2. The percentage of lecithin in the non-ruminant 'ghosts' was over three times as high as that found in ruminant 'ghosts'.

3. In contrast, the concentrations of sphingomyelin and choline plasmalogen were higher in ruminant 'ghosts' and consequently there were no significant differences between the concentrations of total choline-containing phospholipid in the various species.

4. Phosphatidylethanolamine was markedly higher in the 'ghosts' of omnivores than in 'ghosts' of herbivores.

5. Choline-containing phospholipids (mainly lecithin) accounted for over 94% of the plasma phospholipid of all species.

6. Of the possible acidic phospholipids in erythrocyte 'ghosts' which could function as cation carriers, phosphatidylserine was present at a relatively high concentration (8·9–16%) in all species, phosphatidylinositol varied from 1·6% in man to 5·8% in the goat and phosphatidic acid was below 1·5% in all species.

We are grateful to Mr F. Hills for valuable assistance.

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Evidence for the Chemical Interaction of Urease in Solution

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Urease prepared by the method of Sumner (1926) and recrystallized by the modified procedure of Dounce (1941) has in all cases reported shown at least two peaks in the ultracentrifuge (e.g. Sumner, Gralen & Eriksson-Quensel, 1938; McLaren, Sheppard & Wagman, 1948). Enzymic-activity studies (Hofstee, 1949), sedimentation and activity studies (Kuff, Hogeboom & Striebich, 1955), molecular-weight estimations (Setlow, 1952) and kinetic experiments (Kistiakowsky & Lumry, 1949; Ambrose, Kistiakowsky & Kridl, 1950) all provided results which could be interpreted to some degree by assuming that the components observed in the ultracentrifuge are aggregates of the same species. In this connexion it is pertinent that a reaction involving the readily detectable sulphydryl groups in the undenatured enzyme (Hellerman, Chinard & Dietz, 1943) to form disulphide linkages has been discussed (Sizer & Tytell, 1941; Kistiakowsky & Lumry, 1949); this disulphide-bond formation may occur intermolecularly, as such a cross-linkage has been postulated with other protein systems where sulphydryl groups are readily 'available' (e.g., Huggins, Tapley & Jensen, 1951; Steinrauf & Dandliker, 1958). Since an understanding of the nature of this aggregation reaction, if it occurs, is essential to proper formulation of the kinetics, and hence to any interpretation of the mechanism of urease action, a detailed study has been made of the ultracentrifugal characteristics of the substance, and is reported here.

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Secondly, in view of the known ultracentrifugal complexity, the validity of some of the primary physicochemical data for urease can be questioned. Notable in this respect are (*a*) the isoelectric point, found by a minimal solubility method (Sumner & Hand, 1929), and (*b*) the widely used molecular weight, calculated from the Svedberg expression by utilizing diffusion measurements made in the presence of sulphite ions and sedimentation results found in their absence (Sumner *et al.* 1938). We have therefore investigated the electrophoretic behaviour of urease over a wide range of pH, in order to obtain information on electrophoretic complexity and to establish fundamental data on the mobility of the substance with as high an accuracy as the material warrants. By interpolation in these results, the isoelectric point has been estimated more directly. In addition, two diffusion experiments were made to test the effect of sulphite. The physicochemical data obtained present several interesting and unusual features; when correlated with enzymic-activity measurements, they lend strong support to the hypothesis of intermolecular reaction.

EXPERIMENTAL

Materials

Enzyme preparations. The seven samples of urease used in this study were prepared by the acetone-fractionation method of Sumner (1926), from jack-bean meal obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A., and were recrystallized twice by the method of Dounce (1941). Glass-distilled water previously passed through both anionic- and cationic-exchange columns was used throughout the experiments to avoid accidental inhibition of the enzyme by traces of metal ions. The glassware was cleaned with a nitric acid–sulphuric acid bath, rinsed thoroughly and steamed (Henry & Smith, 1946; Kistiakowsky & Shaw, 1953). The specific activity of the samples prepared varied from less than 10 000 to 80 000 Sumner units (S.U.), this being quite typical of other findings (Hellerman *et al.* 1943; Evert, 1952; Kistiakowsky, Mangelsdorf, Rosenberg & Shaw, 1952). The samples, with the exception of sample 5, were stored in solution at 2–4° in the presence of cysteine hydrochloride. After 2 months the enzyme solutions developed a faint turbidity and the activity in some cases had decreased by 50% (Kistiakowsky *et al.* 1952). Consequently, after 1 month, during which time the solutions remained clear and maintained their activity reasonably well, the sample was discarded.

Buffers. The composition of the numerous buffers used in this work is given in the text. The urease was either dissolved directly in the cold buffer, or dialysis in the cold for 7 days was employed to exchange different buffers, keeping the urease in solution.

Sulphite solutions. The solutions used in the experiments performed in the presence of sulphite ions had the following composition (cf. Sumner *et al.* 1938; Epstein, 1959): 0·048 M-Na_2HPO_4, 0·021 M-KH_2PO_4, 0·031 M-Na_2SO_3, 0·013 M-$NaHSO_3$. No special precautions were taken to prevent atmospheric oxidation. The oxidation–reduction potential of the solutions was not measured and, consequently, no quantitative significance was attached to the total sulphite concentration.

