more labile DNP derivatives are not present. The possibility of cysteine as an end group was also considered, for this acid was not investigated in Sanger's original paper. *NS* bis-DNP-cysteine was prepared and found to be as stable to acid as DNP-alanine and to run with an R value of 0·1 on a chloroform column. This acid would thus have been readily detected.

The precedent for the existence of cyclic chains in proteins is to be found in gramicidin S (Sanger, 1946), which is much simpler in structure than anything investigated here. It is not known whether ovalbumin is composed of cyclopeptides, or whether the terminal amino groups are linked to the carbohydrate residues. Such an explanation cannot hold, however, for myosin and tropomyosin in which prosthetic groups are entirely absent. It is encouraging to find that in the case of tropomyosin at least, the concept of a cyclopeptide helps to explain features which would otherwise be puzzling. These aspects are considered in the following paper (Tsao *et al.* 1951).

SUMMARY

1. Sanger's (1945) fluorodinitrobenzene method has been applied to a study of the terminal amino groups in tropomyosin, myosin and fibrinogen.

2. Tropomyosin and myosin appear to consist of cyclopeptides. It is not yet possible to decide whether the terminal residues in fibrinogen arise from the protein itself or from impurities.

3. The lysine side chains in myosin are fully reactive. There is evidence that tropomyosin contains a small proportion of unreactive lysine side chains, and the same is true of fibrin but not of fibrinogen.

4. The end-group method has been used to investigate the stability of the peptide bonds of these proteins in acid and alkaline media. Myosin at 0° is stable to alkali at pH 11 but not at pH 13; tropomyosin is stable up to pH 12.

I am deeply grateful to Dr F. Sanger and Dr R. R. Porter for their unfailing help, and to Mr I. M. Glynn for assistance with fibrinogen preparations.

REFERENCES

Abrams, A., Cohen, P. P. & Meyer, O. O. (1949). *J. biol. Chem.* **181**, 237.
Bailey, K. (1937). *Biochem. J.* **31**, 1406.
Bailey, K. (1944). *Advanc. protein Chem.* **1**, 289.
Bailey, K. (1948). *Biochem. J.* **43**, 271.
Blackburn, S. (1949). *Biochem. J.* **45**, 579.
Desnuelle, P. & Casal, A. (1948). *Biochim. Biophys. Acta*, **2**, 64.
Desnuelle, P., Rovery, M. & Bonjour, G. (1950). *Biochim. Biophys. Acta*, **5**, 116.
Dubuisson, M. (1946). *Experientia*, **2**, 413.
Edsall, J. T., Foster, J. F. & Scheinberg, H. (1947). *J. Amer. chem. Soc.* **69**, 2731.
Jaques, L. B. (1943). *Biochem. J.* **37**, 344.
Mihalyi, E. & Lorand, L. (1948). *Hungarica Physiol. Acta*, **1**, 218.
Morrison, P. R., Edsall, J. T. & Miller, S. G. (1948). *J. Amer. chem. Soc.* **70**, 3103.
Porter, R. R. (1948). *Biochim. Biophys. Acta*, **2**, 105.
Porter, R. R. (1950). *Methods in Medical Research*, **3**, 256.
Porter, R. R. & Sanger, F. (1948). *Biochem. J.* **42**, 287.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Sanger, F. (1946). *Biochem. J.* **40**, 261.
Sanger, F. (1949). *Biochem. J.* **45**, 563.
Snellman, O. & Erdös, T. (1948). *Biochim. Biophys. Acta*, **2**, 650.
Szent-Györgyi, A. (1947). *Chemistry of Muscular Contraction.* New York: Academic Press.
Tsao, T.-C. (1950). Unpublished observations.
Tsao, T.-C., Bailey, K. & Adair, G. S. (1951). *Biochem. J.* **49**, 27.
Weber, H. H. (1950). *Proc. roy. Soc.* B, **137**, 50.
Weber, H. H. & Stöver, R. (1933). *Biochem. Z.* **259**, 269.

The Size, Shape and Aggregation of Tropomyosin Particles

By T.-C. TSAO, K. BAILEY and G. S. ADAIR
Biochemical Laboratory and Low Temperature Research Station, University of Cambridge

(*Received* 13 *September* 1950)

Much of the recent work on the proteins of the myofibril has been devoted to the two major components, actin and myosin. The study of the interaction of these two proteins with each other and with adenosinetriphosphate has led to the belief that this system contains the essential components of the contractile mechanism. A knowledge of the properties of the proteins concerned must be of significance in the interpretation of the process of contraction and, for this reason, we have attempted to define the structure of one of the simpler asymmetric proteins of the myofibril, tropomyosin (Bailey, 1948).

Previous investigations (Bailey, 1948; Bailey, Gutfreund & Ogston, 1948; Astbury, Reed & Spark, 1948) have suggested that tropomyosin is a proto-

type of myosin, similar in physico-chemical properties and amino-acid pattern. Like myosin, the large-angle X-ray diffraction pattern is of α-type. Tropomyosin differs from myosin mainly in its stability to denaturing agents such as ethanol, and is in fact prepared from muscle mince which has first been dried at room temperature in ethanol and ether. In salt-free solutions more alkaline than the isoelectric point, tropomyosin sols have an exceedingly high viscosity which decreases in a dramatic way as small amounts of salts are added. These observations suggested that when salts are removed, the molecules of tropomyosin aggregate to form fibrils. This conclusion was borne out by electron microscope studies (Astbury *et al.* 1948). The interesting features of this aggregation are first that it is fairly regular, giving rise to fibrils 200–300 A. wide and 3000–6000 A. long, and secondly, that it is largely electrostatic in character (Bailey, 1948).

The first estimations of the molecular weight of tropomyosin by ultracentrifugal and osmotic methods gave a value of about 90,000 for a solution of pH 6·5 and ionic strength 0·27 (Bailey *et al.* 1948). Further investigations, described in this paper, were carried out to determine the effects of concentrated urea solutions and of variations in the concentrations of salts and of hydrogen ions. Preliminary experiments (Adair, Bailey & Tsao, 1949) with solutions of ionic strengths from 0·1 to 1·1 at pH 6·5 showed that the average molecular weight varied from 135,000 to 65,000, diminishing with increasing salt concentration. It seems probable that in the most concentrated salt solutions employed, the protein molecules were still associated because a lower value for the particle size, 53,000, was obtained both in 6·7 M-urea and in 0·01 N-hydrochloric acid.

