JOURNAL OF PHARMACY AND PHARMACOLOGY

VOLUME XV No. 12

DECEMBER 1963

Published by Direction of the Council of

THE PHARMACEUTICAL SOCIETY OF GT. BRITAIN

17 BLOOMSBURY SQUARE, LONDON, W.C.1

JOURNAL OF PHARMACY AND PHARMACOLOGY

Editor: George Brownlee, D.Sc., Ph.D., F.P.S.

Assistant Editor: J. R. Fowler, B.Pharm., F.P.S.

Annual Subscription **£5 0s. 0d.** Single Copies **10s.**

17 BLOOMSBURY SQUARE, LONDON, W.C.1

Cables: Pharmakon, London, W.C.1 *Telephone:* HOLborn 8967

Vol. XV No. 12 **December, 1963**

CONTENTS

RESEARCH PAPERS

THE MOLECULAR PROPERTIES OF GHATTI GUM: A NATURALLY OCCURRING POLYELECTROLYTE

By P. H. Elworthy and T. M. George

*From the School of Pharmacy, Royal College of Science and Technology, Glasgow, C.*1

Received July 25, 1963

An authenticated sample of ghatti gum from *Anogeissus latifolia* has been fractionated by ethanolic precipitation (Fraction I), and chromatography on silica gel (Fractions 2 and 3). The equivalent weights found were 1,750, 1,800 and 2,040 respectively. The sodium salts of the fractions have been studied in various salt solutions by viscosity and light scattering methods. From the latter the molecular weights ($= 2{\cdot}1$, $2{\cdot}7$, and $2{\cdot}6 \times 10^6$ respectively), radii of gyration, and some idea of molecular shape have been obtained. The molecule appears to be rod shaped. The expansion factors of the molecules calculated from the light scattering and the viscosity data agree with each other. Reasons for the difference in the values of the calculated and observed second virial coefficients are discussed.

Ghatti gum is obtained from *Anogeissus latifolia* (Combretaceae). During the latter part of the nineteenth century, until British control in 1898, troubles in the Sudan almost stopped the export of acacia. This shortage led to a search for substitutes, and to the introduction of ghatti gum.

Work on the chemical structure of the gum is not complete, but degradation studies have shown it to be a polysaccharide which consists of a backbone of galactose units to which other sugars are attached (Aspinall, Hirst and Wickstrøm, 1955; Aspinall, Auret and Hirst, 1958). The side chains can consist of arabinose residues and also of aldobiuronic acids.

Ghatti gum has a number of industrial applications but very little physico-chemical work has been done. The molecular weight of the neutral dialysed material was found to be 2,878 by freezing-point depression and 11,920 by osmotic pressure measurements (Shaw, 1939). Equivalent weights ranging from 1,340–1,735 were found. Electrophoresis studies on glass-fibre filter paper have been made by Lewis and Smith (1957), and the interaction of the gum with Prussian Blue solution has been observed (Carhart and Shaw, 1936). In this paper attempts to fractionate the gum and to determine its molecular nature in solution have been made. These are necessary preliminaries to studies of adsorption and electrophoresis of suspensions stabilised with this material.

Experimental

Fractionation of Ghatti Gum and Preparation of Sodium Ghattate

The material was first authenticated by comparison with a known sample of gum from *Anogeissus latifolia*. Whole tears of gum were slowly stirred in water at 20° overnight and the solution filtered to remove

any insoluble material. The free acid was prepared by careful acidification of the filtrate with hydrochloric acid and precipitation with ethanol. The precipitate was collected, washed well with ethanol, and redissolved in water. After dialysis for 24 hr. the solution was concentrated at not more than 40° using a rotary film evaporator. The free ghattic acid was then precipitated with ethanol. This precipitation was repeated twice. Finally, the material was dissolved in water, neutralised with sodium hydroxide solution, concentrated, and dried at 35° in a vacuum oven over P_2O_5 (Fraction 1).

A chromatographic fractionation was also attempted. The solution of crude gum was passed down a strong cation exchanger to convert it to the free acid. This solution was then dialysed and evaporated to dryness as before. 16·5 g. of the ghattic acid was dissolved in 525 ml. of a 1 : 2 water : methanol mixture. This solution was then passed down a column of 450 g. silica gel. Elution of the column with 1 litre of 33 per cent water in methanol gave 8 g. of material (Fraction 2). Slow increase of water content and finally elution with 1 litre of water gave, after evaporation, 3·5 g. material (Fraction 3). The remainder of the material on the column could not be eluted with water.

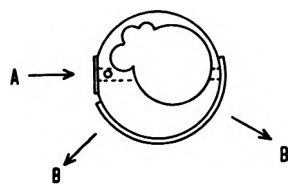


FIG. 1. Top view of light scattering cell. External diameter = 5 cm. A, incident beam. B–B viewing area.

Fractions 2 and 3 were evaporated to a small volume, neutralised as before with NaOH, using a titration curve to determine the end-point on a small part of the sample, and dried.

The equivalent weights of fractions 1, 2 and 3 were found to be 1,750, 1,800 and 2,040 respectively. The sodium hydroxide and sodium chloride used were Analar materials, and the water was twice redistilled from permanganate.

Light-Scattering Measurements

The apparatus described by Elworthy and McIntosh (1961) was used. The turbidity of Analar benzene measured immediately after an extensive calibration with Ludox solutions had been found to be $27{\cdot}2 \pm 0{\cdot}4 \times 10^{-5}$ cm.$^{-1}$; eighteen months later four repeat measurements on benzene gave $27{\cdot}3 \pm 0{\cdot}3 \times 10^{-5}$ cm^{-1}. This agreement indicated that there was no change in calibration constant, within experimental error, over this time period.

The cells originally described have been modified by the inclusion of semi-circular light traps cut into the back wall (Fig. 1), which eliminated stray light when measurements were made down to 30° to the incident

beam. The cell was blackened inside by the Relanol process. Solutions were clarified by filtration through a No. 5 porosity gas filter tube. The concentrations of the solutions were checked on a Hilger-Rayleigh interferometer after filtration.

Specific Refractive Index Increments (dn/dc)

These were determined with a Hilger-Rayleigh interference refractometer using monochromatic light (Bauer, Fajans and Lewin, 1960). In sodium chloride solution the specific refractive index increments were measured at constant chemical potential as described by Vrij and Overbeek (1962).

Viscosity Measurements

A Couette viscometer, based on the design by Ogston and Stanier (1953) was constructed. A constant voltage transformer (Advance 250 w.) was used in addition to a variable speed motor control (Engelhard-Hanovia) to give a constant rotational speed for the outer cylinder. As this cylinder rotated a brass rod attached to it activated a Counting Instruments photo-transistor counter type 500. The speed of rotation could thus be measured at the same time as the deflections of the inner cylinder were observed. A suspended level dilution viscometer was also used.

Densities

The densities were measured by displacement of dry benzene.

All measurements were made at 20°; ± 1° for light scattering, and ± 0·05° for all other experiments.

Results and Discussions

Light scattering measurements were made on all fractions in 0·5N sodium chloride solutions and on fractions 1 and 2 in varying salt concentrations. Two representative Zimm plots are shown in Figs. 2 and 3 for fraction 2, where c in the concentration of sodium ghattate in g./ml. and S_θ is the scattering from the solute at an angle θ to the incident beam. The equations governing the two limiting lines of a Zimm plot are given below.

Zero angle line:
$$\left(\frac{Hc}{T}\right)_{\theta=0} = \frac{1}{M_w} + 2Bc \quad .. \quad .. \quad (1)$$

Zero concentration line:

$$(Hc/T)_{c=0} = 1/M_w.P(\theta)$$
$$= \frac{1}{M_w}\left(1 + \frac{16\pi^2}{3\lambda_1^2} Rg_z^2 \sin^2\frac{\theta}{2}\right) \quad .. \quad .. \quad .. \quad (2)$$

in which $H = 32\pi^3 n_0^2 (dn^*/dc)^2/3\lambda^4 N$

T = turbidity, B = second virial coefficient, P ( ) = particle scattering factor, Rg = radius of gyration, and M = molecular weight.

The asterisk on the (dn/dc) symbol indicates that the measurement was taken at constant chemical potential where salt was present.

The fraction prepared by ethanolic precipitation has the lowest molecular weight. There is considerable variation in the molecular weights determined for fraction 2 in various solvents. Several authors (Benoit, Holtzer and Doty, 1954; Robinson and Saunders, 1959; Elworthy and Macfarlane, 1962) have shown that errors in the determination of M can

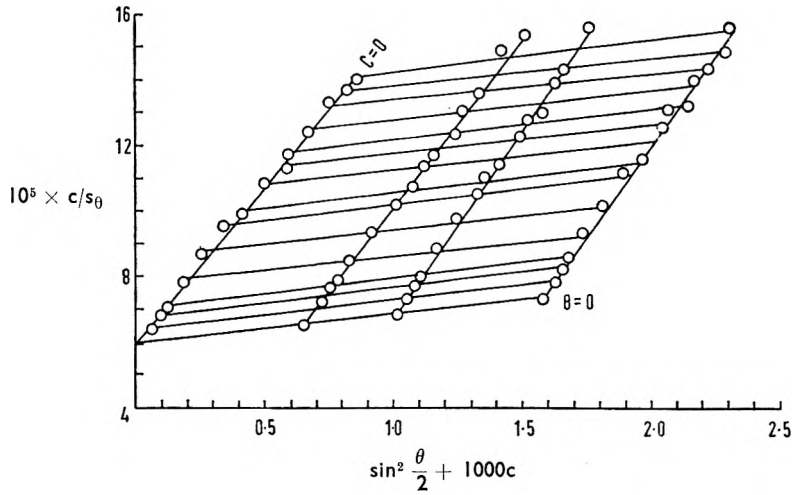


FIG. 2. Zimm plot of fraction 2 in 0·5N sodium chloride solution. See text for symbols.

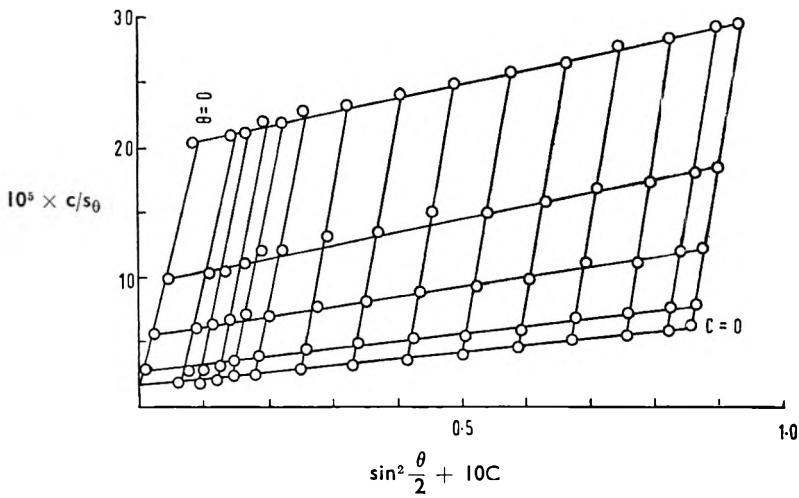


FIG. 3. Zimm plot for fraction 2 in water. See text for symbols.

be as large as ± 10 per cent. Estimates of error have been based on a study of equation (1) using particles small enough for $P(\theta)$ to be taken as unity. The use of a Zimm plot introduces further error as extrapolated values of $(Hc/T)_{\theta = 0}$ must again be used in an extrapolation procedure to obtain M.

The radii of gyration are consistent with the molecular weights of the different fractions. As the salt concentration is decreased Rg increases, which is normal polyelectrolyte behaviour. It is possible that the radii of gyration may have lower limits or error than M as they are obtained from the ratio of the limiting slope of the zero concentration line to the intercept in the Zimm plots. As experimental errors would affect both parts of this ratio in the same sense, it may be that some cancellation is obtained.

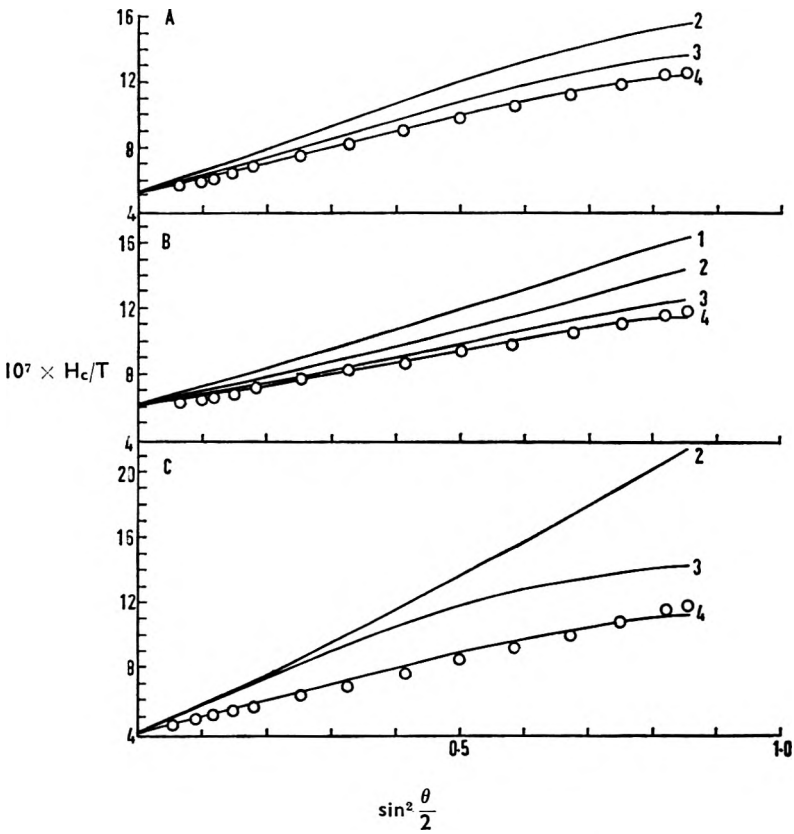


FIG. 4. Comparison of calculated and experimental zero concentration lines of Zimm plots. Curve 1, coils with $M_w:M_n = 2:1$. Curve 2, monodisperse coils. Curve 3, monodisperse rods. Curve 4, rods with $M_w:M_n = 1{\cdot}1:1$ in graph A (fraction 1 in 0·5N NaCl). Rods with $M_w:M_n = 1{\cdot}1:1$ in graph B (fraction 2 in 0·5N NaCl). Rods with $M_w:M_n = 2:1$ in graph C (fraction 2 in water.)

For fraction 1 in 0·5N sodium chloride and fraction 2 in 0·5N sodium chloride and in water, the molecular model which fits the limiting line ($c = 0$) of the Zimm plots was calculated. Using the observed Rg values calculations of particle scattering factors for monodisperse rods and coils, and for coils with a molecular weight distribution $M_z:M_w:M_n = 3:2:1$ were made. In all cases the rod model gave the closest approach to the experimental results (Fig. 4). The fit of calculated to experimental

results was not perfect even for the rod model, and hence the effect of polydispersity was explored using Reichmann's treatment (1959) where $x = (2\pi/\lambda_1)\, L \sin\frac{\theta}{2}$ in which L is the length of the rod (for experimental values see Table I) and z, w, and n are used to indicate the appropriate average.

TABLE I

DATA FROM LIGHT SCATTERING MEASUREMENTS

Fraction	Solvent	dn*/dc	$10^{-6}M$	Rg_z(Å)	10^6B	L(Å)	ρ
1	0·5N NaCl	0·137	2·1	727	4	2,520	0·017
1	0·05N NaCl	0·139	2·1	821	10	2,840	0·016
2	0·5N NaCl	0·136	2·2	768	4	2,660	0·027
2	0·05N NaCl	0·138	3·3	873	10	3,030	0·027
2*	0·005N NaCl	0·143	2·7	1,005	41	3,460	0·020
2	Water	0·148	2·7	1,061	260	3,680	0·048
3	0·5N NaCl	0·130	2·6	740	2	2,560	0·033

* By dissymmetry method ρ = depolarisation

$$\text{The equation}\quad (Hc/T)_{c=0} = \frac{1}{M_w}\left(1 + \frac{x_z^2}{9}\right) \qquad (3)$$

holds up to $x_z = 1{\cdot}5$.

For the upper part of the limiting line, approaching the asymptote where smaller molecules make a significant contribution to the scattered light:

$$(Hc/T)_{c=0} = \frac{1}{M_n}\left(\frac{2}{\pi^2} + \frac{2x_n}{\pi}\right) \qquad (4)$$

This equation holds down to $x = 2$. Its usefulness can be extended if higher terms of the series are included:

$$(Hc/T)_{c=o} = \frac{1}{M_n}\left(\frac{2x_n}{\pi} + \frac{2}{\pi^2} + \frac{2}{\pi^3 x_n} + \frac{2}{\pi^4 x_n^2} + \frac{2}{\pi^5 x_n^3}\right) \qquad (5)$$

Tests showed that this equation held satisfactorily down to $x_n = 1{\cdot}5$. Equation (3) can simply be applied to the results as M_w and Rg_z (hence L_z and x_z) are directly determinable from Zimm plots. As light scattering measurements could not be made at high enough angles to give an idea of the asymptote to the limiting line of the Zimm plot, equation (5) was used by successive approximation. By assuming a molecular weight distribution ($M_z : M_w : M_n$), as M_w was known, M_n could be calculated; the ratio of $L_z : L_w : L_n$ was taken in the same way. Starting with the monodisperse case, $M_z = M_w = M_n$, cases of increasing polydispersity were considered until the best fit to the experimental results were obtained (see Fig. 4).

The figure clearly shows that the rod model gives the best fit to the experimental results, although some deviations from a perfect rod shape could be present, indicating that this is the most probable shape for the molecule, and that it was maintained when the solvent was changed to water. It is interesting that an apparent change of molecular weight distribution occurs on altering the solvent. Both fractions 1 and 2 give a narrow

distribution in 0·5N sodium chloride solution, but in water fraction 2 gives $M_w : M_n = 2:1$. This effect must be bound up with the expansion of the molecules (discussed later); on contraction, in the concentrated sodium chloride solution, differences between the various molecular lengths would be minimised, giving an apparently narrow distribution.

TABLE II

DISTRIBUTION GIVING BEST FIT TO LIMITING LINE OF ZIMM PLOT

Fraction	Solvent	$L_z : L_w : L_n$
1	0·5N NaCl	1·2 : 1·1 : 1·0
2	0·5N NaCl	1·2 : 1·1 : 1·0
2	Water	3·0 : 2·0 : 1·0

Further information on molecular structure can be obtained from the viscosity measurements. From the viscosity results, following Schneider and Doty's procedure of plotting the viscosity intercepts $[\eta]$ against $1/N^{\frac{1}{2}}$ where N = normality of added salt, straight lines are obtained (Fig. 5) and these can be easily extrapolated to infinite ionic strength ($1/N^{\frac{1}{2}} = 0$). In this state the molecule can be treated as being uncharged.

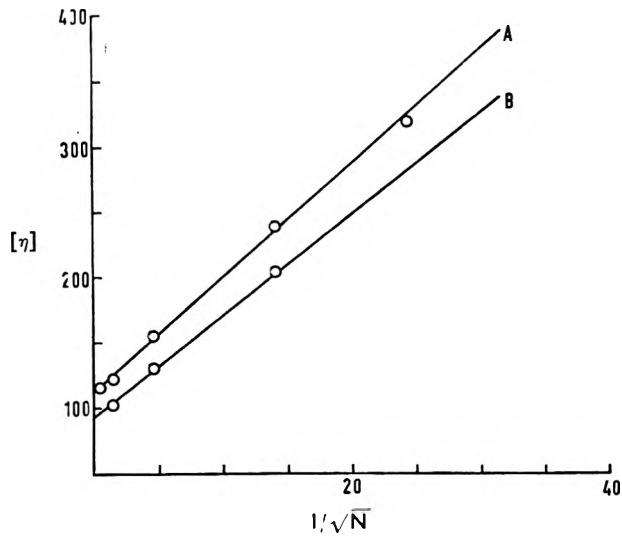


FIG. 5. Plots of $[\eta]$ against $1/N^{\frac{1}{2}}$ for: A, fraction 1; B, fraction 2.

$$[\eta] = \left(\frac{\eta^{sp}}{c}\right)_{c=0} = v(\bar{v}_2 + w_1 v_1^0) \qquad (6)$$

where v is Simha's shape factor (see Mehl, Oncley, and Simha, 1940), $\bar{v}_2$ = specific volume of solute, v_1^0 = specific volume of solvent and w_1 is the hydration of the macromolecule in g. water/g. material. The viscosity intercepts at infinite ionic strength are 112 and 94 c.c./g. for fractions 1 and 2 respectively. The size of those intercepts indicates that the molecule is either highly asymmetric or highly solvated, or both.

Water vapour adsorption studies have been used (Elworthy, 1961) to determine hydration; unpublished studies on sodium ghattate show $w_1 =$ 0·20 g./g. for fraction 1 and 0·19 g./g. for fraction 2. The shape factor is calculated from equation (6) for two conditions, $w_1 = 0$, and for the values of w_1 given above. With this level of hydration it is apparent that the deviation of the viscosity intercept from the Einstein value of 2·5 is due to molecular asymmetry, and since light scattering indicates that rod like particles are present, the viscosity results have been interpreted on this basis. Mehl, Oncley, and Simha's table gives v in terms of the axial ratio a/b of a prolate ellipsoid, and for comparison with light scattering results, which give the length, L, of a rod shaped molecule, a/b is converted to L/d, where d is the diameter of the rod, by Tanford's 1961 (a) procedure:

$$a/b = (0{\cdot}6666).^{\frac{1}{4}}\ L/d \quad .. \quad .. \quad .. \quad .. \quad .. \quad (7)$$

From the light scattering molecular weights, densities, and values of hydration the volumes of the molecules can be calculated, and thus using the viscosity results, the molecular dimensions.

TABLE III

MOLECULAR DIMENSIONS CALCULATED FROM VISCOSITY INTERCEPTS AT INFINITE IONIC STRENGTH

		$w_1 = 0$			$w_1 = 0{\cdot}20$ (fraction 1), $w_1 = 0{\cdot}19$ (fraction 2).		
Fraction	p * (g./ml.)	a/b	L	d	a/b	L	d
1	1·52	49·0	2,190	37	43·4	2,160	41
2	1·54	44·5	2,220	41	38·5	2,190	47

* p = solute density, L and d in Å.

The lengths of the rods at infinite ionic strength (obtained by extrapolating the results from light scattering experiments as a plot of Rg^2 against $1/N^{\frac{1}{4}}$), were 2,500 Å and 2,580 Å for fractions 1 and 2 respectively. Considering the errors when two sets of experimental results are compared, the agreement between these lengths and those calculated from viscosity is good.

It is interesting to compare the structure of arabic acid as determined from light scattering experiments by Veis and Eggenberger (1954) with that found here for ghattic acid. Arabic acid appears to be a stiff coiled molecule with a root mean square distance between its ends varying from 555 Å when uncharged to 1,050 Å when fully charged. This expansion is proportionally greater than that exhibited by sodium ghattate, and may be due to the higher charge density of arabic acid (equivalent weight = 1,200). The molecular weight of arabic acid appears to be half that of the ghatti molecule. No further ideas on the shape of the arabic acid molecule are available as Zimm plots were not used. The viscous behaviour of both materials seems similar, in that the addition of electrolyte greatly reduces viscosity. In the presence of 0·005 N NaCl $[\eta]$ for sodium arabate was 28 c.c./g. (Basu, Dasgupta, and Sircar 1951), compared with $[\eta] = 203$ c.c./g. for sodium ghattate. The higher viscosity of the ghatti solution is due to the larger size of the molecules.

Expansion of Molecule

The viscosity results obtained at different ionic strength are plotted in Fig. 6 for fraction 1, and results in water for the other fractions are given in Fig. 7. Fractions 1 and 2 were examined in water, 0·005 and 0·5N NaCl solution in the Couette viscometer, and showed Newtonian flow over the concentration range studies and up to a velocity gradient of 72 sec.$^{-1}$ (the maximum obtainable on the instrument). The capillary viscometer was used therefore for the remainder of the measurements (average velocity gradient 1,310 sec.$^{-1}$) as this is the more precise instrument. Using the same solution, the results obtained on both instruments agreed well (cf. Fig. 6).

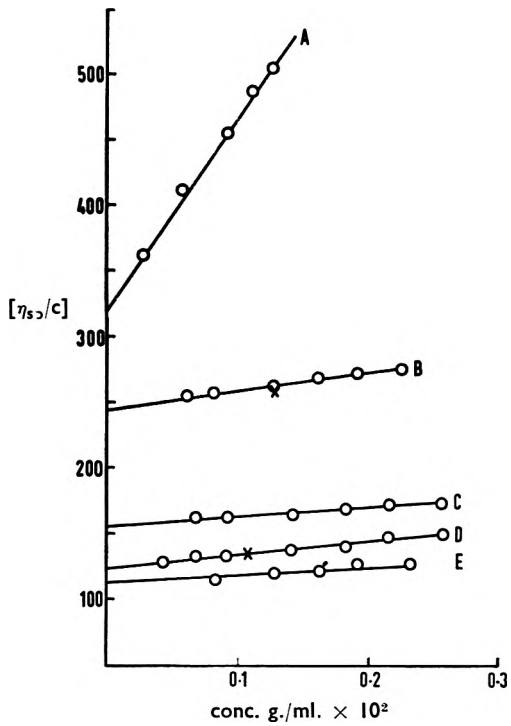


FIG. 6. Plots of η_{sp}/c for fraction 1 in sodium chloride solutions with normalties of: A, 0·0017; B, 0·005; C, 0·05; D, 0·5; E, 2·0. X = points from couette viscometer.