Methods

Enzymic-activity measurements. The assays were performed under the standard conditions specified by Sumner & Hand (1928). The reaction was stopped after 5 min. with 2 N-HCl, and the ammonia formed was determined in accordance with the general method described by Kistiakowsky *et al.* (1952). In this case a Zeo-Karb 225 ion-exchange column was used to adsorb the NH_4^+ ions, which were eluted with KOH and the solution was diluted to 250 ml. Nessler's reagent was added and the extinction was measured with a Unicam SP. 500 spectrophotometer, after 15 min. The absolute amount of ammonia formed was obtained by reference to a standard curve. Each assay reported was performed in duplicate on at least three different dilutions of the enzyme sample, chosen so that 0·1–0·4 μmole of ammonia was produced. Each set of measurements also included blanks, in which the acid was added before the enzyme; since the buffered urea solutions (used as substrate) were freshly prepared for each set of assays, the corrections were negligible.

Electrophoresis. All electrophoresis experiments were conducted at 1–2° in a Spinco model H electrophoresis–diffusion apparatus, employing simultaneously or separately Rayleigh and schlieren optics. The former system yields a record of the refractive index versus vertical-cell co-ordinate in terms of interference fringes; the number, J, of such fringes between successive schlieren minima is accurately determinable and forms a convenient comparative unit to specify concentrations in electrophoresis experiments (Longsworth, 1951). In other aspects of the work described, the concentrations of urease were also determined by counting the fringes observed when a boundary was formed between the dialysed solution and its dialysate; for this reason all concentrations are quoted in terms of J. If the specific refractive increment of all components has the value 0·00180 dl.g.$^{-1}$ (cf. Perlman & Longsworth, 1948; Halwer, Nutting & Brice, 1951) then the relation $c = 5{\cdot}8 \times 10^{-3} J$ (where the concentration c is in g./100 ml.) may be applied to all values of J quoted in this paper (Creeth, Nichol & Winzor, 1958). In the mobility experiments, for which the 11 ml. cell was used, the ascending limb was sealed from the atmosphere to prevent flow through the cell due to the continually changing pressure head. Further, after a 4 hr. electrophoretic migration in one direction, the current was reversed for an equal period of time. At least five exposures were taken at suitable time intervals to record the progress of the boundary in each direction, and the average of the two descending mobilities (determined from the regression of the observed boundary position upon time) was taken as the mobility of the species. This technique, which is applicable only to very dilute solutions, precludes errors due to flow caused by volume changes within the electrode vessels and provides a sensitive means of detecting minor cell leaks. The maximum of the schlieren peak was used to measure the rate of movement of the boundary in mobility determinations. Since the peaks were nearly symmetrical (see below), and the concentrations low, it was considered that deter-

mination of the first moments (Longsworth & Jacobsen, 1949) would not have led to any useful increase in accuracy. The conductivities of the buffer solutions (dialysates) were used in the mobility calculations; they were measured at the temperature of the experiment, with a Philips's conductivity bridge. As the concentration of the protein used was kept at a low value (3–20 fringes), the error in assuming that the conductivities of the buffer and the protein solution are the same is small. The pH values were obtained with a Doran Universal pH meter.

Sedimentation. Sedimentation-velocity experiments were performed in a Spinco model E ultracentrifuge at 47 660 rev./min. and the sedimentation coefficients were corrected to water at 20°. The temperature was recorded, but not controlled, by the RTIC unit and the average value was taken as the temperature of the run. A variation of no more than 0·1° was observed during the normal 40 min. duration of the experiments when performed near room temperature (about 0·3° for the runs at 3°). The required viscosity and density values for the buffers were measured directly with respectively an Ostwald viscometer and a 25 ml. pycnometer. The partial specific volume of 0·73 reported by Sumner *et al.* (1938) was used in each case, assuming that it was independent of buffer composition. With systems that showed several peaks in the ultracentrifuge, the corrected areas under the peaks (Svedberg & Pedersen, 1940), together with the refractometrically determined fringe counts, were used to estimate the required concentrations. Dilutions, where necessary, were made by weight and the dilution factor was applied to the fringe count obtained on the original solution. The procedure is not valid unless all non-dialysable material is visible in sedimentation experiments during photography.

Diffusion. The diffusion experiments were performed in the Spinco model H apparatus referred to above, Rayleigh optics alone being employed. The procedure, together with a detailed discussion, has been described by Creeth *et al.* (1958). Before diffusion all protein samples (which were freshly prepared) were dialysed with periodic gentle agitation and frequent changes of buffer in the cold for 8 days. The temperature of the run was periodically recorded with a previously standardized Beckmann thermometer. The Rayleigh interferograms were measured with a two-dimensional comparator (Optical Measuring Tools Ltd., Maidenhead, Berks.), accurate to 2×10^{-4} cm., fitted with a projection screen.

