STUDIES ON THE ANOMALOUS VISCOSITY AND FLOW-BIREFRINGENCE OF PROTEIN SOLUTIONS

II. On Dilute Solutions of Proteins from Embryonic and Other Tissues

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INTRODUCTION

In the foregoing paper a description has been given of apparatus whereby the flow-birefringence and the relative viscosity of a protein solution may be measured and its anomalous viscosity assessed, careful distinction being made between effects due to the bulk phase and those due to the surface film at the air-water interface. We have now to turn to the more specific detailed results of the investigation of a number of proteins.[1]

Behaviour of Proteins

Tobacco Mosaic Disease Virus Nucleoprotein.—This plant virus protein,[2] (for full descriptions of which see Bawden, 1939; Bernal and Fankuchen, 1941) has already been subjected to measurements of viscosity and birefringence, in the work of Robinson (1939). The specimen used by us gave the flow-birefringence curves seen in Fig. 1; allowing for differences in conditions (in our experiments 1.5 cm. column as against 21 cm.; 0.5 per cent as against 0.02 per cent concentration of virus; shear rate 13.1 as against 18.8; Robinson's sample was more flow-birefringent than ours. In the viscosimeter it was interesting to find that under both low level (film) and flood level (bulk) conditions, the virus gives a strongly anomalous type of flow, see Figs. 2 and 3.) This probably means that the virus aggregates retain their anisometric shape when they enter into the formation of the surface film.

The only difference is that the viscosity curve of 0.025 per cent virus for the bulk descends to its orientation plateau by about 20 R.P.M. while that for the film does not do so until a speed of between 70 and 80 R.P.M. is attained. Orientation within the film must therefore be a good deal more difficult than orienta-

[1] The present work was begun in 1938–39 by Joseph Needham and Shih-Chang Shen (Fellow of The Rockefeller Foundation) with A. S. C. Lawrence as rheological adviser. Margaret Miall joined the group with a Rockefeller Foundation grant in 1941. A preliminary report has already appeared (Lawrence, Needham, and Shen, 1940).

[2] Hereinafter called TMD virus.

tion in the bulk, as would be expected if considerable mutual interference occurred there. So far as the bulk phase is concerned, our data for TMD virus are in agreement with those of Robinson (1939) on the coaxial, and Frampton (1939) on the capillary, viscosimeter.

In the course of further experiments, it was noticed that when samples of TMD virus had deteriorated by ageing (Δ in the microscope cell having dropped

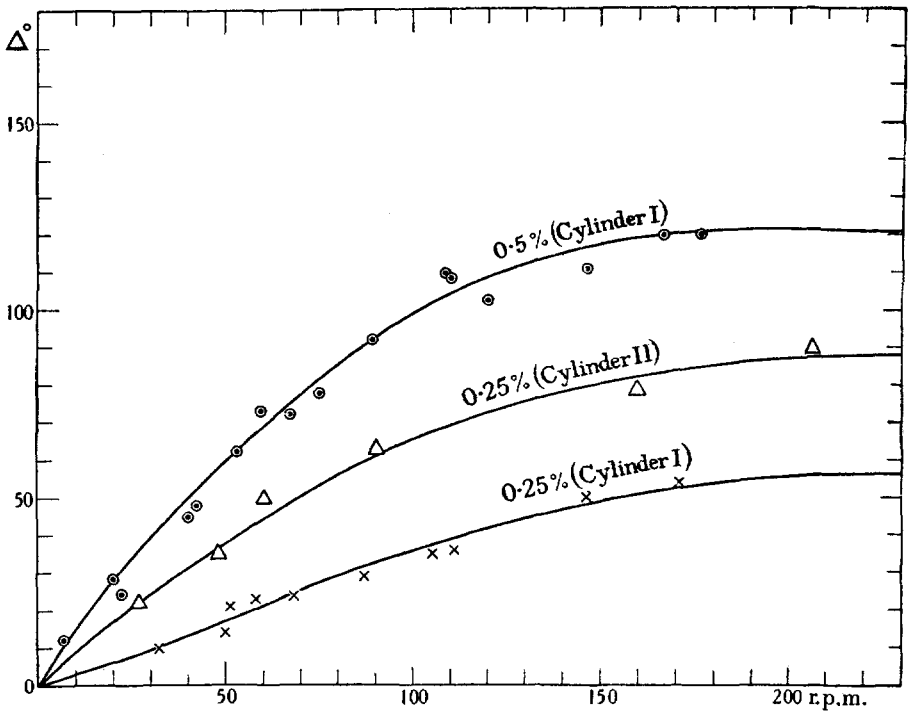

FIG. 1. Flow-birefringence (in the microscope cell) of virus nucleoprotein.

from 80° to 20° for 0.5 per cent solution at 70 R.P.M.) or if samples of fresh virus were "denatured" by the addition of a few particles of copper carbonate causing similar falls of flow-birefringence intensity, anomalous flow was no longer obtained, either in the surface film or in the bulk. Flow became normal or Newtonian and the lines describing sets of deflection readings rose unmistakably from the origin. But at the same time it also became noticeable that under film conditions (low level cylinder position) the phenomenon of anomalous return was now obtained, indicating that a built-up multilayer was being made. In explanation, it may be suggested that these forms of denaturation involve the association of the elongated virus particles into close tangles; these are unable to suffer orientation in the bulk but enter into complex relationships

within the film. It is possible, however, that denaturation involves the breakdown of the long virus particle into shorter, more nearly isometric, lengths, and then film formation reaggregates them, perhaps in some different way.

Crystalline Ovalbumin.—Crystalline ovalbumin was prepared according to the method of Larosa (1927).

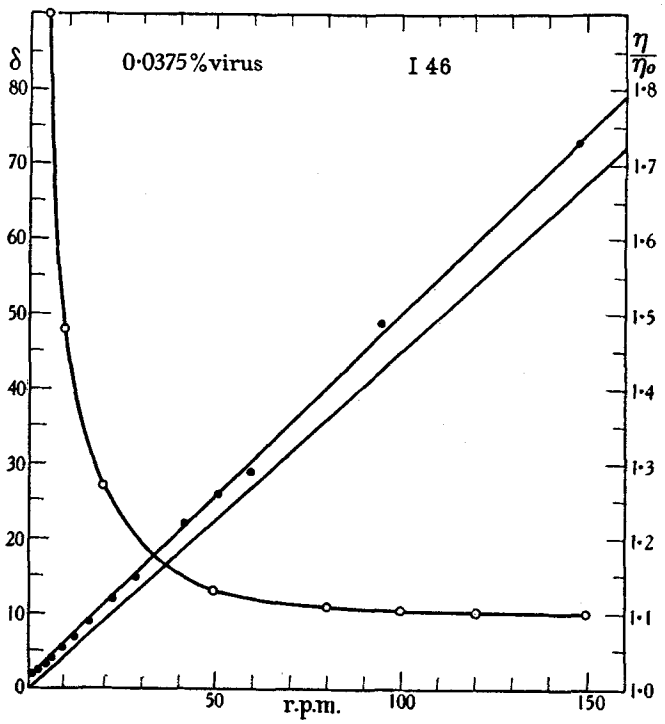

FIG. 2. Anomalous and relative film viscosity of virus nucleoprotein.

When placed in the viscosimeter, whether at low level (film) or at flood level (bulk), normal flow was invariably observed. The absence of any appreciable elongation in the native ovalbumin particles was of course expected in the light of previous knowledge of this protein (*cf.* Böhm and Signer, 1931). But this protein strongly showed the phenomenon of anomalous return, the building up of a solid multilayer (see Fig. 4). In this particular case the concentration of the protein was 0.0283 per cent.

The main interest in these built-up films lies in the fact that after they have been fully developed, it is possible to show by the present methods that elongated particles have been formed in them. Two experiments may be described which illustrate this. In the first (Experiment I 36/28d) a sample of oval-

bumin (0.0565 per cent) placed in the viscosimeter at 20°C. showed first perfectly normal flow, but as the measurements proceeded, showed an anomalous return. The solution was then taken out, stirred, filtered, diluted by half, and returned to the viscosimeter, again at low (film) level. The result was a clearly anomalous flow curve (Fig. 5), with an approximation to complete

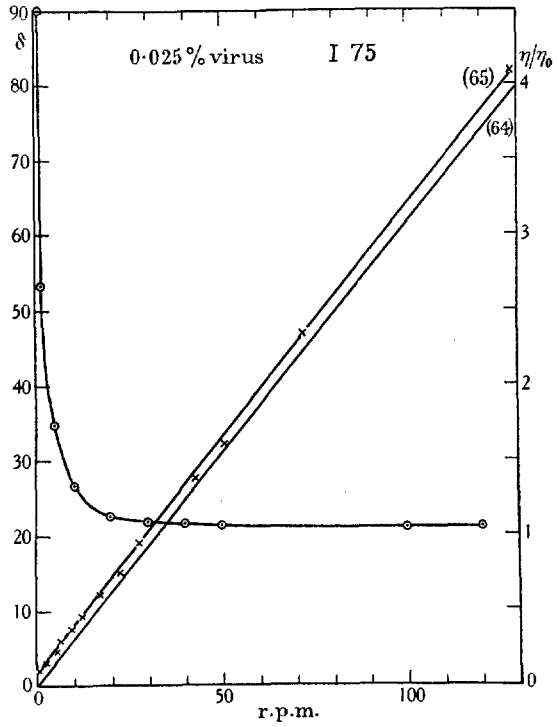

FIG. 3. Anomalous and relative bulk viscosity of virus nucleoprotein.

orientation at 30 R.P.M. Fresh ovalbumin solution measured immediately afterwards as control gave perfectly normal flow.

The second experiment shows that these artificially formed anisometric particles can be detected within the bulk also (Experiment II 103). A 1 per cent solution of crystalline ovalbumin was placed in a Langmuir trough and after the surface film had been repeatedly compressed and released, the film was drawn off, shaken up in water, filtered, and placed in the viscosimeter at flood level. As is shown in Fig. 6, anomalous flow was obtained, the only difference from the previous experiment being that the film had been longitudinally compressed rather than rotationally spun, and that (as has been seen to be the case with virus) the anisometric particles are more easily oriented in

the bulk phase than they are in the film (plateau reached at 10 instead of 30 to 40 R.P.M.) doubtless because of the lack of mutual interference there.

