REVIEW ARTICLE

MOLECULAR SIZE AND SHAPE

A REVIEW OF THE LIGHT-SCATTERING METHOD APPLIED TO SOME IMPORTANT BIOLOGICAL AND OTHER MACROMOLECULES. PART II.

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IT is only within the last generation that it has been possible to study biological and other macromolecules on a new and more fundamental level. This has been achieved by measurements of rates of diffusion, viscosity, sedimentation, and the development of electron microscopy, X-ray diffraction and light-scattering. The study of biological systems in terms of microscopic units has thus now become one of molecular architecture.

In Part I of this review (published in the March issue) the theoretical and practical aspects of the light-scattering method and its application to the study of the physico-chemical characteristics of some important biological compounds were discussed. Other biopolymers and substances having physiological activity and also studies of reaction mechanisms which involve changes in molecular size and shape are reviewed in Part II. Substances of more general chemical interest investigated by the light-scattering method are also mentioned.

BIOPOLYMERS AND SUBSTANCES HAVING PHYSIOLOGICAL ACTIVITY

Muscle Protein

An important group of proteins which have been studied extensively are myosin, actin and actomyosin.

Several investigators have attempted to characterise the myosin molecule but the results so far are inconclusive; one difficulty is probably related to the readiness with which myosin aggregates, particularly in dilute salt solutions. Mommaerts[75] examined a very pure myosin by light-scattering and obtained a molecular weight of 850,000; the length of the molecule, using the rod model, was 1,500 Å. Portzehl[76] reported that the molecular weight by sedimentation-diffusion was 858,000 and by osmotic pressure 840,000; both are in close agreement with light-scattering, but values for the length of the molecule were higher (2,000 to 2,400 Å).

A much lower light-scattering molecular weight of 530,000 was obtained in 1956 by Holtzer and Lowey[77], who also found that the rod-shaped molecule had a length of 1,650 Å (verified by viscosity and sedimentation) which was greater than Mommaerts' value (1,500 Å) by the same method. These workers reported that part of the difficulty of obtaining accurate measurements could be ascribed to spontaneous, temperature-dependent, side-to-side molecular aggregation. Their rabbit muscle protein was extracted, analysed and investigated within 60 hours of the animal's

death; evidence of dimer formation was apparent after keeping for a longer time. Recently, Rupp and Mommaerts[78] have also suggested that myosin solutions have an important ageing effect and temperature dependence. Measurements at a suitable temperature gave a molecular weight of 650,000 and a rod-shape molecular length of 1,600 Å, indicating a diameter of approximately 25 Å and axial ratio of 60. Blum[79] found that the length of the myosin molecule changed with the pH of the solution or on addition of adenosine triphosphate (ATP); the kinetics and mechanisms in muscle action based on Blum's and other light-scattering results have been discussed by Morales and Botts[80].

Preparations of actin appear to be rather ill-defined, making physical characterisation uncertain. Johnson and Landolt[81] studied actin by light-scattering and obtained a molecular weight of 130,000 but suggested that this value could be approximate only, on account of the polydispersity of the preparations. Whilst this value approaches that obtained by Snellman, Erdos and Tenow[82] (150,000) from ultracentrifuge and diffusion measurements, osmotic pressure and fluorescence polarisation studies of Tsao[83] gave a value of 70,000, suggesting that the earlier preparations were in dimeric form.

Johnson and Landolt[64] investigated the transformation of G-actin to F-actin by the addition of salts. On addition of 0·1 M potassium chloride and 0·001 M magnesium chloride a 15-fold increase in scattering intensity occurred and within an hour "activation" to the F-actin appeared complete. The increase in scattering intensity was accompanied by an increase in dissymmetry over the transformation process, indicating a transition from a probable spherical shape to that of a coil having a root mean square distance between its ends of 2,370 Å. In similar studies on the polymerisation of G-actin, Steiner, Laki, and Spicer[85] found from their Zimm plots that the scattering envelope of F-actin best fitted a thin, rigid rod shape; this was confirmed by an intrinsic viscosity of 3·0 in the same medium. They also showed that G-actin was polymerised by decreasing the pH, and the net negative charge could be decreased until maximum polymerisation at the isoelectric point took place. Beyond this point the particles became positively charged and the average extent of polymerisation decreased.

Steiner, Laki and Spicer also found that the size of the complex formed between F-actin and myosin was dependent on the amount of polymerisation of G-actin to F-actin before myosin interaction; Zimm plots from the light-scattering data showed that the F-actomyosin complex decreased in average length and molecular weight with increasing ionic strength or decreasing size of the actin component.

Interest in the nature of the interaction between actomyosin and ATP stemmed from the experiments of Engelhardt, Lyubimova and Meitina[86], who found that ATP caused a strengthening of actomyosin threads produced by injecting a thin stream of the concentrated protein solution into water. Later Szent-Gyorgyi[87] reported that a contraction of threads of actomyosin floating on water took place when ATP was added. It has since been found that, whilst addition of ATP to solutions of actin and

myosin does not cause any observable change in light-scattering intensity, when ATP is added to a solution of the actomyosin complex a noticeable decrease in intensity takes place. The effect of ATP on the viscosity, birefringence and light-scattering intensity of actomyosin solutions has since attracted the attention of many workers whose findings have been interpreted in different ways.

The two-fold importance of the effect has been outlined by Blum and Morales[88]. If the particles of the rabbit muscle protein extracted by the Weber-Edsall method (which Blum and Morales refer to as myosin) undergo deformation, then the theory that muscle action is mediated by a mechanically continuous structure is supported, in which case the elementary action system can be readily studied in solution. If the particles dissociated, however, this theory is weakened and the likelihood of an actin-myosin complex gains ground. The viscosity and birefringence studies of Needham and co-workers[89] indicate that the addition of ATP causes a contraction of the particles whilst the results of ultracentrifugal and viscometric investigations by Mommaerts[90], also Weber[91] and Johnson and Landolt indicate that dissociation takes place.

As previously mentioned, ATP does not cause a noticeable change in light-scattering intensity when ATP is added to solutions of actin and myosin, but the intensity does decrease when added to solutions of the actomyosin complex; the interpretations of this behaviour have likewise been at variance. Johnson and Landolt suggested that the ATP dissociated the actomyosin complex into actin and myosin and this was further

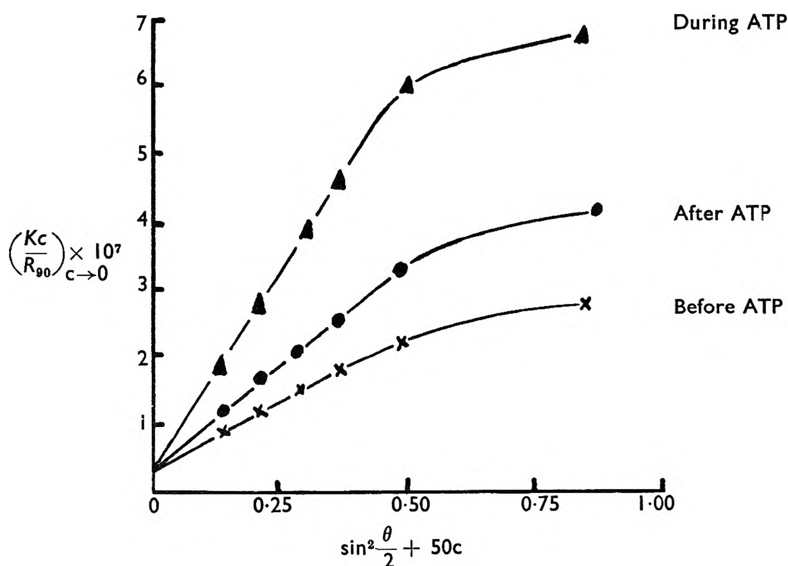

FIG. 7. Limiting curves of the Zimm plots for myosin before, during and after reaction with ATP. The curves show that the addition of ATP causes a change in shape but not in weight of the molecules (after Blum and Morales[88], *Arch. Biochem. Biophys.*, 1953, **43**, 208, with permission.)

substantiated by its effect on similar solutions. However, Jordan and Oster[92] found that the dissymmetry of the scattering envelope increased from 3·4 to 4·7 on addition of ATP and suggested that increased coiling had taken place. A reversible change in shape of the particles was also observed by Blum and Morales[88] (Fig. 7), who suggested that no dissociation into actin and myosin could have taken place. In their extensive study these workers found that the particles of their muscle protein extract were approximately cylindrical and the extension or contraction of cylinders was ATP-concentration dependent.

Tonomura, Wanatabe and co-workers[93,94], investigating the kinetics of the addition of ATP to actomyosin solutions, found that the intensity of scattered light over the whole reaction indicated three phases upon which they have based a mechanism for this reaction.

Arabic Acid

A few light-scattering studies have been made on coiling polyelectrolytes, the most interesting from the pharmaceutical viewpoint being arabic acid. The light-scattering molecular weight for arabic acid was found by Veis and Eggenberger[95] to be one million, whilst Oakley[96] obtained the number average molecular weight of 290,000 by osmotic pressure, both determinations being carried out in 0·02N hydrochloric acid when ionisation of arabic acid was at a minimum. Veis and Eggenberger suggested that the discrepancy in these values could be accounted for in two ways, namely, the solutes were polydisperse and in acid solution hydrolysis probably took place; subsequently Oakley showed that acid hydrolysis did take place to yield a small diffusible component of molecular weight less than 10,000.

Application of the Flory-Fox[97] equation (relating the extension of a coil to its molecular weight and intrinsic viscosity) to the intrinsic viscosity value of 0·329 for the undissociated acid, predicted the shape of the molecule to be a stiff coil of length 548 Å compared with the value of 1,049 Å by dissymmetry of the light-scattering envelope. It was possible that the value of the constant (ϕ) in the Flory-Fox equation was considerably less in Veis and Eggenberger's system where the extension ratio is quite high—allowing for this the two values showed fair agreement. The equivalent radius of the uncharged molecule was calculated to be 555 Å and its effective volume $7 \cdot 2 \times 10^{-16}$ cc./mol. As the concentration of arabic acid was increased Veis and Eggenberger suggested that the randomly-coiled extended molecules would contract, allowing more molecules to fill the empty space; but the closer packing resulted in an increased repulsion between molecules. The contraction process, which would be limited by the bulkiness of the molecule, would lead to an increase in the coil free-energies (gegen-ion effects neglected). The authors considered that the only alternative to allow minimum free energy to be attained would be a change of structure to the rod-shaped form possessing a lower free energy and mutual attraction energy, and also a smaller effective volume. Increasing the concentration beyond available free solvent would bring the molecules within the proximity

of short range electrostatic repulsive and other forces, resulting in the overlapping of the effective volume of one coil by another.

When the arabic acid solution was neutralised and the ionic strength of the solution further increased by the addition of sodium chloride, Veis and Eggenberger found that the reciprocal scattering (Kc/R_{90}) increased with concentration, levelled off, and then increased rapidly to produce a point of inflection. This behaviour, less marked with other polyelectrolytes, was attributed to the highly polar nature of the carbohydrate main chain of arabic acid, its large extension and stiffness, and the presence of bulky polar side chains. The angular dissymmetry of scattered light from arabic acid in the ionised state indicated a considerable extension to 2,400 Å, that is, to approximately twice the extension of the unionised molecule. The variation of the limiting interaction constant (B) with ionic strength showed that the effective volume of the arabic acid molecule also increased as the ionic strength was lowered. There was an unusual variation of the dissymmetry of scattered light with concentration of arabic acid. At different ionic strengths, the curves showed a maximum followed by a minimum (Fig. 8) and were nearly superimposable at lower concentrations, indicating a limiting contraction of the stiff branched molecules.

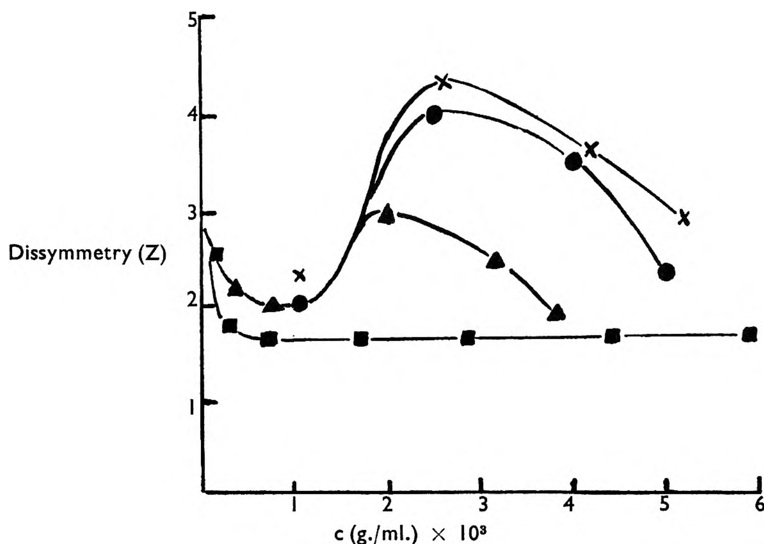

FIG. 8. Dissymmetry of scattered-light for arabic acid in solutions of increasing ionic strength (μ) (after Veis and Eggenberger[95], *J. Amer. chem. Soc.*, 1954, **76**, 1560, with permission.)

× $\mu = 0$. ● $\mu = 3{\cdot}84 \times 10^{-4}$. ▲ $\mu = 2{\cdot}65 \times 10^{-3}$. ■ $\mu = 1{\cdot}99 \times 10^{-2}$

Tobacco Mosaic Virus

The very long rod-like molecules have made tobacco mosaic virus (TMV) an interesting study and workers have gained much information about the behaviour of this virus in aqueous solutions by the light-scattering method.

Particles of TMV, having a maximum dimension at least half the wavelength of the mercury green line (5,460 Å), were found by electron microscopy (Oster[98]) to form rods of uniform thickness of approximately 150 Å and length of 2,700 Å. Older purified suspensions were found to be associated end-to-end in a manner so exact that any repetitive structure was thought to be absent.

Oster, Doty and Zimm[99] measured the dissymmetry of the angular scattering of light by TMV in pure water and buffer solutions; their results are shown in Figure 9. In polar systems the dissymmetry was

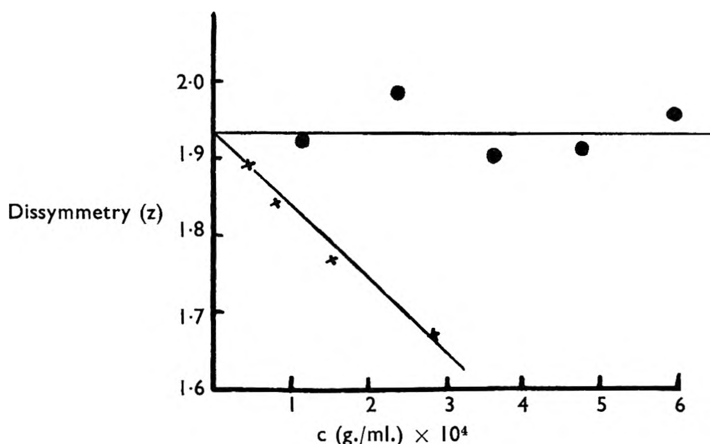

FIG. 9. Dissymmetry of scattered light as a function of concentration of tobacco mosaic virus in water and 0·1M phosphate buffer (after Oster, Doty and Zimm[99], *J. Amer. chem. Soc.*, 1947, **69**, 1193, with permission).

concentration-dependent and any change in dissymmetry was a measure of the particle interaction. In buffer solution (high ionic strength) the dissymmetry: concentration relation was almost constant, indicating a collapse of the electric double layer, resulting in very little interparticle repulsion. In pure water (low ionic strength) an increase in the concentration of the virus brought about a decrease in the dissymmetry; the intermolecular electrostatic repulsion gave rise to a large effective diameter of the particles, producing less randomness and a consequent lowering of the scattering in the forward direction. Extrapolation of the results of Oster and others gave a dissymmetry corresponding to a rod-shaped particle of length 2,700 Å in close agreement with results obtained from the electron microscope and intrinsic viscosity (2,600 Å) measurements. The molecular weight of 40 million also agreed with values calculated from the electron microscope and other methods.

The discrepancy between the scattering dissymmetry from the two solutions led Oster to reinvestigate the turbidity of TMV in water and in 0·1M phosphate buffer; his results are shown in Figure 10. The variation of turbidity with concentration was very much greater in water than in phosphate buffer; the slope of the curves clearly showed the effect

of intermolecular electrostatic repulsion. In buffer, where the interaction constant B was $6{\cdot}3 \times 10^{-7}$, the virus particles behaved (thermodynamically) like particles of twice their volume and in water (where $B = 33 \times 10^{-7}$) nearly ten times the theoretical volume ($B = 3{\cdot}4 \times 10^{-7}$). Dissymmetry measurements corresponded to a rod 2,800 Å in length, in close agreement with the earlier results. Depolarisation of the scattered light indicated that the particles were isotropic.

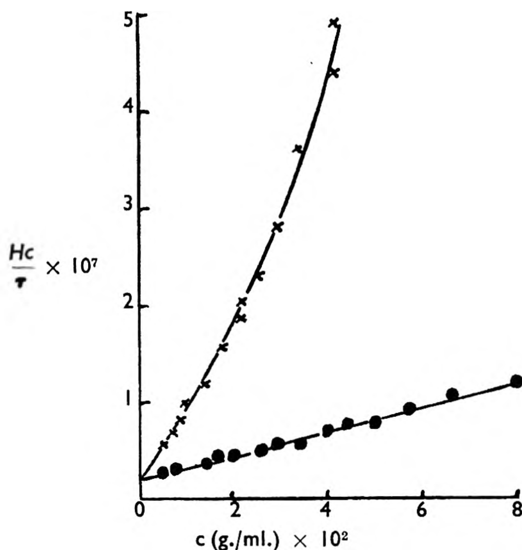

FIG. 10. Reciprocal turbidity: concentration curves for tobacco mosaic virus, showing greater intermolecular repulsion in solutions of smaller ionic strength (after Oster[101], *J. gen. Physiol.*, 1950, **33**, 445, with permission).
× = Water. ● = Buffer.

In 1937 Bawden and Pirie[100] observed that aqueous solutions of the virus, above a concentration of about 2 per cent, separated into two layers on standing for several days. Using crossed polaroid discs they found that the top layer was isotropic and the bottom layer anisotropic (permenently birefringent). Oster[101] used the light-scattering method to study the conditions for two-phase formation with regard to concentration of the virus, concentration of electrolyte and size of particles.