RESULTS

Electrophoresis. All relevant information on the mobility experiments is summarized in Table 1; if the mobility values are plotted against pH a

Table 1. *Electrophoretic-mobility data on urease in buffers at I* 0·10 *and at* 1·0$_0$°

Sample no.	Buffer composition (M)	Concn. of protein (J)	Potential gradient (v cm.$^{-1}$)	pH	Descending mobility ($\times 10^5$, cm.^{2}/sec.$^{-1}$v^{-1})
3	0·08 NaCl 0·02 Sodium acetate 0·18 Acetic acid	5	2·95	$3·6_9$	$6·0_8$
1	0·08 NaCl 0·02 Sodium acetate 0·08 Acetic acid	3	1·98	$3·8_9$	$6·1_3$
3	0·08 NaCl 0·02 Sodium acetate 0·05 Acetic acid	5	2·93	$4·2_3$	$5·0_8$
2	0·06 NaCl 0·04 Sodium acetate 0·03 Acetic acid	3	3·22	$4·7_0$	$2·1_6$
1	0·03 NaCl 0·07 Sodium acetate 0·03 Acetic acid	5	2·48	$5·0_1$	$-0·4$
1	0·03 NaCl 0·07 Sodium acetate 0·01 Acetic acid	12	2·45	$5·4_6$	$-2·3_6$
2	0·08 NaCl 0·02 Sodium cacodylate 0·02 Cacodylic acid	18	3·04	$6·0_1$	$-3·2_5$
1	0·05 NaCl 0·05 Sodium cacodylate 0·01 Cacodylic acid	12	2·31	$6·6_1$	$-5·5_6$
1	0·09 NaCl 0·01 Sodium diethylbarbiturate 0·02 Diethylbarbituric acid	19	1·91	$7·4_5$	$-6·8_5$
1	0·08 NaCl 0·02 Sodium diethylbarbiturate 0·01 Diethylbarbituric acid	14	2·02	$7·9_5$	$-7·1_5$
1	0·07 NaCl 0·01 Sodium tetraborate 0·01 Boric acid	9	2·20	$8·9_0$	$-8·0_8$

smooth curve results, interpolation in which gives the value pH 5·0 ± 0·05 for the isoelectric point in sodium acetate–acetic acid–sodium chloride buffer, I 0·10. This value is in close agreement with other findings (Sumner & Hand, 1929; Wills, 1952). Anderson & Alberty (1948) reported two values of the electrophoretic mobility of urease at I 0·01; in order to compare these with the results reported here, it is necessary to use the procedure outlined by Abramson, Moyer & Gorin (1942), which is based on an inexact microscopic model of electric migration. By the use of the published diffusion coefficient (Sumner *et al.* 1938) to calculate the radius r of a hydrated sphere, $r = 61{\cdot}3 \times 10^{-8}$ cm. is obtained and, for example, the value

$$-4{\cdot}4 \times 10^{-5} \text{ cm.}^2 \text{ sec.}^{-1} \text{v}^{-1}$$

for the 'corrected' electrophoretic mobility at I 0·10, pH 7·4, from Anderson & Alberty's figure. By interpolation from Fig. 1, the corresponding value obtained in this work is

$$-6{\cdot}6 \times 10^{-5} \text{ cm.}^2 \text{ sec.}^{-1} \text{v}^{-1}.$$

Although these calculations are approximate, the discrepancy is large, and probably indicates greater anion-binding at the higher ionic strength.

In the electrophoresis experiments a single relatively symmetrical schlieren peak was obtained at all pH values used. The schlieren pattern shown in Fig. 1 is quite typical of results obtained, but in this case the protein concentration (J 120) is much higher than that used for mobility determinations. The limited solubility of urease at pH

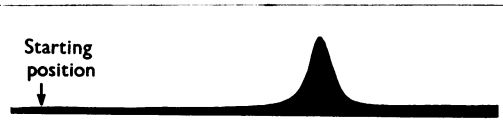

Fig. 1. Electrophoresis pattern (ascending limb) of urease at pH $7{\cdot}4_0$ in diethylbarbiturate buffer, I 0·10, after 8 hr. at a potential gradient of 2·9 v cm.$^{-1}$

