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Physicochemical Studies on Ovalbumin

4. CHARACTERIZATION OF AN IODINE-MODIFIED DERIVATIVE BY ELECTROPHORESIS AND SEDIMENTATION*

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(*Received* 31 *July* 1961)

Winzor & Creeth (1962) described the preparation of an iodine-modified derivative of ovalbumin by reaction with 5 equiv. of iodine at 0°, at pH 6·8 and in the presence of potassium iodide (M). In some respects the derivative was markedly different from the parent material, whereas in others there was a close similarity between the two substances. Since differences in the transport properties are easiest to interpret directly, a com-

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parative study has been made of the sedimentation and electrophoretic behaviour of the two materials, and is reported in this paper. Part of this work has been described by Winzor (1960).

EXPERIMENTAL

Materials

The preparation of the native and iodine-modified ovalbumins has been described by Winzor & Creeth (1962).

Electrophoresis

Electrophoresis experiments were performed at 1·00° in a Spinco Model H electrophoresis–diffusion apparatus with the 11 ml. cell, with Rayleigh optics throughout. The studies were of two types, the first comprising a detailed comparison of the electrophoretic mobilities of the two materials as a function of pH in the range 4–8·5, and the second a comparison of the apparent net charge on the proteins at pH 8·5.

In the mobility experiments, the 'compensation' procedure for bringing the boundaries into view was generally avoided; even when extremely slow, compensation tends to distort and blur the initial symmetrical boundary curve (Longsworth, 1953).

The effect is almost undetectable, at the concentrations used, when viewed by schlieren optics. Since the protein solutions were all of low concentration (0·03–0·10%), the current-reversal method described by Creeth & Nichol (1960) was employed; the mobility value quoted for an experiment is accordingly the mean of the two values obtained from the descending limbs. The conductivities of the protein solutions were measured at the temperature of the experiment with a Philips Model PR9500 conductivity bridge; the mobility values therefore refer to $1{\cdot}0_0°$. All experiments were performed in buffers of stoicheiometric ionic strength 0·100, the exact compositions being reported in Table 2. Only 1:1 electrolytes were used.

The charge values were measured by the method of Charlwood (1950). In this procedure the apparent net charge is calculated from the excess of supernatant buffer concentration required to eliminate the δ-boundary in electrophoresis. Since an accuracy of about 0·02% in the buffer compositions is desirable, unusual precautions are necessary in preparing and handling the solutions. Sufficient protein solution for five electrophoresis experiments (about 65 ml.) was dialysed against veronal–chloride buffer, pH 8·51, I 0·1000 [sodium diethylbarbiturate (0·0400 M), diethylbarbituric acid (0·0100 M), sodium chloride (0·0600 M)] for 4 days at 4°, with constant mild agitation and frequent changes of buffer. The protein solution was then removed from the dialysis sac and divided into five parts, which were stored separately in small air-tight containers. The five samples were then subjected to electrophoresis with supernatant buffer solutions of slightly different composition. These buffer solutions were all prepared in the same 2 l. volumetric flask thermostatically controlled at 1·0°, and all contained the three solute constituents in the same ratio, as a precaution against pH variation.

Particular care was taken to ensure that the shapes of the boundaries were undisturbed by processes other than electrophoretic migration and diffusion. To avoid the skewing effect of compensation (see above) while retaining the essential ability to record the initial boundary before the passage of current, the boundary-sharpening procedure used in diffusion experiments (e.g. Creeth, 1958) was adopted. Thus after the initial formation of the boundaries by shearing, liquid was withdrawn from the system until the boundary initially at the top of one limb of the centre section had moved to within 2 cm. of the bottom. Siphoning was then stopped and the bottom section disaligned. A capillary was then inserted to a point just above this boundary, and intermittent siphoning commenced to remove all protein from the region above the boundary. After readjustment of the levels, the bottom section was aligned, and the boundary was sharpened symmetrically by further flow for 3 min. The capillary was then withdrawn, the initial photograph recorded and electrophoresis commenced so that the sharpened boundary moved upwards. Electrophoresis was continued at 3·4 v cm.$^{-1}$ for 4 hr., when a second series of photographs were recorded, the δ-boundary being completely separated from the protein peaks at this stage.

Measurements and calculations. To establish the differences in the mobilities of the two materials as accurately as possible, the calculations were based on the location of the first moment of the protein boundary; this position corresponds to a weight-average mobility when polydisperse systems are examined (Longsworth, 1941, 1943; Longsworth & Jacobsen, 1949). In view of the electrophoretic complexity of ovalbumin originally reported by Longsworth (1939) and further discussed by Perlmann (1952, 1953), no simpler procedure is applicable; the iodine-modified material (cf. Fig. 1) closely resembled the original in this respect, although the slower component was slightly more difficult to resolve than with the native protein.