Some experiments were made on the behaviour of ovalbumin denatured; *e.g.*, with guanidine. If a solution of this protein is mixed with this base (in

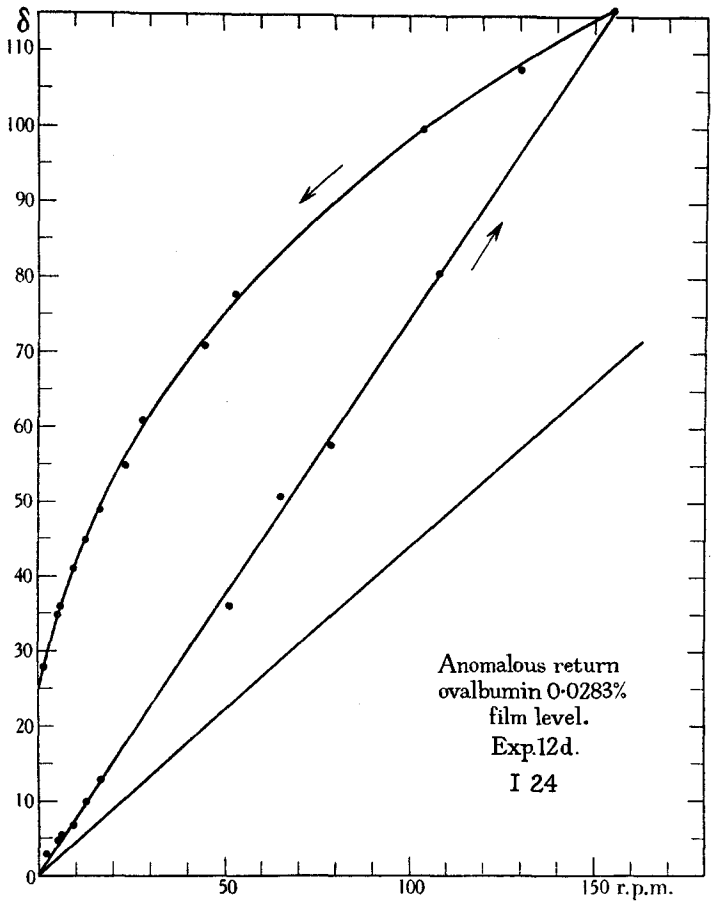

FIG. 4. Anomalous return of ovalbumin at film viscosity level.

the form of its neutralised hydrochloride) the phenomenon of anomalous return ("stretching") is much decreased or abolished. Fig. 7 shows the failure of guanidine-treated ovalbumin to build up the usual film (1 volume 0.05 per cent ovalbumin with 1 volume 3.65 M guanidine HCl). This may perhaps be interpreted by supposing that tangles of unrolled denatured molecules may be formed in the presence of guanidine, which cannot then be easily built into the usual type of film.

Spontaneous decreases of relative viscosity were often observed with this protein (*cf.* Hardy, 1905; Pauli and Valkó, 1933, p. 266).

Myosin.—Myosin was prepared from muscle tissue of rabbit and frog according to the standard methods (Edsall, 1930; Bailey, 1942). If the preparation is a good one, showing flow-birefringence, the viscosimeter behaviour is invariably the same, whether at low (film) level or flood (bulk)

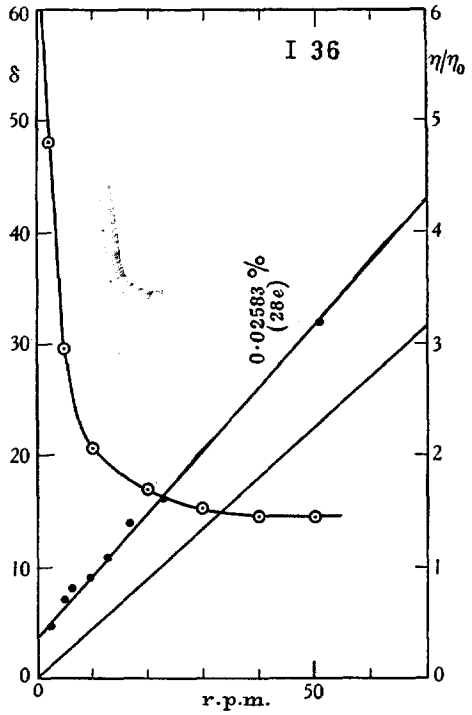

FIG. 5. Anomalous flow of ovalbumin (film) after rotation at film level.

level; it shows marked anomaly of flow. Illustrations are not given here, for the results closely resemble those shown for TMD virus in Figs. 2 and 3. Details differ, thus in one experiment with myosin at low level (Experiment I 17/6b) the protein concentration was 0.0009 per cent in M KCl and orientation was attained at some 70 R.P.M.; while in an experiment with myosin at flood level (Experiment II 99/82b) although the protein concentration was as high as 0.26 per cent in M KCl, orientation was attained already at 30 R.P.M. This brings out well the lack of mutual interference in molecular orientation in bulk as compared with film phase. An illustration of the anomalous flow of myosin has, moreover, already been given in the previous paper (Fig. 10)

in connection with the distinction between bulk and film viscosity. Since further illustrations and much further detail about myosin will be given in the succeeding paper of this series, little more will be said here. It may, however, be added that in poor or ageing preparations of myosin which show no flow-birefringence, flow anomaly may also be absent, and in this case "stretching" and anomalous return may be found; we established that in this case, just as in the case of ovalbumin, replacing the solution in the viscosimeter after the surface film had been fully formed, resulted in evidence of elongated particles by anomalous flow, both under bulk and film conditions. It would appear,

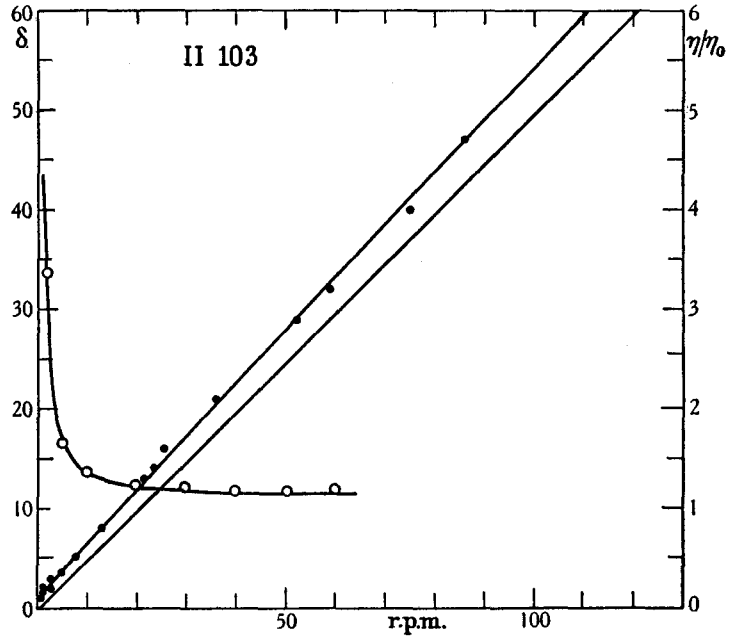

FIG. 6. Anomalous flow of ovalbumin (bulk) after compression in trough.

therefore, that the roughly spherical particles of such preparations may be made to form elongated particles again, either by being unrolled in the film or by being aggregated together in some linear manner. This re-elongation is perhaps analogous to the re-elongations accomplished by exposure of the non-flow-birefringent protein to 37°C., to be described in the succeeding paper. Myosin particles freshly isolated under the best conditions, however, never show any of these phenomena, but simply give repeatable anomalous flow whether in film or bulk.

On treatment with copper carbonate, the flow of virus nucleoprotein passes from anomalous to normal (see p. 234 above); on denaturation with guanidine

the flow of embryo euglobulin does the same (see p. 245 below). Similarly, myosin treated with N HCl forms a gel, and when brought back again to pH 8, its flow is found to be entirely normal (Experiment II 129). This is taken to mean that clumps, roughly symmetrical, have been formed from the anisometric fibrils.

Crystalline Insulin.—The insulin used was a commercial sample, tested in the viscosimeter at 0.025 per cent concentration and 20°C. Its flow in the

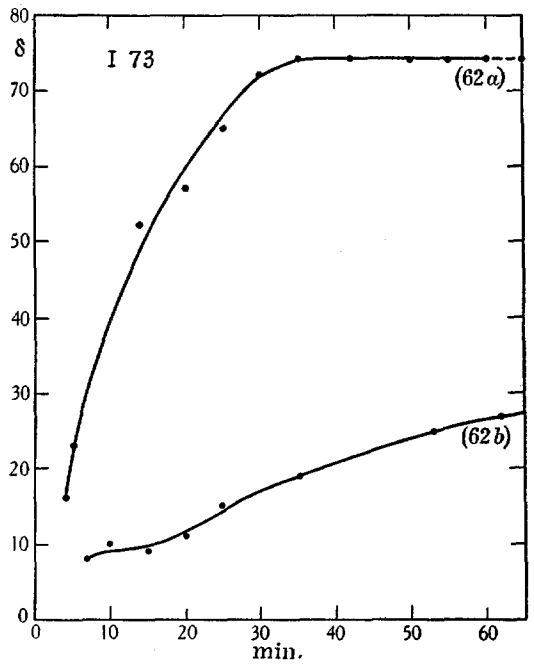

FIG. 7. Failure of ovalbumin denatured in bulk with guanidine to show "stretching" effect; building of multilayer.

surface film was perfectly normal, sets of deflection points giving lines arising unmistakably from the origin. This is in agreement with expectation.

Crystalline Methaemoglobin.—Haemoglobin used was prepared from sheep blood according to the method of Adair and Adair (1934), and converted to methaemoglobin by dialysis against tap water and distilled water at room temperature. Tested at various concentrations, of which the most suitable was 0.0002 per cent at 20°C., its flow in the surface film was perfectly normal, as with insulin.