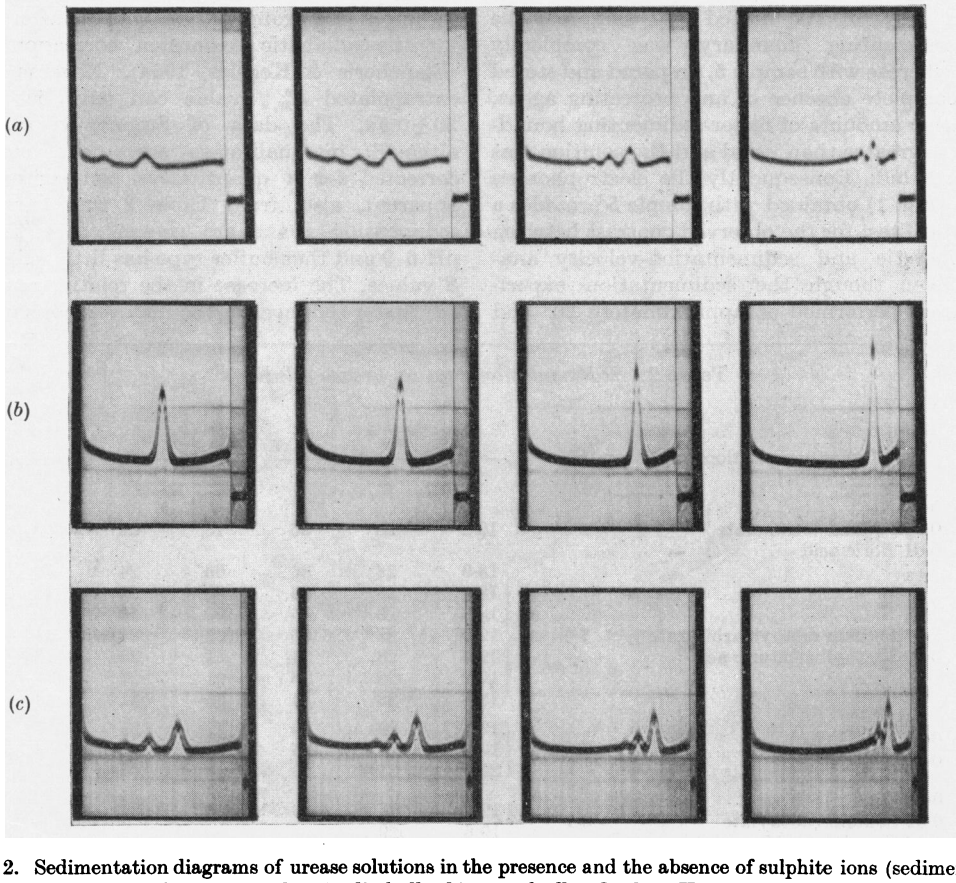

Fig. 2. Sedimentation diagrams of urease solutions in the presence and the absence of sulphite ions (sedimentation from right to left): (*a*) sample 2 in diethylbarbiturate buffer, I 0·10, pH $7{\cdot}4_5$; (*b*) sample 5 in a buffered sulphite solution, pH $7{\cdot}0_9$; (*c*) the same sample as (*b*) after dialysis with 0·96% phosphate buffer, pH $7{\cdot}0_3$.

values near the isoelectric point (Sumner & Hand, 1929; Anderson & Alberty, 1948) prevents similar analytical electrophoresis experiments being performed under these conditions. However it may be concluded from the Rayleigh interferograms that not more than 10% of another resolvable electrophoretic component was present.

Sedimentation. The patterns obtained in sedimentation-velocity experiments provide a striking comparison with those obtained in electrophoresis, where, as we have seen, the absence of appreciable amounts of components with different mobilities was demonstrated. Each sample of urease prepared was sedimented and a typical result (excluding patterns obtained with samples 4 and 5) is shown in Fig. 2 (*a*). At least four peaks are clearly visible. The S of the slowest-moving boundary could only be estimated to lie within the range 4–6 as it was diffuse and never completely resolved during an experiment. (In some experiments at low I, the S 4–6 component was not present and one with S 12 appeared.) The relative areas under the separate peaks varied considerably with different preparations; indeed with sample 4 the fastest-sedimenting boundary was completely absent, whereas with sample 5, prepared and stored in the complete absence of any protecting agent, the relative amounts of faster-sedimenting boundaries were greater than usual and the solution was slightly turbid. Consequently the electrophoresis pattern (Fig. 1) obtained with sample 5 provides a very critical test for the observed contrast between electrophoretic and sedimentation-velocity analyses. Even though the sedimentation experiments were performed at approximately 20° and the electrophoresis analyses at 1°, the comparison is justified as sedimentation of sample 7 at 3° revealed no alteration in the relative amounts of components present in comparison with a control experiment performed at 20°. The appearance of several sedimenting boundaries has been reported by all workers who have analysed urease preparations in this way (Sumner *et al.* 1938; McLaren *et al.* 1948; Kuff *et al.* 1955).

The sedimentation coefficients and the relative amounts of the three major sedimenting boundaries are given, as a function of concentration, in Table 2, where the effect of buffer type, pH and sample (referred to above) is also illustrated. The accuracy of both the estimated concentrations, and (to a lesser degree) the S values, is limited, because no account has been taken of errors due to the Johnston–Ogston effect (Johnston & Ogston, 1946) or those due to the neglect, in area determinations, of any non-dialysable material which was not clearly resolved in the photographs. However, the S values of 18·6 and 28 found by McLaren *et al.* (1948) agree within 1% with values estimated at a comparable protein concentration, after allowing for the adiabatic expansion correction of 0·9° (Biancheria & Kegeles, 1954). Nevertheless, the extrapolated $S^0_{20,w}$ value can only be given as $20 \pm 0{\cdot}5$s. The data of Sumner *et al.* (1938), although in qualitative agreement, cannot be corrected for a quantitative comparison. It is apparent, also, from Table 2 that three major sedimenting peaks are present over the range pH 6–9 and that buffer type has little effect on the S values. The increase in the relative amounts of the faster-sedimenting boundaries observed when

