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Phase relationships in the simple coacervating system isoelectric gelatin : ethanol : water

J. R. NIXON, SALEH A. H. KHALIL* AND J. E. CARLESS

The influence of molecular weight on coacervation in the system iso-electric gelatin : ethanol : water has been studied. The minimum concentration of ethanol required to produce coacervation decreased as the molecular weight of the gelatin increased. Analysis of the coacervate phase showed that the ethanol content was approximately constant and independent of both the molecular weight of the gelatin and the total concentration of ethanol in the system.

UNDER certain conditions macromolecular solutions may separate into two liquid layers. The term coacervation was introduced by Bungenberg de Jong & Kruyt (1929) to denote this kind of phase separation. If the opposition of charges between the colloidal components is the cause of this separation it is termed complex coacervation, whilst if it is due to "desolvation" it is said to be simple coacervation. In this latter instance the coacervate phase is rich in the colloidal component whilst the equilibrium liquid contains only negligible amounts of the colloid. Coacervation has been observed both in solutions of colloidal electrolytes (Bungenberg de Jong, 1937) and non-electrolytes (Dobry, 1938).

Because of its importance in plant and animal biology, complex coacervation has been extensively studied (Bungenberg de Jong, 1949), while few published data refer to simple coacervation. Many patents have been granted in recent years for various coacervating systems, both simple and complex, as coatings for pharmaceutical and other purposes (Green & Schleicher, 1956; Green, 1957). Phares & Sperandio (1964) have prepared samples of pharmaceuticals coated with coacervate layers.

We have examined the location of the coacervate phase obtained from the simple system isoelectric gelatin : ethanol : water in relation to the molecular weight of the gelatin and also the composition of the coacervate and equilibrium liquid.

Experimental

MATERIALS

Gelatin. The samples were alkali-processed hide gelatins having the characteristics given in Table 1. The gelatin samples were dried in thin layers at 110° for 12 hr and stored in air-tight containers. *Absolute ethanol* and *glass distilled water* were used.

METHODS

Determination of the number average molecular weight of gelatin. This was determined from the intrinsic viscosity by the method of Janus & Darlow (1962) using constants obtained by Pouradier & Venet (1950).

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* This work forms part of a thesis (S.A.H.K.) to be submitted for the degree of Ph.D. in the University of London.

TABLE 1. CHARACTERISTICS OF GELATIN SAMPLES

Sample	Number average M	Bloom number	Viscosity (cps, 6·67%)	Isoelectric point	Ash %
A	27,000	55	—	5·1	1·13
B	49,000	106	2·9 (60°)	5·1	1·21
C	60,000	160	4·1 (60°)	5·0	1·19
D	70,000	240	8·1 (40°)	5·2	1·20

Determination of the phase boundaries. A series of gelatin solutions containing from 5 to 30% w/w gelatin were prepared and adjusted to the isoelectric point by the dropwise addition of 2N hydrochloric acid. A weighed quantity of the coacervating agent (ethanol) was added to a weighed quantity of gelatin solution and the mixture equilibrated in a glass stoppered centrifuge tube at 40 ± 0·1°. Further additions of ethanol were made until a phase change was noted. The mixture was equilibrated for 24 hr at 40° and then centrifuged at 5000 rpm for 5 min to achieve separation.

The number and types of phase present at 40° were examined and the percentage composition at which a phase change occurred was calculated.

Measurement of the coacervate volume. The volume of the coacervate phase separated at 40° was measured directly in calibrated 10 ml centrifuge tubes.

Analysis of the coacervate phase and the equilibrium liquid. The method of Phares & Sperandio (1964) was modified to enable mixtures of gelatin: ethanol: water to be assayed. A total gelatin concentration of 5% w/w was used to allow comparison with the results of other workers. The gelatin solution was adjusted to the isoelectric point with 2N hydrochloric acid and the required weight of ethanol added. The stoppered tubes were then equilibrated for 5 days at 40 ± 0·1° to allow complete separation of the phases.

The coacervate phase was sampled and after suitable dilutions with water the refractive index and the specific gravity were determined at 40°. The percentage composition of the dilutions was calculated from the formulae

$$W = 1 - A - G \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

$$G = \frac{\Delta RI - \alpha A}{\beta} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

$$A = \frac{(\Delta \text{ sp.gr.})\,\beta - \Delta RI(\Delta \text{ sp.gr.} + \phi)}{(\beta\psi - \alpha\phi) + \Delta \text{ sp.gr.}\,(\beta - \alpha)} \quad \ldots\ldots \quad (3)$$

Where A = g ethanol/g diluted coacervate phase G = g gelatin/g diluted coacervate phase, W = g water/g diluted coacervate phase, RI = refractive index, sp.gr. = specific gravity, α = slope of refractive index against concentration of ethanol (g/g of solution), β = slope of refractive index against concentration of gelatin (g/g of solution), ψ = slope of specific gravity against concentration of ethanol (g/g of solvent), ϕ = slope of specific gravity against concentration of gelatin (g/g of solvent).

From the value obtained, the composition of the undiluted coacervate phase was calculated.

Samples of the equilibrium liquid were similarly diluted and the refractive index determined. The concentrations of ethanol and gelatin were calculated using the equation of the tie line.

$$A = \gamma + \sigma G \quad .. \quad .. \quad .. \quad .. \quad (4)$$

where γ and σ are constants for each tie line.

By simple mathematical manipulation the concentration of gelatin (G_E) and ethanol (A_E) in the diluted equilibrium liquid can be shown to be given by equations 5 and 6.

$$G_E = \frac{A_E - \gamma}{\sigma} \quad .. \quad .. \quad .. \quad .. \quad (5)$$

$$A_E = \frac{\sigma \Delta RI + \gamma\beta}{\sigma\alpha + \beta} \quad .. \quad .. \quad .. \quad (6)$$

Results

The ternary diagrams for the gelatins examined are shown in Fig. 1. The positions of the various phases produced on transition from a clear

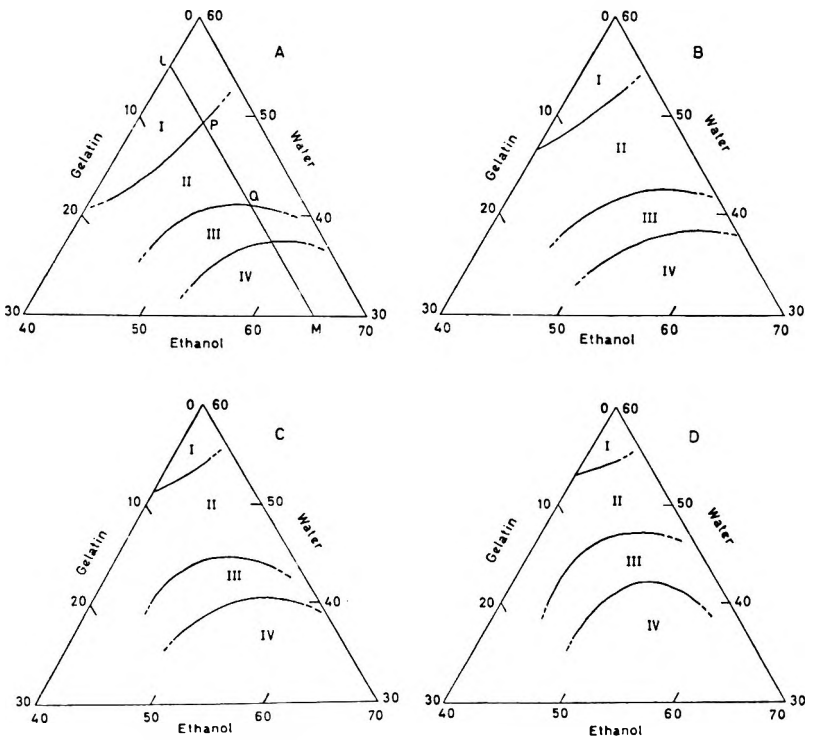

FIG. 1A-D. Ternary diagrams of the phase boundaries in the system iso-electric gelatin : ethanol : water. Gelatin Number Average Molecular Weight : A, 27,000 ; B 49,000 ; C, 60,000 : D, 70,000. I Clear isotropic liquid ; II Coacervate and equilibrium liquid ; III Coacervate, precipitated gelatin and liquid ; IV Precipitated gelatin and liquid. Temperature, 40° ± 0·1°. pH = isoelectric point.

solution to complete flocculation of the gelatin component are shown. In Fig. 1A, the line LPQM represents mixtures containing 5% w/w gelatin. As the concentration of ethanol was increased above 40% w/w, transition into a turbid region (II) occurred at point P. On microscopic examination this was found to consist of coacervate droplets (20–30 μ diameter) in a clear equilibrium liquid. The coacervate phase was viscous and showed no birefringence under polarised light.

Increasing concentrations of ethanol, from 48 to 52%, produced a decrease in coacervate volume (Table 2) and an increase in viscosity. At constant ethanol concentrations the volume of the coacervate phase was proportional both to the amount of gelatin present in the system and to the molecular weight of the gelatin (Tables 2 and 3).

TABLE 2. CHANGE IN COACERVATE VOLUME WITH INCREASE IN ETHANOL CONCENTRATION

Gelatin % w/w *M* 27,000	Ethanol % w/w	Water % w/w	Coacervate volume ml
5	48	47	0·6
	50	45	0·4
	52	43	0·28
10	46	44	1·3
	48·5	41·5	0·9
15	44	41	1·9
	46	39	1·5

TABLE 3. THE EFFECT OF MOLECULAR WEIGHT ON COACERVATE VOLUME AT 40° IN THE SYSTEM GELATIN 5·32% w/w; ETHANOL 46·81 w/w; WATER 47·87% w/w

Number average *M*	27,000	49,000	60,000	70,000
Coacervate volume (ml)	0·65	1·20	1·65	2·20

When the ethanol concentration was further increased, a region (III) was reached in which coacervate and equilibrium liquid existed in equilibrium with precipitated gelatin. Finally, at sufficiently high ethanol concentrations, all the gelatin was precipitated (IV).

The most obvious effect of an increase in the molecular weight of the gelatin was that a lower concentration of ethanol was required to produce any phase change at a given gelatin concentration (Fig. 2, which is based on the positions of the phase boundaries in Fig. 1). These phase boundary lines were parallel, which indicated that the increase in concentration of ethanol required to produce any phase change was independent of the molecular weight of the gelatin. A linear relationship existed between the number average molecular weight and the total ethanol concentration required to produce a phase change.

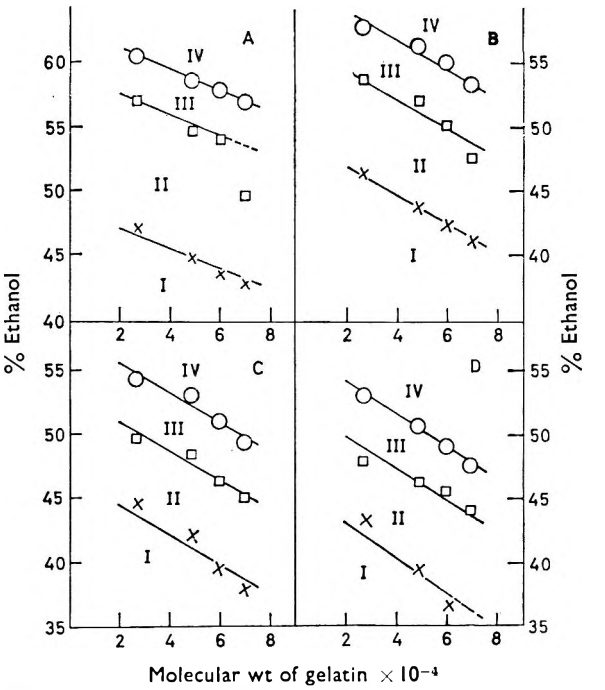

FIG. 2. The effect of molecular weight of gelatin on coacervation. Gelatin concentration % w/w: A, 2·5; B, 5; C, 10; D, 15. I Clear isotropic liquid; II Coacervate and equilibrium liquid; III Coacervate, precipitated gelatin and liquid; IV Precipitated gelatin and liquid. Temperature, 40° ± 0·1. pH = isoelectric point.

Analysis of the coacervate and corresponding equilibrium liquid (Table 4) showed that the ethanol content of the coacervate was approximately constant irrespective of the total ethanol concentration in the mixture and of the number average molecular weight of the gelatin.

TABLE 4. THE EFFECT OF GELATIN MOLECULAR WEIGHT ON THE COMPOSITION OF COACERVATE AND EQUILIBRIUM LIQUIDS

Gelatin Average number M	Percentage w/w compositions								
	Total mixture			Coacervate			Equilibrium liquid		
	Gelatin	Ethanol	Water	Gelatin	Ethanol	Water	Gelatin	Ethanol	Water
27,000	5	48	47	19·3	37·0	43·7	3·1	49·4	47·5
	5	50	45	23·0	38·0	39·0	2·0	52·2	45·8
	5	52	43	25·4	37·4	37·2	1·3	54·7	44·0
	5	54	41	28·0	37·0	35·0	0·7	57·2	42·1
49,000	5	45	50	12·3	36·8	50·9	1·9	48·5	49·6
	5	47	48	15·9	36·3	47·8	1·0	50·6	48·4
	5	49	46	19·3	37·7	43·0	0·7	52·6	46·7
	5	51	44	22·2	37·5	40·3	0·6	54·6	44·8
70,000	5	43	52	15·3	36·3	48·4	0·5	45·9	53·6
	5	44·5	50·5	16·4	36·7	46·9	0·2	47·9	51·9
	5	46	49	17·3	36·9	45·8	0·1	49·6	50·3
	5	47·5	47·5	18·7	36·2	45·1	0·1	51·5	48·4

All figures are the mean of three experiments

Fig. 3 and Table 4 clearly indicate this point, but it should be noted that in the region of the initial formation of coacervate, the composition of coacervate and equilibrium liquid would be approximately the same. This means that the proportion of ethanol in the coacervate would be higher than the equilibrium concentration reached on passing further into the coacervate region, whilst the corresponding equilibrium liquid would contain a proportionately higher colloid concentration.

Discussion

Because of inadequate physico-chemical criteria for defining coacervates and coacervation the terms have been misused in the past and Lawrence (1954) suggested that this nomenclature is not necessary. However, coacervates do have unique characteristics in that they separate out as optically active isotropic liquids in equilibrium with a liquid containing only negligible amounts of the colloid, and the continued use of the term to distinguish them from other colloid-rich phases such as liquid crystals has been supported by Dervichian (1949).

Because of the polydisperse nature of the gelatin it is not strictly accurate to construct a three component phase diagram, but Fergusson & Richardson (1932) have shown that heterogeneous colloids (commercial soaps) behave effectively as a single component, and a triangular diagram showing the regions where one, two or three phases exist in equilibrium is valid (Dervichian, 1954).

The order of occurrence of the different phases in the system studied in this work resembled closely the phase equilibria in the system gelatin : water : ammonium sulphate (Dervichian & Van den Berg, 1948) where in each instance the coacervate was separated from the flocculate region by a zone containing coacervate, solid gelatin and equilibrium liquid

The influence of gelatin average molecular weight on the position of the phases is shown in Fig. 1. The relative position of all the phases moved towards the ethanol corner as the molecular weight decreased. There was no direct relationship between the band width of a phase and the concentration of gelatin in the system. A linear decrease in band width of the coacervate phase was found up to approximately 12% w/w gelatin after which the range of ethanol concentration over which this phase was present sharply increased. The corresponding changes in the width of the three phase system (Fig. 2) were much smaller but whereas the coacervate band showed a minimum width the three phase system had a maximum in the region of 12% w/w gelatin. Fig. 2 also shows that the molecular weight of the gelatin used had no effect on the band width of a particular phase, but only on its relative position within the triangular diagram because the boundaries of the phases were essentially parallel.

Once coacervation had occurred, the further addition of ethanol produced a decrease in coacervate volume (Table 2). It is also possible, from the intercept of the line XA (Fig. 3) with the tie lines, to deduce the relative weights of each of the two phases in equilibrium as the ethanol concentration is progressively increased. The relative weight of the coacervate as a

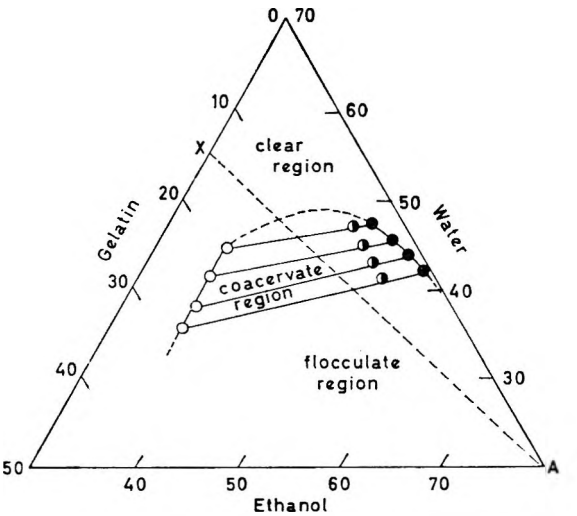

FIG. 3. The composition of coacervate and corresponding equilibrium liquid. Gelatin Number Average Molecular Weight 27,000. ○, Coacervate; ●, Equilibrium liquid; ◑, Total Mixture. Temperature, 40° ± 0·1°. pH = isoelectric point.

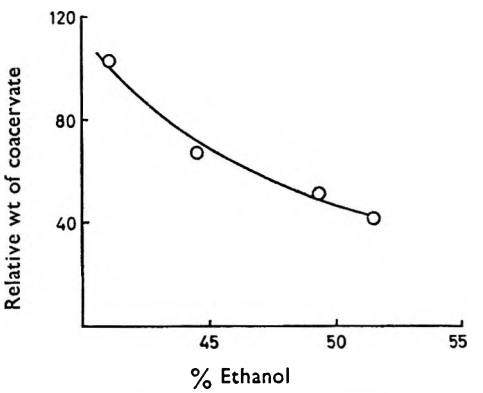

FIG. 4. Variation of the relative weight of the coacervate as a function of total ethanol concentration in the system. Ethanol gradually added to a system containing 15% w/w gelatin: 30% ethanol; 55% w/w water (line XA in Fig. 3).

function of ethanol concentration is shown in Fig. 4. Neither the decrease in relative weight nor volume was directly proportional to the ethanol concentration; an increase in the viscosity of the coacervate was also noticed. As the bulk of the gelatin was contained in this phase and the ethanol content remained constant, the changes must be due to the gradual dehydration of the coacervate as the overall percentage of ethanol in the system was increased.

Analyses of the two phases in equilibrium indicated that the coacervate contained almost a constant percentage of coacervating agent irrespective of the gelatin used or of the total ethanol concentration (Table 4). The

ethanol range was found to be between 36·3 and 38·0% w/w. This was similar to results (36·4–39·9%) obtained by Holleman, Bungenburg de Jong & Modderman (1934) using a less precise method of assay and an alkali processed gelatin of unknown molecular weight. In systems using sodium sulphate as coacervating agent a similar condition can be deduced from results obtained by Holleman & others (1934) and by Phares & Sperandio (1964), but the figures differ depending on the type of gelatin used. With alkali processed gelatin Holleman & others found an average value of 6·7% whilst with acid pretreated gelatin, Phares & Sperandio obtained a value of 9·5%.

With the data available at present it is difficult to be precise about the liquid nature of the coacervate phase or to speculate how the solvent-precipitant liquid is held by the gelatin. The modified theory of coacervation (Bungenberg de Jong, 1949; Basu & Bhattacharya, 1952) suggest that the inclusion liquid is immobilised within the spiral of the flexible colloid molecule. If this is so then the higher molecular weight gelatins, because of their greater flexibility, would be easier to coacervate than the less flexible shorter chain length material, as is shown in Fig. 1. The modified theory of coacervation suggests that the ethanol-water mixture held within the loops of the gelatin molecule can be divided into two parts: an occlusion liquid of the same composition as the equilibrium liquid and an excess of water which is present as water of hydration. Although this type of calculation is obviously imprecise, the amount of water of hydration g per g of gelatin was found to be: 0·2 (*M* 27,000), 0·4 (*M* 49,000), 0·3(*M* 70,000). This is similar to the value of 0·3 reported by Holleman & others (1934).

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The "nicotinic" and "muscarinic" receptors of the urinary bladder of the guinea-pig

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The action of acetylcholine and nicotine on the urinary bladder of the guinea-pig has been examined using three techniques of physiological denervation. Nicotine was neurogenic and equiactive concentrations of acetylcholine were myogenic. Whilst acetylcholine has both "muscarinic" and "nicotinic" effects on the guinea-pig bladder, the concentration required to stimulate the nicotinic receptors was approximately 100 times that required for the muscarinic receptors. These results indicate that the nicotinic receptors are confined to nervous tissue, and do not support the suggestion that there might be non-neuronal nicotinic receptors in the bladder musculature of the guinea-pig. The possibility of a non-cholinergic component in the post-ganglionic parasympathetic fibres to the bladder should still be considered.

THE isolated urinary bladder of the guinea-pig contracts to transmural electrical stimulation and to suitable concentrations of nicotine, acetylcholine or muscarine. The response to acetylcholine or muscarine can be completely abolished by atropine, but that to nicotine or transmural stimulation is resistant to muscarinic blockade (Chesher & Thorp, 1965). This phenomenon of atropine resistance to parasympathetic nerve stimulation in the urinary bladder has been observed in a number of species; it has been reported in the dog, the cat and the rabbit (Langley & Anderson, 1895; Henderson & Roepke, 1935; Edge, 1955; Ursillo & Clark, 1956; Ursillo, 1961); in the possum and the toad (Burnstock & Campbell, 1963; Burnstock, O'Shea & Wood, 1963); and in the rat (Carpenter, 1963; Hukovič, Rand & Vanov, 1965). These studies have provided evidence that a cholinergic component is involved in the response to nervous stimulation. The possibility of the involvement of a non-cholinergic, atropine-resistant component has also been suggested (Henderson & Roepke, 1934; Singh, 1964; Chesher & Thorp, 1965).