Ovoglobulin.—The "ovoglobulin" fraction of the hen's egg white was found by Böhm and Signer (1931) to consist of highly anisometric particles and

considered by them to account for certain properties of fresh egg white such as its very anomalous viscosity first noticed by Rothlin (1919). There has, however, been doubt as to the existence of this protein, and though it is still certainly very difficult to purify from mucins, etc. (*cf.* Needham, 1942, p. 8, 9) it does in all probability exist as a separate entity. We prepared samples for the present purpose by following closely the procedure of Böhm and Signer, which essentially consists in homogenising fresh egg white and collecting the precipitate which forms on half-saturating it with ammonium sulphate. The salt is then dialysed away and the protein purified by reprecipitation.

Tested at low level (film conditions), this protein gave markedly anomalous flow (Experiments I 64/57 and II 83/69b; 0.0212 per cent protein at 20°C.). It was noticeable that exceptionally high speeds—100 R.P.M. and above—were needed before the minimum plateau of relative viscosity corresponding to full orientation was attained.

Tested at flood level (bulk conditions), however, ovoglobulin gives no trace of anomalous flow (*e.g.* Experiment II 83/69a; 0.212 per cent protein at 20°C.). This fact throws doubt on the existence of elongated particles in solutions of the undenatured protein but suggests that as soon as denaturation at the air-water surface takes place, long molecular fibrils are formed. It would agree with this view that we were unable to detect any flow-birefringence in our ovoglobulin preparations.

Serum Globulin.—The globulin fraction of ox serum was prepared following the details in Adair and Robinson (1930), Elford and Ferry (1934), and the review of Cohn (1941). Repeated precipitation by half saturation with ammonium sulphate followed by dialysis through cellophane against weak phosphate buffer solution gave the euglobulin fraction, readily separable from the pseudoglobulins which remain unprecipitated in the supernatant liquid within the dialysis bag.

The results of viscosimeter tests showed a behaviour very similar to that of ovoglobulin (*e.g.* Experiment VI 257). Perfect and repeatable normal flow diagrams were obtained if the test was made at flood level (bulk phase viscosity) while equally definite anomalous flow diagrams were obtained if it was made at low level (film phase viscosity). This must be taken as strong evidence that the globulin particles are not elongated when in bulk solution, but immediately become so when denatured in the surface film. The diagrams closely resemble those already given in this paper (see Fig. 4) except that the relative viscosities were in all cases rather high, higher concentrations than usual being used, with 0.75 M KCl as medium.

The pseudoglobulin fraction also behaved in the same way (*e.g.* Experiment VI 260).

Plasmosin.—The study of tissue globulins, by which we mean tissue proteins soluble in strong salt solutions but not in water, has lagged much behind the

study of the globulins of the body fluids. Such proteins have, however, been examined (Bensley, 1938; Bensley and Hoerr, 1934) and their possible structural importance in cell architecture emphasised (Banga and Szent-Györgyi, 1940). We have made preparations of "plasmosin" from rabbit liver and kidney, but so far have not been able to devote to them the elaborate investigation which they require. We could, however, confirm the descriptions of them given by Bensley. In the case of the liver, we proceeded as follows:—

The perfused blood-free tissue is ground up with sand, squeezed through muslin, washed at 0°C. with 0.85 per cent NaCl, and centrifuged; this is repeated ten times or until no more protein is detectable in the washings. On mixing the residue with about five times its volume of 10 per cent NaCl the majority of the protein slowly dissolved, giving a very slimy opaque liquid. After centrifuging at 3500 R.P.M. for 25 minutes it was thrown into ten times its volume of distilled water. Fibrous filmy strands immediately formed which could be gathered about with a glass rod; these were not intrinsically birefringent under the polarising microscope. After some time, the strands contracted and cohered in the form of a clot, just as Bensley describes.

On dissolving this material in 10 per cent NaCl and centrifuging, a solution was obtained which showed no flow-birefringence and in the viscosimeter at flood level (bulk phase viscosity) gave a quite normal flow diagram. Tests were unfortunately not made at low level to detect the properties of the film phase viscosity. When these are made it will probably give anomalous flow, like ovoglobulin and serum globulin. It seems, at any rate, that by one form of denaturation, elongated particles are obtained, for Banga and Szent-Györgyi (1940, 1941), who extracted the residue from the weak salt extraction with Edsall's fluid (0.6 M KCl, 0.01 M Na_2CO_3, and 0.04 M $NaHCO_3$) in presence of 30 per cent urea, obtained a viscous, thixotropic solution which showed intense flow-birefringence. We were not ourselves able to repeat this observation, with plasmosin either from liver or kidney, but the solutions were difficult to deal with on account of their high light-absorption, and the subject calls for further investigation.

Mucoprotein.—We have examined mucoprotein samples from the human umbilical cord (Wharton's jelly) and from the jelly surrounding anuran amphibian eggs.

The study of mucoproteinases (hyaluronidases) has in recent years given rise to a large literature, most of the phases of which may be followed in the reviews of McClean (1933, 1941). They are of importance in connection with bacteriology, with the physiology of spermatozoa, and with the spread of substances along the skin in mammals. It is now generally accepted since the work of Chain and Duthie (1940) that the "spreading factor," as it was formerly called, is a mucolytic enzyme which attacks the polysaccharide prosthetic group of the mucoprotein molecule (an equimolecular compound of glucuronic acid and N-acetyl-glucosamine). Favilli, McClean, and Hale (1940) point

out, however, that although enzymes of this type cause an immediate and severe fall in the viscosity of their substrates, only a trace of reducing substance can be detected after 4 hours, and the maximum of free glucosamine is not reached till after 24 hours. It was therefore thought worth while to investigate the original mucoprotein preparations in the coaxial viscosimeter, with a view to ascertaining whether perhaps the fall in relative viscosity might be associated with a change in particle shape from anisometric to spherical.

We had two samples of umbilical cord mucoprotein. Sample A was a solution, an extract of 100 gm. dried cord with 2 litres water. Sample B was a dry powder, the former extract having been precipitated with 3 volumes of alcohol in the presence of sodium acetate, washed with acetone, and dried over phosphorus pentoxide, for 18 hours.

We examined the mucoprotein preparations in the viscosimeter at flood level. Neither of them showed the least trace of flow anomaly. But their relative viscosity was very high, thus that of sample A at 0.142 per cent concentration (Experiment II 123) was 15.8 times water and that of sample B at 0.041 per cent (Experiment II 125) was 4.4 times, (all at 20°C.). No trace of flow-birefringence was observable. It is highly probable, therefore, taking all these facts into consideration, that anisometric particles are absent from these preparations and the sharp fall of viscosity which the enzyme brings about must rather be due to decreases in intermicellar forces or some similar factor. The mucoprotein solutions were, however, more stable than any other preparation which we encountered, and preserved their viscosimetric properties unchanged over a long period.

A similar result was obtained with the mucoprotein of amphibian egg jelly. We took advantage of the fact long known to experimental morphologists that anuran jelly is soluble in strong potassium cyanide solution. To 80 cc. of 10 per cent KCN were gradually added 370 cc. of cleaned egg jelly; the final volume was 447 cc. and the KCN concentration 1.8 per cent. The filtered liquid was clear and syrupy. The mucoprotein was precipitable with excess of alcohol but not with acetic acid. In spite of its appearance, the solution was not markedly more viscous than a myosin sol (η/η_0 1.58) and its flow was perfectly normal (Experiment II 115). (It should be noted, however, that the treatment of ovomucoid with KCN may bring about changes in the structure of the protein.)

It is unfortunate that no tests were made of these proteins at low level for film viscosity, for it is likely on histological grounds that mucoproteins may have something to do with fibril formation, and this may involve a kind of denaturation.

Behaviour of Proteins from the Amphibian Embryo

As mentioned in the introductory paragraph of this paper, our interest in the study of protein particle shape was originally aroused by the profound changes

in cell shape which occur in the vertebrate embryo when the cuboidal ectodermal cells and nuclei are elongating to form the constituent cells of the neural plate and tube. In view of the central position which the amphibian embryo has played for many years in the study of causal morphology, it is remarkable that no attention whatever has been paid to its proteins. For the experiments here described we used the embryos of the common frog, *Rana temporaria*, and toad, *Bufo vulgaris*. At first we were careful to use only embryos at the neurula stage, but finding later that the distribution of the proteins does not seem to suffer great changes during development, we also used unfertilised and fertilised eggs, and the earliest hatching stages. Removal from the jelly was accomplished either by dissolving it in strong KCN solution or, more commonly, by snipping the embryos out with scissors, or by allowing hatching to occur and then collecting the free swimming larvae from the jellies, which, with suitable skill, is an easy matter.

Fractionation of the embryo tissue proceeded as follows: The wet material was frozen solid in a vessel surrounded by ether and solid CO_2; *i.e.*, at a temperature of about $-77°C$. and stored over phosphorus pentoxide, calcium chloride, or anhydrous aluminium chloride at a vacuum of 0.01 mm. Hg at 0°C. till needed. Since it was sometimes desired to free it from as much fatty material as possible, it was found that by repeated treatments at $-77°C$. with pure absolute alcohol and anhydrous sodium-dried ether successively, a very large part of the lipoidal constituents and greenish-yellow pigments may be removed without denaturing the proteins. When required for use, the black powder was ground up with fine quartz sand and M KCl, the several extracts mixed together, and centrifuged at 11,000 R.P.M. for about 20 minutes, ice water being run through the Ecco centrifuge-cooling system. The tube will now show the black melanin granules packed together at the bottom while above the yellowish opalescent aqueous solution there is a cap of fat which can usually be removed entire with a ground glass rod or wood splinter fragment. On pouring this solution into ten times its volume of strictly neutral distilled water, the total euglobulin comes down as a voluminous creamy-white precipitate, while the pseudoglobulin remains in solution. Various methods of fractionating the total euglobulin, which must consist largely of vitellin, have been tried, and will be described below. The black residue from the KCl extract and the separated melanin will then yield a small amount of protein to direct aqueous extraction, and a further small amount to extraction with alkaline buffer.