In the present paper, the changes in particle weight have been followed by the use of osmotic pressure measurements in conjunction with viscometric measurements. Experimental data obtained for the particle of molecular weight 53,000, assumed to be the monomer, show that the axial ratio is about 25. Since the average residue weight of the constituent amino-acids is known (Bailey, 1948), and assuming that three amino-acids comprise the fold (Astbury, 1941), the asymmetry can be calculated for a rigid molecule consisting of one, two or more peptide chains. The calculated value for a double-chain model agrees well with the experimental, and this structure is also suggested by the collateral chemical evidence that the monomer consists of a cyclic peptide chain (Bailey, 1951). The magnitude of the asymmetry has been confirmed by Dr G. Weber, who has applied his method of measuring the polarization of a fluorescent dye coupled to the protein (Weber, 1950). These experiments will be published separately.

MATERIALS AND METHODS

Sodium was the cation in all phosphate buffers used.

Preparation of tropomyosin. The protein was prepared from rabbit muscle by Bailey's (1948) method. Slight modifications have been introduced at stages 3 and 4 of the original preparation (see Bailey, 1951). The protein was stored at 0° as an $(NH_4)_2SO_4$ paste, or as an ethanol-ether-dried powder. In the former case, the paste was dissolved in water, dialysed in cellophan in the ice chest until free of $(NH_4)_2SO_4$, and then equilibrated against several changes of the outer liquid to be used in osmotic pressure measurements. Dry tropomyosin was dissolved in water and dialysed against 0·1 M-KCl. Any trace of turbidity at this stage indicates that impurities have not been completely removed.

Osmotic measurements. These were carried out at 0° in the toluene osmometers of Adair (1949). When the pressure head exceeded 20 cm. of toluene, the tetrachloroethylene osmometer was substituted (Adair, unpublished). Collodion membranes (Adair, 1925) were used in all experiments except those employing an alkaline medium, which caused them to become brittle and to collapse. It was found, however, that Visking cellophan tubing (Visking Corporation, Chicago, U.S.A.) withstands alkali well and has a suitable permeability. These tubes (inflated diameter $\frac{18}{32}$ in. (1·43 cm.)) were mounted on rubber in the same way as the collodion membranes. With the latter, osmotic equilibrium was reached quite quickly (sometimes overnight), but in Visking tubes, an equilibrium was established only after several days, because of the extensibility of the material. In all cases, however, readings were taken over quite extended periods to ensure that solutions were truly equilibrated.

Measurement of protein concentration. Protein contents were generally determined by the micro-Kjeldahl method as adapted by Chibnall, Rees & Williams (1943), taking 16·7% as the N content of the protein (Bailey, 1948). For tropomyosin in concentrated urea, a known volume of solution was pipetted into a small cellophan bag, dialysed in several changes of water until free of urea, and the bag plus contents digested in the usual way. An equal volume of diffusate and an equal length of tubing were digested in a similar manner to serve as a blank and to check the thoroughness of dialysis. The analytical values were checked by refractive index measurements in the Zeiss dipping refractometer (Adair & Robinson, 1930*a*). Urea was purified according to Steinhardt (1938).

Calculation of particle weight. The extrapolation procedures of Adair & Robinson (1930*b*) were adopted. The ratio of osmotic pressure (mm. Hg) to protein concentration, i.e. P/C_v, was plotted against C_v, the latter being expressed as g. protein/100 ml. of solvent; C_v was calculated (see Adair, 1928) from the solution constants by using a value of 0·71 for the partial specific volume (Bailey *et al.* 1948) and a hydration figure of 0·248 g./g. protein, this latter determined by the method of Adair & Robinson (1931). Experiments with solutions of haemoglobin (Adair, unpublished) in equilibrium with phosphate buffers at pH 7·4 have shown that a better approximation to a straight line may be obtained by plotting $\log P/C_v$ against C_v. This method was found suitable for the observations on tropomyosin, recorded in Figs. 2 and 3*a*.

Viscosity. Measurements were made at 20°±0·02° in a horizontal capillary viscometer (Tsuda, 1928; Ostwald, 1933) in which the rate of flow can be varied by the use of two 15 l.

aspirators, which maintain a constant pressure during flow. The capillary had the following characteristics: radius, 0·0296 cm.; length, 29·40 cm.; flow time for 1·051 ml. of water at a pressure head of 15 cm. of water, 71·0 sec. The average rate of shear (β) was calculated from the formula of Kroepelin (1929); i.e.

$$\beta = \frac{8}{3}\frac{V}{\pi\gamma^3 t},$$

where V is the volume of liquid flowing through the capillary of radius γ in time t. At the highest shear rate employed in this investigation (1250 sec.$^{-1}$), the kinetic energy correction amounts to less than 0·4% and was neglected.

Axial ratios were assessed from the values of intrinsic viscosity by the use of Simha's equation (Simha, 1940; Mehl, Oncley & Simha, 1940), and an allowance was made for the contribution of hydration to the asymmetry factor (Oncley, 1941).

RESULTS

General considerations

In the past, a native protein in its stability zone has generally been considered to have a particle weight which is little affected by changes either in salt or protein concentration. In a few instances, the degree of polymerization is increased by an increase in protein concentration, as in the case of globin (Roche, Roche, Adair & Adair, 1932), or depends largely upon the concentration and nature of the salt present, as in the case of haemocyanin (Brohult, 1940). Outside the pH stability zone, or in concentrated urea solutions, particles may become more asymmetric, thus increasing the viscosity of the solution (Wu, 1931), and this process may be accompanied by the depolymerization of the protein into smaller units (Wu & Yang, 1932; Adair & Adair, 1938). In those cases in which depolymerization occurs, there has always been some uncertainty whether labile peptide bonds were broken; but the fact that neutral urea or guanidine solutions are as effective as acid and alkali has suggested that the native protein is often built up of several peptide chains which are generally called sub-units. This view has been fully confirmed, first by the identification of free α-amino groups in proteins (Chibnall, 1942), and more elegantly by the fluorodinitrobenzene method of end-group assay (Sanger, 1945). Moreover, there is no indication, by the application of the latter method, that new end groups are produced either in urea, or in acid at pH 2 or in alkali at pH 12 (Bailey, 1951). Two problems must thus be considered: first, the evidence for the existence of a monomer unit, its size and shape; and second, the evidence for the existence of sub-units within the monomer itself.

For convenience, the particle weight of tropomyosin in solvents which are likely to cause depolymerization will first be considered, followed by a study of aggregation phenomena in salt solutions.