Gyermek (1961) examined the cholinergic blockade of cholinomimetic drugs on the bladder *in situ* of the dog and the cat, and showed the response to acetylcholine to have a significant ganglionic component. He suggested that the parasympathetic effector sites of the bladder may differ functionally from those of other organs. He postulated the presence of 'nicotinic' receptors in the bladder musculature and on these grounds considered that the assumption of a non-cholinergic component of the parasympathetic nerves of the bladder was improbable. However, if these atropine-resistant receptors are confined to nervous tissue, the possibility of a non-cholinergic component in the post-ganglionic fibres still exists.

The present work was undertaken to see if the response of the guinea-pig bladder *in vitro* shows a significant ganglionic component in its response to acetylcholine, and to examine the evidence for the presence in this species of non-neuronal "nicotinic" receptors in the bladder musculature.

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Experimental

Guinea-pigs of either sex were killed by a blow on the head and bled. Both ureters were tied and cut and a glass cannula was inserted into the bladder via a cut in the urethra and tied into place. Locke solution was introduced into the cannula to distend the bladder which, in turn, was bathed in Locke solution, in an organ bath of 25 ml capacity, aerated with oxygen containing 5% carbon dioxide. The Locke solution was maintained at 30° unless otherwise stated, and was of the following composition: NaCl, 9·0; KCl, 0·42; $CaCl_2$, 0·24; $NaHCO_3$, 0·3; glucose, 1·0; g/litre. The method of recording was as previously described (Chesher & Thorp, 1965). Under these conditions the intraluminal pressure of the resting bladder was approximately 60 to 80 mm water.

Electrodes for electrical stimulation were placed in the Locke solution, one in the solution in the organ bath, the other in the solution within the lumen of the bladder. Square wave pulses of supramaximal amplitude (20 V) and 2 msec duration were delivered at a frequency of 20/sec.

Dose-response curves for acetylcholine and nicotine were made before and after physiological denervation by the techniques described below. The regression coefficient for each dose-response curve was calculated by the method of Burn, Finney & Goodwin (1950), and the results expressed in terms of the dose ratio (Gaddum, Hameed, Hathaway & Stephens, 1955). The dose ratio is the ratio of equiactive concentrations of the stimulant drug before and after the physiological denervation. For this calculation the concentration of stimulant drug required to produce a response of 40 mm water above the base line was chosen, this being approximately 50% of the maximal contraction under the conditions of these experiments.

"PHYSIOLOGICAL DENERVATION" TECHNIQUES

(*a*) Denervation by storage in the cold (Vogt, 1943; Ambache, 1946; Emmelin & Feldberg, 1947). The preparation was stored in Locke solution for 2 to 5 days at a temperature of 4–5°. After this time it was set up as previously described, at 30°.

(*b*) Denervation by cooling (Innes, Kosterlitz & Robinson, 1957; Gillespie & Wishart, 1957; Beleslin & Varagić, 1958). The temperature of the Locke solution bathing the bladder was reduced to 15°.

(*c*) Denervation by anoxia (Gross & Clark, 1923; Garry, 1928; Prasad, 1935; West, Hadden & Farah, 1951). The gas mixture bubbling through the solution in the organ bath was changed to one containing nitrogen, 95% and carbon dioxide, 5%.

After treatment by each of these techniques an equilibration period of 2 hr was allowed before any drugs were applied to the bladder. *Drugs used* were acetylcholine chloride, nicotine hydrogen tartrate, hexamethonium bromide and atropine sulphate. Doses are expressed as the salt.

Results

DENERVATION BY STORAGE IN THE COLD

In all experiments, storage of the bladder at 4–5° for 2 to 5 days completely abolished the response to electrical stimulation. The denervated bladder responded to acetylcholine though in most cases the dose-response curve was moved to the right. However, in one experiment (CS2) the response to acetylcholine was unaffected by the denervation and in another (CS4) the dose ratio was only 2·5. The response to nicotine, on the other hand, was abolished in all instances.

There were also qualitative differences in the response of the denervated bladder to acetylcholine. The time for the beginning of the contraction after the acetylcholine had been added to the bath was delayed, and both the speed of the response and the time for recovery after the drug was washed from bath were slower than before denervation.

The results expressed as dose ratios are given in Table 1, and the dose-response curves for experiment CS2 are shown in Fig. 1.

TABLE 1. THE RATIO OF EQUIACTIVE DOSES OF ACETYLCHOLINE AND NICOTINE AFTER/BEFORE PHYSIOLOGICAL DENERVATION BY STORAGE IN THE COLD

Experiment No.	Dose ratios		Storage time (days)
	Acetylcholine	Nicotine	
CS1	27·0	no response	5·0
CS2	1·0	,, ,,	1·5
CS3	17·4	,, ,,	4·5
CS4	2·5	,, ,,	3·5
CS5	39·0	,, ,,	5·0
CS6	28·2	,, ,,	3·5

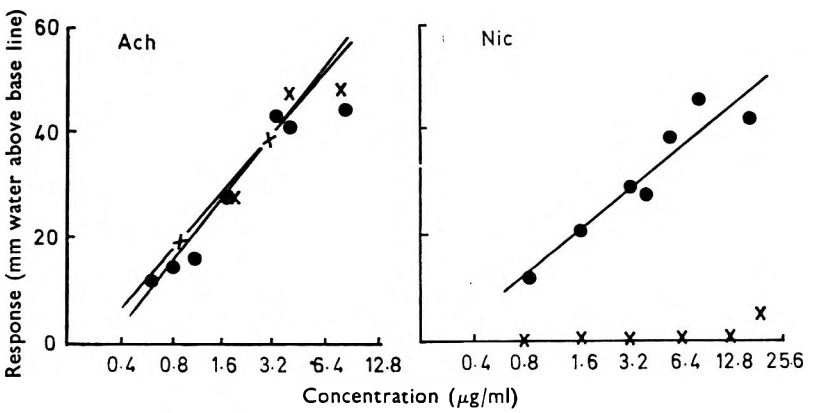

FIG. 1. Guinea-pig bladder. Log dose-response relationship to acetylcholine and nicotine, before (●——●) and after (×——×) physiological denervation by storage in the cold.

DENERVATION BY COOLING

Lowering the temperature of the bath fluid produced an increase in the tone of the bladder which often took more than 2 hr to return to the

baseline. Under the conditions of this experiment it was not possible to completely abolish the response of the bladder to transmural stimulation by cooling to 15°, though in all instances the response was reduced to less than 10% of that at 30°. The response to acetylcholine, though much slower, was little affected by cooling, whilst that to nicotine was significantly reduced, and in two instances was virtually abolished (See Fig. 2 and Table 2, exp. C2 and 4).

The dose ratios are given in Table 2 and the dose-response curves for Experiment C2 are shown in Fig. 2.

TABLE 2. THE RATIO OF EQUIACTIVE DOSES OF ACETYLCHOLINE AND NICOTINE AFTER/BEFORE PHYSIOLOGICAL DENERVATION BY COOLING AND ANOXIA

Experiment No.	Dose ratios	
	Acetylcholine	Nicotine
Cooling:		
C1	1·4	4·8
C2	1·7	*
C3	2·7	4·0
C4	2·1	*
C5	0·7	1·8
Anoxia:		
A1	1·4	9·8
A2	1·0	2·3
A3	0·7	*
A4	2·5	*
A5	1·0	*
A6	0·5	2·8
A7	32·5	*

* Only a small response (less than 20 mm H_2O above base line) could be obtained, and this did not show a dose-response relationship.

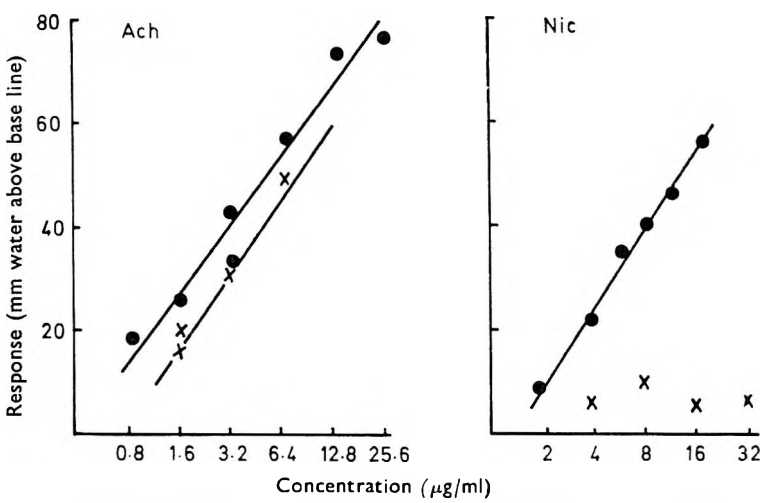

FIG. 2. Guinea-pig bladder. Log dose-response relationship to acetylcholine and nicotine, before (●——●) and after (×——×) physiological denervation by cooling to 15°.

DENERVATION BY ANOXIA

After exposure to the nitrogen-carbon dioxide for 2 hr, the response of the bladder to transmural stimulation was abolished in four experiments and reduced to less than 10% of the pre-treatment response in three (experiments A1, 2 and 6).

The response to acetylcholine was very little affected quantitatively except in experiment A7, though qualitatively the contraction was slower to begin and took longer to reach its peak.

In experiments A3, 4, 5 and 7, where denervation was complete, the response to nicotine was abolished. The preparations which gave a small response to transmural stimulation (experiments A1, 2 and 6) also responded to nicotine, though the dose-response curve was displaced to the right.

The results expressed as dose ratios and the dose-response curves for experiment A4, are shown in Table 2 and Fig. 3, respectively.

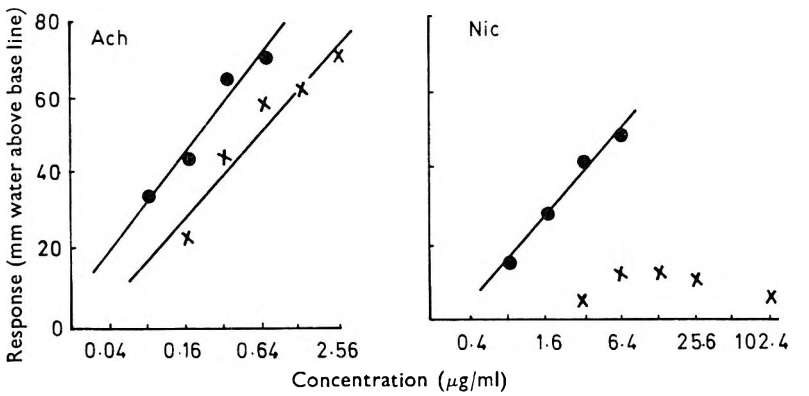

FIG. 3. Guinea-pig bladder. Log dose-response relationship to acetylcholine and nicotine, before (●——●) and after (×——×) physiological denervation by anoxia.

THE EFFECT OF ATROPINE

The atropine-resistant response of the guinea-pig bladder to nicotine permitted a clear demonstration of the "nicotinic" effect of acetylcholine (Chesher & Thorp, 1965). The "muscarinic" effect of acetylcholine was effectively abolished by atropine, though in the presence of this block an increase in the concentration of acetylcholine again produced a response. This contraction was not affected by further additions of atropine, though it was abolished by hexamethonium.

To determine the ratio of "nicotinic": "muscarinic" activity of acetylcholine on the preparation, the following experiment was made.

Equiactive concentrations of acetylcholine and nicotine were selected and, after the addition of atropine (0·16 μg/ml), the concentration of acetylcholine needed to produce a response similar to that produced by nicotine was determined.

In three experiments the acetylcholine concentration had to be increased one hundred times to produce a response which matched that induced by

nicotine. These responses to nicotine, and to acetylcholine in the presence of atropine, were abolished by hexamethonium (40 μg/ml).

Discussion

The denervation techniques we have employed are considered to produce a progressive inactivation of tissue, with the smooth muscle being the most resistant, the post-ganglionic fibre less so and the pre-ganglionic fibre being the most sensitive (Vogt, 1943; Gillespie & Wishart, 1957).

In all the experiments, acetylcholine showed the most resistance to the treatment given, and in a number of instances the response was unchanged or potentiated. In those experiments where the treatment reduced the response to acetylcholine, two possibilities must be considered.

It could be an indication of a nervous component in the response, or it could be due to an effect on the smooth muscle itself. In view of the small dose ratio we found for acetylcholine, which in some experiments was one or less, we consider that the reduced response to acetylcholine was due to an effect of the treatment on the muscle. These results indicate therefore that the response of the isolated bladder of the guinea-pig to acetylcholine is the result of a direct effect on the muscle and does not include a significant ganglionic component. Indeed, much higher concentrations of acetylcholine were needed before an indirect effect was obtained as was shown by the high ratio of "nicotinic" to "muscarinic" effects. In the presence of an atropine block of the muscarinic receptors, the concentration of acetylcholine had to be increased one hundred times before it elicited an equiactive "nicotinic" response. We would have expected this dose ratio to be much lower if acetylcholine, in the concentrations used in the absence of atropine, stimulated ganglion cells.

Further evidence for a purely muscarinic action of acetylcholine at these concentrations has been provided by the observation of Chesher & Thorp (1965) that concentrations of hexamethonium which abolished the response to nicotine had no effect on equiactive concentrations of acetylcholine.

The response to nicotine was sensitive to denervation. In all preparations where the response to transmural stimulation had been abolished, that to nicotine had also been blocked. In those experiments where denervation was not complete, and a small response to transmural stimulation remained, the dose-response curve to nicotine was displaced to the right.

In two experiments no response to nicotine could be elicited even though a small response to transmural stimulation remained. This effect could be explained on the basis of the progressive nature of the denervation. In these instances, the ganglion cells had been rendered insensitive to stimulation by nicotine whilst the post-ganglionic fibres were still responsive, to some degree, to transmural stimulation.

These results indicated that the response of the bladder to nicotine was indirect and was due to the stimulation of the autonomic ganglion cells. Ganglion cells do occur in the bladder musculature of some species

(Gruber, 1933) and we have observed their presence in the guinea-pig bladder, when stained with methylene blue. Apart from the receptors of the intramural ganglion cells, there was no evidence for the presence of nicotinic receptors in the bladder musculature of the guinea-pig. Should such receptors exist, and assuming them to be resistant to the effects of denervation (as were the muscarinic receptors), one would expect that a response to nicotine could still be elicited after denervation. As we found that this was not so, we conclude that the nicotinic receptors in the guinea-pig bladder are confined to the ganglion cells.

The possibility, therefore, that the atropine-resistant response to stimulation of the postganglionic parasympathetic fibres of the bladder might be due to the involvement of a non-cholinergic transmitter, must still be considered.

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Spectroscopic examination of the solubilisation of benzoic acid by a non-ionic surfactant

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Ultraviolet and nuclear magnetic resonance spectroscopy have been used to examine the location of benzoic acid within the cetomacrogol micelle. Evidence has been obtained that the solubilised benzoic acid is probably located at the junction of the hydrocarbon nucleus and oxyethylene palisade layer, supporting the hypothesis advanced earlier on the basis of potentiometric observation.

SOLUBILISATION of organic acids by non-ionic surfactants has been examined in detail by the present authors using solubility and potentiometric methods (Donbrow & Rhodes, 1963a, b, 1964, 1965; Rhodes & Donbrow, 1965). The uptake of benzoic acid by the surfactant observes the Langmuir isotherm, which implies that the acid is located at a surface or pseudo-surface.

Since the micelles of non-ionic surfactants consist of a hydrocarbon nucleus surrounded by a palisade layer of hydrated polyethylene oxide chains, solubilised material could occur in three different regions: (1) within the hydrocarbon nucleus, (2) at the junction of the hydrocarbon nucleus and the palisade layer, (3) within the palisade layer.

Riegelman, Allawala, Hrenoff & Strait (1958) have suggested that ultraviolet absorption spectroscopy may be used to determine the location of solubilised material, as the ultraviolet spectra of many compounds are sensitive to changes in environment. Because micelles are characterised by possession of regions of different polarity, an estimate of the location of solubilised material within micelles can be made from ultraviolet spectra. The aqueous solubilities of the compounds examined by Riegelman and his colleagues were in most instances low. They were therefore able to assume that the ultraviolet spectra they measured in surfactant solutions were entirely due to micellar material. For more polar materials, such as benzoic acid, this assumption cannot be made. In the present paper, possible methods for overcoming this difficulty are proposed and the micellar ultraviolet spectrum of benzoic acid is presented.

The presence of solubilised material within the micelle would also be expected to modify the electronic environment of the protons associated with or influenced by the solubilisation process. Changes in the electron "screening" of protons may be observed by measuring the chemical shift of the protons by means of nuclear magnetic resonance spectroscopy (nmr).

Such changes in chemical shift should be most pronounced for the protons of the surfactant immediately adjacent to the solubilisate, and

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could be used to determine the location of solubilised material within the micelle; nmr data are presented and utilised here for this purpose.

Experimental

Materials. Benzoic acid* A.R.; cetomacrogol B.P.C.†, the mean molecular formula of which was shown by nmr to be $C_{16}H_{33}[C_2H_4O]_{24}OH$; spectroscopically pure cyclohexane and diethylether.

Ultraviolet spectroscopy. The ultraviolet spectrum of benzoic acid, between 250 and 300 mμ, was examined in the following solvents: 0·005N hydrochloric acid, cyclohexane, ether, 1, 5, 10 and 20% solutions of cetomacrogol and 10% solutions of polyethylene glycols 1500 and 3000. All spectra were obtained by use of a Unicam SP800 Recording Spectrophotometer (1 cm cell) and were rechecked using a Hilger Ultrascan (5 mm cell). In all instances the solutions contained about 0·0015M benzoic acid, which gave an absorbance of the order of 1·5 (1 cm cell). Aqueous solutions were acidified with hydrochloric acid. The reference cell contained the appropriate solvent or surfactant solution. Fig. 1 shows the spectra obtained. The spectral characteristics at the maxima are listed in Table 1.

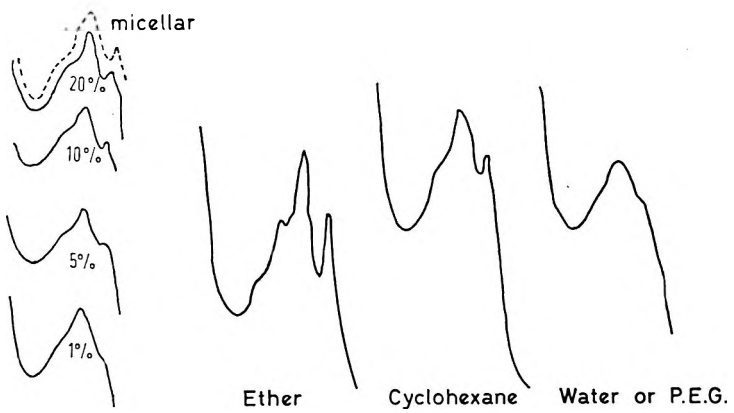

FIG. 1. Ultraviolet spectrum of benzoic acid (0·0015M) in 1, 5, 10 and 20% cetomacrogol solutions, and spectrum of benzoic acid in ether, cyclohexane and water or polyethylene glycol acidified with hydrochloric acid. Scales linear in absorbance (ordinate) and wavelength. (For λ_{max} and ϵ_{max} values see Table 1).

Nuclear magnetic resonance. The nmr spectrum of cetomacrogol, alone and in the presence of benzoic acid, was recorded in D_2O. Tetramethyl silane, in a capillary tube, was used as an external reference. A Varian Associates HR–60 high resolution spectrometer was employed. The changes in chemical shift of the signals of the alkyl protons and polyethylene oxide protons of the surfactant resulting from the presence of benzoic acid: Δ shift in cps, CH_2, 7; OCH_2, 3. Mean molecular

* Supplied by B.D.H. Ltd., Poole, England.
† Supplied by Evans Medical Co. Ltd., Bradford, England.

TABLE 1. ULTRAVIOLET ABSORPTION CHARACTERISTICS OF BENZOIC ACID (0·0015 M APPROX.) IN VARIOUS MEDIA (HILGER ULTRASCAN, 0·5 CM CELL)

Solvent	λ_{max} mμ	ε_{max}	λ_{max} mμ	ε_{max}	λ_{max} mμ	ε_{max}	λ_{min} mμ
Cyclohexane	275	1130	283	980	266–70s	—	258
Ether*	272	895	279	780	265 258s	730	252
Water†	273	990	280s	—	—	—	259
Cetomacrogol 20%†	273	890	280	730	265s	720	257
P.E.G. 1500 10%†	273	1010	280s	—	—	—	259
P.E.G. 3000 10%†	273	1020	280s	—	—	—	259

* Identical results are obtained in ether saturated with water at pH 2.
† Containing HCl to give pH 1·9 to 2·4.
s = shoulder.
P.E.G. = Polyethylene glycol.

formula of surfactant from integrated signals (Donbrow, Molyneux & Rhodes, 1966) $CH_3(CH_2)_{14}CH_2(OCH_2CH_2)_{24}OH$. All changes in chemical shift were upfield.

Discussion

Jaffe & Orchin (1962) state that the amount of vibrational fine structure observed in the ultraviolet spectrum of a compound in a given solvent is related to the degree of interaction between solvent and solute. In general, the degree of interaction increases with increasing polarity of solvent. From these considerations it would be expected that the amount of fine structure observed in the three solvents water, ether and cyclohexane would increase in the order stated. However, from Fig. 1 it can be seen that more fine structure was observed in ether than cyclohexane. This effect may be attributed to dimerisation of benzoic acid in cyclohexane, the intermolecular reaction repressing fine structure. Spectral evidence of dimerisation of benzoic acid in cyclohexane has been obtained by Forbes & Knight (1959) and the spectra in ether were attributed to the formation of a weak hydrogen-bonded ether-benzoic acid complex. The fine structure in ether is similar to that observed earlier by Ungnade & Lamb (1952) in dioxane and also in very dilute solutions of benzoic acid in cyclohexane, the latter showing a progressive loss of fine structure with increase in concentration as the proportion of dimer increased.

