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Review Article

Further aspects of the physical chemistry of some non-ionic detergents*

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SIZE, SHAPE AND HYDRATION OF MICELLES

The most interesting properties of detergents in solution are their surface and micellar behaviour and the factors affecting this. Aqueous solutions of non-ionic detergents are colloidal, thus the techniques applied in their study have been similar to those generally used in colloid science.

It has now been accepted that molecules of non-ionic detergents having a polyoxyethylene chain sufficiently large to produce water solubility of the hydrophobic moiety, orientate themselves in micelles with the hydrophobic moiety inside and the glycol chains outside. The glycol chain confers water solubility by trapping water molecules in some way (Goto, Sugano & Koizumi, 1954; Ferguson, 1955). The exact amount of water trapped and the means by which this is effected is conjectural as, until recently, no independent method of measuring the aqueous co-volume of the micelle had been reported. Hydroxonium ions, hydrogen bonding and various arrangements of the water molecules around the ether oxygens or within the glycol structure have been suggested (Chwala & Martin, 1937, 1947; Wurzchmitt, 1950; Trinchieri, 1952; Hsaio, Dunning & Lorenz, 1956; Kehren & Rosch, 1956; Rosch, 1956; Bailey & Callard, 1959; Schick, 1963b). From viscosity and micellar studies, Kushner & Hubbard (1954) estimated that there were 43 molecules of water per polyoxyethylene chain in a micelle of Triton X 100 (n_{10}). Of this number, they suggested 20 molecules were held by hydrogen bonding to the ether oxygens, the rest being physically trapped by the chain. Nakagawa & Inoue (1958) showed the number of hydrating water molecules per oxygen atom of the polyoxyethylene chain increased with chain length. Other workers (Karabinos, Hazdra & Ballun, 1955; Karabinos & Metziger, 1955; Kehren & Rosch, 1956; Reich, 1956; Rosch, 1956; Boehmke & Heusch, 1960), using data from viscosity, polarimetry, and heat of hydration, have given 1, 2, 3 or 4 water molecules per ether oxygen, depending on the chain length and the workers concerned.

We have recently described a method, based on vapour pressure measurements, of estimating the micellar hydration (Elworthy & Macfarlane, 1964). The vapour pressures over gels and concentrated solutions of detergents were measured as a function of detergent concentration and, by a suitable extrapolation procedure, the concentration determined at which the solution had (within experimental error), the same apparent

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vapour pressure as water. The amount of water in this solution was taken as a measure of micellar hydration as it corresponds to the minimum amount necessary to cause separation of the micelles from one another. Although the method is empirical the values obtained for the hydration (w_1) agree well with those derived from the intrinsic viscosities of micelles which are spherical (Equation 2: Elworthy & Macfarlane, 1965).

As with much of the work on non-ionic detergents, most studies of micellar structure have been made with commercial detergents, and much of the conflicting data in the literature is due to the batch variation of only nominally similar compounds (Dunning, 1957; Dwiggens & Bolen, 1961; Dwiggens, Bolen & Dunning, 1960; Boon, Coles & Tait, 1961). As we have already discussed (Elworthy & Macfarlane, 1965), the polymerisation syntheses give rise to products having a range of ethylene oxide chain lengths; a range which varies depending on the experimental conditions. Although claims of "reproducible heterogeneity" have been made (Becher, 1961) these heterogeneous compounds are far from ideal for physicochemical measurement.

The effect of fractionation of these materials on their physical properties has been studied and serves to further illustrate this point (Kushner, Hubbard & Doan, 1957; Stauff & Rasper, 1957). Molecular distillation of a sample of Triton X 100 gave two fractions, one giving a clear solution in water, the other being classed as "insoluble". Further redistillation of the two fractions gave one insoluble and one soluble subfraction from each. From light scattering studies of the two soluble subfractions, the micellar weights (M) obtained were:

	M	n	Aggregation number
Lower distilling fraction ..	208×10^3	8	373
Undistilled	90×10^3	10	139
Higher distilling fraction ..	53×10^3	12	73

(Where n is the mean number of ethylene oxide units per molecule.)

Recombination of the soluble and insoluble fractions gave a molecular weight the same as that of the undistilled compound.

The above values indicate a trend which is found with all surfactants of the polyoxyethylene type at a given temperature: this is that the micellar aggregation number for a given hydrophobic group decreases with increasing polyoxyethylene chain length (Kushner & others, 1957; Nakagawa & Kuriyama, 1957a; Stauff & Rasper, 1957; Nakagawa, Kuriyama & Inoue, 1960; Becher, 1961; Elworthy & Macfarlane, 1962a; 1963; Schick, Atlas & Eirich, 1962). Also, with standard polyoxyethylene chain length, the aggregation number increases with increasing length of the hydrophobic part of the molecule, i.e., by increasing the length of the paraffin chain (Corkill, Goodman & Ottewill, 1961; Kuriyama, 1962a; 1962b).

Several authors (Balmbra, Clunie, Corkill & Goodman, 1962, 1964; Elworthy & Macfarlane, 1963) have found that micellar weight, as determined from light scattering, appears to vary with solute concentration.

TABLE 1. MICELLAR WEIGHTS OF SYNTHETIC NON-IONIC DETERGENTS

Abbreviations C_{16} = n-hexadecyl, n_6 = six ethylene oxide units.

In each entry the micellar weight (M) is given on the upper line, the aggregation number (m) on the second, and the temperature (T) on the lower line. All micellar weights are $\times 10^5$.

Compound							Reference
C_8n_6 ..	M	0·12	0·16	0·20	0·33	0·82	Balmbra & others, 1964. Corkill & others, 1961
	m	30	41	51	82	210	
	T	18	30	40	50	60	
		0·125					
		32					
		25					
$C_{10}n_6$ ..	M	0·31	1·1	2·7	5·6		Balmbra & others, 1964
	m	73	260	640	1,330		
	T	25	35	45	50		
$C_{12}n_6$ ..	M	0·63	0·84	1·8	3·2		Balmbra & others, 1962
	m	140	186	400	710		
	T	15	18	25	30		
	M	6·3	10	18			
	m	1,400	2,220	4,000			
	T	35	42	45			
	M	0·78					Corkill & others, 1961
	m	173					
	T	25					
$C_{14}n_6$ ..	M	15	26	36	56		Balmbra & others, 1964
	m	3,100	5,400	7,500	11,700		
	T	25	30	35	40		
$C_{16}n_6$ ..	M	51	68	84			*Ibid.*
	m	10,500	13,300	16,600			
	T	28	32	34			
	M	12·3	Infinite dilution				Elworthy & Macfarlane, 1963
	m	2,430					
	T	25					
	M	52	Zimm plot	63	Dissymmetry method		*Ibid.*
	m	10,300		12,500			
	T	25		25			
$C_{16}n_7$ ..	M	0·90	1·14	1·37	3·27		Elworthy & McDonald, 1964. Elworthy & Macfarlane, 1963
	m	162	207	250	590		
	T	15	17·5	20	25		
$C_{16}n_8$ ..	M	1·31	1·43	1·6	4·0		Elworthy & McDonald, 1964
	m	220	240	270	670		
	T	15	25	35	37·4		
	M	6·0	16·7				
	m	1,010	2,800				
	T	39·3	44·3				
$C_{16}n_9$	M	1·4	1·8	3·5	6·4	8·8	Elworthy & McDonald, 1964. Elworthy & Macfarlane, 1962a
	m	220	240	550	1,000	1,380	
	T	25	45	50	53·5	57·5	
$C_{16}n_{12}$ ..	M	1·17					Elworthy & Macfarlane, 1962a
	m	150					
	T	25					
$C_{16}n_{21}$ ..	M	0·82					Elworthy & Macfarlane, 1963
	m	70					
	T	25					

Extrapolations of T–C plots (Elworthy & Macfarlane, 1965) do not pass through the CMC, but through a higher concentration C_L, which may be a second association limit. Thus plots of Hc/T against c curve upwards at low concentrations.