The distribution of nitrogen among these fractions (Table I) clearly shows that much the greatest part of the embryo protein (72 per cent) is in the total euglobulin fraction. Our special interest in the neural tube region led us to separate, in most of the earlier experiments, the neural part from the ventral part of the embryo, but, as Table I shows, the differences between them, as far as this rather crude analysis goes, were insignificant, and later we used the whole embryo throughout.

Total Euglobulin.—Early in the work it was found that when the total euglobulin fraction is examined in the viscosimeter at low level (for film viscosity) it invariably gives diagrams showing anomalous flow, and it is not "stretchable;" *i.e.*, anomalous return is never observed. Since these observations are important, a typical diagram (Experiment I 51/42b for 0.00084 per cent protein at 20°C.) is given in Fig. 8. The slow fall of the relative viscosity, not attaining its minimum plateau till 100 R.P.M. or so, characteristic of film flow anomaly, will be noted. No differences were ever observed between the behaviour of the euglobulin preparations from the neural and ventral portions of the embryos.

TABLE I

Nitrogen Content of Protein Fractions from the Amphibian Embryo

		Neural portion		Ventral portion	
		N/100 mg. dry weight tissue	Protein/ 100 mg. dry weight tissue	N/100 mg. dry weight tissue	Protein/ 100 mg. dry weight tissue
Total nitrogen		10.45		11.60	
		mg.	*mg.*	*mg.*	*mg.*
Total KCl extract	KS	9.69	60.50	10.98	68.50
Pseudoglobulin	A	1.07	6.68	2.77	17.30
Total euglobulin	TG	7.73	48.30	8.06	49.60
Acid-sensitive vitellin fraction	PG	3.31	20.70	8.24	51.60
Acid-resistant "myosin" fraction	SG	0.85	5.31	0.12	0.72
Fraction water-soluble after acid treatment	S	3.18	19.80	0.085	0.53
Aqueous extract of residue from KCl extract	WS	0.07	0.41	0.014	0.09
Final alkaline extract of residue	AK	0.13	0.81	—	—

When later the total euglobulin fraction was examined at flood level (for bulk viscosity) no sign of anomalous flow could ever be obtained and as it was at first thought that this might be due to the presence of lipoidal material in the preparations used, much effort was devoted to their delipidation. Eventually, however, it was found that the same sample of total euglobulin, whether delipidated or not, will regularly give normal or anomalous flow according to whether it is examined in the bulk or in the film (*e.g.* Experiments V 239–243). This fraction, therefore, behaves in a very similar way to serum globulin and ovoglobulin (and probably also the tissue globulins or plasmosins) and we must suppose that the approximately spherical particles of the bulk phase are unrolled into fibres or rods when denatured in the film.

The question of prior denaturation of the euglobulin is interesting. If it is first treated with guanidine in solution (*e.g.* Experiment I 58/51d guanidine

HCl 1.55 M, total euglobulin 0.00201 per cent) the anomalous flow is completely abolished, and the relative viscosity of the film is constant whatever the speed of rotation. This is perhaps because the particles, unrolled in the solution, aggregate into tangles which film conditions can never pull out. Such an explanation is strongly supported by the fact that if the total euglobulin is taken up into Edsall solution to which 30 per cent urea has been added, as in the experiments of Banga and Szent-Györgyi (1940), neither flow-birefringence nor any trace of anomalous flow is to be observed in the bulk phase, although

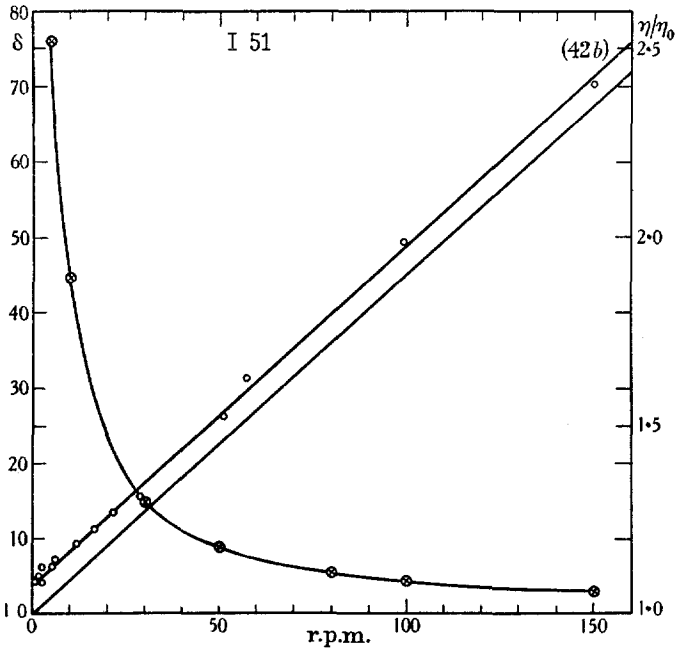

FIG. 8. Anomalous and relative film viscosity of amphibian embryo euglobulin.

the protein is certainly denatured (Experiment V 237/vi b). This is the same behaviour as that described by Banga and Szent-Györgyi (1941—abstract only available) for serum albumin, serum globulin, lactalbumin, ovalbumin, and casein.

The question next arises whether the material with which we are here dealing is wholly vitellin, or whether other globulins of greater structural importance accompany a phosphoprotein which has classically been looked upon as a mere reserve substance. It may be that this attitude to vitellin is a prejudice of which we ought to rid ourselves. We know that phosphorus is removed from it as the yolk is utilised during embryonic development (*cf.* Needham, 1931, p. 1198 *ff.*; 1942, p. 15) but we know nothing of what happens to the rest of the

molecule. There are, moreover, indications that it is capable of participation in orderly structures, for Radlkofer (1859), Schmidt (1924, p. 455, 456), and Fauré-Fremiet (1933) have described weak but definite birefringence in the innumerable yolk platelets which are so prominent a feature of embryonic cells. It may be, therefore, that vitellin is a source of fibre molecules to the embryo as well as phosphorus atoms. The yolk platelets are playing a more and more important part in modern experimental embryology, especially in regard to the mechanism of gastrulation; thus Pasteels (1940, p. 383) writes:

"La distinction faite trop souvent en protoplasme 'vivant' et enclaves 'mortes' semble spacieuse et dangereuse, car la morphogénèse peut être considerée comme le résultat d'un metabolisme complexe et changeant ou peuvent être impliqués tôt ou tard tous les materiaux de l'oeuf, sans distinction de leur état physique ou de leur visibilité microscopique."

Brachet (1940) has described a protein giving a strong -SH reaction and staining characteristically with pyronine and methyl green. It is first seen in the nucleus of the oocyte, its distribution is later coterminous with the organiser region over the dorsal lip of the blastopore, and it eventually appears in the cytoplasm throughout the neural plate and tube. Brachet writes (p. 197):

"Ajoutons que des granulations colorables à la pyronine n'apparaissent à l'intérieur même des cellules qu'au moment où la neurulation débute; elles ont alors l'aspect des fibrilles orientées suivant la longueur des cellules nerveuses. On peut se demander si la présence de ces fibrilles n'a pas d'effet sur l'acquisition de la forme du système nerveux; sa structure palisadique si caractéristique ne résulte certainement pas de l'orientation de mitoses, qui est éminement variable, mais de l'alignement des cellules. Quand on examine le problème à la lumière de nos connaissances récentes sur la structure des proteines, on est en droit de se demander si la forme des cellules nerveuses et les mouvements morphogénétiques que subit le système nerveux, ne proviennent pas de la structure et des déformations que leurs molécules proteiques sont susceptibles de subir."

Attempts to fractionate our total euglobulin fraction were therefore indispensable.

These attempts took four forms: (1) high-speed centrifuging, (2) Tiselius electrophoresis, (3) treatment with acid, (4) fractional dialysis. Up to the present time, only the fourth method has been in any way successful.

We were never able to separate the euglobulin into different fractions by high-speed centrifuging. For example, subjection of the total KCl extract to 16,000 R.P.M. for 2 to 3 hours brought down nothing, and the solution afterwards gave just as marked anomalous flow in the film as it had done before (*e.g.* Experiment I 60/53a). Owing to lack of equipment and spontaneous denaturation of the euglobulin (which readily occurs) during transit to other laboratories,

trials in the Tiselius apparatus were ineffective. This technique, moreover, is particularly difficult with proteins such as these, since they are most stable in M KCl at neutrality, while for electrophoresis ten times less salt and an alkaline pH is required.

Separation by acid treatment was suggested by the possibility that since in the neurula the somites are forming, there might be a small quantity of myosin-like globulin mixed with the vitellin. The total euglobulin fraction was simply treated with N/100 HCl, in which it dissolved, and after standing for varying intervals of time at different temperatures, reprecipitated by neutralisation. Only small amounts of the protein were now soluble in KCl, it being found as expected (Bate-Smith, 1937) that vitellin is denatured by this treatment. However, these soluble "myosin" fractions gave only the same anomalous flow in the film phase as the whole original fraction (*e.g.* Experiment I 52/43b; "myosin" at 0.0017 per cent at 20°C.), so not much advantage was gained. As may be seen from Table I, this "myosin" fraction is not quantitatively very important, though a good deal more of it was obtained, as expected, from the neural than from the ventral part. Another fraction was met with which, though not water-soluble before the acid treatment and therefore following the total euglobulin, remained in the neutralised solution following the acid treatment and would not go down with the denatured vitellin and the undenatured "myosin." This fraction, S, (like the "myosin" fraction itself, SG) was much more plentiful from the neural than from the ventral parts, but it resembled the pseudoglobulin (A) in giving a perfectly normal flow diagram tested under film conditions though differing from it in giving no trace of anomalous return (Experiment I 62/55b; 0.0055 per cent concentration at 20°C.). Owing to lack of material, further information about these fractions could not be obtained.

The spring of 1942 provided exceptionally abundant supplies of amphibian material, so a number of fractional dialyses at neutral pH were carried out, and these effected a better separation than any of the other methods (*cf.* Green's fractionation of horse serum proteins, 1938).