In surfactant solutions of benzoic acid the following equilibrium exists:

$$HA_m \rightleftharpoons HA_w$$

where HA_m is the benzoic acid bound by the micelles and HA_w is the free acid in the water. Fig. 1 shows that the amount of fine structure measured in cetomacrogol solution at constant benzoic acid concentration increases with increasing concentration of surfactant. This is to be expected since increase in the concentration of surfactant will increase the amount of acid bound by the micelles, HA_m, and reduce the amount of unbound material, HA_w. The spectrum observed in any surfactant solution will depend upon the relative amounts of HA_m and HA_w. For the purpose of location of a substance within the micelle we were interested

in the spectrum of HA_m. It is known from previous studies of the benzoic acid-cetomacrogol system that in 20% cetomacrogol solution HA_m is about ten times as large as HA_w (Donbrow & Rhodes, 1964). Thus the spectrum obtained in the 20% surfactant solution could be corrected to obtain the micellar spectrum. Alternatively, when information about the values of HA_m and HA_w was not available, extrapolation from the spectra measured in various strength surfactant solutions to a limiting value representing 100% surfactant enabled the spectrum of HA_m to be obtained.

Though there was evidence of light scatter at lower wavelengths, the peaks shown in Fig. 1 were presumably not appreciably distorted by scatter, since they were reproduced at different absorption cell to photocell distances, in different instruments and at different path lengths.

From Fig. 1 it can be seen that the spectrum of micellar benzoic acid shows much less fine structure than that observed in ether; furthermore the minimum is displaced by 5 mμ (Table 1). The ether spectrum is not affected by saturation with water. It is therefore improbable that the benzoic acid forms an ether-like solution in the palisade layer. Again, the spectrum of benzoic acid in 10% aqueous polyethylene glycol 1500 or 3000 solutions (which have an oxygen content similar to that of 20% cetomacrogol) closely resembles its spectrum in water and does not resemble the micellar spectrum. Hence it is unlikely that there is a similar mode of binding of the benzoic acid to the ethylene oxide groups in the surfactant and the polymer. Since the spectra in ether and polyethylene glycol are entirely different, it is clear that the benzoic acid does not form an ether-like solution in the polyethylene glycol; however, this result could be reconciled with a low affinity of benzoic acid for the polyethylene glycol, particularly as the spectrum in polyethylene glycol resembles that in water (see also Donbrow & others, 1966). In any event the spectral evidence does not support the view that organic acids are wholly located in the palisade layer of the micelles of nonionic surfactants.

The micellar spectrum of benzoic acid is similar to its spectrum in cyclohexane. It is likely that the loss of fine structure accompanying dimer formation is caused by inhibition of vibrational motion (see Jaffe & Orchin, 1962), and that the vibration of the benzoic acid molecules in their solubilised state is also restricted. However the maxima in cyclohexane are displaced bathochromically 2–3 mμ and the absorption is enhanced by about 30% (Table 1). This probably indicates that the micellar benzoic acid is not wholly dissolved in the hydrocarbon core of the micelle but is, at least partially, in a medium of higher dielectric constant.

Further evidence, of value in locating the solubilised benzoic acid, is obtained from the nmr results. If the solubilised benzoic acid were located within, or at the exterior of, the palisade layer it would be expected that the changes in chemical shift of the surfactant protons would be restricted to those of the palisade layer. Similarly if the benzoic acid were incorporated wholly within the hydrocarbon nucleus it would be expected

that the change in chemical shift would be limited to the alkyl protons. From the data it can be seen that though both types of proton show Δ shifts, the Δ shift value is much larger for the alkyl protons (7 cps) than for the polyethylene oxide protons (3 cps). Since the samples were treated in a routine manner, the 3 cps shift is of uncertain significance but the 7 cps is probably significant.

We have previously shown potentiometrically that the uptake of benzoic acid by the micelles does not accord with the possibility of dimerisation occurring in the micelle (Donbrow & Rhodes, 1964), although the method is intrinsically capable of detecting dimerisation and indeed such dimerisation does occur in cetomacrogol dispersions of benzene containing liquid crystal phase (Rhodes & Donbrow, 1965). It is therefore concluded that the most likely location of the solubilised benzoic acid is at the junction of the hydrocarbon nucleus and palisade layer of the micelle, with the lipophilic benzene ring enclosed within the nucleus and the hydrophilic carboxylic acid group protruding into the palisade layer. Benzoic acid so located would lack mobility because of the presence of the polyethylene oxide chains of the surfactant molecules. This location could also allow formation of a hydrogen bond between the acidic hydrogen atom and the innermost ether oxygen atom. It is suggested that these effects may be the cause of the comparative lack of fine structure in the ultraviolet spectrum of micellar benzoic acid.

The uptake of benzoic acid on an interior surface of the micelle, accompanied by a reduction of mobility, fits in with an adsorption model for the solubilisation of this substance. It would therefore account for the observance of the Langmuir isotherm by this and similar systems, which we reported earlier.

Acknowledgements. Thanks are due to Miss Tami Bino, School of Pharmacy, Hebrew University, Jerusalem, for checking the ultraviolet spectra on an Ultrascan instrument, and to the University of London School of Pharmacy, whose instrument was used for the nmr spectra.

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The flow of granular magnesia

T. M. JONES AND N. PILPEL

Investigations of the flow rates of loosely packed magnesia have shown that the general equation developed by Jones & Pilpel (1966)

$$D_o = A\left(\frac{4W}{60\pi\rho_p\sqrt{g}}\right)^{\frac{1}{n}}$$

can be applied to single component and multicomponent mixtures in the size range 0·003 to >0·2 cm. The increase in flow rate caused by mixing coarse and fine particles has been related quantitatively to the size of the particles by the general expression

$$\log \max = -[f(D_{Pc}.D_{Pf})]\, D_o + f(D_{Pc}.D_{Pf})$$

This can be used to calculate the composition for maximum flow in multicomponent mixtures. The mechanism of action of glidants is discussed in the light of the experimental results and a distinction is made between glidants which improve the flow of granulations and those which improve the flow of cohesive powders.

ALTHOUGH there have been many reports on the gravity flow of non-cohesive granular solids, little information is available about the gravity flow of particulate systems in which interparticular forces are operating.

Publications concerned with the improvement of flow of granulations by the addition of fine material (Hammerness & Thompson, 1958; Tucker & Hays, 1959; Fairs, 1960; Vegan, 1960; Gunsel & Lachman, 1963; Krishna & Rao, 1963; Bulsara, Zenz & Eckert, 1964), the addition of glidants to the powders (Munzel & Kagi, 1954; Craik & Miller, 1958) or admixture of coarse particles with powders (Davis, 1943; Hawkesley, 1947; Shotton & Simons, 1950; Nakajima, 1961) to improve their flow characteristics, have been mainly qualitative or comparative. The additives have often had very different chemical and physical properties to the main components; this has made it difficult to elucidate the basic mechanisms of their action.

We have examined the flow characteristics of single component and of multicomponent mixtures of granulated magnesia through circular orifices in order to extend the applicability of a previously developed flow equation (Jones & Pilpel, 1966) to the wider size range 0·003 to 0·3 cm, thus taking into account the effects of interparticular forces which occur when the particles are less than about 0·01 cm in diameter.

By investigating the effects of added fine material on the flow behaviour of coarser granules, it was hoped to gain a clearer understanding of the mechanisms of glidant action. We also hoped to be able to predict how much fine material should be added to a multicomponent mixture of coarser magnesia to increase its flow rate to a maximum.

Experimental

The experimental work consisted of measuring the flow rates of 13 single and 189 multicomponent mixtures of granulated magnesia through

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6 circular orifices from a vertical hopper with a horizontal base. The procedure already reported in detail by Jones & Pilpel (1966) was followed using the same batch of magnesia, particular care being taken to ensure that before the flow started the bed was at its loosest state of packing. Although flow rate is independent of initial voidage for non-cohesive materials, preliminary investigations indicated that the state of packing of the fine fractions had a pronounced effect on flow rate until the bed was fully dilated.

Angles of repose were determined after consolidating the samples. The apparatus used (Fig. 1) consisted of a box constructed with three

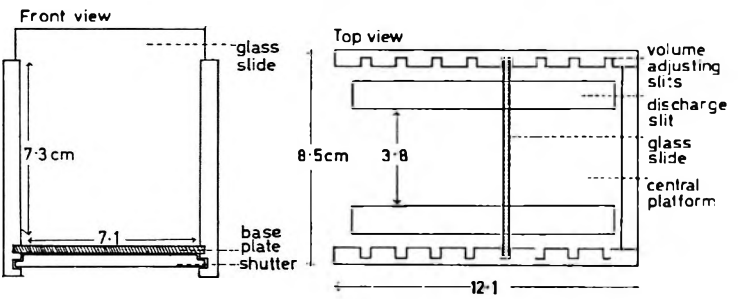

FIG. 1. Apparatus for measurement of consolidated angle of repose.

sides of wood and a laminated plastic base into which two parallel slits had been cut leaving a central platform 3·8 cm wide. The fourth side of the box was a glass slide, the position of which could be adjusted, thereby enabling the volume of the box to be varied.

The technique adopted was to insert a sliding shutter into the base of the box and then pour in the sample from a sheet of demy paper held 2 cm above the box. The bed was then consolidated by dropping the box 50 times from a height of 3 cm on to a flat surface, turning through 90° after each 10 drops. After careful removal of the shutter, the height of the wedge formed was measured and the consolidated angle of repose, θ, calculated from the expression $\tan \theta = 2h/L$ where h is the height of wedge and L, the width of platform.

Results

Fig. 2 shows the effect on the flow rates of coarse sieve fractions ($D_{P_c} = 0{\cdot}0561$, $0{\cdot}0253$ cm) when varying concentrations of fine fractions ($D_{P_f} = 0{\cdot}0090$, $0{\cdot}0071$ and $0{\cdot}0048$ cm) are added.

It is seen that the flow rate reaches a maximum at a particular concentration of fine material. The position of the maximum is dependent upon the size of both the fine and the coarse component.

Fig. 3 shows that the positions of these maxima vary with the diameter of the orifice, the concentration of fine material necessary to produce the maxima decreasing with increase in orifice diameter.

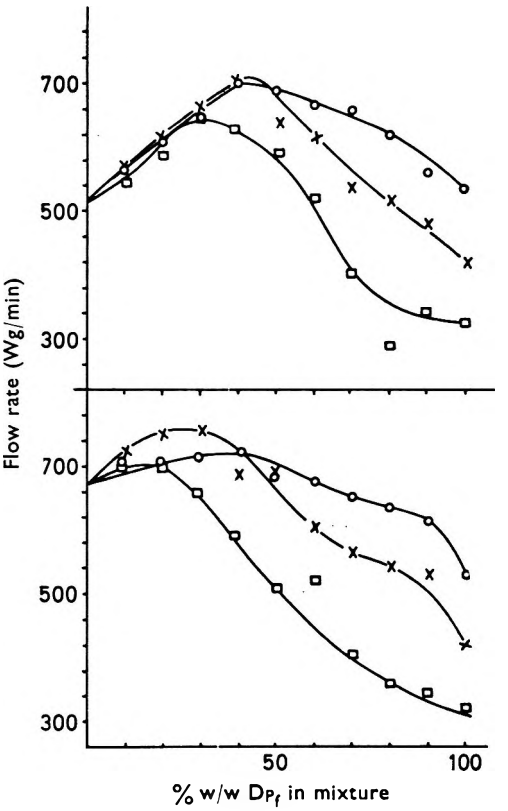

FIG. 2. The effect of particle size on flow rate of binary mixtures of coarse and fine components through a circular orifice D_0 = 0·898 cm. D_{Pc} in top graph = 0·0561 cm and in bottom graph = 0·0253 cm. D_{Pf} = 0·0048 cm (□), 0·0071 cm (×) and 0·0090 cm (○).

Figs 4 (a–d) are representative ternary diagrams showing the variation in flow rate that occurs on the addition of a 3rd (coarse) component to an existing binary mixture of coarse and fine material.

Here too the positions of the maxima depend upon the size of the fine component—D_{Pf} Fig. 4 (a and c), the coarse component Fig. 4 (a and d), the orifice diameter Fig. 4 (a and b) and on the percentage of 3rd (coarse) component present Fig. 4 (a–d).

Discussion

GENERAL FLOW EQUATION

To extend the applicability of the previously developed equation (Jones & Pilpel, 1966),

$$D_0 = A\left(\frac{4W}{60\pi\rho_P\sqrt{g}}\right)^{\frac{1}{n}} \quad .. \quad .. \quad .. \quad (1)$$

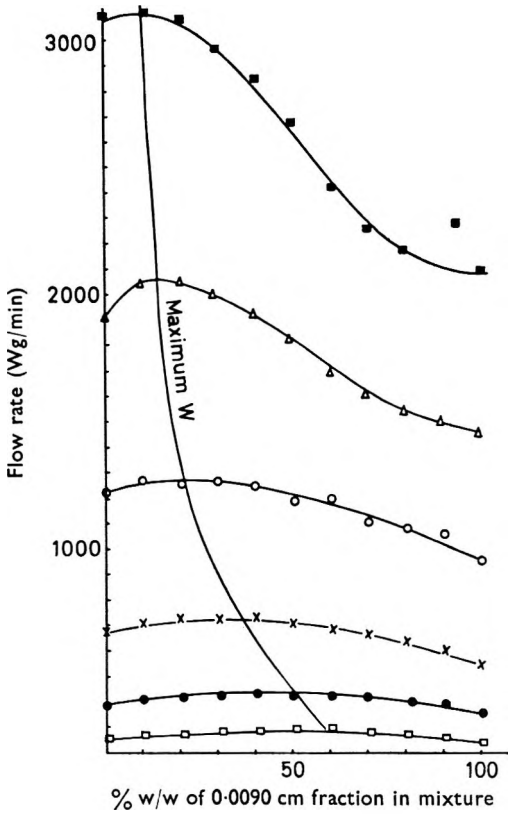

FIG. 3. Effect of orifice diameter on the flow rate of the binary mixture $D_{Pc} = 0{\cdot}0253$ cm, $D_{Pf} = 0{\cdot}0090$ cm. D_o in cm: ■ = 1·686, △ = 1·353, ○ = 1·140, × = 0·398 ● = 0·740, □ = 0·603.

where A and 1/n are functions of particle size, for predicting the flow rate of magnesia over the whole size range 0·003 to 0·3 cm, it is necessary to distinguish between four regions according to the size of the particles concerned.

In region I (particles >0·02 cm) magnesia is non-cohesive and free flowing due to the interparticular forces being ≪ gravitational forces; the equation

$$D_o = (1{\cdot}6822\, D_{Pav} + 1{\cdot}9779)\left(\frac{4W}{60\pi\rho_p\sqrt{g}}\right)^{0{\cdot}2571 - 0{\cdot}0855 \log D_{Pav}} \text{ applies.} \quad (2)$$

Region II ($0{\cdot}02 > D_p > 0{\cdot}01$ cm) represents a transition between region I and region III. Here the flow is beginning to be affected by interparticular forces of friction and cohesion. This can be seen for example by comparing the angle of repose of different sieve cuts of magnesia after consolidation, which causes the particles to pack closely, thereby accentuating the effect of interparticular forces. It is seen from Table 1 that changes in the angle of repose are most apparent in region II.

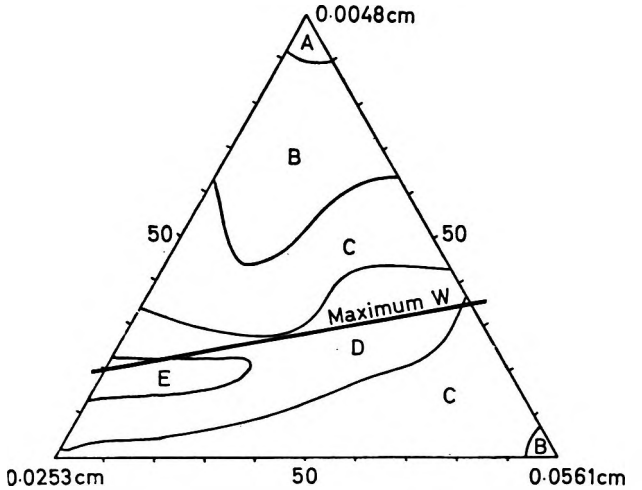

FIG. 4a. Flow contours for ternary system D_{Pc_1} = 0·0561 cm, D_{Pc_2} = 0·0253 cm, D_{Pf} = 0·0048 cm. D_o = 0·74 cm. Flow rate (W g/min): A = <200, B = 200–300, C = 300–400, D = 400–420, E = >420.

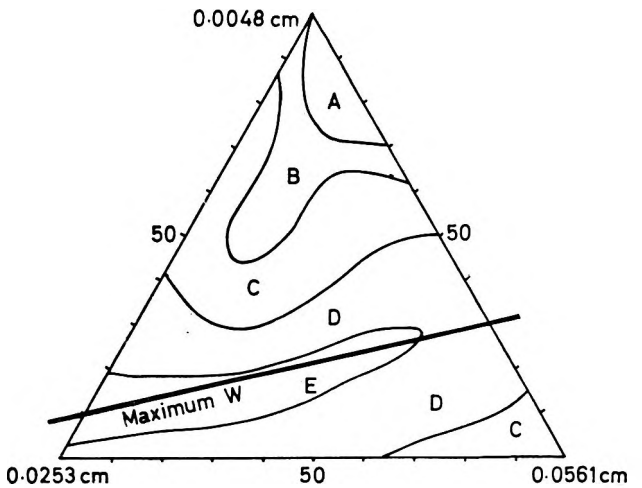

FIG. 4b. Flow contours for ternary system D_{Pc_1} = 0·0561 cm, D_{Pc_2} = 0·0253 cm, D_{Pf} = 0·0048 cm. D_o = 0·898 cm. Flow rate (W g/min): A = <400, B = 400–500, C = 500–600, D = 600–700, E = >700.

Region III covers particles from 0·003 to 0·01 cm. Here the forces between the particles are $\geqslant$ gravitational forces, the flow is still free. Finally in region IV where the particles are <0·003 cm, the predominant interparticular forces are of the van der Waals' type and are $\gg$ gravitational forces; the powder becomes "cohesive". Flow in this region can only be investigated by employing special techniques (Dawes, 1952; Jenike, 1961; Lowes & Perry, 1965) and we do not propose to discuss it further here.

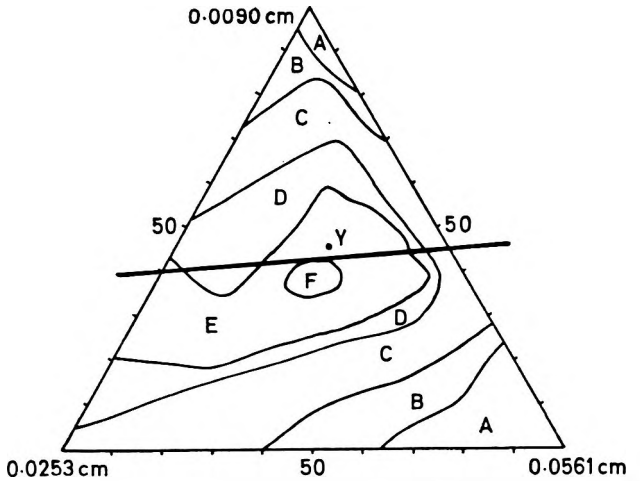

FIG. 4c. Flow contours for ternary system D_{Pc_1} = 0·0561 cm, D_{Pc_2} = 0·0253 cm, D_{Pf} = 0·0090 cm. D_o = 0·898 cm. Flow rate (W g/min): A = <600, B = 600–650, C = 650–700, D = 700–720, E = 720–730, F = >730.

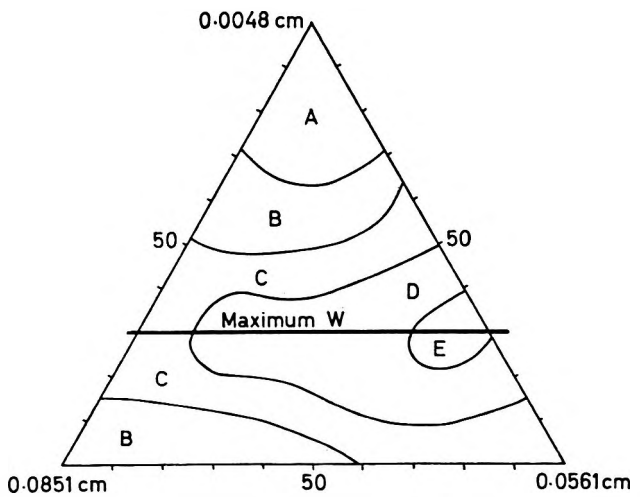

FIG. 4d. Flow contours for ternary system D_{Pc_1} = 0·0561 cm, D_{Pc_2} = 0·0851 cm, D_{Pf} = 0·0048 cm. D_o = 0·898 cm. Flow rate (W g/min): A = <400, B = 400–500, C = 500–600, D = 600–640, E = >640.

We can employ a similar treatment to that used in the previous paper (Jones & Pilpel, 1966) for evaluating the quantities A and 1/n for each region in terms of D_P or D_{Pav} (the geometric mean diameter) where mixtures of particle sizes are being considered.

As A is essentially a measure of the forces between the particles, it varies in different ways with D_P from region to region. It is not therefore considered that a single expression relating A to D_P would be justified over the whole range of particle sizes.

TABLE 1. THE VARIATION OF ANGLE OF REPOSE

Particle size cm	Region	Consolidated angle of repose
0·2435	I	43½
0·1866		43½
0·1340		43½
0·0851		40
0·0561		40
0·0358		40
0·0253	II	37½
0·0158		46½–62
0·0090		65½–68½
0·0071	III	90
0·0059		90
0·0048		90
0·0038		90

The relationships between A and D_P for regions II and III [Region I has already been dealt with (Jones & Pilpel, 1966)] are obtained by plotting A versus log D_P, giving straight lines which on regressional analyses yield

$$\text{Region II} \quad A_2 = -0{\cdot}8469 \log D_P + 0{\cdot}6182 \qquad (3)$$

$$\text{Region III} \quad A_3 = -4{\cdot}606 \log D_P - 6{\cdot}9213 \qquad (4)$$

We now consider the other variable, 1/n. Log W is plotted against log D_o to give a series of straight lines obeying the well established expression

$$W \propto D_o^n \qquad (5)$$

The slopes of these lines (n) obtained by regression analysis are then plotted versus log D_P yielding

$$n = 0{\cdot}6927 \log D_P + 3{\cdot}6325 \qquad (6)$$

Here a single relationship over the whole range of particle sizes is justified since the exponent term in equation (5) is an orifice function and thus not directly related to the forces between the particles. It should be noted that equation (6) yields values of 1/n which differ slightly from those obtained using the previously reported equation but is more accurate over the wider range of particle sizes now being considered.