For the C_{16} n_6 and C_{16} n_7 detergents, Elworthy & Macfarlane (1963), found that, at 25°, the micellar weights at infinite dilutions were $1·2 \times 10^6$ and $0·33 \times 10^6$ respectively, while at higher concentrations, above C_L,

they were ca. 5×10^6 and 1×10^6 respectively. Balmbra & others (1962) report ca. $5{\cdot}1 \times 10^6$ for $C_{16}n_6$ at 28°. Large dissymmetries develop at finite concentrations.

It is not yet clear if the small micelles first formed at the CMC grow steadily in size and reach a constant value at C_L, or whether the first formed micelles aggregate to form the larger ones.

It is undoubtedly time that a more precise experimental technique than light scattering was used in studying these systems.

Table 1 lists the micellar weights of synthetic non-ionic detergents.

Straight line relationships between the aggregation number and the reciprocal of the number of ethylene oxide units per molecule have been obtained for several detergent series, which would make it possible to predict an aggregation number for a given hydrophobe. Little work has been done on detergents containing ethylene oxide chain lengths such that monomers are formed at high concentrations, but it has been shown for some larger compounds (Schick & others, 1962) that, although there is a decreased aggregation number with increasing polyoxyethylene content, the actual micellar weights were greater than of those compounds with short polyoxyethylene chains. The rise in micellar weight together with a CMC lower than expected, has been attributed to a decrease in the solubility of the ethylene oxide moiety as its molecular weight increased. Unfortunately there is insufficient data to show whether a plot of aggregation numbers vs. ethylene oxide units per molecule passes through a minimum, or merely becomes asymptotic.

The shape of the micelle is a further factor to be determined. The three models most frequently used for micellar aggregates are spheres, discs or rods. It has been suggested that, in dilute solution, small micelles of ionic detergents with molecular weights of 10 to 50×10^3 (Debye & Anacker, 1951; Tartar, 1959) are spherical, whereas large micelles, with aggregate molecular weights of greater than 500×10^3, are rod shaped.

From intrinsic viscosity numbers, sedimentation constants, and light scattering dissymmetry ratios on a range of compounds, Schick & others (1962) claimed a similar pattern for non-ionic detergents, i.e., in the range $M = 45$ to 100×10^3, spheres appear the most probable shape, while for large micelles discs or rod-like shapes are likely. For Triton X 100 ($M = 90{,}000$), Kushner & Hubbard (1954), from light scattering and viscosity work, suggested a spherical micelle and Nakagawa & Inoue (1958) also suggested a spherical micelle for a series of compounds of micellar weight from 40 to 60×10^3 on the basis of constant effective specific volume. Becher (1961), on the other hand, pointed out that micelles of this size are so small that the light scattering dissymmetry values (Z_{45} close to unity) do not give much idea about the shape of the micelles. His calculations, based on surface area measurements and the hydrated volume of the micelle, suggested that, for micelles containing molecules with fairly long ethylene oxide chain lengths (n_{15}–n_{30}), the assumption of a rod-like micelle was to be favoured. However, Becher

made no allowance for the variation of hydration with polyoxyethylene chain length.

The use of viscosity in the determination of micellar shape is complicated by the presence of the co-volume of water in the micelle. Viscosity has therefore been used mainly in conjunction with other techniques. Raphael (1954) showed that the viscosity of a given molar concentration of an adduct decreased with decreasing ethylene oxide chain length, passed through a minimum, then rose again. This pattern of behaviour is perhaps illustrated more clearly by studies on a hexadecyl series of synthesised surfactants at 25° (Elworthy & Macfarlane, 1962, 1963; Elworthy & McDonald, 1964); hexadecyl-n_6 and hexadecyl-n_7 gave intrinsic viscosities of 22·7 and 9·7 respectively, and hexadecyl-n_8 gave the smallest value, 3·8, after which the values rose steadily; hexadecyl-n_9, 4·3; up to 6·8 c.c./g for hexadecyl-n_{21}, the largest compound studied.

In the first interpretation of these results, micellar shape factors for the lower members of the series were calculated by assuming maximum and minimum values for their hydration; the minimum level was set by assuming hexadecyl-n_6 to be unhydrated and hexadecyl-n_{21} hydrated and spherical, while the upper limit was obtained by letting hexadecyl-n_{15} and hexadecyl-n_{21} be spherical and hydrated and drawing an asymptote to a plot of g water/g detergent (calculated directly from the intrinsic viscosity) against the number of ethylene units in the detergents.

Correlation of these intrinsic viscosity results with the values obtained for micellar hydration by the vapour pressure method has, however, led to a re-appraisal of these shape factors (Elworthy & Macfarlane, 1964). The micelles formed by the first two members of the series, hexadecyl-n_6 and hexadecyl-n_7, were asymmetric and hydrated to the extent of 0·39 and 0·44 g water/g detergent respectively, but compounds containing more than eight ethylene oxide units per molecule formed spherical micelles, the increase in intrinsic viscosity with increasing chain length being accounted for by the increase in micellar hydration as the series was ascended.

CONFIGURATION OF THE ETHYLENE OXIDE CHAINS

Because of the lack of knowledge of micellar dimensions, much of the early speculation about the configuration of the oxyethylene chain in the micelle was based on viscosity and X-ray diffraction data of the glycols themselves. Lower molecular weight chains, i.e., with not more than ten ethylene oxide units, appeared to exist in the regular extended "zig-zag" or *trans* structure, whereas longer chains occurred as the more condensed "meander" or *gauche* form (Curme & Johnston, 1952). Coils, and "crumpled helical" and "highly convoluted" structures, particularly for very long chains, have also been suggested (Sauter, 1933; Hibbert & Lovell, 1940; Bailey & Callard, 1959).

Rosch (1957) has claimed that the "meander" form is the most likely in aqueous solution; a spiral chain being formed due to carbon-oxygen dipoles (forces which are said to be accentuated in water due to the presence of an onium structure) where each oxygen approached closely

and was attracted to a carbon atom separated from it by three other carbon atoms and one oxygen atom. Despite its being sterically possible, the "meander" form has been described as somewhat "crowded" (Becher, 1962) and may require stabilisation by a chain of hydrogen bond-linked water molecules between the adjacent chain oxygens. The polyoxyethylene chain might even be in a random mixture of both configurations, *trans* and *gauche*, as found in dioxane solution by dipole and infra-red measurements (Uchida, Kurita, Koizumi & Kubo, 1956; Kuroda & Kubo, 1957, 1959).

Although not strictly comparable with micellar structure, application of the Gibbs equation to surface tension data has shown the cross-sectional area of the polyoxyethylene chain in solution to be the controlling factor in the packing of the molecules in the interface (Hsaio & others, 1956; Schick, 1962; Elworthy & Macfarlane, 1962b), but the increment of area per ethylene oxide unit decreased as the number of ethylene oxide units per molecule increased. Coupled with a similar effect for surface potential measurements (Schick, 1963b), this has been interpreted as a coil formation of the polyoxyethylene chain in the aqueous phase, the size of the coils increasing as the chain lengthened without reaching complete randomness even with the longest chains studied by this technique (containing up to one hundred ethylene oxide units).

An expanding coil structure has also been described from measurements of micellar volume (Elworthy & Macfarlane, 1962a, 1964; Schick & others, 1962; Macfarlane, 1963). These measurements showed that, at 25°, the polyoxyethylene chains were contracted to well below their fully extended length but the dimensions calculated for these coils were dependent on the volume assigned to the hydrophobic core of the micelle, that of an oil droplet being considered the most probable.

