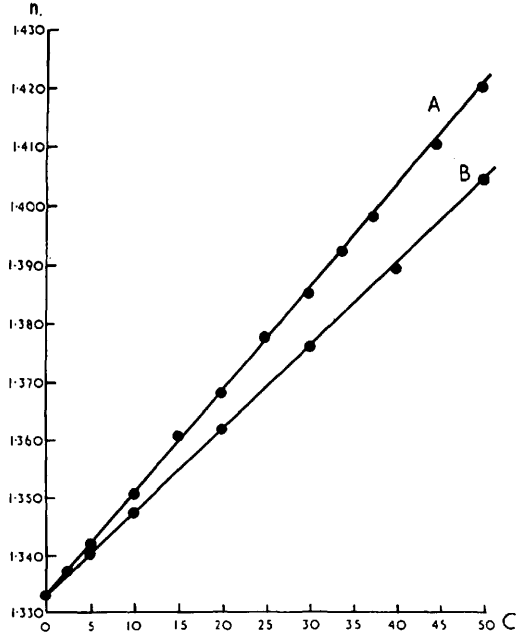

FIG. 3. Variation of refractive index (n) of A, Evans's peptone, and B, glucose, with concentration (C).

appears to contain mainly relatively small molecules capable of passing through a collodion membrane. Measurements were carried out at 20° C with an Abbe refractometer, as already described. The results are summarized in fig. 3. This shows that the refractive index increases linearly with concentration up to 50 per cent. The refraction increment over this range was ·00174. This value refers to the powder as supplied by the manufacturers and is not corrected for water content. The latter is stated to be approximately 5 per cent., so that the true value of α for the dried material would be ·00183. This again is within the range of values found for proteins. At all events the value

for peptone can reasonably be taken as applying to a mixture of non-protein nitrogenous constituents in living cells.

Amino acids. Values of α for four amino acids have been determined experimentally by Adair and Robinson (1930). Their results were: glycine ·00179, alanine ·00171, valine ·00176, and tryptophane ·00252. These values are in accordance with general expectations. Tryptophane contains conjugated double bonds and has a high refractivity. The other amino acids contain no conjugated double bonds and their specific refraction increments are slightly lower than those of proteins. Since proteins contain relatively small amounts of tyrosine, tryptophane, and phenylalanine (which contain conjugated double bonds) one would expect their specific refraction increment to be only slightly higher than the values for non-aromatic amino acids.

Adair and Robinson showed that there is excellent agreement between the observed values of $\frac{n-1}{d}$ and the figures calculated theoretically from values for atomic refractivities given in the literature. The only important deviation was in the case of tryptophane. The same authors also calculated the refractivity of albumin and found fairly good agreement between the observed and calculated values. The agreement would probably have been even better had it been possible to take into account the effect of other aromatic amino acids apart from tryptophane.

Surveying these results it will be seen that there is a remarkable degree of constancy in the values of α for many types of protein derivatives or mixtures of such derivatives. This is in conformity with the behaviour expected from the simple theoretical discussion on page 405.

Nucleic acids and nucleoproteins

According to Tennent and Vilbrandt (1943) the refraction increment of sodium thymonucleate solutions is ·0016. More recent, unpublished measurements by Cecil (1951) on the possibly purer preparations of Gulland and others (1947) gave higher values approaching ·002. Further work on such compounds is evidently desirable, but it is again clear that these results do not differ very greatly from those for proteins. The only figure available for a nucleoprotein is ·0017, obtained by Oster (1954) for tobacco mosaic virus. This again is close to the values for other proteins.

Lipids and lipoproteins

It is virtually impossible to assign any sensible meaning to the specific refraction increment of most fatty substances because they are of course insoluble in water. The refractive indices of most animal and vegetable fats lie between about 1·45 and 1·48. Thus the values for human fat, horse fat, whale fat, and wool fat are given as approximately 1·46, 1·465, 1·47, and 1·48 respectively. If we calculate the value of α from these figures on the assumption that the fats are perfectly water soluble, we obtain, after making due allowance for the