The first experiment was made as follows: About 24 gm. freeze-dried whole-embryo powder (from hatched larvae with external gills) were ground with quartz at 0°C., and delipidated. The delipidation process was the same in all experiments and consisted of three 20 minute extractions of the powder with pure absolute alcohol, followed by four 30 minute extractions with pure sodium-dried ether, all at −77°. The total euglobulin, when extracted and purified, was taken up in M KCl buffered to pH 7.1 with bicarbonate. 30 cc. of this solution were placed in each of eight sacs made from cellophane tubing, and dialysed against various concentrations of KCl so arranged as to give the final equilibria shown in Table II. The sacs were rotated mechanically for the first 6 hours and then remained at 0°C. for 10 hours to attain equilibrium. As expected, increasing amounts of precipitate were thrown out as the final salt concentration diminished; thus in A and B there was but a trace of precipitate; in C, D,

and E the precipitate was flocculent and increasingly massive; while in F, G, and H it was voluminous and creamy, ending in the usual total euglobulin precipitate.

The amounts of protein, determined by Kjeldahls, in each fraction precipitated are shown in Table II. This method has, of course, the disadvantage that each of the later fractions in the series contains all the preceding fractions. Nevertheless it was clear that a certain fractionation had been attained in this

TABLE II

Fractional Dialysis of Total Euglobulin of Amphibian Embryo

Experiment		Final molarity KCl reached by equilibration	Protein N	P/N ratio × 100	Viscosity properties of film phase
			gm.		
I (progressive)	A	0.74	0.005	7.65	Insufficient for test
	B	0.565	0.0115	7.07	" " "
	C	0.443	0.132	4.93	Denatured; no test
	D	0.32	0.228	5.16	Marked anomalous flow
	E	0.224	0.251	5.87	" " "
	F	0.113	0.278	5.96	Not tested
	G	0.0148	0.294	6.04	" "
	H	0.001	—	6.20	" "
II (separated)	A	0.454	0.1805	6.16	Marked anomalous flow
	B	0.403	0.1313	4.78	Slight " "
	C	0.356	0.1135	5.46	Very slight anomalous flow
	D	0.303	0.076	5.40	Normal flow
	E	0.195	0.093	6.37	" "
	F	0.090	0.018	6.44	Not tested
	G	0.009	0.0044	7.54	" "
III (separated)	A	0.453	Trace	7.00	Insufficient for test
	B	0.399	0.052	6.88	Marked anomalous flow
	C	0.329	0.034	6.64	" " "
	D	0.150	0.029	7.58	Normal flow

way, for the precipitates in C, D, and E had a different appearance from the later fractions, and when the phosphorus-nitrogen ratios were obtained, showed a marked difference. As the graph in Fig. 9 shows, the P/N ratio of the small amounts of globulin very precipitable by reduced salt concentration was high, as also was that of the large amounts of globulin which only came out of solution at the low salt levels; but the P/N ratio of the flocculent protein thrown out at the intermediate levels was decidedly low. The value varied from somewhat over 0.07 to somewhat under 0.05. For purified hen's egg vitellin the accepted P/N ratio (Osborne and Campbell, 1900; Jukes and Kay, 1932) is 0.0575, but

from the only modern figure we possess for frog's egg vitellin (McClendon, 1909) its ratio would seem to be considerably higher: 0.0785. The experiment suggests, therefore, that the total euglobulin fraction consists of at least three

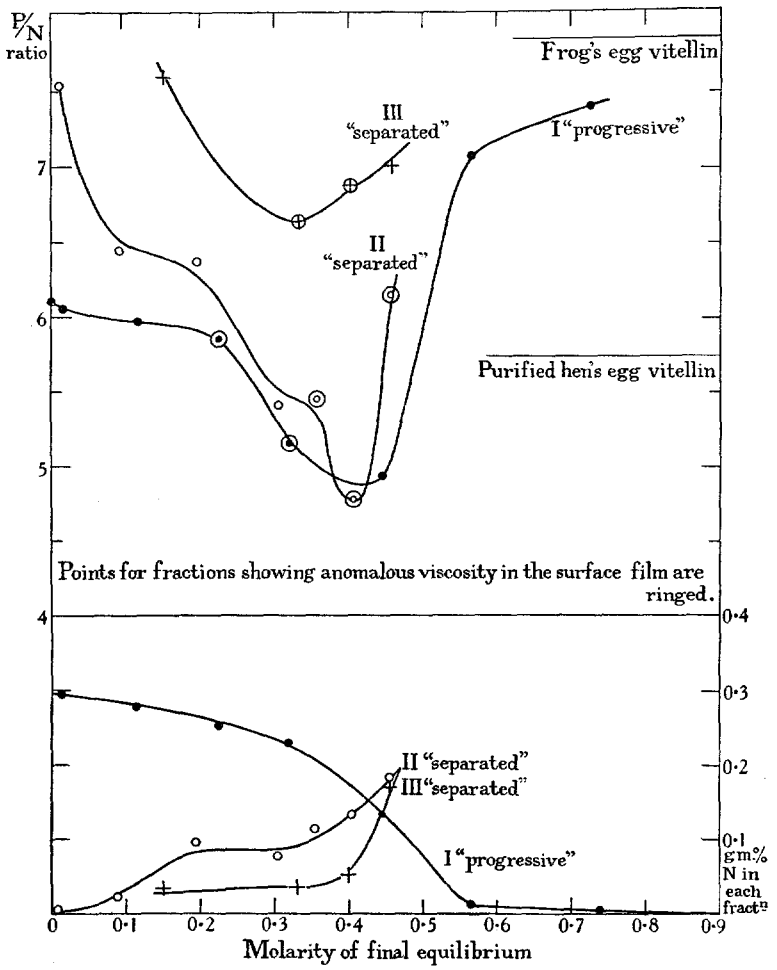

FIG. 9. Separation of fractions of amphibian embryo euglobulin by dialysis to various salt concentrations.

proteins: (1) a protein of high P/N ratio, present only in small quantity, and markedly precipitable by reduced salt concentrations, (2) a protein of low P/N ratio, present in larger amount, not so precipitable by reduced salt concentrations, and (3) a protein of high P/N ratio, present in very large amount, but not coming out of solution till the salt concentration falls below 0.25 M. We

must almost certainly identify the third of these with vitellin. In this experiment, viscosimetric observations were only made on fractions D and E; these corresponded to the second of the three proteins, and in each case gave definite anomalous flow in the film.

These interesting results encouraged us to go further, improving the method by exposing the same sample rather than a series of different samples of euglobulin to successively reduced salt concentrations, so that whatever came out of solution at each equilibrium concentration could be removed by centrifuging and separately examined. Since the critical concentrations at equilibrium seemed to be between 0.5 and 0.25 M, attention was directed especially to this range and to avoid all denaturation, every part of the operations was carried out at 0°C. As Table II and Fig. 9 show, the results of the second and third experiments were in agreement with the conclusions of the first. The starting material of Experiment II was 45 gm. embryo powder from just hatched larvae, and that of Experiment III was 6.5 gm. mixed embryo powders, mostly from neurulae. In both cases the P/N ratio first fell and then rose as the equilibrium salt concentration was reduced. But the new finding here of greatest importance was that in both cases the fractions precipitating at the lower salt concentrations (D, E, F, and G in Experiment II; D in Experiment III), and hence, on the basis of the first experiment, most likely to be vitellin itself, did *not* give the phenomenon of anomalous viscosity in the surface film. This was restricted to the central fractions (A, B, and C in Experiment II; B and C in Experiment III), associated with the lower P/N ratios.

Three fractions thus appear to be present, which we may classify and describe provisionally as follows:

Amphibian embryo euglobulin *a*.	Precipitates between 1.0 M and 0.5 M KCl concentration; present in very small amount; P/N ratio high; viscosity properties unknown.
Amphibian embryo euglobulin *b*.	Precipitates between 0.5 M and 0.3 M KCl concentration; present in considerable amount; P/N ratio lower; shows highly anomalous flow in the surface film.
Amphibian embryo euglobulin *c*.	Precipitates between 0.3 M and zero KCl concentration; present in very large amount; P/N ratio high; shows normal flow in the surface film.

Whether these are three forms of vitellin itself, or whether euglobulin *c* is to be identified with true vitellin and euglobulin *b* with Brachet's protein; what is the nature and significance of euglobulin *a*; and whether still further fractions could be distinguished, must be left for further investigation. We have, however, the clue that Mirsky and Pollister (1942) find the plasmosins to be (cytoplasmic?) nucleoproteins, so that a close relationship may exist between

Bensley's plasmosin, Brachet's gastrular protein, and our embryo euglobulin *b* (*cf.* Schultz, 1941, p. 59).

It has to be remembered that delipidation in the cold, as was necessary here, is not always adequate, however prolonged or repeated. Hence small amounts of lipoid may be present and affect the P/N ratio. These results must therefore be regarded as preliminary, and estimations of the actual lipoid content of the fractions are necessary.

Pseudoglobulin.—The pseudoglobulin is extracted from the embryo powder by the molar salt solution, but remains behind when the total euglobulin is thrown out by dilution with ten times the volume of water. It corresponds to the livetin of Plimmer (1908) and Kay and Marshall (1928) obtained from hen's egg yolk and the corresponding pseudoglobulins which have been described from yolk of many other animals, such as the dogfish egg thuichthin of Needham (1929).

As may be seen from Table I there was rather more of it in the ventral than in the neural portion of the amphibian embryo. It was soon found to differ markedly from the pseudoglobulin of serum (already mentioned) for it never, in the first instance, shows anomalous flow in the viscosimeter at low level (film viscosity). Its normal flow lines are, however, immediately succeeded by anomalous return, and it is, indeed, one of the most eminently "stretchable" of the proteins we examined. Thus (in Experiment I 42/30ccc) at 19.5°C. with the viscosimeter running continuously at 11.9 R.P.M. the mirror deflection rose from 9 to 97 mm. in 50 minutes. It is also worth noting that this protein has more tendency to lose relative viscosity on standing at 0°C. than any other in our experience (*cf.* Pauli and Valkó, 1933, p. 266). Thus, (in Experiment I 47) a concentration of 0.00055 per cent protein, which to begin with had a film viscosity of $\eta/\eta_0 = 4.35$ had fallen after 8 hours to a level barely distinguishable from that of water.