In water the viscosity curves turn upwards as the concentration is reduced (Fig. 7). This behaviour has been observed for other polyelectrolytes in water and the intercepts can be determined (Fuoss and Strauss, 1948) by plotting c/η_{sp} against $c^{\frac{1}{2}}$. In this case the intercept at $c^{\frac{1}{2}} = 0$ is $1/[\eta]$. For fractions 2 and 3, $[\eta]$ = 2,600 c.c./g. and 2,350 c.c./g. respectively.

The increase of both Rg and $[\eta]$ with decreasing salt concentration, can be taken as a measure of the expansion of the molecule. Pals and Hermans

(1952) determined the expansion factor α from $\frac{[\eta]}{[\eta]^\circ} = \alpha^2$, while Schneider and Doty used $\frac{Rg^2}{R\,g_0^2} = \alpha^2$ where $[\eta]^\circ$ and Rg_0 are values obtained by extrapolation to infinite ionic strength.

TABLE IV

EXPANSION FACTOR AT VARIOUS SALT CONCENTRATIONS

NaCl solution, normality	2·0	0·5	0·05	0·005	0·0017
Fraction 1. (visc)	1·01	1·04	1·18	1·46	1·69
1. (L/S)		1·04	1·17		
2. (visc)		1·04	1·18	1·47	
2. (L/S)		1·03	1·17	1·34	

There is excellent agreement between the values of α calculated by the two different methods. Although fraction 1 has the higher charge density, α is very similar for both fractions.

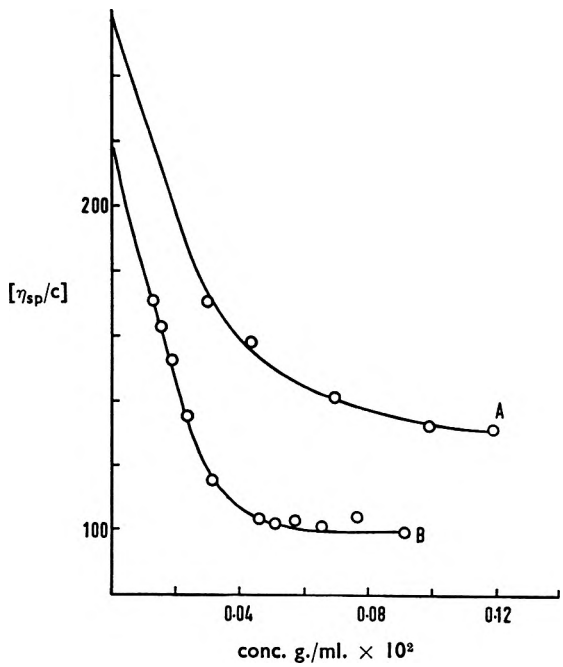


FIG. 7. Plots of η_{sp} /c against c for A, fraction 2; B, fraction 3; both in water.

Schneider and Doty did not obtain agreement between the two different methods of calculating α for the coil like molecule of sodium carboxymethyl cellulose (NaCMC). The square of the radius of gyration is proportional to h^2 for a coil and L^2 for a rod. The comparison of results at low ionic strength with those at infinite ionic strength (where $\alpha = 1$) should lead to a measure of α^2. However $[\eta]$ is proportional to α^3 for a

coiled up molecule, so that the ratio $\frac{[\eta]}{[\eta]^\circ}$ should yield α^3 for this model. A recalculation of Schneider and Doty's results shows that $\sqrt[3]{\frac{[\eta]}{[\eta]^c}}$ gives a value of α in fair agreement with those obtained from light scattering data. The situation is different for rod like molecules. Simha's relationship between the shape factor v and the axial ratio a/b can be generalised, in the range of axial ratios of interest here, to v = constant $(a/b)^{1.8}$ (Tanford 1961b).

As $[\eta]$ is proportional to v we would expect to obtain $\alpha^{1.8}$ from the $\frac{[\eta]}{[\eta]^\circ}$ ratio, provided the molecule is rod like. α^2 is obtained within the limits of experimental error. The discrepancy between these indices can be lessened as we must consider $\alpha^{1.8}$ to be the lower limit for the rod like molecules. It would be expected that as the length of the rod increases during the expansion process, its width would decrease, while it is assumed in the above discussion that as L increases d remains constant. The expansion should lead to a higher power than $\alpha^{1.8}$ and this is observed since α^2 is obtained and agrees with the light scattering data. It would be stretching the results too far to attempt a calculation of the degree of thinning of the rod on expansion, also charged groups present on the side chains may cause a "swinging out" of the side chains during ionisation.

There is a considerable expansion of the molecule length on passing from infinite ionic strength to pure water (from 2,580 Å to 3,680 Å for fraction 2), indicating that a certain amount of flexibility is present, It is possible that electrical forces help to maintain the rod like shape at large extensions, aided by the effect of unionised side chains giving a stiffening to the galactose framework.

Second Virial Coefficients

Firstly the molecule in its uncharged state at infinite ionic strength is considered. The value of B (see eqn. 1) under this condition was determined by extrapolating a plot of B against $1/N^{0.73}$ which gave a convenient straight line. In this state the counterions should be held close to the main part of the molecule, the charge Z does not contribute to intermolecular interactions, and B is expected to be a function of molecular shape and size only. Tanford (1961c) gives

$$B = L\bar{v}_2/dM = LV/dM^2 \quad .. \quad .. \quad .. \quad .. \quad (8)$$

where V is the molecular volume. The length of the rod found from light scattering at infinite ionic strength is used in the calculation.

It can be seen that in uncharged state the value of B agrees reasonably with that calculated for a rod like molecule of dimensions determined by light scattering, particularly as there are large errors in the experimental determination of small values of B.

As the ionic strength is decreased the second virial coefficient increases. The shielding effect of added salt also decreases during this process which gives mutual repulsion between the macromolecules. Donnan-like

conditions should be approached with the counterions distributed between the ionic atmosphere and the solution. Tanford (1961d) gives:

$$B = \frac{1000.\ Z^2 v_1^\circ}{4M^2.\ m_3} \qquad (9)$$

where m_3 is the molality of sodium chloride. The Donnan term has been calculated (Table V) and is considerably larger than the observed second virial coefficient. A similar state of affairs was observed for NaCMC where the Donnan term was 2 or 3 times greater than the observed second virial coefficient. For sodium ghattate the Donnan term is 4 to 40 times greater. This may be a consequence of the much lower charge distribution in the latter molecule than in the NaCMC molecule, and probably indicates that a fair amount of ion-binding exists, even in dilute salt solution. This bound layer of counterions will presumably tend to neutralise most of the macromolecular charge with respect to other large ions. However, the molecule expands as the ionic strength is lowered, so local shielding of one part of the molecule from another cannot be complete. NaCMC, being a coiled molecule, will occupy a much larger volume of space in solution than the sodium ghattate molecule, which is

TABLE V

CALCULATED AND OBSERVED SECOND VIRIAL COEFFICIENTS

Normality	Fraction 1			Fraction 2		
	10^3B expt	10^3B eqn. 8	10^3B eqn. 9	10^6B eqn. 9	10^6B eqn. 8	10^3B eqn. 9
0·5	3	2*		3	2*	
0·05	4		15	4		16
0·005	10		154	10		163
			1,540	41		

* Calculated for unhydrated molecule. No significant change in B is obtained if hydration is considered.

visualised as being a fairly compact rod, although the molecular weight of sodium ghattate is five times that of NaCMC. Much smaller interferences would be expected which is reflected by the value of B for sodium ghattate being 1/30 that of NaCMC in 0·5N sodium chloride solution. This provides an interesting contrast between the two types of polyelectrolyte.

Acknowledgements. We thank Evans Medical Ltd. for the award of an Evans Medical Research Fellowship, and for the gift of ghatti gum. We are indebted to Dr. Kenyon of the Tropical Products Institute and to Professor P. V. Bole of St. Xavier's College for arranging the supply of an authenticated sample of gum, to Dr. F. Fish for authentication and to Mr. G. Cochrane for building the Couette viscometer.

REFERENCES

Aspinall, G. O., Auret, B. J. and Hirst, E. L. (1958). *J. chem. Soc.*, 221–230; 4408–4414.

Aspinall, G. O., Hirst, E. L. and Wickstrøm, A. (1955). *Ibid.*, 1160–1165.

Basu, S., Dasgupta, P. C. H. and Sircar, A. K. (1951). *J. Colloid Sci.*, **6**, 539–548.

Bauer, N., Fajans, K. and Lewin, S. Z. (1960) in Weissburger, '*Physical Methods of Organic Chemistry*', 3rd edition: Vol. 1, Part 2, 1139–1282. New York: Interscience.
Benoit, H., Holtzer, A. M. and Doty, P. (1954). *J. phys. Chem.*, **58**, 635–640.
Carhart, H. C. and Shaw, E. H. (Jr.) (1936). *Proc. S. Dak. Acad. Sci.*, **16**, 34–43; through *Chem. Abstr.* (1938), **32** (1), 3685.
Carhart, H. C. and Shaw, E. H. (Jr.) (1935). *Ibid.*, **15**, 46–50: through *Chem. Abstr.* (1936). **30** (1), 2456.
Elworthy, P. H. and McIntosh, D. S. (1961). *J. Pharm. Pharmacol.*, **13**, 663–669.
Elworthy, P. H. and Macfarlane, C. B. (1962). *J. chem. Soc.*, 537–541.
Elworthy, P. H. (1961). *Ibid.*, 5385–5389.
Fuoss, R. M. and Strauss, U. P. (1948). *J. Poly. Sci.*, **3**, 246–263.
Lewis, B. A. and Smith, F. (1957). *J. Amer. chem. Soc.*, **79**, 3929–3931.
Mehl, J. W., Oncley, J. L. and Simha, R. (1940). *Science*, **92**, 132–133.
Ogston, A. G. and Stanier, J. A. (1953). *Biochem. J.*, **53**, 4–7.
Pals, D. T. F. and Hermans, J. J. (1952). *Rec. Trav. Chim., Pays-Bas*, **71**, 433–457.
Pals, D. T. F. and Hermans, J. J. (1952). *Ibid.*, **71**, 458–467.
Pals, D. T. F. and Hermans, J. J. (1952). *Ibid.*, **71**, 513–520.
Reichmann, M. E. (1959). *Canad. J. Chem.*, **37**, 489–492.
Robinson, N., and Saunders, L. (1959), *J. Pharm. Pharmacol.*, **11**, 115*T*–119*T*.
Schneider, N. S. and Doty, P. (1954). *J. phys. Chem.*, **58**, 762–769.
Shaw, E. H., Jr. (1937). *Proc. S. Dak. Acad. Sci.*, **17**, 27–30. Through Chem. Abstr. (1938), 32 (1) 2002.
Tanford, C. (1961). *Physical Chemistry of Macromolecules*, New York: John Wiley & Sons. a, p. 342. b, p. 336. c, p. 196. d, p. 229.
Veis, A. and Eggenberger, D. N. (1954). *J. Amer. chem. soc.*, **76**, 1560–1563.
Vrij, A. and Overbeek, J. Th. G. (1962). *J. Colloid Sci.*, **17**, 570–588.

THE EFFECT OF IRRITANT SUBSTANCES ON THE DEPOSITION OF GRANULATION TISSUE IN THE COTTON PELLET TEST

BY S. CYGIELMAN AND J. M. ROBSON

From the Department of Pharmacology, Guy's Hospital Medical School, London, S.E.1

Received August 6, 1963

The observation that in a group of substances derived from liquorice, anti-inflammatory activity as determined by the cotton pellet test seemed to be associated with irritant effects at the site of injection, led to the investigation of the effect of known irritant substances. It was found that tartar emetic, croton oil, talc and animal charcoal caused a significant reduction in the granulation tissue deposited around cotton pellets implanted in both intact and adrenalectomised rats. It is suggested that there is some limit to the total number of leucocytes which, in the course of a few days, can be mobilised to take part in an inflammatory reaction. Thus the two sites at which irritant effects have been produced, i.e. the site of implantation of the cotton pellets and the the site of injection of the irritant substance, compete for these cells, giving apparently an "anti-inflammatory" effect. This may well represent an important disadvantage of the cotton pellet test, making it suitable only for the investigation of non-irritant substances.

IN the course of investigating the anti-inflammatory activity of a series of substances derived from liquorice by the cotton pellet test, it was noted that the substances which had a marked anti-inflammatory effect also caused a considerable amount of irritation at the site of injection, whereas compounds which were less potent did not have any adverse local effects (Cygielman, 1963). Most of the compounds in this series were insoluble and, in addition to the increased vascularization apparent at the site of injection, there was a massive influx of white cells. It was of interest to determine the extent to which the anti-inflammatory activity of the compounds tested was associated with their irritant effects. Since there is a possibility that a stress reaction may be produced by the administration of these substances, and that this may be responsible, to some extent at least, for the increased anti-inflammatory effect, experiments were made in adrenalectomised as well as in intact animals.

METHODS

A modification of the method of Meier, Schuler and Desaulles (1950) for the measurement of anti-inflammatory activity was used. Experiments were done on male Wistar rats, weighing 130–190g. Each experiment included one or more treated groups and a control group, and there were five rats in each group. The cotton pellets were weighed individually, and only those within 0·2 mg. of the mean weight were used for any one experiment. The pellets were sterilised by heating for 2 hr. at 150° and implanted subcutaneously, one in each axilla and groin. On the fourth day after implantation the cotton pellets were dissected out, dried for 24 hr. at 60°, and re-weighed. The increase in weight was used as an indication of

the amount of granulation tissue deposited. Test substances were injected subcutaneously daily for four days unless otherwise stated, beginning on the day of implantation. The substances were given as solution or suspension in 0·1 to 1·0 ml. of water, except croton oil, which was injected as such or dissolved in olive oil. Controls received 1 ml. of saline.

The anti-inflammatory effect was calculated as the percentage reduction of the increase in weight of the cotton pellets in the treated as compared with the control group.

The substances chosen for administration were tartar emetic, digitoxin, croton oil, talc and animal charcoal, i.e. substances known to have irritant properties when administered subcutaneously, but believed not to have any anti-inflammatory activity.

Adrenalectomy was performed under ether anaesthesia through a median dorsal skin incision; a slit was made in the body wall over the anterior pole of each kidney, and the adrenal glands were carefully removed by means of curved forceps. Suture clips were used to close the wound. Implantation of pellets was performed at least six days later, and the completeness of the adrenalectomy was confirmed at post-mortem examination.

Results

Local Effects at the Site of Injection

Tartar emetic. 0·1 ml. of a 5 per cent solution/rat/day was found to produce a considerable local inflammatory reaction, consisting mainly of increased vascularisation, and the formation of a yellow watery exudate. This dose was toxic, since in one experiment one out of five intact (i.e. non-adrenalectomised) animals died after the third injection, and in another experiment all adrenalectomised rats died within 24 hr. of a single injection.

At a dose level of 0·03 ml. of the 5 per cent, or 0·1–0·2 ml. of a 1 per cent solution, the local inflammatory effects were greatly reduced, with only an increase in the number of capillaries to mark the site of injection.

Digitoxin. This was administered at two dose levels, 0·5 ml. and 1·0 ml. of a 0·1 per cent aqueous suspension/rat/day. The irritant effect was very small, consisting of some increased vascularization; there was no difference in the effect produced by the two dose levels.

Croton oil. When a single dose of 0·1 ml. of croton oil was injected, the whole surrounding area became grossly necrosed, with enduration of the tissues and sloughing; there were also systemic toxic effects, with the rats losing weight as compared with the control group. When 0·2 ml. of a 10 per cent solution in olive oil was given, again as a single dose, marked necrosis was found at the site of injection, but the toxic effects were reduced, the rats gaining weight, though one out of five in the adrenalectomised group died.

Talc. When administered at a dose level of 10 mg./rat/day, talc greatly increased local vascularity and induced the formation of granulation tissue, the talc finally becoming completely enclosed by the latter. All these effects were increased when the dose was raised to 50 mg./rat/day.

Animal charcoal. The effects were similar to those produced by talc, though the vascularization was more pronounced. 25 and 50 mg./rat/day caused approximately the same degree of irritation.

Effect on the Deposition of Granulation Tissue around the Cotton Pellets. The results obtained are given in Table I and summarised in Table II. As can be seen, digitoxin, which had only a slight inflammatory effect, did not affect the weight of granulation deposited. All the other substances, at dose levels producing a marked effect at the site of injection, also had a marked anti-inflammatory activity not greatly modified by adrenalectomy.

TABLE I

THE EFFECT OF SUBCUTANEOUS ADMINISTRATION OF IRRITANT SUBSTANCES ON THE AMOUNT OF GRANULATION TISSUE DEPOSITED IN THE COTTON PELLET TEST

(Results are expressed as the percentage reduction in the gain in weight of the cotton pellets. All doses are given as the amount of substance injected/rat/day, with the exception of croton oil, which was given as a single dose. There are five animals in each group. The significance is that between the treated group and the control group which was present in each experiment.

Test No.	Treatment		Intact or adrenal-ectomised rats	Reduction of granulation tissue per cent	P
	Compound	Dose			
1*	Tartar emetic	0·1 ml. 5 per cent	Intact	31	0·005
2	"	0·03 ml. 5 per cent	"	20	0·01
3	Digitoxin	0·5 ml. 0·1 per cent	"	10	0·02
	"	1·0 ml. 0·1 per cent	"	0	> 0·1
4	Croton Oil	0·1 ml.	"	49	0·001
5	"	0·1 ml.	"	28	0·02
	"	0·1 ml.	Ad.	27	0·01
	Tartar emetic	0·1 ml. 5 per cent	Intact	28	0·01
6	"	0·1 ml. 1 per cent	"	9	—
	"	0·1 ml. 1 per cent	Ad.	0	—
	Talc	10 mg.	Intact	8	—
	"	10 mg.	Ad.	0	—
	Talc	50 mg.	Intact	44	< 0·001
	"	50 mg.	Ad.	49	< 0·002
	Croton Oil	0·2 ml. 10 per cent	Intact	18	0·02
	"	0·2 ml. 10 per cent	Ad.	21	0·05
	Tartar emetic	0·2 ml. 1 per cent	Intact	10	0·0[illegible]
	"	0·2 ml. 1 per cent	Ad.	27	0·001
8	Animal charcoal	25 mg.	Intact	30	0·001
9	"	50 mg.	"	26	0·001
	"	50 mg.	Ad.	17	0·02

* One animal died after the third injection

DISCUSSION

Since tartar emetic is absorbed from the site of injection, and has at one time been used as an anti-inflammatory agent, and since the composition of croton oil is not certain, and it may have a component which is absorbed, the results obtained with these substances might not be considered convincing. However, neither charcoal nor talc are absorbed, nor are they known to possess any pharmacological activity when given systemically, and it is thus their effects which are of the greatest interest.

It was thought that the most probable explanation of these effects would be that the injection of irritant substances elicited a stress reaction. It has been previously demonstrated by Kellett (1959) that cold stress decreases the amount of granulation tissue formed, and he attributed this to the release of glucocorticoids from the adrenal cortex. However, the

fact that substances which are apparently devoid of any systemic effect nevertheless have an anti-inflammatory action in bilaterally adrenalectomised animals indicates that a different mechanism must be responsible for the effects so obtained.

Both talc and animal charcoal caused extensive infiltration with granulation tissue at the site of injection. In the case of talc this effect was more marked with the larger dose.

The explanation of the apparent anti-inflammatory effect of these substances offered here is that there may be some limit to the amount of granulation tissue which an animal can produce in four days, and when a strong irritant stimulus is produced at the site of injection, the granulation tissue is deposited there rather than at the site of implantation of the cotton pellets. It could be said that the cotton pellets and the irritant substances are competing for the total number of macrophages available for the inflammatory reaction.

The relative intensity of the inflammatory reaction at the site of injection determines the amount of granulation tissue deposited around the cotton

TABLE II

SUMMARY OF THE RESULTS IN INTACT AND ADRENALECTOMISED RATS

Compound	Dose	Reduction of granulation tissue per cent		
		Intact rats		Adrenalectomised rats
Tartar emetic	0·1 ml. 5 per cent	31	28	*
	0·03 ml. 5 per cent	20		—
	0·1 ml. 1 per cent	9		0
	0·2 ml. 1 per cent	10		27
Croton oil	0·1 ml.	49	28	27
	0·2 ml. 10 per cent	18		21
Animal charcoal	25 mg.	30		—
	50 mg.	26		17
Talc	10 mg.	8		0
	50 mg.	44		49

* All animals died.

pellets: thus 50 mg./rat/day of talc had marked irritant and anti-inflammatory effects both in intact and adrenalectomised rats, while 10 mg./rat/day, which had less irritant effect, showed only a weak anti-inflammatory effect in intact rats, and none in adrenalectomised rats. It is suggested that the effect obtained in these experiments are rather similar to those obtained when the body is overwhelmed by bacteria and cannot cope with them.

This may well represent a serious criticism of the cotton pellet test as a means of estimating anti-inflammatory activity, since it means that it is only suitable for substances which are relatively non-irritant, whereas the effects obtained with irritant substances may be due to an artefact.

Acknowledgements. We would like to thank Biorex Laboratories Ltd. for their financial assistance which has enabled us to carry out this work.

REFERENCES

Cygielman, S. (1963). M.Sc. Thesis, University of London.
Kellett, D. N. (1959). Ph.D. Thesis, University of London.
Meir, R., Schuler, W. and Desaulles, P. (1950). *Experientia*, **6**, 469–471.

THE INFLAMMATORY RESPONSE TO IMPLANTATION OF COTTON PELLETS IN THE RAT

BY G. B. PENN AND A. ASHFORD

*From the Pharmacology Department, The Crookes Laboratories Ltd., Park Royal, London, N.W.*10.

Received May 20, 1963

The subcutaneous implantation of a cotton pellet in the rat evokes a short-lasting phase of increased capillary permeability lasting some 20 min. after implantation followed by a more sustained phase which occurs after 2·5–3 hr. The early increase is antagonised by lysergic acid diethylamide, reserpine and 5-HT. The peak granuloma weight is reached at 2 days, and then falls rapidly. Antagonism of oedema and granulation tissue by hydrocortisone and reserpine can be demonstrated at 2 days after implantation.

THE cotton pellet test was introduced by Meier, Schuler and Desaulles (1950) to study the effect of local and systemic applications of cortisone upon developing granulation tissue. Since then this method has been extensively used for the evaluation of anti-inflammatory agents.

The time interval between implantation of the pellet and removal of the granuloma has varied. Singer and Borman (1956) chose 4 days; Meyer, Stucki and Aulsebrook (1953), 5 days; Finney and Somers (1958), 6 days; Dulin (1955), 7 days and Setnikar, Salvaterra and Temelcou (1959), 8 days.

During attempts to shorten the duration of the test we became interested in the inflammatory events occurring in the first 4 days after implantation of the cotton pellet in the rat, and we now describe the results of experiments on the following aspects of the inflammatory response: (*a*) the increased capillary permeability around the cotton pellet during the first 5 hr. after implantation using the vital dye technique, and (*b*) the wet and dry weight of the granulomas, and the effect of hydrocortisone and reserpine upon them in the 4 day period after implantation. The wet weights of granulomas removed at intervals up to 28 days after implantation are also reported.

EXPERIMENTAL METHODS

Female albino wistar rats, 140–180 g. body weight, were used. They were housed at a room temperature of 21°.

One sterile cotton pellet (Johnson & Johnson), weighing from 6 to 10 mg. was implanted subcutaneously in each groin under ether anaesthesia. The pellet was inserted well clear of the skin wound which was then closed with a stainless steel suture clip.

Measurement of Early Changes in Capillary Permeability using Pontamine Sky Blue as Indicator

The pellets were removed from the rat at intervals of from 10 min. to 5 hr. after implantation. Pontamine Sky Blue (0·2 ml. of 2 per cent solution per 100 g. rat) was injected intravenously 15 min. before removal

of the pellets which were then graded for their degree of blueing, by several different observers, against an arbitrary scale ranging from 1 = no blue, up to 6.

Wet and Dry Weights of Granulomas

The two granulomas were removed and were immediately weighed wet in pairs. The dry weights of the paired granulomas were obtained by drying overnight at 60° to constant weight. In the latter instance the initial pellet weight was subtracted from the final dry weight.

Drugs

Reserpine was dissolved in 1 per cent acetic acid and was injected intraperitoneally in 3 doses of 2 mg./kg., over a period of 5 days before the test, the final injection being 2 hr. before implantation of the pellets. Lysergic acid diethylamide (LSD) and 5-hydroxytryptamine (5-HT) were injected intravenously 30 min. before implantation of the cotton pellets. Hydrocortisone acetate was made up as a saline suspension, one injection of 100 mg./kg. being given intraperitoneally at the time of implantation.

All intravenous injections were made into the tail vein.

RESULTS

Increased Capillary Permeability Around the Pellet during the First 5 hr. after Implantation

There was an initial increase in capillary permeability during the first 20 min. after implantation, demonstrated by the blueing of the cotton pellets. This was followed by a phase of impermeability, during which the pellets remained white, which lasted until 2–2·5 hr. after implantation, when a further increase in capillary permeability became evident (Table I).

