REVIEW ARTICLE

MOLECULAR SIZE AND SHAPE

A Review of the Light-scattering Method applied to some Important Biological and other Macromolecules. Part I

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Introduction

Many important biological macromolecules have been studied in more detail in recent years as a result of the development of the light-scattering method. Researches using this technique have contributed not only to the elucidation of the size and shape of biopolymers, high polymers, hydrosols, aerosols, surface active substances and other high molecular weight compounds but also to the study of reaction mechanisms in a range of conditions wider than those obtainable by some other methods. Although substances having molecular weights of less than 1,000 have been examined, the light-scattering method is generally used for the study of macromolecules having weights in excess of 5,000.

Two important differences between solutions of large molecules and "true" solutions are the deviation from ideality and the methods of studying their kinetic and thermodynamic properties. Reliable and accurate information about the physical constants of macromolecules in solution is needed if the mechanism of their reactions is to be understood.

The physical characteristics of macromolecules in solutions as used for measurement by the light-scattering method, are often akin to those existing in natural conditions (*in vivo*) in biological systems, but the concentrations at which such measurements are made are necessarily small so that the results can be extrapolated to infinite dilution and interpreted in terms of thermodynamic functions. Comparison of information obtained by light-scattering under these circumstances with that obtained by other methods, where much higher concentrations and even materials in the dry state are often used, is not always justified.

Several methods for the determination of the molecular characteristics of large molecules are in general use; the classical methods, e.g., cryoscopy, ebulliometry and vapour pressure are often unsatisfactory because of the very high concentrations required to give a measurable effect. The more important methods and the properties on which they depend may be briefly examined.

1. *Osmotic pressure.* This is the most widely used method for the molecular weight range between 5,000 and 100,000. It gives a number average molecular weight and a thermodynamic measure of interaction between molecules (constant B) but no information about shape. Possible errors are attributable to irreversible adsorption of molecules on

membranes and high net surface charges, which set up a membrane potential.

2. *Viscosity.* The method gives a molecular weight approximating to weight average. A model is required (i.e., equations for rods, coils or spheres) to interpret the shape of the molecule which may be difficult to define owing to distortion by the applied force.

3. *Sedimentation, velocity and diffusion constant.* These methods are complementary and depend on the frictional properties and sizes of the molecules. They give a weight average molecular weight and information about size and shape. They are valuable for resolution of a poly-dispersed, non-interacting system into several components.

4. *Dipole moment and relaxation constant.* This method gives information on dipole-ion structure (e.g., of amino acids and peptides) and distribution of surface charges. It is limited to work in solutions of low electrical conductivity.

5. *Ultrafiltration.* Using membranes of different grades the method gives an approximation of size.

6. *Surface films.* Under favourable conditions, this method gives molecular weight, mode of orientation and molecular surface area at an interface. It can be extended to obtain the effects of pH, oxidants, and the presence of electrolytes, on physical and chemical characteristics.

7. *Chemical.* This is by determination of equivalent weight by using gravimetric analysis or by treatment with a compound capable of reacting with certain free groups in the molecule. Thus, Sanger's dinitrofluoro-benzene reagent reacts with amino groups of a protein.

8. *Electron microscope.* The specimens are studied in high vacuum and therefore in a dry state. The molecules are generally required to have dimensions greater than 100 Å. It is difficult to distinguish small structures from background material. This method gives visual evidence of size and shape.

9. *Direct particle counting.* This is effected by counting a weighed number of particles in a known volume of solution; a reference substance is required. Molecular weight only is given.

10. *X-ray diffraction.* The method is to obtain crystallographic unit cell measurements of highly ordered systems. It gives information on molecular size, shape and solvation. The structures of complex proteins and nucleic acids have been examined extensively in this way.

11. *Light-scattering.* A revival of interest in light-scattering as a means of investigating colloidal particles in solution began with the papers of Debye[1,2]. Suitable theoretical treatments were developed and the practical difficulties were studied in many laboratories. The method has been valuable in determining the physical constants and the kinetic and thermodynamic properties of large molecules in solution. It has contributed information on particles of much greater molecular weight ($M_w > 1{,}000{,}000$) and a wider range of temperatures and ionic strengths

of solutions than is possible by osmometry. It can also be used to determine the rates of changes in size and shape when aggregation or disaggregation in solution takes place since it gives instantaneous results; kinetic effects can thus be studied as a function of time. Systems that are deformed by shear or affected by disturbance can be studied to advantage by this method.

Although the theory and practice of light-scattering have been extending rapidly, the quantitative interpretation of results is mainly concerned with the macromolecules in a monodisperse state in dilute solutions, whereas many systems are polydisperse. The light-scattering method measures the weight average molecular weight (M_w) and in a polydisperse system the results are always higher than, for instance, those obtained from osmotic pressure measurements which give a number average molecular weight (M_n). For asymmetric particles the dissymmetry method, where measurements are taken under equilibrium conditions, is better than the streaming birefringence and viscosity methods (dynamic) which depend on setting up shear gradients with a consequent distortion of the particle shape. Cleverden, Harvey, Laker and Smith[3] describe a statistical treatment (random flight) to show a close similarity between viscosity and light-scattering.

Simplified Theory of Light-scattering

When a beam of light passes through a material the electric field associated with the beam induces periodic oscillations in the molecules of the material; the molecules thus become oscillating dipoles which serve as a source of secondary radiation (scattered light) with a wavelength equal to the incident beam. The small fraction of secondary radiation emitted at a different wavelength (Raman effect, Compton effect and fluorescence) is neglected in the theory. The intensity of this scattered radiation increases with the size of the molecules and the readiness with which they respond to the induced periodic oscillations (called the polarisability). If the molecules have a maximum dimension of less than 1/20th the wavelength of the incident beam they are regarded as single dipoles and are called Rayleigh scatterers. Larger molecules are regarded as a fixed array of point sources in which case the light scattered from one part of the molecule may be out of phase with that coming from another, resulting in destructive internal interference and a lower scattering intensity.

The problem of scattering divides itself into two main categories.

1. Scattering of light from solutions of small molecules.

2. Scattering of light from solutions containing molecules comparable in size with the wavelength of light.

1*a*. *Small Isotropic Particles*

Light scattered by small particles randomly disposed is measured experimentally as the "reduced intensity" (sometimes called the Rayleigh ratio) denoted by R_θ. If I_0 is the intensity of the incident beam of

unpolarised light, i_θ the scattered intensity per unit volume at an angle θ to the incident beam, and r the distance between the observer and the scattering system; then:

$$R_\theta = \frac{i_\theta r^2}{I_0} \quad .. \quad .. \quad .. \quad .. \quad (1)$$

For an unpolarised incident beam the angular dependence of R is given by the relation:

$$R_\theta = R_{90}\,(1 + \cos^2\theta) \quad .. \quad .. \quad .. \quad (2)$$

The scatter is therefore symmetrical about 90° to the incident beam. At 90° the scattered light is completely vertically plane-polarised, the polarisation decreasing on either side of 90°, finally becoming zero at 0° and 180°. Figure 1 shows the relative intensity of scattering [$(1 + \cos^2\theta)$ term] about a small isotropic particle.

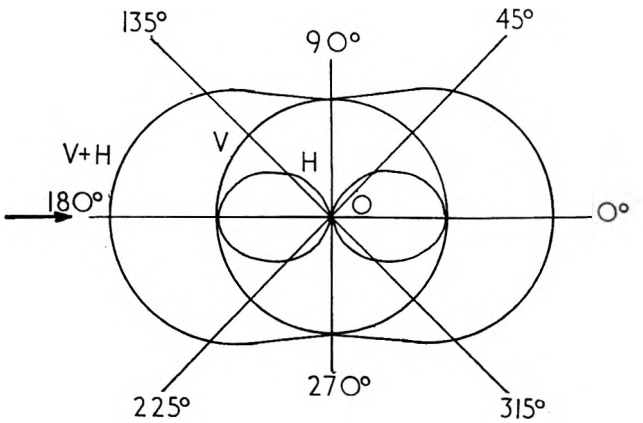

FIG. 1. Angular distribution of scattering of light $(1 + \cos^2\theta)$ by a small isotropic particle at O. V corresponds to the vertically polarised component (the unity term) and H to the horizontally polarised component (which varies at $\cos^2\theta$).

The intensity of light scattered by ν isotropic particles per unit volume of a gas is given by the Rayleigh expression:

$$R_\theta = \frac{8\pi^4 \nu\alpha^2}{\lambda_o^4}\,(1 + \cos^2\theta) \quad .. \quad .. \quad .. \quad (3)$$

where α is the polarisability and λ_o is the wavelength of light in vacuum.

1*b*. *Scattering from Dilute Solutions*

If the molecules are in a highly ordered state as, for instance, in a perfect crystal, total destructive interference takes place and no light is scattered. Pure liquids are ordered, at least compared with the randomness of solute molecules in solution. Smoluchowski[1] explained the scattering in pure liquids by the theory of thermodynamic fluctuations. Molecules of a liquid are in continuous Brownian movement and it is conceivable that within a small element of volume the number of molecules within it, i.e., the density of this element, will vary continuously. In terms

of the whole liquid, density fluctuations occur and the greater the Brownian movement the greater the fluctuations and also the scattering. In solutions, inhomogeneities will be caused by density fluctuations and also by fluctuations in concentration of solute molecules.

Assuming that the solute molecules are large compared with the solvent molecules, equation (3) can be used to obtained a relation between R_{90} (corrected for solvent effects) and the molecular weight of a dissolved macromolecule. The polarisability term (α) is related to dielectric constant (ϵ) which in turn can be put in terms of a measurable quantity called the specific refractive index increment $\left(\frac{n - n_o}{c}\right)$ where n and n_o are the specific refractive indices of the solution of concentration c and pure solvent, according to the relations:

$$4\pi\nu\alpha = \frac{\epsilon - \epsilon_o}{\epsilon_o} = \frac{n^2 - n_o^2}{n_o^2}$$

Replacing ν by $\frac{N_o c}{M}$ where N_o is Avogadro's number, equation (3) becomes:

$$R_{90} = \frac{2\pi^2 n_o^2 (n - n_o/c)^2}{N_o \lambda_o^4} \; cM \quad .. \quad .. \quad (4)$$

or

$$R_{90} = KcM \quad .. \quad .. \quad .. \quad (5)$$

K is a constant for a given solution. By measuring the scattered light at dilute concentrations the molecular weight of the solute can be found.

Alternatively, the scattering may be expressed in terms of diminution in intensity of the incident beam as it passes through the solution. If the intensity is diminished from I_0 to I on traversing a path length l in the solution, then $I = I_0 e^{-\tau l}$. Integrating for scattering (i_θ) over the surface of a sphere gives the result:

$$\tau = \frac{8\pi}{3} R_0 = \frac{16\pi}{3} R_{90} \quad .. \quad .. \quad .. \quad (6)$$

Equation (5) can now be put in the form:

$$\tau = HcM \quad .. \quad .. \quad .. \quad .. \quad (7)$$

where H is the constant for a given solution. Both equations can be used in light-scattering measurements but usually the reduced intensity is preferred since the turbidity of many solutions is very low and difficult to measure accurately.

1c. *Higher Concentrations*

The plot of turbidity: concentration for aqueous sucrose solutions[5] (Fig. 2) shows that at higher concentrations the relationship of equation (7) breaks down. Since the particles are closer together they cease to be independent scatterers and destructive external interference results. In solutions at higher concentrations there will be local fluctuations of the concentration of the solute as well as local fluctuations of the solvent.

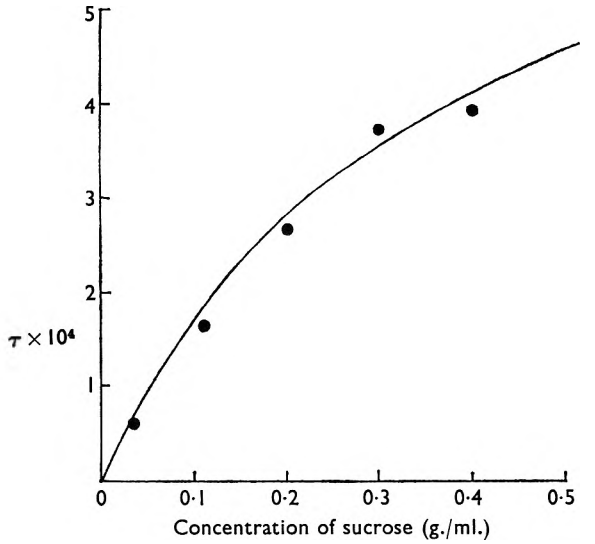

FIG. 2. Variation of turbidity with concentration of an aqueous solution of sucrose (after Debye, *J. phys. Chem.*, 1947, **51**, 18, with permission).

To this additional fluctuation Einstein applied the theory of thermodynamic fluctuations.

Einstein directly related the scattering of light to the osmotic pressure (P) of non-ideal solutions in the Smoluchowski-Einstein equation:

$$\tau = \frac{HRc}{\partial P/\partial c} \quad .. \quad .. \quad .. \quad .. \quad (8)$$

Debye transformed equation (8) into a workable form by resolving the term $\partial P/\partial c$ using the osmotic pressure relation:

$$\frac{P}{RT} = \frac{c}{M} + Bc^2 \quad .. \quad .. \quad .. \quad .. \quad (9)$$

where B is called the interaction constant.

Equation (8) then becomes:

$$\frac{Hc}{\tau} = \frac{1}{M} + 2Bc \quad .. \quad .. \quad .. \quad (10)$$

and also

$$\frac{Kc}{R_{90}} = \frac{1}{M} + 2Bc \quad .. \quad .. \quad .. \quad (11)$$

By taking a series of readings of R_{90} at different concentrations a linear plot is obtained having an intercept equal to the reciprocal of the weight average molecular weight and a slope equal to $2B$.

1*d*. *Anisotropic Particles*

Quite often the particles, although small, are anisotropic. In this case the induced electric moments tend to be of different magnitude in

different directions depending on the shape of the particle. Consequently the 90° scattering will not be completely vertically polarised; as a result the scattering intensity at 90° will be greater than that for an isotropic particle and the Cabannes factor[6] must be applied.

2. *Large Particles*

If any dimension of the macromolecules exceeds $\frac{\lambda}{20}$, the Rayleigh concept of scattering from a single dipole breaks down. Larger particles can be regarded as a fixed array of single dipoles, radiating wavelets in different phases, destructively interfering with each other; multipolar electric and magnetic fields are also set up within the particle. Moreover, the magnitude of interference effects increases with the scattering angle.

Essentially the limiting form of equation (11) for all angles of scattering can be retained by introducing a particle scattering factor $P(\theta)$ defined by the equation:

$$P(\theta) = \sum_i \sum_j \frac{\sin ksr_{ij}}{ksr_{ij}} \quad \ldots \quad \ldots \quad (12)$$

where r_{ij} is the distance between two scattering element i, j, $k = \frac{2\pi}{\lambda}$ (where λ = wavelength of light in solution) and $s = 2\sin\frac{\theta}{2}$.

The values of $P(\theta)$ for a sphere (Rayleigh[7], Gans[8]) of diameter D, a rod (Neugebauer[9]) of length L, and a coil (Debye[5] and also Zimm, Stein and Doty[10]) having a root mean square value of distance between its ends R, are given by the expressions:

Uniform spheres: $$P(\theta) = \frac{3}{x}\left[(\sin x - x\cos x)\right]^2 \ldots x = \frac{ksD}{2} \ldots \quad (13)$$

Rods: $$P(\theta) = \frac{1}{x}\int_0^{2x} \frac{\sin w}{w}\, dw - \left(\frac{\sin x}{x}\right)^2 \ldots x = \frac{ksL}{2}$$

Coils: $$P(\theta) = \frac{2}{x^2}\left[e^{-x} - (1 - x)\right] \ldots x = \frac{(ksR)^2}{6}$$

In practice, $P(\theta)$ can be obtained in either of two ways: (*a*) by the dissymmetry method[5,10], (*b*) by the simultaneous angle and concentration extrapolation of Zimm[11].

The dissymmetry method requires a model. The dissymmetry, previously calculated as a function of some absolute dimension of the model, is measured experimentally and the dimension of the particle determined. For a given angle the dimension gives a value for x in equation (13) and enables $P(\theta)$ to be calculated.

The extrapolation method of Zimm does not require a model. Measurements of R_θ for a wide range of angles and different concentrations are

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plotted $\left(\text{as } \frac{Kc}{R_\theta}\right)$ as a function of $\sin^2\frac{\theta}{2} + kc$ (k an arbitrary constant). Extrapolation along lines of constant concentration to zero angle and constant angle to zero concentration gives an intercept equal to the reciprocal of the molecular weight and a particle scattering factor of unity (after correcting for depolarisation).

So far particles with a relative refractive index (relative to the medium) approaching unity have been discussed. When the relative refractive index is large the scattering pattern is complicated and it is only for spheres that formulae have been developed. Anticipating that the former systems are studied by workers in pharmaceutical chemistry, the reader is referred to Mie's[12] original paper for his calculations on spheres having a high relative refractive index. In multicomponent systems scattering of light will be further complicated by the different refractive index of each component. Treatment of this problem is mentioned in Part II (references 195, 196 and 197).

Practice

The experimental work can be divided into two main parts.

1. Measurement of the reduced intensity and depolarisation of scattered light.

2. Measurement of the refractive index difference between solution and pure solvent.

Reduced Intensity of Scattering

There are variations in design of apparatus for measuring the light scattered by systems of low turbidity but the essential requirements are a source of monochromatic light collimated to give a powerful convergent or parallel incident beam, a glass cell containing the solution, a receiver (usually a 9- or 11-stage photomultiplier) and its signal strength detector (a galvanometer).

Measurements at 90° to the incident beam are inadequate for most work and instruments are adapted to measure scattering over a range of angles on either side of 90°. In the simplest arrangement, the receiving photomultiplier is itself moved, this reduces the optical path lengths of incident and scattered beams to a minimum. The disadvantages are in moving a bulky unit connected to high tension power lines and the large arc described over the range of angles. Alternative designs have a fixed phototube and moveable optical systems which may vary the angle of the incident beam entering the light-scattering cell (see Peaker[13]) or carry the scattered light through a "rotating periscope" into the detector (see M'Ewen and Pratt[14]).

The author uses the apparatus shown in Figure 3, which is described elsewhere[15] and is based on the "Universal" light-scattering apparatus designed at the Colloid Science Department, University of Cambridge[16]. This instrument, elegant in its optical simplicity, enables measurements to be taken every few degrees over a wide range of angles of scattering.

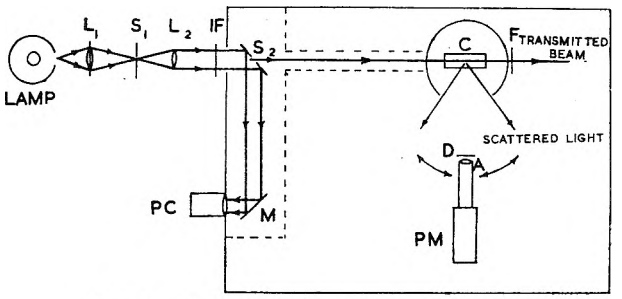

FIG. 3. Diagrammatic sketch of apparatus.

L_1 L_2 = Lenses. S_1 S_2 = Slits.
IF = Interference filter isolating $\lambda = 4358$Å.
F = Neutral density filter.
D = Polaroid disc for measuring depolarisation of scattered light.
A = Aperture. M = Mirror .
C = Cell immersed in thermostat-jacket.
PC = Photocell (connected via switch to galvanometer) for measuring intensity of incident beam.
PM = 11-stage photomultiplier (connected via switch to galvanometer) for measuring intensity of scattered light.

This apparatus has also been adapted by Ottewill and Parreira[17] to follow reaction kinetics. It seems to the writer that the electronics in the "Universal" instrument could be much simplified by use of an 11-stage photomultiplier.