Osmotic pressure of tropomyosin in urea, acid and alkaline solutions

Urea. The protein was equilibrated with a concentrated urea solution for 2 weeks before transference to the osmometers. Making use of the titration curves of Burk & Greenberg (1930) the urea solution (6·67 M) was buffered at pH 6·5 with phosphate (0·075 M, $I = 0·1$). The value of P/C_v extrapolated to infinite dilution (Fig. 1) is 3·21, corresponding to a particle weight of 53,100.

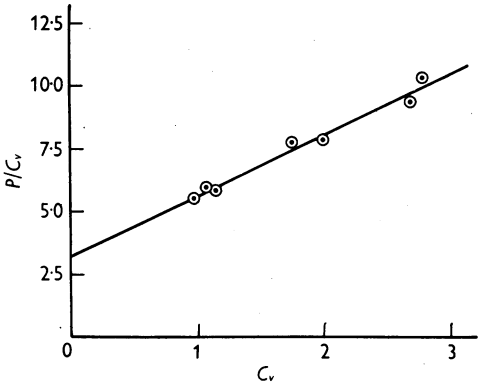

Fig. 1. Osmotic pressure of rabbit tropomyosin in concentrated urea solution at 0°. Urea, 6·67 M; phosphate, 0·075 M; pH 6·5, $I = 0·1$. P in mm. Hg, C_v in g. protein/100 ml. solvent. $P/C_v = 2·42C_v + 3·21$.

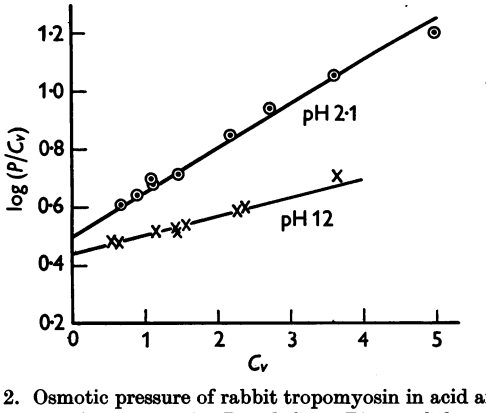

Fig. 2. Osmotic pressure of rabbit tropomyosin in acid and alkaline solutions at 0°. P and C_v as Fig. 1. Solvents: (acid) HCl, 0·01 N; KCl, 0·09 M; $I = 0·1$, pH 2·1; (alkali) NaOH, 0·01 N; KCl, 0·2 M; $I = 2·1$, pH 12. Acid: $\log(P/C_v) = 0·1533C_v + 0·5097$. Alkali: $\log(P/C_v) = 0·06457C_v + 0·4434$.

Acid and alkali. The equilibrating fluids used here were as follows: acid medium, 0·01 N-HCl, 0·09 M-KCl, pH 2·1, $I = 0·1$; alkaline medium, 0·01 N-NaOH, 0·2 M-KCl, pH 12, $I = 0·21$. The curves for both media are given in Fig. 2, in which log P/C_v is plotted against C_v. The extrapolated values of

P/C_v, 3·23 at pH 2·1 and 2·78 at pH 12, correspond to particle weights of 52,700 and 61,400 respectively.

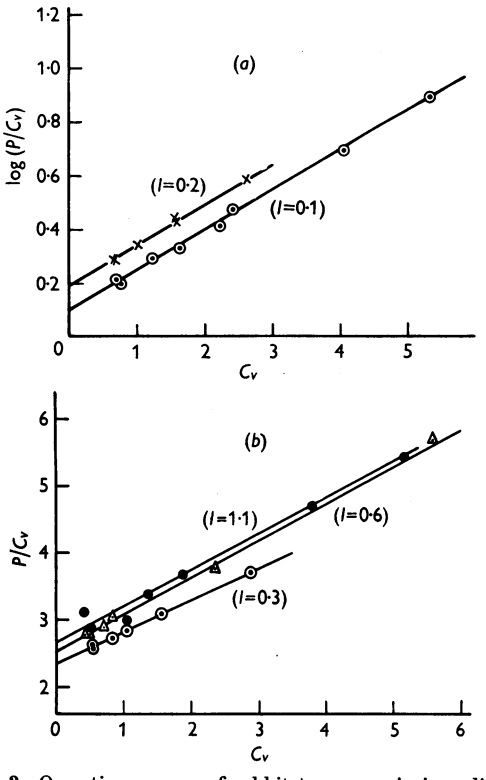

Fig. 3. Osmotic pressure of rabbit tropomyosin in sodium chloride solutions. P and C_v as fig. 1. (*a*) pH 6·5, $I=0·1$ and 0·2. $I=0·1$: log $(P/C_v)=0·150C_v+0·100$. $I=0·2$: log $(P/C_v)=0·153C_v+0·188$. (*b*) pH 6·5, $I=0·3$, 0·6 and 1·1 respectively. $I=0·3$: $P/C_v=0·467C_v+2·36$. $I=0·6$: $P/C_v=0·560C_v+2·54$. $I=1·1$: $P/C_v=0·544C_v+2·64$. Detailed composition of solvents given in Table 1.

Whilst the particle weights in urea and in acid show excellent agreement, that in alkali matches the limiting value obtained in salt solutions (see next section).

Salt solutions. To determine the effect of ionic strength on the aggregation of tropomyosin, the protein was dissolved in sodium chloride solutions of varying concentration, each containing 0·06 M-phosphate buffer, pH 6·5. At a given ionic strength, the protein concentration was varied between about 0·5 and 5·5 g./100 ml. of solvent. The higher concentration represents the limit of solubility of the protein, and below the concentration of 0·5 %, the osmotic pressure is too low to be measured accurately. In solutions where aggregation is most pronounced, the logarithm of P/C_v is plotted against C_v, giving a linear relationship (Fig. 3*a*); in other cases, the conventional plot is adopted (Fig. 3*b*). The extrapolated values of P/C_v, together with the corresponding values of mean particle weight, standard deviation and osmotic coefficient are given in Table 1, which includes the data of Bailey *et al.* (1948).

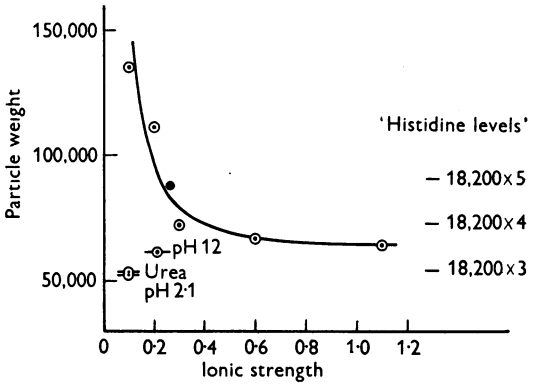

Fig. 4. Particle weight of tropomyosin in salt solutions and other media. 'Histidine levels' represent possible values of molecular weight from the histidine content (Bailey, 1948). ⊙, present work; ●, Bailey *et al.* (1948).