TABLE I

THE INCREASE IN CAPILLARY PERMEABILITY AROUND A COTTON PELLET DURING THE FIRST 5 HR. AFTER IMPLANTATION

Time of removal of pellets	No. of pellets	Degree of blueing ± s.e.
10 min.	54	4·67 ± 0·082
15 ,,	12	5·88 ± 0·135
20 ,,	42	4·45 ± 0·102
30 ,,	6	2·17 ± 0·423
40 ,,	6	1·17 ± 0·112
50 ,,	6	1·17 ± 0·112
60 ,,	6	1·58 ± 0·288
1·5 hr.	6	1·50 ± 0·230
2·0 ,,	12	1·54 ± 0·199
2·5 ,,	6	4·33 ± 0·225
3·0 ,,	6	3·58 ± 0·557
4·0 ,,	12	3·87 ± 0·211
5·0 ,,	6	3·83 ± 0·271

The early blueing was significantly reduced by treatment with LSD and reserpine (Table II), and was abolished by 5-HT in suitable doses (Table III). It is interesting to note that the inhibitory effect of LSD and reserpine was greater at 10 min. than at 20 min.

Granuloma Weights

The paired wet granulomas reached a maximum weight by the 2nd day after implantation. They then decreased in weight until day 4 when there was a levelling off with further falls in weight at day 6 and day 14. The granuloma weight then remained at the same level until day 28 (Table IV).

Hydrocortisone acetate (100 mg./kg.) significantly reduced both wet and dry weights of 1–4 day granulomas (Figs. 1 and 2) and reserpine pre-

TABLE II

THE EFFECT OF LYSERGIC ACID DIETHYLAMIDE AND OF RESERPINE UPON EARLY BLUEING OF AN IMPLANTED COTTON PELLET

Time of removal of pellets	Treatment	No. of pellets	Degree of blueing ± s.e.	P
10 min.	Controls	12	4·89 ± 0·127	
10 ,,	LSD 0·5 mg./kg.	12	2·77 ± 0·161	<·001
20 ,,	Controls	12	4·44 ± 0·195	
20 ,,	LSD 0·5 mg./kg.	12	3·44 ± 0·155	<·001
10 min.	Controls	12	4·04 ± 0·211	
10 ,,	Reserpine	12	1·94 ± 0·134	<·001
20 ,,	Controls	12	4·60 ± 0·139	
20 ,,	Reserpine	12	3·00 ± 0·599	<·01

treatment likewise significantly reduced these weights. The granuloma weights (mg. ± s.e.) for groups of 10 rats at 2 days being: no treatment: 397·19 ± 16·91 (wet) 59·79 ± 5·15 (dry); reserpine: 217·65 ± 5·94* (wet) 19·37 ± 1·84* (dry) *P<·001.

Discussion

From the results of the tests using Pontamine Sky Blue it is evident that in the first 5 hr. after implantation of a cotton pellet there is an inflammatory response consisting of 2 phases of increased capillary permeability,

TABLE III

THE EFFECT OF 5-HT UPON EARLY BLUEING OF AN IMPLANTED COTTON PELLET

Time of removal of pellets	Treatment	No. of pellets	Degree of blueing ± s.e.	P
10 min.	None	12	4·62 ± 0·142	
10 ,,	5-HT 20 mg./kg.	12	1·10 ± 0·062	<·001
10 ,,	5-HT 10 mg./kg.	12	1·37 ± 0·121	<·001
10 ,,	5-HT 5 mg./kg.	12	1·25 ± 0·087	<·001
15 ,,	None	12	5·88 ± 0·135	
15 ,,	5-HT 5 mg./kg.	6	2·42 ± 0·146	<·001
15 ,,	5-HT 2·5 mg./kg.	6	4·08 ± 0·224	<·001
15 ,,	5-HT 1·0 mg./kg.	6	4·67 ± 0·238	<·001

an initial one lasting for about 20 min. and a delayed phase appearing after a lapse of 2·5 hr. In this respect the early inflammatory response to insertion of a cotton pellet closely resembles that caused by other procedures such as mild burning of guinea-pig skin (Sevitt, 1958; Wilhelm and Mason, 1958), bacterial inoculations and exposure of skin to ultraviolet light (Miles and Wilhelm, 1961). Whereas the initial oedema may be due partly to the injury resulting from implantation, and partly to the

irritant effects of the cotton itself, the delayed oedema is most probably a response to the cotton pellet. The duration of the initial increase in capillary permeability is similar to that seen after the intradermal injection of histamine, 48/80 and leukotaxine in the guinea-pig (Miles and Miles,

TABLE IV

WET WEIGHT OF PAIRED GRANULOMAS

Time or removal of pellets (days)	No. of rats	Wet wt. in mg. ± s.e.
1	9	320·10 ± 46·09
2	12	495·30 ± 21·33
3	212	444·40 ± 8·170
4	6	318·70 ± 8·320
5	6	313·20 ± 21·700
6	6	209·10 ± 6·900
7	6	212·70 ± 6·100
10	6	210·20 ± 12·800
14	6	170·10 ± 8·900
17	6	175·80 ± 9·700
21	6	160·00 ± 3·800
28	6	165·90 ± 4·300

1952), and after the administration of 5-HT to the rat (Miles and Wilhelm, 1961). In our experiments the antagonism of the initial oedema by LSD, and by pretreatment with reserpine may mean that 5-HT is involved as a mediator of initial oedema since it is known that LSD antagonises 5-HT-induced oedema of the rat paw (Doepfner and Cerletti 1958), and that

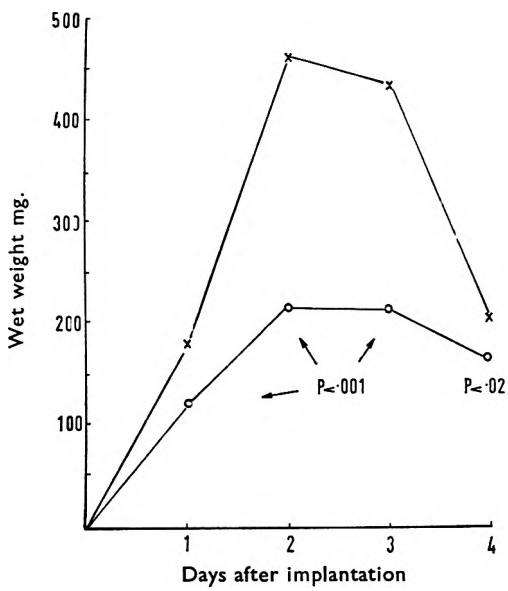


FIG. 1. The effect of hydrocortisone acetate on the development of granulomatous tissue around subcutaneously implanted cotton pellets in the rat. Each point represents the mean wet granuloma plus pellet weight from a group of 5 control and 10 treated rats. Hydrocortisone 100 mg./kg. i.p. was injected at the time of implantation. The probability values refer to the differences between control and treated animals. X—X = Controls. O—O = Hydrocortisone treated.

reserpine will deplete skin and other tissues of its 5-HT content (Parratt and West, 1957).

Rather surprisingly intraperitoneal 5-HT has been shown to inhibit dextran oedema in the rat foot (Georges and Herold, 1957). Similarly Setnikar, Salvaterra and Temelcou (1959) found that iproniazid inhibited formalin-and dextran-induced oedemas, and cotton pellet granulomas in the rat, and suggested that the effect might be due to an anti-inflammatory action of 5-HT. 5-HT also inhibited the formation of fluid in the Selye granuloma pouch (Franchimont, van Cauwenberge and Lecomte, 1961). We have investigated the effect of 5-HT on cotton pellet inflammation and have shown that it antagonised the early increases in vascular permeability, over a very similar dosage-range to that found effective by Georges and Herold (1957).

The mode of action of 5-HT as an anti-inflammatory agent is obscure but may be related to the refractory state of the capillaries which follows

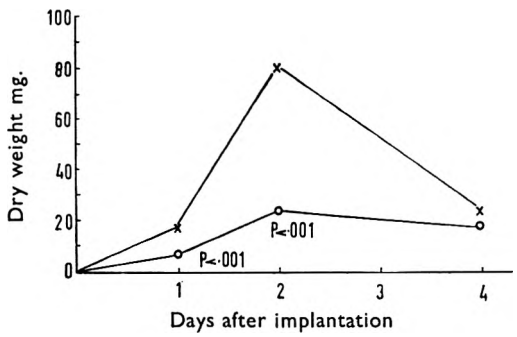


FIG. 2. The effect of hydrocortisone acetate on the development of granulomatous tissue around subcutaneously implanted cotton pellets in the rat. Each point represents the mean weight of dry granulomatous tissue from a group of 5 control and 10 treated rats. Hydrocortisone 100 mg./kg. i.p. was injected at the time of implantation. The probability values refer to the differences between control and treated rats. X—X = Controls. O—O = Hydrocortisone treated.

their stimulation by 5-HT when it is given by intradermal injection (personal observations). A similar refractory state of the capillaries is seen after the intradermal injections of histamine, 48/80 and leukotaxine in the guinea-pig (Miles and Miles, 1952), although it has not yet been shown to follow the administration of 5-HT intravenously. Another possible mechanism of action is via an effect on the adrenals since it has recently been shown that 5-HT stimulates the secretion of hydrocortisone from the perfused adrenal gland of the hypophysectomised dog (Verdesca, Westermann, Crampton, Black, Nedeljkovic and Hilton, 1961). However, Georges (1957) failed to deplete adrenal ascorbic acid in the intact rat using 5-HT.

The rapid increase in wet weight of the cotton pellet granulomas between 0–2 days after implantation may merely represent an extension of the delayed oedema which started at 2·5 hr. after implantation. In this case the equally rapid loss of weight between days 3 and 4 is probably the

result of a loss of fluid from the granuloma. Since the solids contained in such a large amount of fluid in the granuloma at days 2 and 3 will affect the dry weight and therefore the calculated amount of granulation tissue, we determined what proportion of the final dry weight could be accounted for by such solids. Quantities of rat plasma equivalent in weight to that of the 2 day control and hydrocortisone treated granulomas were dried and weighed. The results show that the plasma solids could constitute approximately 1/3 of the control dry weight and approximately half of the hydrocortisone treated dry weight, the remainder being presumably granulation tissue.

The peak weight at day 2 was inhibited by hydrocortisone given in one injection at the time of implantation, and we have subsequently shown that two daily doses (5 mg./kg.) are equally effective at 2 days. The reduction of granulation tissue by hydrocortisone was also apparent visually since granulomas from the treated rats were very thin walled and white compared to the large, hyperaemic granulomas of the controls. It may be possible, therefore, to use a cotton pellet test extending over 2 or 3 days only, for the screening of anti-inflammatory agents.

The antagonism of 2 day old granulomas by reserpine confirms the report recently made by Bhatt and Sanyal (1963) concerning the effect of reserpine on week-old granulomas, and would suggest that the role of 5-HT in the rat is not confined to the mediation of acute inflammatory responses.

Acknowledgement. The authors wish to acknowledge the expert technical assistance of Mr. D. Webb.

REFERENCES

Bhatt, K. G. S. and Sanyal, R. K. (1963). *J. Pharm. Pharmacol.*, **15**, 78–79.
Doepfner, W. and Cerletti, A. (1958). *Int. Arch. Allergy. Appl. Immunol.*, **12**, 89–97.
Dulin, W. E. (1955). *Proc. Soc. exp. Biol. N.Y.*, **90**, 115–117.
Finney, R. S. H. and Somers, G. F. (1958). *J. Pharm. Pharmacol.*, **10**, 613–620.
Franchimont, P., van Cauwenberge, H. and Lecomte, J. (1961). *C. R. Soc. Biol.*, **155**, 432–435.
Georges, G. (1957). *Ibid.*, **151**, 692–695.
Georges, G. and Herold, M. (1957). *Ibid.*, **151**, 695–698.
Meier, R., Schuler, W. and Desaulles, P. (1950). *Experientia*, **6**, 469–471.
Meyer, R. K., Stucki, J. C. and Aulsebrook, K. A. (1953). *Proc. Soc. exp. Biol. N.Y.* **84**, 624–628.
Miles, A. A. and Miles, E. M. (1952). *J. Physiol.*, **118**, 228–256.
Miles, A. A. and Wilhelm, D. L. (1961). *Biochemical Response to Injury. Symposium*, 51–33, Oxford, Blackwell Scientific Publications.
Parratt, J. R. and West, G. B. (1957). *J. Physiol.*, **137**, 179–192.
Setnikar, I., Salvaterra, M. and Temelcou, O. (1959). *Brit. J. Pharmacol.*, **14**, 484–487.
Sevitt, S. (1958). *J. Path. Bact.* **75**, 27–37.
Singer, F. M. and Borman, A. (1956). *Proc. Soc. exp. Biol. N.Y.*, **92**, 23–26.
Verdesca, A. S., Westermann, C. D., Crampton, R. S., Black, W. C., Nedeljkovic, R. I. and Hilton, J. G. (1961). *Amer. J. Physiol.*, **201**, 1065–1067.
Wilhelm, D. L. and Mason, B. (1958). *Brit. med. J.*, **2**, 1141–1143.

ON THE STABILITY OF ADRENALINE IN INJECTIONS: A COMPARISON OF CHEMICAL AND BIOASSAY METHODS

By K. Backe-Hansen,* E. Drottning Aarnes,* A. M. Vennerød† and K. Briseid Jensen†

*From * The Norwegian Government's Pharmacopoeia Laboratory, Blindern, Oslo*
† The Biological Department, The Pharmaceutical Institute, University of Oslo

Received May 14, 1963

The stability of adrenaline in some injections has been investigated by chemical assays based on determinations of adrenochrome and of adrenolutine, while the rat blood pressure method and the rat uterus inhibition method served as biological control procedures. The colorimetric method, in which adrenaline is oxidised to adrenochrome by means of potassium ferricyanide in acid solution, proved unable to detect any deterioration of adrenaline in procaine and adrenaline injections. The corresponding fluorimetric method, in which the adrenochrome formed is rearranged to adrenolutine by addition of strong alkali, gave results which agreed well with the biological results. In injections, adjusted to pH 3 with hydrochloric acid, adrenaline was stable for at least 20 months at 4° in solutions heated at 120° for 20 min., at 100° for 20 min. or unheated. Replacement of the hydrochloric acid by sodium metabisulphite (pH 3·6) prevented the discoloration. In injections of procaine and adrenaline, adjusted to pH 3 with hydrochloric acid, the adrenaline content decreased over the 20 month period, and was most pronounced in the solutions that had been heated before storage. Sodium metabisulphite significantly increased the stability of adrenaline in these injections and also prevented any colour formation.

Few papers have been published on the stability of adrenaline in injections as measured by bioassay procedures, yet the specificity of the chemical methods cannot be generally accepted. A chemical determination of racemisation is only possible in injections with an adrenaline content above 1 mg./ml., and an estimation of a fall in adrenaline content because of deterioration will depend on the method used. Consequently, a bioassay method utilising the effect on the blood pressure of rats, has been adopted for use by the Nordic Pharmacopoeia of 1963.

In the present work we have used some of the experimental conditions adopted by the Nordic Pharmacopoeia. By using chemical and biological methods side by side, we hoped to assess the specificity of the chemical methods used and the role played by the racemisation of adrenaline as a part of the total deterioration processes.

One colorimetric and one fluorimetric method were chosen for the chemical assays, the former based on the oxidation of adrenaline to adrenochrome by potassium ferricyanide (Ehrlén, 1948), the latter being the corresponding adrenolutine method (Hellberg, 1960). The rat blood pressure method of the Nordic Pharmacopoeia, Volume IV, and the modification of Jensen and Vennerød (1961) of the rat uterus method, each based on basically different pharmacological effects of adrenaline, served as reference methods.

EXPERIMENTAL

Adrenaline Injections (1 mg. of adrenaline per ml.)

Series A (pH 3). Adrenaline bitartrate 1·82 g. Sodium chloride 8·00 g. Hydrochloric acid N 3·00 ml. Water for injection to 1000 ml.

Series B (pH 3·6). Adrenaline bitartrate 1·82 g. Sodium chloride 8·00 g. Sodium metabisulphite 0·50 g. Water for injection to 1000 ml.

Procaine and Adrenaline Injections (30 μg. of adrenaline per ml.)

Series C (pH 3·0). Procaine hydrochloride 20·00 g. Adrenaline injection, Series A 30·00 ml. Sodium chloride 4·50 g. Hydrochloric acid N 10·00 ml. Water for injection to 1000 ml.

Series D (pH 3·6). Procaine hydrochloride 20·00 g. Adrenaline injection, Series A 30·00 ml. Sodium chloride 4·50 g. Sodium metabisulphite 0·50 g. Hydrochloric acid N 3·00 ml. Water for injection to 1,000 ml.

The injections were prepared aseptically, dispensed in 10 ml. single-dose containers and immediately sealed by fusion of the glass. No precautions were taken to exclude atmospheric oxygen.

Within each series one batch of sealed containers was heated at 120° for 20 min., another was heated at 98 to 100° for 20 min. and the rest were left unheated. All the ampoules were stored in the dark at 4°.

Chemical Methods

Colorimetric method. The method was that of Ehrlén (1948). To approach the experimental conditions of the corresponding fluorimetric method mentioned below, the pH during oxidation was raised to 6·2 and the concentration of the buffer increased, according to Hellberg (1960). When the oxidation was completed (2 min.), the pH was immediately lowered to about 3·5 with 2 N hydrochloric acid to secure maximal stability of the adrenochrome formed. The extinction was read at 485mμ in a spectrophotometer against the reagent blank.

Each of the results given in Tables I to IV was based on 4 single determinations. The interference of the colour in injections of series A was rendered negligible by appropriate dilution of the solutions; for the procaine and adrenaline injections of series C the interference of colour was eliminated by subtraction of the corresponding blank. The interference observed in the injections of series D is discussed under Results.

Sodium metabisulphite was destroyed before the oxidation of the adrenaline by adding a small excess of iodine 0·22 N to the strongly acid solution; again, the excess of iodine was removed by the addition of arsenite as described by Hellberg (1960).

Fluorimetric method. The method described by Hellberg (1960) was used. Each of the results of Tables I to IV was based on 8 single determinations, the concentrations of the standard solutions being adapted to the level of the test solutions. The average standard deviation, calculated from the autoclaved injections of series D, was 3·6 per cent.

Biological Methods

The rat blood pressure method of the Nordic Pharmacopoeia, Volume IV, based on the modification of Mörch (1960), was used.

The rat uterus inhibition method, as modified by Jensen and Venneröd (1961), was used. In one case (the determination after 20 months of the autoclaved procaine and adrenaline injection, series C, Table III) the

TABLE I

THE STABILITY OF ADRENALINE INJECTIONS, SERIES A (1 MG. OF ADRENALINE PER ML.)
(pH adjusted to 3·0 by addition of hydrochloric acid)

Heat treatment	Storage period (months)	pH	Colour	Adrenaline recovery in per cent of original content			
				Colorimetric method	Fluorimetric method	Rat b.p. method	Rat uterus method
Unheated	0	3·0	colourless	100	—	—	—
	6	3·0	light red	99	102	98 (96–104)*	102 (86–116)
	20	3·0	light red	99	100	94 (95–106)	90 (92–109)
100° 20 min.	0	3·0	colourless	101	106	99 (98–103)	99 (87–115)
	6	3·0	light brown	99	96	99 (97–103)	98 (89–112)
	20	3·0	light brown	98	97	97 (94–106)	104 (92–109)
120° 20 min.	0	3·0	light red	99	100	92 (95–105)	94 (94–106)
	6	3·0	brown, dark ppt.	99	95	93 (97–104)	88 (90–111)
	20	3·0	brown, dark ppt.	96	95	92 (91–110)	83 (88–113)

* Fiducial limits, in parentheses, are expressed as percentages (P = 0·05)

procedure was altered by the addition of procaine to the standard solutions.

RESULTS

The results are given in Tables I to IV.

During the colorimetric analysis of heated adrenaline injections containing procaine hydrochloride and sodium metabisulphite (series D), the

TABLE II

THE STABILITY OF ADRENALINE INJECTIONS, SERIES B (1 MG. OF ADRENALINE PER ML.)
(pH adjusted to 3·6 by adding sodium metabisulphite)

Heat treatment	Storage period (months)	pH	Colour	Adrenaline recovery in per cent of original content			
				Colorimetric method	Fluorimetric method	Rat b.p. method	Rat uterus method
Unheated	0	3·6	colourless	99	102	—	—
	6	3·4	—	100	97	100 (95–105)*	101 (92–109)
	20	3·4	—	100	100	100 (93–108)	87 (89–113)
100° 20 min.	0	3·4	colourless	99	101	96 (94–106)	97 (93–108)
	6	3·3	—	100	96	100 (97–103)	96 (93–108)
	20	3·3	—	100	101	100 (95–105)	98 (90–111)
120° 20 min.	0	3·4	colourless	98	94	97 (93–108)	100 (84–120)
	6	3·3	—	99	95	98 (94–106)	98 (89–113)
	20	3·3	—	99	98	97 (95–106)	94 (83–121)

* Fiducial limits, in parentheses, are expressed as percentages (P = 0·05)

ferricyanide oxidation gave rise to interfering colour reactions, which made the colorimetric method inapplicable. It was shown that a similarly interfering substance (or substances) was formed in solutions of procaine hydrochloride and sodium metabisulphite, upon heating or even when stored in the cold.

Schriever (1956) postulated the formation of a hydroxy derivative of procaine through the action of bisulphite ions and oxygen. The resulting *o*-aminophenol, being a readily oxidizable substance, may be the contaminant involved. Qualitative tests have given some support to this suggestion.

TABLE III

THE STABILITY OF PROCAINE AND ADRENALINE INJECTIONS, SERIES C (30 μG. OF ADRENALINE PER ML.)

(pH adjusted to 3·0 by addition of hydrochloric acid)

Heat treatment	Storage period (months)	pH	Colour	Adrenaline recovery in per cent of original content			
				Colorimetric method	Fluorimetric method	Rat b.p. method	Rat uterus method
Unheated	0	3·0	colourless	101	100	—	—
	6	3·1	light yellow	102	99	96 (96–104)*	93 (88–114)
	20	3·0	yellow	103	77	78 (94–106)	86 (87–114)
100° 20 min.	0	3·0	light yellow	100	94	92 (96–104)	97 (87–115)
	6	3·1	light yellow	102	87	90 (96–104)	91 (87–115)
	20	3·1	yellow	96	72	71 (97–103)	68 (86–117)
120° 20 min.	0	3·0	light yellow	101	86	84 (97–103)	93 (92–108)
	6	3·1	light yellow	100	74	78 (98–103)	77 (90–111)
	20	3·1	yellow	96	65	65 (97–103)	63 (90–112)

* Fiducial limits, in parentheses, are expressed as percentages (P = 0·05)

DISCUSSION

Specificity of the Chemical Methods

Table III shows that the colorimetric method is not a reliable assay method for adrenaline in procaine and adrenaline injections. As the simple adrenaline injections proved to be stable over 20 months (Tables I and II), from the present results it is not possible to claim that the colorimetric method should be abandoned. The results, however, certainly suggest that the specificity of the method should be further controlled.

TABLE IV

THE STABILITY OF PROCAINE AND ADRENALINE INJECTIONS, SERIES D (30 μG. OF ADRENALINE PER ML.)

(pH adjusted to 3·6 by addition of sodium metabisulphite and hydrochloric acid)

Heat treatment	Storage period (months)	pH	Colour	Adrenaline recovery in per cent of original content			
				Colorimetric method	Fluorimetric method	Rat b.p. method	Rat uterus method
Unheated	0	3·6	colourless	102	98	—	—
	6	3·5	—		93	90 (95–105)*	98 (90–111)
	20	3·3	—		77	73 (94–107)	78 (90–111)
100° 20 min.	0	3·5	colourless		90	102 (95–106)	100 (92–108)
	6	3·5	—		88	88 (94–106)	93 (89–112)
	20	3·3	—		88	85 (93–107)	85 (92–109)
120° 20 min.	0	3·5	colourless		88	101 (95–105)	96 (93–108)
	6	3·4	—		90	94 (94–106)	85 (88–114)
	20	3·3	—		82	74 (92–108)	81 (92–109)

* Fiducial limits, in parentheses, are expressed as percentages (P = 0·05)

The agreement between the fluorimetric and the biological results (Tables III and IV) establishes the specificity of the fluorimetric method

and makes clear that no significant racemisation had taken place under the chosen conditions. From an analytical point of view the latter observation is rather interesting. The specificity of the fluorimetric method is limited as it does not differentiate between the optical isomers of adrenaline. If by maintaining a suitable pH the racemisation of adrenaline can be neglected, the fluorimetric method may be regarded as an alternative to the bioassays.

The simultaneous use of the rat blood pressure method and the rat uterus inhibition method assured a safe control of the adrenaline content of the injections examined. While the increase in blood pressure is mainly due to a stimulation of the adrenaline α-receptors in the smooth muscles of the arterioles, the relaxing effect on the rat uterus is caused by a stimulation of the adrenaline β-receptors in the uterus (Ahlquist, 1948). As the range of adrenaline concentrations needed for the rat uterus assay is only 0·1 to 1 ng./ml., compared to 0·5 to 2 μg./ml. for the blood pressure method, the former method must be ascribed a high degree of specificity. The results obtained by the two methods agreed satisfactorily, indicating that any interference from deterioration products was insignificant.

Stability of Adrenaline in Injections

The conditions for optimal stability of adrenaline in injection solutions now seem fairly well established (Heacock, 1959). Of the various factors affecting the stability the pH of the solution is of special interest, as different routes of deterioration of adrenaline have different optimum pH values. It is now generally accepted that, for physiological reasons as well as for pharmaceutical purposes, the pH interval 3 to 3·8, as adopted by the Nordic Pharmacopoeia for adrenaline injections, represents the best possible compromise for the limitation of oxidation and racemisation of adrenaline.

Accordingly, for the series A and C (Tables I and II) the pH was adjusted to 3·0 by hydrochloric acid.

According to Higuchi and Schroeter (1960), the use of sodium metabisulphite as a stabilising agent for adrenaline gave rise to a physiologically inactive sulphonate of adrenaline which may be formed by the action of bisulphite ions. More recently, this substance was claimed to be found in some adrenaline injections (Dibbern and Picher, 1961).