In all light-scattering measurements the elimination of stray scatter is essential to obtain a perfectly linear angular light-scattering envelope. The system must be freed from stray radiation by blackening the surfaces surrounding the cell which contains the solution and also the receiving system, both of which are contained in a light-tight box.

Clarification

The problem of clarification of solutions, particularly when aqueous, is a formidable one. The exclusion of dust, easily seen as bright points when solutions are viewed in the position for small angle scattering, is essential. An all-enclosed glass distillation apparatus, sintered glass or sintered platinum[18] filters, Elford membranes[19], and chemical[20] (precipitation) methods of purification have been described. For aqueous systems, ultrafiltration is probably the most effective but the writer has found that centrifugation and the use of Nos. 4 and 5 sintered glass filters have produced results comparable with other workers.

Calibration

Since the incident and scattering intensities of light are of widely different orders of magnitude, measurement of these to obtain the reduced intensity or Rayleigh's ratio $\left(R_{90} = \frac{i_{90}r^2}{I_0}\right)$ is difficult and most instruments are calibrated.

Several calibrating liquids are in general use. Solutions of polymers (e.g., polystyrene[21] and polymethylacrylic acid[22]) have been found suitable but these substances require careful fractionation and standardisation by other methods. The writer has used a Ludox solution (a colloidal dispersion of spherical silica particles) and benzene and toluene, the organic solvents being preferred for the reasons suggested by Cleverdon, Harvey, Laker and Smith[23]. Corning optical glass and Perspex are used by the writer as secondary standards (suggested by the workers at Cambridge) to check the apparatus at frequent intervals. Correction factors for optical effects of instrumentation are necessary (see Stacey[24]).

Depolarisation

When the incident beam is to be polarised or when measuring depolarisation of the scattered light, polaroid discs with an edge parallel to one axis of transmission are generally used. The presence of dust, secondary scattering and other optical causes can result in false high values of depolarisation. It has been found[25] that photocathodes are not always equally sensitive to light falling upon them in different planes.

Refractive Index

In the Debye interpretation of light-scattering (equation 4) the refractive index difference between solution and pure solvent appears as a squared term and therefore its precise experimental determination is important. Its value is generally of the order 10^{-3} and to obtain an accuracy of 1 per cent difference readings of considerably higher order (approaching 10^{-6}) are necessary. The most suitable instruments are interference refractometers. An adaptation of the Rayleigh interference refractometer for use with monochromatic light is suggested by Grunwald and Berkowitz[26].

Debye[27] and Brice, Halwer and Speiser[28] have designed simple differential refractometers for this purpose.

Accuracy of the Method

The accuracy of light-scattering measurements is largely dependent on the substance investigated and the extent of dilution to which reliable readings can be made, providing the necessary precautions are taken. For dilute solutions of small particles the accuracy would be greater than that at higher concentrations and with larger asymmetric molecules. For most substances which show a tendency to aggregate, for example, proteins and surface-active substances, an accuracy of ± 10 per cent should be possible. Substances that show high dissymmetry would be of a similar order since measurements at low angles of scattered light relative to the incident beam are susceptible to inaccuracies because of the presence of dust.

The International Union of Pure and Applied Chemistry recently organised an investigation on the characterisation of fractions of polystyrene by the light-scattering and other methods. A summarised report by Mark and Frank[21] on a typical sample of polystyrene in toluene showed that errors for the four methods, viz., viscosity, osmotic pressure, light-scattering and sedimentation-diffusion were 1·5, 23·9, 7·5 to 9·0 and

6·6, respectively. The viscosity and ultracentrifuge results were reported as satisfactory, the light scattering discrepancies were attributed to unsatisfactory calibration of the instruments (though consistency on the light-scattering values between the various investigators for the molecular weight and radius of gyration was satisfactory in the writer's view) and the errors in osmometry arising from the unsatisfactory performance of the semi-permeable membranes.

The best values from light-scattering measurements are probably within ± 5 per cent.

APPLICATIONS

Biopolymers and Substances Having Physiological Activity

Light-scattering has made a valuable contribution to the physical characterisation of many biological macromolecules and to studies of the reaction mechanisms which involve changes in molecular weight and other properties.

Some of the more interesting studies are reviewed.

Insulin

On account of its physiological importance insulin has been characterised in detail by many methods. In earlier work discrepancies in the estimates of its molecular weight existed but more recent investigations have led to accurate values being determined.

Molecular weights of 35,000 are now known to be those of aggregates. In 1938 Crowfoot[29] gave a value of 36,000 by X-ray diffraction which clearly indicated from the symmetry of the pattern that the crystals were composed of three sub-units, each of molecular weight 12,000. Detailed amino acid analysis by Sanger[30] in 1949 indicated a minimum molecular weight of either 6,000 or 12,000. More recent investigations by light-scattering[31] give evidence that the minimum molecular weight in aqueous solution is 12,000, which corresponds to a strongly hydrogen-bonded dimer; the reversible association of the protein, the configuration of the aggregates and related thermodynamic properties have since been studied.

Steiner[32] investigated the effect of pH on the tendency of the zinc-insulin to dissociate with increasing dilution (Fig. 4). The curves showed that, at constant ionic strength, dissociation was favoured by an increasing positive charge but the limiting value was the same, giving a minimum molecular weight of 13,000. Increasing the ionic strength was shown to favour association as expected. From these results Steiner considered the dissociation to be stepwise and calculated two dissociation constants for pHs 1·52 and 2·12, indicating trimer formation. At the two higher pH values indications of some association to pentamer formation were apparent. Further measurements at different temperatures gave values for the change in heat content and entropy change associated with the equilibrium states. Similar observations in substantial agreement with Steiner have also been reported by Doty and Myers[33].

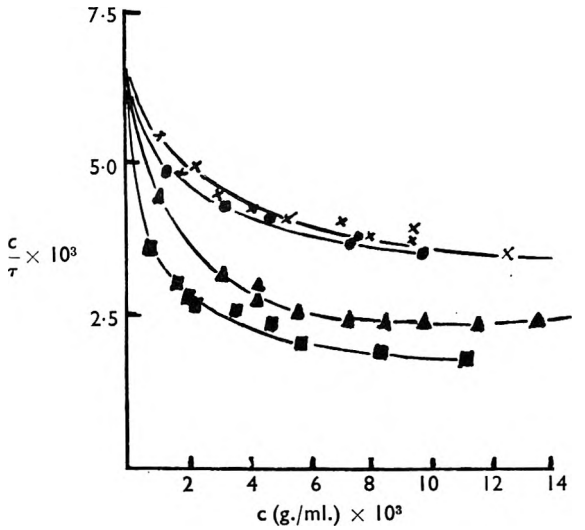

FIG. 4. Dissociation of Zn-insulin on dilution in 0·2M KCl at four different pH values (after Steiner, *Arch. Biochem. Biophys.*, 1952, **39**, 333, with permission).
● pH = 1·52 × pH = 2·12 ▲ pH = 2·65. ■ pH 3·13.

Serum Albumins

Several serum albumins, including horse, pig, human, and bovine have been investigated in some detail by the light-scattering and other methods. Their behaviour in acid solutions, interaction effects in globulin solutions and the influence of salts on the size and shape of many complexes containing a serum albumin component have been examined. The effects of denaturation of serum albumins on their tendency to swelling and aggregation have also been reported.

Results obtained with bovine serum albumin have been found to be reproducible and reliable; this and similar proteins have therefore frequently been used in experiments to study theoretical developments. Doty and Steiner[34, 42] predicted the behaviour and interactions of charged macromolecules and subsequently confirmed their theory experimentally using bovine serum albumin (BSA). The Kirkwood-Shumaker[35] theory was also confirmed experimentally using bovine plasma albumin by Timasheff, Dintzis, Kirkwood and Coleman[36]. It appears that the molecular weights of the different serum albumins obtained by the light-scattering method are generally slightly higher than those obtained by other methods. BSA has probably received most attention and for this substance sedimentation and diffusion studies by Creeth[37] gave molecular weights of 65,400 and 69,000, while osmotic pressure measurements by Scatchard, Batchelder and Brown[38] gave the value of 69,000. Using an absolute photoelectric turbidimeter, a minimum absolute molecular weight of 73,000 for BSA and 79,000 for horse serum albumin were reported by Halwer, Nutting and Brice[39]. They found that the

molecular weight and moisture content of all samples increased by varying amounts with time.

A comparative study of BSA in pure water and 0·15M sodium chloride was made by Danliker[10] to examine the accuracy of measurements on proteins in the absence of supporting electrolytes. By removing salts on mixed-bed ion exchange columns and clearing aggregates by high speed centrifuge, he obtained a molecular weight value for BSA in pure water in good agreement with other methods. The molecular weight in the salt solution was the same as that in pure water within experimental error. In the presence of sodium chloride it was suggested that aggregates were dissociated and were not removed by centrifugation. Although agreement for the molecular weight of the protein in the two solutions was close, the slopes of the reciprocal scattering : concentration curves (Fig. 5) showed a small positive slope for BSA in sodium chloride and a large negative slope for BSA in pure water. The theory of Kirkwood and Shumaker predicted that intermolecular, attractive forces operated over a long range resulting in a negative excess chemical potential for the isoionic BSA in pure water.

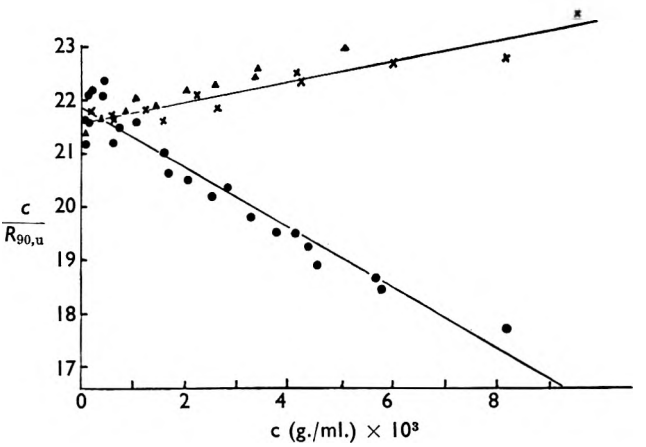

FIG. 5. Light-scattering of bovine serum albumin (after Danliker, *J. Amer. chem. Soc.*, 1954, **76**, 6036, with permission).
○ Centrifuged and measured in water.
× Centrifuged and measured in 0·15M sodium chloride.
▲ Centrifuged in water and measured in 0·15M sodium chloride.

Edsall, Edelhoch, Lontie and Morrison[41] discussed the effects of net proton charge per molecule of BSA and the ionic strength of the solution on the interaction constant B. Measurements at several different concentrations of various salts compared favourably with similar values from osmotic pressure measurements. Better reproducibility, however, could be expected from the light-scattering method (compared with osmotic pressure) where difficulties such as establishing equilibrium between phases across a membrane in the presence of molecules carrying a high net positive charge and at very low ionic strengths, do not arise.

Doty and Steiner[34, 42] observed that, in salt-free acid solutions, the scattering intensity and angular distribution of BSA particles differed considerably from previous observations on this protein. In acid the charged protein molecule becomes surrounded by an electric double layer giving the molecule an effective diameter considerably greater than its molecular diameter, hence interaction between the molecules is then characterised by a distance of closest approach. Due to electrostatic charges on the particles, long-range intermolecular repulsive forces come into play and the particles can no longer be regarded as independent volume elements. External interference of light consequently takes place, resulting in a diminished intensity of scattered light. From their results illustrated in Figure 6 it can be seen that the change in reciprocal scattering

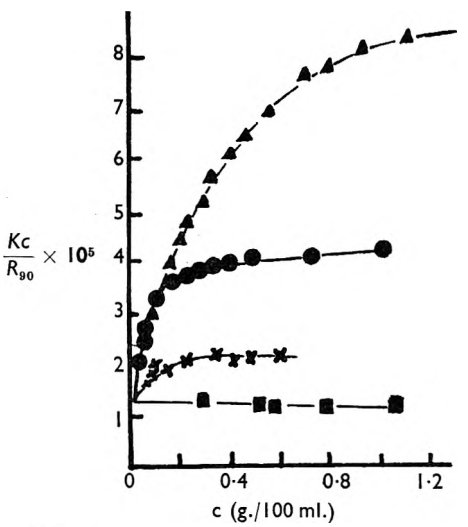

FIG. 6. Variation of $\frac{Kc}{R_{90}}$ with concentration of bovine serum albumin. Deviations from ideal behaviour increase with increasing charge on the particle (after Doty and Steiner, *J. chem. Phys.*, 1949, **17**, 743; 1952, **20**, 85, with permission).

▲ pH = 3·30. ○ pH = 4·10. × pH = 4·36. ■ = pH 5·10.

intensity (Kc/R_{90}) with increasing concentration clearly depends on the charge, the behaviour at pH 3·3 showing a marked deviation from ideality, whilst at pH 5·1 when the protein is isoelectric the change in reciprocal scattering intensity almost vanishes. The increased deviation from linearity with change in pH within the acid range itself is attributed to the change in size of the double layer which varies as the inverse cube of the concentration of gegen ions.

From depolarisation of fluorescence measurements, Weber[43] suggested that the BSA molecule dissociated at pH 1·86 into two sub-units of approximately equal molecular weights. Later Harrington, Johnson and Ottewill[44] carried out further light-scattering studies and found that BSA had a constant molecular weight of 72,000 from pH 4·5 to pH 1·9

(the dissymmetry showing a small change between 1·05 and 1·09); their results from sedimentation velocity also showed no change in molecular weight.

The extent of the process of denaturation of a protein on its physical characteristics (solubility, size and shape, hydration, and electrophoretic phenomena all change to some degree) have been investigated but conclusions are indefinite. The increase in viscosity and decrease in the value of the diffusion constant found when BSA is dissolved in concentrated urea solutions has been attributed to an unfolding or uncoiling of the main polypeptide chains from their native configuration. Doty and Katz[45], however, could not find any increase in dissymmetry of scattered light indicative of such a change. Variation of the pH of the solutions showed that preferential adsorption of water took place on the acid side of the isoelectric point (3,000 molecules of water preferentially bound at pH 3) and preferential adsorption of urea on the alkaline side of the isoelectric point (2,000 molecules urea preferentially bound at pH 8) —neither water nor urea were preferentially bound at the isoelectric point itself. Evidence from these studies led Doty and Katz to the conclusion that the essential change taking place within the BSA molecule is one of isotropic swelling.

In 1950 Greenstein and Hoyer[46] reported that, under special conditions, sodium thymus nucleate prevented the heat coagulation of serum albumin —nearly complete protection of the albumin solutions against heat coagulation was obtained by as little as one part of sodium thymus nucleate to 200 parts of BSA in salt-free solutions.

Geiduschek and Doty[47] investigated the complex formation in solution between BSA and deoxyribonucleic acid (DNA) by light-scattering at room temperature. They mixed the BSA and DNA together and measured the light-scattering by diluting this mixture into a phosphate buffer as solvent. At pH 7·47 and 6·46 no reaction was observed but at pH 5·51 and ionic strength 0·1 DNA was found to bind 35 per cent of its own weight with BSA in a 1 : 1 mixture, i.e., on an average each molecule of nucleic acid bound 11 ± 2 molecules of BSA with no appreciable change in shape of the nucleic acid. Geiduschek and Doty reasoned that, for the protection of BSA by such a small amount of sodium deoxyribonucleate used by Greenstein and Hoyer, the albumin must have been in continuous molecular contact with the nucleic acid. This corresponded to about one serum albumin molecule per nucleotide, representing extremely tight binding in contradiction to the relatively weak interaction shown experimentally by Geiduschek and Doty. From observations of Ambrose and Butler[48] they concluded that strong binding was possible when parts of the protein structure were made accessible by denaturation. The search for a stronger type of binding was subsequently made by Zubay and Doty[49].

In preliminary experiments on individual components, Zubay and Doty showed by light-scattering that the extent to which BSA coagulates was largely dependent on pH or ionic strength. Coagulation was favoured by keeping the negative charge on the albumin at a minimum by either

adjusting the pH to the isoelectric point (4·5) or by the presence of electrolytes. At higher pH values the negative charge rose, electrostatic repulsion between molecules increased and effective contact between them was prevented. No degradation of the DNA took place under the required conditions. When the pH of a mixture having a weight ratio BSA : DNA of 15 was controlled (as in the coagulation of BSA alone) the reaction went to completion, that is, complete binding took place below pH 5, thereby preventing self-aggregation of the serum albumin. Zubay and Doty calculated that at least 1,800 molecules of serum albumin could be bound per DNA molecule. Since the absence of gelation and the presence of a single peak in the ultracentrifuge indicated that there was no cross-linking, the physical characteristics were investigated. The molecular weight determinations by light-scattering were in close agreement with the value calculated, confirming the interpretation of the reaction. Addition of 8M urea to the complex reduced the light-scattering intensity drastically, suggesting that the binding was mainly due to hydrogen bonding.

Lysozyme

Lysozyme was shown to be an enzyme by Meyer, Palmer, Thompson and Khorazo[50] and by Epstein and Chain[51], who suggested that when lysozyme acted on a specific carbohydrate component of cell walls of susceptible micro-organisms it disrupted the cells. The ease with which lysozyme can be obtained in a pure crystalline tetragonal and orthorhombic form lent its readiness to X-ray examination from which a molecular weight of 13,900 was calculated[52]. Values for the wet lysozyme in the orthorhombic and tetragonal crystals was approximately 22,000, indicating about 54 per cent hydration.

The first value for the molecular weight of lysozyme (18,000) was obtained by Abraham[53] in 1939 by ultracentrifugation. Osmotic pressure measurements[54] gave values of 14,700 and later 17,500. Amino acid analysis has also been used, giving molecular weights of 14,700[55] and 14,900[56].

The light-scattering method is capable of giving satisfactory values for the molecular weight of proteins providing that a sufficiently pure and unchanged sample is available. Since lysozyme meets these requirements, its study by the light-scattering method is preferable to osmotic pressure, where, for such small molecules, it is difficult to obtain a semi-permeable membrane which will retain the protein without being so sluggish as to be impractical. Halwer, Nutting and Brice[39] studied this protein in 0·1M sodium chloride at pH 6·2; correcting for a depolarisation factor of 0·03 they gave a molecular weight of 14,800, which was unaltered after centrifuging for one hour at 125,000 *g*. A higher value (15,500) in 0·1M sodium chloride was obtained by Hughes, Johnson and Ottewill[57].

Steiner[58] made a quantitative study by light-scattering of the reversible electrostatic complexing of the two oppositely charged proteins lysozyme (egg white lysozyme has an isoelectric point of approximately 10·9) and bovine serum albumin (isoelectric point of approximately 5·3), and

found that interaction took place at pH values intermediate to the isoelectric points of the proteins; his results also showed that interaction was less as the ionic strength of the solution was increased. Steiner[59] has extended the quantitative aspect of the reversible association processes of globular proteins where association of a single molecule at any instant takes place. He developed equations from information obtainable by the light-scattering and osmotic pressure methods whereby the consecutive association constants of protein associations could be calculated.

Collagen

This protein, regarded as the most prevalent single organic constituent of animal organisms, is essentially a long, thin, partially crystalline, microscopically visible fibre.

In 1927 Nageotte[60] demonstrated that collagenous tissue elements were dispersible in acid solution but on neutralisation or addition of salts the material was reconstituted into an insoluble fibrous or gelatinous state. Since then both the soluble dispersions and the reconstituted fibrous precipitates have received much attention.

Low angle X-ray diffraction studies by Bear[61] and Hall, Jakus and Schmitt[62] showed a filament having a repetitive structure of fibrils. Bear suggested that the significance of this periodic structure may be regarded as either that the primary valency connection of the fibril unit is interrupted at regular intervals to provide a linear string of molecules, or the structure is a repeated pattern of amino acid residues extending along an indefinitely long polypeptide chain.