Table 2. *Sedimentation data on urease solutions*

Sample no.	Buffer composition (M)	pH	$S_{20,w}$* 1	$S_{20,w}$* 2	$S_{20,w}$* 3	Percentage of total area* 1	Percentage of total area* 2	Percentage of total area* 3	Concn. of 1 (J)
7	0·07 NaCl, 0·01 Sodium tetraborate, 0·01 Boric acid	8·9	19·2	28	35	73	22	5	—
2	0·09 NaCl, 0·01 Sodium diethylbarbiturate, 0·02 Diethylbarbituric acid	7·5	18·9	28	36	66	24	10	46
2			18·9	28	36	63	25	12	44
4			18·8	28	—	90	10	0	40
2			19·0	28	35	61	28	11	39
2			19·3	28	34	61	26	13	29
2			19·2	28	36	61	27	12	16
5			18·8	28	34	55	31	14	—
6	0·05 Na_2HPO_4, 0·02 KH_2PO_4	7·0	19·7	29	36	72	21	7	—
7			19·2	28	35	85	12	3	—
7†			21	31	40	83	13	4	—
2	0·08 NaCl, 0·02 Sodium cacodylate, 0·02 Cacodylic acid	6·1	19·3	28	36	60	28	12	13
2			19·6	29	—	—	—	—	10

* The resolved boundaries in Fig. 2 (*a*) are numbered (1, 2 and 3) in order of increasing sedimentation coefficient.

† This experiment was performed at 3° (all others were at ~20°): the discrepancies evident in the S_{20} values probably arise at least partly from the application of a large temperature correction factor.

sample 7 was dialysed from pH 7·0 to 8·9 cannot be taken as evidence for increased aggregation at high pH values as other variables (see below) inherent in the dialysis and handling procedures are introduced. Protein solutions dialysed to pH values near the isoelectric point, or below it, precipitated (the precipitate being insoluble in buffers at pH 7–8) and the resulting solution was too dilute for useful sedimentation-velocity analyses to be made. As is also evident from Table 2, no marked change in the relative areas under the peaks was observed on dilution; in this connexion, the insensitivity of these area determinations should be emphasized. The relative proportions of the components were also unaffected by their solutions standing for periods which ranged from 2 hr. to 30 days after the final recrystallization (for example, with sample 4, six sedimentation-velocity analyses over this period all revealed the amounts 90%, 10% and 0% for the components referred to in Table 2).

If the peaks observed in sedimentation analysis correspond directly with aggregates of well-defined molecular weight, then the effect of modifying agents [preferably group-specific reagents (cf. Putnam, 1953)] on the relative amounts of these sedimenting boundaries should be demonstrable; the experiments described below were therefore performed.

(i) Effect of sulphite ions. When buffered solutions, pH 7·0, of urease (samples 4–7) were dialysed against buffered sulphite solutions and the resulting protein solution was sedimented, the faster-sedimenting boundaries were no longer evident and the amount of the $S^0_{20,w}$ 20 boundary increased markedly. This is shown in Fig. 2 (*b*). With sample 4, this increase in area equalled, within the limits of accuracy of the determinations, the area previously under the *S* 28 peak, which was the only other peak visible in this sample. However, with sample 5 the increase was apparently greater than could be accounted for by the disappearance of the peaks visible in sulphite-free solutions during photography. The effect of sulphite ions was entirely reproducible with all samples tried, and reversible in the sense that when the sulphite was removed by exhaustive dialysis the other peaks returned, as is evident in Fig. 2 (*c*). The relation between overall enzymic activity of the solution and relative amounts of the separate sedimenting peaks is given in Table 3. It is apparent that an increase in activity accompanies an increase in the amount of the $S^0_{20,w}$ 20 component.

The effect of protein concentration on the *S* of the species existing in the presence of sulphite ions is shown graphically in Fig. 3 (data not included in Tables). The determination of concentrations was more accurate in this case as the dilutions were made by weight and thus the extrapolated $S^0_{20,w}$ value can be given as $20·3 \pm 0·1$ and therefore is the same within experimental error as the corresponding value obtained in the sulphite-free system. The solid line of Fig. 3 is an attempt to average the data; its curvature is unexpected, but is apparently outside experimental error.

A diffusion experiment on sample 6 (*J* 110·84) in a buffered sulphite solution, pH $7·0_9$, gave the observed diffusion coefficient referred to as $\mathscr{D}_A$ (Baldwin, Dunlop & Gosting, 1955; Dunlop & Gosting, 1955) of $3·28 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ at 20·00°, corrected to water. The sharpness of the initial boundary was judged satisfactory as the 'zero time' was 165 sec. and there appeared to be no variation of the diffusing solute with time, according to the criteria applied in a previous paper (Creeth *et al.* 1958). The value of $\mathscr{D}_A$, which agrees

Table 3. *Effect of sulphite on the enzymic activity and sedimentation-velocity patterns of urease*

The control sample was urease dissolved in veronal buffer, pH 7·5, *I* 0·1, stored in the cold for 1 week and diluted with phosphate buffer for assay. The sulphite-treated sample was a portion of the solution of urease in veronal buffer, dialysed for 1 week against a sulphite solution and diluted with phosphate buffer for assay. This was performed within 2 hr. of that for the control. These data were found with sample 5, which had a low specific activity (10 000 Sumner units/g.). The effect was reproduced with sample 7. The areas are denoted as in Table 2. Approx. 0·7% solution of protein was used.

