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Experiments on Electrical Migration of Peptides and Proteins inside Porous Membranes: Influences of Adsorption, Diffusion and Pore Dimensions

By R. L. M. SYNGE
The Rowett Research Institute, Bucksburn, Aberdeenshire

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This work was begun in the hope that separations depending chiefly on molecular weight could be achieved in the peptide series by methods of the kind used by Mould & Synge (1954*a*), who were able partially to resolve the homologous series of polymeric straight-chain dextrins under conditions of electrokinetic ultrafiltration in collodion membranes. The use of molecular-sieve effects in membranes for separations according to molecular weight is traditional for protein chemistry, in the techniques of simple dialysis and simple ultrafiltration. Signer, Hänni, Koestler, Rottenberg & von Tavel (1946) and others have developed apparatus, often rather complicated, for multiplying the enrichment effects observed in dialysis of low-molecular compounds so as to obtain actual separations. Craig & King (1955) have reported rates of passage through cellophan of amino acids, peptides, etc., and on the basis of these observations used a repetitive procedure, similar in design to fractional crystallization, to fractionate the partial hydrolysis products of tyrocidine according to their molecular weight. They showed that rates of dialysis are determined not only by diffusion rates and by molecular-sieve effects in the pores of the membrane but that other effects, probably due to electrical charges, must be invoked to explain their results. This point is further discussed below. Lathe & Ruthven (1956) have obtained useful fractionations of proteins, peptides, etc., according to molecular weight by chromatography on starch grains. Here, it seems, molecular-sieve effects cause different proportions of the water-filled spaces within the grains to be accessible to dissolved molecules of different sizes.

Mould & Synge (1954*a*) used the electroendosmotic flow of water inside, and parallel to the walls of, collodion membranes to effect chromatographic separations of dextrins. They concluded that these separations were primarily due to adsorption on the collodion surfaces, although molecular-sieve effects were also present. Exclusion by ultrafiltration seemed to set in rather abruptly for dextrins whose molecular weight exceeded a value which depended on the pore size of the membrane. As most proteins and peptides are positively charged and reasonably stable in weakly acid solutions, it was hoped that, under the conditions of Mould & Synge (1954*a*), they would migrate inside the membranes by electrophoresis and not simply with the electroendosmotic stream, and that the effects of chromatographic adsorption and molecular sieving, superimposed on the ionic migration, could then be studied by varying the porosity of the membranes. However, it was soon found that adsorption effects must be eliminated (see Results and Discussion) for satisfactory use of this type of apparatus.

In order to prevent adsorption, the collodion was denitrated. Solvent mixtures which would swell or dissolve collodion could then be used with the resulting cellulose membranes. The solvent mixture chiefly studied (phenol–acetic acid–water) was expected to displace peptides from the state of adsorption on cellulose. Such solvents tend also to break secondary-valence bonds between peptide molecules and thus act as dissociating agents (cf. Doty, Bradbury & Holtzer, 1956). I have for long doubted whether the substances generally used as protein precipitants or as solvents for extracting or dialysing amino acids and other non-protein material away from proteins can be relied on to extract from biological material peptides other than the simplest ones (Synge, 1953, 1955, 1956). It seems that, for purely chemical studies, solvents which dissociate secondary-valence bonds should be preferred, even

if they denature proteins and destroy biological activity, provided that they do not break primary-valence bonds in the molecules of the substances which are to be isolated and studied chemically. The use of such dissociating solvents thus has additional possible advantages for the extraction and subsequent purification of proteins and peptides.

Under the new conditions in the membranes, very gratifying zone separations dependent on molecular weight were obtained with a number of peptides and proteins. These were chosen because their chemical nature and molecular weights have been rather fully investigated. It has emerged that, on varying the porosity of the membrane, the relative migration rates of peptides and proteins are altered over a wide range of molecular weight. There was no sign of failure to enter the membrane, as with the dextrins in the experiments of Mould & Synge (1954*a*). Although further study is required before the new method can be used preparatively or for quantitative analysis, it seems worth publishing the rather crude observations so far made, so that others may make use of them. Smithies (1955) and Smithies & Poulik (1956) have observed somewhat similar effects on electrophoresis of proteins in stiff starch gels in the presence of borate and other buffers.