The Rayleigh system is, in principle, well adapted for obtaining moments of concentration distributions (e.g. Svensson, 1951), but there seems to have been no report of its application to electrophoretic-mobility determinations. We have found that certain refinements of the simplest approximation procedure are generally necessary if the potential accuracy of the method is to be realized: the basic difficulty arises because the integration limits correspond to cell displacements of $\pm\infty$, so that a simple step-function may introduce serious errors. One solution is to use the measured fringe positions to reconstruct the concentration curve, or its derivative (Longsworth, 1951), which can then be integrated planimetrically; this gives satisfactory results, but is very laborious. It is more

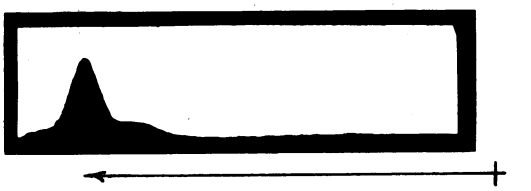

Fig. 1. Electrophoretic pattern (ascending limb) of the 5:1 iodine-modified derivative of ovalbumin, at pH 7·48 in veronal–chloride buffer, I 0·10, after 12 hr. at 3·6 v/cm. Migration is from right to left, the starting position being as indicated. (This experiment was made with a Hilger electrophoresis apparatus.)

desirable to handle the data directly; the following treatment describes the theoretical basis and practical application of a procedure which gives good results.

The first moment about the origin of a boundary where the concentration, c, varies continuously from zero to a constant value, c^*, as a function of the cell displacement, x, is given by:

$$x_m \equiv \int_0^{c^*} x\,\mathrm{d}c \Big/ \int_0^{c^*} \mathrm{d}c. \qquad (1)$$

Assuming that the specific refraction increment of the material is constant, it follows that there is a linear relation between c and the fringe number, j, which runs from 0, at $c = 0$, to J, at $c = c^*$. (J is not in general a whole number.) It is convenient also to refer to fringe positions in terms of their displacements relative to the $(\frac{1}{2}J)$ position, so that equation (1) becomes

$$x_m = x(\tfrac{1}{2}J) + \frac{1}{J}\int_0^J y\,\mathrm{d}j \qquad (2)$$

where $y = x(j) - x(\frac{1}{2}J)$; x is taken positive upwards so that positive values of y refer to j values less than $\frac{1}{2}J$ and vice versa. In the central part of the boundary, it is found that the summation of $y\Delta j$ products accurately reproduces the integral when Δj is taken as 1, but that such an approximation frequently fails when applied to the 'tails' of the boundary. We therefore split the integral into three parts:

$$\int_0^J y\,\mathrm{d}j = \int_0^k y\,\mathrm{d}j + \sum_k^{(J-k)} y\Delta j + \int_{(J-k)}^J y\,\mathrm{d}j \qquad (3)$$

where k is chosen so that the second term on the right-hand side is essentially equal to the corresponding integral. For $J = 20$, trials with planimetry have shown that $k = 3$ is an entirely satisfactory choice. The two integrals corresponding to the 'tails' are evaluated with the aid of an empirical extrapolation function: it has been found that over the range $0{\cdot}5 < j < 3{\cdot}0$ (when $J \simeq 20$), $\log j$ is strictly linear in y. Similarly, it is found that $\log(J-j)$ is linear in y over the range $(J-3) < j < (J-0{\cdot}5)$. It is assumed that these relationships hold down to $j = 0$ in the one case, and $j = J$ in the other (cf. Fig. 2). Thus we may write:

$$j' = A\mathrm{e}^{by} \qquad (4)$$

where j' represents j for positive values of y and $(J-j)$ for negative. The constants, A and b, can be determined from the experimental plots of $\log j$ or $\log(J-j)$ against y; b is negative for positive y and vice versa.

Differentiating equation (4) and substituting, we obtain for the first integral on the right of equation (3):

$$\int_0^k y\,\mathrm{d}j = Ab\int_\infty^{y_k} y\mathrm{e}^{by}\mathrm{d}y = A\mathrm{e}^{by_k}\left(y_k - \frac{1}{b}\right). \qquad (5)$$

Similarly, the integral corresponding to the lower 'tail' of the boundary becomes

$$\int_{(J-k)}^J y\,\mathrm{d}j = A\mathrm{e}^{by_{(J-k)}}\left(y_{(J-k)} - \frac{1}{b}\right). \qquad (6)$$