In the metabisulphite-containing injections, series B and D (Tables II and IV), the pH was initially adjusted to 3·6, to ensure that the pH should not fall below 3·0 during the storage period.

No decomposition could be detected in the adrenaline injections of series A and B, not even in those autoclaved. As the colouring in the series A injections, probably attributable to a very slight degree of oxidation of adrenaline, is pharmaceutically inelegant, the results suggest the use of metabisulphite if atmospheric oxygen is not strictly excluded. The good keeping qualities of adrenaline injections reported by West (1950) were confirmed.

In the procaine and adrenaline injections of series C and D, a significant decomposition was observed in both series, and this was most pronounced when metabisulphite was absent. Thus, while the possibility of the formation of adrenaline sulphonate may not be ruled out, in this experiment the metabisulphite increased the stability of adrenaline. The rate of decomposition observed in the series D injections closely corresponds to results recently reported by Vieillefosse, Hanegraaff and Khalili-Varasteh (1961).

The heat treatment of the series C injections increased the rate of decomposition of adrenaline. This fact may be due to some hydrolytic cleavage of procaine, leading to the formation of diethylaminoethanol (Woolfe, 1941). Still, for the aerobic conditions, oxidative routes of deterioration are possible, e.g. the quinone-amine reaction mentioned by Förster (1961).

References

Ahlquist, R. P. (1948). *Amer. J. Physiol.*, **153**, 586–600.
Dibbern, H.-W. and Picher, H. (1961). *Arzneimitt.-Forsch.*, **11**, 317–323.
Ehrlén, I. (1948) *Farm. Revy*, **47**, 242–250.
Förster, H. (1961). *Arzneimitt.-Forsch*, **11**, 339–343.
Heacock, R. A. (1959). *Chem. Revs.*, **59**, 181–237.
Hellberg, H. (1960). *Svensk Farm. Tidskr.*, **64**, 493–505.
Higuchi, T. and Schroeter, L. C. (1960). *J. Amer. chem. Soc.*, **82**, 1904–1907.
Jensen, K. Briseid and Venneröd, A. M. (1961). *Acta pharm. tox. kbh.*, **18**, 80–88.
Mörch, J. (1960). *Pharm. Acta Helvet.*, **35**, 375–384.
Schriever, K. (1956). *Arch. Pharm. Berl.*, **289**, 343–347.
Vieillefosse, R., Hanegraaff, C. and Khalili-Varasteh, H. (1961). *Ann. pharm. franç.*, **19**, 658–672.
West, G. B. (1950). *J. Pharm. Pharmacol.*, **2**, 864.
Woolfe, G. (1941). *Quart. J. Pharm. Pharmacol.*, **14**, 56–63.

THE ISOLATION OF HORDENINE AND NORSECURININE FROM *SECURINEGA VIROSA* (Baill.). THE STRUCTURE OF NORSECURININE

BY G. O. IKETUBOSIN AND D. W. MATHIESON

From the Schools of Pharmacy, University of Ife, Ibadan, Nigeria, and University of London, Brunswick Square, London, W.C.1,

Received September 20, 1963

From the ground roots of *Securinega virosa*, hordenine and a new alkaloid (norsecurinine) have been isolated. The structure of the latter alkaloid has been elucidated.

BY extracting the ground roots of *Securinega virosa* Baill. with ether, two crystalline bases have been isolated. Of these the first has been shown to be *p*-hydroxy-*NN*-dimethyl-β-phenylethylamine (hordenine). Its identity was established by mixed melting-points of the free base, its hydrochloride and its methiodide, with authentic samples: in no case was any depression noted. Ultra-violet and infra-red spectra were superimposable on those of authentic samples determined under the same conditions. The alkaloid "fluggeine" isolated from the same source by Paris, Moyse, Mignon and Le Men (1955) has also been shown to be hordenine. We are indebted to Professor Paris for his kindness in allowing us to examine a sample of his material.

Analysis of the second base and also of the derivatives detailed in the experimental section, suggested a formula of $C_{12}H_{13}O_2N$: a titration equivalent of 197 and a molecular weight (Rast) of 200 confirmed this. A monoacidic base, this material contains no *N*-methyl groups and forms a quaternary salt with one molecular proportion of methyl iodide thus indicating a tertiary nitrogen: this is confirmed by the absence of NH bands in the infra-red spectrum. Carbon methyl, oxygen methyl, and OH groups are likewise absent.

A similar compound (I; n = 2) has been isolated from *S. suffruticosa* (Pall) Rehd and from *S. virosa* (Murav'eva and Ban'kovskii, 1959; Saito, Kodera, Sugimoto, Horii and Tamura, 1962; Nakano, Yang and Terao, 1962, 1963; Nakano, Yang, Terao and Durham, 1963). The present material differs in that it is a lower homologue (I; n = 1) of the compound isolated by the Russian and Japanese workers. For it we propose the name Norsecurinine.

Absorption at 256 mμ and at 1802, 1770 and 1640 cm.$^{-1}$ is in agreement with an $\alpha\beta$ unsaturated five membered lactone, where the conjugation is extended by a further double bond. The splitting of the carbonyl peak (1802 and 1770 cm.$^{-1}$) implies a hydrogen atom alpha to the carbonyl group.

On catalytic hydrogenation over platinum, one molecule of hydrogen is rapidly absorbed, with a second, more slowly.

The mass spectrum of norsecurinine was recorded on an A.E.I. high resolution mass spectrometer type M.S.9; we are grateful to Dr. Snedden and Dr. Bowen, of Shell Research Limited, for carrying this out and for

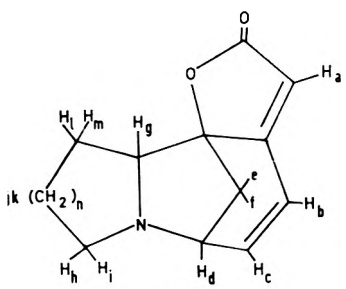
commenting upon the results. The parent peak at m/e 203 confirms the molecular weight. Major peaks occur at m/e 69, 70, 78, 106, 134, 157 and 203. The precise mass of four of these was determined at a resolution of 1 in 15,000 and led to the unequivocal assignments below:

m/e	Formulae
70	$[C_4H_8N]^+$
78	$[C_6H_6]^+$
106	$[C_7H_6O]^+$
134	$[C_8H_6O_2]^+$

As the ratio of peak intensities at m/e 69 and 70 is dependent on instrumental conditions, this is strong evidence that the 70 peak is due to a rearrangement ion and that the true fragment ion has a mass of 69; it is therefore $[C_4H_7N]^+$.

Fragmentation of the molecule on electron impact thus proceeds readily in the following fashion:

$$C_{12}H_{13}O_2N \longrightarrow [C_4H_7N]^+ + C_8H_6O_2$$
$$\text{and } C_4H_7N + [C_8H_6O_2]^+$$



(I)

The $[C_8H_6O_2]^+$ ion then loses CO in two separate stages.

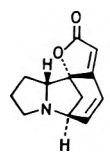
$$[C_8H_6O_2]^+ \xrightarrow{-CO} [C_7H_6O]^+ \xrightarrow{-CO} [C_6H_6]^+$$

The mass spectrum is therefore clear evidence of the absence of a six membered nitrogen containing ring, and is consistent not only with structure I(n = 1) but also with the structure where the nitrogen lies at the alternative bridgehead position between the two five membered rings.

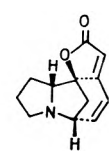
It is on the basis of I(n = 1) alone, however, that the proton magnetic resonance spectrum is interpreted. In this two main groups of lines appear; one between 3 and 4·4 τ represents *three* ethylenic protons (a, b and c), the other between 6·2 and 8·4 τ has an integrated area equivalent to *ten* protons (d to m inclusive). Of the lines at low field, a single peak at 4·33 τ arises from H_a. Protons b and c with d form an ABX system and a pattern of six lines between 3 and 3·6 τ forms the AB part of this. The triplet centred at 6·37 τ constitutes the X part of the system. H_d is coupled in turn to H_b ($J \simeq 0{\cdot}5$ c/s.) to H_c ($J \simeq 6$ c/s.) and also vicinally to H_e and H_f. Since only a *cis* fusion of the methylene bridge

across the seven membered ring can obtain, there are two geometric forms II (a) and II (b).

In both, Dreiding models indicate dihedral angles $\theta \simeq 40°$ and 70° for H_d with H_e and H_f. From the data of Conroy (1960) this yields coupling constants $J \simeq 4{\cdot}5$ and 0·5 c/s. respectively. These lead to the triplet in which unresolved fine splitting is apparent. For H_f which appears at higher field to the triplet the coupling constant J_{fd} may be extracted and the geminal coupling constant for the protons f and e ($J \simeq 11$ c/s.) then follows as in Fig. 1. This is also seen in the five proton multiplet at highest field to which j, k, l and m are also assigned. This leaves protons g, h, and i all positioned α to the nitrogen atom. One of these three gives part of the signal between 7·2 and 7·8 τ, the other two the signal between 6·6 and 7·1 τ. Proton g should be found in the latter group since it is β to the oxygen as well as α to the nitrogen atom: it is thus the least shielded of g, h, and i. Proton h is also assigned with g, these two being *cis* with respect to one another. By elimination H_i falls in the multiplet 7·2–7·8 τ. We conclude from these results that II(a) or II(b) or their mirror images represents the structure of norsecurinine.



(IIa)



(IIb)

EXPERIMENTAL

Isolation of bases from *Securinega virosa*: Powdered whole root (12 kg.) was defatted with light petroleum (b.p. 60–80°). After drying, it was macerated (18 hr.) with 15 per cent ammonia in ethanol (6 litres) then extracted (24 hr.) in a soxhlet apparatus with ether. Total bases were removed from the ether extract with N hydrochloric acid, the acid extract was made alkaline with ammonium hydroxide (0·88) and extracted again with ether. Evaporation of this extract gave a viscous yellow oil (A).

The residual aqueous mother liquors were evaporated to dryness under reduced pressure (temp. $\gtrless 60°$) and the crude mixture of ammonium chloride and organic bases washed with ethanol. The ethanol extract was evaporated to half-volume then diluted with twice its volume of ether and refrigerated. A mixture of ammonium chloride and Base II hydrochloride (1·9 g.) resulted. The mother liquors were again evaporated to half-volume, diluted with thrice their volume of ether and again refrigerated: a further quantity of Base II hydrochloride (4 g.) was obtained. Two more cycles of the mother liquors yielded crops of 2 g. and 8 g. of Base I and Base II hydrochlorides (B) respectively.

Hordenine. The viscous yellow oil (A) crystallised from carbon tetrachloride in colourless prisms m.p. 117·5° (Henry 1949, cites m.p. 117–118°). Found: C, 72·4; H, 9·2; N, 8·9; O, 9·8; NCH_3, 24·1.

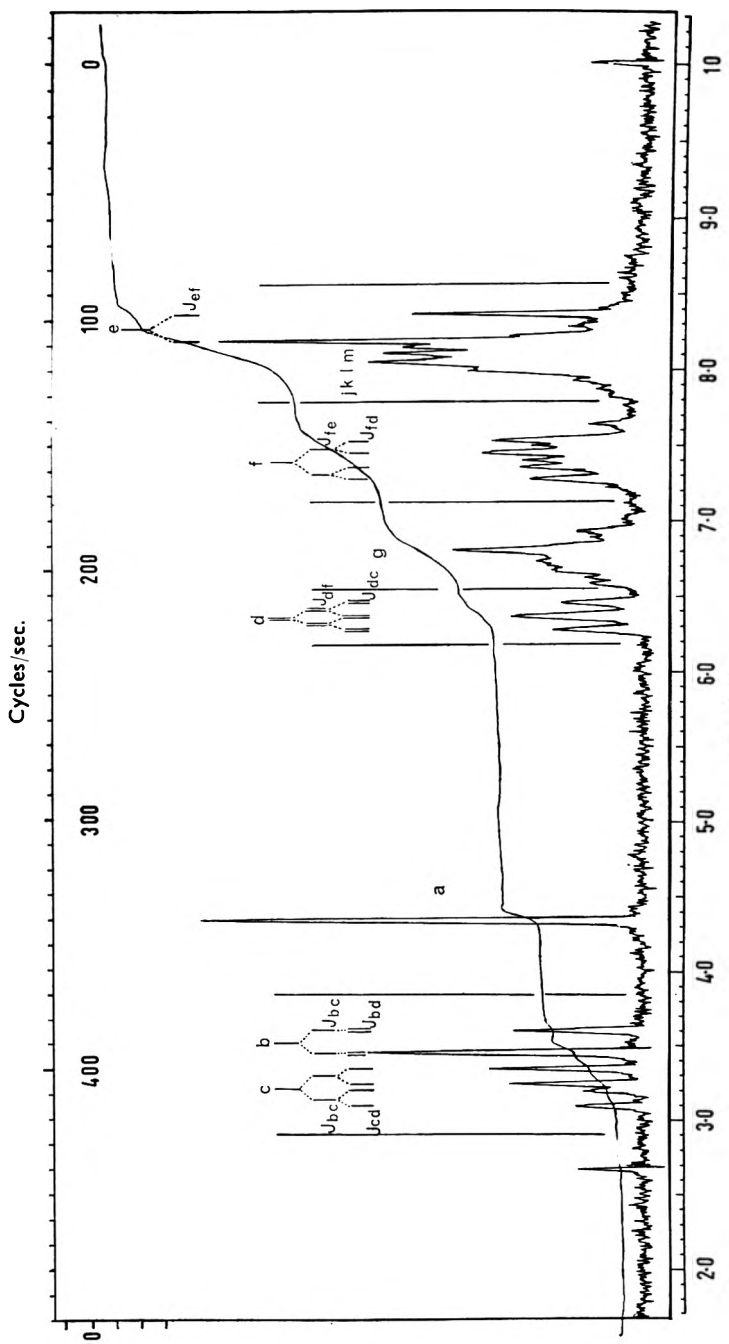


FIG. 1. N.M.R. spectrum of norsecurinine.

Calc. for $C_{10}H_{15}NO$, C, 72·7; H, 9·2; N, 8·4; O, 9·7; NCH_3, 26·7 per cent. The base had a titration equivalent 165; pK_a 8·55; λ_{max} in N HCl 222 mμ (ε 7,700), 275 mμ (ε 1,350), in N NaOH 237·5 mμ (ε 9,500) 293·5 mμ (ε 2,150) ν_{max} 3570 cm.$^{-1}$ (phenolic OH) 1169 cm.$^{-1}$ (phenol) 825 cm.$^{-1}$ (1,4) disubstituted benzene.

The hydrochloride crystallised from ethanol ether in colourless prisms m.p. 177–178° (Henry, 1949, cites m.p. 176·5–177·7°).

The methiodide crystallised from methanol m.p. 226–228° [lit. (Henry, 1949) cites m.p. 229–230°].

Norsecurinine: the hydrochloride (B) (4 g.) was mixed with anhydrous sodium carbonate (4 g.). To this, water (1 ml.) was added and the mixture was dried at 20° *in vacuo*. The dried material was then extracted for several hours in a soxhlet extractor with light petroleum (b.p. 40–60°). On cooling, norsecurinine was obtained in colourless needles, m.p. 81–82°, $[\alpha]_D^{20}$ — 19·5° (*c*, 0·2 in ethanol). Found: C, 70·8; H, 6·4; N, 7·1; O, 16·3. $C_{12}H_{13}NO_2$ requires C, 70·9; H, 6·4; O, 15·8; N, 6·9 per cent. Molecular weight (Rast) 200; titration equivalent 197: pK_a 6·85. λ_{max} (in ethanol) 255·5 mμ (ε 22,000); in 2N NaOH 260 mμ (ε 22,000) ν_{max} (in carbon tetrachloride) 1802 and 1770 cm.$^{-1}$ (CO of an αβ unsaturated lactone) 1640 cm.$^{-1}$ (C=C). In ethanol over Adams' platinum oxide, norsecurinine absorbed 2 molecular proportions of hydrogen, the first rapidly, the second slowly.

Norsecurinine sulphate: a solution of conc. sulphuric acid (4 drops) in ether (1 ml.) was added to the above base (49 mg.) in ether (2 ml.). The precipitated sulphate (57 mg.) was recrystallised from ethanol/ether to give m.p. 224–225°. Found: C, 47·9; H, 4·6; N, 4·8; O, 29·5; S, 11·1; $C_{12}H_{13}NO_2$, H_2SO_4 requires C, 47·8; H, 5·0; O, 31·9; S, 10·6 per cent.

The *methiodide* was made in the usual way from methyl iodide in dry ether and crystallised from ethanol/ether in bright yellow prisms m.p. 194–195°. Found: C, 45·0; H, 4·6; I, 36·9; N, 4·1; O, 9·4; NCH_3, 8·5. $C_{13}H_{16}O_2$ N I requires C, 45·3; H, 4·6; I, 36·8; N, 4·1; O, 9·3 per cent.

The *metho*-p-*toluenesulphonate* prepared in the usual way crystallised from ethanol-ether in colourless needles, m.p. 234–235° C. Found: C, 61·7; H, 6·0; N, 3·7; S, 8·3. $C_{20}H_{23}NO_5S$ requires C, 61·7; H, 5·9; N, 3·6; S, 8·2 per cent.

The *picrate* crystallised from acetone in yellow needles, m.p. 232–233° (decomp.). Found: C, 49·7; H, 3·8; N, 13·3. $C_{18}H_{16}N_4O_9$ requires C, 50·0; H, 3·7; N, 13·0 per cent.

The N.M.R. spectrum was recorded in deuterochloroform with tetramethylsilane as an internal standard (Fig. 1).

Acknowledgements. We are indebted to Miss J. Lovernack of the School of Pharmacy, London, for determining the N.M.R. spectrum on a Varian A.60 Spectrometer. Microanalyses are by Mr. G. Crouch of the School of Pharmacy. One of us (G.O.I.) thanks the Council of the former Nigerian College of Arts, Science and Technology, Ibadan for study leave which enabled this work to be continued in London.

REFERENCES

Conroy, H. (1960). In *Advances in Organic chemistry*, Vol. 2, p. 310. New York: Interscience.
Henry, T. A. (1949). *The Plant Alkaloids*, 4th ed., p. 633. London: Churchill.
Murav'eva, V. I. and Ban'kovskii, A. I. (1956). *Doklady Akad. Nauk, S.S.S.R.*, **110**, 998–1000.
Nakano, T., Yang, T. H. and Terao, S. (1962). *Chem. and Ind.*, 1651–1652.
Nakano, T., Yang, T. H. and Terao, S. (1963). *Tetrahedron*, **19**, 609.
Nakano, T., Yang, T. H., Terao, S. and Durham, L. J. (1962). *Chem. and Ind.*, 1034–1035.
Paris, R., Moyse, H. and Le Men, J. (1955). *Ann. pharm. franç.*, **13**, 245–249.
Saito, S., Kodera, K., Sugimoto, N., Horii, Z. and Tamura, Y. (1962). *Chem. and Ind.*, 1652–1653.

AN ACTIVE GLYCOSIDE FROM *ALBIZIA* SPECIES AND ITS ACTION ON ISOLATED UTERUS AND ILEUM

BY A. LIPTON*

From the Department of Physiology, Makerere College Medical School, P.O. Box 2072, Kampala, Uganda

Received May 21, 1963

An active oxytocic principle was extracted from *Albizia gummifera* (Lipton, 1959) by continuous percolation of the dry bark with methanol. Further purification was by dialysis, fractional extraction and precipitation with organic solvents, and by chromatography on alumina. The concentration of activity achieved was 200 times that of the dried bark, measured *in vitro* on strips of uterus. The active principle is a saponin, giving on hydrolysis an unsaturated triterpene acid (probably $C_{30}H_{48}O_5$), and four sugars, glucose, rhamnose, xylose and arabinose. The principle has a powerful action on isolated uterine tissues from different species, resistant to atropine, antihistamines and brief boiling in aqueous or alcoholic solution, but it fails to excite guinea-pig ileum even in high concentrations. The material, for which the name 'albitocin' is proposed, is thought to be potentially valuable in the study of smooth muscle, particularly uterine muscle. The use of these plants by African native doctors, and their possible implication in the high incidence of uterine rupture in Uganda is discussed.

EXTRACTS of members of the plant genus *Albizia* have been used to accelerate labour and procure abortion in East African women. (Lipton, 1959). These women use native medicines, even when in hospital, in an attempt to accelerate birth at or near term, and the excessively high incidence of uterine rupture in Uganda has been attributed in part to powerful uterine spasmogens in these medicines (Rendle-Short, 1960). Cold aqueous extracts of the bark of *Albizia gummifera* (Gmel.) C. A. Smith var. *gummifera* were previously reported to produce powerful contractions in strips from the gravid uteri of mice, rats, guinea-pigs, sheep, cows and man (Lipton, 1959). The extraction and partial characterisation of the active material, and its actions on smooth muscle preparations is now described.

EXPERIMENTAL METHODS

Isolation of Active Principle

Ground bark (60–80 mesh) (11·36 kg.) was defatted with light petroleum (b.p. 40–60°), then extracted with methanol in a soxhlet-type apparatus until exhausted. The extract, concentrated to 1·5 litres, was then completely precipitated by the addition of acetone. Filtered and dried, this crude solid weighed 661 g. It was dialysed in water (5 litres) for 60 hr. and the residual aqueous liquors treated with charcoal (50 g.). Concentration to 500 ml. *in vacuo* below 35° and finally freeze-drying gave 205 g. of light tan coloured amorphous material. That portion soluble in methanol was again reprecipitated with acetone and the resulting solid in 50 per cent aqueous ethanol passed through a column of deactivated alumina, elution

*Present address: Department of Physiology, University of Queensland, St. Lucia, Brisbane, Australia.

being carried out with the same solvent. Freeze-drying yielded a colourless amorphous Fraction A, melting point 220–225° (softening at 205–210° $[\alpha]_{5461}^{22}$ −23·8° [*c*, 1 in MeOH]). This fraction has pharmacological activity about 200 times that of the original dry bark, and contained more than 80 per cent of all the activity. It was found that concentrations of 4–5 μg./ml. in an isolated organ bath gave approximately equivalent responses to 1·0 milliunit/ml. of Pitocin (Parke, Davis & Co.) with the gravid guinea-pig uterus preparation.

The Fraction A, which has so far not been crystallised, was highly soluble in water, giving a copious stable froth. It was insoluble in ether, acetone and benzene, very sparingly soluble in cold ethanol but readily soluble in methanol. It was precipitated from aqueous solution with basic lead acetate but not by neutral lead acetate and from methanol solution by ethanolic potassium hydroxide or baryta. Precipitates were not obtained with the usual alkaloid reagents and elementary analysis showed the absence of nitrogen and sulphur. Reactions with Fehling's, Barfoed's and Benedict's solutions were negative but a positive Molisch test was given. The Liebermann-Burchardt reaction gave a purple-blue colour, and stannic chloride in thionyl chloride, a blue colour. These tests suggested a saponin, probably triterpenoid in nature. Further confirmation of the presence of saponin was found in that an emulsion of olive-oil in water was stabilised indefinitely by solutions of concentrations greater than 50 μg./ml., while fresh rabbit's blood diluted 1 : 100 with isotonic saline was completely haemolysed *in vitro* but not *in vivo*, by concentrations greater than 10 μg./ml. (Lipton, 1960).

Saponins have been found previously in *Albizia* spp. and a number have been isolated and characterised (Tschirch, 1925; Peyer and Liebisch, 1928; Watt and Breyer-Brandwijk, 1929; Sannil, Lapin and Varshney, 1957; Tschesche and Fortsmann, 1957; Barua and Raman, 1958; Farooq, Varshney and Hasan, 1959).

We also found oxytocic activity in aqueous extracts of three other species of *Albizia*, *A. grandibracteata* (Taub.), *A. chinensis* (Osbeck) Merrill, and *A. isenbergiana* (A. Rich) Fourn., but aqueous extracts of *A. coriaria* Welw. ex. Oliv., *A. schimperiana* (Oliv.) var. *tephrocalyx* (Bren.), *A. ferruginea* (Benth.) and *A. zygia* (DC.) Macbr. gave no responses in doses up to 100 times those required for uterine contractions with *A. gummifera* extracts.

Some preliminary work has been done on the nature of the saponin in Fraction A. Brief boiling in neutral, acid or alkaline solution did not measurably affect the action on the uterus, but prolonged boiling, particularly in acid solution, resulted in a steady decline of activity.

Hydrolysis of Fraction A with dilute aqueous acid gave an insoluble prosapogenin and a mixture of sugars which were characterised by paper chromatography (Whatman No. 1 paper; isopropanol: water, 6 : 4) as arabinose, rhamnose, xylose and a trace of glucose. Further hydrolysis of the prosapogenin with aqueous ethanolic sulphuric acid gave a crude sapogenin and glucose. The saponin was hydrolysed directly to the sapogenin and the four sugars with aqueous ethanolic sulphuric acid.

The sapogenin was purified by conversion to its insoluble potassium salt, which was converted to the free acid and treated with diazomethane and then acetic anhydride in pyridine. Chromatography on alumina gave as the main fraction a crystalline solid, melting point 170–171°$[\alpha]_D^{20} = +76{\cdot}6°$ (*c*, 0·1 in $CHCl_3$) the analysis of which corresponds to the methyl ester triacetate of the triterpene acid $C_{30}H_{48}O_5$. In spite of repeated attempts constant acetyl values could not be obtained. (Found: C, 70·7; H, 9·3 per cent. $C_{37}H_{56}O_8$ requires C, 70·7; H, 8·9 per cent). Prolonged alkaline hydrolysis gave the free acid m.p. 304–305° (decomp.) $[\alpha]_D^{20} = +79{\cdot}4°$ (*c*, 0·2 in MeOH). (Found: C, 74·3; H, 9·7 per cent. $C_{30}H_{48}O_5$ requires C, 73·7; H, 9·9 per cent).