MATERIALS

L-*Arginine.* The monohydrochloride was used.

'Salmine' sulphate. A preparation of the protamine of *Oncorhynchus kisutch* obtained about 15 years ago from the Banting and Best Institute, Toronto, through Dr A. C. Chibnall, was used.

Insulin. Bovine insulin (Boots Pure Drug Co. Ltd., recrystallized, batch CBL 4245 LL), kindly given by Dr F. Sanger.

Gramicidin S hydrochloride. This was prepared from the same crude material as that described by Consden, Gordon, Martin & Synge (1947).

Tyrocidine hydrochloride. The Wallerstein preparation studied by Synge & Tiselius (1947) was used. This is a mixture of tyrocidines A, B, etc. (cf. Battersby & Craig, 1952*a*). In some preliminary experiments tyrothricin was used with similar results.

Cytochrome c. This was a preparation of equine origin (9E40918, E. R. Squibb and Sons, New York).

Ribonuclease. Pancreatic, crystalline ribonuclease (Armour) was used.

Lysozyme. This was prepared from egg white, crystalline (Armour).

Mercuripapain. The crystalline material (Kimmel & Smith, 1954; Smith, Kimmel & Brown, 1954) was used.

The last four preparations were kindly given by Professor Emil L. Smith.

METHODS

Membranes

Collodion membranes. These were prepared and characterized as described by Mould & Synge (1954*a*). Membrane A was prepared in the same way as membrane V there described. It showed similar rate of electroendosmosis and similar R_F value for a dextrin preparation of average degree of polymerization (DP) 33. It probably therefore had 'average pore radius' about 9 mμ. Membrane B was prepared in the same way as II, and showed R_F 0·7 and 0·12 for dextrin preparations of DP 33 and 160 respectively. It was 0·88 mm. thick and had average pore radius (rate of flow of water method) 18 mμ. Membrane C was prepared as for membrane V, but evaporation of the ethanol–ether was allowed to proceed for longer after the collodion had set before it was transferred to water. This gave a finer-pored membrane; dextrin of DP 33 had R_F 0·27, while dextrin of DP 160 was completely excluded. The membrane was 0·50 mm. thick and had average pore radius 6 mμ. It was not always easy to judge the stage of evaporation at which denser membranes such as C should be transferred to water and excessively impermeable membranes sometimes resulted. Perhaps the procedure of Adair (1956) would be more certain (see also Donnet & Roth, 1955).

Denitration of collodion membranes. The denitration procedure of Fuoss & Mead (1943) by the use of ammonium sulphide in excess of ammonia seriously weakened the membranes. On the kind advice of Mr F. D. Miles (cf. Miles, 1955), one of the procedures of Rassow & Dörr (1924), in which ammonium sulphide was used with excess of H_2S, was tried and proved satisfactory. Partial denitration in nitric acid (Alexander & Johnson, 1949) gave a membrane that softened somewhat in phenol–acetic acid–water and gave less good contrast on staining the zones (see below). A piece of collodion membrane (approx. 8 cm. × 20 cm.) was placed in the solution obtained by saturating with H_2S a mixture of ethanol (145 ml.), aqueous NH_3 (sp.gr. 0·880, 75 ml.) and water (to 250 ml.). After keeping for at least 3 hr. at room temp. the membrane was washed with changes of 50% (v/v) aqueous ethanol until no longer yellow, and then with 0·2N aqueous acetic acid, in which it was stored. Collodion membrane A gave denitrated membrane AD, and so forth. Properties of the denitrated membranes are given in Table 1. Koz'mina & Starovoĭtova (1953) observed similar changes of membrane thickness and of pore radius on denitrating collodion membranes.

Migration experiments in membranes

These were done with the apparatus and procedure of Mould & Synge (1954*a*). The membranes were equilibrated with the solvent mixture to be used in the experiment for at least an hour before use, and the same solvent mixture was used in cathode and anode chambers. Equilibration of the

Table 1. *Properties of denitrated membranes stored in* 0·2N *acetic acid*

Freshly blotted membranes were weighed and then dried *in vacuo* in a desiccator over H_2SO_4–soda lime at room temp. 'Average pore radius' was determined on wet membranes as by Mould & Synge (1954*a*), taking 1·53 g. cm.$^{-3}$ as the density of cellulose.