Oster found that in very dilute (0·004 per cent) solutions at the isoelectric point the rod-like molecules aggregated side-to-side as shown by the large dissymmetry ($z = 4{\cdot}8$) of scattered light, breaking down on dilution to a dissymmetry of 2·1. Increasing the concentration to 2·3 per cent the two reversible phases of equal volumes formed on standing for 1 day at 4°. Onsager's[102] analysis predicts that the theoretical minimum concentration for phase separation is $3{\cdot}34/BM$ for the isotropic phase and $4{\cdot}49/BM$ for the anisotropic phase. Substituting his experimental value for B, Oster found that two-phase formation should take place at a minimum concentration of 2·5 per cent, showing close agreement with his experimentally observed value.

The lower, more concentrated phase (by 40 per cent) separated to give a third gel-like phase which showed irridescence when illuminated by white light. Depolarisation of the scattered light indicated that the rod-shaped particles of TMV in the lower, more concentrated phase were not intrinsically anisotropic but that the permanent birefringence was due to orientation of the molecules with their long axes parallel; the low values in turbidity and dissymmetry arose from destructive interference in the fairly ordered system. Electron micrographs showed that the same particle size distribution existed in both phases and also confirmed the length and molecular weight of the particles obtained by light-scattering.

Oster[103] has suggested that since light-scattering of solutions, and therefore light absorptive powers, are sensitive to changes in particle size, shape and interaction, the method could be applied to the determination of the isoelectric point. Adding hydrochloric acid to a solution of TMV, the point of maximum absorption of light was obtained at a pH of 3·9, which agreed well with results obtained from a microelectrophoresis apparatus.

Oster[103,104] has also published light-scattering measurements on solutions of influenza virus and bushy-stunt virus both of which appear to be spherical with molecular weights of 322×10^6 and 9×10^6, respectively. The latter value agreed closely with estimates from ultracentrifugation[105] and diffusion[106].

Turnip-yellow virus was examined by Goring and Johnson[107] in a development study of the light-scattering experimental technique; the particles were found to be spherical with a molecular weight of $5{\cdot}7 \times 10^6$.

Casein

Despite many efforts to obtain the molecular weight of casein the values reported are at such variance that further work on this protein appears to be essential. Early molecular weight determinations were by chemical analysis. The results of Van Slyke and Bosworth[108], based on the supposition that casein molecules contained two atoms each of sulphur and phosphorus, gave a value of 4,444. This, for some obscure reason, was doubled.

Pauli and Matula[109] attempted to estimate the valency of casein by application of the Ostwald dilution rule and gave the valency as 3. The combining weight of casein for base was 1,000, which gave a molecular weight of 3,000.

Yamakami[110] investigated the molecular weight by comparing solutions of equal pressure (Bargers method). For the simplest alkali caseinates which he could prepare he concluded that the mean weight of the ions was approximately 2,000 which, for a caseinate dissociating into only two ions, indicated a molecular weight of about 4,000.

Cohn, Hardy and Prentiss[111] evaluated the molecular weight of casein from its amino acid content and hesitatingly gave a value not less than 96,000, and suggesting that it was most probably twice this value.

Svedberg, Carpenter and Carpenter[112] used ultracentrifugal methods developed in Svedberg's laboratory to study the sedimentation rate and

sedimentation equilibrium of casein prepared by Hammarsten's method. At pH 6·8 (phosphate buffer) they found that casein was a polydisperse mixture of different molecular weights. The fraction soluble in hot 70 per cent ethanol acidified with hydrochloric acid was found to be monodisperse with a molecular weight of 375,000 and a particle size of 83 to 120 Å. The value of 336,000 by osmotic pressure measurements[113] for whole casein in 6·66M urea is regarded by Halwer[114] as one of the most reliable values; urea has a dispersing effect on aggregates which he found were present in solutions of casein and could account for such wide discrepancies in reported values. Burk and Greenberg[113] obtained an even lower value of 24,500 by dissolving the casein in phenol at 42·5°.

In a study of the industrial processing of milk, two Russian workers, D'Yachenko and Vlodavets[115], used the light-scattering method to investigate the molecular weight of casein in solutions of potassium hydroxide (pH 9·0), hydrochloric acid (pH 1·5) and urea (pH 6·2) and obtained values within the limits of 27,000 to 32,000. They found that an increase in temperature in alkaline solution led to aggregation of casein particles which they attributed to traces of calcium, whilst in acid solution dissociation took place. It was suggested that the nature of temperature dependence indicated that the aggregates were linked by hydrogen bonds. In subsequent work[116] they obtained a molecular weight of casein in milk of between 2 and 8 million. This departure from the true molecular weight (approximately 30,000) was again attributed to the presence of calcium ions, which are known to be present in milk in the form of a calcium-caseinate-phosphate complex. Further work on the kinetics of variation of the molecular weight of casein with changes in concentration of calcium ions and hydrogen ions enabled an empirical relation to be calculated. Maximal molecular weights at pCa = 1·5 (negative log calcium ion concentration) and pH approximately 4·6 were found and in more concentrated solutions these maxima corresponded to regions of visible coagulation.

In 1953 Halwer[114] examined α- and β-casein solutions to determine the effect of electrolytes on their size. He considered that this protein was not a "globular" or "native" protein in the sense that the terms are applied to unaltered ovalbumin, serum albumin and others but that the caseins resembled them in the denatured state.

Casein is close to its isoelectric point in pure water and is then unstable and almost insoluble; complexities in measurements then arise as a result of mutual repulsions of the unshielded charge centres. Fuoss[117] has discussed these complexities in osmotic pressure, light-scattering and viscosity measurements of synthetic polyelectrolytes. Halwer suggested that the light-scattering results obtained for α-casein at pH 7 in the absence of added electrolyte were erratic (molecular weights ranged from 25,000 to 65,000) partly because the extent of dilution to which extrapolation for a molecular weight could be accurately made was uncertain and in any case was very high. Doty and Steiner[118] found that the light-scattering results of serum albumin at pH 8 were likewise not reproducible. In the presence of an electrolyte the casein is charged and its solubility

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is increased, a state exhibited by most proteins and which often determines the conditions whereby their physical properties can be investigated. The scattering of the α- and β-caseins increased with increasing concentrations of electrolytes whereas native ovalbumin, serum albumin and lactoglobulin showed independence to electrolyte concentration. The sensitivity of the caseins was akin to that of denatured ovalbumin. The extent of aggregation of the caseins was found to be greater for higher concentrations of the proteins. The two forms of casein differ in their amino acid composition[119] and other properties as shown by the time required for each material to reach a constant light-scattering intensity; on addition of potassium chloride the β-casein showed a much slower reaction. Halwer further showed that aggregation of the α-casein was reversible and that of the β-casein irreversible.

Fibrinogen

Most of the physical techniques have been applied to this important constituent of plasma (0·2 per cent w/v in blood representing 4 per cent of plasma protein content), and the most recent values for the physicochemical constants of human and bovine fibrinogen have been tabulated by Scheraga and Laskowski[120]. From their data it is apparent that the physical characteristics of human and bovine fibrinogen are indistinguishable. Discrepancies between older and more recent values are probably attributable to the states of purity. Scheraga and Laskowski suggest that at least 95 per cent clottability is necessary as a criterion of purity; such preparations are reported to be relatively homogeneous.

Earlier values for the molecular weight of bovine fibrinogen by the light-scattering method were in the region of 400,000 to 500,000, but the more recent value of 340,000, together with information from the angular scattering envelope, obtained by Katz[121,122] and others, suggests that the higher values may have been caused by some polydispersity. This value is in agreement with sedimentation-diffusion[123,124] results (330,000 to 340,000) whilst the earlier values agreed closely with those from sedimentation-viscosity[125] (440,000) and osmotic pressure[125,126] (441,000 to 580,000).

Scheraga and Laskowski regard a molecular dimension of 500 to 600 Å as the best value obtained from their collected data. By comparison, the light-scattering results of Katz and others gave a molecular dimension of 520 Å for a rod-shaped molecule, but assuming an ellipsoid the molecular dimension was 650 Å, which is high by the estimate of Scheraga and Laskowski. An axial ratio of 5 for a prolate equivalent hydrodynamic ellipsoid is reported by Scheraga and Laskoski, calculated without arbitrary assumptions about hydration or volume. In the calculations of Katz where a circular cylinder model was assumed, an axial ratio of 17 was in fair agreement with values obtained by other methods requiring the assumptions mentioned above. All these values were obtained on fibrinogen in dilute solutions. From the results of a dry sample in the electron microscope, Hall[127] calculated a rod-shaped length of 600 Å and diameter 30 to 40 Å; the length and axial ratio were in fair agreement

with the light-scattering results of Katz although there appeared to be greater polydispersity of the dry sample. Later work with the electron microscope by Hall[128] and other investigators has led to evidence of particles containing 3 or 4 small globules "strung together like beads" and having a total length approaching the dimension of the particles in solution.

In a recent investigation, Casassa[129] found that the light-scattering molecular weight of bovine fibrinogen increased by 7 per cent and the molecular length by 12 per cent in hexamethylene glycol at pH 6·2 as solvent. One explanation of the molecular weight increase was attributed to binding with glycol (in the thermodynamic sense), but the 12 per cent increase in molecular length made Casassa incline to the view that swelling of the fibrinogen molecule had taken place. The results showed, however, that hexamethylene glycol produced steric effects by reason of swelling or binding, which were likely to interfere with the clotting process. At pH 9 in salt-glycine buffer Casassa observed that aggregates of fibrinogen were formed in the presence of hexamethylene glycol or upon dilution.

Fibrinogen-Fibrin Reaction

Addition of small amounts of the enzyme thrombin to an appropriately buffered solution of fibrinogen catalyses the solution to produce a fibrin monomer. It is probable that thrombin is a proteolytic enzyme catalysing the hydrolysis of peptide bonds (see Scheraga and Laskowski[120]) The liberation of peptides probably unmasks functional groups (donors or acceptors in the hydrogen bonding process) capable of reacting to form intermediate rod-shaped polymers which undergo further lateral association and cross-linking to form a clot. The properties of the fibrin clot appear to depend on the conditions in which the clot is formed. In general, the reaction proceeds at high pH and ionic strength to form a low turbidity, relatively rigid "fine clot"; lowering the pH or ionic strength increases the turbidity and viscosity and a coarse clot is formed. Some substances are known to inhibit the clotting reaction without denaturing the two reactants.

Using hexamethylene glycol as inhibitor the flow birefringence measurements of Ehrlich, Shulman and Ferry[130] indicated that polymerisation of fibrinogen extends approximately from six to ten times the length of the fibrinogen monomer while the sedimentation constant[130] (combined with the birefringence data) corresponded to a cross-sectional area double and length ten times that of fibrinogen. Viscosity measurements were complicated by this component which had non-Newtonian flow. An independent light-scattering investigation of the intermediate polymer by Ferry, Shulman, Gutfreund and Katz[122] showed that in 0·5M hexamethylene glycol, at pH 6·2 and ionic strength 0·45, about half the protein is converted to a polymer with a weight average of fifteen times the molecular weight of the monomer, a length of 3,500 Å and width double that of fibrinogen. The polymer was found to dissociate sharply on dilution and to have stabilising forces similar to those in micelle formation.

Using urea and guanidine as solvents, Steiner and Laki[131] obtained

a light-scattering molecular weight of 540,000; the length of the extended rod-like model was 840 Å. They reported that, under conditions of high pH and ionic strength, end-to-end association predominated over side-to-side in the early stages. At pH values below 8 lateral association predominated, double and triple parallel fibrils being built up in the clotting process through association of dimers and trimers. The polymerisation process could be stopped completely by oxidation with potassium permanganate, as shown by the lack of further change in the molecular weight. Higher concentrations of potassium permanganate disrupted the aggregates. Evidence from the oxidative reaction led Steiner and Laki to conclude that end-to-end linkages were broken first, indicating a possible difference in bond type between the end-to-end and lateral associations. Light-scattering molecular weight determinations also showed that papain induced a similar clotting of fibrinogen to that shown by thrombin. Casassa[132] came to similar conclusions on the lateral association (dimerisation) and longitudinal polymerisation.

Casassa and Billick[133] studied the clotting of fibrinogen under more realistic conditions by using high concentrations of material. An estimate by ultracentrifugation of the extent of clotting showed that an 80 per cent conversion (in all probability reversible) of 0·4 per cent w/v fibrinogen solution under the given conditions took place for a concentration of 1 unit/ml. of thrombin. The angular distribution of the light-scattering intensity showed polymerisation to an intermediate polymer having a cylindrical rod-shape at least 5,000 Å in length and a mass to length ratio of 2·3 times the monomer units. The effect of variation in composition of the solute components on thermodynamic interactions was also studied. Investigators have contributed greatly to the elucidation of this reaction in recent years but the kinetics of the process is still only partially solved.

Nucleic Acids

One of the main problems in the study of the size and shape of nucleic acids has been the isolation of samples having a native structure. Degradation by depolymerisation takes place easily in acid or alkaline conditions, producing a polydisperse residue of decreased asymmetry. The extent of denaturation and consequent change in physical characteristics of such highly polymeric biological substances depends largely on the method of preparation.

Nucleic acid containing the deoxyribose sugar component (DNA) has been most extensively examined. X-ray diffraction experiments of Franklin and Gosling[134] have shown that it can exist in three main forms which are reversible, the changes in structure depending on humidity. At 75 per cent relative humidity the A form is a stable crystalline helical configuration. At higher humidities the B form is paracrystalline, showing a lower degree of order of the helixes. Each helix of this form is surrounded by a sheath of water molecules which leaves each unit free to adopt a configuration having minimum free energy, independent of its neighbouring units. At low humidities the structure is one of disorder.

Shooter and Butler[135] obtained a sedimentation coefficient by following the path of DNA in the ultracentrifuge using ultra-violet absorption but they reported that, at low concentrations, the sedimentation rate was so dependent on concentration that extrapolation to infinite dilution was not possible. Viscosity measurements and flow birefringence are dynamic methods and distortion of the very large molecules makes calculations based on models difficult. The results of Goldstein and Reichmann[136] on four samples of DNA studied by flow birefringence and light-scattering agreed for lower molecular weight samples (2 to 4 $\times$ 10^6) but higher molecular weight samples showed discrepancies in dimensions.

Very little work has been reported on diffusion studies at low concentrations, but calculations based on diffusion coefficients obtained by James[137] for calf thymus and Goodgall[138] and others for the transforming principle of *Haemophilus influenzae* gave molecular weights of 5 $\times$ 10^6 and 15 $\times$ 10^6, respectively (using a sedimentation coefficient of 20 $\times$ 10^{-13}). Fluke, Drew and Pollard[139] obtained a molecular weight of 6 $\times$ 10^6 for the DNA of pneumococcus transforming principle by electron and deuteron bombardment. Results from preparations from other sources agree more closely to the higher value by Goodgall.

The most recent estimates for the molecular weight of DNA have been by light-scattering. The molecular weight range for most specimens has been narrowed down to 5 to 8 $\times$ 10^6 (Doty[140] reported in 1955 that about 50 preparations of DNA had been examined in his laboratory). Brown, M'Ewen and Pratt[141] examined different specimens of DNA within a molecular weight range of 2·3 to 14·4 $\times$ 10^6 (see also Sadron[142]) They found that the molecular weight increased markedly with the guanine content, their highest values obtained after ageing of the DNA in the solvent for 3 to 8 days before final centrifugation. They explained the significance of the high molecular weight associated with guanine on the basis of the DNA molecule proposed by Watson and Crick[160], where, in the twin helical hydrogen-bonded structure, the 2-amino group of guanine appears to be available for binding two chains or for the binding of different parts of the same chain. Their values of parameters for calf thymus DNA are in excellent agreement with those of Reichmann, Rice, Thomas and Doty[143].

Calculations from the angular distribution of scattering intensity have been variously interpreted for DNA. Different authors have suggested that the particles are flexible and rigid rods[144], stiff coils[145,147] and branched coils[148], statistical coils[145,147] and intermediate shapes. The most recent investigations indicate that the molecules are flexible, probably closest to a coil (greatly extended double helix), their flexibility and size depending on the nature of the aqueous solvent. They are, however, known to be extremely large for their weight, that is, they are highly extended, with characteristics influencing the likelihood of a highly polydisperse system.

When the radius of gyration was plotted against molecular weight for different preparations of DNA[141,142] it was found that a uniform relation existed between the size and shape of the molecules (see Shooter[149]); the higher molecular weight preparations of Brown and

others[141] showed more compactness for their size, indicating a more highly coiled unit. Pouvet, Hermans and Vendrely[150] suggested that salt and alcohol play a predominant role in determining size and shape. This was evidenced by a 66 per cent decrease in the diameter of DNA in methanol to that in water[151]. From an analysis of work up to 1955 Doty concluded that the radius of gyration of the extended chain model was about 2,200 Å and the diameter of the volume of a DNA molecule estimated at 5,500 Å.

Reichmann, Rice, Thomas and Doty[143] examined DNA from calf thymus (molecular weight 6×10^6) and obtained a molecular shape resembling a stiff coil with root mean square end-to-end length of 5,000 Å and a contour length of 20,000 Å. These figures were corroborated by viscosity and sedimentation results. The form of angular scattering envelope depends mainly on the shape of the molecule but also to some extent on the molecular weight distribution of the sample. The observed envelope for DNA corresponded closely to a coil model. However, the envelope was also within the limits that could be reproduced by a polydisperse system alone. Assuming a very narrow molecular weight distribution, the envelope could correspond to a highly swollen symmetrical particle with a centre of high density, or to an ellipsoid or partially extended chain. Alternatively, if the envelope corresponds to a wide molecular weight distribution of randomly coiled chains, it gives the unusually high values of $M_z/M_w = 2$ and $M_w/M_n = 6$, from which it was calculated that about half of the randomly coiled chains were too stiff to be Gaussian in character. For more exact relations on shape it is therefore essential that the molecular weight distribution is known in some detail. Peterlin[152] has put forward a theory for scattering from non-Gaussian random coils where M_w/M_n is not greater than 2. This visualises a narrower range of polydispersity than existed in the experiments of Reichman, Rice, Thomas and Doty.

Recently Doty[153] has reported molecular weights of about 8×10^6 and a chain configuration described by a persistence length of 500 Å for DNA from thymus and salmon sperm. He has probably thus isolated samples of the DNA having a sufficiently narrow molecular weight distribution to apply Peterlin's theory relating the shape of the molecule to the stiffness of the chain by the persistence length parameter.