Assay Methods

Isolated gravid rodent uteri were 5–10 times more sensitive than non-gravid preparations. It was found essential to increase the potassium and decrease the magnesium content of Dale's solution (Dale and Laidlaw, 1912) to ensure low spontaneous activity, good sensitivity to Fraction A and good survival of the gravid guinea-pig uterus. The composition of the 'G.U.' solution is given in Table I together with those of other media used.

TABLE I

THE BATH MEDIA USED

(Salts in g./litre distilled water.)

	G.U. solution	1953 B.P. solution	De Jalon's solution	Rat tyrode	Dale's solution
Sodium chloride	9·0	9·0	9·0	9·0	9·0
Potassium chloride	0·25	0·42	0·42	0·42	0·20
Calcium chloride	0·20	0·24	0·06	0·06	0·20
Magnesium chloride	0·05	0·0025	—	0·0025	0·10
Sodium bicarbonate	1·00	0·50	0·50	0·50	1·00
Sodium acid phosphate	0·05	—	—	—	0·05
Dextrose	0·50	0·50	0·50	0·25	1·00

An automatic apparatus for testing preparations *in vitro* was used, based on earlier designs, which ensured absolute uniformity in dose volume, bath volume, time intervals between doses, and time of exposure to doses (Schild, 1954; Lock, 1961). Recording was by Schild's method (1946).

The required bath solution was freshly prepared each day from stock solutions of the constituent salts (Table I), except for the dextrose and bicarbonate which were kept dry in sealed test tubes and added just before use.

Drugs were dissolved in the appropriate bath medium and checked for neutrality with universal indicator. Doses were warmed to bath temperature before delivery. Washing was by the overflow method to avoid tissue stimulation by stretching and cooling.

Rodent uteri. These were prepared by the standard methods (Dale and Laidlaw, 1912; Holton, 1948; British Pharmacopoeia, 1953; De Jalon, Bayo and de Jalon, 1954) (see Table I). The mouse uteri were suspended in Dale's solution or Rat Tyrode (Table I) with a lever load of about 0·3 g. Strips were obtained from 17 non-gravid guinea-pig uteri, and from

43 gravid guinea-pig uteri in which foetuses varied from recent implantations up to a crown to rump length of 5 cm., and were used for quantitative examination of the various plant extracts. A further 16 gravid uterine strips were obtained from uteri in which the foetuses were of more than 5 cm. crown-rump length, but because of excessive spontaneous activity, or because the slope of the dose-response curve was too steep, only 7 of these were completed as quantitatively successful assays (Lipton, 1960). Nineteen dioestrus rat uteri and 10 gravid rat uterine strips were used, but the latter always showed vigorous spontaneous activity and were of value only for qualitative comparison. Four non-gravid and two gravid mouse uteri were used.

Ungulate uteri. These were obtained from freshly slaughtered animals; thin strips from the myometrium were immersed in aerated Dale's or G.U. Solution (Table I) at 4°. By replacing the solution about every half hr. the strips could be preserved for several hr. without serious deterioration.

Human uteri. These were obtained as described by Robson (1933), and stored at 4° in aerated Dale's solution or G.U. solution. These preparations were at first relatively insensitive, but after remaining in the bath for 30–60 min. with frequent changes of solution and vigorous aeration to remove volatile anaesthetic maximal activity was restored. Robson (1933) and Scott Russell (1943) also observed that the human uterine strips they used did not immediately exhibit maximal activity.

Guinea-pig ileum. Seven of the non-gravid females (weighing 400–600 g.) used for uterine preparations also had short lengths removed from the ileum at least 10 cm. from the ileo-caecal junction, for tests in Tyrode's solution with Fraction A, acetylcholine and histamine.

RESULTS

All the rodent uterus preparations responded vigorously to Fraction A or extracts containing it. Amounts of 0·2 μg./ml. or more for gravid guinea pig uteri, and 1–5 μg./ml. for non-gravid guinea-pig uteri, up to 10μg./ml. for dioestrus virgin rat uteri, and up to 60 μg./ml. for gravid and non-gravid mouse uteri, were needed for assay. The gravid rat uteri with one exception, showed increased rate and force of spontaneous contractions, with doses of more than 2 μg./ml. Fraction A. (Fig. 1 a, c, d).

Although there was no significant difference in the sensitivity of six oestrus and eleven dioestrus guinea-pig uteri, the gravid guinea-pig uteri were always several times more sensitive than the non-gravid uteri; the sensitivity and the log dose : response slope of the gravid preparations increasing as gestation advanced (Lipton, 1960) (Fig. 3).

Three cow uteri, all gravid, (foetuses from 9–20 cm. in crown to rump length), three sheep uteri (two gravid and one virgin), and 2 gravid goat uteri all gave vigorous responses to doses of 20–50 μg./ml. of Fraction A, the sheep uterus showing an increase in force and frequency of spontaneous contractions with 10 μg./ml. or more Fraction A, while a quiescent strip from the lower segment of a gravid goat uterus required 50 μg./ml. or more for powerful responses (Fig. 1b).

Strips from 6 full-term gravid human uteri, four obtained at Caesarian

section and two subsequent to uterine rupture showed increased activity in response to doses as low as 0·2 μg./ml. of Fraction A, and strips from 3 non-gravid human uteri showed increased activity in response to doses from 1–4 μg./ml. (Fig. 2).

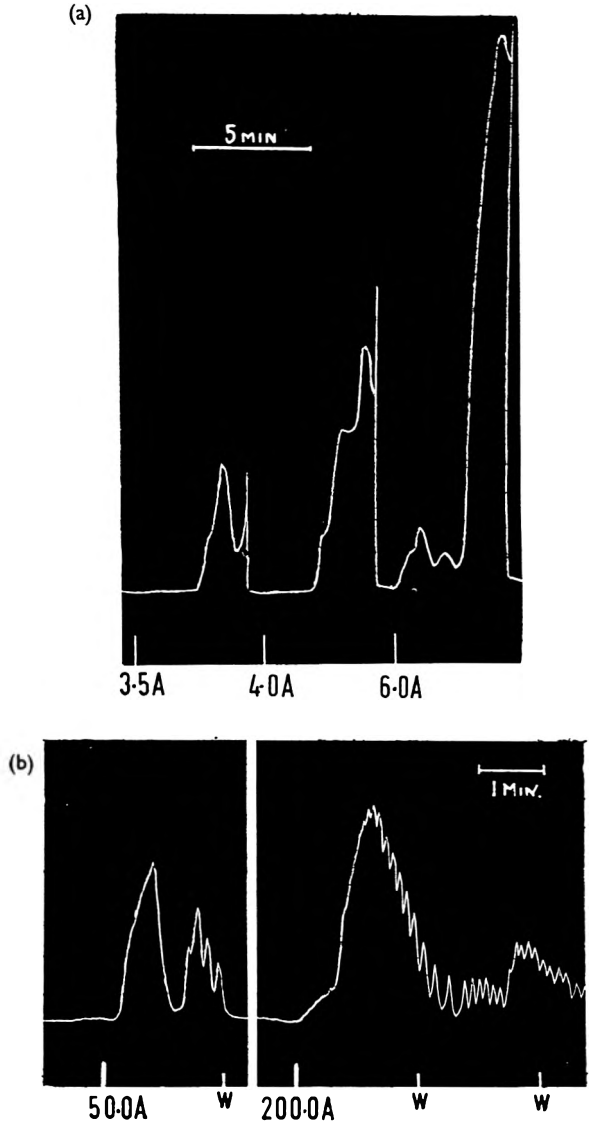


FIG. 1. Responses of various mammalian preparations *in vitro* to extracts of plants of *Albizia* genus. A = μg./ml. albitocin. P = μg./ml. pitocin. w = bath change.
(a) Guinea-pig. Gravid uterus, non-gravid horn, ovarian end, 2 × 2·8 cm. foetuses. Load 1·5 g. G.U. solution* 33° C.
(b) Goat, Gravid uterus, lower segment, 22 cm. foetus, G.U. solution* 36°.
* See Table I for bath media.

All the uterine preparations continued to respond to Fraction A in the presence of sufficient antihistamine (pyranisamine maleate or promethazine hydrochloride) to eliminate the responses to previously adequate doses of

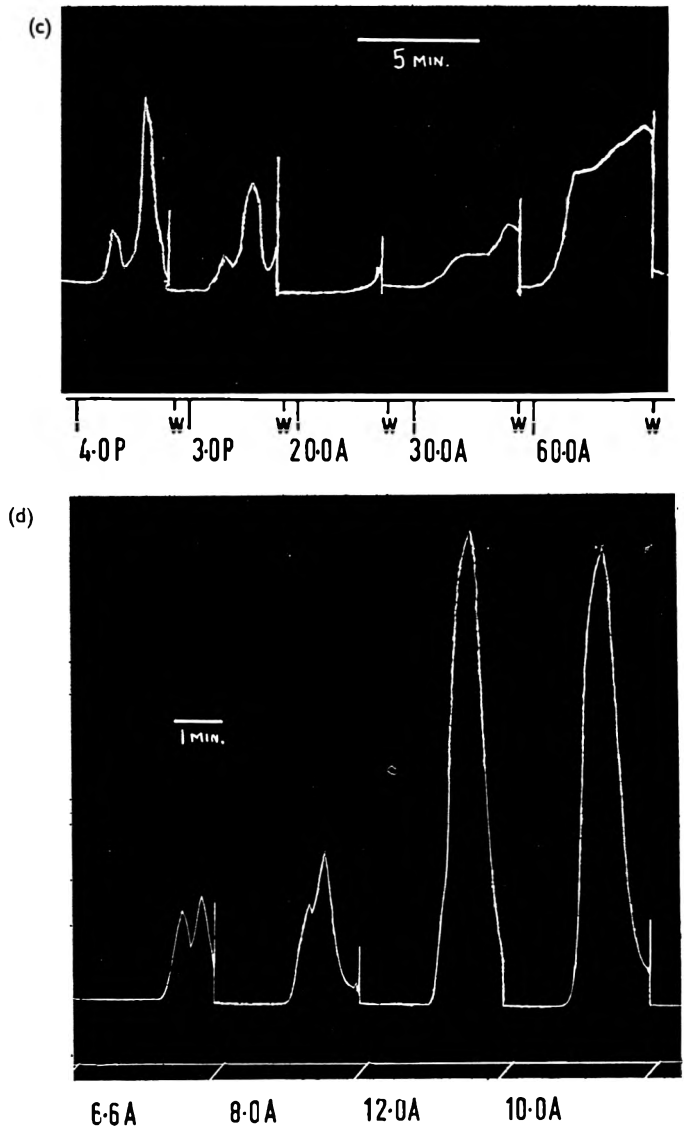


FIG. 1. *continued.*

(*c*) Mouse. Uterus post-partum. Load 0·4 g. Rat tyrode* 32°.
(*d*) Rat. Uterus dioestrus virgin. Load 0·75 g. Rat tyrode* 32°.

histamine, and the rodent uteri continued to respond in the presence of sufficient atropine to prevent responses to acetylcholine.

When chemical studies showed the total absence of nitrogen from Fraction A, similar eliminations of other known alkaloid and polypeptide spasmogenic substances were deemed unnecessary.

The guinea pig ileum preparation. All seven preparations, while exhibiting normal responses to histamine (up to 0·2 μg./ml.) and acetylcholine (up to 0·4 μg./ml.) showed no response to doses of Fraction A of up to 500 μg./ml.; that is 100 to 500 times the dose found effective with uterine preparations from the same non-gravid animals (Fig. 4).

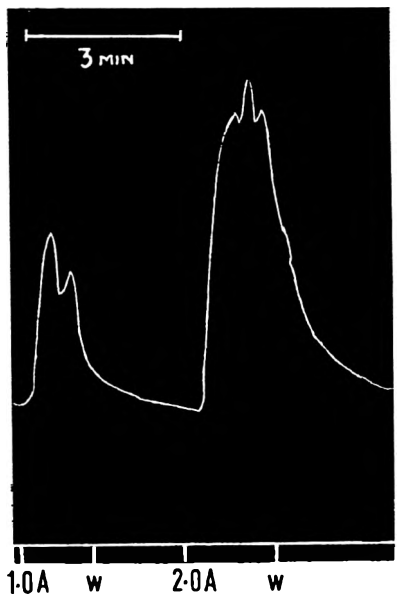


FIG. 2. Human non-gravid uterus response to extracts of plants of *Albizia* genus. Section at hysterectomy for fibroids. Multiparous. Dale's solution* 36·5°. See Fig. 1 for key.

DISCUSSION

The evidence presented shows that the active principle elicits powerful responses in isolated strips of uterine muscle from different mammalian types, acting to induce contractions when the tissue is quiescent and to increase the frequency and force of contractions when spontaneous activity is present.

The exceptionally high incidence of uterine rupture in Bantu women in Uganda (Rendle-Short, 1960) may be partly explained by the known use of this drug and others with similar properties.

There is a difference in sensitivity to the drug in different species, and also between individuals of the same species, depending on whether the uterus from which the strip was obtained was gravid or not, and also on the stage of gestation. The effect of the stage of gestation on the response of the guinea-pig uterus to this drug (Fig. 3) is reported in detail elsewhere (Lipton, 1960). This evidence is of potential value in the design of experiments aimed at greater comprehension of the changes occurring in

the uterine musculature during gestation. Since it also specifically acts on uterine and not on intestinal smooth muscle this would appear to enhance its value in the study of the important differences which undoubtedly exist between these two kinds of muscle.

Work by Csapo and others (Csapo, 1956) and recent work by the present author supports the idea that oxytocic substances in general act on the initiation of contraction, probably by a change at the membrane of the smooth muscle cell, and since other glycosides, e.g. digitalis glycosides, have been shown to have this type of action on isolated heart muscle (Csapo, 1956), this may also be the mode of action of Fraction A. The fact that it does not affect intestinal smooth muscle activity suggests an important difference in the contraction initiation process between this type of smooth muscle and that of the uterus.

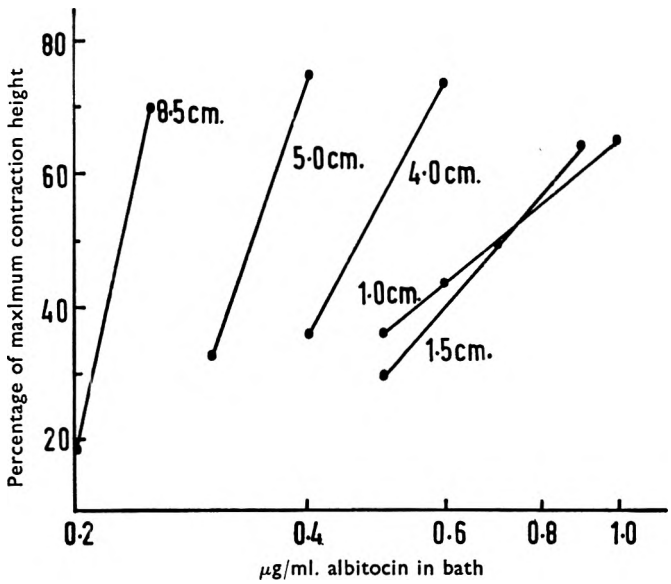


FIG. 3. Log. dose/response relationships of gravid guinea-pig uteri to albitocin *in vitro*, at different stages of gestation. G.U. solution in all cases. Strips from ovarian end, non-placental sites. The response slopes are labelled with the mean crown-rump lengths of the foetuses in the uteri from which the strips were obtained. Each point is the mean of six or more responses.

For convenience in subsequent descriptions of activity we propose the name "albitocin" for the active oxytocic principle in extracts of *A. gummifera*, and our present hypothesis is that it is identical with the Fraction A obtained. This is the first triterpenoid glycoside shown to have this kind of action, to our knowledge, and it would appear to be worth while to examine other compounds of this type for similar activity.

Acknowledgements. The author wishes to express his appreciation of the generosity of the Makerere College Research Grants Committee and the Wellcome Foundation for grants in support of the work. Grateful acknowledgement is also due to Professor J. A. Lock for valuable advice

and guidance, to Professor G. Rendle-Short of Makerere College Medical School, Miss M. Barley of the Lake Bunyonyi Leprosy Settlement, Kabale, Uganda, and numerous African herbalists who provided the first specimens and helped to obtain later supplies of the plants. Thanks are also due to Dr. E. Lind for plant identification, and especially to Dr. S. Wilkinson and other workers of The Wellcome Research Laboratories, Beckenham, Kent, for the extraction procedures and to Mrs. T. Walker for painstaking technical assistance.

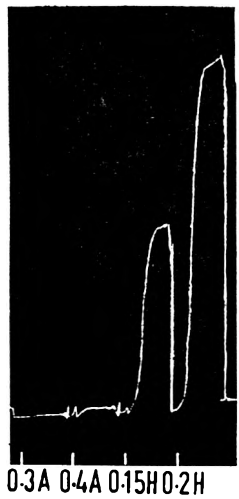


FIG. 4. Response of isolated guinea-pig ileum to albitocin and histamine. The doses of albitocin are about 100 times greater than those required for maximal responses with an *in vitro* uterus preparation from the same animal.
Dale's solution 36°. A = Albitocin in mg./ml. H = Histamine in μg./ml.

REFERENCES

Barua, A. K. and Raman, S. P. (1958). *Science and Culture*, **23**, 435–436.
British Pharmacopoeia (1953). p. 823. London: The Pharmaceutical Press.
Csapo, A. (1956). *Recent Progr. Hormone Res.*, **XII**, 405–427.
Dale, H. H. and Laidlaw, P. P. (1912–13). *J. Pharmacol.*, **4**, 75–95.
De Jalon, Bayo and De Jalon (1954). *Farmacoterap. Actual*, **3**, 313. Quoted by Burn, J. H. (1952). *Practical Pharmacology*. London: Blackwell.
Farooq, M. O., Varshney, I. P. and Hassan, H. (1959). *Arch. Pharm. Berl*, **292**, 57–59.
Holton, P. (1948). *Brit. J. Pharmacol.*, **3**, 328–334.
Lipton, A. (1959). *Nature, Lond.*, **184**, 822–823.
Lipton, A. (1960). Ph.D. Thesis University of London.
Lock, J. A. (1961). *J. Pharm. Pharmacol.* **13**, 378–379.
Peyer, W. and Leibish, W. (1928). *Apothekarztg*, **94**, 1422–1424.
Rendle-Short, C. (1960). *Amer. J. Obstet. Gynec.*, **79**, 1114–1120.
Robson, J. M. (1933). *J. Physiol.*, **79**, 83–93.
Sannie, A. Lapin, V. and Varshney, I. P. (1957). *Bull. Soc. Chim.*, 1440–1441.
Schild, H. O. (1946). *Brit. J. Pharmacol.*, **1**, 135–138.
Schild, H. O. (1954). *Ibid.*, **9**, 24–30.
Scott Russell, C. (1943). *J. Obstet. Gynaec., Brit. Emp.*, **50**, 287–298.
Tschesche, H. S. and Fortsmann, R. (1957). *Chem. Ber.*, **90**, 2383.
Tschirsch, A. (1925). *Handbuch der Pharmakognosie*. II, 2, 1501. III, 1, 26–32. Liepzig: C. H. Tauchnitz.
Watt, J. M. and Breyer-Brandwijk, M. G. (1929). *Arch. int. Pharmacodyn*, **36**, 233–234.

REFRACTOMETRIC DETERMINATION OF THE CRITICAL MICELLE CONCENTRATION OF NON-IONIC SURFACE-ACTIVE AGENTS

By M. Donbrow and Z. A. Jan

From the School of Pharmacy, Chelsea College of Science and Technology, London, S.W.3

Received July 3, 1963

The critical micelle concentrations (CMC) values of some non-ionic surfactants have been determined by refraction and are compared with the results reported earlier by other methods. The slope values have been calculated below and above the CMC and an equation developed to predict the slope below the CMC for any member of the homologous series. The values appear to conform with those expected theoretically from atomic refraction data.

A number of methods have been employed to determine the critical micelle concentration (CMC) of non-ionic surfactants. The most widely used involve the addition of external substances such as iodine or dyes which may influence micelle formation or micelle properties (Ross and Olivier, 1959), whilst others involve measurement of properties such as surface tension or viscosity by dynamic methods, which are liable to disequilibrate the system. Refractive index, however, measures a bulk property under static conditions and should therefore be independent of external influences.

The validity of refraction measurements for the determination of CMC has already been established for ionic surfactants by Klevens (1947), who studied a number of anionic and cationic detergents and soaps. The method has not, however, been applied to non-ionic surfactants and since it appears to offer some important advantages, it is here applied to a number of pure compounds on which data have been reported by other methods.

Experimental

A Hilger Rayleigh Interference Refractometer for liquids (Model M154) was used. It was fitted with a constant temperature water jacket; a tungsten lamp was the light source. Cells had path lengths of 1 and 10 cm.

Chemicals. The following polyethylene oxide alkyl ethers were given by Dr. B. A. Mulley. The preparation and purity of these compounds have been previously described (Mulley, 1958, Mulley and Metcalf, 1960; Carless, Challis and Mulley, 1963).

$C_6H_{13}\cdot[O\cdot CH_2\cdot CH_2\cdot]_4OH$, $C_6H_{13}\cdot[O\cdot CH_2\cdot CH_2\cdot]_5OH$,
$C_{10}H_{21}\cdot[O\cdot CH_2\cdot CH_2\cdot]_5OH$, $C_{10}H_{21}\cdot[O\cdot CH_2\cdot CH_2\cdot]_6OH$,
$C_{12}H_{25}\cdot[O\cdot CH_2\cdot CH_2\cdot]_5OH$, $C_{12}H_{25}\cdot[O\cdot CH_2\cdot CH_2\cdot]_6OH$,
$C_{16}H_{33}\cdot[O\cdot CH_2\cdot CH_2\cdot]_9OH$.

Method. All solutions were prepared using triple-distilled water. A series of dilutions of appropriate concentrations of surfactants were

prepared from filtered stock solutions. Refractive index increments between serial pairs of solutions were measured in terms of instrument scale division differences (ΔR), calculated using the formula:

$$\Delta R = R' - R_0$$

where R' = Instrument scale reading

R_0 = Instrument zero obtained by filling both sides of the cell with the same solution.

All readings were duplicated and an average of three to four readings was taken in every case. ΔR values were summated in the construction of the graphs. Where 1 cm. cells were employed ΔR values were re-calculated for a 10 cm. path length.

The instrument was calibrated with standard sodium chloride solutions and the ΔR values were converted into absolute refractive index differences to give Δn values. In some cases in which the refractive index differences was very small, distilled water was used as a reference solution throughout the experiment.

In the course of the work it was found that the longer-chain surfactants were adsorbed at the surface of the cell, thereby altering the zero and introducing errors into the readings. The adsorbed layer could not be entirely removed by repeated washings and the cell required immersion in water overnight to restore the instrument zero to its normal position.

Results and Discussion

The CMC values obtained are listed in Table I and the refractive index-concentration curves are shown in Fig. 1. The CMC values are indicated by the intersection of two straight lines which govern the slopes below and above the CMC. The values so obtained are compared in Table I

TABLE I

CMC AND SLOPE VALUES FOR POLYETHYLENE OXIDE ALKYL ETHERS

Compounds	CMC (moles/litre at 20° C)			$\Delta(\Delta n)/\Delta c$ litres/mole $\times 10^4$ (Slope $\times 10^4$)	
	Refraction	Surface tension	Iodine solubilisation	Below CMC	Above CMC
$C_6H_{13}\cdot(O\cdot CH_2\cdot CH_2\cdot)_4OH$	$9{\cdot}0 \times 10^{-2}$	$9{\cdot}0 \times 10^{-2}$	$9{\cdot}0 \times 10^{-2}$	326	308
$C_6H_{13}\cdot(O\cdot CH_2\cdot CH_2\cdot)_5OH$	$9{\cdot}25 \times 10^{-2}$	$9{\cdot}0 \times 10^{-2}$		373	356
$C_{10}H_{21}\cdot(O\cdot CH_2\cdot CH_2\cdot)_6OH$	$7{\cdot}8 \times 10^{-4}$	$8{\cdot}6 \times 10^{-4}$	$9{\cdot}5 \times 10^{-4}$	471	365
$C_{10}H_{21}\cdot(O\cdot CH_2\cdot CH_2\cdot)_8OH$	$9{\cdot}6 \times 10^{-4}$	$9{\cdot}2 \times 10^{-4}$	$9{\cdot}5 \times 10^{-4}$	520	442
$C_{12}H_{25}\cdot(O\cdot CH_2\cdot CH_2\cdot)_5OH$	$5{\cdot}00 \times 10^{-5}$	$4{\cdot}0 \times 10^{-5}$	$3{\cdot}5 \times 10^{-5}$	380	492
$C_{12}H_{25}\cdot(O\cdot CH_2\cdot CH_2\cdot)_6OH$	$10{\cdot}00 \times 10^{-5}$	$8{\cdot}2 \times 10^{-5}$	$7{\cdot}8 \times 10^{-5}$	570	418
$C_{16}H_{33}\cdot(O\cdot CH_2\cdot CH_2\cdot)_6OH$	—	$3{\cdot}6 \times 10^{-5}$	$3{\cdot}9 \times 10^{-5}$	—	760*

* Lowest concentration measured 1×10^{-5}M (against water)

with those previously reported for the same compounds by Mulley and Metcalf (1962) and Carless and others (1963) who used the surface tension and iodine solubilisation methods. Agreement is reasonable, though the C_{12} compounds give slightly higher values by the refraction method.