The aggregation of tropomyosin particles as the ionic strength is diminished is best shown graphically (Fig. 4). The most pronounced aggregation occurs in the region $I=0$–$0·3$; in the range $I=0·5$–$1·1$ there is only a slight fall of particle weight, and at these

Table 1. *Particle weight of rabbit tropomyosin by osmotic pressure measurements*

Solvent*	pH	I	$(P/C_v)_{c\to 0}$	Particle weight	S.D.	ϕ† $C_v=1$	ϕ† $C_v=3$
Phosphate (0·06)	6·5	0·10	1·26	135,000	4,500	1·40	2·60
Phosphate-NaCl (0·06, 0·1)	6·5	0·20	1·54	111,000	1,900	1·43	2·88
Phosphate-KCl‡ (0·04, 0·2)	6·5	0·267	1·93	88,000	—	1·14	1·44
Phosphate-NaCl (0·06, 0·2)	6·5	0·30	2·36	72,000	600	1·17	1·51
Phosphate-NaCl (0·06, 0·5)	6·5	0·60	2·54	67,000	1,200	1·22	1·66
Phosphate-NaCl (0·03, 1·05)	6·5	1·10	2·64	64,500	3,400	1·21	1·62
Phosphate-urea (0·075, 6·67)	6·5	0·10	3·21	53,100	1,700	1·73	3·24
KCl-HCl (0·09, 0·01)	2·1	0·10	3·23	52,700	1,800	1·39	2·94
KCl-NaOH (0·2, 0·01)	12	0·21	2·78	61,400	500	1·15	1·62

* Figures in parenthesis, molarity.
† ϕ is a coefficient representing all factors which cause deviation from the simple form of the van't Hoff law.
‡ Data of Bailey *et al.* (1948).

higher salt concentrations the limiting value is virtually the same as that in alkali, but higher by about 10,000 than those in urea and acid. In salt and alkali, therefore, there would appear to be present some particles which are aggregates of those existing in a depolymerizing solvent. The reason for this is not entirely clear. Dr J. Walker has suggested (see Walker, 1949; Neuberger, 1950) that an interaction involving the carboxyl and the amidinium ion might not easily be suppressed by virtue of the formation of a stable ion pair: acids would diminish the interaction by suppressing the ionization of the carboxyl group, reagents such as urea and guanidine by competing for the amidinium ion, and alkali might be effective only at a pH which effectively suppresses the dissociation of the latter group. Such a pH would be higher than that employed here.

Viscosity

The viscous anomaly at low shear rates. The relative viscosity of tropomyosin was determined in the same solutions as those used in osmotic pressure measurements. In the case of solutions in urea, acid

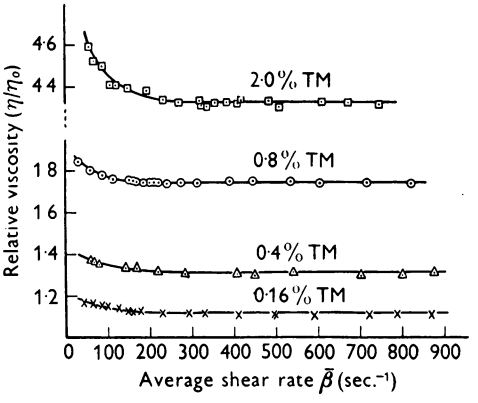

Fig. 5. Relative viscosity of rabbit-tropomyosin solutions (24·4°) at different shear rates. Solvent: KCl, 0·29 M; HCl, 0·01 N; pH 2·1. TM = tropomyosin. % signifies g. TM/100 ml. solution.

and alkali, neutral salts were added to minimize electroviscous effects. At very low rates of shear (about 300 sec.$^{-1}$) the viscosity was found to be anomalous. Typical flow behaviour is shown in Fig. 5 where relative viscosity is plotted against the average rate of shear $\bar{\beta}$. The viscosity-shear rate curves bear a superficial resemblance to those of (acto-) myosin, of tobacco mosaic virus and of other macromolecular systems. Unlike actomyosin, however, the anomaly occurs in regions of very low shear rate, and the curves thereafter are completely horizontal. In the case of actomyosin, the relative viscosity tends to fall continuously with increasing velocity gradient, and reaches a limiting value only at extremely high values of $\bar{\beta}$ (Edsall & Mehl, 1940; Portzehl, Schramm & Weber, 1950). To apply the viscosity data, it was necessary first of all to show that the origin of the anomaly was not in orientation or deformation effects, and that the limiting viscosity for the values of $\bar{\beta}$ plotted was representative of particles exhibiting Newtonian flow.

The theoretical basis of viscous anomaly has been studied from various standpoints (Philippoff, 1935, 1936, 1938; Robinson, 1939; Goodeve, 1939), which indicate that the extra disspiation of energy at low shear rates can arise in several ways. For rigid anisometric particles such as tobacco mosaic virus and mercury sulphosalicylic acid, Robinson (1939) has shown that it is due to the progressive orientation of the particles in the direction of flow. Other types of molecules show deformation during flow, e.g. poly*iso*butylene in light petroleum (Tsvetkov & Frisman, 1945) and polystyrene in *cyclo*hexane (Signer & Sadron, 1936). In these latter cases, however, the molecules examined are long, flexible chains which show the effect only at very high velocity gradients (6,000–30,000 sec.$^{-1}$). The crystallinity of tropomyosin in the intramolecular sense does not favour the idea of a flexible molecule, nor is its asymmetry on any consideration as great as in the cases mentioned; moreover, the anomaly, if due to orientation or to flexibility, should appear at high rather than low shear rates. The following experimental considerations suggest that these factors are not involved:

(1) A solution of tropomyosin in salt-free medium shows flow birefringence between crossed polaroids, whereas in all salt-containing solutions examined viscometrically, whether concentrated with respect to protein or not, no effect was observable even when the shear rates were pushed beyond 2000 sec.$^{-1}$.