The validity of the equation

$$D_o = A\left(\frac{4W}{60\pi\rho_P\sqrt{g}}\right)^{\frac{1}{n}}$$

where $A_1 = 1{\cdot}6822\ D_P + 1{\cdot}9779$ when $D_P > 0{\cdot}02$ cm; $A_2 = -0{\cdot}8469 \log D_P + 0{\cdot}6182$ when $0{\cdot}02 > D_P > 0{\cdot}01$ cm; $A_3 = -4{\cdot}606 \log D_P - 6{\cdot}9313$ when $0{\cdot}01 > D_P > 0{\cdot}003$ cm and $n = 0{\cdot}6927 \log D_P + 3{\cdot}6325$ for predicting the flow rates of mono systems and of binary and ternary mixtures of magnesia through different sized orifices has been tested in Tables 2, 3 and 4. The values of W_{calc} compare with the determined values (W_{obs}) to within the following accuracy: monos, mean 3%; binaries, mean 9%; ternaries, mean 8%. This is considered satisfactory.

TABLE 2. RELATIONSHIP OF W_{calc} TO W_{obs} FOR MONO-DISPERSED MATERIAL D_P <0·01 cm

D_P cm	D_o cm	W_{calc} g/min	W_{obs} g/min	Error %
0·0090	0·898	524	530–543	−1·1
	1·14	889	940–958	−5·4
0·0048	0·898	279	294–345	−5·1
	1·14	453	479–498	−5·4
0·0038	0·898	246	226–239	+2·5
	1·353	519	511–519	0

MAXIMUM FLOW

(a) *Binary systems.* Inspection of Fig. 2 shows that for binary mixtures of coarse, denoted D_{P_c}, and fine, denoted D_{P_f}, components, the flow rate attains a maximum at a definite concentration of the fine component.

The percentage of the fine component (denoted max) required to produce the maximum in the flow rate, increases with the size of the fine component and also depends upon the size of the coarse component. We have

$$\text{max} = f(D_{P_c})(D_{P_f}) \quad .. \quad .. \quad .. \quad (7)$$

where D_{P_c} = the diameter of the coarse component and D_{P_f} = the diameter of the fine component. Now it has been shown previously (Jones & Pilpel, 1966) that in the general expression relating the flow rate of magnesia to the orifice diameter, i.e. equation (5), the exponent n is a function of the geometric mean diameter of the particles. It follows that the quantity designated max should depend on the diameter of the orifice through which the powder is flowing. Fig. 2, which is typical of a number of binary systems, shows this to be the case.

Plots of log max against D_o for binary systems yield straight line graphs showing that

$$\log \text{max} = -mD_o + c \quad .. \quad .. \quad .. \quad (8)$$

where the negative slopes −m and the intercepts c vary with D_{P_c} and D_{P_f}. Combining equations (7) and (8) leads to the prediction that

$$\log \text{max} = -[f(D_{P_c})(D_{P_f})]\, D_o + f(D_{P_c})(D_{P_f}) \quad .. \quad (9)$$

The data obtained in the present investigation have been used to express equation (9) explicitly the first step being to express −m and c as functions of D_{P_f} for fixed values of D_{P_c} and then to evaluate the constants of these functions in terms of D_{P_c}. We find that

$$\log \text{max} = [X - (1061\, D_{P_c} - 118)\, D_{P_f}]\, D_o + (204 - 1506\, D_{P_c})\, D_{P_f} + Y \quad (10)$$

where $X = -3{\cdot}4854\, D_{P_c} + 0{\cdot}1887$

$Y = 14{\cdot}697\, D_{P_c} + 0{\cdot}2364$

Although this equation is empirical, it enables one to estimate the percentage of fine material that is required to produce maximum flow rate

TABLE 3. RELATIONSHIP OF Wcalc TO Wobs FOR BINARY MIXTURES OF D_{Pc} to D_{Pf}

Mixture							
D_{Pc} cm	Conc % w/w	D_{Pf} cm	Conc % w/w	D_o cm	W_{calc} g/mm	W_{obs} g/mm	Error %
0·0851	80	0·0090	20	0·740 0·898 1·353	296 506 1567	300–330 551–576 1874–1871	− 1 − 8 −18
	50		50	0·740 0·898 1·353	387 635 1809	398–402 653–690 1746–1798	− 2 − 3 + 1
	20		80	0·740 0·898 1·353	375 589 1516	383–407 611–639 1488–1560	− 2 − 8 0
0·0851	80	0·0071	20	0·740 0·898 1·353	304 517 1588	308–315 537–546 1763–1896	− 1 − 4 −10
	50		50	0·740 0·898 1·353	405 659 1850	374–378 612–618 1776–1896	+ 7 0 0
	20		80	0·740 0·898 1·353	373 581 1475	377–392 596–604 1440–1460	− 1 − 3 + 1
0·0851	80	0·0048	20	0·740 0·898 1·353	314 530 1618	318–326 576–585 1863–1908	− 1 − 8 −11
	50		50	0·740 0·898 1·353	268 432 1183	311–328 488–525 1188–1310	−13 −11 0
	20		80	0·740 0·898 1·353	293 446 1090	201–216 294–321 661–672	+35 +39 +62
0·0561	80	0·0090	20	0·740 0·898 1·353	342 571 1697	332–343 588–595 1852–1905	0 − 3 − 8
	50		50	0·740 0·898 1·353	417 676 1880	396–410 681–699 1830–1908	+ 2 − 1 0
	20		80	0·740 0·898 1·353	375 588 1526	383–401 609–628 1504–1553	− 2 − 3 0
0·0561	80	0·0071	20	0·740 0·898 1·353	348 580 1713	352–357 620–633 1866–1920	0 − 6 − 8
	50		50	0·740 0·898 1·353	435 699 1913	398–412 635–652 1572–1638	+ 5 + 7 +17
	20		80	0·740 0·898 1·353	373 579 1467	330–346 492–539 1188–1249	+ 8 + 7·4 +17
0·0561	80	0·0048	20	0·740 0·898 1·353	367 606 1759	334–352 615–621 1800–1960	+ 4 − 1 − 5
	50		50	0·740 0·898 1·353	380 605 1615	339–359 575–611 1176–1193	+ 6 0 +35
	20		80	0·740 0·989 1·353	288 439 1072	174–201 275–294 618	+43 +49 +73

TABLE 3—*continued*

Mixture							
D_{Pc} cm	Conc % w/w	D_{Pf} cm	Conc % w/w	D_o cm	W_{calc} g/mm	W_{obs} g/mm	Error %
	80		20	0·740 0·898 1·353	431 695 2920	415–419 705–719 2011–2055	+ 3 − 1 +42
0·0253	50	0·0090	50	0·740 0·898 1·353	378 599 1584	418–430 687–703 1776–1884	−10 −13 −11
	20		80	0·740 0·898 1·353	373 581 1475	390–394 630–641 1518–1578	− 4 − 8 − 3
	80		20	0·740 0·898 1·353	438 703 1919	421–440 747–753 2016–2100	0 − 6 − 5
0·0253	50	0·0071	50	0·740 0·898 1·353	376 591 1538	424–426 690–697 1557–1680	−11 −14 − 1
	20		80	0·740 0·898 1·353	352 541 1344	336–340 531–556 1260–1368	− 3·5 0 0
	80		20	0·740 0·898 1·353	449 718 1942	424–427 693–702 1968–1982	− 5 − 2 − 1
0·0253	50	0·0048	50	0·740 0·898 1·353	374 581 1475	324–340 492–522 1050–1110	−10 −11 −32
	20		80	0·740 0·898 1·353	242 365 872	228–232 345–366 663–738	− 4 0 −18

TABLE 4. RELATIONSHIP OF W_{calc} TO W_{obs} FOR TERNARY SYSTEMS OF D_{Pc_1}–D_{Pc_2}–D_{Pf}

Mixture (% w/w)						
Size						
D_{Pc_1} 0·0561 cm	D_{Pc_2} 0·0253 cm	D_{Pf} 0·009 cm	D_o cm	W_{calc} g/min	W_{obs} g/min	Error %
10	20	70	0·74 0·898	374 586	418–422 678–684	−10 −14
30	30	40	0·898 1·353	630 1736	732–735 1878–1932	−14 − 8
60	20	20	0·74 1·353	363 1750	372–387 1932–2016	− 2 − 9
Size						
D_{Pc_1} 0·0561 cm	D_{Pc_2} 0·0253 cm	D_{Pf} 0·0048 cm				
50	40	10	0·74 1·353	368 1763	376–378 1968–2040	− 2 −10
10	10	80	0·898 0·603	394 168	356–387 170–177	+ 2 − 1
Size						
D_{Pc_1} 0·0851 cm	D_{Pc_2} 0·0253 cm	D_{Pf} 0·0071 cm				
70	10	20	0·898 1·14	539 1028	605–629 1158–1224	−10 −11

in binary systems with a grand mean error of 6% w/w. Allowing for the limitations of the experimental technique, this represents an error of $\pm 10\%$ w/w in the composition of a mixture, which is considered to be satisfactory (Table 5).

TABLE 5. COMPARISON OF MAX_{calc} AND MAX_{obs} FOR BINARY MIXTURES

D_{Pc} cm	D_o cm	D_{Pf} 0·0048 cm			D_{Pf} 0·0071 cm			D_{Pf} 0·0090 cm		
		Max_{obs} % w/w	Max_{calc} % w/w	Error % w/w	Max_{obs} % w/w	Max_{calc} % w/w	Error % w/w	Max_{obs} % w/w	Max_{calc} % w/w	Error % w/w
0·0253	0·603	20	15·9	− 5	35	28·7	− 5	60	46·7	−10
	0·74	20	14·3	− 5	30	24·2	− 5	45	37·2	− 5
	0·898	10	12·8	0	25	19·8	− 5	40	28·6	−10
	1·14	10	10·5	0	10	14·6	+ 5	20	19·2	0
	1·353	10	9·1	0	15	11·2	0	15	13·5	0
	1·686	10	6·5	0	10	7·4	0	10	7·8	0
0·0561	0·603	40	28·9	−10	60	45	−10	65	65·1	0
	0·74	35	26·4	−10	40	39·5	0	55	55·1	0
	0·898	30	23·8	− 5	40	33·9	− 5	45	45·4	0
	1·14	26	20·3	− 5	45	26·9	−15	45	33	−10
	1·353	22	17·6	0	30	21·8	−10	30	26·1	− 5
	1·686	20	14·2	− 5	20	15·9	0	20	17·4	0
0·0851	0·603	40	50·2	−10	75	69·5	− 5	80	89·8	+10
	0·74	35	47·1	−10	65	62·8	0	65	80·4	+15
	0·898	30	43	−10	40	56·4	+15	50	70·5	+20
	1·14	30	37·7	−10	40	47·4	+ 5	40	57·7	+20
	1·353	25	38·4	−15	40	41	0	40	48·3	+10
	1·686	20	27·8	−10	30	32·3	0	30	36·6	+ 5

(b) *Ternary systems.* Equation (10) can now be applied for predicting the percentage of fine material producing maximum flow rate in a ternary system containing two coarse, $D_{P_{c_1}}$, $D_{P_{c_2}}$, and one fine component, D_{Pf}, through any particular sized orifice.

We first construct a co-ordinate system as shown in Fig. 5. The ordinate is log max for one of the binary mixtures (say $D_{P_{c_1}}$, D_{Pf}) present

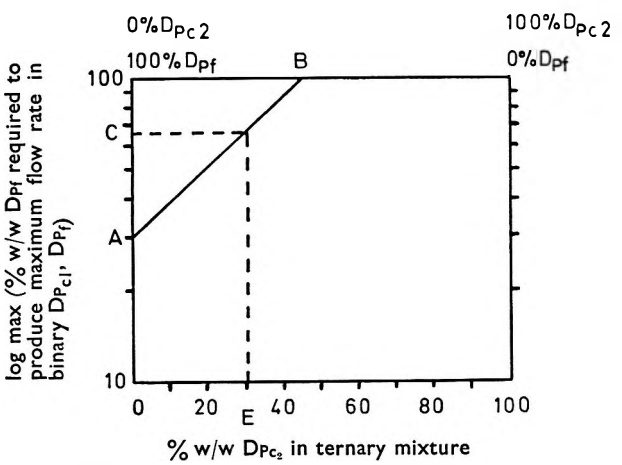

FIG. 5. Method for estimating composition for maximum flow rate in a ternary system. D_{Pc_1} = 0·0253 cm. D_{Pc_2} = 0·0561 cm. D_{Pf} = 0·009 cm.

in the ternary system and ranges from 1·0 representing 10% of the fine component to 2·0 representing 100% of the fine component. The abscissa expressed as % w/w of the second coarse component, $D_{P_{c_1}}$, present in the ternary system ranges from 0 to 100%. Any line emanating from the ordinate shows the variation in the amount of fine component required to produce a maximum in flow rate for the binary D_{c_2} D_f in the presence of gradually increasing amounts of the third component $D_{P_{c_2}}$.

Furthermore, a line drawn parallel to the abscissa through the ordinate 2·0 ($\equiv$100% D_{P_f}) and ranging from 0% to 100% is the max of the second binary ($D_{P_{c_2}}$, D_{P_f}).

We use equation (10) to calculate max values of the two binary systems namely $D_{P_{c_1}}$, D_{P_f} and $D_{P_{c_2}}$, D_{P_f} and plot these points on the respective axes as A and B in Fig. 5.

From a straight line drawn through the points we obtain the max value for the binary $D_{P_{c_1}}$, D_{P_f}, namely the point C, on the ordinate for a particular concentration, say E% w/w of $D_{P_{c_2}}$ in the ternary system. Since the binary $D_{P_{c_1}}$, D_{P_f} represents (100 — E)% w/w of the ternary mixture, the actual concentrations (% w/w) of $D_{P_{c_1}}$ and D_{P_f} in the ternary system can be calculated.

We illustrate the procedure by an actual example. Consider the experimental data given in Fig. 4(c) which show the flow rates of ternary mixtures of 0·0090 cm (D_{P_f}), 0·0253 cm ($D_{P_{c_1}}$) and 0·0561 cm ($D_{P_{c_2}}$) particles through an orifice D_o of 0·898 cm. The problem is to calculate the amount of D_{P_f} (% w/w) which leads to maximum flow when the ternary system contains 30% w/w of the 0·0561 cm ($D_{P_{c_2}}$) material.

We have from equation (10) that the flow rates of binary mixtures of 0·009 cm with 0·0253 cm particles and of 0·009 cm with 0·0561 cm particles are maximal when they contain respectively 30% w/w and 45% w/w of the 0·0090 cm material. In Fig 5, A = 30% w/w and is plotted on the ordinate. B = 45% w/w and is plotted on the line parallel to the abscissa at log max = 2·0. For E = 30% w/w we obtain the point C = 66% w/w D_{P_f} and the % D = (100–66) = 34% w/w. Now since $D_{P_{c_2}}$ = 30% w/w, ($D_{P_{c_1}}$ + D_{P_f}) = 70% w/w of the ternary system, and for maximum flow 66% w/w of the 70% w/w should be D_{P_f}. Therefore total D_{P_f} content = 46·2% w/w. Hence the required composition of the ternary system is % (w/w) $D_{P_{c_2}}$, 30; D_{P_f}, 46·2; $D_{P_{c_1}}$, 23·8. This is the point marked Y on Fig. 4(c), which agrees with the experimentally determined composition having maximum flow rate.

The assumption that has been made in the above treatment is that a straight line can always be drawn between the points A and B in Fig. 5. Justification for this assumption is afforded by an examination of Fig. 4 (a–d). These show the effect of composition on the flow rates of

several ternary systems of 2 coarse and 1 fine component. It is seen that lines connecting the positions of the max in the binary systems (represented by two of the sides of the triangles) pass approximately through the portions of the diagrams where the flow rate is maximum. Thus the assumption seems justified.

THE MECHANISM OF FLOW RATE IMPROVEMENT—GLIDANTS

There have been two different definitions used in the past for the term glidant.

Strickland (1959), has used the term for a fine material which is added to a chemically different cohesive powder to increase its flowability and also for the fine fraction which is commonly added to a coarse granulation of the *same* material for increasing its flow rate (see also Martin, Barker & Chun, 1963).

The mechanism of action in the two instances appears to differ. In the first, the glidant is thought to separate individual powder particles and hence reduce the van der Waals' type cohesive forces which act between them (Munzel & Kagi, 1954; Craik & Miller, 1958; Strickland, 1959). In the second, the improvement is thought to be due to the glidant adhering to the surfaces of the coarser granules reducing their surface rugosity and hence their coefficient of interparticular friction (Crosby, 1960; Martin & others, 1963). Glidants can therefore be divided into (1) those reducing interparticular cohesive forces in powders; (2) those reducing surface rugosity and the coefficient of interparticular friction. Clearly in the present work dealing with the effects of fines on the flow properties of granular magnesia, we have been concerned with the second category.

The findings indicate that, although in practice, category (1) type glidants are often used, this may not always be necessary.

As the size of the fine category (2) glidant particle is increased, its ability to coat the coarser material is diminished and this reduces its efficiency. This can be seen by examining Fig. 2. The smaller the value of D_P the greater the value of max for any particular coarse second component. As the concentration of fine category (2) glidant material is increased, a point is eventually reached when its particles begin to interact with each other. This leads to a reduction in flow rate as can be seen from Figs 2 and 3.

Thus, for every real powder system there should be an optimum combination of (category 2) glidant size and concentration which leads to a maximum in the flow rate. Conversely there will be an optimum concentration of coarse material for improving the flow of a fine powder (a method that was reported by Davis, 1943, and Shotton & Simons, 1950).

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Effect of desipramine on directly or indirectly elicited catecholamine pressor responses in rats*

A. BONACCORSI AND S. GARATTINI

Desipramine enhances the pressor effect induced by noradrenaline, adrenaline, dopamine and dimethylphenylpiperazinium in pithed rats, while indirectly acting sympathomimetic amines, such as tyramine and phenethylamine were inhibited. With a similar degree of noradrenaline potentiation, desipramine was more effective than cocaine as an inhibitor of the tyramine pressor response. Desipramine, but not cocaine, was effective in blocking the hypertension induced by small doses of reserpine in animals pretreated with tranylcypromine.

SEVERAL authors (Sigg, 1959; Sigg, Soffer & Gyermek, 1963; Loew, 1964; Kaumann, Basso & Aramandia, 1965, and others) have observed in cats, dogs and rabbits an increased sensitivity of peripheral adrenergic responses after a treatment with antidepressant drugs such as imipramine and desipramine. On the basis of these observations it was proposed that a potentiation of central adrenergic effects may explain the antidepressant activity of these compounds (Sigg, 1959; Sulser, Bickel & Brodie, 1964). A biochemical basis for this hypothesis was established by experiments which showed an inhibition of the uptake of catecholamines after treatment with imipramine-like agents (Hertting, Axelrod & Whitby, 1961; Thoenen, Huerlimann & Haefely, 1964; Iversen, 1965a,b).

Since previous pharmacological and biochemical studies with antidepressant drugs have been made in these laboratories with rats (Garattini, Giachetti, Jori, Pieri & Valzelli, 1962; Garattini & Valzelli, 1962; Jori & Garattini, 1965; Jori, Paglialunga & Garattini, 1965) it was decided to use rats in an investigation of the influence of desipramine on blood pressure responses to sympathomimetics or to drugs known to act through releasing catecholamines.

Reserpine induces hypertension in animals pretreated with monoamine oxidase inhibitors (Chessin, Kramer & Scott, 1957; Garattini, Fresia, Mortari & Palma, 1960). This hypertension has been interpreted as the result of the peripherally released catecholamines which are not rapidly metabolised at the site of release because of the inhibition of the monoamine oxidase. Since Cuenca, Salvá & Veldecasas (1964) observed an inhibition exerted by desipramine on the initial pressor action of guanethidine and bretylium, whose effect is also thought to be mediated by catecholamines (Gillis & Nash, 1961), it was of interest to investigate the influence of desipramine on this effect of reserpine.

Materials and methods

Male Sprague–Dawley rats, 250–300 g, were anaesthetised with ether. Both carotid arteries were ligated after cannulation of the trachea.

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Animals were then pithed and maintained by artificial respiration. Blood pressure was recorded from a cannulated carotid artery by means of a pressure transducer (Statham P 23A) and displayed on an ink-writing Grass Polygraph. Injections of 0·1 or 0·2 ml were given into the cannulated right femoral vein.

Other experiments were made to observe the pressor effect of reserpine in animals pretreated with monoamine oxidase inhibitors. Sprague–Dawley rats, 300 g, were anaesthetised with urethane, 1·25 g/kg i.p., and the left carotid artery was cannulated for recording blood pressure.

Tranylcypromine, 5 or 10 mg/kg, was injected i.p. either 60 min or 5 hr before the intravenous injection of reserpine. Other monoamine oxidase inhibitors used were pheniprazine, 10 mg/kg, and iproniazid, 100 mg/kg, given 18 hr before the experiment.

Results

EFFECT OF DESIPRAMINE AND COCAINE ON THE BLOOD PRESSURE RESPONSES INDUCED BY INJECTED AMINES OR DIMETHYLPHENYLPIPERAZINIUM

Groups of five or more pithed rats received noradrenaline or adrenaline at doses of 0·05 and 0·1 μg/rat until consistent responses were obtained. Desipramine, 3 mg/kg, was then injected intravenously and the pressor responses elicited by the catecholamines were again determined 30 min after the drug. The responses to the two amines were significantly increased. The pressor response to dopamine, 20–40 μg, was also enhanced by desipramine while the response to dopa, 2 mg, was unaffected. These results are summarised in Table 1.