Membrane	Thickness (mm.)	Dry matter content (%)	'Average pore radius' (mμ.)
AD	0·75	24	6·5
BD	0·69	18	13·4
CD	0·41	32	4·7

liquid paraffin with the solvent was unnecessary. The peptide and protein solutions placed in the anode chamber were 1–2% (w/v). Development was continued with fresh solvent in the anode chamber for not less than 6 times the running-in time. The membrane strips were generally 1·3 cm. broad and about 7 cm. in effective length (anolyte to catholyte). Applied potential was 270 v. Currents were smaller with the solvents used here than with 0·2 N acetic acid. There was considerable browning of the phenolic solvent at the anode, but the resulting pigment showed no tendency to enter the membrane. All the compounds studied except arginine were detected, at the end of the run, by keeping the blotted membrane in 0·1% (w/v) aqueous flavianic acid (2:4-dinitro-1-naphthol-7-sulphonic acid) with gentle agitation for 10 min. and then in several changes of water. When contrast was best, the strip was sketched or photographed and allowed to dry, although with some substances the strips could be stored wet without loss of staining contrast. Arginine was revealed by staining with 0·1% flavianic acid dissolved in 80% (v/v) aqueous ethanol, which solvent was also used for washing off the stain. Cytochrome *c* was visible at all times, and flavianic acid staining of its zones always coincided with the distribution of pink pigment, giving orange-coloured zones easily distinguished from those of other proteins. Moreover, on washing with water, cytochrome *c* appeared to lose flavianic acid more readily than did the other substances studied, giving zones coloured pink which persisted indefinitely on storage under water.

Filter-paper electrophoresis in phenol–acetic acid–water (1:1:1, *by wt.*)

This was done on Whatman no. 1 paper according to Kunkel & Tiselius (1951; cf. Ellfolk & Synge, 1955). Each spot was made with 2 μl. of a 5% (w/v) solution of the substance being studied. *N*-(2:4-Dinitrophenyl)ethanolamine (cf. Mould & Synge, 1954*b*) was used as a marker of electroendosmosis, siphoning, etc. No electroendosmosis was observed. For detection, the undried sheets were stained with ethanolic flavianic acid as above (not suitable for arginine, gramicidin S or tyrocidine), or the dried sheets were sprayed with ninhydrin in the usual way (and heated), or subjected to the Cl_2–starch–KI procedure of Rydon & Smith (1952). Cytochrome *c* was visible at all times. Where a substance being studied was detected by two or more methods, no discrepancies were noted in the positions of the spots.

Expression of mobilities

Ionic mobilities were throughout towards the cathode and are expressed in relation to the potential gradient measured in a straight line in the medium, assuming the ions also to be moving in a straight line. No correction for electroendosmosis was necessary. Migrations were usually measured from the anode end of the membrane to the sharp edge of the zone.

RESULTS

Conditions for electrical migration of peptides in a membrane

All these experiments were done with collodion membrane A or with the derived denitrated membrane AD.

Collodion membrane in saturated urea solution. In 0·2 N aqueous acetic acid 'salmine' migrated at 10×10^{-5} cm.^{2} v^{-1} sec.$^{-1}$. In the same solvent saturated with urea it had the same mobility, but the zone (which had a sharp leading edge in the absence of urea, perhaps due to adsorption), had a sharp tailing edge, suggestive of pH and conductivity effects (cf. Svensson, Benjaminsson & Brattsten, 1949). Gramicidin S in 0·2 N acetic acid saturated with urea entered only the first millimetre or so of membrane, which became shrunken and translucent. During the experiment (17 min.) the current fell from 2 to 1 mA. Tyrothricin gave the same result, except that shrinkage and fall of current were more marked.

Denitrated membrane in saturated urea solution. In 0·2 N aqueous acetic acid sat. with urea, 'salmine' had mobility $4{\cdot}3 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$, showing a sharp trailing edge, whereas gramicidin S had mobility $1{\cdot}2 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$, the zone showing a sharp leading edge and extending diffusely back towards the origin. Tyrothricin did not enter far, causing shrinkage of the 2 mm. of membrane nearest the anode, with fall of current from 1·1 to 0·6 mA.