The slopes of the lines have been calculated below and above the CMC and are given in Table I as $\Delta(\Delta n)/\Delta c$ values, where Δn is the

refractive-index difference between two solutions having a concentration difference of Δc. The C_{12} compound with five ethylene oxide groups is anomalous both in its slope values and their ratios, and although duplicate estimations on the same sample gave concordant values, the

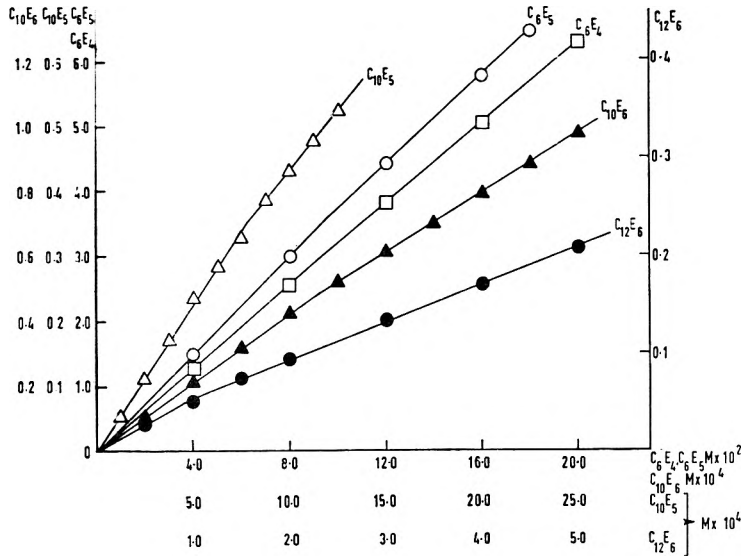


FIG. 1. Variation in refractive indices with concentration. Ordinates: $\Delta n \times 10^4$. Abscissa: concentration (molar)

compound must nevertheless be considered doubtful. Some comparable $\Delta(\Delta n)/\Delta c$ values for sodium alkyl sulphonates taken from the data of Kleven (1947) and some values for long chain alkyl betaines are included

TABLE II

$\Delta(\Delta n)/\Delta c$ VALUES LITRE/MOLE $\times 10^4$ (SLOPE $\times 10^4$)

	Non-ionic surfactants			Sodium alkyl sulphonates		Betaines	
	Below CMC		Above CMC	Below CMC	Above CMC	Below CMC	Above CMC
C_8	E_4 325 E_5 373		308 356				
C_9				303	284		
C_{10}	E_5 471 E_6 523		365 442	343	327	350	293
C_{11}						380	251
C_{12}	E_5 380 E_6 570		492 418	379	367	403	353
C_{14}				424	397	532	304
C_{16}	E_9	—	760	461	438	760	—

in Table II. The values for the betaines have been calculated from the empirical results of Beckett and Woodward (1963), who used the identical instrument and method as a result of this work, and have not previously

been reported. The slope values obtained for the non-ionic surfactants are higher than those for the alkyl sulphonates and betaines, as would be expected if the contribution of the ethylene oxide groups to the molar refractions of the respective systems were greater than those of the polar heads of the ionic surfactants. The change of slope per CH_2 group between members of a homologous series is fairly constant below the CMC though greater in the non-ionic surfactants and betaines (24×10^{-4} approx.) than in the alkyl sulphonates (20×10^{-4} approx.). These slope increments are plotted as a function of the number of CH_2 groups in Fig. 2. The betaine series show a striking anomaly: while the lower members have about the same values as the non-ionic surfactants, the longer chain compounds showed an unexpected break in the slope and much larger slope increments.

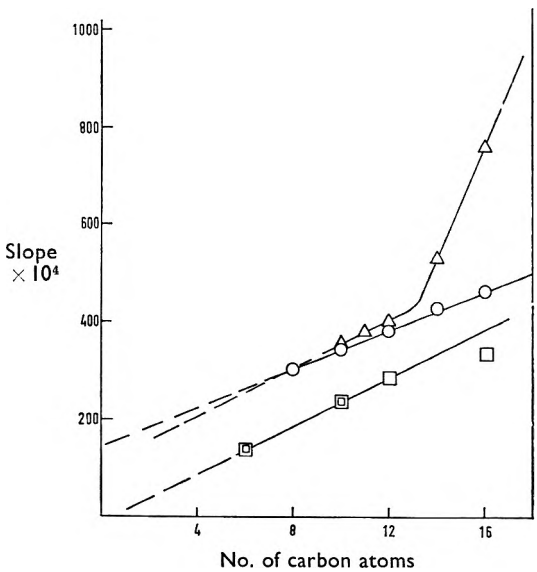


FIG. 2. Refractive index slopes of various types of surfactants as a function of the number of CH_2 groups. △, Betaines. ○, Sodium alkyl sulphonates. □, Polyethylene oxide alkyl ethers. Polyethylene oxide alkyl ethers have been corrected for ethylene oxide groups on the basis of the equation in the text.

Below the CMC, the slope values $\Delta(\Delta n)/\Delta c$ of the non-ionic series would appear to be an additive function of the number of hydrophobic and hydrophilic groups of the compounds and may be predicted for any member of the series having the general formula of $C_nH_{2n+1}[\cdot O\cdot CH_2\cdot CH_2\cdot]_mOH$, with fair accuracy, on the basis of the following equation:

$$\Delta(\Delta n)/\Delta c = l[\Delta(\Delta n)/\Delta c]_C + m[\Delta(\Delta n)/\Delta c]_E$$

where $[\Delta(\Delta n)/\Delta c]_C = 22{\cdot}8 \times 10^{-4}$

$[\Delta(\Delta n)/\Delta c]_E = 47{\cdot}5 \times 10^{-4}$

l = number of C groups (C = alkyl)

m = " " E groups (E = ethylene oxide)

Group slope increments for C and E groups were estimated from the average differences in the values listed in Table I (excluding $C_{12}E_5$). The slopes of various compounds studied were calculated theoretically on the basis of the above equation and are compared in Table III. From this Table it is apparent that the experimental and theoretical values are in good agreement with the exception of $C_{12}E_5$. The experimental slope value obtained for $C_{16}E_9$ was considered to be that above the CMC and has been included in Table III and plotted in Fig. 2 for comparison with the theoretical value below the CMC; it would appear to be of the correct order. Percentage refractive-index increments per CH_2 and ethylene oxide group were also calculated using atomic refraction data (Bauer and Fajan, 1949). The molar refraction for a CH_2 group is 4·62 and for a ($O{\cdot}CH_2{\cdot}CH_2{\cdot}$) group, 10·88. For a unit containing one of each of these groups, the percentage increase in molar refraction on the addition of a further CH_2 is 29·8 while the percentage increase in slope increment calculated from the experimental group increment is 32·4. The corresponding percentage increases per ethylene oxide group are 70·2 from the molar refraction data and 67·5 from the group increments. This is an

TABLE III

COMPARISON OF CALCULATED AND EXPERIMENTAL SLOPE VALUES

Compounds	$\Delta(\Delta r.)/\Delta c$ litres/mole $\times 10^4$ (Slope $\times 10^4$)		Deviation per cent
	Experimental	Theoretical	
C_6E_4	326	327	0·30
C_8E_5	373	374	0·26
$C_{10}E_5$	471	466	1·07
$C_{10}E_6$	520	513	1·36
$C_{12}E_5$	380	510	25·49
$C_{12}E_6$	570	560	1·78
$C_{16}E_9$	760*	790	3·79

* Value above CMC used in this case.

indication that below the CMC the additivity in refraction properties corresponds approximately to that expected theoretically.

Nash (1958, 1959), Becher and Clifton (1959) and Taubman and Nikitina (1960) have observed changes in foam viscosity, fluorescence, light scattering, surface tension and dye titration properties indicative of the existence of non-equilibrium states in micellar solutions, CMC values being obtained over a finite range of concentration determined by kinetic factors. However, the refraction method used here gave single CMC values with abrupt slope changes, readings remaining constant, within experimental limits, for several hours after preparation of the dilutions. Carless and others (1963) obtained evidence of ageing by surface tension measurements with the same $C_{16}E_9$ compound used in this study. The $\Delta(\Delta n)/\Delta c$ values observed for this compound were unchanged, within the limits of experimental error, over a 24 hr. period, but since the CMC was uncertain and the ageing is usually observed below the CMC no clear inference can be drawn.

As the CMC values of non-ionic surfactants are generally much lower than those of ionic surfactants, the concentration range available for plotting the slope below the CMC is somewhat restricted, particularly for the long chain compounds, in which the differences measured approach the order of the accuracy of the instrument. The CMC value obtained is then determined mainly by the accuracy of the backward extrapolation from points near to but above the CMC. In the present work, discrepancies observed in repeated experiments on the C_{16} compound were attributed to adsorption on the cell walls from the very dilute solutions required, and a greater path length or instrument sensitivity is needed to deal with this compound.

Acknowledgments. The authors are grateful to Dr. B. A. Mulley for the presentation of samples of the polyethylene oxide alkyl ethers and to Drs. J. E. Carless and B. A. Mulley for permission to incorporate unpublished data.

REFERENCES

Bauer, N. and Fajan, K. (1949). *Physical Methods of Organic Chemistry*, Editor, Weissberger. Vol. 1, Part II, 1163. New York: Interscience.
Becher, P. and Clifton, N. K. (1959). *J. Colloid Sci.*, **14**, 519–523.
Beckett, A. H. and Woodward, R. J. (1963). *J. Pharm. Pharmacol.*, **15**, 422–431.
Carless, J. E., Challis, R. A. and Mulley, B. A. (1963). *J. Colloid. Sci.*, in the press.
Klevens, H. B. (1947). *J. Chem. Phys.*, **51**, 130–148.
Mulley, B. A. (1958). *J. Chem. Soc.*, **423**, 2065–2066.
Mulley, B. A. and Metcalf, A. D. (1960). *Proc. 3rd Intern. Congr. Surface Activity.* Cologne, Section A, No. 4.
Mulley, B. A. and Metcalf, A. D. (1962). *J. Colloid Sci.*, **17**, 523–530.
Nash, T. (1958a). *J. appl. Chem.*, **8**, 440–444.
Nash, T. (1958b). *Nature, Lond.*, **182**, 1536.
Nash, T. (1959). *J. Colloid Sci.*, **14**, 59–73.
Ross, S. and Olivier, J. P. (1959). *J. Phys. Chem.*, **63**, 1671–1674.
Taubman, A. B. and Nikitina, S. A. (1960). *Doklady Akademii Nauk, S.S.S.R.*, **135**, 1169–1172.

SOME ANTAGONISTS OF ATROPINE-LIKE PSYCHOTOMIMETICS

By W. Lang, S. Gershon and G. Holan*

From the Department of Pharmacology, University of Melbourne, Australia

Received May 20, 1963

The peripheral pharmacological effects of an anti-acetylcholine psychotomimetic drug, ethylpiperidylcyclopentylphenyl glycolate hydrochloride (JB-329) were found to be essentially similar to those of atropine. Both drugs inhibited parasympathetic effects and acetylcholine responses and produced potentiation of the pressor responses to adrenaline and noradrenaline. The compound tetrahydroaminoacridine, shown to be an antagonist of JB-329, was studied and its actions were compared with those of other cholinesterase inhibitors. Certain glycolic acid derivatives, some of which have been shown to antagonise the effects of JB-329, were also studied and their pharmacology and interactions are presented. The mode of action of the antagonists are discussed.

Psychotomimetic effects have been produced by some 3-*N*-substituted piperidyl benzilates in man (Abood, Ostfield and Biel, 1958, 1959; Ostfield, Abood and Mercus, 1958; Pfeiffer and others, 1959). One such compound, JB-329 (*N*-ethyl-3-piperidylcyclopentylphenyl glycolate hydrochloride, Ditran), produces clinical effects simulating some aspects of psychosis more closely than either LSD or mescaline (Gershon and Olariu, 1960; Abood and others, 1958). Behavioural disturbance is also produced in experimental animals (Abood and others, 1959; Biel and others 1961). JB-329 has atropine-like effects in man and animals, an activity demonstrated in the isolated ileum and colon of the rat, and the frog rectus abdominus muscle preparation (Biel and others, 1962).

Atropine in the normal clinical dose has little effect on the central nervous system (Drill, 1958; Goodman and Gilman, 1955) although toxic doses cause hallucinations and disorientation. In large doses it produces behavioural disturbances in animals similar to those seen with JB-329 (Goldenberg, 1957; White and others, 1961; Bell and others, 1963).

The first reported antagonism of the JB-329 syndrome was by Gershon (1960) using 1,2,3,4-tetrahydro-5-aminoacridine (THA). This was found to be an antidote for both the central and peripheral effects of JB-329 in man. THA has marked anticholinesterase activity (Shaw and Bentley, 1953), as well as inhibiting choline acetylase (de la Lande, 1956). Antagonism of the behavioural effects of JB-329 in dogs has been produced with THA and a new series of glycolic acid derivatives (Bell and others, 1963). These compounds significantly reduced the duration of the JB-329 effects on behaviour.

Because of the interest in the atropine-like psychotomimetics and their antagonists it was decided to compare and contrast the actions of JB-329 and atropine, and to investigate the pharmacology of their antagonists.

* Monsanto Chemicals (Australia) Ltd.

To determine the pharmacological basis of the antagonism, the anti-cholinesterase drugs physostigmine, neostigmine and dyflos (DFP) were also used.

Experimental Methods

Conscious dogs were used to observe behavioural and pharmacological effects. Cardiovascular effects were measured in a manner similar to that described by Gershon and Lang (1962). Dogs were surgically prepared beforehand with exteriorised carotid arteries. Arterial pressure was then measured with a Statham transducer using an Offner dynograph recorder. Electrocardiogram, heart rate, salivation, pupillary size and response as well as behavioural changes were also recorded. All drugs were given by intravenous injection using an indwelling polyethylene cannula in the external jugular vein.

Blood pressures from anaesthetised dogs and cats were recorded from carotid or femoral arteries as described above or sometimes with a mercury manometer and kymograph. Drugs were given by intravenous injection; the anaesthetic was normally pentobarbitone or occasionally chloralose.

In the studies on male volunteers, the procedure was that described by Holmberg and Gershon (1961). Intravenous injections of the test drugs were given through indwelling cannulae. During the whole test procedure the ECG was recorded continuously and blood pressure was taken by auscultation every 30 sec.

Isolated strips of fundus from the rat stomach (Vane, 1957) were used to record the effect of the drugs on contractions produced by 5-hydroxytryptamine (5-HT) 30–60 ng.

JB-329 was used in varying doses and its effects were compared and contrasted with those of atropine sulphate. The antagonists were THA, and several members of the series of glycolic acid derivatives (*p*-phenylmandelic acid; 2- and 3-phenanthrylglycolic acids; phenoxymandelic acid) used by Bell and others (1963). Anticholinesterase compounds neostigmine methyl sulphate, physostigmine salicylate and dyflos were also used.

The compounds adrenaline tartrate, noradrenaline bitartrate, acetylchlorine chloride, histamine acid phosphate and 5-HT creatinine sulphate were used to elucidate the pharmacology of the various compounds.

Results

Pharmacology of JB-329 and atropine

In 14 experiments with conscious dogs, JB-329 (0·5 mg./kg. i.v.) produced an increase in both systolic and diastolic blood pressure (average increase 15/20 mm. Hg) and a simultaneous increase in heart rate (120 beats/ min. increase). A gradual return to normal followed and after 4–5 hr. the blood pressure was at control levels although the heart rate was still higher than the control. Administration of JB-329 (0·5 mg./kg. s.c. daily for 7 days) produced no significant effect on blood pressure or heart rate.

TABLE I

MEAN BLOOD PRESSURE RESPONSES BEFORE AND AFTER JB-329 (0·5 MG./KG.) AND ATROPINE SULPHATE (0·5 MG./KG.)

The baseline indicates the basal blood pressure. Change is calculated as the rise or fall in blood pressure to the peak of response

	Before JB-329		After JB-329		Before atropine		After atropine	
	Baseline ±s.e.	Change ±s.e.	Baseline ±s.e.	Change ±s.e.	Baseline ±s.e.	Change ±s.e.	Baseline ±s.e.	Change ±s.e.
A. Conscious dogs—								
Drug alone	118 ± 7·9	—	118 ± 7·9	+20 ± 3·7	109 ± 5·3	—	109 ± 5·3	+22 ± 6·8
Adrenaline 1 μg./kg.	115 ± 5·3	+38 ± 4·5	125 ± 5·5	+65 ± 5·3	110 ± 3·5	+41 ± 3·8	117 ± 3·9	+95 ± 9·9
Noradrenaline 1 μg./kg.	115 ± 5·7	+38 ± 6·4	129 ± 5·5	+71 ± 7·1	113 ± 4·5	+50 ± 10·5	119 ± 4·9	+105 ± 17·5
Acetylcholine 0·5 μg./kg.	117 ± 4·5	−27 ± 3·7	126 ± 3·7	−3·5 ± 2·4	111 ± 1·5	−28 ± 10·7	123 ± 7·7	−9 ± 5·8
B. Anaesthetised cats—								
Drug alone	12·2 ± 16·0	—	12·2 ± 16·0	−3·1 ± 4·2	•155	+20	—	—
					145	−30	—	—
Adrenaline 2 μg./kg.	138 ± 17·2	+32 ± 5·6	124 ± 15·7	+46 ± 8·0	150	+35	+165	+55
					150	+25	125	+60
Noradrenaline 2 μg./kg.	142 ± 17·5	+49 ± 6·1	217 ± 14·5	+65 ± 7·1	160	+75	160	+110
					150	+35	125	+45
Acetylcholine 0·2 μg./kg.	101 ± 11·8	−37 ± 5·4	90 ± 12·2	0 ± 0	170	−80	155	0
					140	−65	125	0

• Results for atropine are actual recordings in two experiments.

TABLE II

ANTAGONISM BY THA OF THE EFFECTS OF JB-329 ON THE RESPONSES OF THE CATECHOLAMINES AND ACETYLCHOLINE

	Response to noradrenaline 1 μg./kg. in mm. Hg			Response to adrenaline 1 μg./kg. in mm. Hg			Response to acetylcholine 0·5 μg./kg. in mm. Hg		
Dogs	Control	After JB-329 0·5 mg./kg.	After THA 1 mg./kg.	Control	After JB-329 0·5 mg./kg.	After THA 1 mg./kg.	Control	After JB-329 0·5 mg./kg.	After THA 1 mg./kg.
1	+27·5	+37·5	+15	+25	+40	+22·5	−10	0	−12·5
2	+37·5	+85	+45	+32·5	+85	+40	−17·5	0	−5
3	+27·5	+37·5	+20	+35	+45	+17·5	−27·5	0	0
4	+57·5	+65	+42·5	+42·5	+57·5	+37·5	−37·5	0	−22·5
5	—	—	—	—	—	—	−15	0	−20

Tolerance to the autonomic effects of JB-329 with chronic daily medication in man was demonstrated by Gershon and Olariu (1960).

Atropine (0·5 mg./kg. i.v. 4 dogs) produced increases in both systolic and diastolic blood pressures as well as tachycardia of the same degree as JB-329. Autonomic changes with both drugs included inhibition of salivation and dilatation of the pupils with marked slowing of the response to light. The hypotensive effect of acetylcholine was inhibited by both JB-329 and atropine in anaesthetised and conscious animals. Both JB-329 and atropine produced potentiation of the pressor responses to adrenaline and noradrenaline (Table I); these pressor responses were even doubled in some dogs.

In anaesthetised cats and dogs the potentiation was less, but did occur. JB-329 and atropine had little action on the effects of histamine 0·5 μg./kg. or 5-HT 5–10 μg./kg. on the blood pressure in conscious dogs. In anaesthetised cats, the primary sharp fall in blood pressure produced by 5-HT 5–10 μg./kg. was inhibited by JB-329 and atropine. This primary fall due to 5-HT is probably a vagal effect (Page, 1952) so that this observation does not necessarily indicate anti-5-HT activity. In isolated strips of fundus from the rat stomach, JB-329 and atropine exhibited anti-5-HT activity only in concentrations about 5×10^{-4} g./ml.

JB-329 (0·15 mg./kg. i.v.) and atropine (0·03 mg./kg. i.v.) were given to three men. In each, a rise in blood pressure and tachycardia occurred after both drugs. Although the increased blood pressure masked the results, the control pressor responses to adrenaline and noradrenaline were found to be potentiated by JB-329 and atropine when the peaks of the blood pressure rises were measured. The depressor response to methacholine chloride was inhibited by both drugs. The response varied between subjects, but each individual responded similarly to both drugs.

Pharmacology of the Antagonists

Tetrahydroaminacrine hydrochloride (THA). In four conscious dogs, THA, 1 mg./kg. i.v., produced no significant change in blood pressure or heart rate, but effects like salivation, vomiting, diarrhoea and twitching were occasionally observed.

In six anaesthetised cats, there was a fall in blood pressure of 30–40 mm.Hg, after which there was a return to normal. In four animals, a secondary rise to approximately 30 mm.Hg above the original level occurred. In two other cats, only the rise in blood pressure occurred. THA produced no apparent modification of the reflex rise in blood pressure occurring after occlusion of the carotid arteries.

The depressor response to acetylcholine 0·2–0·5 μg./kg. was increased in magnitude and duration after THA in conscious and anaesthetised preparations, in keeping with the known anticholinesterase action of the compound.

Variable modifications occurred in the blood pressure responses to injected adrenaline and noradrenaline after THA. In conscious dogs, THA, 1 mg./kg., produced no significant effect on the responses. However,

in anaesthetised cats there sometimes occurred inhibition or reversal of the blood pressure responses.

The diphasic response produced by adrenaline and the magnitude of the noradrenaline-induced rise in blood pressure in the anaesthetised cat, varied with the blood pressure level or the depth of anaesthesia or both

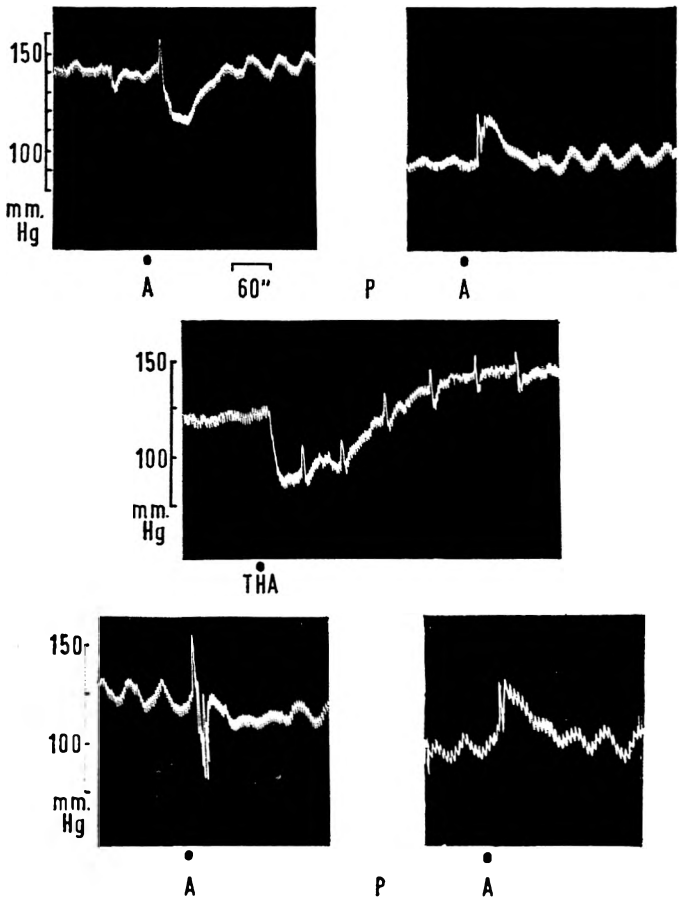


FIG. 1. Blood pressure record in a cat anaesthetised by pentobarbitone 40 mg./kg. i.p. A = i.v. injection of adrenaline 10 μg. THA = i.v. injection of THA 1 mg./kg. P = i.p. injection of pentobarbitone 15 mg./kg. with an interval of 10 min. between the traces. The upper record shows the responses to adrenaline as affected by pentobarbitone-induced changes in depth of anaesthesia and blood pressure base-line before THA injection. The middle record shows the effect of THA on blood pressure. The lower record shows the responses to adrenaline after THA.

(Fig. 1). It appeared from the results that the inconsistent inhibition and reversal of adrenaline and noradrenaline responses produced by THA were due to modifications of the basal blood pressure and depth of anaesthesia. Thus, when the blood pressure was raised or the anaesthesia lightened after THA, then the pressor response to the catecholamines was inhibited

and the depressor response to adrenaline was made apparent or exaggerated. With the dosage of THA used (1 mg./kg.), the experiments on the conscious dog show that no true anti-adrenaline action occurred. Responses to histamine and 5-HT were not significantly affected in the conscious or anaesthetised preparations.

p-*Phenylmandelic acid.* In eight conscious dogs this compound (5 mg./kg. i.v.), an active antagonist of the JB-329-induced syndrome (Bell and others, 1963), produced variable and inconsistent effects on blood pressure. Heart rate was affected only when changes in blood pressure initiated reflex responses. Chronic administration of the same dose daily for one week also produced no significant effects in five dogs. A gradual increase in blood pressure (average value 15–20 mm.Hg) was apparent in seven of eight anaesthetised cats (a similar response was seen with THA). However, no significant changes occurred in the heart rates of these animals.

In the conscious dogs, no apparent behavioural change was seen or autonomic functions affected with either acute or chronic administration of *p*-phenylmandelic acid.