To test the accuracy of this procedure, a comparison was made of the values of the integrals as given by equation (6) and as determined planimetrically. The results are illustrated in Table 1, which shows, for various times, the measured and calculated integrals for both 'tails' of the boundary, and also the central piece [where the 'calculated' value refers to the summation indicated in equation (3)]. Since the agreement is uniformly excellent, the validity of the analytical integration is established. However, as with most of the examples given in Table 1, it was frequently found that the boundary position defined by the full integral $\int_0^J y\,\mathrm{d}j$ differed negligibly from that defined by the 'centre piece' $\sum_3^{(J-3)} y\Delta j$. Accordingly, the full integration procedure was not applied to all exposures in an experiment, but was used as a control to assess the error involved in the use of the expression:

$$x_m \simeq x(\tfrac{1}{2}J) + \frac{1}{J-2k}\sum_k^{(J-k)} y\Delta j. \qquad (7)$$

This approximation is only applicable to fairly symmetrical boundaries, but requires much less computation. Where

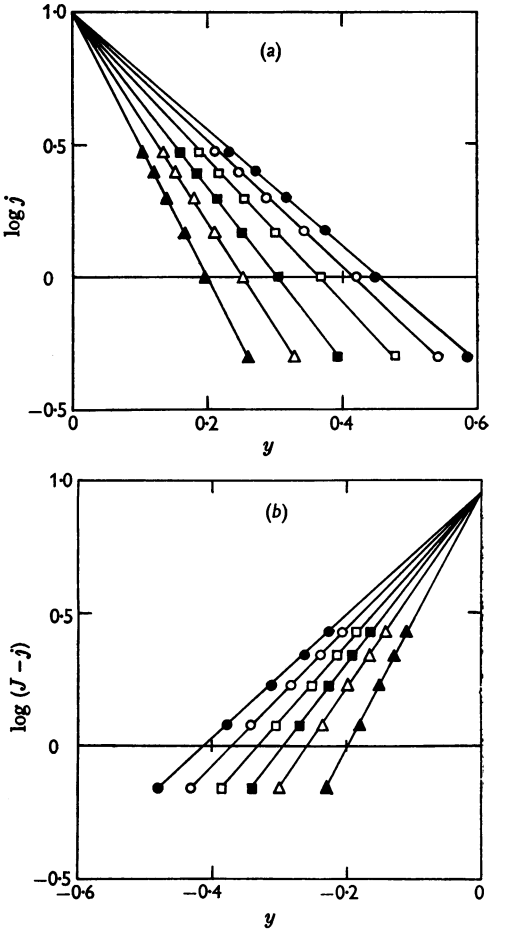

Fig. 2. The linear relation between $\log j'$ and y (see text) obtained in a typical electrophoresis experiment. Both diagrams relate to the descending boundary, each set of points referring to different stages in the progress of the run: (*a*) obtained from the upper region of the boundary ($j = 0{\cdot}5$–3); (*b*) from the lower region ($j = 13$–$15{\cdot}2$), J for the experiment being 15·7.

the full integral showed that the use of equation (7) led to negligible error in the exposures selected, the other exposures were not so treated, provided that all values of x_m [obtained by use of equation (7)] were linear with time.

All measurements on the photographic plates were made with a two-dimensional comparator reading to 0·01 mm., allowance being made for the slight non-linearity of the nominally straight fringes obtained in control experiments (Creeth, 1955). This small correction was of great importance in the δ-boundary estimations, where it is believed that errors were reduced to 0·05 fringe.

Sedimentation

Sedimentation-velocity experiments were performed either in a Spinco Model E, or a Svedberg oil-turbine ultracentrifuge. Sedimentation coefficients were calculated by standard methods, and corrected to water at 20°. It was assumed that the partial specific volumes of the native and iodine-modified ovalbumins were identical and independent of buffer; the value 0·7479 obtained by Dayhoff, Perlmann & MacInnes (1952) was used. In the concentration-dependence estimations, solutions were prepared by weight-dilution from more concentrated stock, the concentration of the latter being measured refractometrically (Winzor & Creeth, 1962).

For the study of sedimentation as a function of the extent of reaction with iodine, portions of a stock ovalbumin solution were treated with appropriate volumes of iodine to give 1·0, 2·0, 3·0, 4·0 and 5·0 equiv./mole of ovalbumin. After standing for 1 hr., the five samples were dialysed against phosphate buffer, pH 6·8, I 0·3; after equilibration they were examined as soon as possible in the ultracentrifuge.

RESULTS

Electrophoretic studies

Mobility measurements. The results of the electrophoretic-mobility determinations on both native and iodine-modified ovalbumins, in the pH range 4–8·5, are shown in Table 2. Both sets of mobility values lie on smooth curves, the one for ovalbumin being closely similar to that reported by Perlmann (1953), after allowance has been made for the temperature difference between the two investigations: our values, determined at 1°, are expected to be some 3½% greater than those at 0°. The mobility values given by Longsworth, Cannan & MacInnes (1940) may only be compared in the pH range 4–5·3 and at 7·8 where univalent buffer ions were used: the agreement is satisfactory. In the pH range 6–8, Longsworth *et al.* (1940) used phosphate buffers, in which the mobility is markedly more negative than in univalent buffers of the same pH.