In some experiments (such as Experiment I 55/50bc, in which 0.011 per cent protein was used), the solution, diluted by half, filtered, and replaced in the viscosimeter immediately after normal flow and a large anomalous return had been observed, gave straightway an anomalous flow diagram, showing presumably that the denatured fibrils of the film retained their character and were ready to form a spontaneous surface film flow anomaly. But if, instead of diluting with M KCl, the contents of the viscosimeter was diluted with M KCl containing a suitable concentration of guanidine HCl (3.15 M in Experiment I 59/52abc), then no anomalous flow in the film was observable upon trial in the viscosimeter. This experiment is shown in Fig. 10. It must again mean that the fibrils in the bulk phase are aggregated into tangles by the dissolved denaturing agent, and perhaps in some way still further denatured, so that they can take no part in forming a new film showing flow anomaly.

It may be concluded that the pseudoglobulin is as capable of forming surface

film fibre particles as the euglobulins, but that the process is much slower under our conditions than with euglobulin *b*, which gives the flow anomaly immediately the film is set up.

Other Fractions.—In order to try to extract mucoproteins, nucleoproteins etc. from the KCl-extracted residue, extracts were made with distilled water (Experiment I 53/44) and phosphate buffer solution at pH 9.0 (Experiment I 65/58). As Table I shows, no appreciable amounts of protein could be extracted by these means. Examined in the viscosimeter both fractions showed

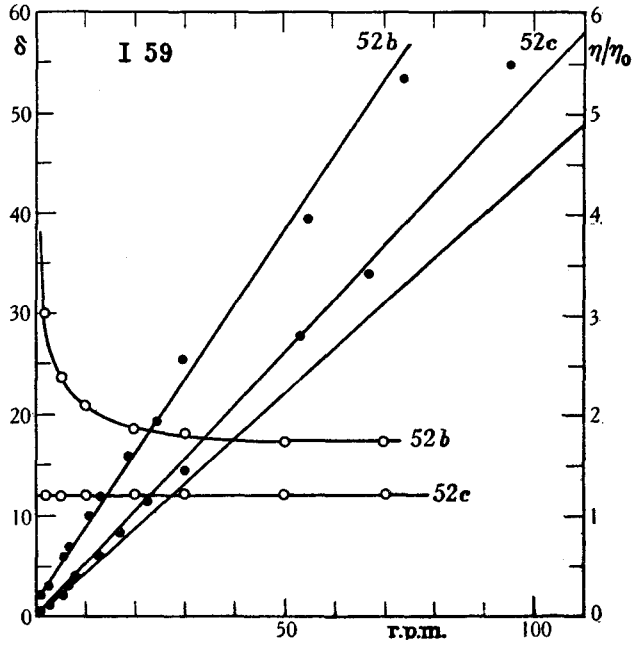

FIG. 10. Anomalous flow of amphibian embryo pseudoglobulin (film) after rotation at film level.

quite normal flow, but the amount of material available was insufficient to establish their properties further.

DISCUSSION

The data reported in this paper, the first extensive survey of the behaviour of dilute solutions of proteins in the coaxial viscosimeter, may be partly summarised in the form of a table (Table III). Group A includes the proteins clearly fibrillar or rod-like in the bulk phase as well as in the film. Probably also belonging to this group are fibrinogen (Wöhlisch and Clamann, 1932; Böhm and Signer, 1932); keratin (if in thioglycollic acid solution; Goddard and

Michaelis, 1935); lens albumoid (Böhm, 1934); the stromatin of erythrocytes, which is thought to form a lattice in the blood corpuscle (Böhm, 1935; Parpart and Dziemian, 1940; Furchgott, 1940); elastoidin (Fauré-Fremiet, 1936, 1937; Champetier and Fauré-Fremiet, 1937); collagen (Mehl, 1938; Champetier and Fauré-Fremiet, 1938); the neuronin of nerve fibre axoplasm (Bear, Schmitt, and Young, 1937); fibroin and sericin (Meyer and Jeannerat, 1939); and a plant

TABLE III

Provisional Classification of Proteins As Studied in the Coaxial Viscosimeter

A	B		C
Proteins which show flow-anomaly in bulk and in film, with flow-birefringence	Proteins which show flow-anomaly in film but not in bulk (except after film)		Proteins which show flow-anomaly neither in film nor in bulk
Fibrillar in bulk and film.	Fibrillar only in film (or in bulk after film)		
	B_1. Immediately (Polyfilm formation very rapid)	B_2. Only after anomalous return ("stretching"). (Polyfilm formation slow)	
Tobacco mosaic virus	Mammalian serum euglobulin	Crystalline avian ovalbumin	Crystalline insulin
			" methaemoglobin
Myosin	Mammalian serum pseudoglobulin	Amphibian embryo pseudoglobulin	Amphibian embryo euglobulin *c* (= vitellin?)
	Avian ovoglobulin		Amphibian egg jelly mucoprotein
	Amphibian embryo euglobulin *b*		Human umbilical cord mucoprotein
	Plasmosins (?)		

protein occurring in heather-honey (Pryce-Jones, 1936). This list includes the fibrillar corpuscular proteins and any fibrillar (normally non-corpuscular) proteins which can be got into solution.

Thyreoglobulin and certain haemocyanins have been stated to show flow-birefringence, but it is extremely weak (Lauffer and Stanley, 1938). Certain fractions of both the serum albumin and the serum globulin group may also be flow-birefringent (Sadron, Bonot, and Mosimann, 1939). Gelatin, as a degradation product of a fibrillar non-corpuscular protein (collagen) stands in a somewhat special position; both flow-birefringence (Umlauf, 1892) and anoma-

lous viscosity (Rothlin, 1919) have been reported for it, but in view of the difficulty of obtaining standard preparations it requires reinvestigation by modern methods.

Bulk anomalous viscosity has been observed for myosin (von Muralt and Edsall, 1930*a*, p. 340; Frampton, 1939; Edsall and Mehl, 1940), for lens albumoid (Böhm, 1934), for erythrocyte stromatin (Böhm, 1935), and for ovoglobulin (Böhm and Signer, 1931); all using flow through capillary viscosimeters at varying pressures. Other proteins tested in this way with negative results are casein (Rothlin, 1919), ovalbumin and ovomucoid (Böhm and Signer, 1931), lactoglobulin and serum pseudoglobulin (Neurath, Cooper, and Erickson, 1942*a*), haemocyanin (Polson, 1939), and myogen (Weber, 1933, p. 128).

We hope in the course of time to investigate as many as possible of these proteins with our methods.

Group B includes all the proteins tested which unfold ("stretch") as denaturation occurs within the surface film. It has long been known that a molecule of ovalbumin, having a radius of 55Å (Adair and Adair, 1940) unrolls into a surface film of but 9.5Å thickness (Astbury, Bell, Gorter, and van Ormondt, 1938). Some members of this group do it instantly (group B_1) giving anomalous flow diagrams in the first instance, while others (group B_2) require a considerable time to do so, and only give anomalous flow diagrams after the film has built itself up for half an hour or more. It may be surmised that the first of these groups includes many globulins and the second many albumins. The time factor in film formation has, of course, been known since the early work of Wilson and Ries (1923) and Gorter and Grendel (1928).

Finally, there is a group of proteins which has not given us at any time anomalous flow, either in bulk or film, (group C). The changes occurring in surface denaturation do not seem in these cases to lead to fibrillar particle formation, or if they do, the fibrils are not orientable. It is possible that this group would merge into group B_2 under conditions other than those used by us.

It is of much interest that the particles of proteins of group A, which are highly elongated in the bulk phase, remain so in the film. Seastone (1938) found that the virus spreads at air-water interfaces very unwillingly, but in our experiment there was no doubt that a film was present. Particles of proteins of group B, which are certainly not elongated in the bulk phase, become so in the film, and can later be detected in the bulk phase if the film is dispersed by shaking (Fig. 6). The distinction between group A and group B is probably the same as that in the now commonly used expressions "fibrillar corpuscular" and "globular corpuscular" proteins (Astbury, 1941).

Certain discrepancies still remain, however. For example, Böhm and Signer (1931) studied the proteins of hen's egg white with a coaxial apparatus similar to that used by Signer (1930) for measuring the flow-birefringence of polystyrols and Vorländer and Walter (1925) for paraffin chains, etc. Viscous anomaly was observed by timing

the speed of flow through a capillary viscosimeter at different pressures. Neither ovalbumin nor ovomucoid showed a trace of flow-birefringence or anomalous viscosity, but ovoglobulin showed both to a marked degree, accounting for the flow-birefringence and anomalous viscosity said to exist in fresh egg white. From the description of their apparatus given by Böhm and Signer it is not clear whether their cylinder was completely immersed in the protein solution or not, but it seems probable that it was. As their preparation of ovoglobulin gave very strong flow-birefringence and bulk phase anomalous viscosity it seems hardly likely that their higher shear rate as against ours (131 to 44) could have been wholly responsible for the absence of these phenomena in our preparations. We did, of course, find anomalous viscosity in the film. The subject requires further investigation but clearly ovoglobulin may belong to group A rather than to group B.