(2) The magnitude of the flow anomaly decreases slightly with dilution as can be seen from Fig. 5, and for a given concentration of protein, the variation in the magnitude of the anomaly from one series to another is not pronounced. The relative viscosity of the solutions, moreover, has a slightly negative temperature coefficient: a 2% (w/v) solution in solvent of pH 2·1 and $I = 0·3$ has a viscosity of 4·42 at 17·2° and 4·32 at 24·4° relative to the solvent at these temperatures. On the assumption that there is no change in hydration with this small increase in temperature, this last observation is directly opposed to the anomaly caused by orientation, for which a positive temperature coefficient of viscosity exists (Robinson, 1939).

(3) It is of interest to note that whereas the flow of the solvent media is perfectly Newtonian down to the smallest rate of shear investigated (approx. 30 sec.$^{-1}$), concentrated urea and guanidine hydrochloride solutions show anomalous viscosity at low shear rates. In Fig. 6, these flow curves are compared

with those of water and of solutions of tropomyosin, one in urea and one in acid. The values for water and buffer were calculated relative to their respective Newtonian levels, which were taken as unity.

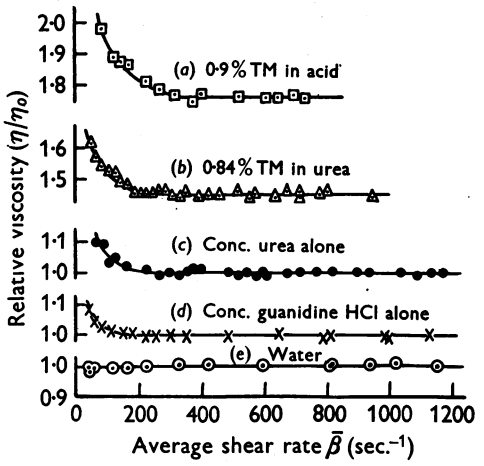

Fig. 6. Relative viscosity of various media (20·0°) at different shear rates. (*a*) KCl, 0·29 M; HCl, 0·01 N; pH 2·1; (*b*) urea, 6·67 M; phosphate, 0·075 M; pH 6·5; (*c*) as for (*b*); (*d*) guanidine HCl, 5·68 M; phosphate, 0·05 M; pH 5·4. TM = tropomyosin.

Anomalous viscosity is usually a property of colloidal solutions and not of crystalloids. (The anomaly found in strong lithium chloride solutions by Scott Blair & Schofield (1931) was later disproved by the same authors (1934).) The effect has been reported—though at high velocity gradients—in pure castor oil (Neale, 1937) and is supposed to be due to molecular association. It is conceivable that urea and guanidine molecules associate to some extent at high solute concentration, presumably by hydrogen bonding, and the anomaly in these systems is of the same magnitude as that of the tropomyosin solutions. This may be relevant with respect to the nature of the anomaly, though at the present stage of theoretical development, it is difficult to pursue the analogy further.

(4) Dr G. Weber has kindly determined the two relaxation times of tropomyosin in phosphate-sodium chloride buffer, pH 6·5 and $I = 0{\cdot}6$ by the method of polarization of fluorescent dyes (Weber, 1950). Assuming an ellipsoid of revolution, these are 8×10^{-8} and $0{\cdot}5 \times 10^{-8}$ sec. respectively, from which the rotational diffusion constants are calculated to be $H_b = H_c = 6{\cdot}3 \times 10^6$ and $H_a = 1{\cdot}9 \times 10^8$ sec.$^{-1}$, where H_b and H_c are rotational constants about the short axes and H_a about the long axis. The ratio $\bar{\beta}/H_b$ at the maximum shear rate of 1250 sec.$^{-1}$ is $\ll 1$, and is small enough to indicate that the rotational Brownian movement of the particles is sufficiently intense to oppose orientation by flow (cf. Boeder, 1932; quoted by Robinson, 1939).

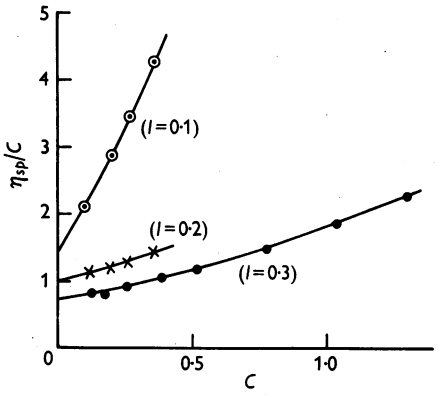

Fig. 7. Viscosity of rabbit tropomyosin in solutions at 20°. *C* in g./100 ml. solution. ⊙—⊙, in phosphate, 0·06 M; $I = 0{\cdot}1$, pH 6·5. ×—×, in phosphate, 0·06 M; NaCl, 0·1 M; $I = 0{\cdot}2$, pH 6·5. ●—●, in phosphate, 0·06 M; NaCl, 0·2 M, $I = 0{\cdot}3$, pH 6·5.

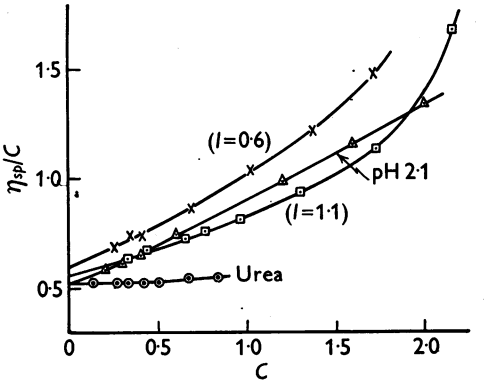

Fig. 8. Viscosity of rabbit tropomyosin in solutions at 20°. *C* in g./100 ml. solution. ×—×, in phosphate, 0·06 M; NaCl, 0·5 M; $I = 0{\cdot}6$, pH 6·5. ⊡—⊡, in phosphate, 0·06 M; NaCl, 1·0 M; $I = 1{\cdot}1$, pH 6·5. △—△, in HCl, 0·01 N; KCl, 0·29 M; $I = 0{\cdot}3$, pH 2·1. ⊙—⊙, in urea 6·67 M; phosphate, 0·075 M; KCl, 0·2 M; $I = 0{\cdot}3$, pH 6·5.