Denitrated membrane in phenol–acetic acid–water (1:1:1, *by wt.*). 'Salmine', gramicidin S and tyrocidine gave fairly compact zones with the trailing edges the sharper, having respective mobilities 2·7, 1·9 and $0{\cdot}9 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$. These are in similar proportion to the relative mobilities determined in the same solvent on filter paper (Table 2). Current was constant (0·3 mA approx.) in all three experiments. A mixture of the three substances under the same conditions gave three well-resolved zones. These observations were taken to indicate that adsorption had been eliminated and that, with the peptides in question, membrane AD did not show much selective retardation dependent on diffusion or molecular-sieving effects.

Effect of porosity of membrane on migration of peptides and proteins

In these experiments, the relative ionic mobilities of several substances of fairly well-established mol.wt. were compared in phenol–acetic acid–water (1:1:1, by wt.) on filter paper, in the coarser membrane BD and in the finer membrane CD. Each substance was compared in mobility with cytochrome *c* (by running a mixture of the two in the membrane experiments, and by having spots side by side on filter paper). By observing the membranes before and after staining with flavianic acid it was easy to distinguish by colour the cytochrome zone from that of the substance being tested, and greater reliance can thus be placed on the relative mobilities than if they had been determined in separate experiments, where fortuitous variations of applied

voltage, membrane porosity, etc., might have misled. As mentioned under Methods, cytochrome *c* showed no sign of dissociation into pigment and protein moieties, as occurs with haemoglobin or myoglobin in acidic organic solvents. This can be attributed to the primary-valence bonding between pigment and protein (Theorell, 1938; Tuppy & Bodo, 1954). The results are set out in Table 2. It is seen that, while in membrane BD there is some indication of influence of molecular weight on mobility, in the finer-pored membrane CD this is very much greater.

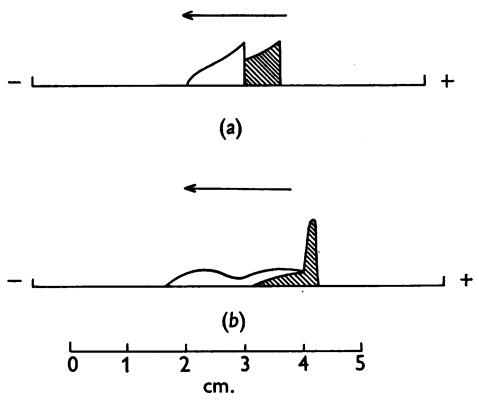

Fig. 1. (*a*) Displacement effect in migration of a mixture of gramicidin S and cytochrome *c* in membrane BD. Run, 53 min. (*b*) Interaction of tyrocidine and cytochrome *c* during migration of a mixture in membrane CD. Run, 210 min. Hatched areas indicate distribution of visible pigment, unhatched of material staining with flavianic acid only, as assessed by eye.

Where, in these experiments, two zones lay close together, displacement effects of the kind observed by Svensson *et al.* (1949) were noticed. With increasing concentration of migrating substance, there are local changes in pH and conductivity (which determines potential gradient), which tend to decrease the migration rate. Zones therefore have sharp trailing edges and diffuse leading edges. Fig. 1 (*a*) shows diagrammatically the displacement effect of gramicidin S on cytochrome *c* in membrane BD. The change of conditions at the trailing edge of the gramicidin S zone 'displaces' what would otherwise be the diffuse leading edge of the cytochrome zone. Fig. 1 (*b*) illustrates the type of interaction between cytochrome *c* and tyrocidine or insulin, which was observed in membrane CD. Approximate migration rates of the intermediate zones are given in a footnote in Table 2.