EFFECTS OF TEMPERATURE

Solubility of detergent compounds, even in dilute solutions, is often limited to certain temperature ranges; only between a lower Kraft Point and an upper cloud point can a solution exist without phase separation. Thus, outside this temperature range, the detergent loses many of its most important properties. Little work appears to have been done on the Kraft Point as few of the non-ionic detergents exhibit this phenomenon, but the cloud point has been studied extensively.

As has been described, the micellar properties are influenced by the hydrophilic properties of the molecule, which, in turn, appear to be a function of the length of the polyoxyethylene chain and the amount of water "trapped". The cloud point has generally been assumed to be caused by a thermal dehydration of the micelle reaching a stage where the hydrophilic properties of the micelle were reduced to such a level that the detergent was thrown out of solution. Above the cloud point a phase rich in detergent separated out of solution (Maclay, 1956; Nakagawa & Tori, 1960), leaving a concentration of detergent in the co-existing aqueous phase which appeared to be dissolved as a monomer dispersion with virtually no micelles present.

Here, as with the other solution properties, altering the length of the polyoxyethylene chain for a given hydrophobe affected this behaviour; the cloud point rising on increasing the number of ethylene oxide units in the chain (Raphael, 1954; Elworthy & McDonald, 1964). For ether-linked adducts of constant glycol chain length, it fell with increasing size of the hydrophobic group in ascending a homologous series. Though cloud points are sometimes found to be constant over a fairly wide range of concentration (Maclay, 1956), some have been shown to vary with concentration (Balmbra & others, 1962), and certain discrepancies in the literature have been attributed to comparison at different concentrations.

Provided the compound is pure, the cloud point of polyoxyethylene ether adducts appears as a sharp change in turbidity, but as the hydrophilic chain is lengthened, the cloud point exceeds 100°. To measure these high cloud points, pressure has been increased to obtain higher temperatures, or a substance which lowered the cloud point to a more easily measured temperature has been added. The values obtained are then extrapolated to zero additive concentration (Maclay, 1956).

For non-ionic detergents, clouding is a reversible procedure, the solution becoming clear again when the temperature drops below the critical point. Clouding is used to follow the polymerisation of ethylene oxide onto a hydrophobic group as the phenomenon can be adapted to give a quick idea of the progress of the reaction. Steele & Berger (1956) have characterised non-ionic detergents from plots of cloud points against apparent density. These plots showed that values for a particular type of detergent fell into a characteristic band which could be used as a method of identification.

In contrast to many ionic detergents (Kuriyama, 1962b, 1962c), the micellar weights of polyoxyethylene type non-ionic detergents increase with rising temperature (Dwiggens & Bolen, 1961; Balmbra & others, 1962), the rate of increase becoming greater as the cloud point is approached (Kuriyama, 1962a, 1962b). It is therefore necessary to consider the effects of temperature on micellar weight and structure.

Kuriyama (1962b) measured by light scattering the micellar weights and apparent second virial coefficients of a methoxydodecyl polyoxyethylene glycol over a range of temperatures up to the cloud point. He found the solute-solvent interaction decreased with increase in temperature, the rate of the interaction decreasing more rapidly as the cloud point was approached. He concluded that the phase separation occurred not because of a simple dehydration of micelles, but rather as a result of the decreased interaction between water and very large micelles.

Diffusion-viscosity experiments on a methoxypolyoxyethylene octanoate (Nakagawa, Inoue, Tori & Kuriyama, 1958) showed the increase in micellar weight with temperature was accompanied by an increase in hydration with the development of asymmetry near the cloud point.

The behaviour of micellar weight with temperature appears to depend on the balance of the hydrophobic and hydrophilic parts of the detergent molecules. Balmbra & others (1962, 1964) have reported an exponential rise of micellar weight with increasing temperature for $C_{10}n_6$, $C_{12}n_6$ and

$C_{16}n_6$. For C_8n_6 there was a more rapid increase at temperatures above 45° than below this temperature.

Elworthy & McDonald (1964) investigated the consequences of varying the hydrophilic part of the monomer on the temperature effects; they studied three synthesised detergents; hexadecyl-n_7, hexadecyl-n_8 and hexadecyl-n_9, by light scattering, viscosity, and vapour pressure techniques. Below temperatures of 22°, 33° and 45°, respectively, for these compounds, a slow increase in micellar weight with increasing temperature was found, but above these transition temperatures (designated T_t) there was an exponential rise in micellar weight which was accompanied by the development of much micellar asymmetry.

It can be envisaged that a compound containing a long polyoxyethylene chain would give a slow increase of micellar weight with temperature, while one containing a short chain would give the exponential increase only.

Below the transition temperature the micelles were reasonably spherical with the polyoxyethylene chains contracted to about 50% of their fully extended lengths but above T_h the micelles appeared to resemble prolate ellipsoids of revolution. The increasing micellar size was accompanied by an increase in micellar hydration, at least up to T_h. This finding, although somewhat surprising, fits in with the marked extension of the polyoxyethylene chains as more space is available for trapping water in the expanded micelles.

The use of commercially produced detergents in studies of the temperature variation of micellar weight obscures the sharpness of the transition at T_h, due presumably to the range of polyoxyethylene chain lengths present.

EFFECT OF ELECTROLYTES

One of the often quoted advantages for non-ionic detergents is their stability even in relatively high electrolyte concentration: this, however, does not mean that they are unaffected by the presence of electrolytes (Maclay, 1956; Bolle, 1960; Kuriyama, 1962b). Early work by Doscher, Myers & Atkins (1951) showed that sodium chloride strikingly raised the viscosity and turbidity of aqueous solutions of non-ionic detergents, whereas calcium chloride had a much smaller effect. The surface tension increased gradually with increase of the ratio of calcium chloride to detergent, whereas it decreased with sodium chloride. These effects were interpreted as sodium chloride "salting out" the non-ionic detergent whereas calcium salts "salted in" the hydrated calcium ions co-ordinating in some way with the ether oxygens. The isolation of a detergent-calcium chloride complex further substantiated this theory. Maclay (1956), and Bolle (1960), in an examination of the factors affecting solubility of non-ionic adducts, showed that, in dilute solution, the lowering of the cloud point by electrolytes was a linear function of the ionic strength, and the observed salting out effect for alkali metals and multivalent cations was roughly in the order of decreasing ion hydration. Kuriyama (1962b), however, pointed out that although calcium chloride exhibited a smaller effect on the cloud point when compared with sodium

chloride at the same ionic strength, if compared at the same molar strength it was equally effective. The effect of electrolytes on the cloud points is obviously integrated with the complex network affecting micellar structure, hydration, and aggregation number at different temperatures, but whether one can obtain an indication of the effects of electrolytes at lower temperatures from their effects on cloud points is doubtful, particularly in the light of studies on the effects of salts on micellar weights at different temperatures.

At 30°, the addition of sodium chloride to a methoxy polyoxyethylene dodecyl ether (n_{12}) gave a linear increase in micellar weight with increase of salt concentration whereas with calcium chloride, although the micellar weight increased initially, it was nearly constant over a range of 0·5–1·0M. At 50°, the effect of sodium chloride was greater than at the lower temperature, but at this higher temperature equimolar concentrations of calcium chloride were equal to or more potent than those of sodium chloride in their effect on micellar weight. To further complicate the issue, other workers have claimed that addition of electrolytes at a given temperature does not necessarily give an increase in micellar weight (Mankowich, 1955; Becher, 1962; Schick & others, 1962). Becher (1962), working on a series of commercial dodecyl ethers, showed that although a slight increase in micellar weight was found for the lower members of the series, the larger detergents showed, if anything, a slight decrease in micellar weight. Schick & others (1962), have shown similar effects with an octadecyl ether series but the decrease in micellar weight with added electrolytes did not appear until a much longer polyoxyethylene chain was present in the adduct (n_{100}). It has been suggested (Schick & others, 1962) that the increased length of the polyoxyethylene chain enhanced the adsorption of electrolyte to such an extent that it increased the overall solubility of the detergent, but it would appear that much depends on the particular series being studied.