Using the light-scattering, electron microscope, and viscosity methods, Rowan, Eden and Kahler[145] found that the molecular configuration of sodium deoxyribonucleate was intermediate between a rod and a coil (molecular weight $4{\cdot}5 \times 10^6$), the molecules undergoing a marked change in size under the influence of ions. As the ionic strength of the solution increased from 0 to 0·02 the long thread-like molecules contracted from 6,800 to 4,500 Å. A similar decrease in size of this substance induced by dilute acid was found by Reichmann, Bunce and Doty[154]. From these and similar observations on later work Rowan[155] suggests that the contraction takes place when the molecule is surrounded by electrical charges of neighbouring ions. Relating his observations to the theory of Hermans and Overbeek[156] he obtained good agreement supporting the concept that

the DNA molecule is flexible enough to contract when surrounded by charged ions. Rowan[157] also suggests that similar contractions of DNA molecules *in vivo* may explain how they are able to be incorporated into small biological substances and impart to the complex changes in size and shape that chromosomes undergo during mitosis.

Alexander and Stacey[158] examined DNA from herring sperm heads under different conditions of pH and electrolyte. In 0·1M sodium chloride at pH 6·6, DNA had a molecular weight of 6×10^6 comparable with the results obtained by Reichmann and others[143] and a root mean square radius of gyration of 1,980 Å. Lowering the pH to 2·8 decreased the size of the coil with no change in molecular weight but on neutralisation the coil recovered to the original radius of gyration. Thomas and Doty[159] similarly found that the light-scattering characteristics of DNA in neutral 0·02M sodium chloride remained unchanged for two weeks, but at pH 2·6 a gradual degradation took place. Below pH 2·6 Alexander and Stacey found that an increase in molecular weight took place, indicating aggregation, but on neutralisation the original molecular weight was regained although the radius of gyration was not restored. Below pH 2·6 and in the absence of salt the molecular weight decreased to approximately half the original value, suggesting that the molecular weight of 6×10^6 represents a dimer. To investigate this possibility further, DNA was dissolved in 4M urea where a lowering of the molecular weight to $2{\cdot}7 \times 10^6$ took place (approximately half the previous value) accompanied by a slight increase (20 per cent) in the radius of gyration (2,100 Å) of the coil. Alexander and Stacey suggested that the conditions of experimentation indicated that native nucleic acid of molecular weight 6×10^6 was a hydrogen-bonded dimer consistent with the assumption that the urea split the hydrogen bonds of the twin-stranded spiral model of Watson and Crick[160].

Further parallel work by Doty and Rice[161] on a sample of DNA from thymus glands showed a contradiction in behaviour; the DNA was not split by urea. On heating samples of herring sperm and thymus gland DNA in aqueous solution another difference became apparent. The herring sperm DNA molecular weight of Alexander and Stacey decreased from 6 to $2{\cdot}5 \times 10^6$ whereas the thymus gland DNA molecule of Doty and Rice contracted without change in molecular weight. Alexander and Stacey[162] have since confirmed the work of Doty and Rice.

Geiduschek and Doty[163] studied the interaction between a low molecular weight DNA and bovine serum albumin and found no reaction in phosphate buffer at pH 7·5 and pH 6·5 but at pH 5·1 DNA bound with bovine serum albumin in a 1 : 1 ratio by weight. The complex gave a molecular weight of 3×10^6, indicating that 1 molecule of DNA bound 11 molecules of bovine serum albumin.

Alexander and Stacey[164] irradiated moist herring sperm DNA samples with gamma rays and 1·2 MeV electrons, and examined the irradiated samples by light-scattering. Equal doses of the different rays were found to produce the same amount of degradation; a minimum molecular

weight occurred at a dose of approximately $10^6 r$, indicating that the effect of radiation was one of degradation and aggregation.

Polysaccharides

The large size and asymmetrical shape of polysaccharides and the importance of the complexes which they form with proteins has stimulated interest in light-scattering studies on some of these compounds; glycogens, amyloses, dextrans, and cellulose and its derivatives have been investigated under various conditions.

Glycogens from several animal and other sources were studied by the light-scattering method by Harrap and Manners[165], who found that the values for the molecular weight ranged between 2·8 and 14·8 millions. These showed a marked deviation from values obtained by sedimentation and diffusion which were consistently lower. Small amounts of very high molecular weight material could affect light-scattering results, and for this reason the authors did not correct for dissymmetry.

Foster and others[166,167] were interested in the state of aggregation and degradation (in a sense akin to the denaturation of proteins) of amylose. An aqueous solution of amylose adjusted to pH 4 was found to have a constant turbidity for 30 days. This indicated that the aggregates were very stable and since disaggregation was a precursor to degradation, presumably the amylose remained in its native state. Foster considered that amylose possessed a helical structure stabilised largely by hydrogen-bonding, similar to that suggested for proteins (see Pauling, Corey and Branson[16]), the extent of any degradation being governed by the rate of disruption of the intramolecular hydrogen-bonds of the helical form. In a solution of normal potassium hydroxide, amylose showed a substantial reduction in turbidity with time and in solutions near neutral pH the decrease in turbidity was even more rapid. This suggested that dissociation of the aggregates preceded a transition from the "native" to the degraded amylose.

Fractional samples of native dextran show a wide range of molecular weights, the highly branched samples showing more compactness than the unbranched dextrans of the same molecular weight. Arond and Frank[169] obtained molecular weights of native dextran ranging from 12·6 to 600 millions, the corresponding radii of gyration varying from 570 to 2,930 Å. Other workers[170] found that clarified fractions of enzymatically synthesised dextrans had molecular weights three times as great as the centrifuged material. Price, Bellamy and Lawton[171] showed that the extent to which a sample of crude high molecular weight dextran underwent degradation by high energy cathode rays was reflected in a molecular weight decrease from 650 million to 29,000. Fishmann and Mastrangelo[172] suggest that the erratic results they obtained for measurements on 0·2 per cent solutions of low molecular weight clinical dextran were due to absorption by filters. In collaboration with other workers[173] they used dextran to investigate a method for evaluating the reliability of routine molecular weights determined by light-scattering. The reproducibility of light-scattering results was also investigated by Graham[174]

using a degraded dextran. He found that determinations on the same sample could be expected to fall within ±10 per cent of the mean value 95 per cent of the time. The high degree of branching found in some branched starch polymers was investigated by Stacey, Foster and Erlander[175].

Cellulose, the most abundant of the polysaccharides, is known to have a fibrillar structure of approximately 200 Å in diameter and indefinite length. A few studies are reported for cellulose but most have been detailed investigations on the size, shape, interaction and hydrodynamic properties of the nitrate, acetate, xanthate and ester derivatives. A wide range of samples have been investigated, to which both the rod and random coil shapes have been assigned.

Badger and Blaker[176] investigated samples of cellulose trinitrate having a range of molecular weights from 9,400 to 518,000 and found that the shapes approached the stiff rod model for a degree of polymerisation less than 100 units; more highly polymerised molecules approached the random coil structure with an effective bond length of 50 Å or a ten-fold length of the monomer. This conclusion was supported by results from diffusion experiments, and the 100-unit particle was suggested as a rod-random coil transition region. Solvent interaction of cellulose trinitrate suggested a ribbon-shaped molecule with polar groups along the edges and with non-polar faces. The extent of the heterogeneity of the higher molecular weight fractions was shown by the ratio M_w/M_n in the fractions of cellulose trinitrate investigated by Holtzer, Benoit and Doty[177], who were able to confirm the unusual stiffness of the cellulose trinitrate chain. Their molecular weight results showed an average error for M_w by light-scattering of approximately 10 per cent and for M_n by osmotic pressure of ±5 per cent.

The physical characteristics of potato[178] and corn[179] amylopectin and carrageenin[180] have also been elucidated by light-scattering.

In contrast to the high molecular weights previously discussed, the molecular weight of 338 ± 6 for sucrose was determined by Maron and Lou[181]. The versatility of the light-scattering method for molecular weight determination is clearly shown.

Penicillin

Investigations on the physico-chemical states of penicillin in aqueous systems is controversial. Hauser and colleagues[182,184] reported that, on the basis of surface tension and ultracentrifuge results, both penicillin and streptomycin salts in aqueous solution were present as colloidal micelles. They found that particle sizes varied from 200 to 5,000 Å at concentrations above 20,000 units/ml.

Conductivity and surface tension results of Kumpler and Alpen[185] showed that both potassium and sodium salts of benzylpenicillin are only monodispersed in water.

McBain, Huff and Brady[186] conducted several experiments from which they concluded that benzylpenicillin above 0·25M is a colloidal electrolyte but below this concentration is an ordinary electrolyte.

Using the light-scattering method Hocking[187] found that potassium benzylpenicillin in a concentration range 3 to 30,000 units/ml. showed less scattering intensity than would be expected if large micelles had been present at that concentration as had previously been reported. In the presence of electrolytes which increase micelle formation, the angular scattering exhibited perfect symmetry and Hocking deduced that the maximum dimension of the particles present did not exceed 300 Å. The system appeared to show small scattering, indicating a maximum molecular weight of 3,000. Although benzylpenicillin is surface active the bulk phase showed very little probability of large aggregates even in conditions favouring micelle formation.

Antigen-Antibody Reactions

Despite the large quantity of literature on immunology, studies of the reactions between antigens and antibodies by physico-chemical methods appear to be few. The light-scattering method has been employed to investigate the kinetics of some antigen-antibody reactions, but the mechanisms have proved difficult to follow on account of the high initial rate of reaction and solubility relations.

Singer and Campbell[188] studied the reaction between bovine plasma albumin and its rabbit antibody at neutral pH by ultracentrifugation and obtained strong evidence for bivalency of the antibodies; the antigen was known to be multivalent. Most antigen-antibody reactions occur in the framework of these combining powers according to the hypothesis formulated by Goldberg[189].

The reaction between bovine serum albumin and its purified homologous rat antibody has been investigated by light-scattering by Goldberg and Campbell[190]. The reduced intensity of scattering as a function of time showed that, for equivalent or excess amounts of antibody, complex formation and subsequent precipitation was rapid. Conversely, maintaining antigen in excess, in which the antigen-antibody complex was soluble, the reduced intensity: time relationship was unchanged.

Gitlin and Edelhoch[191] followed the turbidity changes (measured in terms of R_{90}) in the reaction between human serum albumin and its homologous equine antibody and found that equilibrium was reached more rapidly in the region of antigen excess than antibody excess, but the turbidity for a definite antigen-antibody ratio appeared to be the same in the equivalence region from either side of approach to the final state. Large soluble asymmetric complexes were found to exist for all ratios of antigen-antibody. In contrast with the results of Goldberg and Campbell the precipitation point was reached more rapidly when antigen was in excess.

In further work, Gitlin[192] suggested that formation of antibodies was a two-phase process; that its first phase is the formation of long species-specific peptide chains, and that these chains in a second phase are folded to form globular molecules. The nature of determinant groups of antigens, mode of entry into the reticulo-endothelial cells, and liberation of antibody, were also discussed.

The interaction between diphtheria toxin and antitoxin followed by Pope and Healey[193] showed a similar precipitation curve to the human serum albumin : homologous equine antibody system, with little or no precipitation of a complex when either component was markedly in excess. Using a low salt concentration Johnson and Ottewill[194] were able to show that diphtheria toxin-antitoxin combination was rapid, forming a maximum size of particle at the equivalence point. The overall reaction was found to be approximately second order and at a low ionic strength of solution small additions of salt accelerated the reaction rate and promoted larger particle formation. Flocculation occurred when the equivalence zone was reached. The presence of urea diminished the overall effect, and dispersed the toxin-antitoxin aggregates already formed. These workers clearly demonstrated the presence of stable antitoxin-rich aggregates and were able to suggest a mechanism for the Danysz phenomenon in which the resultant toxicity of the mixture is dependent on the method of mixing the toxin and antitoxin (Fig. 11).

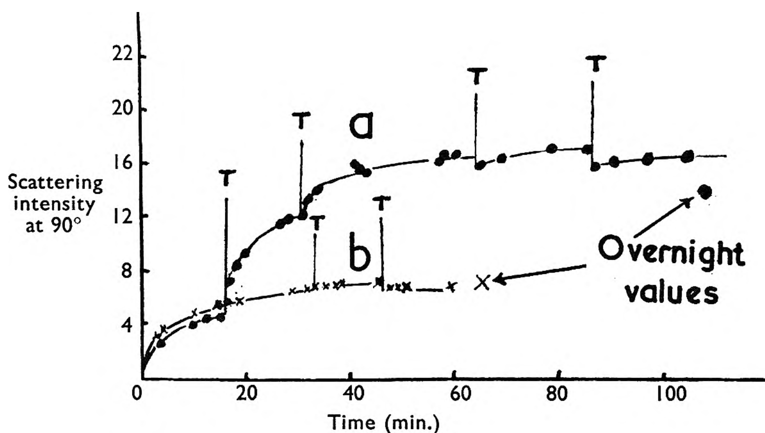

FIG. 11. Scatter from toxin-antitoxin as a function of time. Johnson and Ottewill's light-scattering counterpart of the Danysz phenomenon[194] (*Disc. Farad. Soc.*, 1954, **18**, 327, with permission).

T = Addition of toxin.

Stepwise addition of toxin to antitoxin up to equivalent amounts resulted in large particle size formation (higher values of I_{90} in curve *a*) than a single addition of toxin (curve *b*), indicating solution of large antitoxin-rich aggregates; further stepwise addition of toxin combined only slowly, leaving the solution active in toxin.

A multicomponent system was prepared by Steiner[198] to study the association of human serum albumin and rabbit antibodies. Results from the experimental work were therefore interpreted in terms of the special treatment derived by Stockmayer[195], also Kirkwood and Goldberg[196] and Edsall[197]. Steiner[198] used a lightly iodinated human serum albumin antigen which appeared homogeneous in the ultracentrifuge and had a

light-scattering molecular weight of 79,000. Reciprocal reduced turbidity: concentration curves for the antisera suggested a typical reversibly associating sytems. In general, between pH 6 and 9 the complexes were stable but outside this range a rapid fall in molecular weight, that is to say, rapid dissociation, was apparent. Dissociation was also effected by an increase in ionic strength, addition of urea, or the substitution of sodium iodide for sodium chloride.

While most of the work has been semi-qualitative, the quantitative aspect has also been studied. Goldberg[189] suggested that the reaction sites of an antigen and antibody combined to form a "bond" and on the assumption that this association was a reversible one governed by a single equilibrium constant, Goldberg was able to calculate the distribution of species for a system of uni- and bivalent antibodies and multivalent antigens. Providing the valencies were known, the distribution of the species could be calculated and the average ratios of antibody to antigen, for a critical and maximum extent of reaction between the products, thereby obtained. At these extents Goldberg considered that the aggregates were so large that their ratios approximated to the ratios for precipitation. This theory prompted several investigators to evaluate the equilibrium constant for the reversible reaction and other thermodynamic properties of antigen-antibody associations.

Epstein, Doty and Boyd[199] considered that the multivalency of an antigen, even if this is known, gives rise to diverse reaction products, making the extent of reaction difficult to determine. By replacing the multivalent antigen by a hapten, association with divalent antibody was followed by light-scattering; the molecular weight, equilibrium constant and other thermodynamic properties were subsequently evaluated.

Systems of General Interest

With increasing knowledge of the interpretation of results relating to the behaviour of substances in solution, more complicated systems further removed from ideality are being studied by the light-scattering technique. Some of these have already been reviewed, a few other examples of more general chemical interest will be mentioned.

Emulsions

The scope of investigations on the structure of emulsions by the light-scattering method is limited by particle size and turbidity. Workers in India have concentrated on the property of oil-in-water emulsions to polarise scattered light. Another important contribution has been made by Schulman and others, who used light-scattering to supplement and complete results obtained on emulsion studies by other physical methods.

From the results of X-ray and surface measurements, Schulman and others[200,202] postulated that in oil-water systems the disperse phase was present in spherical oil or water droplets stabilised by an interfacial mixed monolayer of the soap and alcohol molecules used. It was suggested that droplets were arranged in uniform close-packing; the diameters of the droplets and their distances apart were examined by

X-ray analysis. Light-scattering studies subsequently confirmed the model postulated and the dimensions of the particles. Since there is a high degree of orderliness in the liquid lattice of the emulsion, concentration fluctuations would be very small. Consequently, application of the Einstein-Smoluchowski equations would give a low value. Furthermore, the diameters of the particles were known to be too large in the normal conditions to apply the Rayleigh equation, which does not account for interference effects associated with large particles.

Schulman and Friend[202] attempted to achieve a degree of randomness which would give a particle dimension approaching the theoretical value, by increasing the ratio continuous phase : disperse phase of the system. Results calculated from the Rayleigh equation gave diameters of about 100 to 400 Å; these were in good agreement with X-ray and surface chemistry measurements for particles with an apparent molecular weight of under 30 million.

Van der Waarden[203] obtained values for particle diameters by the light-scattering method which he subsequently employed to study the viscosity and electroviscous effect of emulsions. From particle diameter, emulsifier content and viscosity data he calculated an apparent increase in particle diameter during flow which was ascribed to a surface charge originating from the emulsifier. Since the apparent increase in diameter of the particles of oil during flow was peculiarly independent of particle size, van der Waarden suggested that the oil droplets behaved as if they were enveloped by a rigid layer, the charged emulsifier molecules residing in the interfacial layer causing the electroviscous effect. The high electric field at the interface may have influenced adsorption of water molecules, resulting in the apparent increase in radii of the particles.

Sol-Gel Transformation

The process of gelation of agar and gelatin sols has been followed by light-scattering. Katti[204] reported a similarity between agar and gelatin in terms of hydration, increasing scattering and increasing particle size. His results indicated that the particles grew more symmetrical with increasing size up to concentrations of 0·5 per cent but they became more asymmetrical as the concentration was further increased.