Over the pH range 4·0–5·4, the mobility of the iodine-modified protein is virtually identical with that of native ovalbumin, but at higher pH values the modified material has a greater (negative) mobility. At about pH 6·8 the mobility difference reaches $0{\cdot}6 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ v^{-1}, and this difference remains essentially constant up to the highest pH investigated, 8·5.

δ-Boundary elimination and charge calculations. The results of the δ-boundary estimations for the series of experiments on the native protein and its derivative are shown in Fig. 3. The size of the δ-boundary decreases linearly with the excess of buffer concentration in the supernatant, as was found by Charlwood (1950) for a horse-serum-albumin preparation. When the straight lines (determined by the method of least squares) are extrapolated, the values $0{\cdot}1032_0$ and $0{\cdot}1034_5$ are obtained respectively for the ionic strengths at which the δ-boundary is eliminated with ovalbumin (0·138 mM) and the iodine-modified ovalbumin (0·147 mM). Substitution of these values in the relevant equations (Charlwood, 1950), with the buffer ion mobilities given by Alberty (1948) (after correction from 0 to 1°) and the ionic concentrations quoted earlier, gives −10·6 and −11·6 for the apparent net charge of the native and modified proteins respectively.

The mobility values themselves allow a second, and independent, estimate of the charge difference

Table 1. *Test of integration procedure used in determining first moment positions of boundaries in electrophoretic-mobility experiments*

The two sets of values under each integral refer to those determined by planimetry ('Measured') and those obtained by applying the relevant numerical or analytical procedure ('Calc.'). 'Exposure 1' in each case refers to the boundary at the start of the mobility determination, and 'Exposure 6' to the boundary at the end of its forward traverse of the cell.

			$\int_0^3 y\,dj$		$\int_3^{(J-3)} y\,dj$		$\int_{(J-3)}^{J} y\,dj$		$\int_0^J y\,dj$	
Expt.	pH	Exposure no.	Measured	Calc.	Measured	Calc.	Measured	Calc.	Measured	Calc.
1	8·51	1	0·65	0·66	+0·01	0·00	−0·75	−0·76	−0·09	−0·10
		6	1·74	1·75	+0·15	+0·16	−1·11	−1·11	−0·78	−0·77
2	6·08	1	0·68	0·67	0·00	+0·01	−0·95	−0·94	−0·27	−0·26
		6	1·56	1·58	+0·12	+0·13	−1·55	−1·57	+0·13	+0·14
3	4·59	1	0·42	0·44	−0·01	+0·01	−0·37	−0·38	+0·06	+0·07
		6	0·88	0·89	0·00	0·00	−0·65	−0·67	+0·23	+0·22

Table 2. *Electrophoretic-mobility data for ovalbumin and iodine-modified ovalbumin in buffers of ionic strength* 0·100, pH 4–8·5, *at* 1·0$_0$°

With the exceptions marked †, each value quoted is the mean of the two descending mobilities obtained in each experiment. The two values generally quoted for each pH refer to the results of independent duplicate experiments. The column headed 'Δu' refers to the mobility difference between ovalbumin and the iodine-modified derivative at that pH, as determined from smooth curves constructed from all data. The buffers used contained: A, sodium diethylbarbiturate (0·04M), diethylbarbituric acid (0·01M) and sodium chloride (0·06M); B, sodium diethylbarbiturate (0·01M), diethylbarbituric acid (0·02M) and sodium chloride (0·09M); C, sodium cacodylate (0·04M), cacodylic acid (0·01M) and sodium chloride (0·06M); D, sodium cacodylate (0·02M), cacodylic acid (0·02M) and sodium chloride (0·08M); E, sodium acetate (0·07M), acetic acid (0·01M) and sodium chloride (0·03M); F, sodium acetate (0·07M), acetic acid (0·03M) and sodium chloride (0·03M); G, sodium acetate (0·04M), acetic acid (0·04M) and sodium chloride (0·06M); H, sodium acetate (0·02M), acetic acid (0·08M) and sodium chloride (0·08M).