In a further attempt to elucidate salting out mechanisms, the effect of added electrolyte on the CMC has been examined, and here the order of effectiveness in lowering the CMC followed a decrease in lyotropic number (Hsaio & others, 1956; Becher, 1962; Schick, 1962); the effect being more pronounced with the more hydrophilic longer oxyethylene chain compounds. A change in the lyotropic number of the anions had a larger effect in lowering the CMC than that of the cations. Surface moment and surface tension studies have suggested that the addition of sodium chloride produces a collapse of the ethylene oxide coil structure, in a manner similar to that observed for the glycols by Bailey & Callard (1959). Addition of a proton donor or acceptor also effects the CMC (Schick, 1963b), e.g., for a nonylphenyl adduct (n_{15}).

Solvent	CMC
0·86M HCl	150 μmol/litre
Water	110 μmol/litre
0·86M NaOH	80 μmol/litre

Hsaio & others (1956) have attributed this shift to oxonium ion formation in the polyoxyethylene chains; added protons increase the ionic character of the non-ionic detergents and consequently the CMC is increased, and vice versa. This has been taken by these authors to indicate that non-ionic detergents are weakly cationic in aqueous solution, and, by Becher (1962), to explain the electrolyte effects in the terms of a positive double layer in the outer layer of the micelle. Conductivity experiments made to clarify this last point (Becher, 1963) appear to be affected by the presence of polymerisation catalysts in the substance.

Mankowich (1955) has suggested that the lowering of the CMC in the presence of electrolyte does not occur because of any specific electrical property of the electrolyte but rather to its effect on the solvent. On the basis that sucrose would markedly lower the activity without affecting the electrical properties, the CMC has been measured in different concentrations of sucrose solutions (Becher, 1963). The values obtained showed little change, and together with the magnitude of the effects at given electrolyte concentrations, and the apparent specificity of the anions, they did not point to an effect on activity as a likely explanation.

Perhaps the most complete picture of electrolyte effects was that obtained from micellar and surface studies by Schick & others (1962) and Schick (1962; 1963b). On the basis of their results, these authors have postulated the following mechanism for "salting out" non-ionic detergents. The salting out of neutral molecules depends on the concentration and ionic radii of the electrolyte, and the dielectric constant of the non-electrolyte (Bailey & Callard, 1959). Small hydrated ions (low lyotropic number) are more effective in salting out than large hydrated ions (high lyotropic number). This was shown to follow with the non-ionic detergents studied; the salting out increased with increasing electrolyte concentration and with decreasing radii of hydrated ions. The hydration of the anions was more important than that of the cations. Thus, Schick & others claimed that there was first a removal of hydrogen bonded water molecules from the ether oxygens of the ethylene oxide chain by the increased electrolyte concentration, and second, that the extent of the dehydration of the ethylene oxide chain was determined by the closeness of approach of the cations to the ether oxygens, but this was partially counteracted by the tendency of the counter anions to be hydrated.

Undoubtedly, work with synthetic detergents is necessary for a better understanding of salt effects.

SOLUBILISATION

The ability of detergent solutions to dissolve organic compounds which are insoluble, or only slightly soluble in water, is one of their most striking properties. It is termed solubilisation. For ionic detergents, it is generally believed that with non-polar hydrocarbons uptake occurs into the interior of the micelle; for partially miscible polar compounds, such as octanol and phenols, there is adsorption on the micelle surface with the hydrocarbon inside and the polar group of the solubilisate in the aqueous phase. Water soluble polar substances, such as glycerol and

certain dyes, which are insoluble in hydrocarbons, are thought to be adsorbed on to the exterior of the micelles (Harkins, Mittelman & Corrin, 1949; Alexander & Johnson, 1950). With non-ionic detergents of the polyoxyethylene type there is another possible mode—that of incorporation into the polyoxyethylene chain part of the micellar structure.

In the study of solubilisation, as with electrolytes, much of the work and the conclusions from it have been based on the effects of solubilisates on cloud points (Weiden & Norton, 1953; Livingstone, 1954; Maclay, 1956; Bolle, 1960). The cloud point of Triton X 100 (Maclay, 1956) has been shown to be significantly raised by anionic detergents and aliphatic hydrocarbons, but, as the polarity of the solubilisate was increased by the introduction of double bonds or polar constituents, the effect on cloud points was much less marked. Aromatic and polar aliphatic additives caused the cloud points to be sharply decreased (Maclay, 1956).

The mode of incorporation may account for the difference in the effects of such compounds on cloud points. Dodecane is insoluble in a polyoxyethylene glycol and will be taken into the hydrophobic core of micelles; benzene, however, is miscible with the hydrophobic moiety, with polyoxyethylene glycols, and with concentrated aqueous solutions of the glycols, and may thus be partially present in the glycol structure, thereby decreasing the hydrophilic properties of the micelle.

Weiden & Norton (1953) attributed the lowering of the cloud point by benzene and phenol to a decrease of the hydrophilic properties of the micelles, but Livingstone (1954) suggested pH might also play an important part in the effect of phenols although this latter idea was refuted by Maclay (1956). Such effects have been used in attempts to develop methods of assay for non-ionic detergents in aqueous solution, either by ascertaining the length of the oxyethylene chain of the adduct or by gaining an idea of its properties in solution (Davis, Wattman & Speel, 1955; Karabinos, 1955).

The solubilisation of phenol and its analogues has also been said to occur by binding of the phenolic hydroxyl groups with the ether oxygens of the polyoxyethylene chains by hydrogen bonding (Mulley & Metcalf, 1956; Patel & Kostenbauder, 1958). Higuchi & Lack (1954), and Higuchi & Guttman (1956), have reported complex formation of phenolic substances with polyoxyethylene groups, and it has been observed that many phenolic substances were readily dissolved in concentrated solutions of polyoxyethylene glycols (Nakagawa, 1954; 1956).

The solubilisation of iodine (Osol & Pines, 1952; Allawala & Riegelman, 1953a; 1953b; Bartlett & Schmidt, 1957; Brost & Krupen, 1957; Hugo & Newton, 1963), and various dyes (Sheppard & Geddes, 1945; Rigg & Liu, 1953; Nakagawa, 1958; Riegelman, Allawala, Hrenoff & Strait, 1958), with their concomitant spectral changes, have similarly been the subject of varying opinion.

Examination of phase changes (Nakagawa & others, 1963) and construction of phase diagrams (McBain & Marsden, 1947; Winsor, 1960; Mulley, 1961; Balmbra & others, 1962; Mulley & Metcalf, 1962) in the presence of different solubilisates have also been made to try to obtain a

clearer insight to this problem. Solubilisation and some pharmaceutical aspects have been well reviewed by Mulley (1964).

Light scattering measurements have been made in a study of the effect of n-decane and n-decanol on the micellar weights of two methoxypolyoxyethylene glycol monodecyl ethers by Nakagawa, Kuriyama & Inoue, 1960). These authors have shown that the micellar weights increased with the addition of solubilisates up to the saturation limit of the solubilisate, the increase originating not only from the simple incorporation of solubilisate molecules into the existing micelle, but also from the number of detergent molecules in the micelle increasing with added solubilisate. The solubilisation process was therefore accompanied by a reconstitution of the micelles in a manner analogous to that concluded from other techniques (Nakagawa & Inoue, 1957; Brady & Huff, 1958); the rate of increase in micellar weight being more rapid with shorter oxyethylene chain compounds.