An interesting analysis of the gelatin transformation process was reported by Boedtker and Doty[205], who were able to show that, in high salt concentrations at 25° gelatin had a molecular weight of 96,000 ± 3 per cent, the undissociated molecules present having the random coil structure. On dilution of the gel a critical concentration value was evident when the state became one of aggregation rather than gelation. From the values for molecular weight (M) and radius of gyration (ρ) Boedtker and Doty reasoned that, if the ratio $\frac{M}{\rho}$ was constant, aggregation would have been end-to-end whilst a constant ratio $\frac{M}{\rho^3}$ would have indicated spheres of constant density; in fact, the ratio $\frac{M}{\rho^2}$ was nearly constant,

indicating that an intermediate form ("brush heap model") of aggregation resembling cross-linking was most likely. This was supported by nearly constant values for the ratios $\frac{[\eta]}{M^{\frac{1}{2}}}$ and $BM^{\frac{1}{2}}$ and led to the probability that the distribution of mass within the aggregates was Gaussian. Aggregation of gelatin molecules in water at the isoelectric point was complicated by electrostatic attractive forces. In gelatin gels at 18° the intensity of scatter was greater than that of corresponding solutions at 40°, indicating a greater randomness in the gels. Figure 12 shows that the scattering

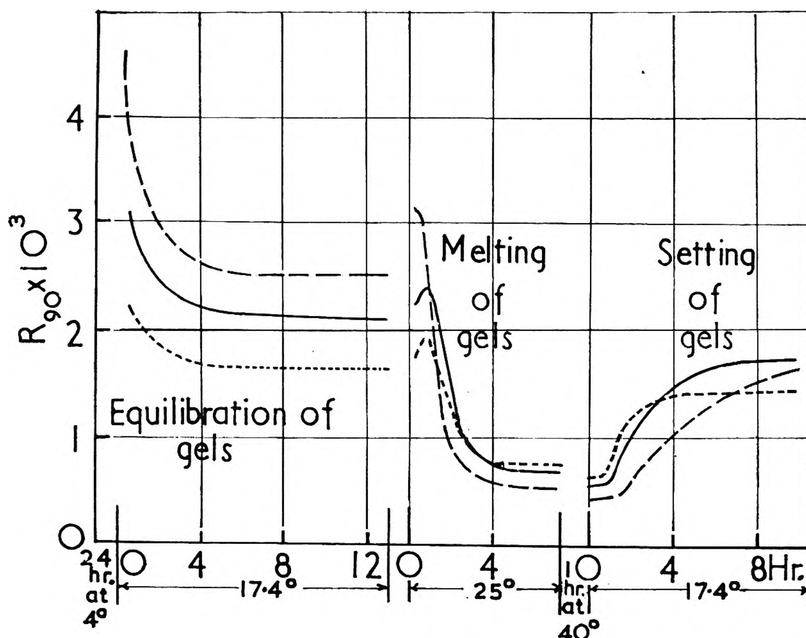

FIG. 12. Variation of scattering intensity with time for gelatin gels in 0·15M sodium chloride at pH 6·5 (after Boedtker and Doty[205], *J. phys. Chem.*, 1954, **58**, 968, with permission).

— — — 0·0090 g./ml.
——— 0·0201 g./ml.
. . . . . 0·0284 g./ml.

of light from the gels decreased with increasing concentration in contrast to the behaviour of the sols. It also shows that gel formation is a slower process than melting, the most concentrated solutions showing the least change in scattering during the transformation. Boedtker and Doty suggest that gelation of gelatin is a process of cross-linking of aggregates which are held together by the formation of crystallites (evidenced by the melting and gelling behaviour and the crystalline structure observed in X-ray diagrams).

Workers in India[206,207] have made an important contribution to the study of sol-gel transformation by investigating hydrophilic (and also a considerable number of hydrophobic) sols by the light-scattering method.

They found that hydrophobic sols showed a general trend; in the sol state, scattering intensities due to particle size and anisotropy were independent of concentration and temperature but transformation to the gel state increased the size of the particles and also their asymmetry, but the anisotropy varied according to the system. Prasad and others[208] have found that shaking during the thixotropic gelation of aluminium molybdate and thorium molybdate did not affect the size and shape of the particles which were isotropic.

Soaps

Light-scattering studies on soaps and detergents have proved valuable in elucidating the structure of micelles, the critical micelle concentration and the effect of different salts on these properties.

Micelles of *n*-hexadecyltrimethylammonium bromide in 0·178M and 0·233M potassium bromide which might perhaps be thought to be spherical were shown by Debye and Anacker[209] to have angular dissymmetry and a rod-like structure. Another investigation by Debye[210] showed which ion of an added electrolyte was the determining factor in its effect on the molecular weight of the micelle of dodecylamine hydrochloride.

Phillips and Mysels[211] found that the effective charge residing on sodium lauryl sulphate molecules in pure water ($M_w = 23{,}000$) and in increasing concentrations of electrolyte ($M_w = 35{,}000$) remained constant at 14 units. The values for the critical micelle concentration were found to agree with conductivity and dye stabilisation methods.

Ludlom[212] was able to show by the light-scattering method that an increase in the hydrophobic portion of a typical detergent (sodium dodecylbenzene sulphonates) increased the size of micelles but lowered the critical micelle concentration.

High Polymers

Some of the more recent advances in the theory and practice of light-scattering have developed mainly through their application to problems in high polymer chemistry.

The volume of researches is extensive and has been of value in solving problems on polymer-solvent interaction. The dependence of the diameter of coiled high polymer molecules on the solvent and temperature has been amply confirmed by light-scattering. The structural changes and degradations arising from chemical reaction, irradiation by electrons and various polymerisation conditions have been reported (see Peterlin[213]).

The weight-average molecular weight obtained by light-scattering is complementary to the number-average molecular weight from osmotic pressure. Stacey[24] has given a good account of the thermodynamics of polymers from light-scattering data and points out that molecular weights from 2,000 to 600 million have been determined by this method. Recently Benoit[214] investigated selected samples of polyoxyethylene glycols within the molecular weight range of 300 to 8000 and obtained good agreement with the values determined by titration of terminal hydroxyl groups. The light-scattering method certainly extends the range of many other methods.

Acknowledgements. The writer would like to thank the authors and publishers who kindly gave permission to reproduce the figures and also Dr. L. Saunders for his interest.

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RESEARCH PAPERS

THE ANTAGONISM OF THE ANTIBACTERIAL ACTION OF MERCURY COMPOUNDS

PART V. QUANTITATIVE ASPECTS OF THE ANTAGONISM OF THE ANTIBACTERIAL ACTION OF MERCURIC CHLORIDE

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Received December 8, 1959

Qualitative experiments previously reported showed dimercaprol to be the most, and thioglycollate the least, efficient antagonist of mercuric chloride. Quantitative experiments reversed this order, but the greater susceptibility of dimercaprol to oxidation is responsible for its apparent lack of efficiency. The recovery of mercuric chloride-treated *E. coli* on media containing an antagonist is affected by the incubation conditions especially when thioglycollate is used as the antagonist. Plots of the percentage of recovered cells against the contact time give different shaped curves for glutathione compared with those for cysteine and thioglycollic acid. Glutathione only inactivates mercuric chloride in the system or adsorbed onto the bacterial cells whereas cysteine and thioglycollic acid, in addition, penetrate the cell and antagonise mercuric chloride within. The incomplete recoveries obtained indicate that the action of mercuric chloride is bactericidal, not bacteriostatic. The rate of kill of *E. coli* by mercuric chloride was logarithmic under the experimental conditions used.

IN the previous work[1] on the antagonism of the antibacterial activity of mercuric chloride by sulphydryl compounds no information was obtained on the number of organisms revived. To obtain such information quantitative work was necessary and experiments involving a counting method appeared to be the most suitable.

The results so far obtained show the action of mercuric chloride on *Escherichia coli* type I to be that of a slow acting bactericide, which is reversible during the initial stages. It was therefore necessary to use methods akin to those for estimating bactericidal activity, which fall into two main groups: (*a*) counting methods, which determine the number of surviving organisms by direct colony counts, turbidimetric or respiratory estimations, and (*b*) end point or extinction methods, which measure the time required for the complete death of an inoculum.

Wills[2] discussed the advantages of extinction methods and regarded them as being more realistic than other methods in assessing the value of disinfectants. As such methods give little or no information on the rate of the killing process a method based upon direct colony counts seemed better. But, the measurement of a percentage mortality yields an estimate of bactericidal efficiency based upon something less than the maximal resistance of the test organism, unlike extinction methods which measure the maximal resistance of the organism to the bactericide.

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Experimental and Results

Dilute suspensions of *E. coli* I were treated with mercuric chloride solution and, after varying contact times, samples of the reaction mixture were dropped on to the surface of overdried plates containing a suitable concentration of antagonist incorporated into the medium. After incubation, the resulting colonies could be counted.

The Effect of Incubation Conditions on the Recovery of E. coli *I Treated with Mercuric Chloride*

It was first necessary to determine whether the recovery of the organisms was affected by either or both of two extraneous factors, (i) the time taken for the drops to be absorbed into the medium and (ii) the length of incubation of the plates; and, if so, to decide upon standard conditions for the later experiments.

By means of a standard dropping needle, 20 ml. of water and 20 ml. of 50 μM mercuric chloride solution were each inoculated with 10 drops of a suspension of *E. coli* I such that the number of viable organisms in the final dilution was about 1–2 $\times$ 10^3 per ml. The reaction mixtures were maintained at 20°. Immediately after inoculation and mixing, a sample of the aqueous dilution was withdrawn and counted by the surface-viable technique, using 10 drops on each of 10 plates. At 5-minute intervals for the first half hour after inoculation and 10-minute intervals for the next half hour, samples were withdrawn from the mercuric chloride solution and dropped on to the surface of peptone agar containing 10 mM thioglycollate.

Of the ten plates for the initial count and those for each of the contact times, half were incubated at 37° immediately and the remainder were allowed to stand at room temperature until drop absorption was complete, and then transferred to the 37° incubator. Incubation was for 7 days, the number of colonies developing being counted each day.

The effect of the incubation conditions upon the recovery of mercuric chloride-treated cells is shown in Figure 1, where the logarithm of the percentage of recovered cells is plotted against the contact time. This shows an approximately linear relationship in each experiment, but the two lines diverge with increasing contact time.

Slightly higher recoveries occurred when the plates were allowed to stand at room temperature until drop absorption was complete than when they were immediately incubated. This effect was more marked with samples after longer contact times, where the number of colonies developing was much lower. Colonies which had developed within 24 hours were larger on the plates incubated immediately than those on the other plates; on further incubation however there was no appreciable difference in colony size.

The effect of prolonged incubation time on the number of colonies developing on thioglycollate agar is shown in Figure 2. This shows that the number of colonies developing increased over the first 72 hours of incubation, remaining approximately constant on further incubation.

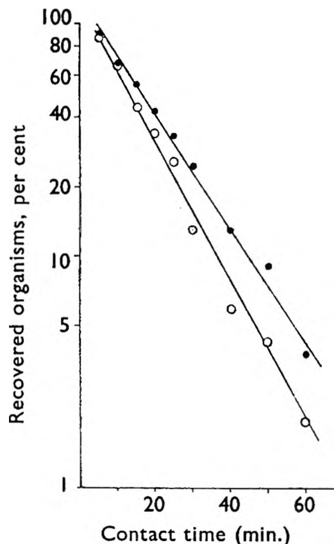

FIG. 1. Effect of incubation conditions on the recovery of mercuric chloride treated *Escherichia coli* developing on thioglycollate agar.

○—○ Plates immediately incubated.
●—● Plates kept at 20° until drops were absorbed.

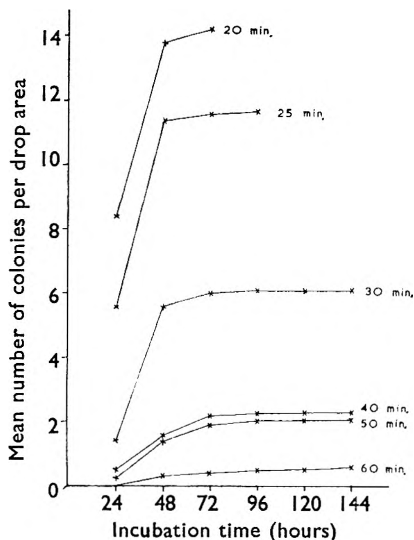

FIG. 2. Effect of prolonged incubation upon mercuric chloride treated *Escherichia coli* growing on thioglycollate agar (6 contact times).

In counting the number of colonies arising in each drop area it was often impossible to repeat the counts after 48 hours. This usually occurred with samples exposed to mercuric chloride for up to 15 minutes, where the number of colonies developing was such as to make growth confluent on prolonged incubation. Repetition of the experiments with a smaller number of organisms showed that the increased count occurring on incubation after 48 hours was negligible with these shorter contact times, and thus any error attached would be small.

With contact times of 20 minutes, or more, the counts obtained after 24 hours incubation bore little resemblance to those obtained after 96, or even 72, hours incubation. In these cases with a smaller number of colonies in the drop areas, the development of one or two additional colonies might make a considerable difference when the results are expressed as percentage recoveries.

The experiments were repeated using peptone agar containing 15 mM cysteine or 10 mM glutathione. Plates incubated immediately at 37° developed fewer colonies, of only slightly if any larger size, than those developing on plates incubated after drop absorption. Neither phenomenon was as marked as with thioglycollate agar.

The counts obtained with cysteine agar were almost complete after 24 hours incubation, with few colonies developing between 24 and 72 hours and none thereafter. The use of glutathione agar resulted in the rapid recovery and growth of the treated organisms, practically no increase in

the colony count occurring after 24 hours incubation. The more rapid growth of mercuric chloride-treated organisms in the presence of glutathione has already been noted[1]. The colonies developed on glutathione agar were much larger than those occurring on cysteine or thioglycollate agar.

Comparison of the Efficiency of the Antagonists in Reviving Mercuric Chloride-treated E. coli *I*

The experiments described above were repeated using peptone agar containing the following concentrations of antagonist, which approximate to the highest concentrations shown[3] to be without adverse effect on the viability of *E. coli* I: cysteine 15 mM, dimercaprol 5 mM, glutathione 10 mM and thioglycollic acid 10 mM. In addition, a second concentration of cysteine (10 mM) was used so that direct comparison of the antagonists at the same concentration could be made. (As the dimercaprol molecule has two sulphydryl groups, a 5 mM solution may be considered as 10 mM with respect to sulphydryl.)

The concentration of mercuric chloride solution used was 50 μM and the inoculum contained approximately 2×10^6 organisms per ml. The reaction temperature was 20°. After sampling and plating, the drops were absorbed at room temperature before transferring the plates to an incubator at 37°. The number of colonies developing on each plate was counted daily and the results were based on the number of colonies present after 4 days incubation.

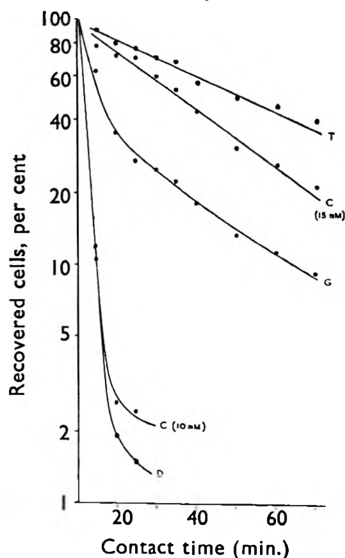

FIG. 3. Recovery of mercuric chloride treated *Escherichia coli* on media containing the antagonists.

T = Thioglycollate.
C = Cysteine.
G = Glutathione.
D = Dimercaprol.

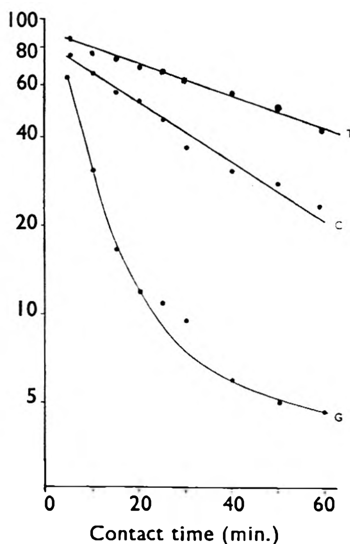

FIG. 4. Recovery of mercuric chloride treated *Escherichia coli* on media containing the antagonists.

T = Thioglycollate.
C = Cysteine.
G = Glutathione.

Typical results are shown in Figure 3, where the logarithm of the percentage of recovered cells is plotted against the contact time.

Media containing the higher concentration of cysteine were found to contain innumerable small particles of cystine, after 24 hours incubation. This tended to make counting difficult, especially with very small colonies; this precipitation did not occur with media containing 10 mM cysteine.

Figure 3 shows that when cysteine (15 mM) or thioglycollate was used as the antagonist, plots of the logarithm of the percentage of recovered cells against the contact time approximated to straight lines, whereas with glutathione as the antagonist an exponential curve was obtained.

Further experiments produced similar results of which Figures 4 and 5 are examples. These are plots of the logarithm of the number of recovered cells against the contact time, or its logarithm respectively. Whilst the plots of Figure 4 closely resemble those of Figure 3, those of Figure 5 are exponential for cysteine and thioglycollate but linear for glutathione.

In experiments of this type where the number of organisms involved is small, the apparent "kill" by the mercuric chloride will be enhanced by the numbers of organisms dying in the aqueous suspension from effects other than those of the antibacterial agent. Control determinations were made in all experiments and these showed that after a period of 1 hour, the number of viable organisms in aqueous suspension varied from 58 to 94 per cent of the original number, with in only one experiment a slight increase (0·5 per cent) in the viable count. The average viability in these controls after 1 hour was about 80 per cent.

Figure 6 shows a typical log survivor : time plot for the aqueous control and a log recovery : time plot for the reaction mixture. In all experiments the slope of the reaction mixture curve was much steeper than that of the aqueous control.

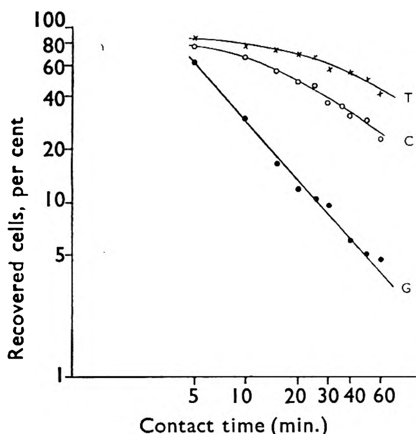

FIG. 5. Recovery of mercuric chloride treated *Escherichia coli* on media containing the antagonists.

T = Thioglycollate.
C = Cysteine.
G = Glutathione.

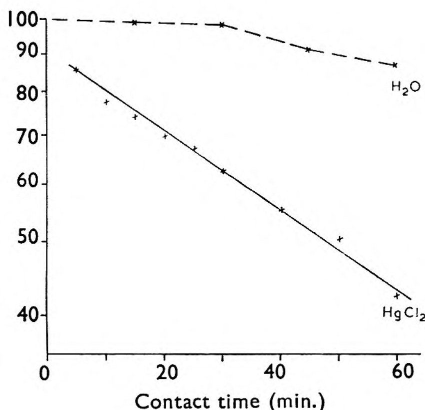

FIG. 6. Effect of mercuric chloride and water on the viability of *Escherichia coli*.

× -- × Aqueous control.
×——× Mercuric chloride reaction mixture.