Our knowledge of the viscosity of protein surface films is not as yet very extensive. The capillary slit surface-viscosimeter and the torsion-ring surface-viscosimeter of Myers and Harkins (1937) have not so far been applied to surface films of proteins. But by measuring the damping of an oscillating platinum disc or vane suspended in the surface film by a torsion wire, it has been possible to measure the absolute viscosities of a number of protein surface films (Langmuir, 1938; Langmuir and Schaefer, 1937, 1939; Fourt, 1939). They vary over a range of about 1:10,000, typical figures being (in μ at pH 5.8 and 25°C. for $F = 6$ dynes) gliadin, casein, and zein about 0.005, up to trypsinogen 1.0, edestin 26, and serum globulin 210. But these correspond to our relative viscosities, and the *relative* viscosity of a protein, though certainly related to the degree of elongation of its particles, is not related to molecular shape in a simple way. It does not give the same conviction of elongated shape which may be derived from the observation of *anomalous* viscosity. Thus Cohn (1939, p. 223) points out that from the pioneer work of Chick (1914) and Chick and Lubrzynska (1914) to the more refined investigations of Fahey and Green (1938) it has been known that the relative viscosities are related as follows:—sodium caseinate > serum euglobulin > serum pseudoglobulin > serum albumin > ovalbumin; and this has long been interpreted as being the order in which the shapes of these molecules differ from spherical symmetry (*cf.* Pauli and Valkó, 1933, p. 240). But, as Cohn says, "a completely satisfactory theoretical equation relating the two properties remains to be developed. The problem has been repeatedly considered, and various extensions of the Einstein equation have been suggested and temporarily employed. Estimates of the relation of the two principal axes of the molecule must be adopted with caution, until they prove to be identical with those derived from diffusion measurements, dielectric constant measurements, and measurements of flow-birefringence." Perhaps this should apply also to the calculations of Neurath (1939) who assesses the axial ratios of the majority of globular corpuscular proteins at from 1 to 10, in contrast with the well established 10 to 100 of the

TMD virus (Lauffer and Stanley, 1939; Kausche, Pfankuch, and Ruska, 1939) and myosin (Mehl, 1938). Moreover, as will be seen in the succeeding paper of this series, in different myosin samples, relative and anomalous viscosity are independent variables. A sample showing normal flow may have a higher relative viscosity than one showing anomalous flow.

This point of view is substantiated by another approach to the nature of the protein surface film, also due to Langmuir (Langmuir, 1938*a*; Schaefer, 1938). The expansion patterns made by dropping oil of known properties on to the centre of a protein surface film, itself already surrounded by an oil of known properties, reveal qualities of cohesion anisotropy in the protein monolayer (*cf.* liquid crystals). The oil spreads from the centre in the form of a sharp pointed star, opening up, as it were, and following "faults" in the protein film;[3] and later, these strips of protein film, separating the oil channels, can be lifted off the surface as visible fibres. Similar fibres will also form parallel to the barrier compressing a protein film. They indicate, says Langmuir (1938*a*, p. 177) "the presence of long chain molecules." Not all protein films behave in this way, however. Some give a smooth circular boundary between the protein and the advancing oil; others, an intermediate form, give a rough circular boundary with a serrated edge. The proteins divide thus (Schaefer, 1938): *star-like expansion pattern*: ovalbumin, pepsin, pepsinogen, tobacco seed globulin, trypsinogen, urease, edestin, and TMD virus; *rough circular expansion pattern*: trypsin, papain, and gliadin; *smooth circular expansion pattern*: insulin, zein, casein, protamine, and gelatin. The relation between these effects and the surface film's viscosity is as follows:—

	Viscosity (in the former units)
Star-like expansion pattern	
Pepsin	0.75
Ovalbumin	0.28
Tobacco seed globulin	0.20
Rough circular expansion pattern	
Trypsin	0.23
Papain	0.18
Gliadin	0.003
Smooth circular expansion pattern	
Insulin	0.028
Zein	0.003
Casein	0.010

[3] Phenomena of this kind may sometimes be seen at the surface of a cup of tea where a film is first formed by tanned plant protein, and this is then split along sharp edges as the oil from the milk expands with greater force beside it.

From this list it is seen that if, as we must suppose, the expansion pattern is a delicate test for molecular elongation the surface film viscosity is not related to it in a simple manner. But it may be significant (although unfortunately we have not worked on many of the same proteins) that ovalbumin, which comes in our group B_2, has a high surface viscosity and a *star-like* expansion pattern, while insulin, which comes in our group C, has a low surface viscosity, and a *smooth circular* expansion pattern.

Schaefer (1938) further reports the extremely interesting fact that if pepsin is subjected to prolonged heat denaturation, its expansion pattern changes from *star-like* to *smooth circular*, suggesting that denaturation has formed elongated fibrils but that these have become clumped in loose tangles as if in a coagulum and can no longer contribute to any cohesion anisotropy of the surface film. This corresponds with our experience, for, as we have seen above, ovalbumin or amphibian embryo euglobulin, denatured by guanidine in the bulk phase, will not at neutral pH form the usual surface film showing anomalous flow. In general, denatured proteins spread on surface films more unwillingly than native ones, but if the pH is adjusted to the acid side, they will do so (Bull, 1938).

Böhm and Signer (1931) denatured ovalbumin with alkali (pH 13). Gel strain-birefringence made its optical examination difficult to interpret, but variable pressure capillary viscosimeter readings indicated highly elongated particles. After some hours, however, this anomalous viscosity quite disappeared, probably owing to tangle formation.

In general, when globular corpuscular proteins are denatured with strong urea solutions (Liu, 1933; Bull, 1940 for ovalbumin; Neurath and Saum, 1939; Neurath, Cooper, and Erickson, 1942*a* for serum albumin; Neurath, Cooper, and Erickson, 1942*b* for serum pseudoglobulin) or by heat (Anson and Mirsky, 1932 for ovalbumin; Loughlin and Lewis, 1932 for haemoglobin), the relative viscosity rises greatly but there is neither anomalous viscosity (measured in the capillary instrument under varying pressures) nor flow-birefringence at any stage. All this agrees with the view that tangles are produced from the fibrils into which the protein cage molecules are first transformed. There would also be a general increase of intermicellar forces.

But when fibrillar corpuscular proteins are denatured (Frampton, 1939, for tobacco mosaic virus nucleoprotein; Edsall and Mehl, 1940 for myosin; and experiments reported in the present paper) anomalous viscosity and flow-birefringence are abolished, and there is a fall of relative viscosity. Increase of intermicellar forces and tangle formation must therefore probably be overcompensated by loss of relative viscosity due to the decreased asymmetry of the particles.

These views somewhat resemble the earlier conceptions of the Peiping school, who greatly emphasised Chick and Martin's distinction (1911) between two stages of denaturation, denaturation proper being followed by coagulation or

agglutination, (Wu and Wu, 1925; Wu, 1927, 1929). Thus it has been stated by Wu and Chen (1929) that denaturation always brings about a large increase of acid- and base-binding power of a protein, but the quickly succeeding coagulation reverses it, ending in a final level less than that of the native protein.

Conversely, insulin, according to Schaefer (1938), if treated with copper or zinc, will give a *star-like* expansion pattern instead of a *smooth circular* one. This would suggest that it has been changed into something capable of forming rod-like or fibre-like particles. That this can easily happen to insulin has been shown by Waugh (1941). If a 2 per cent solution of insulin HCl be boiled for ½ hour it forms a thixotropic gel, and this on dilution gives strongly flow-birefringent solutions, of high viscosity. By alternate freezing and thawing the flow-birefringence and viscosity disappear, and the protein resumes its normal properties. Hormone activity is retained throughout. This can hardly mean anything but reversible linear aggregation.

On denaturation, globular corpuscular proteins give fibre protein x-ray pictures (Astbury and Lomax, 1935); and the Langmuir-Schaefer expansion patterns make it extremely probable that the surface films of corpuscular proteins are composed of unrolled fibrils. Indeed, as long ago as 1882 von Ebner, forcing out ovalbumin solutions into absolute alcohol through a capillary pipette, obtained highly birefringent fibrils. But the evidence which clinches the matter is that of Astbury, Bell, Gorter, and van Ormondt (1938) and Stenhagen (1938), who, building up piles of protein monolayers on a chromium-plated metal slide mechanically moving repeatedly through a protein film at an air-water interface (the technique of Blodgett, 1935; Blodgett and Langmuir, 1937), were able thus to produce multilayers containing from 1400 to 1800 layers of ovalbumin film, each one 9 to 10 Å thick. Such multilayers, stripped from the metal base, were found to be birefringent (as much so as wool), while within them were often contained a multitude of negative tactoids, probably caused by the presence of minute foreign bodies, and showing at their edges an intense birefringence (as high as that of natural silk). Examination by x-rays confirms the presence of innumerable extended polypeptide chains lying roughly parallel to the direction of movement of the slide, with their side-chains roughly perpendicular to the plane of the film. These chains must have pre-existed in the monolayer at the air-water surface, and in forming them the ovalbumin corpuscular cage molecules must therefore have unfolded or uncoiled. Further proof of the fibrous nature of these films was their tendency to tear parallel to the direction in which the slide was moved through the air-water interface, and in some cases they even showed a fringe of delicate fibres along their edges at right angles to this direction. These interpretations are strikingly confirmed by the anomalous viscosities of surface films described by us.

It may be mentioned here that our surface films of proteins are not the first for which anomalous viscosity has been described. Fourt and Harkins (1938)

have given anomalous flow diagrams closely analogous to ours for condensed monolayers of the long-chain alcohols (C14-C18). This non-Newtonian flow only occurs above a certain kink-point as pressure on the monolayer is increased.

In the absence of flow-birefringence observations on the protein surface films, and apart from all other evidence, our measurements of their anomalous viscosity cannot indeed be said to prove the existence of fibrillar particles in them. The anomaly might be a genuine "structural viscosity" due to the existence of intermicellar forces between disc edges, which increasing shear force would gradually overcome. But the occurrence of bulk anomalous flow after the dispersion of the film, makes this point of view difficult to hold, and it would also be hard to believe that in those cases where normal flow is obtained in the film no such intermicellar forces exist.