The concept of "no solubilisation when there are no micelles" (though addition of a third component may lower the concentration at which micelles are formed), is also illustrated by this work. Peaks in the plots of R_{90} (Rayleigh's ratio) vs. concentration below the CMC for the pure compound, increased as the ratio of solubilisate to detergent was raised, and were interpreted as being caused by solubilisate or a solubilisate-surfactant complex, ejected into the aqueous phase by dilution below the CMC, forming small droplets which increased the amount of scattered light. The concentration at which this peak started to develop gradually decreased with the addition of solubilisate in a manner similar to that found for ionic detergents (Ralston & Eggenberger, 1948; Grieger, 1949).

Increasing the temperature of ternary systems, such as n-decane or n-decanol (Kuriyama, 1962a) in an aqueous non-ionic detergent solution, gave a rise in micellar weight, but the patterns of increase varied from each other, reflecting the different effects on the cloud point of the two compounds.

Addition of an anionic (Kuriyama, 1962c) to a non-ionic detergent gave a mixed micelle, the micellar weight showing virtually a straight line relationship between the micellar weights (at 30°) of the two pure compounds in aqueous solution, and increase of micellar weight of the non-ionic detergent with temperature elevation was progressively suppressed by increasing the amount of ionic detergent added, until a stage was reached when the micellar weight decreased on heating. Micellisation in mixed systems has been studied by Corkill & others (1961) and by Corkill, Goodman & Tate (1964).

SURFACE ACTIVITY

The adsorption of surface-active agents at an interface causes a reduction in the surface tension; the surface tension dropping rapidly with increasing concentration until a constant minimum value is reached at the CMC. The general theory of adsorption of surface-active compounds at the interfaces is that it is the result of the opposing tendencies of the hydrophobic and hydrophilic groups, the former tending to escape from

the surface of the water, provided the external phase is sufficiently sympathetic, the latter group tending to pull the molecule into solution.

With non-ionic detergents of the type under consideration it has been shown that for a given hydrophobic group, the surface tension at the air-water interface above the CMC increases with increasing glycol length, the actual value of the surface tension being a property of the hydrophobe in question (Fineman, Brown & Myers, 1952; Raphael, 1954; Schick, 1962), and the temperature; the surface tension decreasing with rise in temperature over moderate ranges (Corkill & others, 1961).

Measurement of the surface tension as a means of determining the CMC has the advantage that the solution may be examined without the addition of any extra component which may itself effect the value obtained. Application of the Gibbs Equation to measurements below the CMC allows the area per molecule at the interface to be calculated.

Surface ageing, though said to be absent with shorter compounds (Mulley & Metcalf, 1962) has been reported by Schick (1962; 1963b), Corkill & others (1961), and others, particularly as the concentration of the solution approached the CMC. Times allotted by different workers for the attainment of equilibrium vary widely and although a time of some 3 hr can be expected for a solution around 10^{-6} molar on the basis of time taken for diffusion of the molecules to the interface (Ward, 1949), times up to several days have been required for hexadecyl-n_6 at concentrations less than the CMC (Corkill & others, 1961; Elworthy & Macfarlane, 1962b). This, with other results, suggests that other factors are involved, e.g., orientation of the polyoxyethylene chains.

A further difficulty which arises in attempting to make measurements of surface activity at low concentrations (10^{-5}–10^{-6}) is that adsorption of detergents on to glass or other vessels may significantly affect the concentration. The equilibration of glassware and solutions by the method suggested by Pethica & Matijevic (1958) is therefore necessary.

Other methods of observing adsorption have been reported (Hsaio & Dunning, 1955; Kuno & Abe, 1961; 1962) but with much variation in the results, arising from the interfacial conditions and the mode of analysis; selective adsorption of shorter length ethylene oxide chain adducts often influencing the results to different extents.

Interfacial tensions between aqueous solutions of polyoxyethylene ether adducts and such substances as vegetable oils, benzene and toluene, gave minima with ethylene oxide contents of 14–20 units (Wrigley, Smith & Stirton, 1957; Raphael, 1954), and 7–12 units respectively (Cohen, 1951), while hexane followed a pattern similar to that existing at the air-water interface.

For single species *p*-t-octylphenoxyethoxyethanol, Crook, Fordyce & Trebbi (1963) have shown that there was a preferential adsorption of the shorter ethylene oxide chain length compounds at the air-water interface. At the iso-octane : water interface, the compounds with longer ethylene oxide chains were preferentially adsorbed.

Elworthy & Florence (1964) studied synthetic detergents with branched hydrocarbon chains at the air-water interface. An equation of the type

$(\pi-\pi_0)$ $(A-A_0)$ = constant fitted the results above $\pi = 2$ dynes/cm (π=surface pressure, A = area/molecule).

Lipophilic substances, such as carbon black, gave a Langmuir type adsorption for a range of nonyl-phenol adducts (Kuno & Abe, 1962), but with a hydrophilic powder, such as calcium carbonate, the type of adsorption depended on the compound examined and was very sensitive to change in chain length (Kuno & Abe, 1961). Compounds with less than four and more than nine ethylene oxide units per molecule showed no adsorption on to calcium carbonate, those with five and six gave Langmuir type, whereas seven and seven point five ethylene oxide units showed multilayer adsorption.

References*

(* References cited by Elworthy & Macfarlane, 1965, are not re-cited here.)

Alexander, A. E. & Johnson, P. (1950). "*Colloid Science*", p. 628, Oxford.
Allawala, N. A. & Riegelman, S. (1953a). *J. Amer. pharm. Ass. Sci. Ed.*, **42**, 267–275.
Allawala, N. A. & Riegelman, S. (1953b). *Ibid.*, **42**, 396–401.
Bailey, F. E. & Callard, R. W. (1959). *J. appl. Polymer Sci.*, 56–62.
Balmbra, R. R., Clunie, J. S., Corkill, J. M. & Goodman, J. F. (1964). *Trans. Faraday Soc.*, **60**, 979–985.
Bartlett, P. G. & Schmidt, W. (1957). *Appl. Microbiol.*, **5**, 355–359.
Becher, P. (1961). *J. Colloid Sci.*, **16**, 49–56.
Becher, P. (1962). *Ibid.*, **17**, 325–333.
Becher, P. (1963). *Ibid.*, **18**, 196–197.
Boehmke, G. & Heusch, R. (1960). *Fette u. Seif.*, **62**, 87–91.
Bolle, J. (1960). *3rd Intern. Congr. Surface Activity, A*, 294–298.
Boon, P. F. G., Coles, C. L. S. & Tait, M. (1961). *J. Pharm. Pharmacol.*, **13**, *Suppl.*, 200*T*–204*T*.
Brady, A. P. & Huff, H. (1958). *J. phys. Chem.*, **62**, 644–649.
Brost, G. A. & Krupen, F. (1957). *Soap Chem. Spec.*, **33**, 93–107.
Chwala, A. & Martin, A. (1937). *Melliand Textilber*, **18**, 725–728.
Chwala, A. & Martin, A. (1947). *Textilrdsch.*, 147–161.
Cohen, M. (1948). *C. R. Acad. Sci., Paris*, **226**, 1366–1368.
Cohen, M. (1951). *Mem. Services, chim. etat.*, **36**, 207–210.
Crook, E. H., Fordyce, D. B. & Trebbi, G. F. (1963). *J. phys. Chem.*, **67**, 1987–94.
Curme, G. O. & Johnston, F. (1952). "*Glycols*", p. 180, New York: Reinhold Publ. Corp.
Davis, B. F., Wattman, K. E. & Speal, H. C. (1955). *Soap & Sanit Chem.*, **31**, 73–77.
Debye, P. J. & Anacker, E. W. (1951). *J. phys. Chem.*, **55**, 644–655.
Doscher, T. M., Myers, G. E. & Atkins, D. C. (1951). *J. Colloid Sci.*, **6**, 223–235.
Dunning, H. N. (1957). *Chem. Eng. Data.* 2, No. 1, 88–92.
Dwiggens, C. W., Bolen, R. J. & Dunning, H. N. (1960). *J. phys. Chem.*, **64**, 1175–1178.
Dwiggens, C. W. & Bolen, R. J. (1961). *Ibid.*, **65**, 1787–1788.
Elworthy, P. H. & McDonald, C. (1964). *Kolloid Z.*, **195**, 16–23.
Elworthy, P. H. & Macfarlane, C. B. (1964). *J. chem. Soc.*, 311–315.
Elworthy, P. H. & Macfarlane, C. B. (1965). *J. Pharm. Pharmacol.*, **17**, 65–82.
Ferguson, L. N. (1955). *J. Amer. chem. Soc.*, **77**, 5288–5289.
Fineman, M. N., Brown, G. L. & Myers, R. J. (1952). *J. phys. Chem.*, **56**, 963–966.
Grieger, P. F. (1949). *Ann. N.Y. Acad. Sci.*, **51**, 827–835.
Harkins, W. D., Mittelman, R. & Corrin, M. L. (1949). *J. phys. Colloid Chem.*, **53**, 1350–1361.
Hibbert, H. & Lovell, E. L. (1940). *J. Amer. chem. Soc.*, **62**, 2140–2143.
Higuchi, T. & Lack, J. L. (1954). *J. Amer. pharm. Ass., Sci. Ed.*, **43**, 465–470.
Higuchi, T. & Guttman, D. (1956). *Ibid.*, **45**, 659–663.
Hsaio, L. & Dunning, H. N. (1955). *Ibid.*, **59**, 362–366.
Hugo, W. B. & Newton, J. M. (1963). *J. Pharm. Pharmacol.*, **11**, 731–741.
Karabinos, J. V. (1955). *Soap Chem. Spec.*, **31**, 50–51.
Karabinos, J. V., Hazdra, J. J. & Ballan, A. T. (1955). *Euclides*, **15**, 145–149.
Karabinos, J. V. & Metziger, M. C. (1955). *Trans. Ill. Stat. Acad. Sci.*, **48**, 118–127.