	Percentage of total area			Enzymic activity of 1 ml. of solution (Sumner units)
	1	2	3	
Control	55	31	14	25
Sulphite-treated urease	100	0	0	50

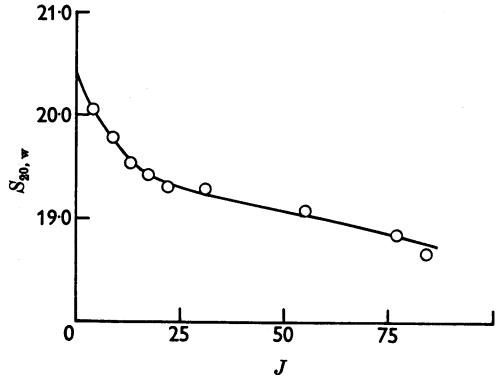

Fig. 3. Variation of sedimentation coefficient with concentration (expressed as fringes, *J*, see text) of the species present in a buffered sulphite solution of urease, sample 6.

with the value of $3{\cdot}46 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ found by Sumner *et al.* (1938) quite closely, is not the diffusion coefficient D_{11} appropriate to the Svedberg molecular-weight expression [cf. the exact form of this expression given by Baldwin (1958)] unless (*a*) flow-interaction effects (Baldwin *et al.* 1955; Dunlop & Gosting, 1955; Dunlop, 1957), (*b*) second-order concentration-dependence effects (Creeth, 1955; Gosting & Fujita, 1957), and (*c*) any impurities are absent. The deviation graph, Fig. 4, which gives the relative fringe deviations, Ω_R, over the whole boundary, as a function of H (z^*) (Creeth, 1958), indicates clearly that some, at least, of the above conditions are not fulfilled. Although an analysis by the curve-fitting procedures described (Creeth, 1958) indicates the presence of a fast-diffusing impurity which could be tentatively associated with the S 4–6 component, such procedures are not justified where flow interactions are likely (Creeth *et al.* 1958; O'Donnell, Baldwin & Williams, 1958).

A second diffusion experiment was performed under similar conditions (veronal buffer, pH 7·5, I 0·10, J 64·66, zero time 334 sec.) but with the sulphite omitted. The results in this case were quite different, the diffusion coefficient being much lower ($\mathscr{D}_A$ $1{\cdot}8 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ at 20°) and the (nearly symmetrical) deviation plot very much greater in magnitude ($\Omega_{R\ max.}$ 680×10^{-4}). The contrast in the results of these two experiments, particularly in the size of the deviation plots, is illustrative of the pronounced effect of sulphite on the system.

(ii) *Effect of cysteine hydrochloride.* This reagent was added during preparation to prevent inhibition by metal ions which could accidentally be present (Shaw, 1954). Accordingly, no fixed amount was added during the preparation of most of the samples. As has been described, the apparent proportions of the components observable in the ultracentrifuge varied widely between different samples. To test whether this variability was the direct result of varying contents of cysteine (i.e. on the assumption that cysteine and sulphite have similar actions), this reagent was not added at any stage of the preparation of sample 5. The resulting preparation had a low activity (Table 3) and relatively greater amounts of the faster-sedimenting components (Table 2). Although not conclusive, this result supports the contention that cysteine and sulphite do have similar actions on urease, in respect of reducing or reversing aggregation.

(iii) Effect of an oxidizing agent. The addition of 0·01 M-potassium ferricyanide caused immediate and complete precipitation of the protein. The product was insoluble in buffer at pH 7·0 and in this respect resembled the precipitate obtained on dialysing a urease solution to pH 5·0.

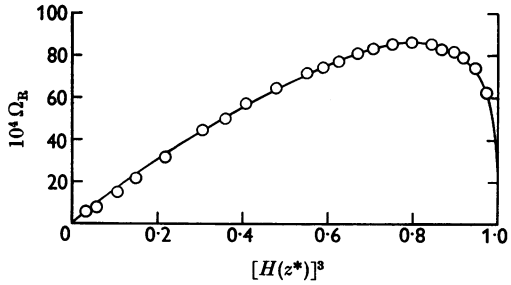

Fig. 4. Fringe-deviation diagram obtained from the diffusion of urease, sample 6, in the presence of sulphite ions at pH 7·0 .

DISCUSSION

The appearance in an electrophoresis experiment of a single peak which approximates to Gaussian form (cf. Fig. 1) cannot, of course, be interpreted as convincing evidence of electrophoretic homogeneity. Boundary-spreading experiments at the isoelectric point would be desirable, so that the extent of heterogeneity could be specified (Anderson & Alberty, 1948); these, however, are prohibited in this case because of the low solubility. Although the mobility experiments described earlier indicate that only one peak is evident over a wide pH range, the experiments were performed at only one ionic strength and do not preclude the possibility of the presence of components with slightly differing mobilities. Nevertheless, it is clear that the marked heterogeneity of the samples in the ultracentrifuge, illustrated by Fig. 2 (*a*), cannot be directly related to the electrophoresis patterns obtained, in terms of individual components. To correlate these results, and to relate the significant finding that sulphite converts all the heavier species into one of $S^0_{20,w}$ 20, only one hypothesis is apparent, namely that urease tends to form dimers, etc., in the absence of reducing agents, these polymers having lower specific enzymic activities, but electrophoretic mobilities equal to that of the monomer. This hypothesis will now be examined in the light of all available evidence. The numerical calculations below have been made by standard procedures, following, for example, Peacocke & Schachman (1954) and Johnson, Shooter & Rideal (1950).