TABLE 1. EFFECT OF DESIPRAMINE ON PRESSOR RESPONSE ELICITED BY ADRENALINE HYDROCHLORIDE, NORADRENALINE BITARTRATE, DOPAMINE, DOPA PHENETHYLAMINE OR DIMETHYLPHENYLPIPERAZINIUM IN PITHED RATS. (TIME BETWEEN DESIPRAMINE AND PRESSOR RESPONSES WAS 30 MIN). DOSES ARE EXPRESSED AS SALTS

No. of experiments	Pressor agent	Dose µg/rat i.v.	Pressor response in mm Hg ± s.e.		Statistical significance (P)
			Saline	Desipramine 3 mg/kg i.v.	
5	Noradrenaline	0·05	39 ± 4	72 ± 8	=0·01
5	"	0·10	55 ± 7	101 ± 13	=0·01
4	Adrenaline	0·05	20 ± 3	38 ± 3	=0·01
4	"	0·10	44 ± 8	76 ± 10	=0·05
5	Dopamine	10	34 ± 3	53 ± 2	=0·01
5	"	20	50 ± 4	70 ± 8	=0·01
5	Dopa	2,000	55 ± 4	58 ± 6	>0·05
10	DMPP	20	33 ± 10	65 ± 13	=0·05
10	DMPP	40	46 ± 6	105 ± 10	<0·01
4	Phenethylamine	50	25 ± 3	4 ± 4	<0·05

The effect of desipramine on noradrenaline was less consistent when animals were not pithed but anaesthetised with urethane.

Dose-response curves to noradrenaline were shifted to the left after treatment with desipramine or cocaine (Fig. 1). On the other hand the pressor effect of tyramine was antagonised (Fig. 2). The dose-response

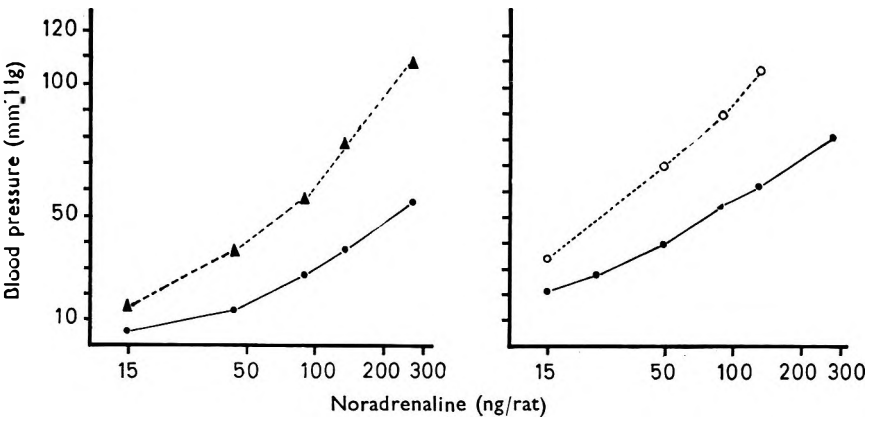

FIG. 1. Potentiation of blood pressure response to noradrenaline by previous treatment with desipramine (▲- - - - -▲), 3 mg/kg, or cocaine (○- - - - -○), 5 mg/kg, in pithed rats. Control ●—●. The curves obtained in a single rat are representative of a typical response.

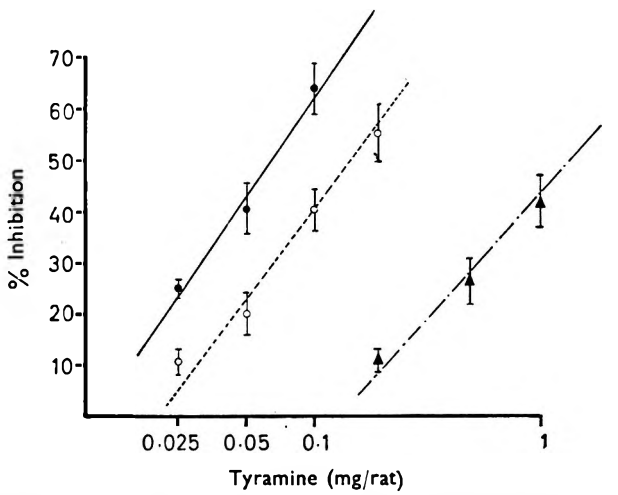

FIG. 2. Inhibition of blood pressure response to tyramine by previous treatment with desipramine (▲- · -▲), 3 mg/kg, or cocaine (○- - - - -○), 5 mg/kg, in pithed rats. Control ●—●. Vertical bars represent the standard error of the mean.

curves are parallel and desipramine shifts the curve more to the right than cocaine. The pressor response to phenethylamine, 50 μg, was also antagonised by desipramine (see Table 1).

In nine experiments desipramine enhanced the blood pressure response induced by 20–40 μg of the ganglion stimulant 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) (see Table 1). This is in agreement with the data obtained by Osborne & Sigg (1960) who worked with imipramine in anaesthetised dogs.

EFFECT OF COCAINE AND DESIPRAMINE ON THE BLOOD PRESSOR RESPONSE INDUCED BY RESERPINE IN RATS TREATED WITH A MONOAMINE OXIDASE INHIBITOR

In intact animals pretreated with tranylcypromine, 5 mg/kg i.p., 60 min before the experiment, reserpine, 250 μg/kg, induced a sustained increase of the systolic blood pressure which was inhibited by desipramine (see Fig. 3) but unaffected by cocaine. Similar results were obtained using pithed rats.

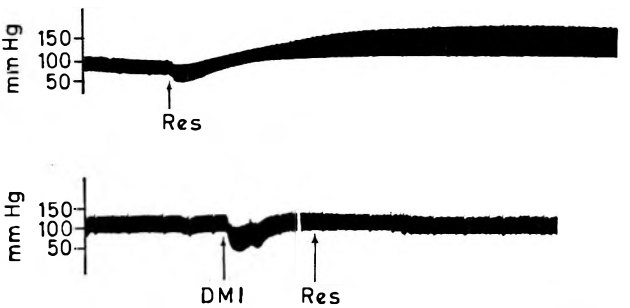

FIG. 3. Effect of desipramine (DMI), 3 mg/kg i.v., on the hypertensive response elicited by reserpine (Res), 250 μg/kg i.v., in rats pretreated 60 min before the experiment with tranylcypromine, 5 mg/kg i.p. Immediately after tranylcypromine administration, animals were anaesthetised with urethane.

When 5 hr elapsed after the tranylcypromine pretreatment, the pressor action of reserpine was smaller and occurred only with a larger dose, 2·5 mg/kg. This response was unaffected by desipramine. Similar results were obtained when pheniprazine and iproniazid were given 18 hr before reserpine (see Table 2).

TABLE 2. EFFECT OF DESIPRAMINE ON THE PRESSOR RESPONSE INDUCED BY RESERPINE IN RATS TREATED WITH MONOAMINE OXIDASE INHIBITOR. (TIME BETWEEN DESIPRAMINE OR COCAINE AND RESERPINE WAS RESPECTIVELY 30 AND 10 MIN)

No. of animals	Pretreatment i.p. mg/kg	Time after MAO inhibitor	Compound mg/kg i.v.	Reserpine mg/kg i.v.	Pressor responses in mm Hg ± s.e.	P
9	Tranylcypromine 5	60 min	Saline	0·25	79 ± 9	<0·01
9	" 5		Desipramine 3	0·25	11 ± 4	
6	Tranylcypromine 5	60 min	Saline	0·25	68 ± 11	>0·05
5	"		Cocaine 3	0·25	50 ± 13	
11	Tranylcypromine 10	5 hr	Saline	2·5	45 ± 7	>0·05
6	"		Desipramine 3	2·5	28 ± 5	
8	Pheniprazine 10	18 hr	Saline	2·5	26 ± 2	>0·05
7	"		Desipramine 3	2·5	27 ± 4	
6	Iproniazid 100	18 hr	Saline	2·5	39 ± 14	>0·05
6	"		Desipramine 3	2·5	20 ± 7	

The difference in response to reserpine between the 60 min tranylcypromine-treated rats and the 5 hr tranylcypromine-treated, 18 hr pheniprazine-treated or 18 hr iproniazid-treated rats may be accounted for by the activity of tranylcypromine itself inhibiting noradrenaline uptake (Iversen, 1965a). This activity may have dispersed 5 hr after administering the drug. Iproniazid does not inhibit noradrenaline uptake (Iversen, 1965a).

Discussion

The ability of desipramine to potentiate noradrenaline and adrenaline and to antagonise the pressor effect of certain indirectly acting sympathomimetic amines such as tyramine and phenethylamine in pithed rats has been demonstrated in the present work.

As previously mentioned desipramine prevents the uptake of catecholamines (Hertting & others, 1961; Iversen, 1965a,b), particularly at low concentrations (Iversen, 1965c) presumably by inhibiting the active transport mechanism. The potentiation of noradrenaline and adrenaline may therefore be explained at least in part by the inhibition of uptake, an important mechanism for terminating the action of catecholamines (Koelle, 1959; Dengler, Spiegel & Titus, 1961; Rosell, Kopin & Axelrod, 1963).

Dimethylphenylpiperazinium is known to act at sympathetic ganglia to induce a release of catecholamines at the nerve ending. The potentiation of the dimethylphenylpiperazinium pressor response by desipramine may therefore also be related to an inhibition of catecholamine uptake. Furthermore it has been demonstrated that desipramine enhances the effect of electrical stimulation of the peripheral sympathetic system (Haefely, Huerlimann & Thoenen, 1964).

The effect of indirectly acting sympathomimetic amines is generally attributed to a release of noradrenaline from tissue stores (Carlsson, Rosengren, Bertler & Nilsson, 1957; Burn & Rand, 1958). The inhibition by desipramine or cocaine of responses to indirectly acting sympathomimetics may therefore be related to a decreased access of these amines to the noradrenaline storage sites. Imipramine congeners inhibit catecholamine depletion induced by tyramine (Kaumann & Basso, 1965) and desipramine prevents the uptake of tyramine (Matsumoto, Costa & Brodie, 1964).

From these data it is evident that desipramine shows a pattern of effects comparable with that shown by cocaine. Data from the literature, confirmed by this experimental work, demonstrate that cocaine potentiates the action of noradrenaline, adrenaline and dimethylphenylpiperazinium but inhibits the effect of tyramine and phenethylamine. In these experiments desipramine can be distinguished from cocaine only by its inhibition of the pressor effect of reserpine elicited 60 min after administration of a monoamine oxidase inhibitor.

Both desipramine and cocaine only slightly affect the hypertension induced by reserpine in rats when reserpine was given several hours after the monoamine oxidase inhibitor.

Further work is required to elucidate the reason for the hypertension induced by reserpine being greater—60 min after, compared with 5 hr after—than treatment with a monoamine oxidase inhibitor.

In conclusion the data presented show that in rats the interaction of desipramine and catecholamines or drugs acting through a catecholamine release is compatible with the hypothesis that this antidepressant drug inhibits amine uptake.

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Effect of solubilisation on the antibacterial activity of hexachlorophane

R. A. ANDERSON AND K. J. MORGAN

The solubilisation of hexachlorophane by various non-ionic agents has been studied at pH 2·5 and 8·0. The interaction of the phenol with these agents in unsaturated systems at pH 8·0 has been determined by a dialysis technique using rubber latex membranes. The bactericidal activity is shown to depend on the concentration of unbound hexachlorophane, but this is not simply related to the degree of saturation of the total system. The results of agar-plate diffusion tests cannot be correlated with either the concentration of unbound hexachlorophane or the total concentration.

MANY phenolic antiseptics have a low water solubility and because of this are often used together with surface-active agents which greatly increase the amount of the phenol in solution. Hexachlorophane is a sparingly-soluble bis-phenol, the mono-sodium salt of which is also only slightly soluble in water. At the near neutral pH of many pharmaceutical systems, it exists as the singly charged anion.

The inactivation of hexachlorophane by surface-active agents has been reported many times and a non-ionic surfactant, polysorbate 80, has been used to neutralise this and other phenols in microbiological media (Lawrence & Erlandson, 1953). Agar plate diffusion techniques have given results suggesting that surface-active agents enhance the antibacterial activity of hexachlorophane (Gregg & Zopf, 1951).

The present paper gives results showing the degree of interaction of hexachlorophane with representative non-ionic agents, and relates some of these results to the effect of the interaction on microbiological activity.

Experimental and results

MATERIALS

Hexachlorophane was recrystallised twice from benzene (m.p. 164–165°). The solubilising agents shown in Table 1 were used as supplied without further purification. Water was distilled from a Scorah all-glass still.

TABLE 1. SOLUBILISING AGENTS USED

Name used	Composition and other names	Manufacturer of sample used
Lauromacrogol	Lauryl polyoxyethylene ether; Brij 35	Atlas Powder Co.
Macrogol	Hard Macrogol, B.P.C.; Polyethylene glycol 4000; Carbowax 4000	Union Carbide
Polyethylene polypropylene glycol	Oxyethylene-oxypropylene polymer; Pluronic F 68	Wyandotte Chemicals Corp.
Polysorbate 20	Polyoxyethylene sorbitan monolaurate; Tween 20	Atlas Powder Co.
Sucrose laurate	Sucrose monolaurate	Colonial Sugars Co. ("purified", Lot 27, sample 2037)

From the Pharmacy Department, University of Sydney, Australia.

SOLUBILISATION

Solutions of the solubilising agents were prepared by dilution of a concentrated solution with 0·05M aqueous Tris buffer (pH 8·0) or 0·003N hydrochloric acid. Approximately 20 ml samples were added to about 200 mg of hexachlorophane in glass stoppered containers and agitated at 25 ± 0·1°. An equilibration time of at least 3 days was allowed and then analyses were made on each of several successive days to ensure that equilibrium had been reached, the solutions being filtered through Millipore pads of 0·45 μ pore size. The hexachlorophane concentration was determined spectrophotometrically at 303 mμ after suitable dilution in 0·05M Tris buffer in 95% methanol (pH 8).

The results are graphed in Fig. 1A and B. In acid solution, macrogol and polyethylene polypropylene glycol did not increase the solubility of hexachlorophane.

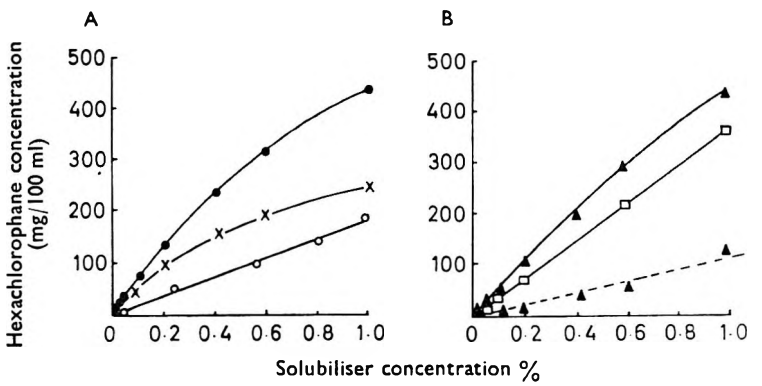

FIG. 1. Solubility of hexachlorophane at 25° in the presence of solubiliser. In A: —○— sucrose laurate, —●— polyethylene polypropylene glycol, —×— macrogol, all in aqueous 0·05 M Tris buffer at pH 8·0. In B: —▲— lauromacrogol and —□— polysorbate 20 in aqueous M/20 Tris buffer at pH 8·0, - - - - ▲ - - - - lauromacrogol in 0·003 N hydrochloric acid.

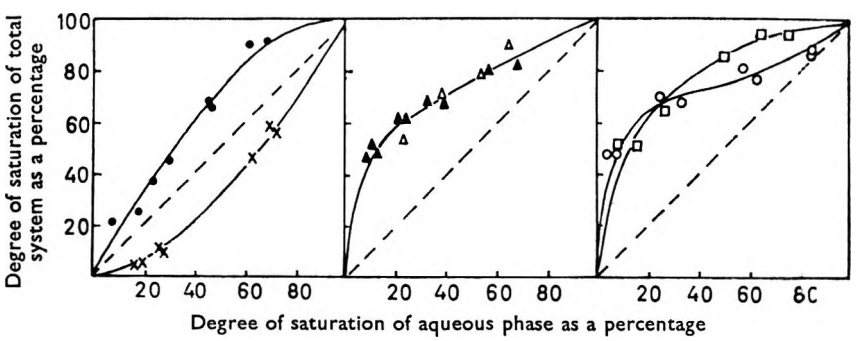

FIG. 2. Distribution of hexachlorophane in solutions of various agents in aqueous 0·05M Tris buffer, pH 8·0 at 25°. ○, sucrose laurate 1%. □, polysorbate 20 1%. △, lauromacrogol 0·1%. ▲, lauromacrogol 1%. ×, macrogol 1%. ●, polyethylene propylene glycol 1%.

The solubility of hexachlorophane in aqueous 0·05M Tris buffer pH 8 is 6·6 mg/100 ml and in 0·003N hydrochloric acid is $0{\cdot}1_4$ mg/100 ml.

DIALYSIS

Dialysis cells were made according to specifications kindly supplied by Dr. H. B. Kostenbauder and were similar to those used by Patel & Foss (1964). Membranes were of rubber latex washed initially in a solution of the surfactant to be used and then in successive changes of distilled water until the washings were clear and surfactant was completely removed. Tests were made to confirm that hexachlorophane passed through the rubber from an aqueous solution to a surfactant solution and *vice versa*. All solutions were made in 0·05 aqueous Tris buffer at pH 8. For each series, four different concentrations of hexachlorophane were used with the one concentration of solubilising agent on one side of the membrane and an aqueous solution of approximately similar degree of saturation, to hasten equilibrium, placed on the other. The systems were equilibrated by intermittent agitation in a tumbling shaker at $25 \pm 0{\cdot}1°$ for six days. Equilibrium was reached in about three days for solutions of similar saturation. The concentration of hexachlorophane on each side of the membrane was determined as described above.

The distributions of hexachlorophane between the solubilising agents and Tris buffer are shown in Fig. 2.

BACTERICIDAL ACTIVITY

A strain of *Escherichia coli* was maintained on agar slopes, being transferred at weekly intervals. As required, a week old slope was used to inoculate a new slope and after 48 hr a tube of nutrient broth (peptone 5, "Lab-lemco" 1, dextrose 1, water to 1000) was inoculated from this slope. After incubation at 37° for 24 hr, the broth was diluted 10,000 times in saline and 0·2 ml of this dilution was added to 20 ml of test solution held at $25 \pm 0{\cdot}1°$. The test solutions contained selected concentrations of hexachlorophane and solubilising agent in aqueous Tris buffer (pH 8).

At selected time intervals the test solution was shaken and appropriate volumes spread on the surface of nutrient agar containing 2% polysorbate 80; the plates were incubated overnight at 37°. Three replicates were taken for each time of each sample. Control samples were taken in the absence of hexachlorophane to check the original number of organisms per ml and to show that no appreciable kill due to the vehicle occurred during the time of the experiment.

Rates of kill are shown in Table 2 as decimal reduction times for the various combinations.

AGAR PLATE DIFFUSION

The antibacterial activity of hexachlorophane against *Bacillus pumilus* NCTC 8241 was tested by an agar plate diffusion method similar to that used by the *British Pharmacopoeia* (1963) for the biological assay of antibiotics. Filter paper discs (Carl Schleicher & Schuell Co. No. 740 E)

TABLE 2. DECIMAL REDUCTION TIME FOR *E. coli* IN THE PRESENCE OF HEXACHLOROPHANE AND SOLUBILISER AT pH 8 AND 25°

Solubiliser	Lauromacrogol 1%						Macrogol 1%				No solubiliser	
Concentration hexachlorophane (mg/100 ml)	400	350	300	250	200	150	250	200	150	50	5	2
Decimal reduction time (min)	7	8	17	59	130	500	4	6	7	9	5	13

were used as a reservoir for the test solution instead of cylinders or holes in the agar.

A suspension of spores of *B. pumilus* was prepared and stored as recommended by the *British Pharmacopoeia* (1963) for microbiological assay. This was heated at 50° for 10 min immediately before use to kill any organisms in the vegetative form, and then 2·6 ml was added to 260 ml lots of nutrient agar made by adding 1·15% agar to Medium A (pH 7·8) of the *British Pharmacopoeia* (1963). The seeded agar was poured onto a shallow flat plate measuring 12 by 12 inches placed on a horizontal glass slab. The surface was left to dry for 15 min before placing the discs which had been dipped in test solution. The plate was then covered and allowed to stand at room temperature for 2 hr before being incubated at 37° overnight. The zones of inhibition were measured in millimetres with the aid of a magnifying device.

Dose-response curves were obtained for hexachlorophane in Tris buffer (pH 8) and in solutions of a solubilising agent in Tris buffer (pH 8) by using a suitable serial dilution involving nine doses over the required concentration range. Nine replicates were used for each dose level and the 81 doses were randomised on a 9 × 9 Latin square. A straight line dose-response curve can be calculated from the 81 responses and deviations of the average of the responses for each dose level tested for significance using an analysis of variance technique. Significant deviations from linearity were found for the hexachlorophane in lauromacrogol solutions.

The dose response curves are shown in Fig. 3. The curve for sucrose laurate is shown as a broken line because circular zones could not be obtained for this surfactant; consequently the values are less reliable and have not been subjected to statistical analysis.

Discussion

The pK_1 value for hexachlorophane has been estimated as 5·4 (Mahler, 1954). This value was obtained in 30% methanol as solvent and, although the value in water will differ from this, it is probable that at pH 8 the phenol exists almost entirely as the singly charged anion, and that it is unionised in 0·003N hydrochloric acid (at about pH 2·5).

At either pH, hexachlorophane is solubilised by the surface-active agents, although the increases in solubility due to these agents are less in the acid solution than at pH 8. The unionised species might be expected to hydrogen bond to the ether oxygens of macrogol or polyethylene polypropylene glycol, but this does not occur under the conditions used in

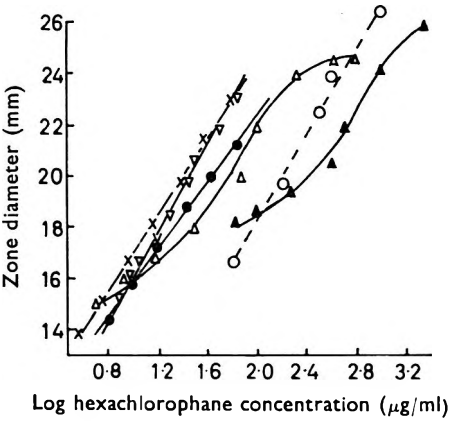

FIG. 3. Zone diameter/concentration curves for hexachlorophane in solutions of various agents in aqueous 0·05M Tris buffer pH 8·0. ▽, buffer alone. ×, macrogol 1%. ●, polyethylene polypropylene glycol 1%. △, lauromacrogol 0·1%. ▲, lauromacrogol 1%. ○, sucrose laurate 1%.

this study, probably because in acid solution the ether oxygens interact with hydronium ions more strongly than with hexachlorophane molecules.