Kehren, M. & Rosch, M. (1956). *Melliand Textilber*, **37**, 434.
Kuno, H. & Abe, R. (1961). *Kolloid-Z.*, **177**, 40–44.
Kuno, H. & Abe, R. (1962). *Ibid.*, **181**, 70.
Kuriyama, K. (1962b). *Ibid.*, **181**, 144–149.
Kuriyama, K. (1962c). *Ibid.*, **183**, 68–71.
Kushner, L. M. & Hubbard, W. D. (1955). *J. Colloid Sci.*, **10**, 428–435.
Kuroda, Y. & Kubo, M. (1957). *J. Polymer Sci.*, **26**, 323–327.
Kuroda, Y. & Kubo, M. (1959). *Ibid.*, **36**, 453–459.
Livingstone, H. K. (1954). *J. Colloid Sci.*, **9**, 365–368.
Macfarlane, C. B. (1963). Ph.D. Thesis, Glasgow.
Maclay, W. N. (1956). *J. Colloid Sci.*, **11**, 272–285.
Mankowich, A. M. (1955). *Indust. Engng Chem.*, **47**, 2175–2181.
Mulley, B. A. (1964). *Advances in Pharmaceutical Science*, Vol. 1. p. 87–194, New York: Academic Press.
Mulley, B. A. & Metcalf, A. D. (1956). *J. Pharm. Pharmacol.*, **8**, 774–780.
Nakagawa, T. (1954). *J. pharm. Soc., Japan*, **74**, 1116–1119.
Nakagawa, T. (1958). *Ann. Report Shionogi, Res. Lab.*, **8**, 886–890.
Nakagawa, T. (1956). *J. pharm. Soc., Japan*, **76**, 1113–1115.
Nakagawa, T. & Inoue, H. (1957a). *J. chem. Soc., Japan*, **78**, 636–640.
Nakagawa, T. & Inoue, H. (1958). *Ibid.*, **79**, 345–348.
Nakagawa, T., Inoue, H., Tori, E. & Kuriyama, K. (1958). *Ibid., Ind. Chem. Sect.*, **79**, 1194–1198.
Nakagawa, T. & Tori, K. (1960). *Kolloid-Z.*, **168**, 132–139.
Nakagawa, T., Shinoda, K., Tammanshi, B. & Isamura, T. (1963). "*Colloidal Surfactants*", p. 135, London: Academic Press.
Osol, A. & Pines, C. C. (1952). *J. Amer. pharm. Ass., Sci. Ed.*, **41**, 289–293.
Patel, N. R. & Kostenbauder, H. B. (1958). *Ibid.*, **47**, 289–293.
Pethica, B. A. & Matijevic, E. (1958). *Trans. Faraday Soc.*, **54**, 1382–1389.
Ralston, A. W. & Eggenberger, D. C. (1948). *J. Amer. chem. Soc.*, **70**, 983–987.
Raphael, L. (1954). *Proc. 1st Intern. Congr. Surface Activity*, Vol. 1. p. 36–49, Paris.
Reychler, A. (1914). *Kolloid-Z.*, **13**, 252–254.
Riegelman, S., Allawala, N. A., Hrenoff, M. K. & Strait, L. A. (1958). *J. Colloid Sci.*, **13**, 208–217.
Rigg, M. W. & Liu, F. W. J. (1953). *J. Amer. Oil Chem. Soc.*, **30**, 14–17.
Rosch, M. (1956). *Kolloid-Z.*, **147**, 78–81.
Rosch, M. (1957). *Ibid.*, **150**, 153–156.
Sauter, E. (1933). *Z. phys. Chem.*, **21**, 161–185.
Sheppard, S. E. & Geddes, A. L. (1945). *J. chem. Phys.*, **13**, 63–65.
Stauff, J. & Rasper, J. (1957). *Kolloid-Z.*, **151**, 148–154.
Steele, A. B. & Berger, L. D. (1956). *Soap and Sanit. Chemicals*, **32**, 48–50.
Tartar, H. V. (1959). *J. Colloid Sci.*, **14**, 115–122.
Trinchieri, G. (1952). *Amer. Dystuff. Reporter*, **41**, 729–740.
Uchida, T., Kurita, Y., Koizumi, N. & Kubo, M. (1956). *J. Polymer Sci.*, **21**, 313–322.
Ward, A. F. H. (1949). *Surface Chemistry*, p. 55, London: Butterworths.
Weiden, M. H. J. & Norton, L. B. (1953). *J. Colloid Sci.*, **8**, 606–610.
Winsor, P. A. (1960). *Chem. & Ind.*, 632–644.

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Research Papers

Actions of hemicholinium and triethylcholine on responses of guinea-pig colon to stimulation of autonomic nerves

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Hemicholinium caused a failure of responses of the guinea-pig colon to stimulation of extrinsic parasympathetic and sympathetic nerves: failure of the parasympathetic responses occurred the more readily. In seven of 20 experiments, hemicholinium did not block the inhibitory response to sympathetic nerve stimulation but the latent period between the start of a train of stimuli and the first sign of relaxation was prolonged after repeated stimulation in the presence of hemicholinium in all 20 experiments. Triethylcholine caused failure of responses of the guinea-pig colon and a reduction of responses of rabbit ileum to sympathetic nerve stimulation. Choline sometimes reversed the blocking action of hemicholinium on responses to parasympathetic and sympathetic nerve stimulation.