$S_{20,w}$ values. These are approximately in the ratio 1:1·4:1·8. Two interpretations of these figures may be made on the basis of models representing extremes in shape. Thus if 'urease monomer' (the $S^0_{20,w}$ 20 component) is considered to be spherical and the dimer and trimer to be cylinders of axial ratio 2 and 3 respectively, it may be shown

on the basis of elementary theory and the equations of Perrin (1936) (tabulated by Svedberg & Pedersen, 1940) that the three sedimentation coefficients would lie in the ratio $1:1{\cdot}5_2:1{\cdot}8_7$. These differ little from the values for spheres quoted by Kegeles & Rao (1958). The alternative interpretation is based on very similar reasoning but correlates the observed frictional-coefficient ratio of 1·19 [Sumner *et al.* (1938) and this work] with asymmetry alone. By this means one obtains about 4 for the axial ratio of the 'monomer'. If the polymers are taken to have axial ratios of 8 and 12, the corresponding values of the sedimentation coefficients lie in the ratio $1:1{\cdot}3_1:1{\cdot}5_4$. Although both of these calculations must be considered as approximate, the similarities in the predicted ratios with those observed lends considerable support to the initial hypothesis. On some models, the $S_{20} = 36$ component could correspond to a tetrameric unit.

Electrophoretic-mobility values. Calculations here are somewhat more uncertain owing to the approximations involved in the theory, but applying the equations referred to by Abramson *et al.* (1942) to the case of a spherical monomer and cylindrical dimers and trimers, as before, and assuming that the total charge is conserved, one obtains mobility values in the ratio $1:1{\cdot}0_9:1{\cdot}1_2$. The second model described (cylinders of axial ratio 4, 8 and 12) gave lower mobilities, but these lay in the same ratio as for the first model. This result is partly coincidence, and does not imply that the mobility ratios are independent of the geometry of the model; thus a change from spherical monomer to dimer of axial ratio 4 produces mobilities in the ratio $1:0{\cdot}8_9$. In short, the theory predicts relatively small changes in mobility as a consequence of aggregation, and that these could either increase or decrease the mobility relative to that of the monomer, depending on the model used.

To sum up, it is clear that if the aggregation process is visualized as taking place between nearly spherical monomer units and that if the essential structural integrity of the monomer is largely or wholly preserved in the dimers and trimers (or tetramers), then this picture is compatible with both the ultracentrifuge and electrophoresis results. Analogous behaviour has been recorded for several other systems, e.g. L-amino acid oxidase (Blanchard, Green, Nocito, Ratner & Moore, 1945), haemocyanin (Svedberg, 1937), ground-nut globulins (Johnson *et al.* 1950; Johnson & Naismith, 1953), α-keratose (O'Donnell & Woods, 1956) and soya-bean proteins (Briggs & Wolf, 1957).

Effect of sulphite. The apparent conversion of heavier material into one of $S^0_{20,w}$ 20, caused by this reagent, is most directly explained on the assumption that the dimers, trimers etc. arise by the formation of intermolecular disulphide linkages; this linkage is known to be cleaved in mild and specific fashion by sulphite ions at pH 7. Although bisulphite ions must be present to a minor extent at this pH (Ambrose *et al.* 1950), it is not thought that bisulphite is concerned in the postulated disulphide-bond cleavage, in view of its relative inefficiency (compared with sulphite) in this respect (Cecil & McPhee, 1955*a*, *b*; Parker & Kharasch, 1959). Clearly, however, the action of bisulphite may be important at lower pH values, or generally as an inhibitor (cf. Ambrose *et al.* 1950).

The increase in area of the $S^0_{20,w}$ 20 component cannot always be correlated quantitatively with the disappearance of faster components. This might be due to neglect of material of very high molecular weight, evident at the bottom of the cell, which may also be affected by the sulphite. The most probable interpretation is that the species present in sulphite solutions has the same molecular weight as the $S^0_{20,w}$ 20 component in untreated solutions; this, if substantiated, has the consequence that either there can be no structurally important disulphide linkages in urease, or that, if they are present, they are considerably less 'accessible' than the intermolecular linkages.