The lauromacrogol, which forms micelles, does solubilise unionised hexachlorophane. This may be a measure of the amount of hexachlorophane which dissolves in the oleophilic centres, but it is also possible that when the surfactant molecules aggregate to form micelles the polyoxyethylene chains not only dehydrate but also release hydronium ions allowing hexachlorophane molecules to hydrogen bond with ether oxygens in the polyoxyethylene layer. The results with polysorbate 20 were essentially similar, although there was evidence of liquid crystal formation, opalescent solutions which could not be clarified being obtained.

The critical micelle concentration value for the polyethylene polypropylene glycol has been determined by Saski & Shah (1965) who found a value of 0·1%. The inability of 1% of this agent to solubilise hexachlorophane in acid solution suggests that under these conditions it does not form micelles. Protonation of the oxygen atoms probably occurs in the acid solution and this would retard micelle formation.

At pH 8, hexachlorophane interacts to an appreciable extent with polyoxyethylene compounds whether in the form of micelles or as randomly dispersed macrogol chains. It is possible that the negative charge on one of the phenolic oxygen atoms does not prevent hydrogen bonding through the second phenolic group to ether oxygens of the polyethylene glycols. Such a mechanism would also have to be postulated for systems containing the polyoxyethylene surfactants because polysorbate 20 and lauromacrogol effect much greater increases in solubility than sucrose laurate. Also polyethylene polypropylene glycol in neutral solution increases the solubility of hexachlorophane to a greater extent than any of the other agents; this is best explained as being due to strong interaction with the oxygen atoms of the ethylene oxide and propylene oxide chains.

Because the hexachlorophane is present in the aqueous phase almost entirely as the anion, it does not necessarily follow that the species interacting with the surfactants is also the charged anion. It is possible that the hexachlorophane is solubilised as the uncharged molecule.

The determination of the amounts of hexachlorophane interacting with the solubilising agents in unsaturated systems at pH 8 has been made by dialysis across rubber membranes which are impermeable to the solubilising agents but permeable to hexachlorophane. In dilute solutions at equilibrium, the concentration of drug in the buffer solution will be the same as that in simple aqueous solution in the solubilised system (Patel & Kostenbauder, 1958). It is probable that only the uncharged molecule can pass through the rubber so that a pH change might result if diffusion of large amounts of material is necessary to arrive at equilibrium. For this reason and to hasten attainment of equilibrium, solutions with approximately similar degrees of saturation were placed on each side of the dialysis membrane.

The dialysis data do not support the concept of a simple partition between the micellar pseudo-phase and the true aqueous phase as suggested by Evans (1964) for *p*-hydroxybenzoic acid. Fig. 3 shows the distribution of hexachlorophane plotted as the degree of saturation of the true aqueous phase as suggested by Allawala & Riegelman (1953) and Mitchell (1964). This method of plotting allows the effective concentration in the true aqueous phase to be calculated, which is important if it is accepted that only the free phenol is able to exert an antibacterial effect.

When hexachlorophane is allowed to partition between aqueous buffer on one side of the membrane and a 1% solution of macrogol on the other side, the buffer solution and hence the true aqueous phase have a higher degree of saturation than the total system. In all other instances the true aqueous phase is less saturated than the total system.

Lauromacrogol was investigated at two concentrations (0·1 and 1·0%) and the distribution seems to be essentially unaffected by this change.

ANTIBACTERIAL ACTIVITY

A solution containing 400 mg of hexachlorophane in 100 ml of 1% lauromacrogol is seen (from Fig. 1B) to be about 90% saturated, and one might expect its antibacterial activity to be approximately equal to that of an aqueous solution with the same degree of saturation. However, Table 2 shows the activity to be less than that of an aqueous solution containing 5 mg/100 ml (which is 76% saturated), and this is consistent with the results in Fig. 2, where a system with a total saturation of 90% is shown to have an aqueous phase which is only about 70–80% saturated. Similarly, the 2 mg per 100 ml standard which is about 30% saturated has bactericidal activity which corresponds to that of a solution in 1% lauromacrogol containing between 300 and 350 mg/100 ml hexachlorophane. These solutions are about 70% and 80% saturated with respect to the total system whereas Fig. 2 shows that the aqueous phases are only 30 and 40% saturated respectively.

There is a similar correlation for the solutions in 1% macrogol. It

may be predicted from Figs 1A and 2 that to obtain 2 and 5 mg/100 ml of free hexachlorophane, the total concentration in the presence of 1% macrogol must be about 50 and 150 mg/100 ml respectively, and these values are in reasonable agreement with the results shown in Table 2.

It is apparent from these examples that predictions based on the total degree of saturation of systems containing solubilised hexachlorophane often give a very poor estimate of the bactericidal activity which may be expected. When an estimate is based on the concentration of bactericide which has not interacted with the solubilising agent, the predicted value is much closer to the measured antibacterial activity.

Although macrogol reduces the bactericidal activity of hexachlorophane against *E. coli* it does not seem to affect markedly the activity shown against *B. pumilus* using the agar plate diffusion method (Fig. 2). The polyethylene polypropylene glycol also does not have an appreciable effect on the activity shown by this procedure. However, 1% of either sucrose laurate or of lauromacrogol reduces significantly the activity of hexachlorophane, although the curve for the lauromacrogol systems seems to be approaching the standard at lower hexachlorophane concentrations. This trend is confirmed by the curve obtained for solutions containing 0·1% surfactant; in this instance the curve crosses that of the standard at low concentrations, indicating that for certain combinations increased activity may be shown.

The agar plate diffusion results do not seem to bear any relationship to the solubilisation or dialysis data. This method gives a measure of the diffusion and bacteriostatic activity of hexachlorophane in the presence of the solubilising agent. Attempts to determine minimum inhibitory concentrations of hexachlorophane in the presence of selected amounts of the solubilising agents have been thwarted by the obvious interaction of these agents with broth components and consequent interference with their interaction with hexachlorophane. The same broth components are likely to reduce the effect of the solubilising agents on hexachlorophane in the agar plate diffusion technique.

Obviously results obtained from the agar plate diffusion method must be of limited application and cannot be expected to give a good estimate of the activity of a preparation in actual practice. The results obtained from methods measuring the rates of kill of appropriate organisms are much more useful. The use of diffusion methods to study interaction with solubilisers has been questioned by Wedderburn (1964) when discussing the anomalous results obtained by Gregg & Zopf (1951). Our results show that for tests involving a single dose level, either reduced or increased activity might be demonstrated, depending on the concentrations of solubiliser and antibacterial agent which happen to have been chosen.

In a situation where some of the antibacterial agent will be used up by interaction with bacteria, the skin or foreign substances, it is necessary to take into account the capacity of the system to maintain the required concentration. Allawala & Riegelman (1953) have shown that the capacity of such systems is increased when an increased concentration of

antibacterial agent is used together with a solubiliser. This effect will work most efficiently when for a given change of total saturation there is a relatively small change in the degree of saturation of the aqueous phase and this condition obtains for those combinations for which the slopes in Fig. 2 are greatest. Thus, if a saturated solution of hexachlorophane in 1% macrogol at pH 8 is used under conditions where the phenol is being lost, a fifth of the phenol can be removed and the residual activity is equivalent to that of an aqueous solution 85 to 90% saturated with respect to hexachlorophane; on the other hand, a similar loss of a fifth of the total phenol from a saturated solution in 1% lauromacrogol reduces the activity to that of a 60% saturated aqueous solution.

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Water vapour transmission properties of free polymer films*

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The water vapour transmission properties of selected hydrophilic, lipophilic, and mixed hydrophilic-lipophilic polymer systems, cast as free films, have been examined as a function of time, film thickness, plasticiser concentration, and film formulation. An inverse linear relationship was established between the logarithm of the water vapour transmission rate (R_{wvt}) and the logarithm of film thickness in the presence of the various plasticiser concentrations for the three systems studied. The lipophilic n-butyl methacrylate films were found to be less permeable to moisture than either the hydrophilic hydroxypropyl cellulose or mixed methyl hydroxypropyl cellulose-ethyl cellulose films. The butyl methacrylate films closely follow Fickian diffusion, whereas the permeability constants of the other two films increased linearly with film thickness over the range of thicknesses studied. These phenomena are discussed with respect to the relative attractions these films have for water vapour.

ONE way of combatting drug instability has been to coat pharmaceutical products to prevent access of moisture or air or both. The shortcomings of the conventional methods of coating have led to the development of newer techniques, prominent among which is film coating. The many advantages of this process have been reviewed by Gross & Endicott (1960) who also discussed the desirable properties of an ideal polymer film suitable for pharmaceutical application. One such property of major importance is the permeability of these films to moisture.

An inverse linear relationship between the rate of water vapour transmission and film thickness has been shown for ethylcellulose and methylcellulose free films (Kanig & Goodman, 1962). In agreement with Higuchi & Aguiar (1959), it was found that the more polar the film, the greater was the rate at which water vapour was transmitted. Similar findings have been made by Munden, DeKay & Banker (1964). Patel, Patel & Lemberger (1964) observed that the permeability constant of cellulose ester films decreased with increasing chain length of the acid moiety used and that the plasticiser could enhance or retard moisture permeation, depending upon its concentration. The optimum plasticiser concentration at which a minimum water vapour transmission rate is obtained was found to be similar for all the plasticisers examined by Lachman & Drubulis (1964), working with cellulose acetate phthalate films. The rate of water vapour transmission was directly proportional to the relative humidity for both low and high plasticiser concentrations. A significant relationship between the number of carbon atoms in the alcohol chain of esterified polymers cast as films and the water vapour transmission properties of the films was established by Lappas & McKeehan (1965). But no detailed investigations of the water vapour transmission across polymer films deposited on single dose preparations

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have been reported. Also, the results of water vapour transmission rates across free films may not necessarily reflect the water vapour transmission properties across films applied to a solid dosage form.

We present the results of work undertaken to elucidate the effect of time, film thickness, plasticiser concentration and film formulation on the water vapour transmission properties of selected hydrophilic, lipophilic and mixed hydrophilic-lipophilic polymers cast as free films.

Experimental

SELECTION AND PREPARATION OF FREE FILMS

A range of polymers in combination with various compatible plasticisers were screened for their film forming properties using FDA food-additive approved solvents. The films were evaluated on the basis of clarity, uniformity, flexibility, plasticiser compatibility and absence of stickiness. From these data, three polymer-plasticiser systems, representative of a hydrophilic, a lipophilic and a mixed hydrophilic-lipophilic system, were selected (Table 1).

Unplasticised and plasticised films of various thicknesses, with different plasticiser concentrations, were prepared by pouring solutions of the selected polymer-plasticiser-solvent combinations on to a pool of mercury contained in a petri dish, or on to a glass plate. In the mercury technique, the film thickness was controlled by the area of the petri dish and the volume of solution used. In the glass plate technique plastic rings limited the spread of solution and thereby controlled the film thickness. The solvents were allowed to evaporate overnight at ambient temperature after which the films were removed. The film samples were stored in air-tight containers in the presence of anhydrous calcium sulphate at ambient temperature.

Coherent films of the methyl hydroxypropyl cellulose-ethyl cellulose mixture could not be prepared in the absence of a plasticiser (propylene glycol).

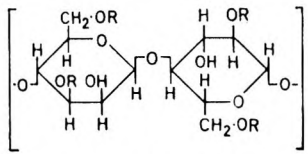
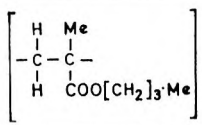
DETERMINATION OF FILM THICKNESS

The film thickness was initially determined by five or six different measurements over the film samples using a micrometer screw gauge. The results showed large variations, due apparently to the compressibility of the film. The mean film thickness was therefore calculated from a knowledge of the density, weight and area of each film. The density was determined by weighing the film sample first in air and then in a non-solvent liquid. For hydroxypropyl cellulose and the mixed polymer films, n-hexane was used and for butyl methacrylate films water was used. No visible swelling of the films in these non-solvents was observed.

APPARATUS

Transmission cell. The transmission cell, similar in design to that used by Patel & others (1964), had a capacity of approximately 23 ml and contained 5 ml of a saturated sodium tartrate solution in contact with

TABLE 1. DESCRIPTION AND PREPARATION OF FILM FORMULATIONS

Type of polymer and chemical name	Monomer unit composition	Plasticiser concentration used (% w/w of polymer)	Composition of solvent system (parts by volume)	Polymer concentration in film coat solution (% w/v)	Method of preparation of film samples
Hydrophilic Hydroxypropyl ether of cellulose[1]	Anhydroglucose unit  where R = $-CH_2CH(OH)Me$ at a concentration not exceeding 4·6 groups per anhydroglucose unit	Propylene glycol 0, 10, and 20%	Methylene chloride 50 Methanol .. 50	10	Glass substrate technique
Hydrophilic : lipophilic Mixture of: Methylhydroxypropyl cellulose[2]—3 parts Ethyl cellulose[3]—1 part	Anhydroglucose unit as above, with: R = Me (28–30%) = $CH_2CH(OH)Me$ (7–12%) R = $-CH_2Me$ (48–49·5%)	Propylene glycol 25 and 50%	Methylene chloride 40 Methanol .. 30 Isopropanol .. 30	3	Glass substrate technique
Lipophilic n-Butyl methacrylate[4]		Diethyl phthalate 0, 2·5, and 5%	Methylene chloride 50 Ethyl acetate .. 50	10	Mercury substrate technique

[1] Klucel-L, Hercules Powder Company, Wilmington, Delaware, U.S.A.
[2] Methocel 60HG, 50 cps, Dow Chemical Company, Midland, Michigan, U.S.A.
[3] Ethocel, Type N, Dow Chemical Company, Midland, Michigan, U.S.A.
[4] Lucite 2044, E.I. Du Pont, De Nemours and Co., Inc., Wilmington, Delaware, U.S.A.

excess solid phase. This system had a vapour pressure of 29·0 mm Hg at 30° (Lowry & Morgan, 1924) which was maintained throughout the experiment. The area of the film available for permeation was 1·77 cm^2.

Temperature and humidity controlled chamber. This was an air-tight glove box. The temperature inside was controlled by circulating water from an external water bath to a radiator inside the chamber. A small fan behind the radiator circulated the air and maintained the temperature at 30 $\pm$ 1°. The humidity inside the chamber was kept at 0% by the presence of sufficient anhydrous calcium sulphate. This was checked by a relative humidity indicator inside the chamber.

DETERMINATION OF WATER VAPOUR TRANSMISSION

The assembled transmission cell, containing the film sample and the sodium tartrate solution, was placed inside the chamber and immediately weighed on an analytical balance in the chamber. It was also weighed at known time intervals over 72 hr. The amount of moisture transmitted through the film at any one time was given by the loss in weight of the cell at that time when compared to the weight at zero time. The balance was placed to allow the sample to be weighed without disturbing the temperature and humidity conditions. Each film sample was weighed just before, and immediately after, the transmission study to determine the amount of moisture held by the film as a result of sorption. Cells containing discs of aluminium, 0·015 cm thick, were used as controls; the weight loss after 72 hr was less than 1% of that with a polymer film present. All weight losses with polymer films were therefore attributed to the permeation of moisture.

Results and discussion

A number of equations, based on Fick's law of diffusion, have been used to obtain the rate of permeation through organic film coatings (American Society for Testing Materials, 1961; Utsumi, Ida, Takahashi & Sugimoto, 1961; Rains, 1962; Patel & others, 1964). Higuchi & Aguiar (1959) combined Fick's law with Henry's law of solubility of gases, since water permeability was held to involve diffusion of the vapour through the film and sorption of the vapour by the film. If, as was so in the present work, the amount of moisture sorbed is low in relation to the amount transmitted then it may be neglected (Patel & others, 1964). Even if this were not so, sorption of moisture by the film must occur before transmission begins and, under equilibrium conditions, the amount of moisture passing into the proximal surface of the film will equal that passing out of the distal surface.

The following relationship, based on Fick's law but modified to exclude film thickness, was used by us to calculate the water vapour transmission rates of the polymer films under investigation.

$$R_{wvt} = \frac{W}{A.\Delta P} \quad .. \quad .. \quad .. \quad .. \quad (1)$$

where R_{wvt} is the rate of water vapour transmission, W the amount of moisture transmitted through the film, in g/hr, A the area of the film exposed, in cm^2, and ΔP the vapour pressure difference across the film, in mm Hg. The water vapour transmission rate, R_{wvt}, is defined as the amount of moisture transmitted per unit time through a film of unit area subjected to a vapour pressure difference of unity. R_{wvt} is expressed as $g/hr/cm^2/mm$ Hg.

The weight in g of moisture transmitted through all the films studied was found to be directly proportional to time, over the period of 72 hr, when the temperature, vapour pressure, and area of the film exposed were held constant.

Curvi-linear relationships were apparent between R_{wvt} and film thickness for the various systems examined. However, log–log plots of R_{wvt} against film thickness were linear, as shown in Fig. 1. These relationships may be expressed by the following equation:

$$\log R_{wvt} = \log C_1 + m_1 \log t \qquad (2)$$

which reduces to:

$$R_{wvt} = C_1 \cdot t^{m_1} \qquad (3)$$

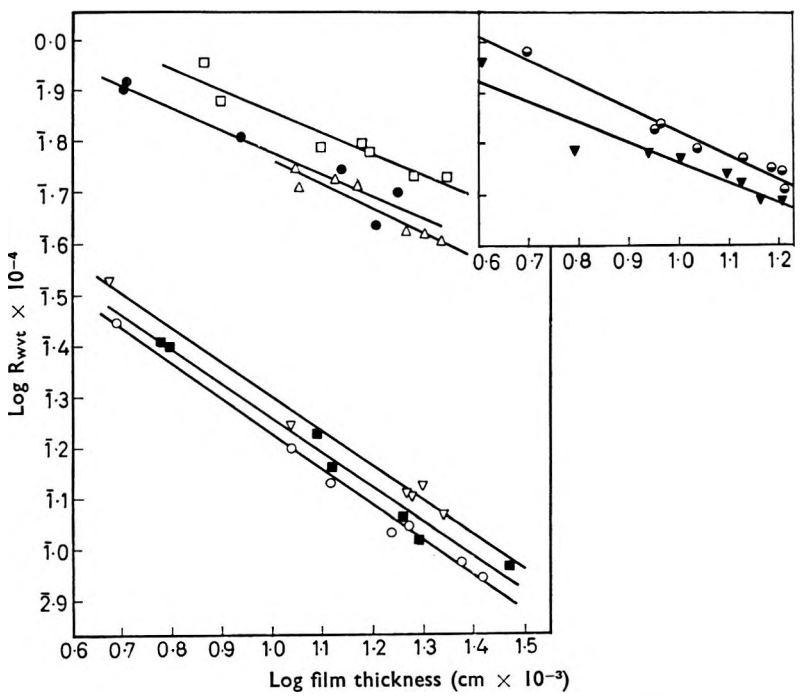


FIG. 1. Relationship between logarithm of water vapour transmission rate (R_{wvt}) and logarithm of film thickness for polymer films at 30° and under a vapour pressure difference of 29·0 mm Hg. Key: butyl methacrylate (unplasticised), ■; butyl methacrylate + diethyl phthalate 2·5%, ○; 5·0%, ▽; hydroxypropyl cellulose (unplasticised), ●; hydroxypropyl cellulose + propylene glycol 10%, △; 20%, □; methylhydroxypropyl cellulose : ethyl cellulose + propylene glycol 25%, ▼; 50%, ◒.

where R_{wvt} is as previously defined, t is the film thickness in cm, C_1 the extrapolated ordinate intercept in water vapour transmission units, and m_1 the slope of the line. The statistical constants for these plots are given in Table 2.

It is apparent from the data in Fig. 1 that the water vapour transmission rates at equivalent film thicknesses through the hydroxypropyl cellulose and mixed polymer films are higher than those of the butyl methacrylate films. For example, at an equivalent film thickness of 0·01 cm, the R_{wvt} values are about $5{\cdot}7–7{\cdot}4 \times 10^{-5}$, $5{\cdot}7–6{\cdot}9 \times 10^{-5}$, and $1{\cdot}7–2{\cdot}0 \times 10^{-5}$ g/hr/cm²/mm Hg, for the hydroxypropyl cellulose, mixed polymer and butyl methacrylate films respectively. It is also apparent that the *rate* at which R_{wvt} decreases with increasing film thickness is greatest in the lipophilic butyl methacrylate systems (Table 2).

TABLE 2. STATISTICAL CONSTANTS FOR RELATIONSHIP BETWEEN LOG WATER VAPOUR TRANSMISSION RATES AND LOG FILM THICKNESS

System	Plasticiser concentration (% w/w of polymer)	Correlation coefficient r	Slope of line m	Ordinate intercept log C_1 ($\times 10^{-4}$)
Butyl methacrylate + diethyl phthalate	0	−0·988	−0·687	$\bar{1}$·944
	2·5	−0·996	−0·701	$\bar{1}$·927
	5·0	−0·983	−0·672	$\bar{1}$·964
Methylhydroxypropyl cellulose: ethyl cellulose (3 : 1) + propylene glycol	25	−0·943	−0·389	0·257
	50	−0·979	−0·456	0·384
Hydroxypropyl cellulose + propylene glycol	0	−0·959	−0·420	0·203
	10	−0·935	−0·468	0·233
	20	−0·954	−0·405	0·267

The % increases in weight per unit dry volume of film due to moisture sorption during the water vapour transmission studies are shown in Table 3. Moisture sorption is here compared on the basis of equal film thickness since the total cross-sectional area of all films used was constant. The weight increase per unit volume was used in preference to the weight increase per unit dry weight of film because the densities of the various film formulations were different. It may be seen from Table 3 that the amount of moisture sorbed decreases in the same order as R_{wvt}, i.e. hydroxypropyl cellulose, mixed polymer films and butyl methacrylate.