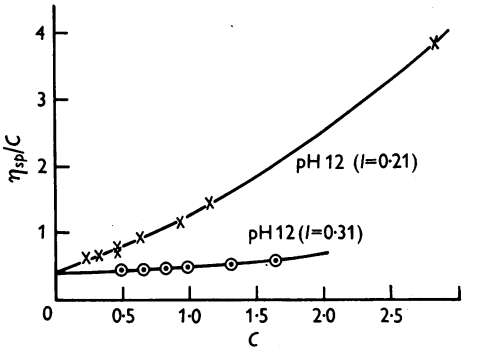

Fig. 9. Viscosity of rabbit tropomyosin in solutions at 20°. *C* in g. protein/100 ml. solution. ×—×, in NaOH, 0·01 N; KCl, 0·2 M; $I = 0{\cdot}21$, pH 12. ⊙—⊙, in NaOH, 0·01 N; KCl, 0·3 M; $I = 0{\cdot}31$, pH 12.

Taken in all, the anomalous viscosity which has been observed in the present study seems due to factors other than orientation or deformation, and the constant relative viscosity for values of $\bar{\beta}$ above 300 sec.$^{-1}$ is taken as a region in which flow behaviour is perfectly Newtonian.

The asymmetry of tropomyosin from intrinsic viscosity. The relative viscosity of tropomyosin, (*a*) in various solvents and (*b*) at varying concentrations of protein, was derived from the horizontal portion of the curves discussed above. The ratio of specific viscosity, η_{sp} (defined in Table 2), to concentration was plotted against C giving rise to sets of curves shown in Figs. 7–9 (C is here defined as g. protein/100 ml. solution). In Figs. 7 and 8 it is seen that the intrinsic viscosity falls as I increases, indicating a corresponding diminution in asymmetry as the particle weight falls. In Figs. 8 and 9 it is shown that the intrinsic viscosity is the same in acid as in urea, the two media in which the particle weight is also the same. However, particle weight and intrinsic viscosity do not follow each other in parallel fashion throughout the series; in alkali, where the former is higher than in urea and acid, the asymmetry is considerably less. Fig. 9 also shows that the dependence of viscosity upon salt concentration is very great, yet the curves extrapolate to the same value at infinite dilution.

It must be admitted that electroviscous effects may come into play in the more dilute salt solutions investigated, and the higher intrinsic viscosity obtained by extrapolation would arise from a combination of this factor and polymerization. The extent of the electroviscous contribution will alter only the absolute magnitude and not the qualitative trend of changes in asymmetry during aggregation, since evidence for polymerization is given both by osmotic pressure measurements and the electron microscope.

The calculated axial ratios derived from the viscosity increment ν by Simha's equation are given in Table 2, together with these values corrected for the estimated hydration of 25%. In the salt series there is a fall of axial ratio with increase of ionic strength, and the limiting value is very close to that in urea and in acid. Although the Simha equation cannot legitimately be employed for those solutions where there is more than one molecular species, nevertheless, the pronounced changes in the apparent axial ratio in the salt series runs parallel with the diminution of the particle weight, and can only be interpreted on the assumption that aggregation is initially an end to end and not a side to side process. Moreover, the identical values of asymmetry and of particle weight in urea and in acid lend great support to the view that in these media the protein exists in its monomeric state.

The calculation of asymmetry from X-ray data. The calculations previously applied to tropomyosin of supposed molecular weight 90,000 (Bailey *et al.* 1948) are now re-examined in the light of the newer data. From the average amino-acid residue weight of 116·4, the number of amino-acids in a molecule of the probable molecular weight 52,900 is 455. Since the intramolecular configuration is of α-type, and assuming three amino-acid residues in the fold, a single polypeptide chain would have a length of $455 \times 5{\cdot}11/3 = 775$ A. Independent chemical evidence (Bailey, 1951) suggests that tropomyosin possesses no terminal amino group, and consists of a cyclic peptide chain. The configurations that can be envisaged for such a structure are either a double polypeptide chain, or such a chain folded further to give effectively some multiple of two chains aligned side by side. A double chain can possess two configurations, one in which the peptide chains are linked through the side chains to give a grid fragment

Table 2. *Shape of rabbit-tropomyosin particles in solution*

Medium				Axial ratio				
				Simha equation		Calc. from particle wt., analysis and X-ray		
					Hydrated 25%		Hydrated 25%	
pH	Solvent and ionic strength	Intrinsic viscosity* ([η])	Viscosity increment* (ν)	Unhydrated		Unhydrated	Ellipsoid	Cylinder
6·5	Salt (0·1)	1·40	197	53	45	67	54	58
6·5	Salt (0·2)	1·00	141	44	37	55	45	47
6·5	Salt (0·3)	0·70	99	35	30	39	32	33
6·5	Salt (0·6)	0·59	83	32	27	33	27	29
6·5	Salt (1·1)	0·57	80	31	26	32	26	28
6·5	Urea (0·3)	0·523	74	30	25	26	22	23
2·1	HCl (0·3)	0·523	74	30	25	26	22	23
12	NaOH (0·2)	0·39	55	25	21	30	25	26
12	NaOH (0·3)	0·39	55	25	21	—	—	—

* $[\eta] = \lim_{c \to 0} \eta_{sp}/C$ and $\nu = \frac{[\eta] \times 100}{\bar{V}}$, where C = g./100 ml. of solution, η_{sp} is $(\eta_r - 1)$, η_r being the relative viscosity η/η_o, and $\bar{V}$ is the partial specific volume.

20 A. in the plane of the side chains and 9·5A. perpendicular to them; or one in which the aggregation is effected through the backbone, giving corresponding dimensions of 10 and 19A. respectively. The overall dimensions of the two models are thus virtually identical. The mean axial ratio would thus be 775/2 (the length) divided by the mean width of 14·5A., giving a value of 26 for the unhydrated molecule.

Although tropomyosin crystals contain as much as 90% of water, the low hydration figure of 25% determined by the method of Adair & Robinson (1931) probably represents the water bound by the molecules in solution, and held on the surface. If this is the case, it can be calculated that the thickness of the hydration layer is 1·7A. if the rod is a prolate ellipsoid, and 1·1–1·2A. if cylindrical. The axial ratio would then be reduced from 26 to 22 and 23 respectively.

Owing to the many assumptions involved, and the fact that viscosity for mixtures measures the weight average and osmotic pressure the number average, it would be unwise to assume that the good agreement between these calculated values of axial ratio (about 23) and that deduced from viscosity (about 25) has no fortuitous element. But it does help to eliminate other configurations. The calculated axial ratio for a single chain would be roughly 78, for a triple chain (which seems unlikely on the chemical evidence) 13, and for a quadruple chain even less. Taken together, the data resolve the structure of tropomyosin as a double chain in the α-configuration, and the one remaining ambiguity is whether the linkage is through the side chains or the backbone.