		$10^5 \times$ mobility (cm.^{2} sec.$^{-1}$ v^{-1})		
Buffer	pH	Ovalbumin	Iodine-modified ovalbumin	Δu
A	8·51	$-5·9_0$	$-6·4_5$	$0·6_0$
		$-5·9_5$†	$-6·5_7$†	
B	7·48	$-5·8_7$	$-6·4_1$	$0·6_0$
		$-5·9_5$	$-6·4_9$	
C	6·58	$-5·2_2$	$-5·8_4$	$0·4_8$
		$-5·3_0$†	$-5·9_3$†	
D	6·08	$-4·8_2$	$-4·9_5$	$0·2_0$
E	5·43	$-3·5_1$	$-3·5_6$	$0·0_5$
		$-3·2_2$	$-3·2_4$	
F	4·98	$-1·8_5$	$-1·7_8$	0·0
G	4·59	$-0·4_2$	$-0·4_7$	0·0
		$-0·3_6$	$-0·3_9$	
H	3·95	$+2·4_2$	$+2·4_8$	0·0
		$+2·5_0$	$+2·4_1$	

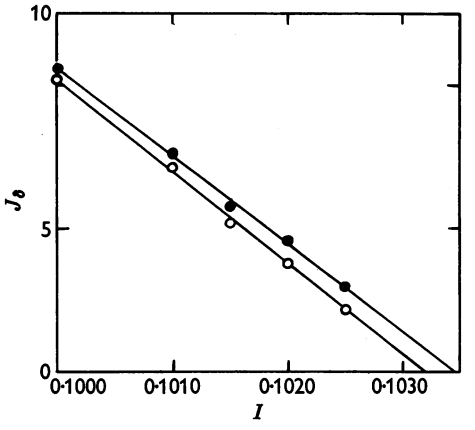

Fig. 3. The relationship between size of the δ-boundary in electrophoresis experiments and ionic strength of the buffer used as upper solution for ovalbumin (○) and iodine-modified ovalbumin (●) at pH 8·5 in veronal–chloride buffer. The valence values are obtained from the intercepts on the I axis.

to be made, although this method rests on a microscopic model of electric migration which cannot yet be considered well established in its application to proteins (see below). The equation of Abramson, Gorin & Moyer (1939) may be written in terms of the valence, z:

$$z = u \frac{6\pi\eta r(1+\kappa r+\kappa r_i)}{f(\kappa r)\,(1+\kappa r_i)} \times 6·25 \times 10^9 \qquad (8)$$

where r is the radius of the protein particle, assumed spherical, and the other quantities have their usual significance. With $r = 2·90 \times 10^{-7}$ cm. (derived from the sedimentation data reported below), the values $-10·7$ and $-11·8$ are obtained for the valences of ovalbumin and the derivative respectively; the former agrees well with the value $-10·6$ interpolated from the Table of Abramson, Moyer & Gorin (1942), as expected from the essential similarity of the data. If a moderate degree of asymmetry of the molecule is assumed (cf. Abramson *et al.* 1942), the valence values are somewhat higher, but the difference is essentially unaltered. Thus both physical measurements agree in indicating that the charge difference between the two substances is unity.

Sedimentation studies

Sedimentation-velocity experiments were performed on both materials at two different pH values: (*a*) 4·6, where both proteins are essentially isoelectric; (*b*) 7·5, where both possess a net

negative charge. As observed by Creeth, Nichol & Winzor (1958), the patterns obtained with ovalbumin at pH 4·6 showed considerable proportions of aggregated material (dimer, etc.) and the sedimentation coefficients were not reproducible; the values of S are therefore not reported. By contrast, the sedimentation behaviour of the modified material at this pH was quite satisfactory, a symmetrical peak was always observed, and the sedimentation coefficients agreed quite closely with those found at pH 7·5. Comparative sedimentation patterns for the two materials at pH 7·5 are shown in Fig. 4.

In the study of sedimentation behaviour as a function of the extent of reaction with iodine, single symmetrical peaks were observed in all cases; the sedimentation coefficients of the various samples (whose concentration varied between 0·35 and 0·21 g. dl.$^{-1}$) were all in the range

$$S_{20,w} = 3{\cdot}3 \pm 0{\cdot}1\,\text{s}.$$

The results of the two main series of sedimentation-coefficient measurements are summarized in Fig. 5.

Since no significant difference between the two materials is revealed by these data, all the values at pH 7·5 were employed in a least-squares calculation to give the result:

$$S_{20,w} = 3{\cdot}42 - 0{\cdot}26c, \quad \text{with} \quad 0 < c < 1{\cdot}0,$$

when c (corrected for radial dilution) is expressed in g. dl.$^{-1}$. This result is in quite good agreement with the more extended findings of Miller & Golder (1952).

The data for the iodine-modified derivative thus lend quantitative support to the claim made by Anson (1942) that the sedimentation coefficient of ovalbumin is unchanged by iodine modification; Cunningham & Nuenke (1959) reported that the derivative obtained by their procedure (the 6:1 product) also had the same sedimentation coefficient as ovalbumin.

To test the comparative homogeneity of the two proteins as critically as possible, the schlieren patterns obtained in the centrifuge experiments were used to calculate the diffusion coefficients of the proteins. The procedure is based on the mathematical analysis of Fujita (1956) for the shape of the gradient curve and has been described fully by Baldwin (1957). This method is appropriate to the case investigated (where S varies appreciably with c, but D can justifiably be taken as constant), and its advantage of greater sensitivity outweighs the disadvantage that only qualitative characterization of the heterogeneity is obtained.