TABLE 3. MOISTURE SORPTION BY POLYMER-PLASTICISER FORMULATIONS CAST AS FREE FILMS

System	Plasticiser concentration (% w/w of polymer)	Moisture sorption (% w/v of dry film)
Butyl methacrylate + diethyl phthalate	0	0·68 ± 0·29
	2·5	0·63 ± 0·48
	5·0	0·23 ± 0·37
Methylhydroxypropyl cellulose : ethyl cellulose (3 : 1) + propylene glycol	25	1·27 ± 0·67
	50	0·22 ± 0·15
Hydroxypropyl cellulose + propylene glycol	0	4·01 ± 0·72
	10	2·73 ± 0·78
	20	2·55 ± 0·90

For Fick's law of diffusion to be valid, the following relationship should be obeyed:

$$P = R_{wvt} \times t \qquad .. \quad .. \quad .. \quad (4)$$

where P is the permeability constant, and R_{wvt} and t are as previously defined. The permeability constant should be independent of thickness in those films which exhibit Fickian diffusion. Values of P, calculated from equation 4, have been plotted against film thickness in Fig. 2. With lipophilic butyl methacrylate films, the permeability constant is nearly independent of film thickness. This contrasts with the relationship obtained with the two relatively hydrophilic films, where P increases rapidly with film thickness.

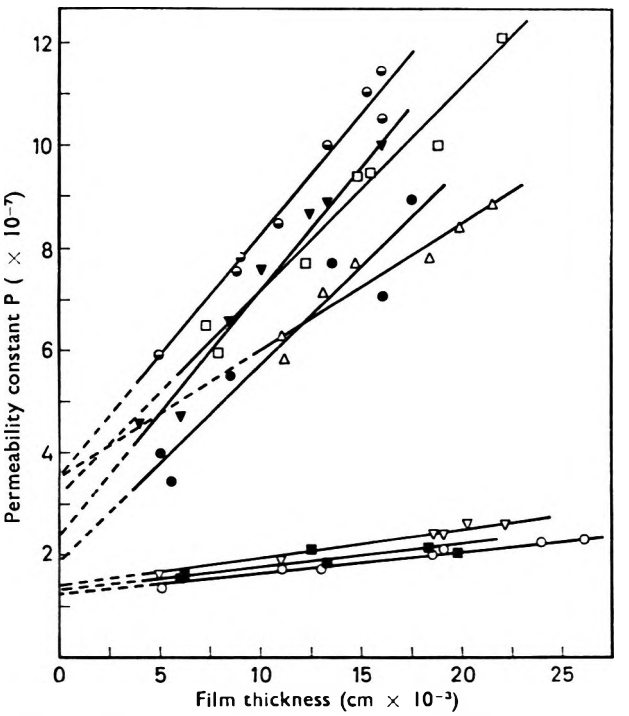


FIG. 2. Variation of permeability constant (P) with film thickness for the three film systems. Key: as in Fig. 1.

The observation that the diffusion of water vapour through the lipophilic butyl methacrylate films approximates to that predicted by Fick's law, implies that the vapour pressure gradient across the film is the major driving force. By the same reasoning, the increase in P with film thickness for the other two systems means that factors other than those considered in Fick's law are operative and are, presumably, superimposed on the diffusion due purely to the vapour pressure difference existing across the film.

Patel & others (1964) have also reported increases in P with film thickness for cellulosic films. The magnitude of this effect increased with decreasing chain length of the acid moiety used in the selected cellulose ester films, i.e. as the components of the film became more hydrophilic. The results of these workers show that P tended to reach a limiting value at film thicknesses in excess of approximately 0·025–0·03 cm. The range we investigated was from 0·004 to 0·026 cm, which encompasses the usual film coating thickness range. Over this range P was found to be linearly related to film thickness.

Two main mechanisms have been proposed to account for the permeation of materials through polymer films, namely a sieving mechanism and a solution process. Michaels & Parker (1959) consider the polymer as a porous medium in which the crystallites constitute the particulate skeleton and the amorphous phase the interstitial phase. According to Baddour, Graves & Vieth (1965), the evidence accumulated thus far indicates that the crystallites are impermeable to moisture. These workers point out that since the amorphous regions are networks of tangled chains, it is hardly likely that such regions would have precise pore diameters. Accordingly, the amorphous regions of the membrane may be viewed as a solvent in which the permeant dissolves and then moves under the influence of a concentration gradient to re-evaporate on the distal surface as proposed by Tuwiner (1962). This would seem to account for most of the transmission properties observed in view of the correlations between the ability of a polymer to hydrogen bond and its water vapour permeability and moisture sorption (Reid & Breton, 1959; Baddour & others, 1965). The volume fraction through which this mechanism could act would depend on the relative amounts of the crystalline and amorphous phases. Moisture sorption presumably leads to swelling of the amorphous regions of the film and this facilitates permeation. Other factors which may be involved are the degree of cross-linking, chain branching, the degree of substitution of the polymer and the molecular weight and shape of the permeant.

In the present work, the deviations from Fick's law observed for hydroxypropyl cellulose and the mixed polymer systems are probably associated with the attractive forces existing between these films and water molecules. As shown in Table 1, the cellulosic polymers consist of chains of variously substituted anhydroglucose units bearing a large number of hydroxyl groups. Propylene glycol, the common plasticiser, also possesses hydroxyl groups. The environment is therefore highly polar and affords ample opportunity for the existence of van der Waals' forces and hydrogen bonds. This, together with the swelling accompanying the sorption, facilitates water permeation. The butyl methacrylate films, on the other hand, are considerably less polar. Sorption, on the whole (Table 3), is less than that which occurs with the other two systems and the water vapour transmission is lower. The normal process of diffusion is not reinforced greatly by the presence of attractive forces and the permeability properties of these films more closely approach Fickian diffusion.

The reciprocal of the rate of water vapour transmission is indicative of the resistance of the film to moisture permeation. Fig. 3 shows the results obtained when R_{wvt}^{-1} is plotted against t. The theoretical plot

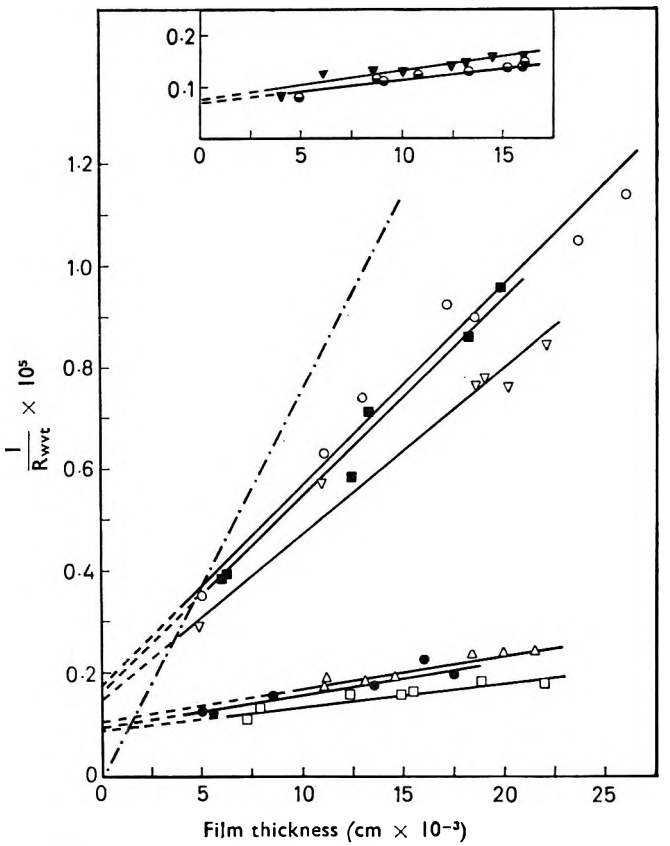


FIG. 3. Variation of the reciprocal of the water vapour transmission rate (R_{wvt}^{-1}) with film thickness for the three film systems. Key: as in Fig. 1; ●—— ●, theoretical plot for Fickian system having permeability constant of 0·013 × 10^{-5}.

for ideal Fickian diffusion is also shown for the case where P = 0·013 × 10^{-5} (the extrapolated permeability constant for butyl methacrylate films at zero thickness, taken from Fig. 2). Similar theoretical plots may be readily calculated for the other polymer systems. In all instances, the observed negative deviation of the experimental plots is an indication of the relative non-Fickian behaviour of the various films, the actual film resistance to moisture permeation being less than that predicted by Fick's law. Positive intercepts at zero film thickness, as found in Fig. 3, have also been observed by Patel & others (1964). These workers suggested that, with cellulosic films, this may be due to shrinkage of the fibres on the distal film surface when this surface is exposed to low

humidity conditions. The presence of a positive intercept with the non-cellulosic butyl methacrylate polymer films suggests that this is due to interactions between permeant and film, the intercept decreasing as the interaction decreases.

The slopes and ordinate intercepts contained in Table 2 are such as to preclude any absolute, quantitative evaluation of the data obtained. However, it appears that the mixed polymer and hydroxypropyl cellulose films are of a similar hydrophilic nature since the plot for the mixed polymer + 25% propylene glycol system is similar to that for the hydroxypropyl cellulose + 20% propylene glycol film. Examination of the structures of these polymer systems (Table 1) shows that this is not an unexpected result. The data also indicate that the plasticiser, on a % concentration basis, exerts a greater influence upon the water vapour transmission rates of the hydrophilic films than the lipophilic films studied. Insufficient plasticiser concentrations were investigated to establish the possible existence of minima in R_{wvt} as the plasticiser concentration is increased, as has been reported by Lachman & Drubulis (1964) and Patel & others (1964) for cellulose acetate films.

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Aloe-emodin glycosides of senna leaf

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A new glucoside has been isolated from senna leaf (*Cassia senna* L.) and shown to be identical with the aloe-emodin-8-mono-β-D-glucoside recently isolated from *Rheum palmatum*. This new quinone glucoside is insoluble in water and is present in only small quantities and therefore unlikely to contribute much to the activity of the drug. Confirmation of the presence of a second, highly water soluble glycoside based on a reduced form of aloe-emodin is given; this glycoside may well be responsible for the reported "synergistic effect" of the non-rhein glycosides of senna leaf.

IN 1961, Crellin, Fairbairn, Friedmann & Ryan published a preliminary report on the presence in senna leaf of two glycosides based on aloe-emodin, one of which had been isolated in sufficiently pure form to indicate it to be an aloe-emodin glucoside. A counter-current method for its purification is now described. Using this method sufficient of the glucoside was obtained to establish its structure. We also obtained further evidence for the presence of the other aloe-emodin glycoside. The term "glucoside" is used for the substance of established structure and "glycoside" for the second substance, or a mixture of both.

Experimental

CHROMATOGRAPHY

Examination of numerous systems showed that the upper phase of n-butanol-ethanol-water (5:1:4), the single phase system ethyl methyl ketone-methanol-water (20:1:5) and the lower phase of water-acetone-benzene (2:1:4) gave the best results. The Rf values for the aloe-emodin glucoside in the three systems were 0·48, 0·66 and 0·33 respectively; in the first two systems the sennosides had very low Rf values (0·22 to 0·05) and in the third system high values (0·85 each). Whatman Paper No. 20, ascending technique and room temperature were used.

PRELIMINARY FRACTIONATION OF THE LEAF COMPONENTS

Moderately fine leaf powder was percolated with chloroform to remove free compounds and pigments. The marc was dried, and extracted with 10 to 12 volumes of methanol by percolation; the percolate was evaporated to dryness *in vacuo* yielding a yellow brown residue containing 9·5% rhein glycosides and 2·5% aloe-emodin glycosides, both calculated as sennosides. Further enrichment of the latter was effected by dissolving the extract in the lower phase of n-butanol-ethanol-water (5:1:4) and extracting about 10 times with equal volumes of the upper phase. Most of the aloe-emodin glycosides passed into the upper phases which on evaporation to dryness *in vacuo* and exhaustion with benzene to remove any newly formed aglycones yielded a solid containing 4·4% aloe-emodin glycosides, and small quantities only of sennosides. This fraction was used for the subsequent counter-current work.

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COUNTER-CURRENT SEPARATION

Batches of 9 g of the solid fraction already referred to were distributed between ethyl methyl ketone-0·05% sodium chloride* in water (4 : 3) on a counter-current machine till 60 transfers had been effected. Paper chromatographic examination showed that the aloe-emodin glucoside had been carried to the end of the train. Contents of the appropriate tubes were removed and evaporated to dryness *in vacuo* to give 3 g of a yellow residue containing 7·92% of aloe-emodin glycosides. This residue was distributed between n-butanol-ethanol-0·05% aqueous sodium chloride solution (5 : 1 : 4) till 60 transfers had been effected. After paper chromatographic examination the contents of appropriate tubes were evaporated to dryness *in vacuo* to yield 1·2 g solid. Further impurities were removed by washing the residue with ethanol-methanol (1 : 1) till the washings were colourless. The residue from 3 batches (200 mg) gave a single spot for aloe-emodin glucoside in the systems already referred to.

The residue (100 mg) was dissolved in 0·6 ml dimethylsulphoxide; carbon tetrachloride (10 ml) was gradually added and the solution was then warmed and filtered. After storage at 5° for a few days, 75 mg of crystals were deposited. These were separated and recrystallised in the same manner and dried *in vacuo*.

PROPERTIES AND IDENTITY OF THE ALOE-EMODIN GLUCOSIDE

The yellow, needle-shaped microcrystals were insoluble in ether and chloroform, almost insoluble in water, sparingly soluble in methanol, ethanol and acetone, more soluble in 70% methanol and very soluble in dimethylsulphoxide.

The glucoside gave an orange red colour with alkali and a bluish red colour with concentrated sulphuric acid. The glucoside had a m.p. of 237–238°; λ max (methanol), 222 mμ ($\log \epsilon = 4{\cdot}52$), 255 m$\mu$ ($\log \epsilon = 4{\cdot}41$), 410 m$\mu$ ($\log \epsilon = 3{\cdot}93$); υ max (KBr): C = O (free) 1670 cm^{-1}; C = O (chelated) 1624 cm^{-1}; C = C, 1580 cm^{-1}. Paper chromatography (see before); thin-layer chromatography, ethyl acetate-methanol-water (100 : 16·5 : 13·5) Rf = 0·50.

Combustion analysis (after drying over $MgClO_4$ *in vacuo* 100–102°). Found: C, 56·8; H, 4·8; calc. for $C_{21}H_{20}O_{10}$, $\frac{1}{2}H_2O$; C, 57·1; H, 4·8; calc. for $C_{21}H_{20}O_{10}$: C, 58·3; H, 4·6.

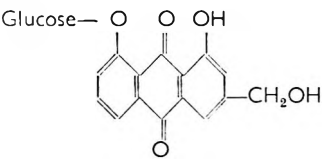
HYDROLYSIS

A known weight of the dried glucoside was hydrolysed in 3N hydrochloric acid and the aglycone separated by extraction with ether, a part of which was evaporated to dryness. The yellow residue was recrystallised from toluene and the crystals sublimed by gently heating in a high vacuum. Examination in several chromatographic systems showed aloe-emodin only was present. The m.p. was 223–224° (lit. 223–224°). Amount present by colorimetric assay in sodium hydroxide [E(1%, 1 cm) for aloe-emodin = 320] was 60·9%.

* The use of water alone causes emulsions.

The aqueous phase was examined chromatographically and shown to contain glucose only; the amount present was estimated by the oxidase method of Hugget & Nixon (1957) giving 37·0% and the *o*-toluidine method of Hultman (1959) giving 36·8%. Anhydrous monoglucoside of aloe-emodin should yield aloe-emodin 60% and glucose 40%.

Our glucoside is identical with the aloe-emodin monoglucoside isolated from *Rheum palmatum* by Hörhammer, Farkas, Wagner & Müller (1964). Comparison with a sample supplied by Prof. Hörhammer showed no depression of melting-point on admixture, and the absorption spectra were also identical. We therefore conclude that our glucoside is aloe-emodin-8-mono-β-D-glucoside.



A SECOND ALOE-EMODIN GLYCOSIDE

A methanolic extract of the leaf was fractionated on a polyamide column (Ultramid K228 BM2, Badische Anilin & Soda Fabrik) by elution with water. The aloe-emodin glucoside was retained on the column but chemical assay (*Analyst*, 1965) of the first fractions of the eluate showed that significant amounts of "non-rhein" glycosides were present along with water-soluble rhein type glycosides. These fractions were hydrolysed with acid, the aglycones extracted into ether and the rhein-type aglycone removed by extraction with aqueous sodium bicarbonate solution. The aglycone remaining in the ether was purified by band chromatography and shown to be a reduced form of aloe-emodin by fluorescence in ultraviolet light, by air oxidation to aloe-emodin in sodium hydroxide solution and by ultraviolet spectrum. The latter, however, differed from that described by Lemli, Dequeker & Cuveele (1964) for a dianthrone of aloe-emodin which they isolated from senna leaf, so that it is not possible at this stage to be certain that both aglycones are derived from the same glycoside. A preliminary report on the presence of a "gluco-aloe-emodin" from senna leaf is given by Romanova & Bankovskii (1965) but no chemical data are given.

Discussion

Since aloe-emodin-8-mono-β-D-glucoside is in the quinone form and is insoluble in water it is most unlikely that it will possess much purgative activity (Fairbairn, 1965). The second aloe-emodin glycoside, on the other hand, is in the reduced form, is highly water soluble and is present to a much greater extent (about ten times the amount of the quinone glucoside). It may therefore be responsible for the synergistic effect reported earlier (Fairbairn & Saleh, 1951). Unfortunately we cannot identify any observed chromatographic spot with this glycoside;

only after hydrolysis is it possible to identify the aglycone. The isolation of the parent glycoside therefore presents considerable difficulty which is not lessened by its high water solubility.

Recently Lemli & Cuveele (1965) and Schmid & Angliker (1965) have isolated two new glycosides (sennosides C and D) from senna leaf, both of which are based on the heterodianthrone of aloe-emodin and rhein. The presence of rhein renders the aglycones soluble in sodium bicarbonate solution so that our previous work on the biological activity of the ' non-rhein" glycosides is not affected by the discovery of these new glycosides.

Acknowledgements. We would like to thank Prof. L. Hörhammer for the sample of his glucoside used in this work; the British Council (Colombo Plan) for financial support to one of us (A.B.S.); Badische Anilin & Soda Fabrik for a generous supply of polyamide powder and the Science Research Council for a grant to purchase a Steady State machine which was used in some of the stages of counter-current investigation. This work forms part of a thesis presented by one of us (A.B.S.) for the Degree of Ph.D., University of London.

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Isolation and characterisation of hepatotoxins from *Penicillium rubrum*

SIR,—It is known that an acidic toxic material can be extracted from cultures of *Penicillium rubrum* grown on a corn-sucrose medium (Wilson & Wilson, 1962). We have now found that when a strain of *P. rubrum* M. R. 043 (kindly supplied by Mr. P. Austwick, C.V.L., Weybridge) is grown for 11–13 days on a Raulin Thom medium enriched with $2\frac{1}{2}\%$ malt extract, a complex of toxins may be produced and isolated in good yields.

The mould was grown as stationary cultures at 28–30°. After the culture fluid had been harvested from 13 day old cultures and concentrated to a small volume under reduced pressure, much atoxic material was precipitated sequentially, first by ethanol, and then by acetone, each solvent being removed by evaporation after the precipitate was recovered by filtration.

The toxic concentrate remaining was distributed between ethyl acetate and water. The ethyl acetate phase, after drying over anhydrous sodium sulphate, was evaporated to dryness to yield a pale orange gum which solidified to a buff coloured powder after washing out residual solvent with sodium-dried diethyl ether.

The results of a typical isolation from $1\frac{1}{2}$ litres of culture filtrate are shown in Table 1.

TABLE 1. FRACTIONATION OF METABOLISM SOLUTION FROM CULTURES OF *P. rubrum*

	Average lethal dose oral mg/kg	Total dry weight	Dry weight recovered %	Toxicity recovered %
Metabolism solution	1850	46·3 g	100	100
Ethanol ppt	Atoxic	5·6 g	12·1	0
Acetone ppt.	Atoxic	19·1 g	41·2	0
Aqueous soluble fraction	2000*	15·6 g	33·6	31·2
Ethyl acetate fraction	120	1·41 g	3·0	47
Recovery		41·7 g	90·0	78·2

* Whether the toxicity of the aqueous residue is due to a distinct toxin or to one or other of the solvent soluble toxins bound to a water soluble constituent of the culture filtrate is not yet known.

The crude toxin is a buff amorphous powder, m.p. 126–146° (with decomposition) $[\alpha]_D^{13} + 32{\cdot}5°$ (*c.* 2% acetone), LD50 3·75 mg/kg (intraperitoneally in mice). It is very soluble in acetone, soluble in alcohols, ethyl acetate, dioxan or *NN*-dimethylformamide, slightly soluble in ether but insoluble in chloroform, carbon tetrachloride, benzene or light petroleum. The toxin is insoluble in cold water but soluble in sodium bicarbonate or alkali solution.

The crude material contains only carbon, hydrogen and oxygen and gives the following analysis: C 58·7%, H 6·3%. Analysis for *C*-methyl gave a result of only 2·84% which corresponded to a minimum molecular weight of 528. Back titration of a solution of toxin in excess of cold sodium hydroxide gave an equivalent weight of 180 which indicated the presence of a tri-basic acid with a molecular weight of 540. Heating the alkaline solution resulted in a further uptake of sodium hydroxide and a complex of reactions which will be discussed in more detail in another publication.

At least two distinct highly toxic components can be isolated from the crude toxin either by careful precipitation by ether and light petroleum from a solution in acetic acid, or by slow evaporation of a saturated solution of crude toxin in diethyl ether.

The more easily obtained of these toxins, which we suggest be called Rubratoxin A, has been obtained as a white waxy looking material crystallising in rosettes of needles from acetone by slow evaporation at room temperature. Rubratoxin A has the following characteristics: m.p. 214° (decomp.) $[\alpha]_D^{15}$ + 86·6°. C, 59·6, H, 6·1, O, 34·6%. The ultraviolet absorption spectrum in ethanol has a broad peak at 206–209 mμ [E(1%, 1 cm) 351] and in alkali a peak appears at 262 mμ [E(1%, 1 cm) 150] with a shoulder at 300 mμ [E(1%, 1 cm)80], both of which disappear on acidifying.