Discussion

There is little published information on the effects of incubation conditions on the results of a count of organisms surviving the action of an antibacterial substance. Wills[4], investigating the resistance of *E. coli* I to moist heat, found that agar plates kept at room temperature for a time after inoculation before transfer to the incubator gave a lower count than those transferred immediately after inoculation. This effect which was not noted with unheated control suspensions is contrary to our findings. In our experiments, prolonged incubation did not increase the count when there were a large number of survivors, but with smaller numbers the count increased with increase of incubation time. Wilson and others[5] noted a similar effect in plate counts on milk samples.

In attempt to explain the discrepancy in the recoveries obtained by the different incubation conditions, the following points were noted.

If drops of water are placed on the surface of overdried plates containing one of the antagonists, and immediately removed, the liquid gives a positive reaction for sulphydryl. From this it is inferred that some antagonism of mercuric chloride will occur as soon as the treated organisms are placed on the surface of the medium, by diffusion of the antagonist into the drop of reaction mixture.

Drops are absorbed more rapidly at 37° than at 20° and hence the organisms will be in contact with the mercuric chloride for a shorter time at 37° than at 20° before complete absorption ensures intimate mixing with the antagonist.

The antibacterial action of mercuric chloride is more rapid at 37° than at 20°.

Oxidation of sulphydryl compounds occurs more rapidly at 37° than at 20°, and possibly more of the antagonist will be oxidised, rendering it useless as an antagonist, before the treated organisms come into contact with it.

Viable counts of untreated organisms gave identical results whether incubated before or after drop absorption.

From these experiments it is concluded that in work of this nature the absorption of the drops should be allowed to occur at room temperature before the plates are incubated for a period of up to 96 hours, depending upon the particular antagonist used.

From the results shown in Figure 3 it was concluded that neither 5 mM dimercaprol nor 10 mM cysteine in peptone agar were suitable media for the determination of the number of organisms recovering from mercuric chloride treatment. This was believed to be due primarily to oxidation of the sulphydryl compounds rather than to their chemical inefficiency as antagonists. The rapid oxidation of these concentrations of cysteine and dimercaprol when incorporated into solid media has been shown[6], and overdrying of the plates was the main factor in accelerating the oxidation. The oxidation occurring leads to the belief that little if any sulphydryl compound remained to act as an antagonist. In such experiments drops of the reaction mixture on the surface of the medium will still be subjected to the action of the mercuric chloride. Glutathione,

thioglycollate and the higher concentration of cysteine were shown to be less susceptible to oxidation, and this is reflected in their efficiency in allowing the recovery of large numbers of the treated organisms.

From the results obtained it appears that thioglycollate was the most efficient antagonist and reviver of mercuric chloride-treated *E. coli* I. Its main disadvantage is the length of incubation necessary to ensure that the majority of the cells which have been inhibited, are revived and develop.

The revival and growth of mercuric chloride-treated cells caused by glutathione was the most rapid and this might indicate its efficiency as an antagonist. The much smaller number of organisms recovered, however, compared with thioglycollate, point to the fact that antagonism is incomplete.

Cysteine (15 mM) appeared to occupy an intermediate position between the other two antagonists, in both the number of organisms recovered and the time required for their recovery.

The difference in shape of the recovery:time plots (Figs. 4 and 5) for glutathione compared with those for cysteine and thioglycollate may point to some differing mechanism of antagonism. It is known that thioglycollic acid is miscible with both water and many organic solvents whereas glutathione is water-soluble only; cysteine again occupies an intermediate position being soluble in water and some organic solvents.

A possible explanation of the differing antagonistic efficiency of these three compounds may lie in their differing lipid solubility. Glutathione may act by mere chemical combination with mercuric chloride in the reaction mixture and adsorbed on the bacterial surface. Its marked loss of activity after prolonged contact of the organisms with mercuric chloride may be an indication of its inability to antagonise the antibacterial agent which has penetrated the interior of the bacterial cell. Cysteine and thioglycollic acid having some degree of lipid solubility, may be able to enter the cells and antagonise the mercuric chloride which has penetrated. This is contrary to the theory of Hess and Speiser[7], who believe that the action of mercurial compounds is irreversible once they have penetrated the cytoplasmic membrane.

Qualitative experiments[1] showed dimercaprol to be the most efficient reviver of mercuric chloride-treated cells, and thioglycollate the least. These quantitative experiments have reversed the order.

Consideration of the acid strength of sulphydryl groups raises an important question whether the antagonism of the antibacterial action of mercuric chloride proceeds by molecular or ionic reactions. Danielli and Davies[8] stated that in biological systems, the pH is such that the sulphydryl group is practically unionised. They distinguished two types of sulphydryl compounds: nonionogenic, which are without ionising groups other than sulphydryl and ionogenic, which have ionising groups other than sulphydryl. Of the sulphydryl compounds used as antagonists in this investigation, only dimercaprol is nonionogenic; cysteine, glutathione and thioglycollic acid being ionogenic. Benesch and Benesch[9] reported ionisation of the sulphydryl group of the latter three compounds to occur at pH 7·4 to an extent of 6, 1 and 0 per cent, respectively. If

antagonism proceeds through the mercaptide ion, then cysteine would be expected to be the most efficient.

Until the antagonism of the antibacterial action of mercuric chloride has been studied more fully it is felt desirable to refer to organisms recovered from the mercuric chloride reaction mixtures as "recoveries" rather than "survivors."

Figure 6 showed the divergence between the number of organisms which could be recovered from a reaction mixture compared with those recovered from aqueous suspension. If the action of mercuric chloride was that of a bacteriostat, then the number of organisms recoverable should be the number originally present, less the smaller number which had died in the mixture from other causes. As this was not found to be the case, it implies that mercuric chloride acts as a bactericide towards *E. coli* I rather than as a bacteriostat. On the basis of these observations with only one antibacterial agent and one test organism, it is not proposed to cite this in favour of Price's hypothesis[10] that there is no such thing as a state of bacteriostasis. It is reasonable, however, to assume the revival: time curves obtained are analogous to survivor: time curves of disinfection studies.

If it is assumed that, in the case of thioglycollate, revival of the mercuric chloride-treated organisms was complete, then the results obtained give some information on the bactericidal action of mercuric chloride. For example, under the conditions of the experiments, the rate of kill of *E. coli* I appeared to be logarithmic.

It is possible, however, that other sulphydryl-containing compounds could be found which would produce greater recoveries from the reaction mixtures, and so any conclusions drawn as to the bactericidal activity of mercuric chloride must of necessity only be provisional. For this reason, no attempts have been made to discuss the distribution of resistances of the bacteria which could be deduced from plots of the percentage recoveries against the logarithm of the contact times[11].

Most reports in the literature dealing with revival of mercuric chloride-treated organisms refer to Gram-positive organisms and, even so, few quantitative data are given. McCalla and Foltz[12], however, claimed 100 per cent revival of mercuric chloride-treated *E. coli* after an hour's contact by the use of a sulphide as antagonist. Such high recoveries have not been obtained in this investigation.

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THE STABILITY OF OILY CREAM B.P.

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Received December 1, 1959

Oxidation of Wool Alcohols B.P. in stored Oily Cream B.P. is insignificant and does not affect the stability of the cream, but the method of preparation, and the amount of autoxidation in the wool alcohols used in making the cream, do affect stability. Storage of wool alcohols before use, as a mixture with Liquid Paraffin B.P. or Liquid Lanolin "60" (a solvent segregation product of Anydrous Lanolin) greatly reduces autoxidation. Liquid Lanolin "60" also acts as an auxiliary emulsifier and imparts stability. Modifications to the official method of preparation are suggested.

Oily Cream B.P. formerly known as Hydrous Ointment B.P. is known sometimes to separate during storage. Muirhead and others[1] found that the breakdown was caused by oxidation of wool alcohols in the cream. Insolubility of the alcohols in the paraffins also present ensued, and thus the amount of active emulsifying agent was reduced.

Muirhead and others[1] found the oxidation of the wool alcohols to be rapid in an emulsion similar to this cream, as shown by changes in acid value. Since the rate of oxidation far exceeded that encountered by the present authors, it was decided to investigate the subject further.

The present work is concerned with distinguishing between the oxidation of wool alcohols in the cream, and their autoxidation during storage before use. It is also concerned with minimising autoxidation without resorting to antioxidants, and improving the stability of the cream with an auxiliary emulsifying agent and by modifying the official method of preparation.

Since Oily Cream B.P. contains a high proportion of liquid paraffin, the effect on autoxidation of storing wool alcohols, before use, as a mixture with liquid paraffin, has been studied, as softening wool alcohols in this manner avoids brittleness and the formation of small pieces which if they remain in storage may oxidise extensively as a result of their having relatively large surface areas.

Because the emulsifying system of the cream was not considered to be satisfactory, and easily affected by oxidation or unsuitable technique of preparation, the effect of adding an auxiliary emulsifying agent has been examined. A liquid was chosen because it could also replace liquid paraffin for eliminating brittleness during storage. The auxiliary emulsifier was a solvent segregation product* of anhydrous lanolin derived from the same parent substance as wool alcohols, and is completely miscible with it as well as with the other ingredients of the cream. Mixtures of it with wool alcohols yield emulsions of greater stability than those given by either substance alone. Also it is chemically similar to anhydrous lanolin, and is equally acceptable or beneficial dermatologically.

* Liquid Lanolin "60", supplied by Westbrook Lanolin Company.

Experimental and Results (I)

The work was in two stages, the first consisting of 4-month storage tests at room temperature on anhydrous wool alcohols† and blends of this with both liquid paraffin and Liquid Lanolin "60", in good and bad storage conditions. The second stage was storage and stability tests on Oily Cream prepared from each of the 4-month old samples.

The tests were made with the following systems.

(i) Wool alcohols filled whilst molten into a 1-kg. lever-lid tin to leave the minimum air-space. The lid was hermetically sealed with adhesive tape.

(ii) Wool alcohols scraped into thin shavings, spread in a shallow layer on a tray and loosely covered with a sheet of paper.

(iii) A mixture of the wool alcohols with Liquid Paraffin B.P. in the ratio 1:2 by weight, hermetically sealed into a tin as in (i).

(iv) A mixture similar to that of (iii) but as a layer ¾ in. (19 mm.) deep in a 56 lb. (25 kg.) anti-corrosive-coated steel drum, the lid being loosely fitted.

(v) A mixture of the wool alcohols with Liquid Lanolin "60" in the ratio 1:2 by weight, hermetically sealed in a tin as in (i).

(vi) A mixture similar to (v) filled into a drum as in (iv).

The acid values of all six systems were determined at the beginning of the storage period, and at the end were re-determined both on the upper ⅛ in. (3 mm.) of the sample and on the total sample after melting and mixing. (A test on the surface of the flaked wool alcohols was not feasible.) The results are listed in Table I.

TABLE I

Increase in acidity of anhydrous cream bases after 4 months storage

System	Acid value		
		After storage	
	Original	Surface	Mixed
(i) Wool alcohols	1·14	1·56	1·22
(ii) Wool alcohols, flaked	1·14	—	12·20
(iii) Wool alcohols + liquid paraffin	0·38	0·44	0·38
(iv) " " " "	0·38	0·48	0·42
(v) Wool alcohols + Liquid Lanolin "60"	1·42	1·58	1·44
(vi) " " " " "	1·42	6·64	1·94

Discussion (I)

(i) Autoxidation of the surface of the wool alcohols stored under good conditions for 4 months is appreciable, although the final acidity is well within the B.P. limit. The acid value of the mixed sample indicates the main bulk to have been unaffected.

† "Golden Dawn" Wool Alcohols B.P. supplied by Westbrook Lanolin Company.

(ii) The large increase in acidity under these drastic conditions confirms earlier findings[2–4], as does the fall in cholesterol content of the sample from 36 to 16 per cent.

(iii) and (iv) The blending with liquid paraffin had a pronounced inhibiting action on autoxidation of the wool alcohols. Even under the severe conditions prevailing in system (iv) the oxidation was less than that of the pure wool alcohols hermetically sealed in a tin. Under good conditions (system iii), no increase in acid value could be detected in the melted and mixed sample.

(v) The rise in acidity here also is less than that of the pure wool alcohols, notwithstanding that Liquid Lanolin "60" and similar products, like their parent substance anhydrous lanolin, are liable to autoxidation, although to a lesser degree than wool alcohols.

(vi) Under these severe conditions autoxidation of either the Liquid Lanolin "60" or the wool alcohols, or both, is much increased on the surface. The acidity of the mixed sample, however, showed the affected surface to have been less than 2 mm. deep.

Experimental (II)

Storage of Oily Cream Samples

A batch of 400 g. of Oily Cream B.P. 1958 was prepared from each of the samples used in the storage tests described. This formula differs from that used by Muirhead and others, but we preferred to base our work on the official preparation.

Since the method of preparation can affect the stability of the cream, the details are given here.

All components of the oil phase were melted together and adjusted to 50° in the bowl of an electric food mixer fitted with twin beaters. With the mixer operating at full speed the water, at room temperature, was added gradually, mixing continuing for 2 minutes. In addition, the bowl was rotated by hand and the mixing assisted by guiding the emulsion into the beaters with a spatula. The emulsion, cooled to room temperature, was given a final full-speed mix for 2 minutes.

The creams so prepared were packed into glass-stoppered, clear glass jars of 250 g. capacity. One jar of each cream was stored at 38° for 5 months, a second jar of each being kept at room temperature protected from direct sunlight for the same period, after which each cream was examined. A portion of the oil phase from the incubated creams stored at 38° was used for the determination of acid value, using the method of Muirhead and others.

Results (II)

Examination of Creams Stored at Room Temperature

Consistency. This was assessed by "feel", using a spatula. System (ii) produced the softest cream, the other five preparations being similar to each other.

Fineness of emulsion. No significant differences could be seen microscopically. All emulsions were coarser, and the variation in particle size greater, than when first prepared.

Free water. By squeezing a spot of emulsion between two microscope slides, any free water appeared as a film around the edge of the squeezed emulsion. System (ii) showed more free water than any of the others, which all showed similar small quantities.

General stability. All creams were similar in appearance, no appreciable separation of the phases into layers being visible in any.

Examination of the Creams Stored at 38°

The appearance of the emulsion was as follows.

(i) Approximately 20 per cent of the sample was a clear layer, partly water, partly melted fats, which had settled. The supernatant emulsion had yellowed slightly and its surface exhibited a few cracks or lines of separation.

(ii) Again the emulsion was slightly yellowed and "cracked," with phase separation. The clear lower layer formed 5 per cent of the bulk.

(iii) and (iv) Phase separation occurred in each, the lower layers representing 20 to 25 per cent of the bulk. The supernatant emulsions were similar to those of systems (i) and (ii).

(v) and (vi) No visible settlement or separation of the emulsions, even the fine cracks shown in other samples were absent. Yellowing was very slight.

Acid values of the oil phases of creams stored at 38° are given in Table II.

TABLE II

CHANGES IN ACID VALUE OF THE OIL PHASES OF OILY CREAM DURING STORAGE AT 38° FOR 5 MONTHS

System	Acid value of total oil phase		Acid value calculated on wool alcohols content	
	Original	Final	Original	Final
(i) Wool alcohols	0·07	0·08	1·17	1·33
(ii) Wool alcohols, flaked	0·73	0·46	12·17	7·67
(iii) Wool alcohols + liquid paraffin	0·07	0·07	1·17	1·17
(iv) " " " "	0·08	0·12	1·33	1·87
(v) Wool alcohols + Liquid Lanolin "60" ..	0·26	0·41	1·44*	2·28*
(vi) " " " " " ..	0·35	0·46	1·94*	2·56*

* Calculated on content of wool alcohols plus Liquid Lanolin "60".

DISCUSSION (II)

The cream prepared from the highly oxidised flaked wool alcohols showed less separation at 38° than that prepared from relatively unoxidised alcohols, although its stability at room temperature was inferior. This was probably due to the higher viscosity of the oxidised, compared to the unoxidised alcohols (a recognised property of the substance).

The acid value of the oil phase containing the flaked wool alcohols was lower than the initial value. This may be due partly to the fact that a proportion of these highly oxidised alcohols was insoluble in the paraffins (as a result of oxidation) and a part was not incorporated in the cream, but adhered tenaciously to the mixer blades. The insoluble portion had a much higher acid value than the remainder, and the loss in the mixer could account for a lowering of the acid value. Another possible reason is that polymerisation or inter-esterification of the free fatty acids in the oxidised wool alcohols occurred during storage of the cream, and that the rate of this polymerisation was greater than the production of new acids by oxidation. Such polymerisation or inter-esterification is associated with the autoxidation of wool alcohols.

The outstanding aspect of the results is their difference from those of Muirhead and others, in particular the relatively small increases found by us in acidity of the oil phases. These authors also found that the separation of their emulsions occurred in as little as 26 hours. This may be accounted for by the different formula used, which contained as much as 68 per cent of water and only 2 per cent of wool alcohols. This formula might be considered too unstable for reliable comparisons were it not for the effectiveness of the 0·007 per cent of Progallin in preventing breakdown. Also this difference would not account for the rapid rise in acid value that they observed. Unfortunately they gave no details of preparation. Even if abnormal amounts of entrapped air were present, the rates of increase in acidity were far beyond our experience.

This point was investigated further. Four batches of emulsion were prepared to the formula of Muirhead and others. Although they quote a viscosity of 74 Redwood seconds for their white oil, no temperature is specified. The oil we used had a viscosity of 121 seconds at 38°, this being the thinnest oil obtainable. The emulsions were made with our technique from four different lots of wool alcohols, representing three different manufacturers. Antoxidants were incorporated in two further emulsions, and all were stored in half-full cans at 38°. Acid values of the oil phase were determined at the start, and again after 48 hours. The results in Table III are calculated on the wool alcohol content only, the paraffins having an insignificant original acid value.

TABLE III

CHANGES IN ACID VALUE OF OIL PHASE AT 38°

Wood alcohols Reference	Acid value	
	Initial	After 48 hours
A	1·98	2·43
B	1·74	1·84
C	0·27	0·30
D	0·90	0·90
B + 0·007 per cent P.G.*	1·85	1·84
B + 0·05 per cent B.H.A.†	1·90	2·00

* Propyl gallate. † Butylated hydroxyanisole.

The emulsions showed a varied separation, but none separated completely, and the antoxidants did not appear to have a significant effect upon stability.

The increases in acidity were much less than those found by Muirhead and others, even though storage at 38° was over a longer period. The reason for this difference remains obscure, but the wool alcohols they used must have been exceptionally susceptible to oxidation. Also there has been an improvement in the quality of pharmaceutical wool alcohols since their paper appeared.