THE action of hemicholinium on the responses to stimulation of sympathetic adrenergic nerves was examined by Chang & Rand (1960) to test for the presence of a cholinergic link in the release of noradrenaline. They found that hemicholinium indeed caused a failure of response. Others have observed a failure (Brandon & Rand, 1961; Wong & Long, 1961), or reduction (Bentley & Sabine, 1963; Birmingham & Wilson, 1963; Bevan & Su, 1964) of sympathetic responses, however, there are reports that hemicholinium is ineffective (Wilson & Long, 1959; MacIntosh, 1959; Gardiner & Thompson, 1961; Bentley, 1962; Long & Highgenboten, 1964).

The only tissue in which the effects of hemicholinium have been tested on responses to both cholinergic and adrenergic nerve stimulation is the dog bladder (Wong & Long, 1961). These authors found that the contractions of the bladder in response to stimulation of the hypogastric (adrenergic) nerve failed with lower frequencies of nerve stimulation and smaller doses of hemicholinium than did the contractions induced by stimulation of the pelvic (cholinergic) nerves.

Recently, Huković showed us how to prepare the guinea-pig isolated colon with both sympathetic and parasympathetic nerves. It was decided to test the action of hemicholinium on the responses of the colon to stimulation of each nerve.

Triethylcholine acts like hemicholinium in producing failure of response to stimulation of cholinergic nerves at a sufficiently high rate (Bowman & Rand, 1961), and it has been shown that it impairs the synthesis of acetylcholine (Bull & Hemsworth, 1963). Therefore experiments were also made with triethylcholine.

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Finally, we wish to report a few additional experiments using preparations of sympathetically innervated rabbit intestine.

Methods

Huković's preparation of the isolated guinea-pig colon with both the sympathetic and parasympathetic extrinsic nerves intact is essentially similar to the preparation of the dually innervated rabbit colon described by Garry & Gillespie (1955). Adult male guinea-pigs were killed by a blow on the head and bled out. The nerves were identified and dissected free from the surrounding fascia. As long lengths as possible were removed together with 3 to 4 cm of the terminal colon. The colon was suspended anal end down in McEwen's (1956) solution at 32° in a 50 ml bath, bubbled with 95% oxygen and 5% carbon dioxide. Bipolar stimulating electrodes of the type described by Burn & Rand (1960) were placed on the nerves as far away from the colon as possible, in order to reduce current spread and to allow free movement of the tissue. Stimuli were given from an electronic stimulator generating rectilinear pulses. In every instance the voltage was adjusted to be supramaximal, usually 10 V was used, 6 to 8 V giving a maximal response. Other details of the parameters of stimulation are given in Results. Movements of the colon were recorded using a frontal writing lever with a magnification of 8 times and exerting a tension of 1·5 g. A few observations were made with guinea-pig ileum, without extrinsic nerves, in Tyrode solution in a 10 ml bath. Segments of rabbit colon and ileum were used with sympathetic, but not parasympathetic nerves. They were set up in a 50 ml bath of McEwen's solution at 37°. The writing lever had a magnification of 4 times and exerted a tension of 3·5 g.

The drugs used were hemicholinium dibromide (Aldritch Chemical Co.), triethylcholine chloride (Ward Blenkinsop), choline chloride, acetylcholine chloride, noradrenaline bitartrate, nicotine acid tartrate, atropine sulphate, hyoscine hydrobromide, guanethidine sulphate and hexamethonium bromide. The amounts stated refer to these salts.

Results

RESPONSES OF THE COLON TO NERVE STIMULATION

Stimulation of the pelvic nerve produced a contraction which rapidly reached a peak and was not sustained (Figs 1 and 2), occasionally a small component of relaxation was seen (Fig. 2), which was presumably due to the inclusion of stray sympathetic fibres in the electrodes. The threshold pulse duration was 200 μsec and the threshold frequency was 5/sec. The maximal response was obtained by stimulation with pulses of 2 msec duration at a rate of 50/sec in a train lasting 10 or 20 sec. This could be repeated at 2 min intervals without any decline in response during more than 4 hr.

Stimulation of the sympathetic nerve caused the colon to relax, and this response often lasted for a minute or more after stimulation had ceased (Figs 1 and 2). The threshold pulse width and frequency, and the

parameters of stimulation giving maximal relaxation, were the same as those for the pelvic nerve. Constant responses to regular periods of stimulation of the sympathetic nerve (2 msec, 50/sec for 10 sec every 2 min) could be obtained for more than 7 hr.

There was a difference in the latency of the responses to stimulation of the pelvic and sympathetic nerves. The contraction caused by the pelvic nerve started immediately the stimulation was begun, but the sympathetic relaxation started only after an appreciable latent period (2 or 3 sec). The persistence of the response during stimulation differed between the two nerves. As noted above, the contraction to pelvic nerve stimulation was not sustained, and if the train of stimulation was continued the contraction began to decline after about 10 sec, and had often fallen back to the baseline by 20 sec. However, the sympathetic relaxation was remarkably well sustained. For example, during stimulation at 50/sec for 30 min the colon remained relaxed; the tone recovered when the stimulation was stopped, and then the colon responded again to stimulation as well as it had done before.

Acetylcholine in a concentration of 0·02 μg/ml regularly caused contraction to about the same height as the maximal contraction to pelvic nerve stimulation. Noradrenaline (0·1 μg/ml) regularly caused relaxation.

EFFECTS OF ATROPINE, HYOSCINE, HEXAMETHONIUM AND GUANETHIDINE

Atropine or hyoscine in a concentration of 1 μg/ml blocked completely the responses to pelvic nerve stimulation and to acetylcholine, but were without effect on the responses to sympathetic stimulation.

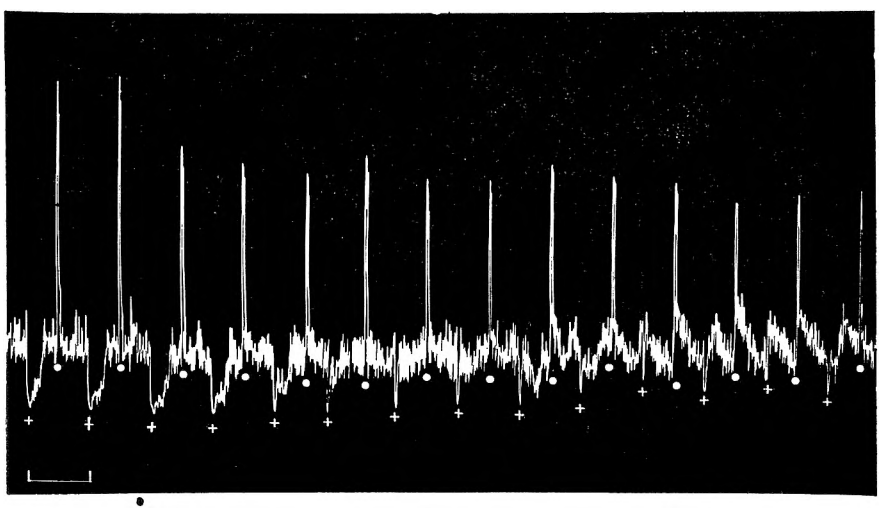

FIG. 1. Responses of guinea-pig colon to alternate stimulation of pelvic nerve (at white dots) and of sympathetic nerve (at +). Each nerve was stimulated with 2 msec pulses at 50/sec for 10 sec every 4 min. Guanethidine (2 μg/ml) almost abolished the inhibitory responses to sympathetic stimulation and reduced contractions to pelvic nerve stimulation.

Guanethidine (1 to 2 μg/ml) reduced the response to sympathetic nerve stimulation and the blockade was almost complete after 30 min (Fig. 1). This blockade persisted after washing out the guanethidine, but was reversed by dexamphetamine. The responses to pelvic nerve stimulation were reduced by guanethidine (Fig. 1), but were restored after washing it out.

Hexamethonium (1 to 5 μg/ml) blocked the contractions produced by pelvic nerve stimulation but was without effect on responses to sympathetic stimulation (Fig. 2).