Bresler, Finogenov and Frenkel[63] studying an homogeneous extract of soluble rat skin collagen in citrate obtained a molecular weight of 70,000 $\pm$ 3,500 by sedimentation and diffusion and dimensions corresponding to a cylinder $380 \times 16{\cdot}7$ Å. The authors suggest that this shape must appear coiled since the degree of polymerisation per residue of 4 Å was calculated to be about 600, which indicates a total polypeptide chain of approximately 2,400 Å.

Osmotic pressure measurements by Matthews, Kulonon and Dorfman[64] gave a number average molecular weight of 74,000 but the authors thought that large aggregates were probably present which made their estimates low compared with those obtained by other methods.

Several recent investigations by light-scattering have been made; most of them giving values for the molecular weight higher than 70,000. In 1954 Gallop[65] investigated an acid-soluble collagen (ichthyocol) from carp swim bladders by sedimentation, viscosity and light-scattering. He concluded that acid dispersions were long, thin, filamentous particles called "protofibrils" (observed previously by M'Ewen and Pratt[14]). His viscosity and sedimentation data gave a tentative axial ratio of the long, filamentous particle about 190 Å assuming it to be extended, but Gallop pointed out that a partial random coil shape was more probable. The mass per unit length was 88 avograms (one gram mass divided by Avogadro's number, see *Chem. Engng News*, 1950, **28**, 1841), that is, approximately one amino acid residue per ångström of particle contour length.

Since a residue can extend up to 3·67 Å along a contour axis, Gallop reasoned that the acid-dispersed particles contained up to three separate polypeptide chains in lateral aggregation. This is in agreement with Bear[66], who suggested that one residue occupies about an ångström of protofibril, the residues being connected end-to-end to form a single regularly coiled polypeptide chain capable of extension to many times its length. By light-scattering the acid-dispersed particles had an effective length of 4,100 Å and a contour length of about 13,400 Å, which is equal to about twenty times the disc-like repetitive structural unit in fibrils observed by electron microscope and small angle X-ray diffraction. From the mass : length value obtained by sedimentation and viscosity measurements Gallop calculated that the protofibrils would contain 50,000–80,000 avograms having a mass : unit length of approximately 120 avograms. He obtained a light-scattering molecular weight of $1{\cdot}67 \times 10^6$ for the protofibril which was somewhat less than the value of M'Ewen and Pratt (3 to 10×10^6) but enormously greater than the result obtained by Bresler and others. In addition, Gallop showed that on heating to only 40° at pH 3·7 the material underwent an irreversible change yielding a product called parent gelatin which had a light-scattering and sedimentation-diffusion molecular weight of nearly 70,000.

At first it may appear that on account of the mild treatment of collagen required to produce the parent gelatin it was the latter substance that gave Bresler his low molecular weight value of 70,000. Although such a large discrepancy existed in the light-scattering molecular weights, the intrinsic viscosities of these samples remained constant.

Boedtker and Doty[67] reinvestigated the problem of the molecular weight and dimensions of soluble collagen in 1956 by several physico-chemical methods; some of their results are tabulated below.

SUMMARY OF MOLECULAR CONSTANTS OF ICHTHYOCOL COLLAGEN
(after Boedtker and Doty, *J. Amer. Chem. Soc.*, 1956, **78**, 4267, with permission)

Method	Molecular weight	Length Å	Diameter Å
Osmotic pressure	310,000	—	—
Light-scattering	345,000	3,100	12·8
Intrinsic viscosity and molecular weight	—	2,970	13·6
Sedimentation and viscosity	250,000	—	12·0
Flow birefringence and viscosity	350,000	2,900	13·3

Attributing the discrepancies between constant values of the hydrodynamic characteristics and the very high light-scattering molecular weights to insufficient optical clarification, Boedtker and Doty carried out a rigorous examination of various samples of collagen solutions at different concentrations and after different times of ultracentrifugation. Their results showed that a stepwise removal of a high scattering component took place with an increase in time and speed of centrifugation, the light-scattering molecular weight decreasing also stepwise to the minimum value of 340,000. If the value of parent gelatin was 70,000 (Gallop) the collagen molecule would probably be made up of 5 basic units of the former material. Boedtker and Doty found, however, the

light-scattering molecular weight of the parent gelatin to be 135,000 (confirmed by osmometry and sedimentation-viscosity) after complete denaturation giving a ratio of collagen : parent gelatin of 2·5 : 1, indicating that the molecules of parent gelatin must be of substantially different weights. It is likely that the discrepancy between the light-scattering molecular weights of 70,000 (Gallop) and 135,000 (Boedtker and Doty) for parent gelatin was caused by hydrolysis. Boedtker and Doty found that the thermal and ageing effects of this process were rather critical. Possible structures of the collagen molecule interpreted from their measurements and those of X-ray studies have been suggested.

Haemoglobin

The physiological importance of haemoglobin and the ease with which it can be prepared in a pure crystalline form has led to its extensive study by various physical methods. The first accurate value for the molecular weight of haemoglobin (67,000) was reported by Adair[68] in 1925 in his investigations on the osmotic pressure of solutions of haemoglobin from different sources. At that time analysis of the iron content gave a precise estimate of the minimum molecular weight (16,700, giving a molecular weight of $4 \times 16{,}700 = 66{,}800$) and this fact made haemoglobin a useful model in the kinetics and thermodynamics of protein solutions. Subsequent work has elucidated the shape and extent of hydration of haemoglobin and also the structural and other changes which take place under different conditions (see Gutfreund[69]). A recent survey of the properties and genetic control of human haemoglobin is given by Itano[70].

By suitable adjustment of conditions, the properties of mammalian haemoglobin indicated that the molecule consisted of four sub-units. Reichman and Colvin[71] tested this supposition by light-scattering experiments on horse haemoglobin. The results showed that in dilute salt solutions below pH 2·5 the haemoglobin dissociated into four units of approximately equal molecular weight; these were subsequently separated by electrophoresis into two different components. In the crystalline form, X-ray analysis showed[69] that the molecule consists of two identical asymmetric halves related by the dyad or two-fold rotational axis of symmetry. The light-scattering weight average molecular weight of horse haemoglobin in 0·05M sodium chloride and pH 2 was 21,000 to 23,000 (osmotic pressure gave 16,000 and equilibrium sedimentation 22,000) rising to 38,000 on increasing the pH to 2·65. At this pH, performic acid oxidation of horse-globin slightly increased the molecular weight to 41,000 (osmotic pressure gave 21,000). Decreasing the pH to the original value of 2·0 did not decrease the molecular weight.

Further light-scattering studies by Haug and Smith[72] on the two electrokinetically different components of horse haemoglobin showed that the faster component with molecular weight of 25,000 associated readily as the ionic strength and pH were increased. The slower component with molecular weight of 17,000 showed little tendency to associate. Evidence from the unfractionated globin indicated that the two components interacted with each other.

Determinations of the dimensions of the haemoglobin molecule have been complicated by the extent of hydration. Bragg and Perutz[73] derived an ellipsoidal shape with dimensions 71 × 53 × 53 Å for horse haemoglobin by X-ray diffraction but the observations Crick[74] suggest that the molecule is probably more irregular than an ellipsoid.

[Other important biological substances and substances having physiological activity will be discussed in Part II of this review which will appear in the April issue of the Journal. Macromolecules of more general interest will also be briefly discussed.]

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RESEARCH PAPERS

THE EFFECT OF HORMONES AND THEIR ANALOGUES UPON THE UPTAKE OF GLUCOSE BY MOUSE SKIN *IN VITRO*

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An experimental system is described for measuring the uptake of glucose by mouse skin *in vitro*. Insulin is found to stimulate and hydrocortisone to depress the glucose uptake of this system when added to the medium in concentrations comparable to those which may occur *in vivo*. Oestrogens, androgens and progestational agents, in contrast, have no effect at approximately physiological levels, but cause some depression of glucose uptake at high concentrations.

Our object was the development of an *in vitro* technique for studying the influence of steroid hormones upon an aspect of skin metabolism. A major contribution to this problem had previously been made by Bullough,[1] who developed a method for studying the effect of hormones upon the rate of mitosis in a preparation of the skin of the mouse. Employing this technique Bullough was able to demonstrate a stimulatory effect of insulin and of oestrogens, and an inhibitory effect of cortisone, upon the rate of mitosis in mouse ear epidermis. He ascribed the stimulatory effects to direct intervention in the glucokinase system. A method based upon determination of mitotic activity, however, is not readily adaptable for routine screening of a large number of compounds. We therefore turned our attention to the measurement of glucose uptake of mouse skin, hoping thereby to develop an assay based upon a more convenient parameter.

Mouse ear shows a very slow rate of glucose uptake, and difficulty was experienced in the development of a method of preparation of the skin which would allow comparable samples of adequate size to be taken for test and control experiments. This was accomplished by mechanical chopping of the skin, by thoroughly mixing the strips of skin from a large number of animals, and by taking large replicate samples. This procedure took longer than would be acceptable with more delicate tissues, but no evidence was obtained that it led to a serious deterioration in response of the skin. A number of experiments were carried out using single pairs of flasks containing samples rapidly prepared in an oxygen atmosphere, but no alteration in sugar uptake or response to oestrone was observed.

Experimental

Mice

Male albinos of the Schofield strain aged 9 to 12 weeks were used. All had been employed five times previously for routine insulin assays. They were starved for 24 hours before use.

Procedure

(i) The mice were killed by breaking the neck, and the ears were removed and cut into strips 1 mm. wide with a mechanical tissue chopper (Hospital and Laboratory Supplies, Ltd.). Strips from 40 pairs of ears were bulked in ice-cold Krebs-Ringer phosphate buffer pH 7·4, and thoroughly mixed. Samples weighing 250 to 350 mg. were then removed, blotted on filter paper, weighed on a torsion balance, and transferred to eight 50-ml. conical flasks each containing 8 ml. of a solution of glucose (0·4 mg./ml.) in Krebs-Ringer phosphate buffer. 0·04 ml. of a solution of the material under test was added to four flasks, and 0·04 ml. of the solvent was added to the remaining four. Propanediol was used for steroids and 0·01N hydrochloric acid for insulin. The flasks were then incubated for four hours at 37° with lateral shaking in an atmosphere of air. Three 2-ml. samples of fluid were taken from each flask, pipetted into 15-ml. centrifuge tubes, and 1 ml. of 0·3N barium hydroxide solution and 1 ml. of 5 per cent w/v zinc sulphate heptahydrate solution were added. After centrifugation, 2 ml. of the supernatant solution was taken for glucose determination by the method of Somogyi[2], using Nelson's colour reagent[3].

(ii) A number of experiments were made in which the conditions used by Bullough and Johnson[4] were followed as closely as possible. Six mice were killed, the ears were chopped rapidly, and the strips were mixed in oxygenated saline. Two 100-mg. samples were rapidly weighed, and transferred to Warburg flasks containing 4 ml. of a medium identical with that used by Bullough and Johnson, except that it contained a lower glucose concentration of 0·4 mg./ml. The flasks were attached to manometers, gassed with oxygen, and incubated at 38° for 4 hours. Three 1-ml. samples were then removed from each flask for glucose assay.

Results

Glucose uptake under the conditions used was found to be approximately linear over 6 hours. The rate of uptake varied from experiment to experiment within the range 0·5 to 1·5 mg. glucose/g. wet tissue/hour. The method of calculation was as follows. For each experiment the ratio of the glucose uptake in each flask containing test material to that of each control flask was calculated; an experiment comprising four control and four test flasks would thus yield sixteen ratios. The ratios of similar experiments were then combined to give a single mean. Results are expressed in Table I in terms of the percentage alteration of glucose uptake at each concentration of test substance in the medium. The limits given are the standard deviations of the means. N is the number of ratios used in the calculation of the results.

No effect of insulin was demonstrable at a concentration of 0·1 μg./ml., but from concentrations of 1 μg./ml. upwards an increasing stimulatory effect on glucose uptake was observed.

Hydrocortisone and prednisolone both markedly inhibited sugar uptake, the effect being demonstrable at concentrations down to 0·01 μg./ml.

TABLE I

THE EFFECT OF ADDED SUBSTANCES ON THE GLUCOSE UPTAKE OF MOUSE EAR *in vitro* USING EXPERIMENTAL PROCEDURE (i)

Substance	Concentration μg/ml.	N*	Per cent change in glucose uptake
Insulin	100	16	+29·2 ± 5·4
	10	20	+19·8 ± 4·7
	1	16	+ 5·4 ± 3·9
	0·1	16	0
Hydrocortisone	10	32	−53·6 ± 6·6
	1	52	−47·3 ± 13·4
	0·1	24	−41·1 ± 16·9
	0·01	32	−11·2 ± 5·0
	0·001	16	0
Prednisolone	1	34	−42·2 ± 10·6
	0·1	16	−39·6 ± 2·7
	0·05	16	−27·2 ± 1·6
	0·01	37	− 5·2 ± 7·5
Cortisone	1	27	−26·5 ± 5·3
	0·1	4	−33·1 ± 3·3
Cortisone acetate	1	9	−30·4 ± 14·0
Desoxycorticosterone acetate	10	16	−29·0 ± 5·1
	1	32	− 7·2 ± 10·6
	0·1	28	0
4-Methylcortisone acetate	1	16	− 6·7 ± 5·5
2-Chloroprednisone acetate	1	4	−21·4 ± 5·6
	0·1	4	0
Oestradiol	10	16	−42·0 ± 18·6
	5	16	−10·7 ± 5·4
	1	96	0
Oestrone	10	20	−16·5 ± 7·9
	1	32	− 4 ± 5·8
	0·1	16	0
Testosterone	10	16	−19·9 ± 12·9
	1	16	0
Pregnenolone	10	32	− 2·8 ± 6·2
	1	16	0
Progesterone	10	32	− 6·2 ± 7·3
	1	16	0
Cholesterol	10	12	0
Glycyrrhetinic acid	10	16	− 6·6 ± 5·8

* N = Number of ratios used in calculating the results.

Cortisone and its acetate were also active. 2-Chloroprednisone acetate, 4-methylcortisone acetate, and desoxycorticosterone acetate showed inhibitory activity only at relatively high concentrations.

Oestrone, oestradiol and testosterone all inhibited glucose uptake at a concentration of 10 μg./ml., but showed little or no effect at lower concentrations. In addition, a further series of experiments showed that no effect of oestrone at a concentration of 1 μg./ml. was demonstrable when experimental procedure (ii) was used.

Progesterone and pregnenolone had a slight inhibitory effect at a concentration of 10 μg./ml. Glycyrrhetinic acid also had a slight inhibitory effect at this concentration, but cholesterol had no effect at a concentration of 10 μg./ml.

The following experimental compounds, not listed in the Table, showed no activity at concentrations of 1 or 10 μg./ml. 3β-Naphthol-Δ^2-*cyclo*penten-1-one-2-acetic acid: 21-acetoxy-16α-methylprogesterone: 16α-methoxydesoxycorticosterone: 1,2-dimethyloestradiol diacetate: 2-chloro-1-methyloestradiol dipropionate: 4-chloro-1-methyloestradiol dipropionate: 4-chlorotestosterone: 11β,20β-dihydroxypregn-4-en-3-one: 3β-hydroxy-6-methylpregn-5-en-20-one.

The last three compounds, at a concentration of 1 μg./ml., did not inhibit the action of cortisone at a concentration of 0·1 μg./ml.

Discussion

The glucose uptake of mouse skin *in vitro* under the conditions used is influenced both by insulin and by the glucocorticoids at concentrations similar to those found *in vivo*. Thus the normal concentration of hydrocortisone in peripheral blood lies within the range 0·02 to 0·23 μg./ml.[5], while mouse skin responds to concentrations below this range. The minimum concentration of insulin at which a response of skin is detectable is just above the upper limit of normal serum concentrations, which are believed to lie within the range 0·005 to 0·5 μg./ml.[6], but is not so high as to be of doubtful physiological significance. Thus these two hormones show effects *in vitro* in accord with their actions *in vivo*, and in agreement with Bullough's observations of their effect upon mitosis in skin *in vitro*. It is therefore of interest that similar agreement is not obtained with Bullough's observations of the effect of oestrogens. Whereas Bullough found that stimulation of mitosis was caused by oestrogen, we find that the only demonstrable effect on glucose uptake is an inhibitory one at high oestrogen concentrations. Our findings are more in accord with those of Shelley and Hurley[7], who found that implanted oestrogens had no demonstrable effect upon human skin.

The authors wish to thank Dr. D. Burn, Dr. B. Ellis, Dr. D. N. Kirk, and Mr. D. M. Williamson for the preparation of some of the compounds tested. Their thanks are also due to Dr. A. David for providing facilities for the tissue studies.

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WATER-SOLUBLE CELLULOSE DERIVATIVES*

Uses as Primary Emulsifying Agents. Part I

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The ability of methyl-, methylethyl-, and sodium carboxymethylcellulose to promote emulsions with liquid paraffin and arachis oil is largely independent of the viscosity of their mucilages. Methyl- and methylethyl-cellulose are more efficient emulsifying agents than is sodium carboxymethylcellulose. Emulsifying efficiency increases with the concentration of the derivative, and low viscosity grades are more efficient than high viscosity grades. Mixtures of these derivatives have little advantage as emulsifying agents over methyl- and methylethyl-cellulose used singly.

Earlier work[1,2] was concerned with determining the factors influencing the viscosity of aqueous dispersions of certain water-soluble cellulose derivatives. The purpose of this investigation was to study the use of these same derivatives as emulsifying agents, and, in the light of our previous findings, to attempt to clarify the role of viscosity and other factors in emulsion formation and stability. The effects of varying the concentration, viscosity grade and type of derivative are discussed.

Materials

The cellulose derivatives used included six viscosity grades of methylcellulose,† one viscosity grade of methylethylcellulose,‡ and three viscosity grades of sodium carboxymethylcellulose.§ The substances emulsified were liquid paraffin B.P. and arachis oil B.P. Chemicals were of A.R. quality.

Methods

Our previous work showed that the usual accelerated storage tests would be unsuitable for emulsions made with the cellulose derivatives. In the experiments described below, emulsions were stored for periods of up to 1 year, during which globule counts and macroscopic examinations were made at frequent intervals.

Preparation of Emulsions

Mucilages were prepared as previously described[1] and were allowed to stand for 24 hours. Emulsions (200 ml. quantities) containing 25 per cent v/v of liquid paraffin or arachis oil were made by adding the mucilage to the oil and mixing with a propeller at a speed of 3000 revs./min. for

* 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.

† Celacol M20, M50, M100, M450, M2500 (British Celanese Ltd.); Methocel 4000 (Dow Chemical Co.).

‡ Edifas A (Imperial Chemical Industries Ltd.).

§ Cellofas B, low, medium and high (Imperial Chemical Industries Ltd.).

10 minutes. The product was passed once through a hand-operated homogeniser and left to stand for 24 hours before examination.

Globule Counting

The method of globule counting was based on the techniques described by Cockton and Wynn[3] and Levius and Drommond[4], using a haemocytometer of 1/10 mm. depth and a ruled disc in the microscope eyepiece. Results were expressed as the value of "H", which is the number of millions of globules into which 1 cu. mm. of oil has been subdivided.

Reproducibility of Results

Some emulsions, particularly those which were later found to be the least stable, broke on dilution. The most stable emulsion appeared to be that containing a high concentration (3·375 per cent w/v) of a low viscosity grade (Celacol M20) of methylcellulose. Counts on 40 samples of a single batch of this emulsion gave a coefficient of variation of 10·75 per cent, and on single samples of 20 different batches 10·25 per cent. This indicated that the variation resided in the sampling, diluting and counting, rather than in the preparation of the emulsions. We attribute it largely to a small but variable degree of break-down on dilution.