Two points of interest in connexion with the polymerization remain. These are (*a*) whether it is an equilibrium reaction (in the sense that comparable proportions of e.g. monomer and dimer can exist at equilibrium at a realizable concentration) and (*b*) the related question of the mechanism. Concerning point (*a*), the effects of standing, of dilution and of temperature variation all indicate that, if an equilibrium exists, the kinetic constants regulating the interconversion are rather small. This conclusion is supported by the appearance of the centrifuge diagrams, which show clearly that the concentration gradient falls to zero between the peaks, thereby implying independence of the flow of each component (Gilbert, 1959; Williams, Van Holde, Baldwin & Fujita, 1958). This behaviour may be compared with that of such systems as α-chymotrypsin (Massey, Harrington & Hartley, 1955) and β-lactoglobulin (Ogston & Tilley, 1955), where rapid equilibration occurs between monomer and polymers. In respect of (*b*), the mechanism of polymerization, intermolecular disulphide bridges could arise either by direct oxidation of sulphydryl groups on separate molecules or by the mercaptan–disulphide interchange, originally proposed by Huggins *et al.* (1951) to account for the gelation phenomena encountered with plasma albumin in urea solutions. The evidence presently available does not allow a choice to be made between these possibilities and further discussion on this point will be deferred.

Urease solutions containing sulphite are colourless and relatively stable, and accordingly more

confidence can be placed in the quite reproducible physicochemical data obtained with them. Nevertheless, the results do not allow a reliable value of the molecular weight to be derived. The extrapolated sedimentation coefficient (in sulphite) $S^0_{20,w}$ could be combined with a similarly extrapolated $\mathscr{D}_A$ if such a value were available, or combination of S and $\mathscr{D}_A$ at the same finite concentration could be attempted. The latter procedure, which is normally a good practice, when there are grounds for assuming that $\mathscr{D}_A$ approximates to D_{11}, is not justified in this case because (*a*) the concentration dependence of S is rather pronounced, (*b*) even more doubts than usual must arise about whether an expression valid for two components can justifiably be applied to the system urease–buffer–sulphite–water, (*c*) $\mathscr{D}_A$ must differ from D_{11} by an amount at present unknown, but certainly significant. Confirmation of the presently accepted value of the molecular weight is evidently desirable.

SUMMARY

1. Electrophoretic-mobility data on urease have been obtained over the range pH 3·5–9·0; by interpolation it is confirmed that the isoelectric point of the enzyme in 0·10 ionic strength acetate buffers is 5·0 ± 0·05. The material gave a single, rather symmetrical peak at all pH values.

2. Sedimentation-velocity experiments generally gave at least three peaks, the relative proportions varying somewhat with the particular sample: the S_{20} values were approximately 19, 28 and 36 s.

3. In the presence of sulphite, samples which previously gave three peaks gave only the one with $S_{20} \sim 19$, and the specific enzymic activity increased. The concentration-dependence of the S of this species has been determined.

4. Diffusion experiments gave much lower values of $\mathscr{D}_A$ in the absence of sulphite than in its presence, and the deviations from Gaussian form of the diffusion curves also changed markedly.

5. The data are interpreted as indicating that dimers and trimers tend to be formed in urease solutions, but that the system is not one in rapid equilibrium: the polymers are probably formed by intermolecular disulphide cross-linkages.

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Carbohydrates in Protein

2. THE HEXOSE, HEXOSAMINE, ACETYL AND AMIDE-NITROGEN CONTENT OF HEN'S-EGG ALBUMIN*

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One of us described more than 20 years ago (Neuberger, 1938) the isolation, from an enzymic hydrolysate of crystalline egg albumin, of a polysaccharide containing mannose, glucosamine and some unidentified nitrogenous material. Comparison of the hexose and hexosamine content of the protein and of the isolated polysaccharide together with the estimation of the molecular weight of the latter indicated that all the carbohydrate was present as one unit in the egg-albumin molecule. The analytical data obtained also suggested that, on the basis of the then accepted value of 40 000 for the molecular weight of the protein, egg albumin contains four residues of mannose and two of glucosamine. As the methods of isolation used involved treatment with acetic anhydride, no information could be obtained as to whether the glucosamine residue was present in the protein as its *N*-acetyl derivative. By the methods then available it was not possible to obtain any indication of the linkage between the carbohydrate and the peptide chain. Some revision of the data is also necessary in view of the fact that the molecular weight of egg albumin is now generally believed to be 45 000 (see Warner, 1954).

This work was taken up again some years ago and a short report of some new findings has appeared (Johansen, Marshall & Neuberger, 1958). Preliminary communications on the same subject from other Laboratories have also been recently published (Cunningham, Nuenke & Nuenke, 1957; Jevons, 1958). In the present paper the analytical methods used are critically examined and some of the earlier figures revised. Chromatographic evidence obtained with a hydrolysate of the polysaccharide, the preparation of which from egg albumin has been briefly described (Johansen *et al.* 1958), indicated that the only sugars present are mannose and glucosamine.

Mannose

Carbohydrate in proteins has generally been determined by reactions involving heating the material with conc. H_2SO_4 in the presence of an appropriate phenol, nitrogen base or related compound, giving rise to products, the intensities of the colours of which are measured. These methods have been applied to egg albumin. The orcinol–H_2SO_4 method as described by Winzler (1955) was used; but the extinction was read at a wavelength of 505 mμ, since it was found that, with a mannose standard, the spectrum of the coloured product had a steep curvature at a wavelength of 540 mμ but not at 505 mμ, and secondly that rectilinearity was achieved on plotting absorption against amount of mannose at 505 mμ but not at 540 mμ. A five-times recrystallized [$(NH_4)_2SO_4$] preparation

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