Experiments were made at concentrations of 0·9 and 0·3 g. dl.$^{-1}$ in both cases, giving the results $D_{20,w} = 6{\cdot}92 \times 10^{-7}$ and $6{\cdot}99 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ for ovalbumin, and $7{\cdot}08 \times 10^{-7}$ and $6{\cdot}97 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ for the derivative. The internal consistency of the data from the centrifuge experiments, and the absence of time-dependence of the derived diffusion coefficients, are demonstrated in Fig. 6.

Thus the proteins are homogeneous in sedimentation within the limitations of the test. It is expected that heterogeneity corresponding to a standard deviation of 5 % of the mean sedimentation coefficient would be detected by this procedure. The close similarity between all diffusion coefficients may be to some extent fortuitous, in view of the known experimental uncertainties: the mean of all corrected values is $6{\cdot}99 \times 10^{-7}$ cm.^{2} sec.$^{-1}$, referring to 20°, which becomes $3{\cdot}80 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ at 1°, and is thus within the expected 5 % of the value for ovalbumin determined in free diffusion experiments (e.g. Nichol, Winzor & Creeth, 1960).

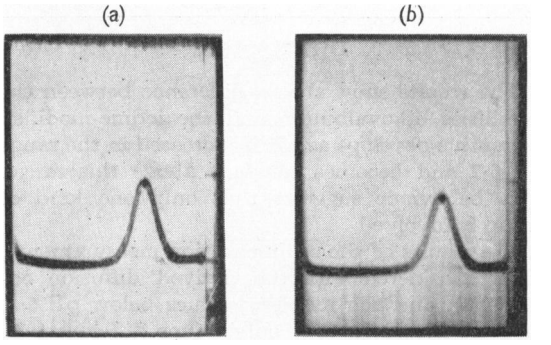

Fig. 4. Comparative sedimentation-velocity patterns obtained with: (*a*) ovalbumin (0·93 g. dl.$^{-1}$), and (*b*) iodine-modified ovalbumin (0·89 g. dl.$^{-1}$), in veronal–chloride buffer, pH 7·5, I 0·10, after 2 hr. at 60 000 rev./min.

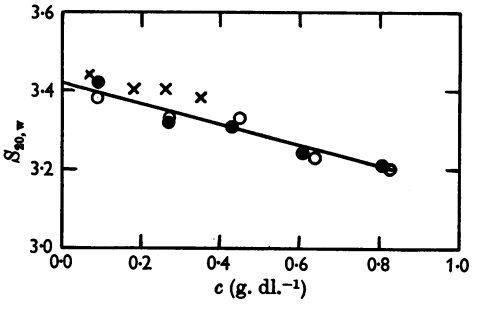

Fig. 5. The concentration dependence of the sedimentation coefficients of native ovalbumin (○) and of iodine-modified ovalbumin (●) at pH 7·5, I 0·10. The line corresponds to the relationship $S_{20,w} = 3{\cdot}42 - 0{\cdot}26c$. The points marked × refer to the modified material at pH 4·6.

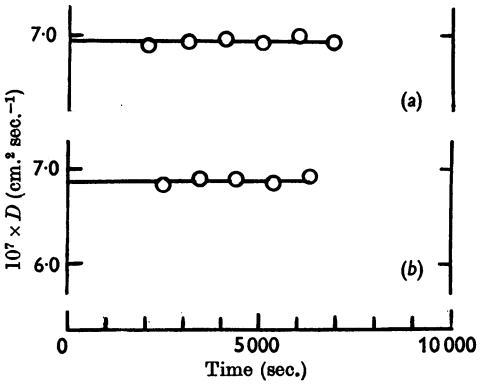

Fig. 6. Detection of heterogeneity in ovalbumin and the iodine-modified derivative: the points represent apparent diffusion coefficients calculated from the individual exposures of the sedimentation experiments, and the horizontal lines are derived from the slopes of the appropriate functions of height–area against reduced time (Baldwin, 1957). (*a*) Ovalbumin, 0·93 g. dl.$^{-1}$; (*b*) iodine-modified ovalbumin, 0·89 g. dl.$^{-1}$.

DISCUSSION

The results show that a difference between the mobilities of ovalbumin and the iodine-modified derivative develops as pH is increased in the range 5·4–6·7 and becomes constant above this range. This behaviour suggests that only one kind of group is involved.