The inferences that may be drawn from our results are that oxidation of Wool Alcohols B.P. 1958 whilst in Oily Cream B.P. is insignificant and has little if any effect upon stability, but the oxidation which may occur in wool alcohols before being incorporated in the cream can have a deleterious effect and must be avoided. Storage of the alcohols as a mixture with liquid paraffin or with a solvent segregation product of anhydrous lanolin, such as Liquid Lanolin "60", reduces autoxidation. The latter provides the additional advantage of increasing the basic stability of the Oily Cream without altering its consistency.

Technique of Preparation of Oily Cream

The B.P. 1958 specifies no temperatures to be observed when preparing the cream, only that the water should be added to the melted fats with constant stirring, followed by mixing vigorously until a smooth cream is obtained and stirring until cold.

A batch of the cream was so prepared, melting the fats to 80° and adding the water at room temperature, with constant stirring. The emulsion was transferred to an electric mixer and vigorously mixed for 2 minutes, after which it was stirred at intervals by hand until cold.

From similar ingredients the cream was also prepared by our technique, but in addition was passed through a piston-type homogeniser.

The two creams were stored at room temperature for 4 weeks, when the B.P. preparation was found by the microscope slide test to contain appreciably more free water and also to have a distinctly coarser emulsion structure than the cream prepared by our method.

Therefore a more detailed specification of the method of preparation seems necessary, and a modification on the lines we have described would be beneficial to the stability of Oily Cream.

References

1. Muirhead, Oberweger, Seymour and Simmonite, *J. Pharm. Pharmacol.*, 1949, **1**, 762.
2. Gillam, *Austr. Chem. Inst. J. Proc.*, 1948, **14**, 361.
3. Janecke and Senft, *Arch. Pharm., Berl.*, 1957, **290**, 472.
4. Horn, *J. Sci. Food Agric.*, 1958, **9**, 632.

A NOTE ON AUTOXIDATION AND ITS INHIBITION IN WOOL ALCOHOLS B.P.

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Received December 1, 1959

The effects on Wool Alcohols B.P. of gradual autoxidation during storage at room temperature have been compared with the known effects of accelerated oxidation. There is a decrease in emulsification value, a bleaching of the surface, and, contrary to previous reports, a rapid fall in melting point. Autoxidation, even under bad conditions, has been inhibited, apart from a slight fall in melting point, for at least 23 months by the addition of 500 p.p.m. of butylated hydroxyanisole, but certain other antoxidant systems had only a limited effect.

The oxidation of Wool Alcohols B.P. has been studied by a number of workers[1–4]. Muirhead and others[2] investigated the antoxidant properties of α-tocopherol and pyrogallol, whilst Janecke and Senft[3] used butylated hydroxyanisole, α-tocopherol, citraconic acid, β-conidendrol and nordihydroguaiaretic acid.

Accelerated methods of oxidation were used in both of these investigations, ultra-violet irradiation was used by Muirhead and others, while Janecke and Senft used a hot air oven, as did Gillam[1] in studying the oxidation of wool wax alcohols. No previous study of the oxidation and its inhibition of wool alcohols stored for long periods under normal conditions has been reported. This we have done.

Experimental and Results

The storage tests, over almost 2 years, were made in good and bad storage conditions.

Firstly, 100 g. amber glass jars were almost filled with molten Wool Alcohols B.P.* and securely closed with lacquered, tin-plate screw-caps. Secondly, some of the same batch of wool alcohols was scraped into very thin shavings and packed loosely into similar jars, with loose fitting caps.

Wool alcohols containing four different antoxidant systems and a control were included in the storage tests. The four systems were as follows. (1), ascorbyl palmitate† 77 p.p.m., (±)-α-tocopherol (free)† 23 p.p.m.; (2), ascorbyl palmitate 70 p.p.m., (±)-α-tocopherol (free) 20 p.p.m., citric acid 10 p.p.m.; (3), (±)-α-tocopherol (free) 100 p.p.m.; (4), butylated hydroxyanisole‡ 500 p.p.m.

All but one of the antoxidants were dissolved in a little of the wool alcohols whilst hot, the resultant concentrate being thoroughly mixed into the bulk of the molten alcohols. The exception, citric acid, was dissolved in the minimum of hot water and this solution stirred into the molten alcohols, which were then passed through a piston-type homogeniser.

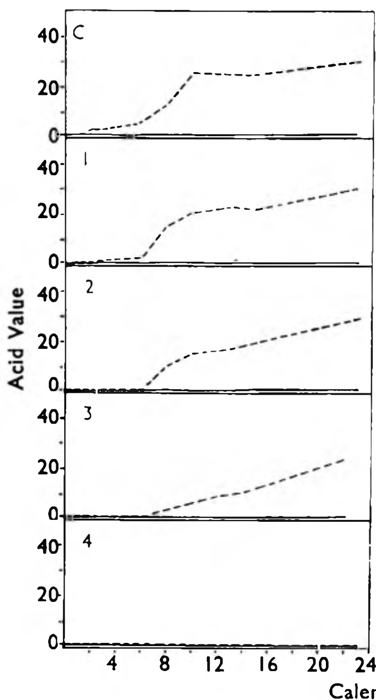
* "Golden Dawn" Wool Alcohols B.P. supplied by Westbrook Lanolin Company.
† Supplied by Roche Products, Ltd.
‡ "Embanox," supplied by May and Baker, Ltd.

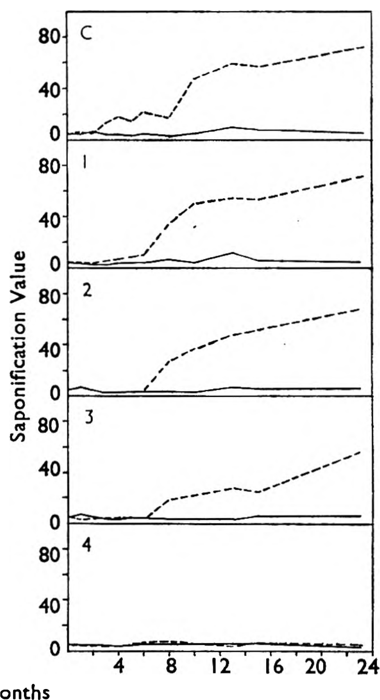
Methods of Examination

Jars of each test system were removed from storage at regular intervals. The contents of each were melted and mixed, and submitted to the following tests.

1. *Acid value.* The method of the B.P. 1958.
2. *Saponification value.* The method of the B.P. 1958, modified by extending the time of reflux to 4 hours and adding a little purified carborundum powder as an aid to boiling.
3. *Emulsification value.* The per cent by volume of water emulsified at 25° (using a twin-beater, electric food mixer) by a mixture of 5 g. of wool alcohols with 40 g. of Liquid Paraffin B.P.
4. *Melting point.* The method of the B.P. 1958.
5. *Cholesterol content.* Determined colorimetrically by the Liebermann-Burchard reaction. The method was previously calibrated on the same wool alcohols against cholesterol determined by digitonin. Owing to the known interference with this colorimetric method by oxidative degradation products the results are only an indication of the trend of the changes.
6. *Colour.* Determined hot with a Lovibond Tintometer, using a ¼ in. cell and measuring yellow and red units. Most samples required the superimposition of neutral tint glasses.

The results are included in Figures 1 to 5.





Calendar Months

FIG. 1. Changes in acid value. FIG. 2. Changes in saponification value.
——— Solid wool alcohols. - - - Flaked wool alcohols.
The numbers in the graphs refer to the systems in the text. C = Control.

FIG. 3. Changes in emulsification value.
——— Solid wool alcohols.
– – – Flaked wool alcohols.

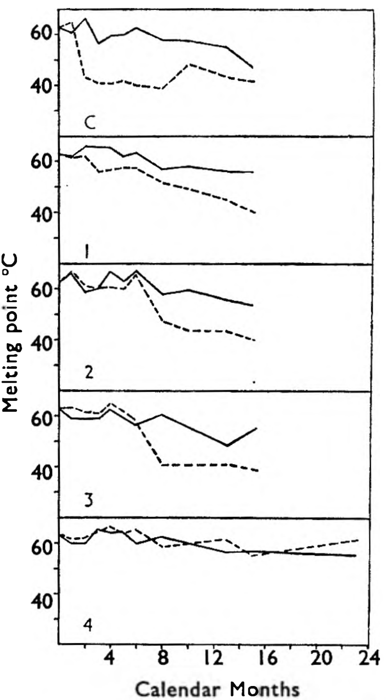


FIG. 4. Changes in melting point. Melting points were not taken after 15 months, owing to the difficulties created by the extremely viscous nature of the molten oxidised samples.

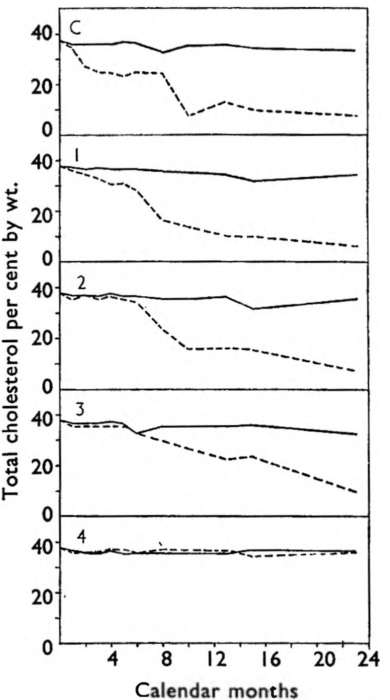
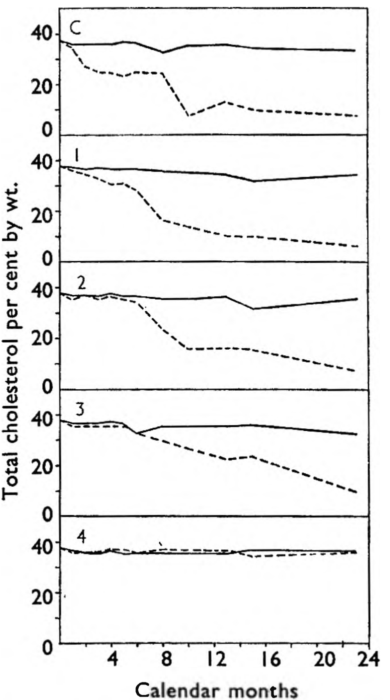



FIG. 5. Changes in cholesterol content.

The numbers inside the graphs refer to the systems described in the text. C = control.

Discussion

The Figures show that the changes in wool alcohols as a result of gradual autoxidation at room temperature are similar to those occurring during accelerated methods of oxidation. When stored under good, normal conditions, however, the autoxidised surface layer is indicated to be extremely shallow even after 23 months. No measurement of the thickness of the layer was attempted, but from visual judgment it was less than 1 mm. Some surface bleaching occured but light was non-essential for autoxidation.

The results confirm earlier findings that autoxidation may be effectively inhibited by a suitable antoxidant such as butylated hydroxyanisole, using 500 p.p.m., the other antoxidants tested having a very limited effect. Concentrations of butylated hydroxyanisole lower than used here may be sufficiently effective for practical purposes. Janecke and Senft showed 200 p.p.m. to be almost as effective as 500 p.p.m., but further work is contemplated to extend the range of antoxidants tested, and also to determine how the natural susceptibility to autoxidation of wool alcohols varies between different production batches.

References

1. Gillam, *Austr. Chem. Inst. J. Proc.*, 1948, **14**, 361.
2. Muirhead, Oberweger, Seymour and Simmonite, *J. Pharm. Pharmacol.*, 1949, **1**, 762.
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WATER-SOLUBLE CELLULOSE DERIVATIVES*

USES AS PRIMARY EMULSIFYING AGENTS. PART II

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Received November 16, 1959

A study has been made of the effects of heat, acid and alkali, salts and alcohol on the stability of methyl-, methylethyl- and sodium carboxymethyl-cellulose emulsions of liquid paraffin. All were stable for 4 to 8 weeks at 40°, but at 80° deteriorated rapidly, the order of decreasing stability being methyl-, methylethyl- and sodium carboxymethylcellulose emulsions. Heating at 115° for 30 minutes decreased the stability of sodium carboxymethylcellulose, but not of methyl- and methylethyl-cellulose emulsions. Stability in the presence of added substances depended largely on the physical properties of the reaction products. The results are related to the behaviour under similar conditions of aqueous dispersions of the three derivatives, and the influence on emulsion stability of dehydration and degradation of the emulsifying agent is discussed.

IN the first part of this work we showed that the emulsifying properties of the water-soluble cellulose derivatives depend upon the concentration, viscosity grade and type of derivative used[1]. The effects of heat and various added substances have now been examined.

MATERIALS AND APPARATUS

Materials, apparatus, and the methods of preparing and assessing the emulsions were as previously described[1]. The disperse phase was liquid paraffin 25 per cent v/v. The chemicals were of A.R. quality. pH measurements were made on the Cambridge bench pH meter using a glass electrode and a saturated calomel reference electrode.

EXPERIMENTAL AND RESULTS

Repeated Gelling and Cooling of a Methylcellulose Emulsion

An emulsion made with methylcellulose M20 (4·7 per cent in the aqueous phase) was divided into two samples. Each, after determination of their "H" values, was rapidly heated in a water bath to 50°, when they completely gelled. They were then removed and allowed to stand at room temperature for 24 hours to cool and revert to their original fluid state, briefly shaken, and their "H" values redetermined.

This procedure could be repeated six times without the emulsions deteriorating. After the seventh treatment some small oil globules were visible on the surface of the emulsions.

* The subject-matter of this communication forms part of a thesis by one of us (R.E.M.D.) accepted by the University of Wales for the degree of Master of Pharmacy.

Emulsions Stored at Elevated Temperatures

Emulsions made with methylethylcellulose and low viscosity grades of methyl- and sodium carboxymethyl-cellulose were distributed into 10-ml. neutral glass ampoules which were sealed and then stored in hot air ovens at 40° and 80° for 6 months. The emulsions were examined initially, and, thereafter, at 1, 2, 4, 8, 12 and 24 weeks, being allowed to stand for 24 hours at room temperature after removal from the ovens. The results (Table I) show that: (*a*) All the emulsions deteriorated more rapidly at 40° than at room temperature. Nevertheless, at 40° they all remained stable for 4 to 8 weeks. (*b*) Emulsions stored at 80° deteriorated rapidly; within a week there was loss of viscosity, accompanied by creaming and oil separation. The order of increasing stability was sodium carboxymethylcellulose, methylethylcellulose and methylcellulose emulsions.

TABLE I*

STORAGE OF LIQUID PARAFFIN EMULSIONS AT ELEVATED TEMPERATURES

	40°			80°
	Value of "H"			
Derivative	Initial	Final	Remarks†	Remarks†
Methyl M20 4 per cent	48·1	50·3 (8)	A thin film of oil appeared between 8 and 12 weeks. By 24 weeks the emulsion had become less viscous	Rapid creaming after 1 week's storage, when emulsion became very fluid. Oil separation began between 2 to 4 weeks. Surface of emulsion "browned" between 4 to 8 weeks
Methylethyl 3·5 per cent	25·5	24·5 (4)	Oil separation began between 4 to 8 weeks. A thin oil film covered the surface after 12 to 24 weeks	Oil separation began within a week, and a thin continuous oil film had formed after 2 weeks. Emulsion became very fluid
S.C.M.C. 3·25 per cent	53·9	53·4 (8)	Oil separation began between 8 and 12 weeks	Oil separation began within 24 to 48 hours. A continuous layer of oil formed between 72 to 96 hours, when sample creamed and darkened in colour. There was an obvious loss of viscosity after 1 week

† The appearances are those of the emulsions 24 hours after removal from the ovens.

Emulsions Heated and Subsequently Stored

Emulsions made as above, after their "H" values had been determined, were distributed into 10-ml. neutral glass ampoules and heated in an

EXPLANATORY NOTES

* TABLES I TO IV

Concentrations. These refer to the percentage w/v of derivative in the mucilage used to prepare the emulsions. (Emulsions contain 25 per cent v/v of oil.)

Final value of "H." Figures in parenthesis indicate stage, in weeks, of last globule count.

Coefficient of variation. Calculated on all globule counts made during the period of storage (see also previous note).

Cream volume. Figures in parenthesis indicate stage, in weeks, of observation. The volumes were assessed by means of a rule placed against the sides of the jars.

Oil separation. Unless otherwise stated, this refers to the separation of small isolated globules, visible to the naked eye, on the surface of the emulsion.

autoclave at 115° for 30 minutes. They were re-examined 24 hours after heating, and at 4, 12 and 24 weeks. The results (Table II) show that: (*a*) The stability of methyl- and methylethyl-cellulose emulsions was not reduced by autoclaving. (*b*) The stability of sodium carboxymethylcellulose emulsions was reduced by autoclaving, gross deterioration becoming apparent within the first 4 weeks of storage.

TABLE II

EMULSIONS OF LIQUID PARAFFIN HEATED FOR 30 MINUTES AT 115° AND SUBSEQUENTLY STORED

Derivative	Value of "H"		Coefficient of variation	Remarks
	Initial	Final		
Methyl M20 4 per cent	47·8	49·1 (24)	1·8	No oil separation after 24 weeks' storage. Creaming behaviour similar to unheated emulsion
Methylethyl 3·5 per cent	29·9	27·5 (24)	6·5	As above
S.C.M.C. (low) 3·25 per cent	51·3	53 (24 hours)	—	Oil began to separate within 4 weeks and increased over the storage period. The unheated control sample neither creamed nor showed oil separation

TABLE III

STORAGE OF LIQUID PARAFFIN EMULSIONS MADE WITH MUCILAGES HEATED FOR $3\frac{1}{2}$ HOURS AT 115°

Derivative	Sample	Efflux time of mucilage (secs.)	Value of "H" (initial)	Cream volume (per cent)	Stage of oil separation (weeks)	Remarks
Methyl M20 4 per cent	Unheated mucilage	360	48·6	95 (4, 24)	—	Thick, pourable
	Heated mucilage	270	50·4	85 (12, 24)	8–12	Less viscous than control sample
S.C.M.C. (low) 3·25 per cent	Unheated mucilage	400	56·9	0	8–12	Thick, pourable
	Heated mucilage	147	53·2	85 (4, 24)	0–4	Much less viscous than control. Traces of oil apparent within 36 hours of preparation. The volume of oil separating on storage was greater than in the control sample

Emulsions Prepared with Heated Mucilages and Subsequently Stored

Mucilages containing the low viscosity grades of methyl- and sodium carboxymethyl-cellulose were each divided into two portions, one of which was heated at 115° for $3\frac{1}{2}$ hours to reduce its viscosity. The heated and unheated mucilages were used to make emulsions of liquid paraffin which were stored at room temperature for 6 months, being examined initially and, thereafter, at 4, 8, 12 and 24 weeks. The results (Table III) show that: (*a*) Although the efflux time, measured with a Redwood viscometer, of the methylcellulose mucilage had been reduced by 25 per cent, both

batches of emulsion had, initially, similar "H" values. That made with the autoclaved mucilage was less viscous and oil began to separate between the second and third month of storage, whereas the "control" sample remained stable. (*b*) Although the efflux time of the heated sodium carboxymethylcellulose mucilage had been reduced by over 60 per cent, both batches of emulsions had, initially, similar "H" values.