DISCUSSION

Adsorption effects in membranes

In the experiments aimed at establishing conditions for the ionic migration of peptides in collodion membranes, the pore size used (membrane A) permitted passage of dextrins up to at least mol. wt. 5000 and should therefore have been ample for the passage of gramicidin S and tyrocidine, if not for salmine. In fact, salmine was found to migrate freely in the membrane in dilute acetic acid whether or not urea was present, while gramicidin S and tyrocidine did not migrate in the same solution saturated with urea (in order to dissolve them). With

Table 2. *Relative mobilities of substances in filter paper and in membranes of different porosity, with phenol–acetic acid–water* (1:1:1, *by wt.*) *as solvent*

A, mobility relative to that of cytochrome *c* in same membrane. *B*, mobility relative to that of substance itself in filter paper. Cytochrome *c* had average mobility: 2·9 (range 2·8–3·0) $\times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$ in filter paper; 1·6 (range 1·3–1·9) $\times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$ in membrane BD; 0·47 (range 0·28–0·82) $\times 10^{-5}$ cm.^{2}v^{-1} sec.$^{-1}$ in membrane CD.

		Filter paper		Membrane BD		Membrane CD	
	Mol.wt.	*A*	*B*	*A*	*B*	*A*	*B*
L-Arginine*	174‡	1·27	1	1·57	0·68	2·60	0·33
Gramicidin S	1 142‡ (1)	0·86	1	1·20	0·77	2·57	0·48
Tyrocidine	1 270‡ §(2)	0·58	1	0·72	0·68	1·75†	0·49
Insulin	5 734 (3)	0·89	1	0·94	0·58	1·79†	0·33
Ribonuclease	14 000 (4)	0·92	1	1·00	0·60	0·87	0·15
Cytochrome *c*	13 000–16 000 (5)	1	1	1	0·55	1	0·16
Lysozyme	14 000–17 000 (6)	0·96	1	0·96	0·55	0·94	0·16
Mercuripapain	>21 000 (7)	0·91	1	0·85	0·52	0·61	0·11
'Salmine'	3 800–6 800 (8)	1·32	1	—	—	—	—

* This gave very spreading zones. Figures refer to trailing edge only.

† These figures refer to the fastest, cytochrome-free zone. There was also an intermediate, mixed zone, having $A = 1{\cdot}29$ for tyrocidine and $A = 1{\cdot}21$ for insulin. See Text.

‡ Free base. § Tyrocidine A.

(1) Battersby & Craig, 1951; (2) Battersby & Craig, 1952*b*; (3) Ryle, Sanger, Smith & Kitai, 1955; Sluyterman, 1955; (4) Hirs, Stein & Moore, 1954; Edsall, 1953; (5) Wyman, 1948; Edsall, 1953; (6) Fevold, 1951; Edsall, 1953; (7) Smith, Kimmel & Brown, 1954; Smith, Stockell & Kimmel, 1954; (8) Phillips, 1955.

these last peptides, the current fell and the membrane shrank seriously at the anode end. An obvious explanation could be that gramicidin S and tyrocidine, but not salmine, are adsorbed on the collodion surfaces, conferring a positive charge and reversing the direction of electroendosmosis in that portion of the strip of membrane. Water continues to move towards the cathode from ahead of the adsorbed zone but back towards the anode inside the zone, so that shrinkage of the membrane, with drying out and total blockage, occurs. The next step was thus to try to eliminate adsorption. Denitration of the membrane was found to permit passage of gramicidin S in dilute acetic acid saturated with urea (adsorption on cellulose being presumably less than on cellulose nitrate), but tyrocidine still did not migrate and caused shrinkage of the membrane. As the membrane had been converted into cellulose, organic solvents could now be used in it without swelling or weakening it, and a mixture of equal parts of phenol, acetic acid and water was found to permit migration of tyrocidine without shrinkage of the membrane.