The results of the sedimentation measurements, when considered with the derived diffusion coefficients, and the mobility values below pH 5·5, show conclusively that only minor differences in the molecular weight or frictional coefficient can exist between the two substances. The mobility difference at higher pH values must therefore be ascribed very largely to a difference in charge, and the results of the independent procedure for charge determination are in agreement with this conclusion. Moreover, both methods agree in indicating that the charge difference is unity: a whole number is to be expected in view of the evidence that the group or groups concerned are fully ionized at pH values greater than about 7.

Before this interpretation of the results can be accepted, the validity of the methods must be examined. Charlwood (1950) found that results by his procedure were always high, relative to values determined in other ways, and ascribed this to non-fulfilment of some of the assumptions involved. On this basis, the method would be useful only to define an upper limit to the valence. Since the use of Rayleigh optics enabled us to work with concentrations of protein (in terms of electrochemical equivalents) generally much lower than those used by Charlwood, the assumptions must be more closely fulfilled and the errors correspondingly reduced.

The calculation of charge from mobility values is at best only approximate because of the simplifications in the theory (cf. Overbeek & Lijklema, 1959; Brown & Timasheff, 1959) and has often been criticized [see, for example, Barnett & Bull (1960) who also list many of the recent applications]. However, with bovine plasma albumin (Waldmann-Meyer, 1960) charge values calculated from the mobilities were in agreement with those obtained from titration data, after correction for salt-ion binding. Thus the validity of the method is established for bovine plasma albumin, and it is unlikely that ovalbumin would behave very differently.

It is therefore concluded that the result given by the direct charge determinations is correct, and that the iodine-modified ovalbumin differs from the original by the introduction of a singly ionizable group of pK about 6. This conclusion contrasts with that of Perlmann (1952, 1953) for ovalbumin and the products of enzymic dephosphorylation; here a mobility difference of $0·6 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ v^{-1} was interpreted as indicating a charge difference of 2, on the basis of a comparison with titration data uncorrected for salt binding.

The iodine-modified derivative prepared in this work may not be identical with that studied by Fredericq & Desreux (1947) in view of the different conditions of preparation; their titration data indicated identical base-binding power for ovalbumin and the derivative where our mobility values show a difference. However, in this region (pH 5·5–8·5) Fredericq & Desreux show only one point on the titration curve for the derivative, so comparison of the data may not be justified.

The experimental findings which must be taken into account in formulating a reaction mechanism may be summarized: (i) the stoicheiometry of the initial reaction involves 5 iodine atoms/molecule of ovalbumin, but 1–2 more atoms of iodine react slowly if the 5:1 product is allowed to stand with excess of iodine; (ii) 2 sulphydryl groups/molecule disappear in the initial reaction, but the number (2) of disulphide groups is not changed; (iii) 1 iodine atom is incorporated into the molecule of the 5:1 product; (iv) no significant change in molecular weight or shape occurs; (v) the derivative possesses an additional singly ionizable group, of pK about 6; (vi) the solubility of the derivative is much lower, but its optical rotation is unchanged.

The most direct explanation of an odd-numbered stoicheiometry is the union of two free radicals, but this is not allowable, at least in the final product, in the light of (iv) above. Partial iodination is unlikely to be the explanation, in view of the well-defined end point in the titration and the other evidence presented. A detailed mechanism cannot

yet be advanced, but the following scheme is consistent with all the evidence now available and with many of the suggestions made by Cunningham & Nuenke (1959, 1960):

$$2Pr(SH)_2 \xrightarrow{I_2} HS\cdot Pr\cdot S\cdot S\cdot Pr\cdot SH \xrightarrow{2I_2}$$
$$IS\cdot Pr\cdot S\cdot S\cdot Pr\cdot SI \xrightarrow{2I_2+3H_2O} Pr(SI)\cdot S\cdot OH + Pr(SI)\cdot SO_2H,$$

where Pr represents the whole of the protein not taking part in the reaction, and thus includes 2 sulphydryl and 2 disulphide groups. Each of the steps is to be anticipated on other grounds: (*a*) dimers are formed spontaneously in ovalbumin solutions near the isoelectric point (Foster & Rhees, 1952; Creeth *et al.* 1958) and therefore must be expected under mild oxidizing conditions at other pH values; (*b*) disulphide bonds in proteins are known to be oxidized further by iodine (e.g. McLaren, Leach & O'Donnell, 1959); (*c*) a sulphinic acid was isolated from the products of iodine oxidation of cysteine, and evidence for the formation of a sulphenic acid was also found (Simonsen, 1933); (*d*) Cunningham & Nuenke (1959, 1960) have shown the presence of one stable sulphenyl iodide (SI) group in the 6:1 iodine-modified product.

The lack of noticeable colour in the product is presumably due to the relatively low proportion of sulphenyl iodide groups (cf. Fraenkel-Conrat, 1955). Since no dimer could be detected at any stage in the reaction, the first step must be considerably slower than the last.