The only modifications of neurohumoral responses on blood pressure were an increase in magnitude and duration of the depressor effect of acetylcholine 0·2 μg./kg. and the secondary fall after adrenaline 2 μg./kg. in anaesthetised cats. There was no effect on 5-HT-induced contraction of rat stomach muscle strips.

2-*Phenanthrylglycolic acid,* 3-*phenanthrylglycolic acid and phenoxymandelic acid.* The first two compounds (effective antagonists of the JB-329-induced syndrome) and the third compound (ineffective against JB-329, Bell and others, 1963) in single doses of 10 mg./kg. i.v. in anaesthetised cats produced increases in blood pressure of approximately 20 mm.Hg. The depressor response to acetylcholine 0·2 μg./kg. was potentiated in magnitude and duration by the compounds, while that to histamine was antagonised by both 2- and 3-phenanthrylglycolic acids but not by phenoxymandelic acid. The 3-phenanthrylglycolic acid appeared to have the greater antihistamine effect and completely abolished the fall in blood pressure induced by histamine 0·2 μg./kg. No significant effects were produced on the responses produced by the other neurohumors or the reflex rise in blood pressure after occlusion of the carotid arteries.

Interactions between Psychotogens and Antagonists

JB-329 and THA. The cardiovascular changes produced by JB-329, 0·5 mg./kg., were antagonised by THA, 1 mg./kg., in five conscious dogs. The blood pressure was lowered, pulse pressure was increased and tachycardia was lessened. In addition the autonomic changes produced by the anticholinergic actions of JB-329 were antagonised. The potentiated pressor responses to injected adrenaline and noradrenaline, 2 μg./kg., were returned to control values and in some cases were even reduced. Blockade of the response to acetylcholine 0·5 μg./kg. was partially or completely antagonised in four of the five dogs (Table II).

In anaesthetised cats, the results were similar except that the blood pressure was increased slightly by THA after JB-329.

Overall results indicated complete antagonism by THA of the effects of JB-329.

JB-329 and other anticholinesterases. Neostigmine methyl sulphate, 0·03 mg./kg., given before or after JB-329 partly antagonised the potentiation of the responses to adrenaline and noradrenaline and the blockade of responses to acetylcholine resulting from JB-329.

In both conscious and anaesthetised animals, a larger dose of neostigmine, 0·05 mg./kg., given after JB-329, produced a complete antagonism of all peripheral pharmacological and autonomic changes in the same way as did THA.

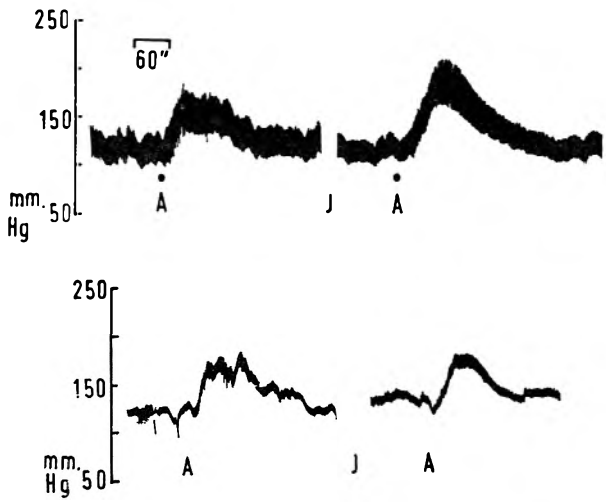


FIG. 2. Blood pressure record in a conscious dog. A = i.v. injection of adrenaline 1 μg./kg. J = i.v. injection of JB-329 0·5 mg./kg. The upper record shows the response to adrenaline before and after JB-329. The lower record, from the same dog, shows the response to adrenaline before and after JB-329, following treatment with *p*-phenylmandelic acid (5 mg./kg. i.v. daily for seven days). Potentiation of adrenaline pressor response by JB-329 does not occur after *p*-phenylmandelic acid.

Physostigmine salicylate, 0·04 mg./kg., in conscious dogs produced the same degree of peripheral antagonism as did THA and neostigmine. Dyflos, 0·3 mg./kg. produced the same peripheral antagonism.

JB-329 and p-phenylmandelic acid. In five conscious dogs given chronic medication with *p*-phenylmandelic acid (5 mg./kg. i.v. daily for seven days), JB-329 produced its usual effects: tachycardia, a rise in mean arterial pressure of 10–30 mm.Hg, and a decreased pulse pressure. However, the potentiation of the pressor responses to adrenaline and noradrenaline normally found after JB-329, 0·5 mg./kg. i.v., did not occur in four of the five dogs tested (Fig. 2). Despite this, the response to acetylcholine 0·5 μg./kg. was still abolished by JB-329 and the responses to other neurohumors were not significantly affected.

In single doses of 5 mg./kg. i.v., *p*-phenylmandelic acid in conscious dogs did not significantly alter any of the effects of JB-329. The same result was obtained in anaesthetised cats given 5–10 mg./kg. *p*-phenylmandelic acid.

Atropine and antagonists. THA produced antagonism to the peripheral effects of atropine similar to that against JB-329 described above.

Single doses of *p*-phenylmandelic acid, as with JB-329, produced no antagonistic effect to the peripheral actions of atropine.

Discussion

Atropine and JB-329 produce essentially the same peripheral pharmacological effects. In the same dose, both slightly raised blood pressure, reduced pulse pressure, induced marked tachycardia and produced the other autonomic changes expected of atropine-like compounds. The responses to the neurohumoral amines were modified in the same way.

Both compounds are stated to produce similar behavioural responses in unrestrained conscious dogs (Bell and others, 1963). JB-329 (0·5 mg./kg.) produced anti-acetylcholine effects on autonomic responses, followed by incoordination, ataxia, apparent disorientation and agitated behaviour. These authors reported that atropine 0·5 mg./kg. produced only autonomic changes, but that larger doses (1 mg./kg.) produced a syndrome identical to that of JB-329. In other behavioural studies with dogs (Goldenberg 1957; White and others, 1961) large doses of atropine (1·5 mg./kg.) produced similar results, although 0·5 mg./kg. did not induce bizarre behaviour. Edery (1962) stated that injection of 0·5 mg. atropine into the cerebral ventricles was without effect, but 1 mg. produced disturbed behaviour suggestive of the production of hallucinations. Horowitz and Chow (1962) concluded that JB-329 appeared to have a more potent central effect than atropine but about the same magnitude of peripheral side-effects. It appears that whilst the effects of atropine and JB-329 in animals are similar, the atropine effects on behaviour appear only with higher doses than are required with JB-329.

Various clinical reports of atropine poisonings (Alexander and others, 1946, Grossier 1956, Welbourn and Buxton 1948) describe the patients as suffering from disorientation, confusion, delirium, visual hallucinations and muscular incoordination. Many other synthetic atropine-like compounds produce similar behavioural alterations (Fink 1960, Pfeiffer and others, 1959).

The antagonism of the central effects of JB-329 by THA (Gershon, 1960; Gershon and Olariu, 1960; Bell and others, 1963) and the series of glycolic acid derivatives (Bell and others, 1963), is of interest because it may give insight into the mode of action of atropine-like compounds. In view of the atropine-like activity of JB-329 and the apparent involvement of acetylcholine in the syndrome, the anticholinesterase activity of THA seemed a logical basis for explaining the antagonism. We have shown that the anticholinesterases neostigmine, physostigmine and DFP parallel the peripheral antagonism of JB-329 shown by THA. However, neostigmine in man (Abood and others 1959), dyflos, physostigmine in rats

(Abood and others, 1959; Abood, 1959) and dogs (Bell and others, 1963) have proved ineffectual in antagonising the severity of the central disturbances produced by JB-329. Non-penetration originally seemed a possible explanation for the lack of antagonism, but there is much evidence that all these compounds exert an inhibitory action on the cholinesterase within the brain (Douskaya and Khaunina, 1961; Irwin and Hein, 1962).

THA is a monoamine oxidase inhibitor (Kaul, 1962), but another monoamine oxidase inhibitor, nialamide has been found to be ineffective in antagonising the behavioural changes produced by JB-329 in dogs (Bell, 1962). It is therefore difficult to see how the effects of THA can be attributed to its inhibition of cholinesterase or monoamine oxidase.

In view of the reported choline acetylase inhibition produced by THA (de la Lande, 1956) it is of interest that some members of the series of glycolic acid derivatives also inhibit choline acetylase (Garratini and others, 1958). Several of this latter series produce antagonism to the behavioural effects induced by JB-329 in dogs (Bell and others, 1963). It seems unlikely, however, that this action could be of importance, because JB-329 and atropine are very powerful acetylcholine antagonists.

The pharmacology of the members of the series of the glycolic acid derivatives studied here did not indicate any very strong cardiovascular or autonomic effects. The only obvious effects were potentiation of the depressor responses to acetylcholine and adrenaline by *p*-phenylmandelic acid, the 2- and 3-phenanthrylglycolic acids and phenoxymandelic acid. These compounds, with the exception of phenoxymandelic acid, were found by Bell and others (1963) to be active against behavioural changes induced by JB-329.

The responses to noradrenaline and adrenaline were shown to be markedly potentiated by JB-329. Chronic *p*-phenylmandelic acid medication in conscious dogs antagonised this adrenergic potentiation (Fig. 2). This effect was also produced by THA but also by the other anticholinesterases. It is interesting to note in this context that adrenaline blocking agents have been shown to antagonise the psychotomimetic action of lysergic acid diethylamide (Murphree, 1962; Elder and Dille, 1962).

It is apparent that the pharmacological basis for the action of the acetylcholine blocking psychotomimetic compounds and their antagonists is as yet not established. Abood (1961) pointed out the possibility that psychotropic agents may exert a direct action at central receptor sites and that the so-called neurohumoral amines merely define the chemical configuration of these receptor sites. Receptor site action by JB-329 was also favoured by Bell and others (1963) in explaining the action of the behavioural antagonists.

The findings in this study show that in addition to direct cholinergic effects, the antagonists inhibit the adrenergic responses potentiated by JB-329 and atropine. These effects should be considered in the light of Biel's proposal (1962) of a balance existing between sympathetic and para-sympathetic centres or functions in the central nervous system. However, the failure of all anticholinesterases to counteract central effects produced by these acetylcholine antagonising psychotomimetics

still cannot be explained. The hypothesis of Abood (1961) that psychotropic substances, in general, may exert a direct action at receptor sites deserves greater attention.

Acknowledgements. This work was supported in part by a grant from the University of Melbourne Medical Research Fund.

REFERENCES

Abood, L. G. (1959). *A Pharmacological Approach to the Study of the Mind*, pp. 234–7. Springfield, Ill.: Charles C. Thomas.
Abood, L. G. (1961). *J. med. pharm. Chem.*, **4**, 469.
Abood, L. G., Ostfield, A. M. and Biel, J. H. (1958). *Proc. Soc. exp. Biol., N.Y.*, **97**, 483–486.
Abood, L. G., Ostfield, A. M. and Biel, J. H. (1959). *Arch. int. Pharmacodyn.* **70**, 186–20.
Alexander, E., Morris, D. P. and Eslick, R. L. (1946). *New Eng. J. Med.*, **234**, 258–259.
Bell, C., Gershon, S., Carroll, B. and Holan, G. (1963). *Arch. int. Pharmacodyn.*, in the press.
Biel, J. H., Abood, L. G., Hoya, W. K., Leiser, Helen A., Nuhfer, P. A. and Kluchkesky, E. F. (1961). *J. org. Chem.*, **26**, 4096–4103.
Biel, J. H., Leiser, H. A., Nuhfer, P. A., Hoya, W. K. and Abood, L. G. (1962). *Ann. N.Y. Acad. Sci.*, **96**, 251–262.
De La Lande, I. S. (1956). Ph.D. Thesis, University of Melbourne.
Douskaya, L. V. and Khaumina, R. A. (1961). *Bijullet. Eksperim. noi Biol. i Med.*, **52**, 69–72.
Drill, V. A. (1958). *Pharmacology in Medicine*, 2nd ed. New York: McGraw-Hill.
Edery, H. (1962). *Brit. J. Pharmacol.*, **18**, 19–28.
Elder, J. T. and Dille, J. M. (1962). *J. Pharmacol.*, **136**, 162–168.
Fink, M. (1960). *Electroenceph. Clin. Neurophys.*, **12**, 359–369.
Garattini, S., Morpingo, C. and Passerini, N. (1958). *Experientia*, **14**, 89–91.
Gershon, S. (1960). *Nature, Lond.*, **186**, 1072–1073.
Gershon, S. and Lang, W. J. (1962). *Arch. int. Pharmacodyn.*, **135**, 31–56.
Gershon, S. and Olariu, J. (1960). *J. Neuropsych.*, **1**, 283–292.
Golenberg, M. (1957). *Novosibersk Govt. Med. Inst. Publ.*, **29**, 7.
Goodman, L. S. and Gilman, S. (1955). *The Pharmacological Basis of Therapeutics*, 2nd ed. New York: Macmillan.
Groissier, V. W. (1956). *Ann. int. Med.*, **44**, 1020–1024.
Holmberg, G. and Gershon, S. (1961). *Psychopharmacologia*, **2**, 93–106.
Horovitz, Z. L. and Chow, M. (1962). *J. Pharmacol.*, **137**, 127–132.
Irwin, R. L. and Hein, M. M. (1962). *Ibid.*, **136**, 20–25.
Kaul, P. N. (1962). *J. Pharm. Pharmacol.*, **14**, 243–248.
Murphree, H. B. (1962). *Clin. Pharmacol. Therap.*, **3**, 314–320.
Ostfield, A. M., Abood, L. G. and Mercus, D. A. (1958). *Arch. Neurol. Psych.*, **79**, 317–322.
Page, I. J. (1952). *J. Pharmacol.*, **105**, 58–73.
Pfeiffer, C. C., Murphree, H. B., Jenney, E. H., Robertson, M. G., Randall, A. H. and Bryan, L. (1959). *Neurology*, **9**, 249–250.
Shaw, F. H. and Bentley, G. A. (1953). *Austral. J. exp. Biol.*, **31**, 573–576.
Vane, J. R. (1957). *Brit. J. Pharmacol.*, **12**, 344–349.
Welbourn, R. B. and Buxton, L. D. (1948). *Lancet*, **2**, 211–213.
White, R. P., Nash, C. B., Westerbeke, E. J. and Possanza, G. J. (1961). *Arch. int. Pharmacodyn.*, **132**, 349–363.

A SPECTROPHOTOMETRIC METHOD FOR THE DETERMINATION OF β-GLYCYRRHETIC ACID (ENOXOLONE) AND ITS ESTERS IN BIOLOGICAL MATERIALS

BY T. J. COLEMAN AND D. V. PARKE

From the Department of Biochemistry, St. Mary's Hospital Medical School, University of London, London, W.2

Received October 7, 1963

A method for the determination of β-glycyrrhetic acid (enoxolone) and its readily-hydrolysable esters in biological materials is described. The material containing the glycyrrhetic acid or its esters is hydrolysed with ethanolic sodium hydroxide, the glycyrrhetic acid is extracted from the acidified hydrolysate, submitted to two-dimensional, thin-layer chromatography on alumina, eluted with ethanol and estimated spectrophotometrically at 248 mμ. Mean recoveries of 100 μg. β-glycyrrhetic acid were 102 per cent (s.e.m. ± 4 per cent) from pure solution; 92 per cent (s.e.m. ± 4 per cent) from rat blood; 90 per cent (s.e.m. ± 4 per cent) from human urine; and recoveries of 20 μg. and 50 μg. from rat bile were 91 to 95 per cent. Mean recoveries of 100 μg. of β-glycyrrhetic acid hydrogen succinate were 95 per cent (s.e.m. ± 5 per cent) from pure solution; 100 and 110 per cent from rat blood; and 92 and 109 per cent from human urine.

β-GLYCYRRHETIC acid (enoxolone) is a triterpenoid obtained from liquorice root which has anti-inflammatory properties (Finney and Somers 1958). It has been used in the treatment of dermatitis (Colin-Jones, 1960) and more recently in the treatment of gastric ulcer (Doll, Hill, Hutton and Underwood, 1962). Recent metabolic studies with tritium-labelled glycyrrhetic acid in the rat have shown that metabolites are excreted mainly via the bile and less than 2 per cent of the activity is found in the urine (Parke, Pollock and Williams, 1963). It is therefore desirable to have a method for the determination of glycyrrhetic acid, which could be applied to bile and faeces as well as to blood and urine.

Several colorimetric methods for the determination of glycyrrhetic acid have been described, the colours being developed with vanillin and sulphuric acid (Weist, 1949), 2,6-di-t-butyl-*p*-cresol and sodium hydroxide (Brieskorn and Mahran, 1960) or by heating with a solution of antimony pentachloride in chloroform (Dr. S. Gottfried, personal communication). Because of lack of specificity or sensitivity, none of these methods is suitable for the determination of glycyrrhetic acid in biological materials.

Spectrophotometric methods have been described for the determination of glycyrrhetic acid in urine (Van Katwijk and Huis in't Veld, 1955) and in blood (Finney, Somers and Wilkinson, 1958), but both of these methods are unsatisfactory because of the lengthy chromatography procedure in the former and the absence of specificity in the latter.* Because of this lack of specificity, a rigorous separation of the β-glycyrrhetic acid is an essential preliminary to any estimation. Its separation from cholesterol

* Since the completion of this work a similar method for the determination of 18-β-glycyrrhetic acid in faeces has been published (Helbing, 1963).

and other steroids which in its chemical structure and solubilities it closely resembles, is particularly important.

A method has now been devised for the spectrophotometric determination of glycyrrhetic acid following a simple thin-layer chromatographic separation from sterols and other biological substances. This method is both sufficiently sensitive and specific for the determination of this triterpenoid in biological materials.

Experimental

Materials

β-Glycyrrhetic acid (3-hydroxy-11-oxo-18β-olean-12-en-30-oic acid), m.p. 285–7°, $[\alpha]_D^{19°}$ + 163° (Biorex Laboratories); β-glycyrrhetic acid hydrogen succinate (3-*O*-β-carboxypropionyl-11-oxo-18β-olean-12-en-30-oic acid), m.p. 315°, $[\alpha]_D^{20°}$ + 130°, light absorption in ethanol, λ_{max} 248 mμ, ϵ_{max} 12,100 (Biorex Laboratories); cholesterol, m.p. 148°, and coprostanol (coprosterol), m.p. 101°, were used for the estimation procedures. Alumina (Aluminiumoxid G nach Stahl, Merck), silica gel (Kieselgel G nach Stahl, Merck) and Polyamide (Merck) were used as adsorbents in the thin layer chromatography.

Ultra-violet Absorption Spectra

The ultra-violet absorption spectrum of β-glycyrrhetic acid and subsequent quantitative estimations were determined with a Unicam SP 500 spectrophotometer. The light absorption of β-glycyrrhetic acid in ethanol (99·7 per cent, R.R. Grade for spectroscopy) showed maxima at 204 mμ (ϵ_{max}, 11600) and 248 mμ (ϵ_{max} 18700). The absorption at 248 mμ obeyed the Beer-Lambert Law over the range of 1 to 25 μg. of β-glycyrrhetic acid per ml. Previous spectrophotometric determinations have also been made at 248 mμ (Van Katwijk and Huis in't Veld, 1955; Finney, Somers and Wilkinson, 1958).

Chromatography

The principal problem was the separation of β-glycyrrhetic acid from cholesterol and other sterols which would interfere in the spectrophotometric determination of glycyrrhetic acid. β-Glycyrrhetic acid, β-glycyrrhetic acid hydrogen succinate, cholesterol, coprostanol, and extracts of biological materials containing β-glycyrrhetic acid were chromatographed on thin-layer plates (Desaga, Heidelberg) of alumina, silica gel and Polyamide. The chromatography plates were developed in various solvent systems and some R_F values are given in Table I.

The most efficient separation of β-glycyrrhetic acid from cholesterol, coprostanol and other biological substances was achieved by two-dimensional chromatography on thin-layer plates of alumina, developed first in acetone-chloroform (1 : 1 for 30 min.) and then at right angles in methanol-ammonia (s.g. 0·88) 4 : 1 for 90 min.). The α- and β-isomers of glycyrrhetic acid could be separated on alumina in methanol-ammonia (4 : 1), the R_F value for α-glycyrrhetic acid being 0·82.

The extracts of β-glycyrrhetic acid in solution in chloroform were applied to the adsorbent as a single spot of 2-5 mm. diameter. After development in the two solvent systems the chromatography plates were dried in air at room temperature to ensure the removal of any ammonia and the β-glycyrrhetic acid was located under ultra-violet light (254 mμ, Hanovia "Chromatolite") as a dark ultra-violet-absorbing spot at the correct R_F values (see Table I). This method of detection was sensitive to as little as 4 μg. of glycyrrhetic acid per cm.² and in practice 2 μg. could be chromatographed and subsequently detected.

The β-glycyrrhetic acid was eluted from the alumina with ethanol, but the efficiency of the elution was found to be dependent on the pH of the eluent. At pH values of less than 2·0 the elution of β-glycyrrhetic acid was quantitative (100 ± 5 per cent), but became less at higher pH values (pH 4, 78 per cent; pH 5, 65 per cent; pH 6, 62 per cent).

TABLE I

APPROXIMATE R_F VALUES OF β-GLYCYRRHETIC ACID AND SOME STEROLS ON THIN-LAYER CHROMATOGRAPHY

Adsorbent	Solvent	R_F Values of			
		β-Glycyrrhetic acid	β-Glycyrrhetic acid hydrogen succinate	Cholesterol	Coprostanol
Alumina	n-Hexane(100)chloroform(1) ..	0	0	0·12	0·31
,,	Chloroform(1)acetone(1) ..	0	0	0·96	0·92
,,	Toluene(4)piperidine(1) ..	0·14	0	0·93	0·93
,,	n-Butanol(10)ammonia*(1) ..	0·96 (1) 0·36 (10)	0·96 (5) 0·36 (1) 0 (5)	0·89	0·89
	Methanol(20)ammonia*(1) ..	0·95 (1) 0·77 (10)	0·66 (1) 0·29 (5)	0–1·0†	0–1·0†
,,	Methanol(10)ammonia*(1) ..	0·92	0·92 (1) 0·66 (5)	0‡	0‡
,,	Methanol(4)ammonia*(1) ..	0·95	0·95 (5) 0·66 (1)	0	0
Silica gel	Toluene(2)piperidine(1) ..	0·50	—	0·92	0·92
,,	n-Butanol(10)ammonia*(1) ..	1·0	0·88	0·84	0·84
,,	Methanol(10)ammonia*(1) ..	0·98	0·98	0–1·0‡	0–1·0‡
,,	n-Hexane(8)ammonia*(1)-ethanol(1)	0	0	0·95	0·95
Polyamide	Chloroform(1)acetone(1) ..	0·55	—	0·91	—

* s.g. 0·88. † Continuous streaking. ‡ Streaking

Hydrolysis of Esters of Glycyrrhetic Acid

To simplify the chromatographic separation, extracts of glycyrrhetic acid were submitted to a preliminary hydrolysis procedure to convert esters of glycyrrhetic acid and sterols into the free compounds. The hydrolysis procedure, namely heating in 2N sodium hydroxide-ethanol (1 : 2 by vol.) for 5 min., quantitatively converts glycyrrhetic acid hydrogen succinate into free glycyrrhetic acid and has no destructive effect upon the glycyrrhetic acid (recoveries, 98 ± 5 per cent).

The removal of gross amounts of sterols before chromatography was necessary for efficient separation in the chromatography procedure and this was achieved by extraction of the saponified solutions with n-hexane. To prevent entrainment of glycyrrhetic acid into the hexane phase it is necessary to remove the ethanol from the hydrolysed solution. When

the ethanol is allowed to evaporate during the hydrolysis, extraction of the saponified solution (4 ml.) with n-hexane (5 ml.) removes most of the sterols but less than 3 per cent of the glycyrrhetic acid (recoveries, 97 per cent).

The glycyrrhetic acid may be quantitatively recovered from the hydrolysed solution (4 ml.) by acidification with 2 ml of 2N sulphuric acid, followed by a single extraction with 5 ml. of chloroform. By this procedure recoveries of β-glycyrrhetic acid were 97 ± 4 per cent of theoretical.

The Standard Procedure

Gently boil a solution of biological fluid (1 ml.) containing 20–100 μg. of β-glycyrrhetic acid or its esters with 2N sodium hydroxide (1 ml.) and ethanol (2 ml.) in a 10 ml. test-tube, allowing the ethanol to evaporate. Cool the solution, add water (4 ml.) and shake the mixture in the stoppered test-tube with n-hexane (5 ml.) for 5 min. Centrifuge the mixture to separate the two phases and remove and discard the hexane. Acidify the aqueous phase with 2N sulphuric acid (2 ml.) and extract the glycyrrhetic acid by shaking in the stoppered test tube with chloroform (5 ml.) for 5 min. Centrifuge the mixture again to separate the two phases and remove and discard the aqueous phase. Dry the chloroform by adding anhydrous sodium sulphate (0·25 g.), centrifuge, and remove the chloroform. Wash the sodium sulphate with chloroform (1 ml.) and add to the chloroform extract; concentrate to about 0·2 ml. Transfer the concentrated chloroform extract quantitatively to a thin-layer chromatography plate of alumina and develop first in one direction in acetone-chloroform (1 : 1) and then at right-angles in methanol-ammonia (4 : 1). Dry the plate for 10 min. in air at room temperature and detect the glycyrrhetic acid under ultra-violet light at the appropriate R_F value. Remove the area of adsorbent containing the triterpenoid and transfer to a 10 ml. stoppered test-tube containing 3 ml. of ethanol containing 1 per cent v/v of 2N hydrochloric acid. Shake the mixture for 5 min. and centrifuge to deposit the alumina. Measure the light-absorption of the supernatant ethanol at 248 mμ and evaluate the content of β-glycyrrhetic acid from a calibration curve. Obtain a blank by eluting an equivalent amount of chromatographed alumina. In our work the blank gave E (1 cm.) 248 mμ = 0·02, equivalent to 1·5 μg. β-glycyrrhetic acid, and this was subtracted from the experimental determinations.