The case of tropomyosin in alkali is exceptional, for, although the osmotic pressure measurements indicate that the average particle weight is higher than the minimum, the viscosity data suggest that the asymmetry is less than that of the monomer or of its aggregates. The molecules may assume a slightly kinked or looped configuration. Whatever changes do occur are necessarily reversible, since the protein can be recovered in the native form and recrystallized (see next section).

The stability of tropomyosin with respect to pH *and solvents*

The equilibration of tropomyosin solutions in osmotic experiments lasted for periods extending from a few days to a few weeks. Both before and after the experiment, samples were examined by electrophoresis, and in the case of exposure to solutions of high or low pH, a check was made that no peptide bonds had been split (Bailey, 1951). General properties such as solubility and crystallizability were also tested.

Solutions of tropomyosin in salt, alkali and acid. There was no evidence that tropomyosin recovered from any of these solvents differed either in solubility or viscosity from the original specimen. It was surprising to find, moreover, that the amide N, as determined by the method of Bailey (1937), was stable to alkali at pH 12 over a period of 2 weeks at 0°. In all cases, the recovered protein could be crystallized, and no breakdown could be detected by end-group assay. The phenomena of aggregation and disaggregation are thus freely reversible, and do not depend in any way upon secondary chemical changes.

Solutions of tropomyosin in urea. The intrinsic viscosity and particle weight of tropomyosin are similar both in acid and in strong urea, yet in the latter solvent the protein is irreversibly changed and can no longer be crystallized. It is still freely soluble in water, the solutions being more viscous and showing a more pronounced flow birefringence than solutions of the native protein. The increased fibrosity of the aggregates was confirmed by examining the fibrils in the electron microscope, which revealed filaments of enormous length (2–6 μ) and quite regular width. These experiments were done in collaboration with Prof. W. T. Astbury and Dr R. Reed. Prof. Astbury also found that there is no recognizable change in the intramolecular pattern after urea treatment, and if the changes induced are slight, as they appear to be, it is difficult to understand why the ability to crystallize is lost.

DISCUSSION

The present study adds little to our knowledge of the exact role of tropomyosin in muscle; the emphasis is placed upon features of structure and behaviour which seem to have some significance in problems of the biogenesis of protein fibres. It must be admitted, of course, that the aggregation phenomena are largely electrostatic in origin, and thus impermanent, but it is possible that the initial steps in the condensation of protein monomers to give true fibres arises in a somewhat comparable fashion. If the interpretation of the data is correct, there are indications that some part of the interaction persists at salt concentrations high enough to suppress the purely electrostatic attractions, for while the particle weight is at a minimum in urea and in acid, it reaches an asymptotic value some 20% higher in neutral salt solutions of quite high ionic strength. The effect is closely similar to the action of salts on the fragmentation of *Helix* haemocyanin into half molecules, where the extent of depolymerization becomes limiting at a value of 75% as the ionic strength of the medium is increased (Brohult, 1940, 1947).

The disaggregation in salt solutions has a bearing upon the state of tropomyosin in muscle. It will be recalled that the protein is soluble in water after

extraction, but is only given up by the fibres when these are immersed in M-salt solutions. It is probable that the effect of salt is to produce a depolymerization of tropomyosin ordinarily present in an aggregated condition.

The asymmetry of the monomer as deduced from viscosity data is in excellent agreement with the value calculated for a double chain in the α-configuration. As the molecules aggregate to give a variety of polymeric forms, the Simha equation cannot legitimately be applied, but for reasons given earlier (p. 33) it does appear that aggregation is end to end in the initial stages. The former calculations of tropomyosin of particle weight 90,000 (Astbury *et al.* 1948) in solutions of ionic strength 0·27 showed that the calculated dimensions for a double chain agreed only approximately with diffusion data, and even less with viscosity. (The viscosity increments used then were tentative values, lower than those reported here.) Nevertheless, the data sufficed to indicate that only a double chain was feasible.

The findings reported in the accompanying paper (Bailey, 1951) seem to be of importance in interpreting some of the unusual features of the stability of tropomyosin. It is shown there that tropomyosin contains no terminal amino group, and must thus be regarded, in the absence of detectable prosthetic group, as a cyclopeptide. This finding lends great weight, of course, to the conclusions made about the exact configuration of tropomyosin, but it also helps to explain why the ordinary denaturing processes have such little effect. If the molecule is restrained from opening out into a single chain, there is presumably little tendency for acid- and alkali-treated molecules to assume more random configurations, as do the majority of proteins. Even so, it is difficult to understand why some degree of randomness is not introduced into parts of the molecule. This might indeed be found if we had more sensitive means of detection.

The idea that still more asymmetric proteins such as myosin are built from units similar to tropomyosin has already been discussed (Bailey, 1948) and need only be amplified a little. Current work (Tsao, 1950) undoubtedly indicates that the particle which is considered to be the native myosin molecule (see Weber, 1950) can be depolymerized and, once again, no terminal amino-acids can be detected. How far this general plan extended to other fibrous proteins is a matter of very great interest, and it may be significant that the end groups found in wool keratin (Middlebrook, 1949) are of great variety and quantitatively rather meagre, more consistent with the view that they arise from non-keratinous cell inclusions.

SUMMARY

1. Rabbit tropomyosin has been studied by osmotic pressure and viscometric methods under varying conditions.

2. In salt solutions (pH 6·5) the average particle weight falls as the ionic strength of the solute increases, and approaches the value found in a depolymerizing solvent such as 6·7 M-urea. The particle weight in acid (pH 2) is the same as in urea, i.e. 53,000; in alkali (pH 12) it is somewhat higher (61,000).

3. Tropomyosin recovered from salt solutions, acid or alkali behaves in every way like the original material and can be crystallized. This is true even when the protein is stored at pH 2 or 12 for several weeks at 0°. End-group assay (Bailey, 1951) shows that no peptide bonds are split.

4. Tropomyosin recovered from urea no longer crystallizes, and after removal of urea will aggregate into fibrils of indefinite length.

5. At extremely low shear rates, the viscosity of tropomyosin is anomalous. This is due, not to orientation, but to structural factors, which are evident also in strong protein-free solutions of urea and of guanidine hydrochloride. The intrinsic viscosity of tropomyosin has been obtained from values of relative viscosity determined at moderate shear rates under conditions of Newtonian flow. The asymmetry of the particles in various solvents has been calculated from viscosity data by means of the Simha equation.

6. In its monomeric form, tropomyosin has a particle weight of 53,000 and consists of a cyclic peptide chain. The calculated asymmetry for a fully extended loop in the α-configuration agrees with that obtained from viscosity data.