The mobility studies indicate that the new group or groups in the 5:1 product titrate over a small pH range; it follows either that the sulphenic acid and sulphinic acid groups have rather similar pK values, or, more probably, that atmospheric oxidation of the sulphenic acid group occurs during the inevitably prolonged dialysis period between preparation and electrophoretic examination. Data on the dissociation constants are not available.

Item (vi) above indicates that a change in the tertiary structure (Linderstrøm-Lang, 1952) of the native protein has been brought about, resulting in the exposure of previously masked non-polar residues. The secondary structure, however, remains essentially intact.

Other possible reaction mechanisms involve the substitution of iodine in the tyrosine residues or the liberation of a hitherto-masked histidine group. These are considered less likely, but cannot yet be dismissed.

SUMMARY

1. The 5:1 product of iodine oxidation of ovalbumin has been studied in electrophoresis over the pH range 4–8·5. A new procedure is described for obtaining more accurately the first moment positions of the boundaries, and has been used in the determination of mobilities. The mobility–pH curve is identical with that of ovalbumin between pH 4 and 5·4, but is more negative at higher pH values. Above pH 6·7 the mobility difference is constant, and has the value $0·6 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ v^{-1}.

2. From electrophoresis experiments under modified conditions, the relation between the size of the δ-boundary and the excess buffer concentration in the supernatant solution has been established for both proteins: these relations have been used to calculate the valences of the proteins at pH 8·5. The values obtained were $-10·6$ and $-11·6$ for the native and modified materials respectively. The mobility values were also used to calculate valences, giving corresponding values of $-10·7$ and $-11·8$.

3. The sedimentation coefficient–concentration relationship was established for the modified product at pH 7·5. No difference from the behaviour of the native protein was detectable, both sets of data fitting the equation $S_{20,w} = 3·42 - 0·26c$.

4. With Fujita's (1956) expressions, diffusion coefficients were calculated from the sedimentation patterns as a sensitive test for heterogeneity: identical behaviour indicating homogeneity within the limits of the test was found for both materials.

5. A mechanism for the iodine–ovalbumin reaction is suggested to account for these observations and the previously determined sulphydryl and disulphide content. It involves the initial formation of a mixed product, half containing sulphenic acid groups and half sulphinic acid groups, each half being substituted with a single sulphenyl iodide (SI) residue. Subsequent aerobic oxidation of the sulphenic acid portion to yield a single final product is thought to occur.

Grateful acknowledgement is made to the Commonwealth Scientific and Industrial Research Organization for a studentship held by D. J. W. during the period 1957–59. The authors are indebted to Dr R. A. Kekwick of the Lister Institute for the gift of one sample of ovalbumin and for his criticism of the manuscript; Mr H. Murray provided technical assistance in some of the ultracentrifuge work. The encouragement and support of Professor D. O. Jordan during the part of the work carried out in Adelaide are gratefully acknowledged.

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Studies on Protein and Nucleic Acid Metabolism in Virus-Infected Mammalian Cells

5. THE KINETICS OF SYNTHESIS OF VIRUS PROTEIN AND OF VIRUS RIBONUCLEIC ACID IN KREBS II MOUSE-ASCITES-TUMOUR CELLS INFECTED WITH ENCEPHALOMYOCARDITIS VIRUS*

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(*Received* 16 *January* 1962)

Martin & Work (1961) have shown that during the course of a single growth cycle of encephalomyocarditis virus in Krebs II mouse-ascites-tumour cells there is a striking change in the pattern of ribonucleic acid metabolism as compared with the normal uninfected cell. In particular there was a 340% increase in the rate of ribonucleic acid turnover in the mitochondrial fraction coincident in time with the appearance of complete virus in this fraction. However, not more than 5–8% of this ribonucleic acid could be virus nucleic acid.

A simple method has now been developed for the isolation of complete virus from cell homogenates and from culture supernatants. The method depends on adsorption of the virus on sheep red cells and subsequent isolation of the sheep red-cell stroma with virus still attached to it.

By application of this method to virus grown for different times in Krebs cells in the presence of a suitable radioactive tracer it has been possible to follow the complete course of synthesis of virus ribonucleic acid and of virus protein. To do this, it was necessary, however, to measure the specific radioactivity of the precursor pool of amino acid and of ribonucleotide in the host cell. Phenylalanine and uridylic acid were the immediate radioactive precursors of virus protein and of virus nucleic acid and methods were devised to measure the specific radioactivity of the intracellular pools of these compounds and the effects of virus infection thereon. It has thus been shown that the synthesis of virus ribonucleic acid takes place early in the growth cycle and that it precedes the synthesis of virus protein by about 1 hr. The massive synthesis of ribonucleic acid in the cytoplasm of infected Krebs cells late in the growth

* Part 4: Martin & Work (1961).