The following procedure was adopted. Emulsions were prepared in duplicate and three counts were made on each batch. If the coefficient of variation of the six counts was less than 10 per cent, the mean was recorded; if not, the emulsions were examined macroscopically only. Subsequently, one sample of each batch was counted. If the variation was within the above limits the mean was recorded; if not, a further three samples of each batch were examined before discontinuing the counts and relying solely on macroscopic observations.

Experimental and Results

(I) Cellulose Ethers Used Singly

Liquid paraffin emulsions. Emulsions of liquid paraffin made with methyl-, methylethyl-, and sodium carboxymethyl-cellulose were stored for 1 year at room temperature and examined at 1 day, 1, 2, 4, 8, 12, 24, 36 and 52 weeks. The results (Tables I to III) show that: (*a*) The higher the concentration of emulsifying agent, the larger was the "H" value and the greater the stability of the emulsion. (*b*) The lower the viscosity grade of the emulsifying agent, the higher was the "H" value and the greater the stability of the emulsion. This is so whether the emulsions are compared for concentration or the viscosity of the continuous phase. (*c*) Methylethylcellulose yielded coarser emulsions than the low viscosity grades of methyl- and sodium carboxymethyl-cellulose. They were, however, more stable than methylcellulose emulsions, and sodium carboxymethylcellulose emulsions were the least stable.

Arachis oil emulsions. Emulsions of arachis oil made with methylethylcellulose, and the low viscosity grades of methyl- and sodium carboxymethyl-cellulose were stored for 6 months at room temperature and

TABLE I*

SHELF STORAGE OF LIQUID PARAFFIN EMULSIONS MADE WITH METHYLCELLULOSE

Derivative grade	Concentration (per cent)	Efflux time of mucilage (sec.)	Value of "H"		Coefficient of variation	Cream volume (per cent)	Stage of oil separation (weeks)	Remarks
			Initial	Final				
M20	4·5	540	46·3	48·1 (52)	7·5	50 (24, 52)	—	Thick, pourable
	4·0	345	46·9	48·2 (36)	10·9	50 (24, 52)	—	Thick, pourable
	3·0	145	35·2	33·3 (36)	9·6	50 (24) 25 (52)	36–52	Fluid
	2·0	63	18·4	19·5 (24)	6·8	33 (1) 25 (52)	24–36	Very fluid
	1·0	38	13·9	16·0 (1)	—	33 (1) 25 (52)	1–2	Very fluid
M50	2·5	510	9·3	8·9 (12)	8	75 (1, 52)	12–24	Thick, pourable
	2·0	246	9·1	6·9 (4)	11	50 (1) 33 (52)	8–12	Thick, pourable
	1·5	122	6·0	7·3 (1)	—	50 (1) 33 (24, 52)	8–12	Fluid
	1·0	59	—	—	—	50 (1) 25 (52)	2–4	Very fluid
	0·5	39	—	—	—	50 (1) 25 (52)	0	Incompletely emulsified
M100	2·25	648	5·5	4·9 (2)	14·5	66 (4, 52)	8–12	Thick, pourable
	2·0	417	5·7	5·1 (4)	11	50 (12, 52)	8–12	Thick, pourable
	1·5	180	6·8	—	—	33 (2, 52)	8–12	Fluid
	1·0	75	—	—	—	33 (2, 52)	1–2	Very fluid
	0·5	41	—	—	—	33 (2, 52)	0	Incompletely emulsified
M450	1·5	422	Not examined					Good appearance but broke down on dilution
M2500	1·0	450	Not examined					Incompletely emulsified
4000	0·9	450	Not examined					Incompletely emulsified

*EXPLANATORY NOTES

TABLES I TO V

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.

TABLE II

SHELF STORAGE OF LIQUID PARAFFIN EMULSIONS MADE WITH METHYLETHYLCELLULOSE

Concentration (per cent)	Efflux time of mucilage (sec.)	Value of "H"*	Cream volume (per cent)	Stage of oil separation (weeks)	Remarks
4·0	449	—	75 (24) 50 (52)	—	Thick, pourable
3·5	240	—	75 (8) 50 (52)	—	Thick, pourable
3·0	138	20·6 20·8 (4 weeks)	75 (1) 50 (12,52)	—	Readily pourable
2·0	60	15·5	50 (1, 52)	36–52	Fluid
1·0	36	10·7	50 (1, 52)	36–52	Very fluid

* In many instances clumping of globules around the partially-etherified fibre particles in the thick emulsions and in the cream layers of the less viscous samples made it impossible to prepare acequately dispersed dilutions for counting purposes. Unless otherwise stated, the value quoted is the initial "H" value.

examined at 1 day, 1, 2, 4, 8, 12, and 24 weeks. The results (Table IV) show that (*a*) Although methyl- and methylethyl-cellulose promoted emulsions which had higher "H" values than the corresponding liquid paraffin emulsions, they were generally much less stable. Methylethyl-cellulose appeared to be the better emulsifying agent for arachis oil. (*b*) Generally, the higher the concentration of emulsifying agent, the greater was the stability of the emulsion. (*c*) Sodium carboxymethyl-cellulose failed to completely emulsify the arachis oil.

(II) CELLULOSE ETHERS USED IN COMBINATION

Emulsions of liquid paraffin and arachis oil made with various mixtures of the ethers were stored for 6 months at room temperature and examined at 1 day, 3 months and 6 months. Comparison of the results, summarised in Table V, with those set out in the earlier tables leads to the following general observations.

Liquid paraffin emulsions. The combination of methylcellulose M20 with higher viscosity grade derivatives made for far greater emulsifying efficiency than would have been achieved by the latter components alone in the concentrations used in the mixture. If mucilages of approximately equal viscosities are considered, methylcellulose M20 used alone promoted more stable emulsions than did any combination of it with the other derivatives. Similarly, if the basis of comparison is mucilages of equal concentration the mixed emulsifying agents do not offer any advantage over methylcellulose M20 used alone.

Methylethylcellulose mixtures gave similar results, except that those containing low or medium grades of sodium carboxymethylcellulose as the other component gave more stable emulsions than did 1 per cent methylethylcellulose alone.

Arachis oil emulsions. Although all the emulsions made with the methylcellulose mixtures showed oil separation after 12 weeks' storage, they were more efficient emulsifying agents for arachis oil than any of the components used singly.

TABLE III

SHELF STORAGE OF LIQUID PARAFFIN EMULSIONS MADE WITH SODIUM CARBOXYMETHYLCELLULOSE

Derivative grade	Concentration (per cent)	Efflux time of mucilage (sec.)	Value of "H"		Coefficient of variation	Cream volume (per cent)	Stage of oil separation (weeks)	Remarks*
			Initial	Final				
Low	3·675	573	47·8	53·3 (24)	9	75 (36) 50 (52)	12–24	Thick, pourable
	3·25	387	54·2	52·4 (2)	2·2	75 (36) 50 (52)	12–24	Thick, pourable
	2·5	160	—	—	—	50 (24) 33 (52)	4–8	Pourable. Incompletely emulsified before homogenisation
	1·5	64	—	—	—	—	0	Incompletely emulsified
	1·0	42	—	—	—	—	0	Incompletely emulsified
Medium	1·3	575	40·5	41·8 (4)	1·7	0	4–8	Thick, pourable. Incompletely emulsified before homogenisation
	1·1	340	31·9	31·8 (1)	—	95 (8) 75 (24, 52)	2–4	As above
	0·9	182	—	—	—	—	0	Incompletely emulsified
	0·7	103	—	—	—	—	0	As above
	0·5	63	—	—	—	—	0	As above
High	1·0	536	18·9	18·4 (1)	—	0	2–4	Incompletely emulsified before homogenisation. Pools of oil had separated by 24 weeks
	0·9	349	18·4	—	—	50 (2) 25 (24, 52)	1–2	As above
	0·7	207	—	—	—	—	0	Incompletely emulsified
	0·5	101	—	—	—	—	0	As above
	0·3	51	—	—	—	—	0	As above

* There was no difference in the behaviour of emulsions stored in the light and in the dark.

The mixtures containing methylethylcellulose were more efficient emulsifying agents than any of the components alone in the concentrations used in the mixture. However, they were generally no more efficient than methylethylcellulose used alone in equivalent concentration, and much less efficient than this derivative used in concentrations yielding mucilages of equivalent viscosity.

DISCUSSION

Influence of Viscosity Grade

Because the cellulose derivatives have the common property of yielding viscous dispersions in water, they have been regarded as emulsion-stabilisers rather than emulsion-promoters. Our results show, however,

TABLE IV

SHELF STORAGE OF ARACHIS OIL EMULSIONS

Derivative	Concentration (per cent)	Efflux time of mucilage (sec.)	Value of "H"*	Cream volume (per cent)	Stage of oil separation (weeks)	Remarks
Methyl M20	4·5	546	141·6 147·6 (8 weeks)	0	—	Thick, pourable. The surface became oily and granular by 24 weeks
	3·0	149	136	0	—	As above
	2·0	61	—	25 (24)	12–24	Frank breakdown on storage
	1·0	37	—	25 (24)	12–24	As above
Methyl-ethyl	4·0	452	231·6	0	—	Thick, pourable. Counts discontinued because of dispersion difficulties
	3·0	145	176	75 (24)	12–24	As above
	2·0	60	—	50 (24)	12	Fluid. Granular appearance and thin continuous oil film by 24 weeks
	1·0	35	—	50 (12, 24)	12	Very fluid. "Pitted" surface with free oil by 24 weeks
S.C.M.C. (low)	3·675 and under	570 and under	—	—	0	All incompletely emulsified

* Unless otherwise stated the value quoted is the initial "H" value.

that some of them are efficient primary emulsifying agents and, further, that their ability to promote emulsions owes little to the viscosity, as such, of their mucilages.

One of the most interesting aspects of this work was the higher emulsifying efficiency of low as compared with high viscosity grade derivatives. This was particularly well marked in the methylcellulose series. The explanation may lie in the rates at which the various derivatives diffuse to the interface. It is known that some low molecular weight agents diffuse more rapidly to the interface than high molecular weight agents of analogous structure, although the latter may ultimately be more strongly adsorbed[5]. It is for this reason sometimes considered advantageous to use a mixture of low and high molecular weight agents as an emulgent.

Influence of Concentration

An increase in the concentration of emulsifying agent led to a decrease in the size of the dispersed particle. At the same time, irrespective of the viscosity of the continuous phase, the "H" values of any group of emulsions increased as the viscosity grade of the emulsifying agent decreased, and there were also differences in the "H" values obtained with the various derivatives. Also, mucilages which had been appreciably

TABLE V

SHELF STORAGE OF LIQUID PARAFFIN AND ARACHIS OIL EMULSIONS MADE WITH MIXTURES OF DERIVATIVES

Derivatives	Efflux time of mucilage (sec.)	Disperse phase*	Value of "H"†	Cream volume (per cent)	Stage of oil separation (weeks)	Remarks
Methyl M20 1 per cent and S.C.M.C. (low) 2·5 per cent	633	A	23·5	50 (24)	24	Thick, pourable. Oil film on surface by 24 weeks
		B	252·0	0	12	Thick, pourable. Frank breakdown by 12 weeks
Methyl M20 1 per cent and S.C.M.C. (medium) 0·8 per cent	567	A	—	80 (12) 50 (24)	24	Thick, pourable. Oil film by 24 weeks
		B	249·2	0	12	Thick, pourable. Frank breakdown by 12 weeks
Methyl M20 1 per cent and S.C.M.C. (high) 0·6 per cent	632	A	—	75 (24)	24	Thick, pourable
		B	194·2	0	12	Thick, pourable. Frank breakdown by 12 weeks
Methyl M20 1 per cent and Methyl 4000 0·65 per cent	520	A	—	40 (12, 24)	12	Thick, pourable
		B	58·8	0	12	Thick, pourable. Frank breakdown by 12 weeks
Methylethyl 1 per cent and S.C.M.C. (low) 2·5 per cent	646	A	26·2	75 (24)	—	Thick, pourable
		B	182·4	0	12	Thick, pourable
Methylethyl 1 per cent and S.C.M.C. (medium) 0·8 per cent	530	A	16·4 15·3 (12 weeks)	75 (12) 60 (24)	—	Thick, pourable
		B	47·2	25 (24)	12	Thick, pourable
Methylethyl 1 per cent and S.C.M.C. (high) 0·6 per cent	680	A	4·3	75 (12) 60 (24)	12	Thick, pourable
		B	72·0	25 (24)	12	Thick, pourable
Methylethyl 1 per cent and Methyl 4000 0·65 per cent	502	A	—	75 (12) 50 (24)	12	Thick pourable Very coarse
		B	39·8	50 (24)	12	Thick, pourable. Granular appearance by 24 weeks

* A = liquid paraffin, B = arachis oil.
† Unless otherwise stated the value quoted is the initial "H" value.

reduced in viscosity by heating yielded emulsions which had "H" values similar to others made with the undegraded mucilages[6]. The degree of dispersion, therefore, must be chiefly determined by some factor other than viscosity but which varies with the concentration of the emulsifying agent. Interfacial tension experiments suggest that this factor is the rate and extent of adsorption of the emulsifying agent at the interface[7,8].

Fordham[7] found that the interfacial tension between a solution of

sodium carboxymethylcellulose and liquid paraffin decreased as the strength of the solution increased until a concentration of between 0·88 and 1·4 per cent was reached. For still stronger solutions the interfacial tension was independent of concentration. With methylethylcellulose solutions interfacial tension remained independent of concentration when a strength of 0·38 per cent was reached. Although the range of concentrations over which interfacial tension decreases is narrower than that over which we have observed variations in "H" values, Fordham's records of interfacial tension as a function of time showed that the initial rate of fall was greatest for the strongest solutions. This would explain why we continued to obtain progressively higher "H" values as the concentration of emulsifying agent was increased to relatively high levels.

Influence of Derivative

Methyl- and methylethyl-cellulose were much more efficient emulsifying agents for both liquid paraffin and arachis oil than was sodium carboxymethylcellulose. Since the sample of methylethylcellulose contained a proportion of only partially-etherified fibre[1] it is difficult to compare its efficiency with that of methylcellulose on a concentration basis. Aesthetically, methylethylcellulose emulsions were unsatisfactory. Not only did the derivative produce more foam than methylcellulose, but, on storage, the partially-etherified fibre separated to give a third and sometimes even a fourth layer in creaming emulsions. Nevertheless, it emulsified very readily and emulsions containing it in adequate concentration were extremely stable. For arachis oil it was the best emulsifying agent, and it is possible that a "purer" sample would have proved generally superior to methylcellulose. Morrison and Campbell[8] also considered methylethylcellulose a better emulsifying agent than sodium carboxymethylcellulose, particularly at low concentrations, and both they and Fordham[7] have related their observations to the results of interfacial tension studies. Morrison and Campbell preferred a high rather than a low grade of sodium carboxymethylcellulose for emulsifying liquid paraffin, but they found that when the concentration was reduced to 1 per cent or less emulsification became progressively more difficult. Our results show that higher concentrations of lower viscosity grades are far more efficient as emulsifying agents than lower concentrations of higher viscosity grades. Like us, Morrison and Campbell found that the presence of methylethylcellulose improved the emulsifying ability of sodium carboxymethylcellulose, but apart from the fact that such mixtures foamed less we are not convinced that they offer any special advantage over methylethylcellulose used alone. Sodium carboxymethylcellulose itself is unsatisfactory as an emulsifying agent for arachis oil; indeed, it would seem that liquid paraffin is one of the few substances with which it can be used with any degree of success[8].

Degree of Dispersion and Stability

The stability of an emulsion is not necessarily a function of its degree of dispersion. An emulsion promoted by a mucilage which had been

reduced in viscosity by heating deteriorated very much more rapidly than one made with the unheated mucilage, although both, initially, had similar "H" values[6]. Again, arachis oil emulsions had considerably higher "H" values initially than did liquid paraffin emulsions, yet the former were much less stable than the latter. And although in the liquid paraffin series, some of the sodium carboxymethylcellulose emulsions yielded the highest "H" values recorded, and the methylethylcellulose emulsions the lowest, the former were generally the most unstable and the latter the most stable of the series.

Observations on the Globule Counting Procedure

Because of the tendency of the emulsions to break down on dilution, the method as applied has not proved a generally suitable one for long-term stability studies on cellulose derivative emulsions. Only with a few emulsions was it possible to continue the counts over the entire storage period, and, since these were in fact the most stable emulsions, the globule counts remained largely unaltered. Those emulsions which subsequently proved to be unstable broke on dilution before the incipient instability could be demonstrated. In no instance, therefore, has it been possible to detect any deterioration by microscopical examination. These difficulties apart, the quantitative results have provided some support for the conclusions drawn from the macroscopic observations, and the initial counts, in particular, have shed an interesting light on the comparative emulsifying properties of the various derivatives studied.

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ON THE PHARMACOLOGY OF PETALINE CHLORIDE, A CONVULSANT ALKALOID FROM *LEONTICE LEONTOPETALUM* LINN

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The alkaloid petaline chloride, obtained from extracts of *Leontice leontopetalum* Linn. is a more potent convulsant poison than leptazol. At lower dose levels it appears to reduce the convulsant activity of leptazol and apparently gives some protection from electrically induced seizures. Petaline chloride depresses both the patellar tendon reflex and the crossed extension reflex; it also has muscle relaxant activity and increases the rate, force and amplitude of the beat of the isolated auricle depressed in a low calcium medium. These properties are discussed in relation to its use in folk medicine.

EXTRACTS made from the fresh tuberous roots of *Leontice leontopetalum*, a plant which grows wild in the Lebanon, are used there as a folk remedy for *grand mal* epilepsy. An account of their preparation, actions, dosage and use is given by McShefferty and others[1] who, quoting a personal communication by Dr. W. M. Ford Robertson and Dr. A. S. Manugian of the Lebanon Hospital for Mental and Nervous Disorders, Asfuriyeh, Beirut, state that initially the juice from the fresh tubers is given in a dose of one teaspoonful three times daily for 3 days. This induces *status epilepticus*. After the initial treatment, large quantities of an aqueous extract of the marc left after preparation of the juice are given each day for several months. McShefferty and others[1] have investigated the chemistry of *L. leontopetalum* and Nelson and Fish[2,3] have described its history, sources, macroscopical and microscopical characters. A preliminary pharmacological investigation has also been made of the alkaloids petaline chloride and leonticine[1].

Petaline chloride, 0·05 mg. caused sedation and slowed respiration in 25 g. mice. Larger doses (0·075 mg.) induced respiratory distress and varying degrees of central nervous system stimulation. 0·08 or 0·1 mg. caused death from acute respiratory failure. It was concluded that the MLD of petaline chloride in the mouse was approximately 3·1 mg./kg. In the rabbit a total dose of 30 to 40 mg. of petaline chloride caused respiratory depression, mild clonic convulsions and death and the approximate MLD was 15·6 mg./kg. Petaline chloride was also shown to antagonise the stimulant actions of acetylcholine on the isolated frog rectus abdominis muscle. On this preparation it had about 65 per cent of the potency of gallamine triethiodide and its effects were antagonised by eserine.

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Extracts of the tubers of *L. leontopetalum* are undoubtedly complex mixtures and probably contain several pharmacologically potent compounds each of which may possess different types of activity. Results obtained from a study of one compound can naturally not be taken as expressing the whole activity of the plant, but the bizarre manner in which extracts are made use of by local inhabitants, the reports of confirmed cures of epilepsy[1] and the convulsant activity of the extracts seemed of sufficient interest for us to investigate more fully the properties of petaline chloride before considering the properties of extracts and other active principles. We are much indebted to Dr. J. B. Stenlake of the School of Pharmacy, Royal College of Science and Technology, Glasgow, for supplies of petaline chloride.