Fridrikhsberg & Gutman (1953; cf. Toman, 1954; Fridrikhsberg & Gutman, 1954) have stressed the idea, apparently not previously enunciated, that, where ionic material in solution is migrating in a coarsely porous membrane and is also strongly adsorbed on the pore walls, it will confer a charge on the walls such as to promote electroendosmotic flow of liquid relative to the membrane exactly equal and opposite to the migration velocity of the dissolved ions relative to the liquid. They showed that various dyes could not migrate across collodion diaphragms under conditions of free electroendosmosis, although they migrated readily when electroendosmosis was prevented by hermetically sealing the two chambers of their diaphragm cell. They observed similar effects with serum proteins. Thus, under conditions where electroendosmosis can occur freely, as with the apparatus used in the present experiments, it is useless to expect ionic transport of substances which are appreciably adsorbed on the membrane. The same applies to most arrangements using electrical transport in diaphragm cells, and an example can usefully be given from work done in this Laboratory. Synge (1951) described a fractionation of grass juice by electrical transport in a diaphragm cell. Most of the 'bound' amino acids present did not migrate towards the anode through a membrane of formolized parchment at pH 6, although glutamic acid, aspartic acid and various plant acids migrated readily. As the bound amino acids also did not migrate towards the cathode through cellophan at pH 3, they were judged to be devoid of ionizing groups. More recent experiments by Mr J. C. Wood and me (unpublished work) have shown that much of this 'bound amino acid fraction' is actually acidic in nature and migrates freely towards the anode on electrophoresis in silica jelly at pH 6. Presumably adsorption of these compounds on the parchment membrane produced electroendosmosis sufficient to prevent their own migration, while not preventing that of anions of greater mobility. Although the effect in the present instance was deleterious, there seem to be possibilities, based on the ideas of Fridrikhsberg & Gutman, for making membranes for diaphragm cells that will not simply be selective for cations or anions as such but will permit selective passage of ions exceeding a definite threshold of mobility, which can be controlled by the chemical nature of the membrane or of substances adsorbed thereon.

Adsorption effects of this kind should not seriously hinder passage of materials by diffusion or mechanical ultrafiltration through relatively coarse membranes, but in fine-pored membranes they can be expected to hinder the passage of the compound in question because the concentration of dissolved ions will be greatly diminished in the region of the electrical double layer which occupies an increasing proportion of the volume of the pores. The observations of Craig & King (1955) on relative rates of diffusion of differently charged amino acids and peptides through cellophan bear out the occurrence of such effects, and it is interesting to note how slowly tyrocidine passed through cellophan, even in 50 % (v/v) aqueous acetic acid, in view of the strong adsorption of tyrocidine suggested by the present experiments (see also Grandjean, 1952).

Other factors influencing migration in porous media

The experiments on the influence of membrane porosity on migration rates were all done with phenol–acetic acid–water, in view of the good results with tyrocidine and because the mixture is an excellent solvent for all the substances studied so far, is volatile and free from nitrogen (which will be useful in analytical and preparative work) and is unlikely to attack primary-valence bonds in the compounds being studied. Doubtless, future work will reveal other useful solvent media. With filter paper, there was negligible electroendosmosis in phenol–acetic acid–water [with *N*-(2:4-dinitrophenyl)ethanolamine as marker], nor was there noticeable electroendosmotic movement of solvent in the experiments with denitrated collodion membranes. The ionic migration on filter paper of the compounds studied showed a surprisingly slight range of mobilities in view of the great range of the ratio of number of ionizing basic groups in the molecule to molecular weight (cf. Table 2). Perhaps, in such solvents, dielectric effects decrease greatly the extent of ionization of basic groups when they are close together in the molecule, or there may be

rather tight binding of oppositely charged (acetate) ions.