The work of the past 20 years has familiarised us with the conception of protein denaturation as essentially the transformation of compact, rigid, almost crystalline, coiled or folded polypeptide-chain molecules with a highly specific configuration, maintained by secondary valencies, hydrogen bonds, sulphydryl linkages, etc.; into unrolled, more flexible, fibrillar molecules with a less specific configuration. Obviously this might involve profound chemical changes in the molecules. Of several papers outstanding from the mass of literature, the pioneer work of Wu (1931) and Mirsky and Pauling's continuation of it (1936) and the work of Astbury, Dickinson, and Bailey (1935) may be mentioned here. The decrease in solubility on denaturation is explained by the assumption that in the native molecule the polar groups are mostly on the external surface of the framework, but when the molecule unrolls, the non-polar hydrophobe groups are bared. The appearance of -SH groups is explained by their being no longer employed in cross-chain linkages. The crystallisation of native proteins and the incapacity of denatured proteins to crystallise is explained by the view that they are no longer rigid and compact structures. On the other hand the denatured fibrils may participate in forming regular submicroscopic bundles, or liquid crystals, and it is possible that the behaviour of the surface films described in the present paper should be interpreted from this point of view (*cf.* Lawrence, 1938). In the modern view, all the diverse methods of denaturation can be understood. Drying withdraws water molecules from the framework causing it under some conditions to crumble; heat and radiant energy shatter the secondary valency bonds; shaking and gas bubbling uncoil the frameworks at the air-water interfaces; urea and guanidine derivative molecules penetrate into the frameworks and disrupt them; organic solvent molecules also penetrate and by dissolving the non-polar groups, turn the frameworks inside out, and so on. The denaturation process may be up to a certain stage reversible, as Anson and Mirsky (1925, 1931) and Wu and Lin (1927) were the first to point out. Little, of course, is as yet known about

the actual structure of the framework; if the geometrical formulations of Wrinch (1937), which have aroused so much interest, are untenable, some other such formulations will have to be proposed. The framework conception does, however, enable us to understand how isotopic amino acids can so quickly interchange with the amino acids in the protein molecule (*cf.* Rittenberg, 1941), for if the main chain were not coiled and held together by many secondary valencies and similar bonds, the peptide linkages in the main chain could hardly open and close as frequently as it seems they do.

According to the view adopted here, it may be a great mistake to suppose that the uncoiled protein chains have no physiological significance. As was indicated in the introduction, the assumption of a "dynamic framework or lattice" (*cf.* Needham, 1942, page 658; Schmitt, 1939) of fibre molecules in the cell fills a real need in causal morphology, experimental embryology, histology, and the like; and there are numerous facts, especially concerned with the existence of liquid crystalline phases in the cell, which support it. Most germane to the present paper is the direct proof which Pfeiffer (1937) has given that fibril molecules are contained in living protoplasm. If naked cytoplasm of animal eggs (molluscs, echinoderms) or of plant cells (such as the liquefying pericarps of *Solanum* or the parenchyma cells of hyacinths and orchids) is caused to flow through a minute capillary viscosimeter under different pressures, curves exactly analogous to those reported in the present paper, showing marked anomalous viscosity, are obtained. Moreover, we have evidence that such forced flow of protoplasm may have important biological consequences; thus P. E. Lindahl found that if echinoderm eggs are squeezed through a capillary tube, the leading end of the "sausage" always afterwards becomes the ventral pole.

The question of the biological status of proteins in the denatured condition is important. When Banga and Szent-Györgyi (1940) find flow-birefringence in urea-treated tissue globulin solutions; when we, as here, find anomalous viscosity in protein surface films—are these phenomena of physiological significance or not? There is considerable reason to think that they are. We are not without evidence that an appreciable proportion of the protein in the living cell is in the denatured state.

If denaturation be taken as synonymous with the unrolling of a corpuscular protein, this must occur at all oil-water interfaces in the cell, *e.g.* at the surfaces of mitochondria, the cell membrane, the intracellular oil droplets, etc. The very low surface tension (< 1 dyne per cm.) found by Harvey and Shapiro (1934) and Harvey and Schoepfle (1939) for intracellular oil droplets was shown by Danielli and Harvey (1934) to be due to protein, and Danielli later (1938*a*) found that these low tensions are produced at a wide variety of oil-water interfaces by a wide variety of corpuscular proteins. There is little specificity. Lastly, Askew and Danielli (1936, 1940) proved that the protein absorbed at the oil-water interface is actually unrolled.

If denaturation be taken as synonymous with loss of solubility, there is direct evidence that forms of the same protein with different solubilities occur in the living cell. Many workers have established that in alkaline rigor, frog and rabbit myosin is less soluble than in resting muscle (Mirsky, 1938). The same thing occurs in limb muscle of crabs in iodoacetate rigor, or as a result of exercise to exhaustion (Danielli, 1938*b*). Mirsky, too, (1936) has shown that a certain protein fraction in sea urchin eggs becomes less soluble after fertilisation.

The question of the retention of specificity and biological activity by surface-denatured uncoiled protein is also relevant, and we now have a certain amount of information on it. It is true that the differences between haemoglobins of different animal species (crystal form, solubility, gas affinities, position of absorption bands, etc.) disappear on denaturation to their haemochromogens by acid and alkali (Anson and Mirsky, 1925); that denatured proteins lose immunological specificity (Zinsser and Ostenberg, 1914; Wu, TenBroeck, and Li, 1927) and that pepsin and trypsin denatured irreversibly with heat or acids can perform proteolysis no more (Northrop, 1930, 1932). But these transformations were not carried out by surface film unrolling. It may indeed be that it is only upon tangle formation (see foregoing) that biological activities and specifications are finally lost. Pepsin has been obtained by Gorter (1937) and Langmuir and Schaefer (1939) in the unfolded surface film state—it retained its activity. Catalase retains in the film 20 per cent of the activity it possessed when in solution (Langmuir and Schaefer, 1938; Harkins, Fourt, and Fourt, 1940), and though urease is apparently inactivated (Langmuir and Schaefer, 1938), saccharase is not (Sobotka and Bloch, 1941). Rothen, Chow, Greep, and van Dyke (1941) studied insulin and certain pituitary hormones. The activity of insulin they found to be unimpaired by surface denaturation, but that of the posterior pituitary oxytocic pressor hormone and that of the pituitary gonadotrophic hormone were markedly reduced or destroyed. On the other hand, the latter protein hormone had in no way lost its capacity to combine with homologous antibodies. Again, though surface films of Pneumococcus antibody cannot combine with their specific polysaccharide (Danielli, Danielli, and Marrack, 1938) avian ovalbumin in the surface film does not lose its property of combining specifically with antibodies of homologous sera (Rothen and Landsteiner, 1939) and a streptococcal agglutinogen nucleoprotein remains immunologically active in the monolayer (Chambers, 1939). Finally, myosin shows adenosine triphosphatase activity, not only in the bulk phase, but also when partially dried and in visible fibre form (Engelhardt, Ljubimova, and Meitina, 1941). One may conclude that although some biological specificity and activity may be lost when the protein cages unroll, by no means all of it disappears until the unrolled fibres finally intertwine chaotically.

If then we may adopt the hypothesis that protein molecules unrolled into elongated fibrils by denaturation have a part to play in the dynamic architecture of the cell, as well as those which are elongated before denaturation, the question arises as to how they are produced in the living organism? Is it possible that the rotational churning and streaming motion of protoplasm has some connec-

tion with this? The streaming of protoplasm has often been considered only in connection with amoeboid movement, but the classical cyclosis of the cytoplasm in plant cells and the extraordinary churning movements in eggs, especially before cell division (see, for example, the descriptions of Harris, 1935) cannot have this significance. It is worth asking whether they might not play a part in the orienting of surface-denatured fibrils by shear forces into those anisometric micelles which the cell shape itself (as in the neural plate of the embryo), or the egg's polarity and symmetry properties, or the different times of determination of limb bud spatial axes, seem all to imply. It may be said that the speed of cytoplasmic streaming is low, and hence the shear forces associated with it would be much lower than those which we have in our instruments, but this difference is offset by the probability that on the other hand the effective near-molecular annulus equivalent in which the shear forces would act in the cell would be enormously smaller than in our instruments, so that the actual shear rate might, on balance, be very much higher. The spinning of long protein molecules by protoplasmic streaming is a conception analogous to that which Preston (1941) has invoked to account for the "crossed fibrillar" structure of cellulose chains in the walls of plant cells; he supposes that it derives from alternate contractions and relaxations of protein chains in the cytoplasm. This affords another instance of the universally felt need for chain structures in cells.

Lastly, if the above argument is sound, it is not without interest that in modern human technology, the preparation of actual fibres such as the artificial wool (lanital) from casein (von Weimarn, 1927; Sutermeister and Browne, 1939, p. 215), plant seed globulins such as edestin and excelsin (Astbury, Dickinson, and Bailey, 1935; Labarre and Dostert, 1942), and soya bean protein (Satô, 1923; Horvath, 1938) would be an instance of man having unknowingly made use of certain properties of proteins which have been, since the beginning of living things, essential to the life and development of cells and organisms.

SUMMARY

1. An extensive investigation has been made of protein particle shape using the methods of flow-birefringence and anomalous viscosity measurement in the coaxial cell.

2. As a result of investigations on a number of proteins, it is concluded that they may be divided into four groups.

Group A consists of those which show flow-anomaly both in the bulk phase and in the surface film. These also show flow-birefringence in the bulk phase. Examples: tobacco mosaic disease virus nucleoprotein; myosin. Though corpuscular proteins, they have elongated particles before denaturation.

Group B consists of those which show flow-anomaly only (in the first instance) in the surface film, and no flow-birefringence in the bulk phase. They are

probably close to spherical in shape in solution, but form elongated particles as they denature in the surface film. After this process has been completed, they may show flow-anomaly also in the bulk phase. Some proteins show flow-anomaly in the surface film immediately it forms, others only show it after a certain time has elapsed for the building up of the film. We designate the former as group B_1 and the latter as group B_2.

Group B_1, immediate surface film flow-anomaly. Examples: serum euglobulin, amphibian embryo euglobulin *b*.

Group B_2, slowly appearing surface film flow-anomaly. After the film has once been fully formed and then dispersed by shaking, the solution may have the properties of that of a protein in group B_1; *i.e.*, anomalous flow in the film may occur immediately on testing in the viscosimeter. Examples: avian ovalbumin, amphibian embryo pseudoglobulin.

Group C consists of those proteins which show flow-anomaly neither in the bulk phase nor in the surface film, under the conditions used by us. They are probably close to spherical in shape. Examples: insulin, methaemoglobin, amphibian embryo euglobulin *c*, mucoproteins.

3. The theoretical significance of protein fibre molecules, whether native or formed by denaturation in the living cell, is discussed, especially in relation to experimental morphology and cytology.

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