Methods and Results

Convulsant: Anticonvulsant Activity

Comparison with leptazol. The method used was based on that of Goodman and others[4]. Male albino mice weighing 20 ± 1 g., or 25 ± 1 g., were used. Each group contained not less than 10 mice, the weight of the individuals remaining constant. Drugs in aqueous solution were injected into the tail vein. The volume of solution injected at each dose level was constant and the duration of injection did not exceed 2 to 3 seconds. The range of doses used is shown in Table I and the approximate CD50 and LD50 were obtained graphically for petaline chloride and leptazol. (Figs. 1 and 2). The CD50 of leptazol was found to be 40 mg./kg. and that for petaline chloride 6·6 mg./kg. The LD50 of leptazol was 56 mg./kg. and the LD50 of petaline chloride was 9·2 mg./kg. Petaline chloride is clearly a more potent convulsant and is more toxic than leptazol.

Effect of pre-treatment with petaline chloride on the response to leptazol. Three groups, each containing not less than 50 mice, were used. The weights of individuals in each group were constant at 20 ± 1 g. or 25 ± 1 g.

TABLE I

Convulsant activity and mortality in mice after petaline chloride or leptazol (figs. 1 and 2)

Petaline chloride dose mg./kg.	Per cent convulsing	Per cent mortality
4	10	—
6	40	—
8	70	20
10	100	70
12	100	100
Leptazol dose mg./kg.	**Per cent convulsing**	**Per cent mortality**
30	13	—
40	51·4	11·4
50	83·3	22·2
60	92·8	57·1
80	100	100

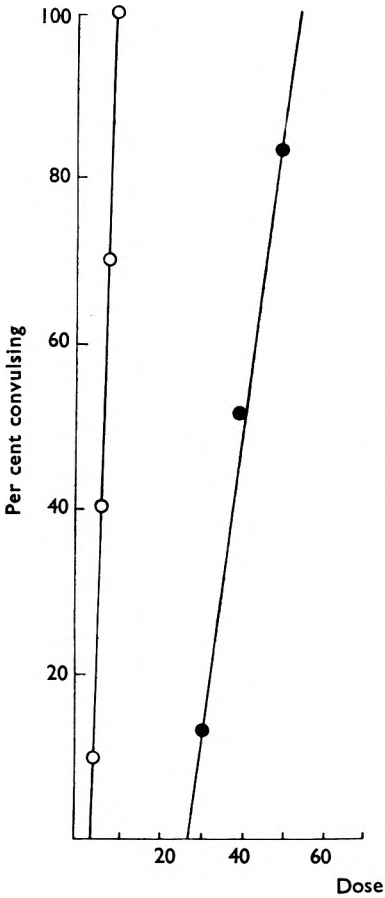

FIG. 1. Convulsant activity of petaline chloride (O—O) and leptazol (●—●) on the mouse.

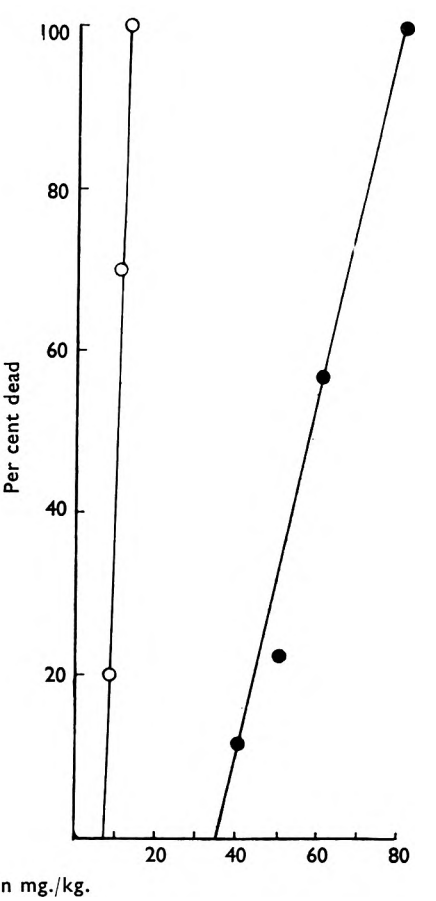

FIG. 2. Mortality in mice from petaline chloride (O—O) and leptazol (●—●).

The groups were given respectively 2·5, 5·0, or 8·0 mg./kg. of petaline chloride by intravenous injection. After an interval of 45 to 60 minutes the survivors of each large group were divided into four groups of about 10 and each group was given leptazol by intravenous injection. The doses of leptazol used were from 20 to 75 mg./kg. (Table II). The CD50 and LD50 of leptazol were calculated for the pre-treated animals and found to be respectively 42·8, 43·5 and 35 mg./kg. and 64, 70·7 and 48 mg./kg. (Figs. 3 and 4). Lower doses of petaline chloride may, therefore, give some protection but higher doses increase both convulsant activity and toxicity.

Effect of petaline chloride on electro-shock seizures. The method used was based on that of Swinyard[5] but ear clip electrodes[6] were employed. A supra-threshold current intensity of 20 mA applied for 5 seconds caused tonic extension of the hind limbs, which was taken as the end point.

TABLE II

CONVULSANT ACTIVITY AND MORTALITY OF LEPTAZOL GIVEN 45 TO 60 MINUTES AFTER PETALINE CHLORIDE. (FIGS. 3 AND 4)

Leptazol dose mg./kg.	Petaline chloride dose mg./kg.			Petaline chloride dose mg./kg.		
	2·5	5·0	8·0	2·5	5·0	8·0
	Per cent animals convulsing			Per cent mortality		
20	—	—	0	—	—	—
25	0	—	10	—	—	0
30	—	0	—	—	—	—
37·5	20	40	—	0	—	—
40	62·5	—	68·75	—	—	12·5
50	90	60	100	10	0	58·3
60	—	—	—	42·8	—	70·5
62·5	—	100	—	71·4	30	—
75	100	—	—	80	60	—

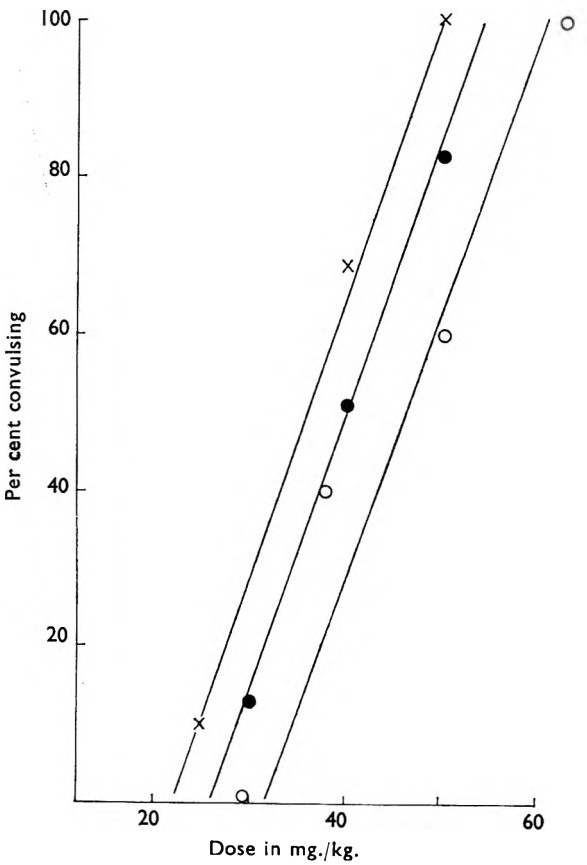

FIG. 3. Effects of pretreatment with petaline chloride, 5 (O—O) or 8 (×—×) mg./kg. on convulsant activity of leptazol (●—●).

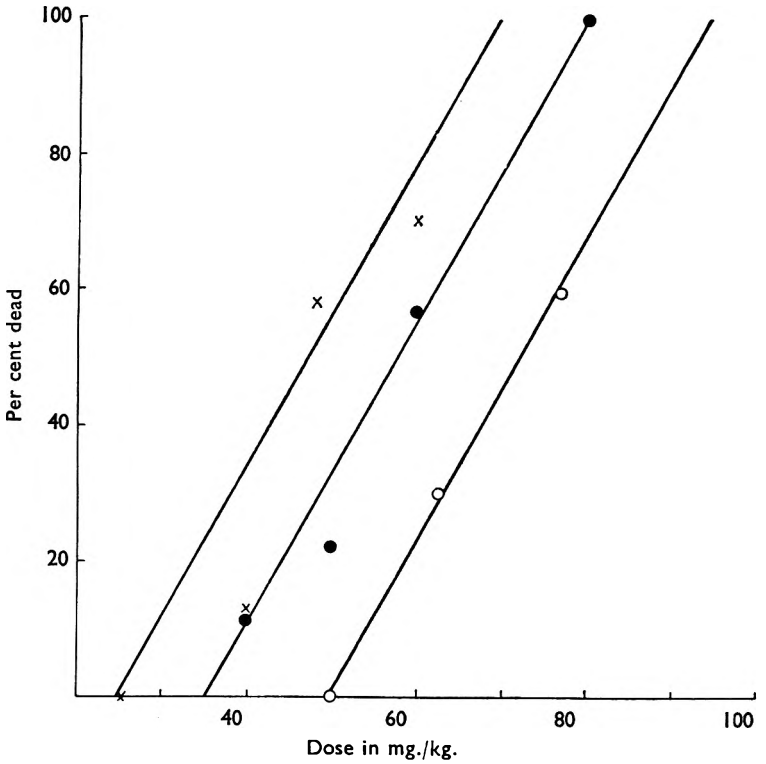

FIG. 4. Effects of pre-treatment with petaline chloride 5 (O—O) or 8 (×—×) mg./kg. on mortality due to leptazol (●—●).

Seven groups of 10 male albino mice were employed, the weights of the members of each group were constant at 10 ± 1, 15 ± 1 or 20 ± 1 g. All animals to be used for the tests were treated with a supra-threshold current of 20 mA for 5 seconds, 24 hours before the experiment and those which did not give the end point were discarded. Three groups of 10 mice were pre-treated with 4·0, 5·0 or 6·6 mg./kg. of phenobarbitone by intraperitoneal injection. The remaining four groups were given by the same route 1·66, 2·5, 3·3, or 5·0 mg./kg. of petaline chloride. After an interval of 1 hour electroshock treatment was repeated. The number of mice in each group failing to show the end point was counted and the percentage protection calculated (Table III, Fig. 5). Petaline chloride is apparently capable of conferring some protection but it is very much less potent than phenobarbitone. At higher doses petaline chloride protection was reduced.

Muscle Relaxant Activity

Neuromuscular transmission in the cat. The method adopted was that described by Lewis and Muir[7]. Cats weighing from 2·0 to 3·0 kg. were anaesthetised by intraperitoneal injection of 60 mg./kg. of sodium pentobarbitone. The gastrocnemius muscle was partially freed from the

TABLE III

PROTECTION OF MICE FROM ELECTROSHOCK BY PRE-TREATMENT WITH PETALINE CHLORIDE OR PHENOBARBITONE (FIG. 5)

Petaline chloride dosage mg./kg.	Per cent protection
1·6	10
2·5	25
3·3	37·5
5·0	20
Phenobarbitone dosage mg./kg.	**Per cent protection**
4	20
5	50
6·6	80

surrounding tissues and the achilles tendon severed near to its insertion into the calcaneus. The tendon was attached by means of a strong linen thread and pulley system to a myograph lever. The sciatic nerve was exposed on the lateral aspect of the thigh and stimulated by means of platinum electrodes using square impulses at a frequency of 8 to 10 per minute, 15 to 20 volts, pulse width 3 to 4 msec. In any given experiment frequency, voltage and pulse width were constant. In some experiments,

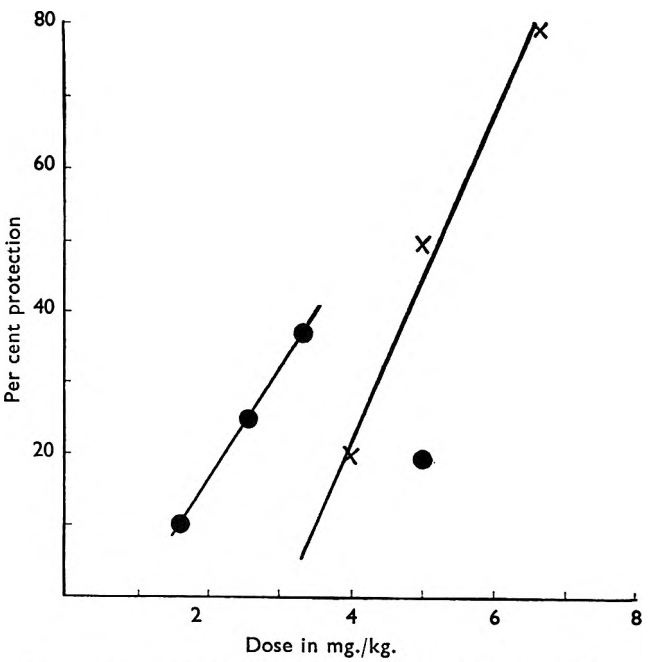

FIG. 5. Protection of mice from electroshock seizures by treatment with petaline chloride (●—●) or phenobarbitone (×—×).

however, a tetanizing current of 1,500 square impulses per minute was applied, voltage and pulse width remaining constant at the values stated. Drugs in aqueous solution were administered by way of the cannulated external jugular vein. Petaline chloride (3·0 to 10·0 mg./kg.) reduced twitch height; recovery occurred in about 15 to 20 minutes and the effects of petaline chloride were reversed by edrophonium (1·0 mg./kg.) or neostigmine methylsulphate (0·10 mg./kg.). When a tetanizing current was applied to the nerve of a petaline chloride treated muscle, the tetanus was not maintained—an effect similar to that seen when tubocurarine is used.

Neuromuscular transmission in the rat diaphragm. A method similar to that described by Bülbring[8] was used. The preparation was set up in a 100 ml. bath containing "double glucose" Tyrode's solution (NaCl, 0·8; KCl, 0·02; $CaCl_2.6H_2O$, 0·02; $MgCl_2$, 0·01; $NaHCO_3$, 0·1, NaH_2PO_4,$2H_2O$, 0·005 and glucose 0·2 per cent) at 29°. The solution was gassed with oxygen or a mixture of 95 per cent oxygen and 5 per cent carbon dioxide. The electrode described by Bell[9] was used so that the muscle could be stimulated both directly and indirectly. Stimulation of the nerve was by square impulses at a frequency of 6 to 8 per minute, 10 to 12 volts, pulse width 0·5 to 1·0 msec. When the muscle was stimulated directly the frequency was 6 to 8 per minute at 25 to 50 volts, pulse width 1·5 to 2·0 msec. In any given experiment, frequency, voltage and pulse width were constant. Drugs in aqueous solution were added directly to the bath. Petaline chloride (0·2 to 0·3 mg./ml.) abolished the response of the diaphragm to indirect stimulation but when the muscle was stimulated directly at a higher voltage and with a wider pulse, regular contractions were produced (Fig. 6).

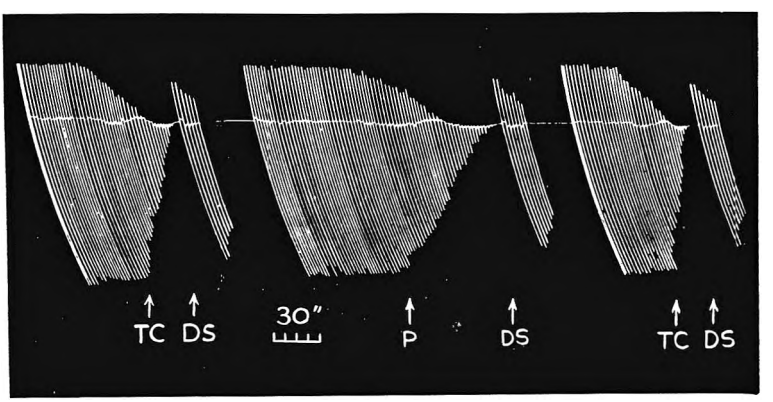

FIG. 6. Rat phrenic nerve-diaphragm preparation. Contractions induced by indirect stimulation of the phrenic nerve or by direct stimulation of the muscle.
Bath 100 ml., temperature 29°, bath fluid, oxygenated "double glucose" Tyrode's solution.
At TC, 3·0 μg./ml. tubocurarine added to bath.
At P, 0·20 mg./ml. petaline chloride added to bath.
At DS, direct stimulation of the muscle.

Frog rectus abdominis muscle. The method used was essentially that of de Jalon[10]. Frogs of either sex were decapitated and pithed. The rectus abdominis muscle was dissected and set up in a 10 ml. bath containing oxygenated frog Ringer's solution (NaCl, 0·65; KCl, 0·014; $CaCl_26H_2O$, 0·012; $NaHCO_3$, 0·02; $NaH_2PO_42H_2O$, 0·001 and glucose 0·2 per cent) at room temperature. Acetylcholine chloride (0·10 to 1·0 μg./ml.) was added to the bath and left in contact with the tissue for 90 seconds, the contraction being recorded. Drugs were added to the bath 30 seconds before a subsequent addition of acetylcholine and the contraction again recorded for 90 seconds. The bath was then washed out. Between successive additions of acetylcholine, there was an interval of 7 minutes. 5 to 20 μg./ml. of petaline chloride antagonised contractions caused by acetylcholine chloride (0·1 to 1·0 μg./ml.); a graded effect was seen. Petaline chloride was found to have about 50 per cent of the potency of gallamine triethiodide on this preparation. (Fig. 7).

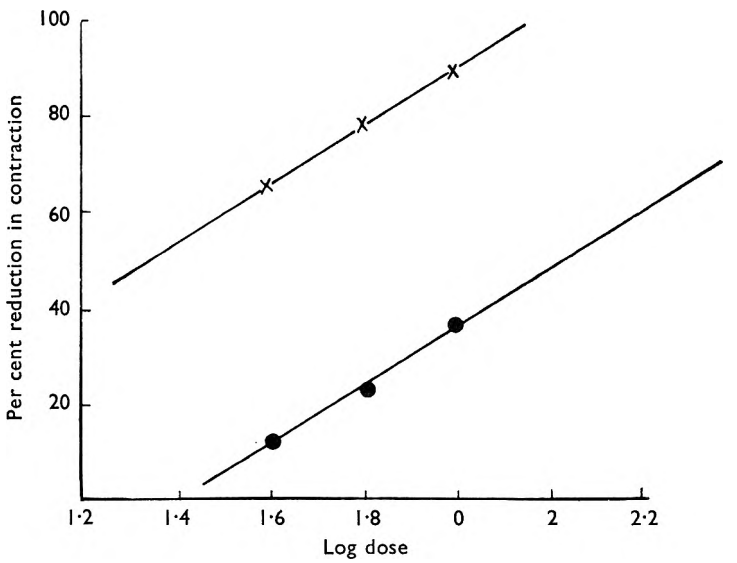

FIG. 7. A comparison of the effects of gallamine triethiodide (×—×) and petaline chloride (●—●) on acetylcholine-induced contractions of the frog rectus abdominis muscle.

Other Effects

Cardiovascular Actions

Blood pressure of anaesthetised cat. Cats weighing from 2·0 to 3·0 kg. were anaesthetised by intraperitoneal injection of 60 mg./kg. of sodium pentobarbitone. Blood pressure was recorded from the common carotid artery using a mercury manometer and drugs in aqueous solution were injected by way of the cannulated external jugular vein. Petaline chloride (2·0 to 4·0 mg./kg.) caused an immediate fall of blood pressure. The

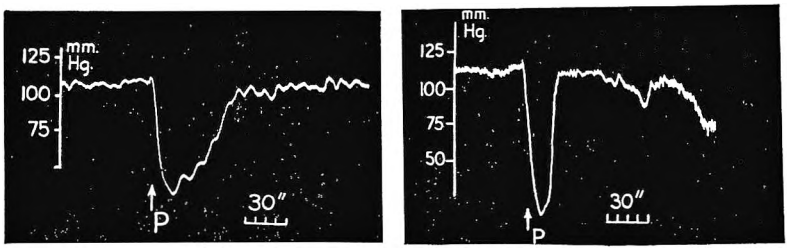

FIG. 8. Effect of petaline chloride on arterial blood pressure. Left hand record, cat carotid artery blood pressure. At P, 2 mg./kg. petaline chloride. Right hand record, rat carotid artery blood pressure. At P, 58 μg./100 g. petaline chloride.

level returned to normal in from 5 to 10 minutes (Fig. 8). In some cases there was a slow secondary fall. This was only seen with higher doses. There was no effect on the blood pressure level of spinal cats even at doses of up to 8·0 mg./kg.