It is clear that the effects on migration rates due to the use of a more finely pored membrane (Table 2) are closely correlated with molecular weight. It is not as yet clear what is the mechanism of this effect. Westhaver (1947; cf. Synge, 1949) discussed formally the migration of ions in a packed column against counterflowing liquid, in relation to the separation of potassium isotopes, and used a 'theoretical plate' treatment to take account of diffusion and convection in the flowing liquid as factors broadening the zones. However, he did not pay attention to the possible influence of the packing on the absolute or relative migration rates of the zones of solutes undergoing separation. Kunkel & Tiselius (1951) have treated the effect of the packing as increasing the path length that must be travelled by the migrating ion while at the same time decreasing the potential gradient, which is less when measured along this path than when measured in a straight line along the column. This idea has been attacked by McDonald (1955; cf. McDonald, Lappe, Marbach, Spitzer & Urbin, 1953), who proposes a 'barrier' theory, in which the electrical field in the liquid is considered to be uniform in strength and direction throughout; migrating ions encounter mechanical obstructions to their progress, however, at more or less frequent intervals. McDonald criticizes Kunkel & Tiselius on the practical ground that their theory does not predict differences in the relative retardation of molecules of different molecular weight. He gives (p. 60) a diagram of molecules of different sizes hitting paper fibres with different frequencies, which is so grossly distorted in relative scale as to be misleading; he also gives examples of differential retardation of different substances by different types of filter paper, ignoring the points: (i) that small molecules can penetrate cellulose fibres while large ones cannot; (ii) that electrical transport of small molecules within cellulose fibres will be much influenced by the electrochemical nature of the small molecule, as is well known from experiments on transport of ions across cellophan membranes. McDonald further criticizes Kunkel & Tiselius on theoretical grounds which may be valid if the packing of the column itself has appreciable conductivity, but are certainly not valid if the packing is an insulator. McDonald complicates his arguments by using the expression 'thermodynamic activity' in a sense quite different from that in general use.

The essential difference between these two approaches is whether the field is regarded as curving round the packing (an insulator) or passing straight through it (in which case it must be penetrable by the majority of migrating ions). There will be limiting cases where one or the other approach is more strictly applicable. For the practical objective of relating mobilities in packed media to those in free solution, conductivity measurements have been used by both groups as the basis of conversion, with essentially similar results.

A reasonable explanation for the effects seen in the present experiments could be that during ionic migration the larger molecules are obstructed on encountering barriers of regenerated cellulose through which hydrogen and acetate ions, carrying the greater part of the current, can migrate freely. The larger molecules would then escape from these situations by circuitous routes, at rates depending on their diffusion coefficients. Information on the detailed structures of collodion membranes has been given by Bugher (1953), Beutelspacher (1954), Helmcke (1954), Maier & Beutelspacher (1954*a*, *b*) and Spandau & Zapp (1954). When the pore size approaches molecular dimensions still more closely, the 'barrier' effect of McDonald or 'frictional' effects of Tiselius & Flodin (1953) must come into operation, and are probably tautologous with the expression 'molecular-sieve' effects. Even here, diffusion presumably plays a part in extricating molecules from situations in which they have become immobilized. This is implicit if one extends to ionophoretic migration the treatment by Mackie & Meares (1955) of the diffusion of ions inside ion-exchange resins.

Applicability of the new procedure

Detailed analysis of the mechanism of electrical transport in porous media must clearly await further experimental and theoretical studies. The present work shows, however, that, regardless of mechanism, resolutions dependent mainly on molecular weight or diffusion behaviour can be realized with peptides and proteins in such media. Reasonably well-characterized chemical entities were selected for the present study, and it was pleasing that they arranged themselves mostly in the sequence to be expected from what is known of their molecular weights. All the substances studied, moreover, migrated as reasonably compact, single zones (although, in view of the slight range of mobilities in phenol–acetic acid–water, migration as a single zone is not a very serious test of homogeneity). With the mixtures (cytochrome *c* + insulin) and (cytochrome *c* + tyrocidine) the formation of mixed zones of intermediate mobility (cf. Grassmann, 1953; Grassmann & Hübner, 1953; Synge, 1956) suggests reversible complex formation, perhaps with dissociation of the cytochrome into submolecules which combine with tyrocidine or insulin; the resultant complexes would migrate faster than the cytochrome in membrane CD but at a similar rate to it in membrane BD. This phenomenon seems worthy of more detailed study, since

both tyrocidine and insulin have loci in their molecules at which aromatic amino acids are grouped closely together. These loci might enter into combination with similar loci in cytochrome submolecules which normally combine with one another. Diffusion experiments in free solution should yield relevant results. Tint & Reiss (1951) reported the formation of a complex between cytochrome *c* and the peptide antibiotic bacillomycin *b*, and studied its electrophoretic behaviour.

The new procedure has obvious possibilities for fractionating according to molecular weight polymeric electrolytes which, in free solution, migrate with ionic mobility independent of molecular weight. It should also be useful in analysing whole tissues for polypeptides (cf. Synge, 1953, 1955, 1956). Compounds migrating faster than cytochrome *c* in membranes such as CD can be expected to be substantially more basic in character, or of lower molecular weight, or both. It might be possible to increase the scale of working by conducting the migrations with the field perpendicular to membranes packed tightly together in a pile. Improved arrangements are also needed for preventing electrode reactions from attacking solvents and materials undergoing analysis.