7. The approximate dimensions of the monomer are as follows: length 385 A., mean width 14·5 A., axial ratio 25. Whether the double chain is held together through the side chains or the backbone linkage is unknown.

We should like to thank Prof. W. T. Astbury, F.R.S., for his continued help and criticisms and for the financial aid from his Rockefeller grant to one of us (T.-C. T.) which made the work possible. We also wish to thank Dr G. Weber and Dr P. Johnson for the invaluable discussions we have had with them, and Dr E. C. Bate Smith for the cold-room facilities in the Low Temperature Research Station.

REFERENCES

Adair, G. S. (1925). *Proc. roy. Soc.* A, **108**, 627.
Adair, G. S. (1928). *Proc. roy. Soc.* A, **120**, 573.
Adair, G. S. (1949). In *Haemoglobin*, ed. F. J. W. Roughton and J. C. Kendrew. London: Butterworth's Scientific Publications.
Adair, G. S. & Adair, M. E. (1938). *C.R. lab. Carlsberg*, **22**, 8.
Adair, G. S., Bailey, K. & Tsao, T.-C. (1949). *Biochem. J.* **45**, v.
Adair, G. S. & Robinson, M. E. (1930*a*). *Biochem. J.* **24**, 993.
Adair, G. S. & Robinson, M. E. (1930*b*). *Biochem. J.* **24**, 1864.
Adair, G. S. & Robinson, M. E. (1931). *J. Physiol.* **72**, 2*P*.
Astbury, W. T. (1941). *J. Soc. Chem. Ind., Lond.*, **60**, 491.
Astbury, W. T., Reed, R. & Spark, L. C. (1948). *Biochem. J.* **43**, 282.
Bailey, K. (1937). *Biochem. J.* **31**, 1406.
Bailey, K. (1948). *Biochem. J.* **43**, 271.
Bailey, K. (1951). *Biochem. J.* **49**, 23.
Bailey, K., Gutfreund, H. & Ogston, A. G. (1948). *Biochem. J.* **43**, 279.
Boeder, P. (1932). *Z. Phys.* **75**, 259.
Brohult, S. (1940). *Nova Acta Soc. Sci. upsal.* **12**, no. 4.
Brohult, S. (1947). *J. phys. colloid. Chem.* **51**, 206.
Burk, N. F. & Greenberg, D. M. (1930). *J. biol. Chem.* **87**, 197.
Chibnall, A. C. (1942). *Proc. roy. Soc.* B, **131**, 136.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Edsall, J. T. & Mehl, J. W. (1940). *J. biol. Chem.* **133**, 409.
Goodeve, C. F. (1939). *Trans. Faraday Soc.* **35**, 342.
Kroepelin, H. (1929). *Kolloidzschr.* **47**, 294.
Mehl, J. W., Oncley, J. L. & Simha, R. (1940). *Science*, **92**, 132.
Middlebrook, W. R. (1949). *1st Int. Congr. Biochem. Abstr.*, p. 141.
Neale, S. M. (1937). *Chem. & Industr.*, p. 140.
Neuberger, A. (1950). *J. Sci. Food Agric.* no. **3**, 80.
Oncley, J. L. (1941). *Ann. N.Y. Acad. Sci.* **41**, 121.
Ostwald, Wo. (1933). *Kolloidzschr.* **63**, 61.
Philippoff, W. (1935). *Kolloidzschr.* **71**, 1.
Philippoff, W. (1936). *Kolloidzschr.* **75**, 142.
Philippoff, W. (1938). *Kolloidzschr.* **83**, 163.
Portzehl, H., Schramm, G. & Weber, H. H. (1950). *Z. Naturforsch.* **5***b*, 61.
Robinson, J. R. (1939). *Proc. roy. Soc.* A, **170**, 519.
Roche, J., Roche, A., Adair, G. S. & Adair, M. E. (1932). *Biochem. J.* **26**, 1811.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Scott Blair, G. W. & Schofield, R. K. (1931). *Phil. Mag.* **11**, 890.
Scott Blair, G. W. & Schofield, R. K. (1934). *Phil. Mag.* **17**, 225.
Signer, R. & Sadron, C. (1936). *Helv. chim. Acta*, **19**, 1324.
Simha, R. (1940). *J. phys. Chem.* **44**, 25.
Steinhardt, J. (1938). *J. biol. Chem.* **123**, 543.
Tsao, T.-C. (1950). Unpublished observations.
Tsuda, S. (1928). *Kolloidzsch.* **45**, 325.
Tsvetkov, V. N. & Frisman, E. (1945). *Acta Physicochimica, U.R.S.S.*, **20**, 61.
Walker, J. (1949). *J. chem. Soc.* p. 1996.
Weber, G. (1950). Unpublished observations.
Weber, H. H. (1950). *Proc. roy. Soc.* B, **137**, 50.
Wu, H. (1931). *Chin. J. Physiol.* **5**, 321.
Wu, H. & Yang, E. F. (1932). *Chin. J. Physiol.* **6**, 51.

Studies on the Analysis of Vitamins D

1. THE REACTION OF VITAMINS D AND RELATED SUBSTANCES WITH IODINE TRICHLORIDE

BY J. GREEN
Walton Oaks Experimental Station, Vitamins Ltd., Tadworth, Surrey

(*Received* 18 *September* 1950)

The problem of the chemical analysis of vitamins D in natural oils and synthetic irradiation products of ergosterol and 7-dehydrocholesterol has received considerable attention over the past 15 years. Relatively little success, however, has been achieved in attempts to find a substitute for bioassays for determining vitamin D potencies, although some progress has been made with the analysis of high potency synthetic concentrates. Ewing, Kingsley, Brown & Emmett (1943) have given a very full summary of the literature dealing with the chemical analysis of vitamin D. So far, the most useful reagent has undoubtedly been the improved antimony trichloride reagent of Nield, Russell & Zimmerli (1940). Since the paper by Ewing *et al.* (1943), the main advances have been due to Sobel, Mayer & Kramer (1945) with their discovery of the glycerol dichlorohydrin reaction, and De Witt & Sullivan (1946), who used a chromatographic separation on magnesia and diatomaceous earth and a modified antimony trichloride-acetyl chloride reagent in ethylene dichloride for determining vitamin D in several products. Ewing, Powell, Brown & Emmett (1948) developed two methods for determining vitamin D in irradiated ergosterol products, one by direct estimation with the improved antimony tri-