Blood pressure of anaesthetised rat. The technique employed was similar to that described by Dekanski[11]. Rats weighing 300 to 350 g. were used. Anaesthesia was induced by subcutaneous injection of 175 mg./100 g. of urethane. Blood pressure was recorded from the cannulated common carotid artery by means of Condon's manometer[12] and drugs in aqueous solution were administered by way of the cannulated external jugular vein. Petaline chloride (20 to 60 μg./100 g.) caused an immediate but short lived fall in blood pressure (Fig. 8). In some experiments when higher doses of petaline chloride were used there was a gradual secondary fall. There was slight antagonism by petaline chloride (30 to 40 μg./100 g.) to the pressor effects of 0·05 to 0·10 μg./100 g. of adrenaline bitartrate or noradrenaline bitartrate.

Rat hind-quarters preparation. The isolated hind-quarters of rats were perfused with oxygenated Locke's solution (NaCl, 0·9; KCl, 0·042; $CaCl_26H_2O$, 0·024; $NaHCO_3$, 0·05 and glucose 0·1 per cent) at room temperature, using the method described by Burn[13]. Outflow was recorded, using Stephenson's[14] recorder and drugs in aqueous solution were administered by way of the perfusion cannula. Petaline chloride (2·0 to 3·0 mg.) caused slight vasodilatation and slight antagonism to the vasoconstrictor actions of adrenaline bitartrate (1·0 μg.) or noradrenaline bitartrate (1·0 μg.) (Fig. 9).

Isolated perfused rabbit or kitten heart. Hearts were perfused using Langendorff's method[15]. The perfusion fluid was well oxygenated "double glucose" Locke's solution at 37°. Outflow was recorded by Stephenson's[14] recorder and drugs in aqueous solution were injected into the perfusion cannula. Petaline chloride 2 to 4 mg. increased slightly the rate and amplitude of the ventricular contractions and caused a slight increase in outflow. A similar effect was seen when the heart was perfused with 200 μg./ml. of petaline chloride in Locke's solution.

Isolated guinea pig auricles. The auricles were set up in a 10 ml. bath containing well oxygenated Locke's solution at 29°. Drugs in aqueous

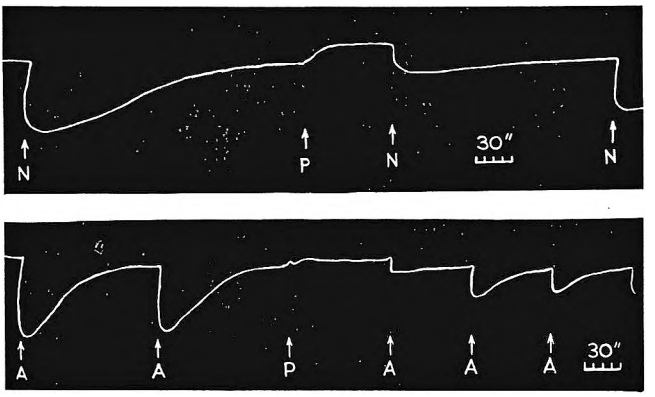

FIG. 9. Perfused rat hind quarters. Perfusion with Locke's solution at room temperature. Outflow recorded by Stephenson's outflow recorder. Drugs in aqueous solution injected into the perfusion cannula.

At N, 1·0 μg. noradrenaline bitartrate.
At A, 1·0 μg. adrenaline bitartrate.
At P, 2·0 mg. petaline chloride.

solution were added directly to the bath and kept in contact with the auricles for 60 seconds. Petaline chloride (200 to 400 μg./ml.) increased the rate and amplitude of the contractions but after washing they returned to normal. There was no antagonism or very slight antagonism to the effects of adrenaline bitartrate (0·10 μg./ml.), noradrenaline bitartrate (0·10 μg./ml.) or histamine acid phosphate (0·10 μg./ml.), but the effects of acetylcholine chloride (0·02 μg./ml.) were antagonised. When the auricles were perfused with Locke's solution containing half the usual amount of calcium chloride the amplitude and rate were reduced and the auricles gradually came to rest. Petaline chloride (200 μg./ml.) added to the bath restored the rate to normal and the amplitude to supranormal levels (Fig. 10).

Effects on Smooth Muscle

Guinea-pig ileum. The method used was a modification of that described by Guggenheim and Loffler[16]. A 5 ml. bath containing oxygenated Tyrode's solution at 37° was used. Drugs in aqueous solution were added to the bath and kept in contact with the tissue for 15 seconds. Large doses of petaline chloride (1 mg./ml.) caused a slight increase in spontaneous activity. The responses to acetylcholine chloride (1·0 μg./ml.), histamine acid phosphate (0·3 μg./ml.), 5-hydroxytryptamine creatinine sulphate (1·0 μg./ml.), barium chloride (0·6 mg./ml.) and potassium chloride (5·0 mg./ml.) were antagonised by petaline chloride (100 μg. to 1·0 mg./ml.), added 30 seconds beforehand. The effect was reversible on washing and there was a graded inhibition according to the dose.

Rat uterus. The method adopted was a modification of that described by Amin, Crawford and Gaddum[17]. Virgin female rats weighing from 150 to 180 g. were employed and brought into oestrus by subcutaneous

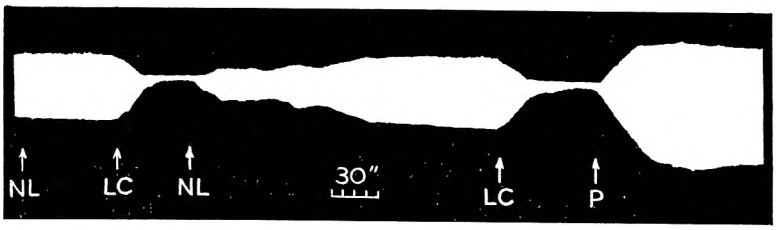

FIG. 10. Isolated guinea pig auricles. Record of spontaneous contractions. Bath 10 ml., temperature 29°. Bath fluid oxygenated Locke's solution. At NL, Locke's solution.
At LC, the bath fluid changed to "half calcium" Locke's solution.
At P, 200 μg./ml. petaline chloride added to bath.

injection 24 hours before use of 0·10 mg./100 g. of stilboestrol in arachis oil. One horn of the uterus was set up in a 5 ml. bath containing de Jalon's solution (NaCl, 0·9; KCl, 0·042; $CaCl_26H_2O$, 0·006; $NaHCO_3$, 0·05 and glucose 0·1 per cent) at 29°. The solution was gassed with a mixture of 95 per cent oxygen and 5 per cent carbon dioxide. Drugs in aqueous solution were added to the bath and allowed to remain in contact with the tissue for 30 seconds. In doses of 20 to 60 μg./ml. petaline chloride reduced the height of contractions produced by 0·10 to 0·50 μg./ml. of acetylcholine chloride. There was a graded inhibition according to dose and recovery to control levels was usually complete. No direct effects were seen.

Toxic Effects in the Rabbit

Only two rabbits were used. One (2·75 kg.) was given 30 mg. petaline chloride by intravenous injection. It immediately developed convulsive jerky movements, respiration stopped and about 3 minutes later the heart ceased to beat. After death the animal was flaccid. Similar effects were seen in a second rabbit (2·25 kg.) given 35 mg. of petaline chloride. These results are similar to those of McShefferty and others[1].

DISCUSSION

Petaline chloride is a potent convulsant and is apparently 5 to 7 times as powerful as leptazol. Lower doses (2·5 and 5 mg./kg.) may reduce the convulsant activity of a subsequent dose of leptazol and apparently give some protection from electrically induced seizures, but higher doses (8 mg.) are additive with leptazol both with respect to convulsant activity and acute toxicity. Petaline chloride has muscle relaxant activity, it also depresses the patellar tendon reflex and the crossed extension reflex and is more effective than mephenesin with respect to the latter. Similar effects to these are seen when tubocurarine is used and the effect is probably due to the muscle relaxant activity. When these actions are taken, in conjunction with the stimulant effects upon isolated cardiac muscle, in particular the effect on the isolated auricles depressed in a low calcium medium, then there may be some basis for its use in folk medicine. It

must not, however, be forgotten that the extracts used undoubtedly contain other substances[1] and that the disease reported to have been treated may not have been correctly diagnosed by the native physicians or the sufferers' relatives. We hope to have the opportunity of testing other extracts and pure substances.

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POLAROGRAPHIC ESTIMATION OF AMPHENONE B AND SU 4885

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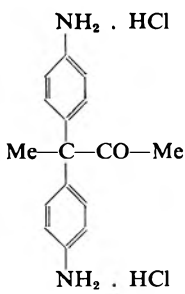
The polarographic behaviour of the compounds Amphenone B (3,3-bis(*p*-aminophenyl)-butan-2-one) and Su 4885 (1,1-dimethyl-2-oxo-ethylenebis-3-pyridine) in aqueous medium is described. Both substances are reduced at the dropping mercury electrode by two electrons and produce well-developed diffusion cathodic waves of a height proportional to the concentration of the depolariser. Su 4885 can be estimated polarographically also in adrenal homogenates in Ringer-Krebs phosphate buffer.

The insecticide DDD, the active component of which is 1,1-dichloro-2, 2-bis(*p*,-chlorophenyl)ethane, causes a decrease of the corticosteroid excretion and extensive atrophy of adrenals, accompanied by destruction of the adrenal cortical tissue[1,2].

Among other substances capable of inhibiting the production of some physiologically active corticosteroids by direct action in the adrenal glands are the compounds Amphenone B (3,3-bis(*p*-aminophenyl)-butan-2-one dihydrochloride)[3] (I) and Su 4885 (1,1-dimethyl-2-oxo-ethylenebis-3-pyridine)[4] (II). These compounds have been utilised in the study of the biochemistry of the corticosteroid metabolism and physiology of the adrenal cortex, as well as for the diagnosis and experimental therapy of some endocrine disorders. Amphenone B inhibits the enzymatic oxygenation of the steroid molecule at the 11-, 17- and 21-positions[4–6] and Su4885 acts as a specific inhibitor of 11-hydroxylation[6–8]. In contrast to the effect of DDD, the adrenals do not atrophy but hyperplasia develops.



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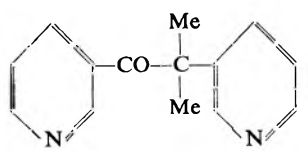

II

Hitherto no attention had been paid to the analytical estimation of Amphenone B or Su 4885 nor to their polarographic behaviour.

Experimental

The polarographic curves were recorded on a Heyrovský polarograph, Type V 301 (ČZ Brno). The measurements were made in a Kalousek vessel with a separate saturated calomel reference electrode. The rate of

mercury flow through the capillary used at the reservoir height $h = 60$ cm. was $m = 3{\cdot}05$ mg.sec.$^{-1}$ and the drop time was $t = 2{\cdot}6$ sec. (in 0·1 N LiCl). The galvanometer sensitivity was $2{\cdot}2 \cdot 10^{-9}$A/mm. The half-wave potentials were measured with an accuracy of $\pm$ 10 mV.

As the supporting electrolyte 0·1 N lithium chloride, McIlvain's buffer or 0·1 N lithium hydroxide were used. The oxygen was removed from the solution by bubbling a stream of nitrogen through it for 3 minutes before the curve was recorded. The standard aqueous stock-solution of Amphenone B and Su 4885 was $5{\cdot}10^{-3}$M.

RESULTS

The compounds are polarographically reduced at a dropping mercury electrode in a solution of 0·1 N lithium chloride. One very well-developed cathodic wave was recorded. The polarographic waves are limited by diffusion; this was confirmed by studying the dependence of the limiting

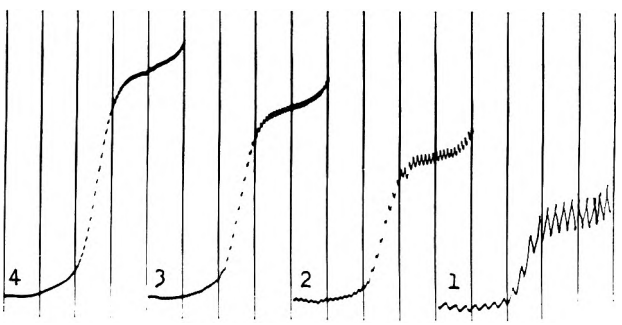

FIG. 1. Dependance of the height of the amphenone wave on the height of the mercury reservoir.

$3{\cdot}5 \times 10^{-4}$ M Amphenone B in 0·1 N LiCl; from about—1·0 V against standard calomel electrode, 200 mV/absc., height of the reservoir h = 1. 15 cm. 2. 30 cm. 3. 55 cm. 4. 80 cm., sensitivity 1 : 15.

current on the square root of the height of the mercury reservoir (Fig. 1). The dependence is linear from the origin. The temperature coefficients $1/i \cdot \triangle i/\triangle T$ of the waves of both substances also provide evidence of diffusion character of limiting current (see Table I).

The two-electron reduction, common also in other ketones, was confirmed by comparing the height of the waves with the height of a thalium (I) wave under analogous conditions. Amphenone B is reduced at a more

TABLE I

THE VALUES OF SOME POLAROGRAPHIC CONSTANTS

	Half-wave potential $E_{\frac{1}{2}}$ V(SCE) in 0·1 N LiCl	Diffusion current constant $d = id/m^{2/3}t^{1/6}c$	No. of electrons n	Diffusion coefficient D . 10^6 (cm.^{2}sec.$^{-1}$)	Temperature coefficient $1/i.\ \Delta i/\Delta T$ grad^{-1} per cent
Amphenone B ..	−1·40	1·8	2	2·2	1·59
Su 4885	−1·22	2·3	2	3·7	1·28

negative potential than Su 4885 which would be expected as the carbonyl group of the latter compound conjugates with the aromatic pyridine nucleus while in Amphenone B the carbonyl is isolated from the aromatic system. The difference of 180 mV between the half-wave potentials of the two substances in 0·1 N LiCl makes it possible to estimate them simultaneously.

When investigating the influence of pH of the solution over the range of pH 3 to 8 (McIlvain buffer) and pH 12 respectively (LiOH) the half-wave potential of Su 4885 is shifted by 83·6 mV per pH unit to the more negative potentials (Fig. 2). The effect of pH on Amphenone B could not be

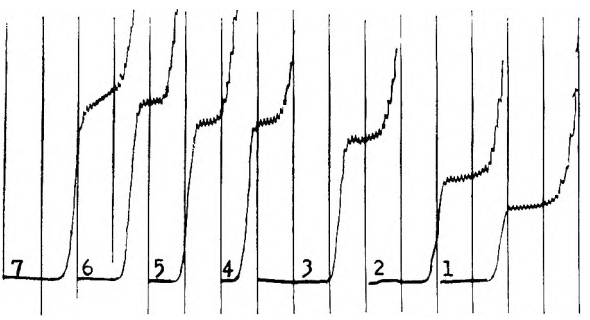

FIG. 2. Effect of pH on the Su 4885 wave.
$2{\cdot}5 \times 10^{-4}$ M Su4885 in McIlvaine buffer; pH = 1. 2·2. 2. 3·0. 3. 4·0. 4. 5·0. 5. 6·0. 6. 7·0. 7. 8·0, from about 1 to 3. − 0·4 V, 4 to 8. − 0·8 V, h = 60 cm. 200 mV/absc, sensitivity 1:15.

ascertained because the dihydrochloride of this substance in the buffered aqueous solution did not produce measurable polarographic waves. Neither the shape nor the half-wave potential of the Su 4885 wave in 0·1 N LiCl were markedly influenced by the presence of deformable ions Ca^{++} and Cs^{+}.

The height of the polarographic wave of Amphenone B and Su 4885 in 0·1 N lithium chloride and of the latter compound also in other media studied is a linear function of the depolariser concentration (Fig. 3).

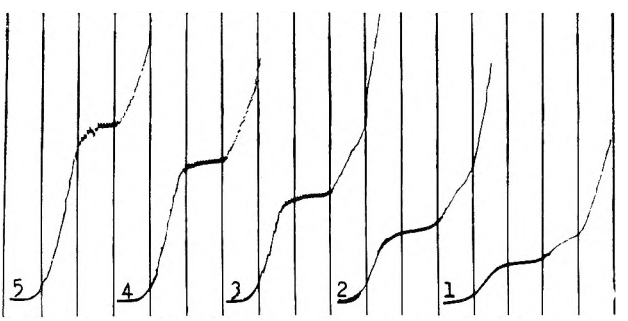

FIG. 3. Dependance of the height of the wave on the concentration of Su4885 in 0·1 N LiCl.
1. $0{\cdot}6 \times 10^{-4}$M. 2. $1{\cdot}2 \times 10^{-4}$M. 3. $1{\cdot}8 \times 10^{-4}$M. 4. $2{\cdot}4 \times 10^{-4}$M. 5. $3{\cdot}0 \times 10^{-4}$M of Su4885. 200 mV/absc., h = 60 cm., sensitivity 1:20, from about − 1·0 V against standard calomel electrode.

This fact can be utilised for the analytical estimation of these compounds. That the height of the wave does not change with time is also of advantage analytically. Polarographic curves rarely show maxima which can be suppressed by a gelatine solution but not by acid fuchsin, and then at higher concentrations only.

The effect of the hydrogen ion concentration in McIlvain's buffer on the height of the wave was studied in Su 4885. On the acid side the height of the wave rises sharply with increasing pH, but over the range of pH 4 to 8 changes in the hydrogen ion concentration have less influence. (Fig. 4).

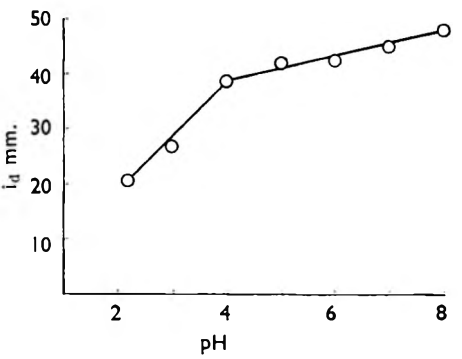

FIG. 4. Effect of pH on the height of polarographic wave of Su4885 in McIlvaine buffer.

These findings are of importance in the polarographic estimation of Su 4885 in aqueous solutions and of Amphenone B in neutral aqueous non-buffered solutions with the usual accuracy of polarographic estimation, of 2 per cent. In addition to the possibility of estimating these compounds in the pure crystalline state and in pharmaceutical preparations, the polarographic method of estimating Su 4885 can be used when investigating its fate during its action as the biochemical inhibitor of the oxygenation of the steroid molecule in the adrenal cortical tissue. For this purpose Ringer-Krebs phosphate buffer either alone or with homogenised adrenals proved very satisfactory.

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SPASMOLYTIC ESTERS OF *N*-SUBSTITUTED α-AMINOPHENYLACETIC ACIDS

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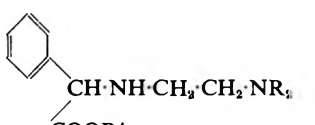
A series of alkyl and cycloalkyl esters of α-(2-pyrrolidin-1′-ylethylamino-), α-(2-piperidinoethylamino)- and α-(2-diethylaminoethylamino)-phenylacetic acid has been synthesised and examined for spasmolytic activity. The isopentyl- and cyclohexyl esters of α-(2-pyrrolidin-1′-ylethylamino)phenylacetic acid are new compounds possessing spasmolytic properties greater than papaverine and, in addition, 1 per cent and 4 per cent respectively of the antimuscarine activity of atropine as measured on the guinea pig ileum.