low density (approximately ·93) of fats, a value of about ·0015. This is rather lower than the figures for proteins, but its meaning is obscure. On the whole it seems more reasonable to assume that fats will exist in living cells mainly in two forms: (*a*) as actual fatty droplets of microscopic size (in which case their presence will scarcely affect the values obtained for refractive index of the cytoplasm, since measurements can be made on regions free from such droplets), and (*b*) combined with protein as lipoproteins. There is an increasing body of evidence in support of this view (see Faraday Society Discussion on Lipoproteins, 1949). According to Oncley and others (1950), 70 per cent. of the normal plasma lipids are contained in the β_1 lipoprotein fraction of the plasma proteins. The chemical constitution of this lipoprotein is very remarkable in that it contains approximately 23 per cent. protein, 29 per cent. phospho-lipids, 39 per cent. cholesterol esters, and 8 per cent. free cholesterol. In other words, more than three-quarters of the weight of this substance is accounted for by lipids. Another plasma fraction, α_1 lipoprotein, contains 65 per cent. protein and 35 per cent. lipide. Clearly such complexes could contain enough lipid to account for the quantities present in living cells. Fortunately, figures are available for the specific refraction increment of these lipoproteins. According to Armstrong and others (1947), the value of α for β_1 lipoprotein is ·00171, for α_1 lipoprotein ·00178. Adair (1952) obtained values of between ·00170 and ·00171 for plasma lipoprotein fractions. It thus appears that the presence of even very considerable amounts of lipids has only a small effect on the refractile properties of proteins. Oncley and others remarked that the solubility and amphoteric properties of lipoproteins are characteristic of the protein fraction rather than of the lipids; the same is apparently true for refractivity. Another piece of evidence which has some bearing on this question is that Armstrong and others found that although the figures for α for human plasma from cases with lipaemia were lower than the normal average of ·001833, even the lowest value did not fall below ·00176. From the point of view of the present work, therefore, it seems improbable that the presence of bound lipids in living cells would be likely to alter the refraction increment by more than a small amount, probably less than 5 per cent.

Carbohydrates

Refractometry is a standard method of estimating sugar concentration in industry, and the refractive indices of several sugar solutions have been measured in great detail over a very wide range of concentrations. Unfortunately, most of the published tables (see Honig, 1953, and International Critical Tables) give sugar concentrations in terms of grams per 100 grams of solution. With these units the refractive index does not vary linearly with concentration, so that α is not constant. If, however, one recalculates the concentration in terms of grams per 100 ml. of solution, using published density tables, it is found that α is constant over a very wide range with very nearly the same value (approximately ·0014) for different sugars. We have our-

selves verified this experimentally for glucose, obtaining $\alpha = \cdot 00143$ in the range 0–50 per cent. concentration (see fig. 3). Such values are distinctly lower than those for proteins and might be a cause of error when attempting to measure solid concentrations of living cells by refractometry. However, as in the case of lipides, it is improbable that all intracellular carbohydrates are present in simple solution. Some will no doubt occur in the form of glycogen granules, and some are probably bound to proteins. Unfortunately no figures are available for the refraction increment of glycogen or other polysaccharides or for glycoproteins and mucoproteins, but serum globulins contain about 2 per cent. of carbohydrates. Armstrong and others (1947) obtained values for α of $\cdot 00183$ for α_2 globulin and $\cdot 00185$ for β_1 globulin. These results are not markedly different from those for other proteins. Although there are many gaps in our knowledge of the refractile properties of carbohydrates, it seems on the whole improbable that their presence in moderate amounts would cause the refraction increment of protoplasm to deviate markedly from that of pure proteins.

Inorganic constituents

Many investigations have been carried out on the refractive indices of salt solutions, and extensive summaries are given in the International Critical Tables. Care has to be taken in using such tables that concentrations must be expressed in terms of grams per 100 ml. of solution. Baxter and others (1911) found that the refractive index increased linearly with concentration over a wide range. They found $\alpha = \cdot 0016$ for sodium chloride and $\alpha = \cdot 0012$ for potassium chloride. Other workers have obtained similar results. The figures given for calcium and magnesium chlorides in the International Critical Tables are $\cdot 0011$ and $\cdot 0012$ respectively. Although in most cases the values of α for salts are less than those for proteins, the concentration of intracellular salt is usually low, and in any case the immersion medium contains similar salts, so that in general one can probably ignore the contribution to the total refractive index made by intracellular salt.

The refraction increment of protoplasm

At first sight it might appear impossible to suggest a mean value of α for so complex a mixture as living protoplasm. Undoubtedly α must vary to some extent with different types of cells. Fortunately, however, the main solid organic constituent of the cytoplasm of nearly all cells is protein and, as already discussed, at least some of the lipides and carbohydrates are probably present in the form of complexes with protein, and the refraction increments of such complexes do not differ markedly from those of other proteins. Nucleic acids, amino acids, and other non-protein nitrogenous constituents also have very nearly the same refraction increments as proteins. Until more is known about the chemical analysis of living cells and about the forms in which various substances exist in such cells, it seems reasonable to take the

mean value of α for protoplasm as ·00180. This is very slightly less than the average for most proteins and takes into consideration the possible lowering effect of lipides and carbohydrates. For the majority of cells it seems unlikely that this figure would be in error by more than perhaps 5 per cent., but of course ignorance of the exact value of α must always remain a fundamental error of the method. It may be that in some cells of very unusual chemical composition the error may be greater, but even if the cell were composed entirely of carbohydrate α would not fall below ·0014. At the other extreme, so far as we are aware, no values greater than ·0020 have ever been reported for aqueous solutions of organic substances.

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Fig. 1 is reproduced by kind permission of the Editor of *Nature*.

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