TABLE IV

STORAGE OF LIQUID PARAFFIN EMULSIONS OF VARYING pH

Derivative	pH	Value of "H" Initial	Value of "H" Final*	Coefficient of variation	Cream volume (per cent)	Stage of oil separation (weeks)	Remarks
Methyl M20 4 per cent	1·54	50·8	53·5	4·9	33 (4, 24)	—	Thick, pourable
	2·82	53·0	51·8	3·7	33 (12, 24)	—	As above
	4·66	54·8	51·6	3·4	As above	—	As above
	9·08	52·4	55·0	3·7	As above	—	As above
	12·8	52·0	52·1	4·7	As above	—	As above
Methyl-ethyl 3 per cent	1·52	20·3	19·5	6·4	33 (4) 50 (8, 24)	—	Readily pourable
	3·29	21·1	18·6	6·1	As above	—	As above
	4·94	22·1	20·9	5·2	As above	—	As above
	9·7	20·0	19·6	2·8	As above	—	As above
	12·9	17·5	16·3	5·8	50 (24 hours, 24)	—	As above
S.C.M.C. (low) 3·25 per cent	1·55	—	—	—	0	—	Thixotropic. No creaming or oil separation
	2·98	—	—	—	0	—	As above
	7·1	51·1	54·7 (4)	—	90 (24)	8–12	Thick, pourable
	8·88	53·1	56·2 (4)	—	50 (4) 33 (24)	4–8	As above
	12·5	55·8	—	—	50 (0) 33 (4, 24)	0–4	As above

* Value of "H" after 24 weeks unless otherwise indicated.

The emulsion made with the heated mucilage was much more fluid, creamed more readily and oil separation was greater and occurred earlier than with the "control" sample.

Storage of Emulsions of Varying pH

Hydrochloric acid or sodium hydroxide solution was added to the emulsions to yield a range of pH values. Where necessary, water was also added so that each emulsion was diluted by the same amount. The emulsions were stored at room temperature for 6 months and examined at 24 hours, 4, 8, 12 and 24 weeks. The results (Table IV) show that: (*a*) The most acid methylcellulose emulsion creamed more rapidly than the other samples. (*b*) The most alkaline emulsion of methylethylcellulose creamed more rapidly than the other samples. (*c*) Although both the

acidified emulsions of sodium carboxymethylcellulose were thixotropic, microscopically they appeared no different to the other samples, but they neither creamed nor showed any separation of oil. The three remaining samples creamed and oil separated on storage, the degree of deterioration increasing with the alkalinity of the sample. The most alkaline sample was the least viscous.

Storage of Emulsions Containing Sodium Chloride

Emulsions made with low viscosity grades of methyl- and sodium carboxymethyl-cellulose were diluted with equal volumes of water containing varying concentrations of sodium chloride (0 to 30 per cent w/v), stored for 6 months at room temperature and examined at 1 day, 4, 8, 12 and 24 weeks. The results can be summarised as follows: (*a*) The stability of methylcellulose emulsions was unaffected by up to 5 per cent of sodium chloride. Emulsions containing 10 and 15 per cent of sodium chloride were thixotropic and did not cream; microscopically, they appeared similar to the other samples. None of the emulsions showed any separation of oil. (*b*) All the sodium carboxymethylcellulose emulsions creamed to the same extent. At the end of 6 months' storage, oil had separated from all the emulsions, but its volume decreased with increasing sodium chloride content.

Storage of Emulsions containing Other Chlorides

Emulsions made with sodium carboxymethylcellulose (low) were diluted with equal volumes of water containing various monovalent and bivalent chlorides to yield a salt concentration of 0·2M in the emulsion. They were stored at room temperature for 6 months and examined at 1 day, 4, 8, 12 and 24 weeks.

Less oil separated from those emulsions containing salts than from the control samples.

Storage of Emulsions Containing Alcohol

To emulsions made with low viscosity grades of methyl- and sodium carboxymethyl-cellulose were added quantities of industrial methylated spirit (74 o.p.) and, where necessary, sufficient water to dilute the emulsions by 50 per cent. The concentration of I.M.S. in the emulsions varied from 0 to 50 per cent. The emulsions were stored at room temperature for 6 months and examined at 1 day, 4, 8, 12 and 24 weeks. The results can be summarised: (*a*) Alcohol did not influence the creaming behaviour of methylcellulose emulsions but it did effect separation of traces of oil after 6 months' storage. With 50 per cent of alcohol the ether partially precipitated. (*b*) As little as 10 per cent of alcohol profoundly affected the stability of sodium carboxymethylcellulose emulsions and considerable oil separation occurred within 24 hours. Fifty per cent of alcohol precipitated the ether.

Discussion

Influence of Temperature

When methyl- and methylethyl-cellulose sols are heated their viscosity gradually falls until a temperature is reached (about 50°) at which the

ether precipitates. At this point, depending on the concentration of the sol, either discrete gel particles or a continuous gel is formed, and, in the latter case, the viscosity rises sharply. The viscosity of sodium carboxymethylcellulose sols decreases progressively with rise in temperature. If the heating period is not prolonged and the temperature not excessive, both these changes are reversible and the sols regain their original viscosity on cooling. Prolonged heating at high temperatures, however, leads to irreversible viscosity changes[2].

When a methylcellulose emulsion is heated to 50° only the reversible changes have to be considered. If 50° is reached rapidly, the possibility of aggregation and coalescence of oil globules during the brief pre-gelation period of lowered viscosity can be ignored. But when the particles of the emulsifying agent are dehydrated sufficiently to form a gel, it might be expected that changes would occur at the interface to alter the stability of the emulsion. As shown, however, the sol/gel/sol transformation can be accomplished without deterioration. Since the oil globules are immobilised while the emulsion remains gelled, two explanations suggest themselves: the first, that the interfacial film remains intact in its partially dehydrated state, or the second, that the film is disrupted as dehydration proceeds, but rapidly reforms as the system cools. In the latter instance, some coarsening of the emulsion would be inevitable unless there was close correlation between interfacial readsorption and break-down of the gel structure. The first explanation, therefore, seems the more likely. That the emulsion will not withstand repeated treatments of this kind indicates that in spite of the apparent rigidity of the film some points of weakness are induced by dehydration.

In the long-term storage tests, 40° was chosen in the expectation that for both the methyl- and methylethyl-cellulose emulsions it would be near the point of minimum viscosity (that is, just below gel point). Both emulsions gelled at this temperature, however, while the sodium carboxymethylcellulose emulsions became less viscous. The results show that methyl- and methylethyl-cellulose emulsions can be maintained in the gelled state for 4 to 8 weeks without deterioration. Some breakdown occurs eventually, indicating that prolonged dehydration weakens the interfacial film. The methylethylcellulose emulsion was the least stable of the two. Gels of the methylethyl- derivative are less rigid than those of methylcellulose and therefore coalescence is easier. Deterioration of the sodium carboxymethylcellulose emulsion results from the lowered viscosity which facilitates aggregation and coalescence of the disperse phase.

In the emulsions stored at 80° the effects of the irreversible viscosity changes became apparent. After storage for a week, emulsions of methyl- and methylethyl-cellulose creamed rapidly on cooling, indicating that the derivatives had undergone degradation. Although the reduced viscosity of the continuous phase must have contributed to the instability, degradation (and, in the case of the methyl- and methylethyl-cellulose emulsions, dehydration) of the derivative at the interface may be an equally important factor. As expected, sodium carboxymethylcellulose emulsions proved to be the least stable under these conditions.

The results obtained after emulsions were autoclaved and then stored also agree with previous findings. The amount of degradation which the methyl- and methylethyl- derivatives underwent was too small to affect the viscosity of the continuous phase. Sodium carboxymethylcellulose, however, degrades far more readily.

As previously mentioned[1], the emulsions made with heated mucilages were less stable than similar emulsions made with unheated mucilages. This instability is largely due to the reduced viscosity of the continuous phase, although other factors, such as the ability of the degraded derivative to form a stable interfacial membrane, may also be involved.

Influence of Acid and Alkali

The behaviour of methyl- and methylethyl-cellulose emulsions in the presence of acid and alkali can also be directly related to that of the mucilages under similar conditions[2]. Thus, creaming was most rapid in the most acid methylcellulose emulsions and the most alkaline methylethyl-cellulose emulsions. The behaviour of sodium carboxymethylcellulose preparations, however, is paradoxical. When sufficient acid was added to the mucilages to precipitate carboxymethylcellulose the viscosity fell immediately; but when precipitation occurred in the emulsion a product with thixotropic properties was formed which neither creamed nor showed oil separation. The other emulsions in this group behaved as expected, with stability declining with degree of alkalinity.

Influence of Salts

Work on the mucilages[3] suggested that the main effects of electrolytes on methylcellulose emulsions would be those due to dehydration, and this was so. Thus, concentrations of sodium chloride too low to affect the viscosity of mucilages had no effect on emulsions, while concentrations sufficient to cause the mucilage to gel rendered the emulsions thixotropic. The behaviour of sodium carboxymethylcellulose emulsions was not predictable, for salts which reduced the viscosity of its mucilages appeared to enhance the stability of its emulsions. However, the addition of salts to systems containing anionic dispersing agents can result in a lowering of interfacial tension[4], and here the effect on stability of a reduction in the viscosity of the continuous phase may be more than counterbalanced by a closer packing of the emulsifying agent at the interface.

Influence of Alcohol

Alcohol added to mucilages of the cellulose derivatives first led to a progressive dehydration of the particles and a thickening of the system; larger volumes precipitated the derivatives without gel formation. This explains why, in contrast to the effect of strong solutions of salts, alcohol decreased rather than enhanced the stability of methylcellulose emulsions. Sodium carboxymethylcellulose emulsions were particularly sensitive to alcohol, precipitation of the derivatives being accompanied by syneresis as well as oil separation.

General Observations

An attempt has been made to relate alterations in emulsion stability to changes which occur under similar conditions in aqueous dispersions of the emulsifying agent, and which are made manifest by variations in viscosity[2,3]. This has been done because the viscosity of the continuous phase is an important factor in emulsion stability, and because a change in viscosity signifies an alteration in the state of the emulsifying agent, and may therefore be indicative of simultaneous but less obvious changes in the properties of the interfacial film. While recognising the complexity of these reactions, it seems reasonable to draw the following conclusions from the results.

(i) Interfacial films of methyl- and methylethyl-cellulose withstand short periods of dehydration without disruption, but prolonged dehydration weakens the film.

(ii) When dehydration is accompanied by degradation of the derivative the instability of the emulsion is increased. This is because the reduction in viscosity of the continuous phase permits the dispersed oil globules to aggregate, and, if the interfacial film has been sufficiently weakened, to coalesce. Degradation may also affect the ability of the derivative to form stable films at the interface.

(iii) When dehydration is effected by a dehydrating agent, the stability of the emulsion depends on the physical properties of the reaction product. If the derivative forms a gel the emulsion will remain "stable", because the globules are immobilised.

(iv) Because sodium carboxymethylcellulose undergoes degradation more readily than do the two other derivatives, emulsions containing it are less stable to heat. This is due not only to the high irreversible viscosity losses in the continuous phase, but also to the fact that the reversible change on heating is not from sol to "stabilising" gel but to a more fluid preparation.

(v) As with methyl- and methylethyl-cellulose, the stability of sodium carboxymethylcellulose emulsions in the presence of other substances appears to depend largely on the physical properties of the product of the reaction.

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A NOTE ON CORIANDER OF COMMERCE

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Received January 15, 1960

As a result of the examination of a number of samples of coriander it has been shown that it is possible to divide the commercial varieties into at least three groups and to give some indication of the geographical origin of the samples.

The variation in the price of samples of coriander and in the quality and quantity of the oil distilled from them, has led to enquiries for a simple means of distinguishing between the commercial varieties. These varieties are usually named according to their geographical source.

Botanically, de Candolle[1] in 1830 distinguished two varieties. He gives the source as *Coriandrum sativum* Linn., and in a note adds the variety *microcarpum*, described as having fruits smaller by about one half. Alefeld named the first of these *Coriandrum sativum* L. var. *vulgare*. That the difference is recognised commercially is noted by Berger[2] and Heeger[3] who write that the fruit of *Coriandrum sativum* L. var. *vulgare* Alef. has a diameter of 3 to 5 mm. and that of *Coriandrum sativum* L. var. *microcarpum* has a diameter of 1·5 to 3 mm. The former would include Moroccan or Mogadore coriander and the latter Russian coriander. Two Polish specimens of the larger size which had been grown in the Pharmacognosy Gardens at Warsaw and Krakow respectively were labelled as being *Coriandrum sativum* L. var. *macrocarpum*.

Varentzov[4] found that, in general, the smaller fruits contained more essential oil than did the larger ones. Guenther[5] gives figures which support this, for example, Moroccan 0·2 to 0·3 per cent and Russian 0·8 to 1·0 per cent of oil.

Althausen and others[6] established standards such as ultra-violet adsorption spectra of the oil and emission spectra of the fruit ash, by which it is possible to "approximate or define the geographical origin of a sample of coriander fruit subject to inspection." The methods when applied to whole fruit are lengthy and it was decided to see if a sample method of distinction could be devised.

Diameters of these small spherical fruits are difficult to measure and there is some overlap of the diameters of the different varieties. Since the volumes of spheres of different diameter vary much more widely than do their diameters, and the mass of a sphere is proportional to its volume, the weight of a number of fruits (say 100), might prove a useful and easily applied means of differentiation of the varieties. The average mass (or weight) of 100 fruits of each available variety was therefore determined. By expressing the results as the number of fruits per gram, whole numbers are obtained and these give the most suitable means of comparison.

Experimental and Results

Materials. Recent samples of fruit were obtained through the kindness of Mr. G. R. A. Short, Dr. T. E. Wallis and Mr. E. J. Shellard. Museum specimens were also examined from the Museum of the Pharmaceutical Society of Great Britain and from the Museum of the Chelsea School of Pharmacy.

Method. Wherever possible, 10 batches of 100 fruits were counted from each sample and each batch was then weighed. Table I summarises the results obtained, both in terms of the minimum, average and maximum weights of 100 fruits and also of the minimum, average and maximum number of fruits per gram.

TABLE I

Weight of 100 fruits and the number of fruits per gram of 17 samples of different varieties of coriander

Variety	Source	Weight of 100 fruits g.	Number of fruits per per gram
1. Moroccan	* Wholesaler 1959	1·6234–1·7506–1·8675	54– 57– 62
2. Moroccan	† Drug Broker No. 1, 1959	1·3907–1·4714–1·6195	62– 68– 72
3. Mogadore	Chelsea Museum *c.* 1930	1·4528–1·4889–1·5859	60– 67– 69
4. Mogadore	† P.S.G.B. Museum Box c. 1900	1·2210–1·3636–1·4616	69– 73– 82
5. German (?)	† P.S.G.B. Museum 1909	1·3482–1·4313–1·5234	66– 72– 74
6. English	† Drug Broker No. 1, 1959	1·1372–1·1613–1·2496	80– 86– 88
7. Polish	‡ Macrocarpum from Warsaw 1953	1·1003–1·1260–1·1894	84– 89– 91
8. English	† P.S.G.B. Museum Jar	0·9464–1·0025–1·0554	95–100–106
9. English	† P.S.G.B. Museum Box	0·9184–0·9747–1·0176	98–102–108
10. Roumanian	† Drug Broker No. 1, 1959	0·8974–0·9422–0·9902	101–106–111
11. Polish	‡ Macrocarpum from Krakow 1958	0·9154–0·9354–0·9716	103–107–109
12. German	" Drug Broker No. 2, 1959	0·6540–0·7042–0·7688	130–142–153
13. Hungarian	* Wholesaler 1959	0·6314–0·6702–0·7010	143–149–158
14. Russian	Chelsea Museum 1942	0·5894–0·6208–0·6712	149–161–170
15. Russian	† P.S.G.B. Museum Jar 1933	0·5930–0·6098–0·6396	156–164–169
16. Russian	† P.S.G.B. Museum Box *c.* 1900	0·5310–0·5702–0·6080	166–175–188
17. Polish	‡ Microcarpum from Krakow 1958	0·5218–0·5481–0·5704	175–182–191

* through Mr. G. R. A. Short
† through Dr. T. E. Wallis
‡ through Mr. E. J. Shellard

Discussion

The figures in Table I show that there is a clear division between samples 11 and 12. It seems reasonable to assume that samples 1 to 11 are derived from *Coriandrum sativum* L. var. *vulgare* Alef. and that samples 12 to 17 are derived from *Coriandrum sativum* L. var. *microcarpum* D.C. A smaller break occurs between samples 5 and 6 which suggests putting the samples into three groups. The fruits of samples 1 to 5 were found to have purple patches on the surface, a feature which was absent from samples 6 to 11. The purple patches appear to be confined to Moroccan or Mogadore varieties. The labelling of sample 5, therefore, needs comment. There is no evidence that this sample of fruit was grown in Germany. It is almost certain that it is Mogadore coriander which was probably obtained through the port of Hamburg.

Conclusions

The following conclusions were drawn.

1. Samples with less than 75 (average 66·5) fruits per gram and showing purple patches are Moroccan or Mogadore coriander.

2. Samples between 80 and 110 (average 98) fruits per gram are English or Roumanian coriander (the Polish samples 7 and 11 were not commercially available material.)

3. Samples with over 130 (average 161) fruits per gram are German, Hungarian, Polish or Russian coriander.

Acknowledgements. My thanks are due to Dr. T. E. Wallis for suggesting this work, for helpful advice and for access to some of the samples; also to Mr. G. R. A. Short and Mr. E. J. Shellard for obtaining other samples.

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BOOK REVIEWS

PROGRESS IN DRUG RESEARCH. Vol. I. Edited by Ernst Jucker. Pp. 607, including 60 figures and 124 tables. Birkhäuser Verlag, Basle, 1959. Sw. fr. 68.00.