A Current hypothesis[1] of antimuscarinic action is that acetylcholine-sensitive receptor-surfaces of parasympathetically innervated organs can be effectively blocked only by a compound which has two foci for attachment to the surface. One of these is a quaternary or tertiary nitrogen atom, and the other, which must be between 5 and 10 Å distant from the nitrogen atom, may be one of a number of, as yet, ill-defined groups to which is attached one or more groups large enough to form a protective shield over the receptors and prevent access to them of the natural neurohormone. By considering the structure of the widely used antispasmodics it appears that the unshared electrons of a polarised carbonyl or ethylene group, or the lone pair on a hydroxyl oxygen or second nitrogen atom, may constitute the second focus for attachment to the receptor surface[2].



I

In compounds based on I the pKa of the terminal tertiary nitrogen atom is above 7 and, accordingly, at physiological pH values the compounds is partially protonised and possesses in part a terminal cationic group which is known to increase antimuscarinic activity. The protecting or umbrella groups in these compounds comprise the phenyl group and the large alkoxycarbonyl-CO·OR′ group.

The present communication relates to compounds based on I, in which the esterifying group is ethyl, isopentyl, phenyl, cyclohexyl or 3,5,5-trimethylcyclohexyl, and the terminal tertiary nitrogen group-NR_2 is diethylamino, piperidino or pyrrolidin-l-yl. In one series the non-terminal secondary amino group has been converted into a tertiary group.

The compounds were prepared by the Hell-Volhard-Zelinsky bromination of phenylacetic acid and reaction of the resulting α-bromophenylacetyl bromide with the appropriate alkanol. Condensation of the α-bromo

esters with the required 2-dialkylaminoethylamine gave the amino esters of type I: these were distilled and converted into a salt. Isopentyl α-(2-diethylaminoethylamino)phenylacetate (compound 3) has been prepared previously by a similar route[3,4] and also by the diethylaminoethylation of isopentyl α-aminophenylacetate[5].

Spasmolytic Activities

The spasmolytic activities of the amino esters against acetylcholine, histamine, 5-hydroxytryptamine, nicotine and barium-ion spasm of isolated guinea pig ileum were determined by the Magnus method and are recorded in Table I. The spasmogen dilution (pD) given at the head

TABLE I

SPASMOLYTIC ACTIVITIES OF COMPOUNDS OF GENERAL FORMULA
$Ph{\cdot}CH(COOR'){\cdot}NR''{\cdot}CH_2{\cdot}CH_2{\cdot}R$
AGAINST ACETYLCHOLINE, HISTAMINE, 5-HYDROXYTRYPTAMINE, NICOTINE AND BARIUM ION ON THE ISOLATED GUINEA PIG ILEUM

Cpd. No.	R''	R'	R	Acetylcholine pD* 7·08†	Histamine pD 7·08†	Nicotine pD 5·34†	5-HT pD 5·78†	Barium chloride pD 3·7†
1	H	isopentyl	piperidino	6·24	5·36	6·2	6·08	5·14
2	H	,,	pyrrolidin-l-yl	6·75	5·70	6·56	6·05	5·04
3	H	,,	diethylamino	6·71	5·49	6·7	6·2	5·28
4	Me	,,	diethylamino	5·87	5·42	5·86	5·33	5·28
5	H	cyclohexyl	piperidino	6·69	5·38	6·69	6·06	5·34
6	H	,,	pyrrolidin-l-yl	7·43	5·57	6·76	6·26	5·38
7	H	,,	diethylamino	7·38	5·26	6·65	5·87	5·30
8	Me	,,	diethylamino	6·23	5·42	5·87	5·51	5·22
9	H	3,5,5-trimethylcyclohexyl	piperidino	6·08	5·95	5·79	5·43	5·26
10	H	,,	pyrrolidin-l-yl	6·48	6·79	5·87	5·26	5·40
11	H	,,	diethylamino	5·99	5·37	5·87	5·38	5·36
12	Me	,,	diethylamino	5·60	5·62	5·70	5·54	5·49
13	H	ethyl	piperidino	5·36	5·15	5·43	5·08	4·78
14	H	,,	pyrrolidin-l-yl	5·45	5·67	5·08	4·81	4·75
15	H	,,	diethylamino	5·48	5·19	5·39	5·15	5·13
16	Me	,,	diethylamino	5·46	4·94	5·46	5·10	4·98
17	H	phenyl	diethylamino	4·94	4·86	4·85	4·59	4·56
		atropine		8·85	5·53	7·77	7·17	Nil
		papaverine		5·04	5·40	5·40	5·10	4·90

* Dilutions given on the pD scale. pDx means 1 part of compound in 10^x parts of solution.
† Dilutions effecting 40 per cent reduction of the normal length of ileum strip.

of each column produced about a 40 per cent reduction of the normal length of the ileum strip. The activities of the spasmolytic agents are recorded as the dilution which gave a 50 per cent reduction of the spasm; the figures are the means from 6 ileum strips. Dilutions are given on the pD scale; pDx means 1 part of spasmolyte in 10^x parts of solution.

The following structure-activity observations may be made from the results in Table I. For a given group $-NR_2$ (i) the activity against acetylcholine, nicotine and 5-hydroxytryptamine spasm decreases along the series of ester groupings (–CO·OR'), cyclohexyl, isopentyl, trimethylcylcohexyl, ethyl, phenyl; (ii) the activity against histamine spasm is substantially the same for the cyclohexyl, isopentyl, trimethylcyclohexyl

and ethyl esters and lower for the phenyl esters; (iii) the activity against barium ion spasm is about the same for the cyclohexyl, isopentyl, trimethylcyclohexyl esters and is lower for the ethyl and phenyl esters. For a given ester grouping –CO·OR′ (i) the activity against acetylcholine and histamine spasm is higher for the pyrrolidin-l-yl than for the piperidino and diethylamino compounds; (ii) the activity against nicotine, 5-hydroxytryptamine and barium ion spasm does not vary with a discernible pattern in the series pyrrolidin-l-yl, piperidino and diethylamino. Conversion of the central secondary amino to a tertiary group lowers activity.

Toxicity of Isopentyl α-(2-pyrrolidin-1′-ylethylamino)phenylacetate Di-(hydrogen maleate)

The toxicity of this compound was determined in groups of four mice using closely spaced dosages. The LD50 values were: 1,100 to 1,200 mg./kg. (oral); 250 mg./kg. (intraperitoneal); 70 mg./kg. (intravenous). A dose of 50 mg./kg. i.p. to guinea pigs made the animals flaccid and tranquil: this effect disappeared in 1 to 2 hours. In the rabbit 10 mg./kg. i.v. was just tolerated; at 15 mg./kg. the animals died quickly; at 25 mg./kg. the animals died instantly, apparently by auricular-ventricular block.

For purposes of comparison the LD50 of papaverine hydrochloride in mice was found to be 300 mg./kg. (oral); 120 mg./kg. (intraperitoneal) and 33 mg./kg. (intravenous). Rabbits tolerated 10 mg./kg. i.v. of papaverine hydrochloride; at 20 mg./kg. there were violent tetanic spasms frequently followed by death. The LD50 for atropine sulphate in mice was 400 mg./kg. (oral); 250 mg./kg. (intraperitoneal) and 90 mg./kg. (intravenous).

Mydriatic Activity of Isopentyl α-(2-pyrrolidin-l′-ylethylamino) phenylacetate Di(hydrogen maleate)

Six adult rabbits were used for comparing the mydriatic activity of this compound and atropine sulphate. Two drops (0·2 ml.) of a 1·0 per cent aqueous solution of the ester were instilled into the left eye of three rabbits and the same volume of a 0·1 per cent solution of atropine sulphate into the left eye of the other three rabbits; the right eye of each rabbit was used as control. The pupillary diameters of the test and control eyes were measured at suitable time intervals and the average mydriasis expressed as the per cent increase or decrease of the test pupil diameter relative to the control pupil. The ester effected a 25 per cent mydriasis which lasted for 5 hours; in 6 hours the eye was normal. Atropine sulphate effected 42 per cent mydriasis which persisted for 50 hours; the eye was not normal until after 60 hours.

Nine adult rabbits were used for comparing the activities of the ester and atropine sulphate in the eserinized eye. In all animals the left eye was treated, the right eye being used as a control. Three rabbits received 0·2 ml. of 0·5 per cent solution of eserine; three rabbits received 0·2 ml. of a 0·5 per cent solution of eserine followed immediately by 0·2 ml. of a

1 per cent aqueous solution of the ester; the other three rabbits received 0·2 ml. of 0·5 per cent eserine followed by 0·2 ml. of a 0·1 per cent solution of atropine sulphate. The average results are shown in Table II: the mydriasis is expressed as the per cent increase or decrease in average test

TABLE II

MYDRIATIC EFFECT OF ISOPENTYL α-(2-PYRROLIDIN-1′-YLETHYLAMINO)PHENYLACETATE DI(HYDROGEN MALEATE) AND ATROPINE SULPHATE IN ESERINISED RABBIT EYE

Time in hours	Per cent mydriasis		
	Eserine	Eserine + ester (1·0 per cent)	Eserine + atropine sulphate (0·1 per cent)
0	0	0	0
1/4	−29	+22	+58
½	−50	+22	+86
1	−50	+24	+86
2	−50	+22	+86
4	−21	+20	+57
6	−20	+15	+56
8	−14	+14	+50
24	0	0	+36

pupil diameter relative to the average control pupil diameter. It is evident that the mydriatic activity of the ester is less than that of atropine sulphate, but has the advantage that the mydriasis is of shorter duration.

The isopentyl and cyclohexyl esters of α-(2-pyrrolidin-1′-ylethylamino) phenylacetic acid (Compounds 2 and 6 of Table I) are new compounds possessing spasmolytic properties greater than papaverine; in addition they have 1 and 4 per cent respectively of the antimuscarine activity of atropine as tested on the guinea pig ileum.

CHEMICAL

Isopentyl α-bromophenylacetate. Phenylacetic acid (68 g.; 0·5 mol) and red phosphorus (5 g.; 0·166 atom; washed with water and dried at 90°) were placed in a flask and heated on the water bath to 80 to 85° (int.) until the phenylacetic acid was molten. Dry bromine (160 g.; 53 ml.; 2 atom) was added dropwise during 1 hour, the flask being occasionally swirled and the escaping HBr led away. After the addition, the flask was heated on the water bath for a further 3½ hours, then cooled in an ice bath and dry isopentyl alcohol (88 g.; 109 ml.; 1·0 mol) added in a slow stream over 10 minutes with swirling. When the vigorous evolution of HBr had ceased, the mixture was heated on the water bath for a further 20 minutes, cooled and poured into stirred benzene (400 ml.) and water (400 ml.). The mixture was stirred vigorously and solid potassium bicarbonate added until there was no more effervescence and the pH value was 8. The benzene layer was separated and the aqueous layer washed with benzene (100 ml.). The combined benzene solutions were washed with water (50 ml.), dried over sodium sulphate, filtered and the benzene and excess isopentyl alcohol removed by distillation from the water bath at 16 to 20 mm. The residual oil was distilled, a small fraction b.p. 170 to 184°/18 mm. being rejected; the main fraction (125 g.) b.p. 184 to 187°/18 mm.

was collected. This was redistilled, the fraction b.p. 125 to 138°/1 mm. being rejected; pure isopentyl α-bromophenylacetate b.p. 138 to 144°/1 mm. (150 to 154°/3 mm.) came over as a pale yellow oil (120 g.; 85 per cent).

Ordinary isopentyl alcohol contains 80 per cent of isopentyl alcohol $Me_2CH{\cdot}CH_2{\cdot}CH_2OH$ and 20 per cent of 2-methylbutanol, the latter boils 2·5° lower than the former and is more soluble in water. The Isopentyl alcohol used in this preparation was commercial material which had been washed with a large volume of 2 N sodium hydroxide and then with a large volume of 2 N sulphuric acid. The alcohol was then distilled in steam, the distillate dried and redistilled through an efficient column rejecting about one quarter as forerun. The alcohol used had b.p. 129·5 to 130·5°; it probably still contained a few per cent of the isomer.

The following esters of α-bromophenylacetic acid were prepared in the same manner; the yields are in parentheses. Ethyl: b.p. 120 to 122°/10 mm. (86 per cent). Found: Br, 33·1. Calc. for $C_{10}H_{11}O_2$ Br: Br, 33·0 per cent. *Cyclohexyl*: b.p. 194 to 196°/10 mm. (82 per cent). Found: Br, 27·2. $C_{14}H_{17}O_2Br$ requires Br, 26·9 per cent. 3,5,5-*Trimethylcyclohexyl*: b.p. 176 to 182°/1·5 mm. (80 per cent); m.p. 72 to 74°. Found: C, 60·8; H, 6·8; Br, 24·2. $C_{17}H_{23}O_2Br$ requires C, 60·2; H, 6·8; Br, 23·6 per cent. 3,5,5-*Trimethylhexyl*: b.p. 198 to 200°/11 mm. (91 per cent). Found: Br, 24·5. $C_{17}H_{25}O_2Br$ requires Br, 23·5 per cent. *Phenyl*: b.p. 194 to 196°/1·5 mm.; m.p. 46 to 48° (53 per cent). Found: C, 57·2; H, 3·5; Br, 27·9. $C_{14}H_{11}O_2Br$ requires C, 57·8; H, 3·8; Br, 27·5 per cent).

2-*Pyrrolidin*-1′-*ylethylamine*. Pyrrolidine (177·5 g.; 205 ml.; 2·5 mol) was placed in a 3-necked flask and stirred at −1° (int.) in an ice-salt bath. Solid 2-bromoethylamine hydrobromide[6] (102·5 g.; 0·5 mol) was added in 6 portions at intervals of 10 minutes; the maximum internal temperature following each addition was +15° and a further portion was not added until the temperature had again dropped to −1°. After the whole addition, the mixture was stirred at 15° for 30 minutes and then distilled from a graphite bath to recover some of the excess pyrrolidine, the distillation being stopped when the *internal* temperature of the liquid reached 160°. (The pyrrolidine recovered at this stage was 78 g.; b.p. 85 to 88°.) The residual pale yellow liquid was cooled to 5° and a solution of sodium hydroxide (50 g.; 1·25 mol) in water (50 ml.) added. The mixture was chilled, the supernatant oil poured off, and the sludge of sodium bromide and caustic soda washed with ether (3 × 100 ml.). The combined oil and ether washings were dried three times over sodium hydroxide pellets; after each drying the residual sludge of caustic pellets was washed with 3 × 100 ml. of dry ether. The dry ethereal solution (vol. 1,200 ml.) was distilled through a 30 cm. column of Fenske glass spirals; ether, containing a little pyrrolidine, came over at 34 to 36° and then pyrrolidine (54 g.) at 87 to 88°/747 mm. (The total recovery of pyrrolidine was 132 g., 93 per cent of the excess used in the reaction.) The residual oil was distilled through a 10 cm. Fenske glass spiral column and gave 46·2 g. (81 per cent) of 2-pyrrolidin-1′-ylethylamine, b.p. 166 to

167°/747 mm. 2-Piperidinoethylamine was obtained in the same manner in 80 per cent yield.

(2-*Diethylaminoethyl*)*methylamine*. The following is more convenient than the literature[7] method. 2-Diethylaminoethyl chloride hydrochloride (129 g.; 0·75 mol) was added during 5 minutes to a stirred mixture of 25 per cent aqueous methylamine (120 ml.; 3 mol) and 10 N potassium hydroxide (150 ml.; 1·5 mol) at 0 to 5°. The mixture was stirred at this temperature for 1¼ hours and then heated to 60°; an exothermic reaction resulted, the temperature rising to 70°. The mixture was kept at 70° for a further 2½ hours; cooled and set aside overnight. Flake sodium hydroxide (about 250 g.) was added until the solution was almost saturated. Ether (200 ml.) was added to dissolve the supernatant oil and the whole filtered to remove inorganic solid, the latter being washed into the filtrate with a mixture of 10 N sodium hydroxide and ether. The ether layer was separated and the aqueous layer extracted with ether (6 × 100 ml.). The combined ethereal extracts were *thoroughly* dried over potassium hydroxide and the filtered solution distilled, the fraction b.p. 130 to 170° being collected. Refractionation of the latter gave the product b.p. 152 to 156° as a pungent colourless oil (54 g.; 55 per cent).

Isopentyl α-(2-*pyrrolidin*-1′-*ylethylamino*)*phenylacetate*. 2-Pyrrolidin-1′-ylethylamine (23 g.; 0·2 mol) was added to isopentyl α-bromophenylacetate (57 g.; 45·2 ml.; 0·2 mol) during 5 minutes, the temperature not being allowed to rise above 40°. The residue of base was washed in with benzene (10 ml.) and the mixture heated on the water bath for 4 hours. The resulting brown oil was dissolved in water (200 ml.), the solution stirred, cooled to 0°, and 10 N sodium hydroxide (20 ml.; 0·2 mol) added dropwise, keeping the temperature at 0°. Sodium bicarbonate (8 g.) was added and the solution extracted with ether (3 × 100 ml.). The combined ether extracts were washed with water (50 ml.), dried over sodium sulphate, the ether removed and the residual oil distilled. A small forerun was rejected, the main fraction b.p. 180 to 188°/1·0 to 0·5 mm. being collected (52 g.; 82 per cent); redistillation gave the *product* as a colourless oil (46 g.; 73 per cent), b.p. 184 to 188°/1·0 to 0·5 mm.

The foregoing ester (40 g.) was dissolved in hot ethanol (40 ml.) and to this solution was added a solution of maleic acid (30·6 g.; 1·05 mol) in hot ethanol (70 ml.). The resulting clear solution was allowed to stand overnight and then dry ether (150 ml.) added; the *di*(*hydrogen maleate*) separated as colourless lustrous flakes (60·6 g.; 88 per cent) m.p. 122 to 123°. Found: *M*, 550; N, 5·0. $C_{27}H_{38}O_{10}N_2$ requires *M*, 550; N, 5·1 per cent. The following esters were prepared in similar manner: the yields obtained being in parentheses. *Isopentyl* α-(2-piperidinoethylamino)phenylacetate (80 per cent); pale yellow oil b.p. 198 to 202°/2 mm. Found: *M*, 340. $C_{20}H_{32}O_2N_2$ requires *M*, 332; the *di*(*hydrogen oxalate*) separated from ethanol as colourless micro needles m.p. 154 to 156° (decomp.). Found: *M*, 512. $C_{24}H_{36}O_{10}N_2$ requires *M*, 512.

Cyclohexyl α-(2-diethylaminoethylamino)phenylacetate (67 per cent); colourless oil b.p. 200 to 204°/1 mm. Found: *M*, 334. $C_{20}H_{32}O_2N_2$

requires *M*, 332; the *di(hydrogen oxalate)* separated from ethanol as needles m.p. 154 to 156° (decomp.). Found: N, 5·5. $C_{24}H_{36}O_{10}N_2$ requires N, 5·5 per cent.

Cyclohexyl α-(2-*piperidinoethylamino)phenylacetate* (73 per cent); colourless oil b.p. 214 to 216°/2 mm. (Found: *M*, 346. $C_{21}H_{32}O_2N_2$ requires *M*, 344.) The *dihydrochloride* separated as hygroscopic colourless microneedles, m.p. 88 to 90° (decomp.). Found: *M*, 424. $C_{21}H_{34}O_2N_2Cl_2$ requires *M*, 417.

Cyclohexyl α-(2-*pyrrolidin*-1′-*ylethylamino)phenylacetate* (60 per cent); pale yellow oil b.p. 208 to 212°/2 mm. (Found: *M*, 324. $C_{20}H_{33}O_2N_2$ requires *M*, 330.) The *di(hydrogen oxalate)* separated from ethanol as colourless plates m.p. 172 to 174°(decomp.). Found: *M*, 514. $C_{24}H_{34}O_{10}N_2$ requires *M*, 510.