SUMMARY

1. In order to obtain ionic migration of peptides and proteins inside porous membranes, adsorption of the migrating substance on the structure of the membrane must be eliminated.

2. Favourable conditions for migration resulted from using a phenol–acetic acid–water mixture as solvent inside membranes of denitrated collodion.

3. In free solution in the same solvent a number of polypeptides and simpler proteins had unexpectedly similar cationic mobilities.

4. Experiments on ionic migration in finely pored membranes showed a strong selective-retardation effect; those peptides and proteins of higher molecular weight were the more strongly retarded. Possible mechanisms for this effect are discussed.

5. Evidence was found for complex formation by cytochrome *c* with insulin and with tyrocidine.

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Improved Method for the Preparation of Crystalline β-Lactoglobulin and α-Lactalbumin from Cow's Milk

By R. ASCHAFFENBURG and J. DREWRY
Chemistry Department, National Institute for Research in Dairying, University of Reading

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Crystalline β-lactoglobulin is usually prepared from cow's milk by modifications of the method of Palmer (1934), based on exhaustive dialysis of the 'lactalbumin' fraction isolated by various salting-out procedures. A full description of this type of preparation has recently been given by Larson & Jenness (1955), who list earlier references. The 'lactalbumin' fraction contains another major whey protein, α-lactalbumin, which can be isolated from the β-lactoglobulin mother liquor (Gordon & Semmett, 1953; Gordon, Semmett & Ziegler, 1954; Gordon & Ziegler, 1955).

It has been noted (Gordon & Ziegler, 1955) that 'incomplete removal of β-lactoglobulin appears to hinder the subsequent crystallization of α-lactalbumin', and we have found that the presence of α-lactalbumin tends to interfere with the formation of β-lactoglobulin crystals, and to affect their purity adversely. Furthermore, the lengthy process of crystallizing the β-lactoglobulin is time-wasting when preparation of the α-lactalbumin is the sole objective. A clean separation of the two proteins at an early stage in their preparation would thus provide obvious advantages. Zweig & Block (1954) attempted segregation of the proteins after precipitation with ferric chloride, but achieved only partial separation and a much lower yield of α-lactalbumin than that obtainable by the improved procedure of Gordon *et al.* (1954).

The present paper, which forms part of a general study of whey proteins, describes a simple method for the early and practically complete separation of β-lactoglobulin from α-lactalbumin, and for the crystallization of the separated proteins by steps which can be carried out simultaneously or independently with a considerable saving in time. The milks of individual cows are now known to contain either a mixture ($\beta_{1,2}$) of two different β-lactoglobulins, or only one (β_1) or the other (β_2) of these (Aschaffenburg & Drewry, 1955). The method is equally applicable to such milks and to their mixture in bulk milk, except for minor modifications during the final stages of the preparation of crystalline β_2-lactoglobulin.

EXPERIMENTAL

Milk. All samples were of fresh, whole milk from cows of the Institute herds. Fractionation of 2–10 l. of the uncooled milk was started within a few hours of milking.

Reagents. All chemicals used in the fractionation procedures were of analytical reagent quality. This avoids loss of time through re-working, which was found to be necessary when less pure reagents were employed. Distilled water was used in all operations, as the presence of heavy metal ions in tap water led to the formation of discoloured protein precipitates.

Dialysis. This was done in narrow bags made from seamless Visking cellulose tubing by tying one end into a double knot, filling the bag, and securing the other end in the same manner. By leaving no air-space, undesirable increases in volume were reduced to a minimum. Although pressure inside the bags may increase considerably, no bursts have occurred with narrow tubing (not exceeding $\frac{3}{4}$ in. inflated diameter). If necessary, more than one narrow bag was filled rather than one of larger diameter. The bags were made to rotate during dialysis.

pH. This was determined with a glass electrode and a Pye Universal pH meter. All values refer to a temperature of 20°.