Recoveries of β-Glycyrrhetic Acid

From pure solution. In a series of five estimations using the full standard procedure of hydrolysis, chromatography and spectrophotometric determination, the mean recovery of 100 μg. of β-glycyrrhetic acid from solution in ethanol was 102 per cent (s.e.m. ± 4 per cent). The mean recovery of 100 μg. of glycyrrhetic acid from ethanolic solutions containing also 100 μg. of cholesterol was 98 per cent (s.e.m. ± 4 per cent) and the recoveries from solutions containing 1·00 mg. and 10·0 mg. of cholesterol were 96 and 92 per cent respectively.

From blood. β-Glycyrrhetic acid in amounts of 100 μg. and 20 μg. were added to 1 ml. of heparinised rat blood. Estimation of the glycyrrhetic acid by the standard procedure gave mean recoveries for four

experiments of 92 per cent (s.e.m. ± 4 per cent) for 100 μg. and 104 per cent (s.e.m. ± 10 per cent) for 20 μg.

From urine. β-Glycyrrhetic acid in amounts of 100 μg. and 20 μg. were added to 5 ml. of human urine. The glycyrrhetic acid was estimated by the standard procedure except that the 4 ml. of water usually added after the alkaline hydrolysis was omitted. Mean recoveries for four experiments were 90 per cent (s.e.m. ± 4 per cent) for 100 μg. and 102 per cent (s.e.m. ± 8 per cent) for 20 μg.

From bile. β-Glycyrrhetic acid in amounts of 20 μg. and 50 μg. was added to 1 ml. of rat bile, collected by biliary cannulation, and the glycyrrhetic acid was estimated by the standard procedure. Recoveries were 91 and 93 per cent for 50 μg. and 94 and 95 per cent for 20 μg. Recoveries from concentrated rabbit bile were unsatisfactory due to the high mucin content and the material coagulated on addition of acid.

From faeces. Recoveries of β-glycyrrhetic acid from rat faeces were unsatisfactory (40–50 per cent) and location of the triterpenoid on the thin-layer chromatogram was difficult. Much of the added β-glycyrrhetic acid was not to be found at the expected R_F value. It is possible that the glycyrrhetic acid is changed chemically by the faecal bacteria into a substance with different chromatographic and light absorption characteristics.

Recoveries of β-Glycyrrhetic Acid Hydrogen Succinate

From pure solution. In a series of four estimations using the standard procedure the mean recovery of 100 μg. of β-glycyrrhetic acid hydrogen succinate from solution in ethanol was 95 per cent (s.e.m. ± 5 per cent).

From blood. β-Glycyrrhetic acid hydrogen succinate in amounts of 100 μg. and 20 μg. were added to 1 ml. of heparinized rat blood. Estimation of the glycyrrhetic acid by the standard procedure gave recoveries of 100 and 110 per cent for 100 μg., and 89 and 112 per cent for 20 μg.

From urine. Recoveries of 100 μg. of glycyrrhetic acid hydrogen succinate from 5 ml. of human urine were 92 and 109 per cent.

Acknowledgements. The authors are indebted to Professor R. T. Williams for his interest in this work and to Biorex Laboratories Ltd., for providing the β-glycyrrhetic acid and its derivatives.

References

Brieskorn, C. H. and Mahran, G. H. (1960). *Arch. Pharm. Berl.*, **293**, 1075–1078.
Colin-Jones, E. (1960). *Postgrad. med. J.*, 36, 678–682.
Doll, R., Hill, I. D., Hutton, C. and Underwood, D. J. (1962). *Lancet*, **2**, 793–796.
Finney, R. S. H. and Somers, G. F. (1958). *J. Pharm. Pharmacol.*, **10**, 613–620.
Finney, R. S. H., Somers, G. F. and Wilkinson, J. H. (1958). *Ibid.*, **10**, 687–695.
Helbing, A. R. (1963). *Clin. chim. Acta.*, **8**, 756–762.
Parke, D. V., Pollock, S., and Williams, R. T. (1963). *J. Pharm. Pharmacol.*, **15**, 500–506.
Van Katwijk, V. M. and Huis in't Veld, L. G. (1955). *Rec. trav. chim. Pays-Bas*, **74**, 889–896.
Weist, F. (1949). *Kolorimetrische Bestimmung der Glycyrrhizinsaure als Aglukon*, Dissertation, Zurich.

LETTERS TO THE EDITOR

The Electrically Stimulated Circular Muscle Strip of the Rabbit Ileum

SIR,—The circular muscle strip of the guinea-pig ileum is insensitive to acetylcholine, compared with a longitudinal muscle strip under identical experimental conditions, and is wholly insensitive to histamine, 5-hydroxytryptamine and nicotine. After cholinesterase inhibition the response to acetylcholine is greatly potentiated and the preparation becomes responsive to histamine, 5-hydroxytryptamine and nicotine (Harry, 1963). I have found that the circular muscle strip of the rabbit ileum will respond invariably only to carbachol (from 100 μg./ml.), but not to acetylcholine, methacholine, histamine, 5-hydroxytryptamine or nicotine. It has not been found possible to change this pattern by inhibiting cholinesterase in the preparation with mipafox.

However, circular muscle strips from the rabbit ileum can be made to respond regularly by applying transmural square wave electrical stimulation (Paton, 1955) from a Multitone stimulator at supramaximal voltage (usually 80–90 V), 0·3 msec. duration and a frequency of 50/sec. for 15 sec. not more frequently than once every 10 min. The electrodes consist of two vertical, parallel platinum wires fixed one on either side of a perspex channel with the muscle strip between the wires (Birmingham and Wilson, 1963). The response to stimulation, which consisted of a large twitch followed by a gradual diminution in the height of contraction, developed after a latent period of at least 5 sec. and in some preparations did not develop until stimulation had ceased. Such responses could be elicited for more than 6 hr.

The pulse width of stimulation used in these experiments was indicative of the activation of a nervous mechanism in the tissue. This was confirmed by the use of procaine or lignocaine which consistently abolished the response to transmural stimulation in concentrations which did not reduce the response of the muscle to carbachol. This inhibition could be reversed by washing the preparation for 1 hr. If, in the presence of lignocaine or procaine, the parameters of stimulation were increased to 120 V and 10 msec., at the same frequency as before, then the preparation responded with a maintained contracture of immediate onset. This response, attributed to direct stimulation of the muscle cells was not affected by concentrations of local anaesthetic ten times or more in excess of that required to abolish the nervous response.

The exact nature of the nervous response is under investigation, but the results so far obtained suggest that it may be mediated by acetylcholine.

Department of Pharmacology,
King's College,
London, W.C.2.
September 30, 1963

M. G. TWEEDDALE

REFERENCES

Birmingham, A. T. and Wilson, A. B. (1963). *Brit. J. Pharmacol.* (in press).
Harry, J. (1963). *Ibid.*, **20**, 399–417.
Paton, W. D. M. (1955). *J. Physiol.*, **127**, 40–41P.

The Role of Adrenal Medulla in Maintenance of Cardiac Catecholamine Levels in the Rat

SIR,—It is known that tissues with postganglionic sympathetic innervation, including the heart, can take up noradrenaline from the blood or from the surrounding fluid (Raab and Gigee, 1955; Axelrod, Weil Malherbe and Tomchick, 1959; Bhagat, 1963; Bhagat and Shideman, 1963b,c). This indicates that catecholamines discharged into the blood stream from the adrenal gland and sympathetic nerve endings could supply the heart. Bhagat and Shideman (1963a) have shown that the heart is probably capable of synthesizing noradrenaline from its precursors dopa and dopamine in intact animals. We now describe work undertaken to obtain information on the role of the adrenal medulla in the maintenance of the neurotransmitter store in the intact animal. 1-(5,6-Dimethoxy-2-methyl-3-indole)-ethyl-4-phenylpiperazine (Win 18501–2, Oxyperitine) was employed as a pharmacological tool for depleting the heart of its catecholamine stores and the influence of a reduced supply of circulating catecholamines, as produced by adrenal demedullation, on repletion of these stores was determined.

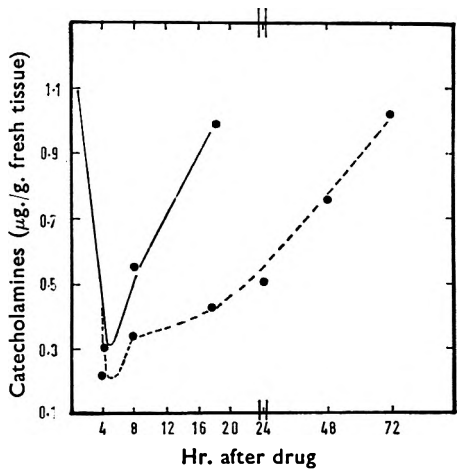


FIG. 1. Effect of adrenal demedullation on the repletion of cardiac catecholamines. Solid lines indicate normal animals. Broken lines indicate demedullated animals. Each amine level is mean of 5 or 6 values.

Male rats (Holtzman strain) weighing 200 to 225 g. were either demedullated or sham operated as described by Bhagat and Shideman (1963a). Ninety-six hr. after the operation, the rats were given Win 18501–2, 50 mg./kg. intraperitoneally, and killed at various times thereafter. The concentrations of catecholamines in the ventricular myocardium were determined by the trihydroxyindole fluorimetric procedure of Shore and Olin (1958) and expressed as μg. of noradrenaline per g. of fresh tissue.

The results (Fig. 1) show that noradrenaline returned to control value in the sham operated rats within 18 hr. after Win 18501–2. This suggests that turnover of noradrenaline in the heart is rapid. While demedullation retarded significantly the restoration of cardiac catecholamine in the rats after Win 18501–2 treatment, thereby showing the importance of an extra cardiac source in the maintenance of the neurotransmitter store, it had no effect on normal levels of catecholamines in the heart. There was no significant difference in myocardial

catecholamine levels between sham operated and demedullated animals. These data indicate that the maintenance of normal levels of myocardial catecholamines is not totally dependent upon deposition of circulating catecholamines of adrenal origin or upon the presence of a functioning adrenal gland. This supports the view that the body has the ability to adjust itself to environmental conditions and this might be considered as a kind of compensation mechanism in the organism. When, for example, uptake of noradrenaline from the circulating blood was reduced by demedullation of the suprarenals, the heart, in order to maintain normal levels, probably increased the rate of synthesis of noradrenaline. Similarly, nervous excitation increases the rate of synthesis of catecholamines, for prolonged stimulation does not alter the noradrenaline content of nerves (Luco and Goni, 1948) or of ganglia (Vogt, 1956). Also, prolonged splanchic stimulation brings about the release of catecholamines from the adrenal medulla but the concentration in the medulla decreases little (Elliott, 1912; Hökfelt and McLean, 1950; Holland and Schümann, 1956). On the other hand, infusion of both adrenaline and noradrenaline, at rates which may be considered to be physiological, inhibits the adrenal medullary secretion of catecholamines (Robinson and Watts, 1962).

Thus, the present study demonstrates that the turnover of heart noradrenaline in the rat is rapid and that uptake of catecholamine from the circulating blood is an important factor in maintaining the normal levels of catecholamines in the heart, though the heart is not totally dependent on extraction of noradrenaline from the blood.

Acknowledgement. I wish to thank Dr. Sidney Archer of Sterling Winthrop for the generous supply of Win 18501-2.

Department of Pharmacology,
University of Minnesota,
Minneapolis, 14.
October 3, 1963

B. BHAGAT*

REFERENCES

Axelrod, J., Weil Malherbe, H. and Tomchick, R. (1959). *J. Pharmacol.*, **127**, 251–256.
Bhagat, B. (1963). *Arch. int. Pharmacodyn* (in press).
Bhagat, B. and Shideman, F. E. (1963a). *J. Pharmacol.* (in press).
Bhagat, B. and Shideman, F. E. (1963b). *Ibid.*, **140**, 317–323.
Bhagat, B. and Shideman, F. E. (1963c). *Brit. J. Pharmacol.*, **20**, 56–62.
Elliott, T. R. (1912). *J. Physiol.*, **44**, 374.
Hökfelt, B. and McLean, J. M. (1950). *Acta physiol. scand.*, **21**, 258–270.
Holland, W. C. and Schümann, H. J. (1956). *Brit. J. Pharmacol.*, **11**, 449–453.
Luco, J. V. and Goni, F. (1948). *J. Neurophysiol.*, **11**, 497–500.
Raab, W. and Gigee, W. (1955). *Circul. Res.*, **3**, 553–558.
Robinson, R. L. and Watts, D. T. (1962). *Amer. J. Physiol.*, **203**, 713–716.
Shore, P. A. and Olin, J. S. (1958). *J. Pharmacol.*, **122**, 295–300.
Vogt, M. (1954). *J. Physiol.*, **123**, 451–481.

* Present address: Howard University, School of Medicine, Dept. of Pharmacology, Washington 1, D.C.

A New Method of Administering Drugs by Mouth to Animals

SIR,—Since animals, even more than children, frequently refuse to take a drug by mouth, we have developed a method which can help overcome their reluctance. It is based on the fact that rats like chocolate (Teitelbaum and Epstein, 1962).

The drug is put up in a chocolate paste (Fry's chocolate spread 65 per cent, starch 35 per cent), stiff enough not to stick to the animal's paws or the cage, but not so stiff as to crumble. The paste is kept in an ointment tube; the dose can be gauged by measuring the length of the column extruded, or more precisely by weighing the tube before and after squeezing out the dose. A trained rat eats the paste off a spatula cleanly and completely in a few seconds, even when it is on a free diet of rat cubes.

Before a drug can be given by this method the animal must be offered control chocolate paste daily for two or three days after being without food for a few hours. It will then readily accept the paste. Training takes longer with more than one or two animals in a cage. The next step is to find a concentration of the drug in the paste which the animal will accept. The human sense of taste is a poor guide for this purpose. Thus hydrocortisone 12 mg./g. chocolate paste is accepted by rats, but tastes bitter to man; and stilboestrol 10 mg./g. paste is rejected by rats, but tastes like control chocolate paste to man.

We have successfully used the method to give hydrocortisone to rats for several weeks. The paste was put in a tube of nozzle diameter 5 mm.; the length of the column squeezed out was measured with a ruler. The mean weight of six

TABLE I

AMOUNTS OF QUININE DIHYDROCHLORIDE TAKEN IN CHOCOLATE PASTE AND IN SOLUTION. (MEANS FOR TWO RATS)

Concentration of drug	Amount of preparation consumed	Total dose of drug consumed
In chocolate paste*		
None	3·9 g. } in	
1 mg./g.	2·6 g. } 10	2·6 mg.
3 mg./g.	2·5 g. } min.	7·5 mg.
5 mg./g.	less than 0·4 g.	
In aqueous solution		
None	26·5 ml. }	
0·1 mg./ml.	22 ml. } in	2·2 mg.
0·3 mg./ml.	16 ml. } 24	4·8 mg.
2·0 mg./ml.	3·5 ml. } hr.	7 mg.
2·0 mg./ml. together with sucrose 250 mg./ml.	13·5 ml. }	28 mg.

* Presented in "doses" of 250–300 mg.

5 mm. lengths was 117 mg., with a standard deviation of ±6 mg.; of six 4½ mm. lengths, 105 ± 4 mg. These figures correspond respectively to doses of 1·40 ± 0·07 mg. and 1·26 ± 0·05 mg. hydrocortisone.

Can a rat be induced by this method to take more of a drug than it takes when the drug is put in its drinking water? Observations to answer this question were made on two singly housed male rats, fed rat-cubes *ad libitum*. On different days they were offered chocolate paste containing varied concentrations of quinine dihydrochloride, or control chocolate paste. For 10 min. each day they were allowed to eat as much paste as they wanted; the amount eaten was measured by weighing the tube. The rats drank either distilled water or a solution of quinine dihydrochloride. To minimize adaptation, successive presentations of quinine were separated by one or more days on which the paste or water was given without the drug. Chocolate paste containing 1 or

3 mg./g. of the quinine salt was eaten readily, though a little more slowly than the unmedicated paste. Higher concentrations were either consumed hesitantly and only in part (5 mg./g.), or rejected after sampling (10 mg./g.) (see Table I). The rejection behaviour was characterized by slow and intermittent chewing, followed by prolonged rubbing of the lower jaw along the floor or sides of the cage (cf. Teitelbaum and Epstein, 1962). When quinine was given in solution, a concentration of 0·1 mg. of the salt per ml. was accepted without any change in fluid intake; with 0·3 mg./ml. or more the fluid intake decreased, and with 2 mg./ml. it was only 10–20 per cent of the control value. When sucrose was added in high concentration (230 mg./ml.) in the presence of 2 mg./ml. quinine dihydrochloride, the fluid intake increased, but was still much below the control level. Table I shows that the rats took a similar total daily dose of quinine, in similar concentration, whether the drug was in chocolate paste or in solution. But all the paste was eaten at one time, whereas the solution was consumed gradually in the course of 24 hr. Normal rats will drink solutions and eat food containing higher concentrations of quinine, about 10 mg./g., but only when they have no choice (Teitelbaum and Epstein, 1962).

The advantages of our method are that it is relatively pleasant for the animal, and interferes minimally with its normal feeding and drinking behaviour. The method is convenient for the operator, and can be used on behalf of a licence holder, but in his absence, by a person unlicenced under the 1876 Act. Drugs keep well in a capped metal tube, and the dose can be accurately measured. However, the method becomes cumbersome with doses approaching 30 mg. and cannot be used for bulky medication.

In principle the method can be adapted to any species including man, with modifications to take account of differing food preferences.

Dept. of Pharmacology,
London Hospital Medical College,
London, E.1.

A. HERXHEIMER

London Hospital,
London, E.1.

M. B. DOUGLAS

October 11, 1963

REFERENCE

Teitelbaum, P. and Epstein, A. N. (1962). *Psychol. Rev.*, **69**, 74–90.

Thermodynamics of Micelle Formation of Non-Ionic Detergents

SIR,—In view of recent interest in the thermodynamics of micellisation of non-ionic detergents (Corkill, Goodman, and Ottewill, 1961; Schick, 1963) we present some results for the molar heats and entropies of micellisation, ΔHm and ΔSm respectively, obtained as part of a study of synthetic detergents, in which some compounds containing branched hydrocarbon chains have been synthesised. The thermodynamic properties were obtained from the temperature dependence of the critical micelle concentration, using the conventional equations (e.g. Schick, 1963).

The results show that increasing the length of the hydrophobic group for a constant polyoxyethylene chain length gives an increase of both ΔHm and ΔSm. The branched chain compounds yield slightly smaller values than their straight chain isomers. Calculations of ΔHm and ΔSm from Schick's (1962) results on ethylene oxide dodecanol, nonyl phenol, octyl phenol, and tridecanol condensates, show that *in general*, these thermodynamic properties become less positive as the polyoxyethylene chain is lengthened, the hydrocarbon moiety being held constant.

The factors controlling ΔHm and ΔSm are complex. The structure of water (iceberg effect of Frank and Evans, 1945) about the hydrocarbon chain of the

TABLE I

THERMODYNAMIC PROPERTIES

Compound	ΔHm k.cal. mole^{-1}	ΔSm cal. mole^{-1}deg^{-1}
$Me(CH_2)_3n_6$	0·8	2·7
$Me(CH_2)_7n_6$	2·2	7·4
$Me(CH_2)_{11}n_6$* ..	3·5	11·7
$(Me)_2CHCH_2n_6$ ..	0·6	2·2
$(Et)_2CH{\cdot}CH_2n_6$ ..	1·2	4·0
$(Pr)_2CH{\cdot}CH_2n_6$ ..	2·0	6·7
$(Bu)_2CH{\cdot}CH_2n_6$ ..	2·9	9·7
$(Bu)_2CH{\cdot}CH_2n_9$ ..	2·3	7·7

* Data of Corkill, Goodman, and Ottewill, 1961.
n represents number of ethylene oxide units in molecule.

monomer will be lost when it enters the micelle, giving a positive entropy change. Configurational entropy changes on micelle formation provide an opposite effect. Soap monomers are considerably contracted in aqueous solution (e.g. Elworthy, 1963) while in the micelle they will be more extended. The configuration of the polyoxyethylene chains in the monomer is unknown, but in the micellar form it has been shown (Schick, Atlas, and Eirich, 1962; Elworthy and Macfarlane, 1963) that they are contracted to the extent of about 50 per cent of their extended length. Lack of knowledge of the hydration of polyoxyethylene chains in the monomer compared with their state in the micelle prevents definite assignment of the entropy change for this transference. However, it seems likely that the mesh of polyoxyethylene chains present in the micelle can trap more solvent than the single chain of the monomer, especially as the latter is fairly short (by polymer standards) and cannot be expected to form a true coil in the polymer sense. Also Elworthy and Macfarlane (1963) and Elworthy and McDonald (1963) have shown that extension of this chain increases with temperature, leading to greater micellar solvation at higher than at lower temperatures, due to the trapping effect.

Considering the above factors it seems likely that the increasing positive value for ΔSm with increasing hydrocarbon chain length (n constant) is due to the entropy change on desolvating the hydrocarbon part of the monomers on

entering the micelle, and that this is the predominant effect. The general trend apparent in Schick's (1962) results and for our branched chain $C_{10}n_3$ and $C_{10}n_9$ compounds, of ΔSm decreasing with increasing polyoxyethylene chain length, may be due to the increased solvation shown to occur when the polyoxyethylene chain is lengthened. Due to the trapping effect, there is likely to be more ordering of the water molecules in the polyoxyethylene region of the micelles, compared with this region in the monomers, and in general this difference should increase with chain length.

Acknowledgement. We thank the Medical Research Council for the award of a Scholarship to A.T.F.

School of Pharmacy,
Royal College of Science & Technology,
Glasgow, C.1.

P. H. ELWORTHY
A. T. FLORENCE

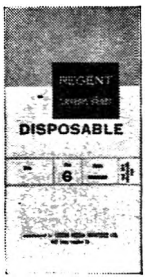
October 25, 1963

REFERENCES

Corkill, J. M., Goodman, J. F. and Ottewill, R. H. (1961). *Trans. Faraday Soc.*, **57**, 1627–1636.
Elworthy, P. H. (1963). *J. chem. Soc.*, 388–392.
Elworthy, P. H. and Macfarlane, C. B. (1963). *ibid.*, in the press.
Elworthy, P. H. and McDonald, C. (1963). *Kolloid Z.*, in the press.
Frank, H. S. and Evans, M. W. (1945). *J. Chem. Phys.*, **13**, 507–513.
Schick, M. J. (1962). *J. Colloid Science*, **17**, 801–813.
Schick, M. J. (1963). *J. phys. Chem.*, **67**, 1796–1799.
Schick, M. J., Atlas, S. M. and Eirich, F. R. (1962). *Ibid.*, **66**, 1326–1333.

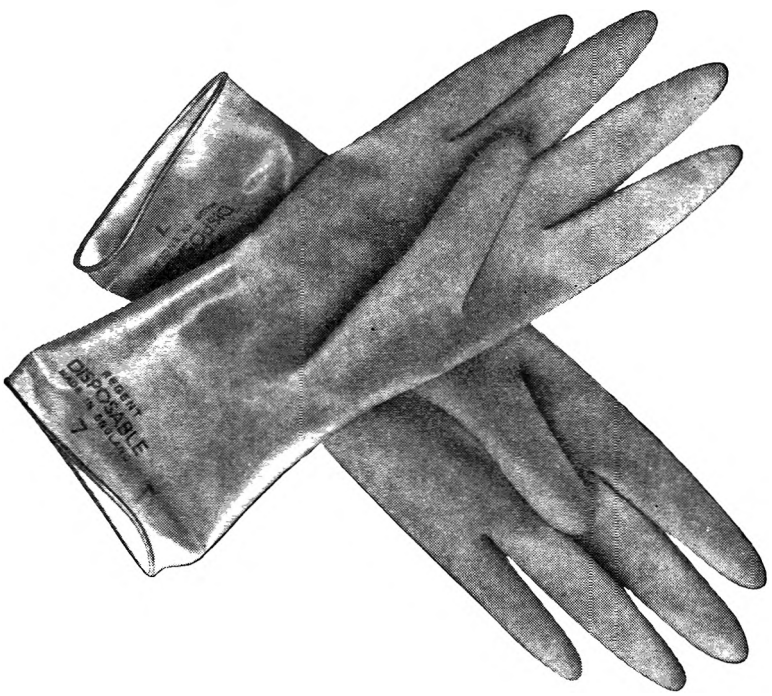
PRINTED BY W. HEFFER & SONS LTD., CAMBRIDGE, ENGLAND.

Save time and money with Regent Disposable Gloves by eliminating multi-stage reconditioning processes, thus simplifying sterilisation and liberating valuable skill for nursing ▪ Because hands are assumed to be an important vehicle of cross-infection, the use of a new glove of guaranteed integrity each time also makes a contribution towards the elimination of this problem ▪ Regent Disposable gloves reduce hand fatigue and provide 'bare hand' tactile sensitivity ▪ They are supplied packed ready for instant sterilisation, complete with Ethicon Bio-sorb powder.



Regent Surgeons' Disposable Gloves

British Standard 1803 : 1952



Available under Ministry of Health Central Contract

Manufactured by LONDON RUBBER INDUSTRIES LIMITED

HALL LANE · LONDON E4

(Surgical Division)