Isopentyl α-(2-diethylaminoethylamino)phenylacetate 64 per cent; pale yellow oil b.p. 174 to 178°/1·5 mm. Found: *M*, 327. calc. for $C_{19}H_{32}O_2N_2$, *M*, 320. The dihydrochloride had m.p. 172°. (Lit. m.p. 173°.)

3,5,5-*Trimethylcyclohexyl* α-(2-*piperidinoethylamino)phenylacetate* (60 per cent); pale yellow oil b.p. 218 to 222°/2 mm. (Found: *M*, 386. $C_{24}H_{38}O_2N_2$ requires *M*, 386.) The *di(hydrogen oxalate)* separated as soapy microplates from ethanol-ether m.p. 110° (decomp.). Found: *M*, 568; N, 4·6. $C_{28}H_{42}O_{10}N_2$ requires *M*, 566; N, 4·9 per cent.

3,5,5-*Trimethylcyclohexyl* α-(2-*pyrrolidin*-1′-*ylethylamino)phenylacetate* (69 per cent), b.p. 206 to 210°/2 mm. Found: *M*, 372. $C_{23}H_{36}O_2N_2$ requires *M*, 372; *di(hydrogen oxalate)* m.p. 139 to 140°. Found: *M*, 546. $C_{27}H_{40}O_{10}N_2$ requires *M*, 552.

3,5,5-*Trimethylcyclohexyl* α-(2-*diethylaminoethylamino)phenylacetate* (60 per cent); b.p. 200 to 202°/1·5 mm. Found: *M*, 384. $C_{23}H_{38}O_2N_2$ requires *M*, 374; *di(hydrogen oxalate)* m.p. 102 to 104°. Found: *M*, 540; N, 5·2. $C_{27}H_{42}O_{10}N_2$ requires *M*, 554; N, 5·1 per cent. Ethyl α-(2-diethylaminoethylamino)phenylacetate; colourless oil (52 per cent) b.p. 156 to 158°/1·5 mm. Found: *M*, 278. $C_{16}H_{26}O_2N_2$ requires *M*, 278; *di(hydrogen oxalate)* colourless needles from ethanol, m.p. 174° (decomp.). Found: *M*, 454; N, 6·4. $C_{20}H_{30}O_{10}N_2$ requires *M*, 458; N, 6·1 per cent.

Ethyl α-(2-*pyrrolidin*-1′-*ylethylamino)phenylacetate*: pale yellow oil (80 per cent) b.p. 163 to 166°/1·5 mm. Found: *M*, 272. $C_{16}H_{24}O_2N_2$ requires *M*, 278; *di(hydrogen oxalate)* (from ethanol) m.p. 158 to 160° (decomp.). Found: *M*, 452. $C_{20}H_{28}O_{10}N_2$ requires *M*, 456.

Ethyl α-(2-*piperidinoethylamino)phenylacetate*: pale yellow oil (76 per cent) b.p. 172 to 176°/2 mm. Found: *M*, 292. $C_{17}H_{26}O_2N_2$ requires *M* 290; *di(hydrogen oxalate)* m.p. 160 to 162°. Found: *M*,472; N, 6·1. $C_{21}H_{30}O_{10}N_2$ requires *M*, 470; N, 6·0 per cent.

Phenyl α-(2-*diethylaminoethylamino)phenylacetate*: yellow oil (20 per cent) b.p. 222 to 224°/1·5 mm. Found: *M*, 338. $C_{20}H_{26}O_2N_2$ requires *M*, 326.

The following esters of α-(2-diethylaminoethylmethylamino)phenyl acetic acid were obtained by condensation of the appropriate α-bromo ester with (2-diethylaminoethyl)methylamine:—*isopentyl*—: colourless oil

(74 per cent) b.p. 174 to 176°/1 mm. Found: *M*, 332. $C_{20}H_{34}O_2N_2$ requires *M*, 334. *Cyclohexyl*—: colourless oil (77 per cent) b.p. 194°/1·5 mm. Found: *M*, 344. $C_{21}H_{34}O_2N_2$ requires *M*, 346. 3,5,5-*Trimethylcyclohexyl*—: pale yellow oil (82 per cent) b.p. 196 to 198°/2 mm. Found: *M*, 384. $C_{24}H_{40}O_2N_2$ requires *M*, 388. *Ethyl*—: yellow oil (45 per cent) b.p. 148 to 152°/1 mm. Found: *M*, 290. $C_{17}H_{28}O_2N_2$ requires *M*, 292.

α-(2-*Diethylaminoethylamino*)*phenylacetamide*, b.p. 200 to 206°/1 mm. crystallised from ethanol as pale yellow needles m.p. 56 to 60°. Found: *M*, 245. $C_{14}H_{23}ON_3$ requires *M*, 249.

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PHARMACOPOEIAS AND FORMULARIES

THE BRITISH PHARMACEUTICAL CODEX, 1959*

REVIEWED BY EDWARD G. FELDMANN, Ph.D.

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Examination of the latest edition of the British Pharmaceutical Codex emphasises the tremendous strides made in therapeutics during the past five years. One need only compare the preparations found in the current volume with those carried in the previous edition in order to document this statement. At no other time in the history of drug standardisation have bodies charged with the responsibility for revision of a pharmacopeia, formulary, or codex been faced with a task of such proportions as that encountered within the last few years. Pharmacopoeial revision is normally a difficult and painstaking project; because of the recent rapid advances in therapeutics, this is more true now than ever before.

For the reasons noted above, the Codex Revision Committee is to be particularly congratulated on the occasion of the publication of the 1959 edition of the British Pharmaceutical Codex. It might be generally stated that any book of a pharmacopeial nature serves a useful purpose only so long as it continues to reflect current interest and usage of drugs, coupled with consideration of good medical practice. It is all too easy for a revision committee to allow itself to become so enamoured with the past glories of those drugs which have since dropped into oblivion that it refuses to exclude such agents from a new revision, and to replace them with items currently recognised as pharmacologically superior. If this is allowed to transpire, however, the volume no longer can be regarded as a working book of standards, but merely as a reference item of historical interest.

The Revision Committee has firmly come to grips with this problem. Inspection of the current edition reveals that monographs for 99 basic drugs, 139 formulary preparations, and 11 miscellaneous items have not been carried over from the 1954 edition or 1957 Supplement. Furthermore, new monographs have been added for 70 drugs, 78 formulae, and 15 miscellaneous items. This is, of course, the most significant feature of the current revision—the attempt to maintain it as a timely, up-to-date book of drug standards. This feature is particularly impressive when the titles of the deletions and additions are examined. While it is true that there will be some minor disagreement with a few of the choices, this will generally reflect personal opinion, and for the most part, the selections have been made with a most critical eye and in keeping with good therapeutic knowledge.

Items retained from the previous volume, moreover, have not been allowed to stagnate. In some cases, the content of the individual monograph has been amended to such a degree that practically only the title is recognisable. In carrying these sections over, the respective sub-committees have revised many of the actions and uses statements, assays, identity tests, and other valuable information.

The general make-up of the present edition follows that previously employed for the Codex, and needs no introduction to those familiar with the earlier works. Following a relatively short statement of general notices, there are six main sections or "Parts" devoted respectively to (1) general monographs, (2) antisera, vaccines, and related products, (3) preparations of human blood, (4) surgical

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ligatures and sutures, (5) surgical dressings, and (6) formulary. Finally, there is a section of useful appendices, which has been expanded to cover 17 topics.

Looking more closely at Part I, General Monographs, it is immediately apparent that the present edition continues a trend established in the previous revision; namely, the bulk of the new admissions constitute synthetic organic drugs covering the entire gamut of therapeutic classes, while the majority of deletions are botanical items which over the years have given way to newer agents of generally greater medicinal potency and specificity. Several new antibiotic forms have also been added.

The monographs themselves are generally quite complete and well written. The pharmacological data ("actions and uses") appears to be especially well considered and provides a good coverage of this aspect of each drug in a rather concise form. The chemical considerations of each monograph are also substantially well done, and the only serious criticism might be levelled at the assay procedures. Of the large number of assay methods described, practically all are either gravimetric or volumetric in nature, including many elemental analyses. Consequently, an unfortunately large percentage are relatively nonspecific and open to serious error. Only a very small sprinkling of the newest monographs contain assay methods which employ newer methods of instrumental analysis. Spectrophotometric (ultra-violet, infra-red, and colorimetric) and fluorimetric assay procedures are often rapid, selective, precise, and well adapted for the determination of small quantities of drugs or contaminants. Competent analysts recognise their proven value and have widely employed them in the course of drug analysis. Greater use of such procedures in the Codex would represent a very substantial improvement over the rather large number of gravimetric and Kjeldahl nitrogen methods presently utilized.

Part VI has again undergone extensive revision, with an apparent attempt to reduce it in size, and at the same time, to improve it in quality. Over one-half of the 78 new preparations listed are of similar or identical composition to those dropped from the latest British Pharmacopoeia. Conversely, 19 of the preparations no longer carried in the Codex may now be found listed in similar or identical composition in the 1958 Pharmacopoeia. The balance of the 139 dosage forms have entirely passed from the scene. Most of these items were of such infrequent use and of such questionable therapeutic merit, that it is quite doubtful that they will be at all missed. By this careful scrutiny, the Revision Committee has quite materially enhanced the content and value of the formulary section.

As in previous editions, a set of appendices is provided. These cover a variety of useful subjects, and include three new headings: (1) milliequivalents, (2) biological assays and tests, and (3) uniformity of diameter of tablets. It would appear that with the inclusion of several spectrophotometric assay procedures in the monographs and formulary sections, it would have been desirable to have added an appendix on this subject. This might have discussed theory, defined terms and symbols, noted precautions, explained calculations, and described acceptable details of technique. However, this can be left for a subsequent edition.

The task undertaken by the Codex Revision Committee was a formidable one. In reviewing the 1959 edition of the book, the reader is impressed by the high degree of success which has been achieved. Pharmacists, physicians, and other members of the health team will benefit immeasurably from the results of this work. While this is particularly true of individuals in the British Commonwealth, the British Pharmaceutical Codex will continue to occupy a place of high prestige with others throughout the world who are also concerned with the public health and safety.

LETTERS TO THE EDITOR

The Paired Tracheal Chain Preparation

SIR,—The guinea pig tracheal chain preparation described by Castillo and de Beer in 1947[1] possesses a quality which recommends this tissue for the study of spasmolytics, and of the inhibitory actions of sympathomimetic drugs. This quality is the high natural tone of the muscle, allowing relaxations to be obtained without prior addition of spasmogen.

A number of experimental difficulties arise when the tissue is prepared as the authors describe. These include the small size of the maximum possible relaxation, the wide variation in the sensitivity of individual animals, a very slow response to drugs and recovery therefrom, and an inability to assess the effect of the spontaneous relaxation of tone, which is often seen, on subsequent drug responses. The first of these difficulties has been overcome by Akcasu[2], in 1959, who opened the rings of trachea by cutting through the cartilage. This manoeuvre increased the magnitude of the recorded responses to drugs by a factor of three. By a very simple expedient, it has now proved possible to overcome the other difficulties.

Two guinea pigs (600 to 800 g.) are killed by stunning and bleeding. The tracheae are removed from larynx to carina, and each is cut into eight rings of

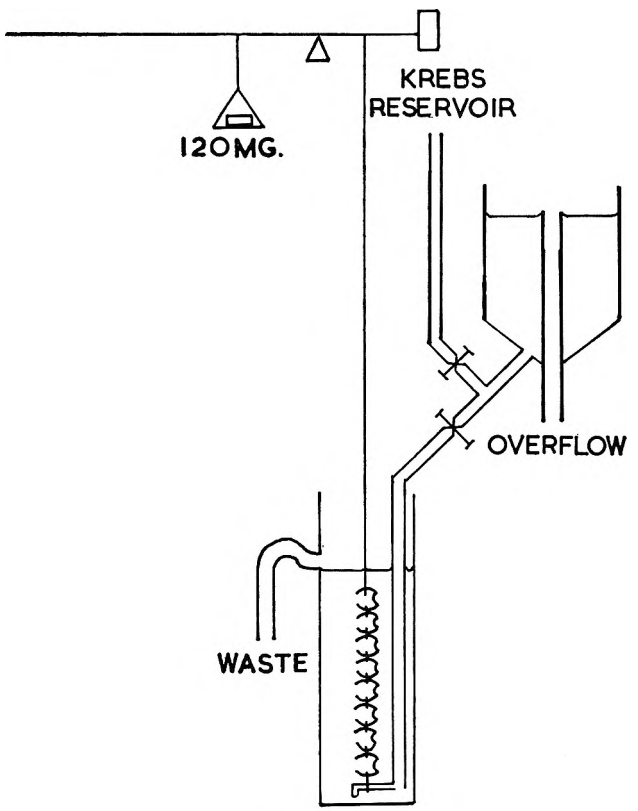

FIG. 1

equal width with scissors. Each ring is opened by cutting through the cartilage and, working from the laryngeal end, the odd numbered rings from one animal and the even numbered ones from the other are tied together to form a chain. The remaining pooled rings form a second preparation which, by this construction, is identical with the first. If, as often happens, only smaller guinea pigs are available, the same effect may be achieved by cutting each trachea into six rings, and using a third guinea pig to provide the other two rings in each preparation.

Each of the two preparations is set up in an apparatus like that shown in Figure 1. Though, for clarity, water jackets and gas lines to both baths have been omitted, each tissue is immersed in Krebs's solution at 38° aerated with oxygen and 5 per cent carbon dioxide. Washing is by displacing the bath fluid with fresh prewarmed and pre-aerated Krebs's solution. Responses to drugs are recorded by a lightly weighted, isotonic, balsa-wood side writing lever, so arranged that the tension applied to the preparation is about 240 mg.

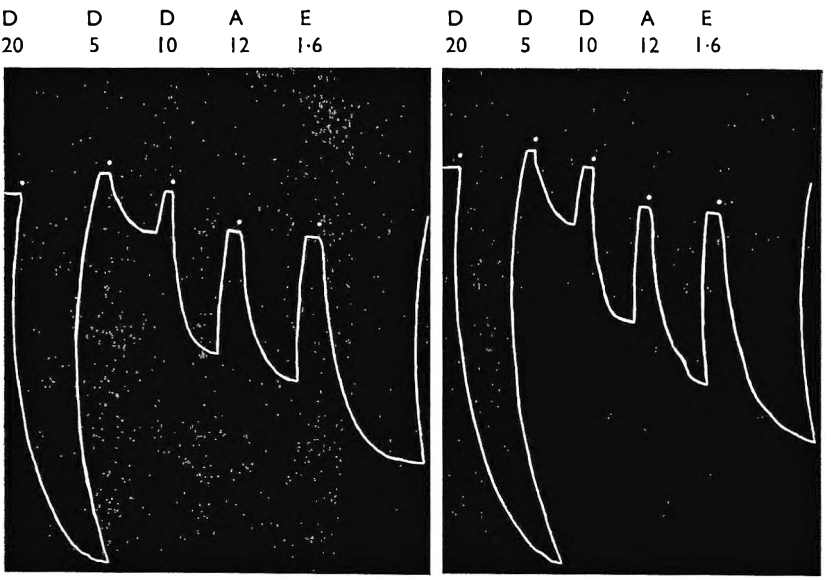

FIG. 2. The similarity of response obtained with the paired tracheal preparation. All doses are in μg. of base per ml. of final bath concentration.

D = Dopamine A = Aminophylline E = (−)-Ephedrine

The spontaneous elongation of the tissue, often seen when it permanently supports this load, may be minimised by removing all tension from it during the period of washing-out the drugs. The load is then reapplied five minutes before the next addition of drug. That such pairs of preparations do, as intended, behave very similarly is illustrated by Figure 2. The great similarity of behaviour of the two preparations to doses of dopamine, aminophylline, and (—)ephedrine is shown. Very important is the fact that the small spontaneous elongation of the tissue is similar in both.

This similarity of behaviour of a pair of preparations reduces the duration of an experiment, as one may be used as a control for drug procedures applied

to the other. The responses of the two preparations may be compared directly so overcoming the need for excessive repetition.

Prepared in the way described in this paper, the guinea pig tracheal chain has proved a consistent and useful tissue in the analysis of the inhibitory actions of sympathomimetics. It lends itself particularly well to the study of blocking and potentiating agents.

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Relation between Dispersibility and Adherence of Powdered Medicinal Substances in Liquid Medium

SIR,—In my recent communication[1] I have reported a method for measuring the dispersibility of powdered medicinal substances by the passage of part of a sample through hydrophilic gauze under the influence of liquid falling in drops. Zinc oxide, talc, bismuth subcarbonate, bismuth subgallate, bismuth subnitrate, bismuth subsalicylate, magnesium subcarbonate, kaolin, precipitated calcium carbonate, precipitated sulphur, mercury amidochloride, yellow mercury oxide, wheat starch, sulphacetamide, sulphadimidine, sulphaguanidine, sulphanilamide, and sulphathiazole, were investigated in this way. Three liquids, i.e. water, 1 per cent solution of Tween 80 in water, and ethanol (95 per cent w/w) were the dispersing media.

I have now determined the "tear off angle" ("Abreisswinkel") of the same substances in the same liquids by a method the principle of which had been suggested by von Buzágh[2]. Thus the adherence of powdered medicinal substances was studied and the relation between dispersibility and adherence was treated by regression analysis[3,4]. The rectification of the data and the due linear regression $\frac{\log u - \log u_k}{d} = 0{\cdot}4343b + 0{\cdot}4343cd$ (d was plotted as abscissa and $\frac{\log u - \log u_k}{d}$ as ordinate) served as fundamentals for finding the values of parameters and the final form of regression equation which in the case of all three liquids proved to be of the following exponential type:

$$u = k.\exp(bd + cd^2) \quad .. \quad .. \quad .. \quad (1)$$

The meaning of symbols is as follows: u = "tear off angle"; u_k = selected reference value of "tear off angle" (in this instance the value for precipitated sulphur), d = dispersibility; k, b, c = parameters relative to the liquid used. The values of parameters for coded units of dispersibility are indicated in Table I (D = actual values of dispersibility); the coding was used in order to facilitate the computation.

TABLE I

PARAMETERS AND SIGNIFICANCE OF THE EXPONENTIAL REGRESSION: ADHERENCE UPON DISPERSIBILITY

Liquid	Parameters			Coding $d =$	Significance of regression (probability of null hypothesis)
	k	b	c		
Water	71·67	$-640{\cdot}7 \times 10^{-6}$	$0{\cdot}3355 \times 10^{-6}$	30D—174	$P < 0{\cdot}01$
1 per cent solution of Tween 80 in water	63·58	$226{\cdot}1 \times 10^{-6}$	$-0{\cdot}4260 \times 10^{-6}$	30D—543	$P < 0{\cdot}01$
Ethanol (95 per cent w/w)	48·25	$323{\cdot}9 \times 10^{-6}$	$-0{\cdot}5511 \times 10^{-6}$	30D—636	$P < 0{\cdot}10$
	(48·25)	$(130{\cdot}1 \times 10^{-6})$	$(<0{\cdot}8755 \times 10^{-6})$	(30D—636)	$(P < 0{\cdot}01)$

As far as suspensions of the powdered medicinal substances in alcohol are concerned, the values in parentheses in Table I have been obtained by omission of two unreliable results.

The above mentioned equation (1) can be used for predicting the value of one property from the knowledge of the other (as both variables are on an equal footing from a statistical point of view, the reverse form of the regression, i.e., the one in which the dispersibility is the dependent variable and the adherence the independent one, could also be calculated by means of pertinent procedures) with proper precaution. Furthermore, despite its statistical character and the absence of an immediate proof of causal relationship between the two variables, the equation may be relevant when considering the theory of pharmaceutical suspensions.

Experimental and computational details of this work will be published later.

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