The infrared spectrum indicates the presence of hydroxyl functions (3450 cm^{-1}), carboxylic acid (1710 cm^{-1}) and double bond (1630 cm^{-1}) functions, as well as showing interesting absorption at 1850 and 1770 cm^{-1}.

Rubratoxin A has an LD50 3·5 mg/kg given intraperitoneally in mice.

A second component, Rubratoxin B, has also been isolated from saturated ethereal solutions of crude toxin as a white amorphous powder but has not been so well characterised. It has the following properties: m.p. 167–168° (decomp.). C, 58·9, H, 5·8%.

Although the infrared spectrum of Rubratoxin B is essentially the same as that of Rubratoxin A it differs in the following aspects. There is a peak at 970 cm^{-1} in the spectrum of Rubratoxin A which is missing in that of B while the peak at 920 cm^{-1} increases in intensity, and there is a peak at 720 cm^{-1} in the spectrum of Rubratoxin B which is missing in that of A.

Details of the toxicity of the crude toxin are in Table 2. The crude toxin was always more toxic when given in propylene glycol than in 0·1% sodium bicarbonate solution.

TABLE 2. LD50 VALUES FOR CRUDE TOXIN

	Route	Dose mg/kg	Solvent
Mouse	oral	120	Propylene glycol
	i.p.	3·75	Propylene glycol
	i.v.	6·5	0·1% $NaHCO_3$
Duckling	oral	60	0·1% $NaHCO_3$
	i.p.	5	0·1% $NaHCO_3$

Doses well above the LD50 level in mice caused death within 2–4 hr of administration. Before death the activity and respiration of the mice were decreased, there was dilatation of the subcutaneous blood vessels and finally complete prostration, followed by death. Post-mortem examination of the mice revealed livers which were extensively haemorrhaged and had a mottled appearance which we have come to regard as characteristic of lethal doses of *P. rubrum* toxins. The kidneys were slightly anaemic and the lungs occasionally showed haemorrhages. Mice which remained alive for longer than 10 hr had their eyelids stuck together by dried exudate.

Post-mortem examination of mice killed seven days after a sublethal dose of the toxin showed no macroscopic liver damage. Mice dosed similarly, but killed 24 hr after the dose had the typically mottled liver. It seems therefore that the liver tissue is able to regenerate over a period of 7 days. Similar observations have been made with ducklings, guinea-pigs and rats.

That the normal metabolic processes of the liver are adversely influenced by doses of toxin which do not cause macroscopic lesions has been conveniently shown by the sleeping time test with pentobarbitone sodium (Plaa, Evans & Hine, 1958). Sleeping time was measured after a 45 mg/kg dose of pentobarbitone sodium given intraperitoneally to groups of 10 mice. The sleeping time was taken as the time between loss and subsequent gain of the righting

reflex by the mice. The control sleeping time for a group of 150 mice was 18½ min.

An oral dose of 75 mg/kg of crude toxin was given to seven groups, each of 10 mice. The mice were left for periods of 1·5, 3, 6, 12, 24, 48, 96 hr after the dose and then each group was tested for the pentobarbitone sleeping time.

The curve obtained indicated a rapid onset of interference with the normal detoxicating mechanism. This reached a maximum two days after administration and then decreased (see Fig. 1).

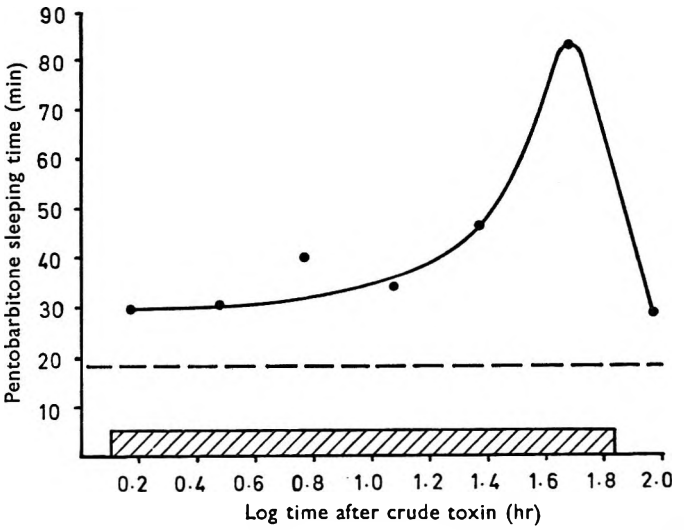

FIG. 1. The prolongation of the pentobarbitone sleeping time at intervals of 1·5, 3, 6, 12, 24, 48 and 96 hr after administration of 75 mg/kg of crude toxin. - - - - - Control value for the pentobarbitone sleeping time in mice. Hatched area indicates the presence of macroscopic liver damage.

These results show that the compounds we have described are distinct from the pigments produced by *P. rubrum* and in particular are consistent with the report of Büchi, White & Wogan (1965) that the pigments from this mould now characterised by them did not contribute to the toxicity of the mould.

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The fibrinolytic activity of anti-inflammatory drugs

SIR,—Buffered solutions of certain organic acids can bring about the thrombolysis of human plasma clots when incubated with them *in vitro* (von Kaulla, 1962), and some derivatives of biphenylcarboxylic acid (Gryglewski, 1966), *N*-phenylanthranilic acid (Gryglewski & Gryglewska, 1966) and 5-benzyloxysalicylic acid (von Kaulla, 1965a) are highly active fibrinolytic agents.

The formation, deposition and resolution of fibrin in the intercellular space is believed to be a regulating factor in the development of inflammation (Astrup, 1966). Since some non-steroidal anti-inflammatory drugs are acidic it was tempting to check their influence on fibrinolysis. A modification of von Kaulla's (1965b) method was used (Gryglewski, 1966) to test the sodium salts of six well known analgesics (Table 1).

TABLE 1. FIBRINOLYTIC ACTIVITY OF SIX ANTI-INFLAMMATORY DRUGS. THE DISSOLUTION OF HUMAN PLASMA CLOTS AFTER 24 HR INCUBATION AT 37° IN 0·2 M TRIS-BUFFER pH 7·4 CONTAINING DIFFERENT CONCENTRATIONS OF DRUGS.

Compound	The range of fibrinolytic concentrations in mM/litre
Salicylic acid	Trace of activity 200–250
Acetylsalicylic acid	Trace of activity 150–200
Amidopyrine	Trace of activity 100–150
Phenylbutazone	Full activity 9–18
Mefenamic acid	Full activity 3–7
Flufenamic acid	Full activity 1·5–4

A relation between anti-inflammatory and fibrinolytic potency is seen. Winter, Shen & Sarett (1964) compared the anti-inflammatory activity of aspirin, phenylbutazone, flufenamic acid and indomethacin in cotton-pellet induced granuloma in rats. Comparable effects were achieved with aspirin, 150 mg/kg, phenylbutazone, 30 mg/kg, flufenamic acid, 3·3 mg/kg, and indomethacin, 0·4 mg/kg. Unfortunately indomethacin was not available to me but if indomethacin also proves to be a potent fibrinolytic agent the fibrinolytic activity of analgesics could be considered to be an indicator of their anti-inflammatory mechanism of action.

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The effect of pregnancy on the central sympathetic components in angiotensin-induced hypertension

SIR,—Numerous reports have demonstrated that angiotensin has less pressor activity in pregnant than in non-pregnant animals. Mackaness (1959) observed that pregnant rats are insensitive to pressor doses of renin. Also, McCaa, Douglas & Richardson (1966) found the pressor action of angiotensin to be decreased in pregnant dogs. Others found pregnant women resistant to the pressor effects of angiotensin (Chesley, Wynn & Silverman, 1963; Abdul-Karim & Assali, 1961), although renin levels appear to be increased during pregnancy (Brown, Davies, Doak, Lever & Robertson, 1963).

There are also numerous reports which suggest that an intact sympathetic nervous system is required for optimal angiotensin pressor activity, and several laboratories have reported that the peptide may generate increased sympathetic activity by a central mechanism (Buckley, Bickerton, Halliday & Kato, 1963; Benetato, Haulica, Uluitu, Bubuianu, Mocodean, Stefanescu & Suhiciu, 1964; Laverty, 1963; Benelli, Della Bella & Gandini, 1964).

We have observed that the injection of angiotensin into the perfused lateral ventricles of cats anaesthetised with chloralose consistently produces sympathetic activation via a central mechanism (Severs, Daniels, Smookler, Kinnard & Buckley, 1966; Smookler, Severs, Kinnard & Buckley, 1966). We have injected angiotensin, 4 μg, into the perfused lateral ventricles (Bhattacharya & Feldberg, 1958) of approximately 40 cats. Nine of these animals were either in a late stage of pregnancy or were lactating and all were hyporesponsive to the central pressor effect induced by angiotensin. The difference between the mean pressor response of the nine pregnant or lactating cats (19/12 $\pm$ s.e. 2/1) and the mean pressor response of nine cats randomly selected from the non-pregnant animals (58/44 $\pm$ s.e. 5·5) was statistically highly significant ($P = <0{\cdot}001$).

This observation indicates that the decreased pressor response to angiotensin during pregnancy may be due, at least in part, to a decreased sympathetic component in the overall angiotensin effect.

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Rabbit reactivity to cannabis preparations, pyrahexyl and tetrahydrocannabinol

SIR,—Recent work on the chemistry of cannabis constituents and synthesis of tetrahydrocannabinols (Gaoni & Mechoulam, 1965; Taylor, Lenard & Shvo, 1966) will certainly renew an interest in their pharmacological activities, relative potency and stability. Among the biological tests so far proposed for cannabis and related principles, the abolition of the rabbit blink reflex appears to be one of the more sensitive (Gayer, 1928). We believe this test to be suitable for the estimation of one of the central actions of marihuana.

The assay is made in groups of 3–6 rabbits repeatedly injected with a preparation until the blink reflex is completely abolished. In preliminary tests a rough estimate of the potency is made and less reactive animals are discarded. The selected rabbits are restrained in wooden cages, with the head out, and maintained in a noiseless room. The test solution is made in saline with polysorbate 80 and the emulsion slowly injected into the ear vein. Twenty stimuli in each eye are made with a horse hair at 10 min intervals and the number of injections (0·2 ml/kg each) to completely abolish the blink reflex in both eyes is determined. For the estimation of the relative potency of the unknown preparation in terms of a standard, two groups of at least 6 animals each should be employed.

The dried and powdered flowering tops and leaves of the plant cultivated in north-east Brazil or in the neighbourhood of these laboratories were extracted with light petroleum for 4–6 hr. The extract was filtered through activated charcoal, washed with water and evaporated to dryness. The residue was dissolved in acetone and kept in the refrigerator overnight to separate wax constituents. After acetone evaporation the crude resin was dissolved in light petroleum and chromatographed on an alumina column. Elution with light petroleum, light petroleum with benzene, benzene, and benzene with sulphuric ether afforded fractions which were examined in a Beckman DU spectrophotometer at 250 and 280 mμ. Details and properties of the main components are given elsewhere (Valle & Hyppolito, 1964).

The most active fractions obtained after chromatography on alumina columns were those designated Cr19Fr10 and Cr23Fr14. Besides these cannabis preparations, synthetic samples of pyrahexyl and tetrahydrocannabinol (THC) were also assayed.

A sample of cannabis crude resin (0·8 mg/ml) was assayed against THC (0·08 mg/ml) as standard. The results (mg/kg $\pm$ s.d.) were 0·107 $\pm$ 0·013 (6 animals) and 1·143 $\pm$ 0·335 (6 animals) respectively and indicated a relative potency of 0·093 and that the solutions tested did not differ significantly in their activities ($F = 0{\cdot}105$, $P < 0{\cdot}05$). Then, by our procedure, the selected cannabis

resin exhibited about 10 times less activity, on a weight basis, than the sample of synthetic THC, adopted as a reference compound. We have also assayed our fractions Cr19Fr10 and Cr23F14, obtained strictly in the same way from the same batch of starting drug material. Solutions of both preparations, containing 20 μg/ml each, showed similar potencies: 0·031 $\pm$ 0·011 and 0·034 $\pm$ 0·010 mg/kg.

TABLE 1. REPEATED ASSAYS OF A CANNABIS CRUDE RESIN AND OF TETRAHYDROCANNABINOL

Date	No. of animals	Mean and limits of body weight (kg)	Test solution	Activity mg/kg (Mean and limits)	F‡
May 25, 1965	4	2·6 (2·0–3·6)	Can.* (2 mg/ml)	3·19 (2·32–3·50)	
Jan. 28, 1966	4	3·1 (2·7–3·5)	,, ,,	2·99 (2·40–3·60)	0·29
Nov. 9, 1965	3	2·8 (2·6–3·0)	THC† (0·1 mg/ml)	0·16 (0·12–0·19)	
Nov. 17, 1965	4	3·0 (2·6–3·5)	,, ,,	0·09 (0·09–0·12)	
Dec. 10, 1965	6	2·8 (2·5–3·6)	,, (0·08 mg/kg)	0·11 (0·08–0·19)	
Jan. 28, 1966	5	2·7 (2·3–2·9)	,, (0·1 mg/kg)	0·10 (0·06–0·13)	2·16

* Cannabis crude resin dated April 1962: ethanolic solution (10 mg/ml) kept at 4°. Dilution with saline plus polysorbate 80 before using. Animals intravenously injected (0·2 ml/kg) every 10 min until the blink reflex is completely abolished.

† Stock solution in ethanol (10 mg/ml) kept for 5 months in dark glass container at room temperature

‡ Values of F at a probability level of 0·05, not significant.

The sample of pyrahexyl was active at 0·68 $\pm$ 0·29 mg/kg rabbit weight. The results of repeated assays of a cannabis crude resin and of THC are given in Table 1 from which the stability of the preparations used may be deduced.

In conclusion, rabbit reactivity to cannabis, tetrahydrocannabinol and pyrahexyl showed that the ethanolic solutions of these agents maintained their activities for months.

Acknowledgements. We wish to thank Rockefeller Foundation (RF-58217) and the "Fundação de Amparo à Pesquisa do Estado de São Paulo" (Proc. 71/62 & 63/337) for their financial support.

A ten year old sample of Pyrahexyl was kindly supplied by Dr. R. K. Richards, Research Division, Abbott Laboratories, North Chicago, Ill. Tetrahydrocannabinol was obtained through the courtesy of Dr. Milton Joffe and Dr. Francis Morthland, U.S. Defense Office (AROLA).

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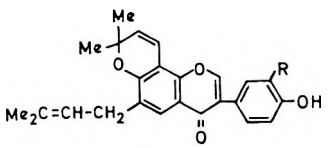
Inhibition of valyl[5] angiotensinamide II by osajin

SIR,—Specific receptors for vasoactive polypeptides have not been demonstrated beyond doubt because of the lack of specific antagonists. Recently, Walaszek & Dyer (1966) offered good but indirect evidence for such receptors.

We now report the antagonism of angiotensinamide II by an isoflavone derivative called osajin (I) isolated from the fruit of *Maclura pomifera* (hedge apples or osage oranges) (Geissman & Hinreiner, 1952) found in the mid-western United States.



I

R = H Osajin
R = OH Pomiferin

Isolated segments of the ileum of guinea-pigs weighing 180–200 g were suspended at 37° in a 10 ml bath of Tyrode solution gassed with oxygen and carbon dioxide. The contractions caused by the various agonists used were recorded on a kymograph. The sensitivity of the preparation to the agonists was first tested, after which osajin was added to the perfusion fluid to give a final concentration of 5 μg/ml. At this point the reactivity to the agonists was reassessed over 2 hr followed by a 2-hr period of recovery. Ten separate experiments were made, five with polypeptides and five with biological amines.

The findings in Fig. 1 demonstrate that osajin antagonised the musculotropic activity of valyl[5] angiotensinamide II but not that of bradykinin or eledoisin. There was no major change in the myotrophic activity of 5-hydroxytryptamine, acetylcholine or histamine.

The mechanism of the antagonist action produced by osajin is not clearly understood. Recently it was reported that valyl[5] angiotensinamide II formed amorphous precipitates with Zn^{++} and Cu^{++} and that perhaps the peptide could

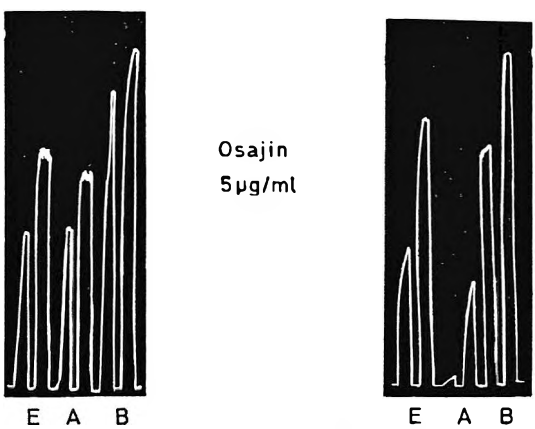


FIG. 1. Influence of osajin on the musculotropic activity of polypeptides. E. Eledoisin, 5 and 10 ng/ml. A. Valyl[5] angiotensinamide II, 10 and 20 ng/ml. B. Bradykinin, 10 and 20 ng/ml.

interact with its receptors in a chelated state (Schwyzer, 1963). The fact that the chelation complex is formed in a 1 to 1 ratio would indicate that the hydroxyl group of the tyrosine residue would be involved. This hydroxyl group has also been found essential for the biological activity of the polypeptide (Bumpus, Khairallah, Arakawa, Page & Smeby, 1961) and also could be involved in the formation of the drug-receptor complex (Walaszek & Dyer, 1966).

The flavonoid compounds have the ability to chelate bivalent metals and it could be possible that osajin irreversibly antagonises the action of valyl[5] angiotensinamide II by complexing a metal which is an integral part of the angiotensin receptor since, like the polypeptide, osajin also contains in its molecular structure a free hydroxyl group.

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Book Review

REMINGTON'S PHARMACEUTICAL SCIENCES, 13th Edition. (RPS XIII.) Pp. xii + 1954 (including Index and over 1000 illustrations). Mack Publishing Company, Easton, Pa., U.S.A., 1965. In Great Britain: John Wiley & Sons, Ltd., London. 212s.

In this new edition of *Remington's Pharmaceutical Sciences* there are 100 chapters, written mostly by separate authors, covering the economic, professional and scientific aspects of pharmacy. The volume is intended both as a text book and as a reference book but as these two functions are clearly separate one wonders what advantage is to be gained by retaining a single mammoth volume. The physical bulk of Remington demands a substantial space on a firm table and this restricts its use as a convenient reference book.

The book is divided into nine parts: Orientation, Physical Pharmacy, Pharmaceutical Manufacturing, Pharmaceutical Chemistry, Pharmaceutical Products, Biological Products, Radiopharmacy, Testing and Analysis, Professional Practice. The layout of material follows the previous editions.

One cannot fail to be impressed by the coverage achieved and the enormous effort involved in the preparation of this book. It is unfortunate however that there is some unnecessary duplication of material particularly in the Physical Pharmacy section. Three different authors have contributed chapters on Surface Activity, Colloidal Dispersion and Emulsification respectively and the same concepts of surface tension are discussed on pages 254 and 284. Gibb's Adsorption theory appears in three different places, pages 257, 273 and 284. On page 286 neither the definition of zeta potential nor Fig. 257 is clear.

The chapter on Rheology (pp. 305–316) would give a student an elementary introduction to the interpretation of flow curves of non-Newtonian materials and emphasises the limitations of "one-point" methods such as the use of the Ostwald viscometer. The latter part of the chapter would have been improved if line diagrams had replaced reproductions from manufacturers' catalogues. It is implied on p. 308 that kinetic energy corrections are required only for kinematic viscosity measurements but not for dynamic viscosity measurements.

In the following chapter on Separation Methods, much of the material in earlier editions is retained, for instance under Filtration one is informed pictorially of the difference between a plain funnel, a ribbed funnel and a hard rubber funnel. This type of catalogue information could well be deleted.

The term "biopharmaceutics" has been coined in the United States for the study of the relationship between chemical and physical properties of a drug and the biological effects observed following administration of the drug in its various dosage forms. In the chapter under this heading, Doluisio and Swintosky discuss the general theory of penetration of drugs through biological membranes based on the lipid solubility of un-ionised drug moieties. The metabolic, storage and transport phenomena which may occur after a drug is administered are briefly discussed together with the factors influencing the route of administration. The factors involved in the design, preparation and evaluation of prolonged action pharmaceuticals are intelligently discussed by Ballard and Nelson although much of the basic information on the nature of membrane permeability duplicates material in the biopharmaceutics section. There are 264 references and tables of proprietary slow release products.

An interesting chapter on the formulation of new drugs in suitable dosage forms is contributed by T. J. Macek. Problems of particle size, crystal form and interaction with excipients are discussed and should alert the student to the kinds of problems he may meet in practice. The section entitled Pharmaceutical Manufacturing (Part III), describes the small scale manufacture of solutions, extracts, powders and tablets. The chapter on ophthalmic solutions includes a discussion on the anatomy and physiology of the eye so that formulation of eye preparations is related to the biological aspects. Details of buffers, viscosity adjustment, preservation and contact lens solutions are given.

Part V has nearly 500 pages and is devoted to Pharmaceutical Products, grouped in relation to their main pharmacological properties and therapeutic uses. Products in the U.S.P., N.F., B.P. and Ph.I. are included, together with explanatory information which is complementary to the official monographs. The comprehensive compilation of pharmaceutical products in this section and in the section on Biological Products make Remington an essential reference book for those engaged in formulation.

Pharmacy students in this country will find certain sections particularly useful as there is no equivalent treatment in British textbooks, e.g. colouring and flavouring agents, tablet coatings, sustained release products, quality control in production and pesticides.

On the title page Remington is described as "A treatise on the manufacturing, standardising, dispensing of pharmaceutical products with biological and chemical properties and tests, assays uses, and doses; also a guide to the legal obligations of the pharmacist and the professional services rendered in helping to maintain community health. . . . A textbook and reference guide for pharmacists, physicians and other medical scientists". This is a fair description.

J. E. CARLESS

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Letters to the Editor

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