Pharmaceutical research has reached a stage of rapid development where the specialist investigator, absorbed with his own immediate interests, is finding it increasingly difficult to keep abreast of the general advance of the subject. *Progress in Drug Research,* which is to be published as an annual series under the editorship of Dr. Ernst Jucker, represents a constructive attempt to fill the gap which lies between the publication of original work and the specialist monograph. The aim is to present each year a select group of reviews on topics of current interest in pharmaceutical research, each by a recognised authority. In particular it is hoped to devote special attention to the subject of structure-action relationships. The first volume clearly demonstrates the international character of pharmaceutical research today. Of the seven contributions three originate from Germany, two from Switzerland, one from Great Britain and one from the United States, and the articles are published in the author's own language. The subjects range from the clinical problems associated with the use of placebos by Drs. H. Haas, H. Fink and G. Härtfelder to the physico-chemical properties of ion-exchangers and their use in Pharmacy and Medicine by Professor J. Büchi. The importance of fundamental physiological and biochemical studies to drug research is emphasised in the contribution of Drs. Tsung-Min Lin and K. K. Chen in cholesterol in relation to arteriosclerosis. Reviews of specific aspects of chemotherapy are provided by Dr. H.-A. Oelkers on worm infestation and Dr. J. Bally on chemical anthelmintics, whilst Dr. W. Kunz has produced an excellent review of the newer medicinal agents of the last five years. The latter will be of particular interest to British readers in view of the attention it draws to developments of continental origin, which may not be quite so familiar as those which derive from this country or the United States. Special interest also attaches to Dr. A. H. Beckett's interesting and thought-provoking contribution on stereochemical factors in biological activity. The importance of stereochemical factors in drug-action has long been known, and the value of the present article lies as much in the attention which it focusses on the problem, as in its lucid approach to the subject itself. The Editor of this volume is to be congratulated in producing a work of wide interest and great value to those engaged in pharmaceutical research. It is excellently referenced. Formulae, diagrams and tables are freely used and greatly assist the clear presentation of information throughout.

J. B. STENLAKE.

BEHAVIOUR OF ENZYME SYSTEMS. By John M. Reiner. Pp. xii + 317. Mayflower Publishing Co. Ltd., London, 1959. 52s.; and Burgess Publishing Co., Minneapolis.

This book has been written, in the words of the author in his "Forward for Timid Souls," "for those research workers who feel the need of tools for quantitative interpretation of the work . . . but . . . are in some doubt as to whether their mathematical training and facility are good enough for the mastery and use of a mathematical analysis of enzyme activity."

The text consists of the development of mathematical equations to describe the behaviour of enzyme systems in terms of the simple rate equations of chemical

kinetics. This of course leads to some very elaborate expressions for overall reaction velocities, involving eight or more "velocity constants," and the situations become somewhat complicated when, as on p. 178, "neutral anti-inhibitor" is considered as "activator."

This work probably forms a useful collection of mathematical equations for workers in the field of enzyme kinetics, and the final chapter on the mechanism of enzyme action is particularly interesting though some might think that the author has dismissed the theory of protein semi-conduction somewhat too cursorily.

The book does raise the question whether the best method of analysis of these complex systems is by repeated application of simple kinetic equations, leading to expressions with a large number of hypothetical velocity constants. The systems themselves are so often, as the author points out, ill-defined and are made up of macromolecular substances; it may be stretching laws which were developed to explain the rates of reaction of simple compounds, rather far to apply them too freely to these complex systems in which mechanisms such as protein conduction could be important.

Although many of the mathemetical equations in the book seem somewhat complicated, no very extensive knowledge of mathematics is needed to follow their development, and the text is well illustrated with graphs showing the form of plots to be expected under various circumstances. It is perhaps a pity that the author has devoted so much space to the development of theories that there is very little treatment of actual experimental results although many references to the literature are given. In a subject such as enzyme kinetics where the interpretation of results is still in the state of hypothesis rather than of theory, it is perhaps more sound to maintain greater emphasis on practical observations and their reliability than on purely theoretical treatment of mechanisms.

L. SAUNDERS.

CLINICAL TOXICOLOGY. By C. J. Polson and R. N. Tattersall. Pp. xi + 589 (including Index). English Universities Press, Ltd., London, 1959, 42s.

This is a fascinating book by the Professor of Forensic Medicine at Leeds University and the Assistant Physician at Leeds General Infirmary. The authors deal with about 65 poisons which they describe as being common, of practical importance or of personal interest. The treatment of each poison is discussed from statistical, historical, clinical and pathological viewpoints. There is an introductory chapter which concerns English Law on Poisons and includes a section on the conduct of a doctor when poisoning is suspected. Although recourse to the doctors defence society is suggested there is no mention of a consultation with a senior police officer. The outstanding features of this book are its literary style and the wealth of interesting information on poisons that it contains. Case histories cover the literature from 1752 to the present day and include many from the extensive experience of both authors. The reader can discover in the book not only what to expect if he accepts a Siberian's invitation to "come in and have a toadstool", but also what it is likely to cost him.

Only 9½ pages out of the 580 are devoted to the barbiturates which are at the moment the most common poisons and of these, 3½ pages describe 12 case histories. It is disappointing to the reviewer to see that the help that blood barbiturate levels can give to the clinician in assessing the length of time the patient will be in coma is not discussed at all. Similarly, salicylate and DNOC

blood concentrations are not mentioned. Elsewhere the chemist may be irritated by the wide divergence in the manner of expression of concentrations (10 p.p.m., 0·001 per cent, 1 mg. per cent, ½ grain per ounce, etc.). The authors are obviously experts in their own fields and it is almost inevitable that, in this wide subject, when they stray into the edges of other sciences, errors or controversial opinions creep in. Evaluations of the reported concentrations of poisons in body fluids are in several places insecure; boron and boric acid (0·04 per cent representing 61 mg./100 g.) are typical examples and the section on ethyl alcohol needs revision. The presence of fluoride in early preparations of fluoroacetates has not been recognised, and surely fluoride produces a lowered, not a raised blood calcium level!

It is, however, these excursions into the many fields of toxicology that make this book what it is—a fascinating, readable book produced in excellent type at the reasonable price of 42s. Not only physicians and pathologists will enjoy and profit from this book; it is more than a textbook filling a gap in toxicological literature, it is a book that will be read with interest by all scientists and would-be murderers.

A. S. CURRY.

LETTERS TO THE EDITOR

Analgesic-Antipyretics

SIR,—A number of compounds with diverse chemical properties have been widely used for many years to relieve the discomfort of mild pain such as that caused by headache, toothache, and various rheumatic conditions.

These compounds can be divided into three main chemical groups: the salicylate derivatives, the *p*-aminophenol derivatives, and the pyrazole derivatives.

They have become known collectively as the Analgesic-Antipyretics, and are usually grouped together in most textbooks of pharmacology.

Over a number of years we have been investigating the activities of many pharmacological agents against an experimental inflammation, an erythema in the guinea pig, produced by ultra-violet irradiation. In the course of this work we have examined the inhibitory effects of the best known and most widely used representative members of the salicylates, pyrazoles, and *p*-aminophenols against this erythema, and the results are reported here. In addition, the effects of two isophthalic acids which have been shown experimentally to possess analgesic and antipyretic properties, have been examined.

For the determination of anti-inflammatory activity a modification[1] of the method first described by Wilhelmi[2] was used. A small area of the back of a depilated albino guinea pig was exposed to the ultra-violet radiation from a Hanovia Kromayer lamp for 20 seconds. Animals received test-substances or saline by mouth 30 minutes before irradiation, and the degree of erythema (0–4) was estimated 120 minutes later by a trained observer who was unaware of the dosage schedules. Saline-dosed animals invariably produced a degree 3 or degree 4 erythema. By using graded doses of active compounds it was possible to obtain an "Effective Dose" for erythema inhibition. This was defined as that dose of a compound which, in a group of animals, reduced the standard erythema to a mean response of 2. A compound which in a dose of 160 mg./kg. failed to reduce the mean erythema response below 3 was considered to be inactive.

Of eight of the clinically most important representative members of the Analgesic-Antipyretics, four had no anti-inflammatory activity (Table I). It is particularly significant that none of the *p*-aminophenol group was active. Salicylamide, a very old compound, re-introduced a few years ago because it was a form of salicylate which was well tolerated, lacked the anti-inflammatory activity which both aspirin and sodium salicylate exhibited. This compound was shown by Bavin and others[3] to be three times more active than aspirin as an analgesic in rats, although it was a weaker antipyretic.

Collier and Chesher[4] found that the two hydroxyisophthalic acids (Table I) showed antipyretic and analgesic activity at least as good as aspirin and placed them "in the group of analgesic-antipyretics of which aspirin is the most widely used". The results reported here indicate that this statement might now be considered inadequate, since neither of these compounds, unlike aspirin, produced any inhibition of guinea pig erythema.

It would appear therefore from our results that the group of compounds known collectively as Analgesic-Antipyretics can, experimentally at least, be further differentiated into those which have anti-inflammatory activity and those which have not. It is possible that this differentiation is also true clinically, and it is significant that only the active compounds in Table I have proved anti-rheumatic activity.

TABLE I

THE ANTI-INFLAMMATORY ACTIVITIES OF A NUMBER OF ANALGESIC-ANTIPYRETIC COMPOUNDS IN GUINEA PIGS WITH ULTRA-VIOLET LIGHT-INDUCED ERYTHEMA

Compound	Number of animals	Oral dose mg./kg.	Mean erythema response	Approximate "Effective Dose"* mg./kg.
Phenylbutazone	10 10 10	30 15 7·5	0·5 1·9 3·1	10
Amidopyrine	16 17 14	160 80 40	0·8 1·8 3·0	80
Aspirin	10 9 10	160 80 40	0·3 1·8 3·5	80
Sodium salicylate	9 9 9	320 160 80	0·4 1·6 2·9	120
Salicylamide	8	320	3·5	Not active at 320
Acetanilide	8	240	3·0	Not active at 240
Phenacetin	8	240	3·0	Not active at 240
N-Acetyl *p*-aminophenol ..	8	240	3·5	Not active at 240
4-Hydroxyisophthalic acid	6	320	4·0	Not active at 320
2-Hydroxyisophthalic acid	6	320	3·2	Not active at 320
Saline controls	20	—	3·8	

* As defined in text.

This may be an over-simplification of a complex problem, but the results recorded here suggest that there may no longer be any justification for classifying the so-called Analgesic-Antipyretics into a single pharmacological group.

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February 11, 1960.

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The Separation of Mixed Phosphatides

SIR,—Chromatographic methods for separating phosphatide mixtures have largely superseded the solvent fractionation and complex formation methods. Separations have been achieved using cellulose, silica, alumina and magnesium acid silicate. In the preparation of egg lecithin described by Hanahan, Rodbell and Turner[1] and modified by Saunders[2], alumina is used to remove the cephalins from the mixed phosphatides. However, this treatment requires a large quantity of fine alumina powder and involves extensive washing of the powder if high losses of lecithin are to be avoided.

To simplify the separation of the cephalin fraction we have examined the possibility of replacing alumina by an anion exchange resin. Mixed egg phosphatides were dissolved in methanol to give a 2·5 per cent solution. This was passed through a column of Dowex 1 × 7·5 resin in the carbonate form. The effluent contained much ninhydrin-reacting material and so this high cross-linked resin was replaced by a more porous one, Dowex 1 × 4. A column of the carbonate form of the resin removed all ninhydrin reacting materials and also all colouring matter from the phosphatide solution. But the product contained a considerable amount of ether-insoluble phosphatide, and this was probably lysolecithin formed by hydrolysis of the lecithin on the column. This difficulty was overcome by using the bicarbonate form of the resin.

We have found that a column containing about 50 g. of the bicarbonate form of Dowex 1 × 4 50–100 mesh completely removes the cephalins from 7g. of mixed egg phosphatides, prepared by acetone precipitation. The solution can be run rapidly through the column and only a small amount of washing is necessary to give a recovery of 5·8 g. of cephalin-free phosphatides. This process is much quicker than the alumina method and more suitable for large-scale work since a free-running column is used which can easily be washed. In addition the resin column can be regenerated and used again.

The separation has been found to be equally effective with yeast, soya-bean, ground-nut and cotton-seed phosphatides.

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February 19, 1960.

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LETTERS TO THE EDITOR

Histamine and 5-Hydroxytryptamine Content of Tissues after Prolonged Treatment with Polymyxin B

SIR,—Bushby and Green[1] in 1955 were the first to show that polymyxin B releases histamine in rats and this antibiotic was later used by Parratt and West[2] to produce a maximal depletion of histamine from some rat tissues. These latter authors gave five doses intraperitoneally over three days and, in addition to obtaining a depletion of histamine from some tissues, recorded a loss of 5-hydroxytryptamine but this was restricted to the inner layers of the skin and the pads of the feet. This dose schedule has been extensively used in the past two years to deplete the skin of its histamine without altering its 5-hydroxytryptamine concentration. We have now found that, when the treatment with polymyxin B is continued for a longer period than three days, there is a reduction in the skin 5-hydroxytryptamine content and a further loss of histamine from tissues other than the skin.

TABLE I

COMPARISON OF THE DOSES (MG./KG.) OF POLYMYXIN B USED IN THE PRESENT WORK (TREATMENT A) WITH THOSE USED BY PARRATT AND WEST (TREATMENT B)

Day of treatment	Treatment A	Treatment B
1	1	2·5
2	2·5, 2·5	5, 5
3	5, 5	7·5, 7·5
4	7·5, 7·5	—
5	7·5, 7·5	—
6	10	—
7	10, 10	—
Total dose of polymyxin B	76	27·5

Female albino rats (180–190 g.) were injected with polymyxin B according to the dose schedule shown in Table I and killed 24 hours later. Extracts of tissues both rich in mast cells (e.g. skin) and deficient in mast cells (e.g. jejunum and pyloric stomach) were then assayed for histamine and 5-hydroxytryptamine as previously described.[3] The results are compared in Table II with those of previous authors[2] who used fewer doses of polymyxin B. The histamine levels in the jejunum and pyloric stomach of those rats receiving 12 doses (treatment A) were found to be considerably lower than those of rats receiving 5 doses (treatment B). Whereas the 5-hydroxytryptamine content of the jejunum and pyloric stomach is only slightly reduced, that of the skin tissues (abdominal skin, ears and feet skin) is markedly lowered, the average reduction being 44 per cent.

TABLE II

THE HISTAMINE AND 5-HYDROXYTRYPTAMINE CONTENTS OF SOME TISSUES OF RATS RECEIVING DIFFERENT TREATMENTS OF POLYMYXIN B, AS SHOWN IN TABLE I. ALL VALUES ARE EXPRESSED AS PERCENTAGES OF THE CONTROL LEVELS

	Histamine		5-Hydroxytryptamine	
	Treatment A	Treatment B	Treatment A	Treatment B
Feet skin	5	6	46	83
Ears	8	4	65	102
Abdominal skin	23	8	56	73
Pyloric stomach	33	75	72	95
Jejunum	41	60	83	95

It is important, therefore, to choose a suitable dose schedule of polymyxin B for the preferential depletion of tissue histamine in the rat, for prolonged treatment reduces the 5-hydroxytryptamine content of tissues as well as that of histamine. Other potent histamine liberators such as compound 48/80[2,4,5] also release both histamine and 5-hydroxytryptamine, but whilst the release of 5-hydroxytryptamine by compound 48/80 usually precedes that of histamine, with polymyxin B the reverse occurs.

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February 26, 1960.

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The Determination of Meprobamate as the Dixanthyl Derivative

SIR,—Roth and others[1] have characterised meprobamate (2,2-di(carbamoyloxymethyl) pentane) by means of its dixanthyl derivative and report only a melting point of 182°. Algeri and others[2] also used the dixanthyl derivative to identify meprobamate, but give no constants for their derivative. We obtained a higher melting point product, by dissolving meprobamate and xanthenol in glacial acetic acid and allowing the solution to stand for 10 hours or more at room temperature[3]. After recrystallisation from hot methanol and drying to constant weight, the nitrogen content of the crystals, which melted at 188° to 189°, was found to be 4·80 per cent. This value agrees with the calculated value of 4·84 per cent for a product of molecular weight 518·42 derived from the reaction of 2 moles of xanthenol and 1 mole of meprobamate.

The spectral absorbance of dixanthyl meprobamate in various solvents showed two maxima at 240 and 289 mμ similar to those shown by solutions of 9-xanthenol. An E (1 per cent, 1 cm.) (λ289 mμ) of 142·5 was found for solutions of the dixanthyl derivative in methanol, ethanol and isopropanol, and was used to calculate the solubility of the compound in saturated solutions of the various solvents shown in Table I.

The best yields of dixanthyl meprobamate were obtained by reacting 0·100 g. of meprobamate with 0·3 g. of 9-xanthenol in 5 ml. of glacial acetic acid, and seeding with 5 to 10 μg. of dixanthyl meprobamate crystals. After standing for 16 hours, 45 ml. of 80 per cent aqueous isopropanol was added to the reaction flasks and the solutions refrigerated for 1 hour. The crystals were transferred to a tared sintered glass filter with 15 ml. of the aqueous isopropanol and dried to constant weight at 100°. The weight of the crystals multiplied by 0·3769 gives

TABLE I

SOLUBILITY OF DIXANTHYL MEPROBAMATE IN VARIOUS SOLVENTS AT 25°

Solvent	g./100 ml.
Methanol	0·040
Ethanol	0·022
Isopropanol	0·013
Butanol	0·027
Glacial acetic acid	0·25
Ether	0·22
Acetone	2·0
Dimethyl formamide	2·0
Dioxane	over 10·0
Chloroform	over 10·0
Benzene	5·0
Isopropanol 72 per cent v/v Glacial acetic acid 10 " " v/v Water 18 " " v/v	0·008
Water	0·0005

the weight of meprobamate recovered as the dixanthyl derivative. The actual yields and the yields corrected for a loss of 4 mg. of dixanthyl meprobamate by solubility are shown in Table II.

TABLE II

RECOVERY OF MEPROBAMATE AS THE DIXANTHYL DERIVATIVE

Test	Recovery per cent	Recovery corrected for solubility per cent	Melting-point uncorrected °C
1	94·48	95·91	187
2	95·05	96·50	188
3	94·78	96·23	188
4	94·78	96·23	188
5	95·39	96·84	188
6	94·29	95·72	188

The nitrogen values for the products from 3 of these tests were 4·90, 4·85 and 4·75 per cent.

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January 28, 1960

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