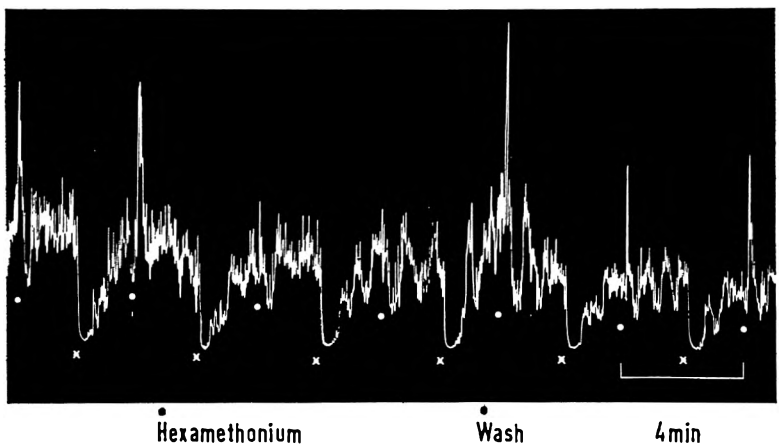

FIG. 2. Guinea-pig colon: nerve stimulation as in Fig. 1. Hexamethonium (5 μg/ml) blocked the contractions to pelvic nerve stimulation.

EFFECTS OF HEMICHOLINIUM AND TRIETHYLCHOLINE ON RESPONSES TO PELVIC NERVE STIMULATION

Hemicholinium in concentrations of 20 to 100 μg/ml caused a gradual failure of the contractions in response to pelvic nerve stimulation in 13 experiments. In one experiment 50 μg/ml did not cause failure, and in two experiments 10 μg/ml of hemicholinium were without action. There was little or no correlation between the concentration and the time taken for the contractions to fail. Table 1 gives the total number of pulses applied until failure developed with stimulation at 50/sec for 10 sec every 2 min. The responses did not recover after washing out the hemicholinium from the bath.

Triethylcholine (100 to 200 μg/ml) caused an immediate blockade of the responses to pelvic nerve stimulation, which were restored on washing out the bath. This effect can possibly be attributed to blockade of the parasympathetic ganglia, since a lower concentration of triethylcholine (10 to 50 μg/ml) blocked the contractions of ileum caused by nicotine (6 μg/ml). The contractions of ileum caused by acetylcholine were not affected by triethylcholine (200 μg/ml) present in the bath for 2 min. Triethylcholine caused a slow contraction of ileum and of colon in a concentration of 300 μg/ml.

EFFECTS OF HEMICHOLINIUM AND TRIETHYLCHOLINE ON RESPONSES TO SYMPATHETIC NERVE STIMULATION

Hemicholinium (20 to 100 μg/ml) caused a slowly developing failure of the extent of the relaxation produced by sympathetic nerve stimulation in 13 out of 20 experiments. The shortest time for complete failure of the inhibitory response, with stimulation at 50/sec for 20 sec periods every 4 min, was 58 min (Fig. 3). The degree of stimulation necessary to cause

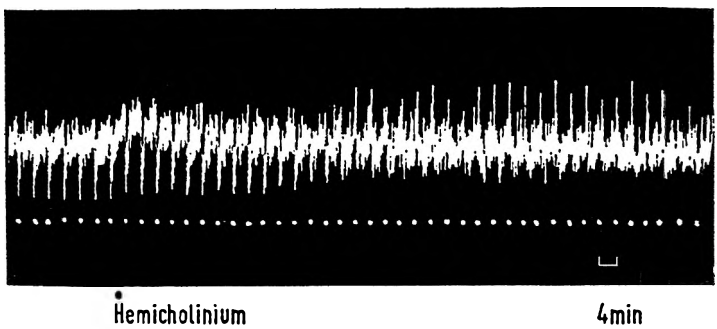

FIG. 3. Guinea-pig colon. Stimulation of sympathetic nerves with 2 msec pulses at 50/sec for 20 sec every 4 min at white dots. Hemicholinium (20 μg/ml) caused a reduction in inhibitory responses and the appearance of a motor response.

failure varied greatly. In some experiments prolonged periods of continuous stimulation lasting for up to 30 min were necessary to produce failure. However, even this was not always successful. If there were no definite signs of a reduction in response within 6 hr of adding hemicholinium the experiment was terminated. There was no consistent relationship between the concentration of hemicholinium and the number of pulses given until complete failure of response (Table 1).

TABLE 1. NUMBER OF PULSES APPLIED TO THE NERVES UNTIL THE RESPONSE FAILED COMPLETELY

Pelvic nerve		Sympathetic nerve	
Concentration of hemicholinium (μg/ml)	Total number of pulses $\times 10^3$	Concentration of hemicholinium (μg/ml)	Total number of pulses $\times 10^3$
20	80	20	14
40	10·5	20	56·5
50	10	40	16
50	15·5	50	43
50	18	50	48
50	22	50	50
50	24	50	51·5
50	30	50	86
50	37	50	95·5
50	48	50	100
50	50	75	30
75	9	100	32
75	15·5	100	36

In every experiment the latent period between the start of a train of stimuli and the first sign of relaxation was prolonged after repeated stimulation in the presence of hemicholinium. This occurred even in

preparations in which the extent of the inhibitory response was not diminished by hemicholinium. Sometimes the prolongation of the latent period was so marked that the relaxation only began at the end of a 20 sec train of stimulation. Nevertheless, experiments in which the extent of the delayed relaxation was the same as in the control period have been counted as ones in which no failure was produced. The prolongation of the latent period was the first effect observed, even in experiments in which relaxation ultimately failed completely.

In seven of the experiments in which the relaxations were blocked by hemicholinium a transient motor response occurred on stimulation of the sympathetic nerve (Fig. 3). Sometimes, at first, this was followed by relaxation, and then the relaxor component slowly failed (Fig. 4). It seemed unlikely that the motor response was due to stimulation of cholinergic fibres for the following reasons. It persisted after hemicholinium had caused a failure of contractions to pelvic nerve stimulation, as shown in Fig. 4, and it was unaffected by atropine or hyoscine in concentrations up to 10 μg/ml. The threshold frequency for eliciting the motor response differed from that for the usual responses of the two automatic nerves; it did not appear at less than 20/sec. The pulse width for eliciting it was almost the same as for the usual responses.

Washing out the hemicholinium did not result in restoration of inhibitory responses when these had been lost or replaced by motor responses.

Noradrenaline had the same action after failure was produced with hemicholinium as it had before.

Triethylcholine in concentrations of 100 to 250 μg/ml caused a slowly developing failure of responses to sympathetic stimulation in five out of seven experiments. The relaxations partially returned on washing out triethylcholine. A motor response, similar to that seen in some experiments with hemicholinium, appeared in one experiment.

EFFECTS OF HEMICHOLINIUM AND TRIETHYLCHOLINE ON PREPARATIONS WITH BOTH NERVES

In two experiments in which the pelvic and sympathetic nerves to the colon were stimulated alternately a parallel failure of the two responses was seen. Thus, in the experiment illustrated in Fig. 4, the contractions caused by pelvic nerve stimulation and the relaxations caused by sympathetic stimulation were diminished at about the same rate. In this experiment a motor response to sympathetic stimulation was observed. In three experiments in which the two nerves were stimulated alternately the responses to sympathetic stimulation failed more slowly and in two experiments they did not fail.

The latent period between the beginning of a train of sympathetic stimulation and the first appearance of the response was prolonged after hemicholinium, but, with parasympathetic stimulation, there was little prolongation of the latent period, the failure being manifested from the start as a diminution in the height of the contraction.

It was not possible to do experiments with triethylcholine using both sympathetic and parasympathetic nerves, because the ganglion blocking

activity of triethylcholine resulted in an immediate blockade of responses to pelvic nerve stimulation.

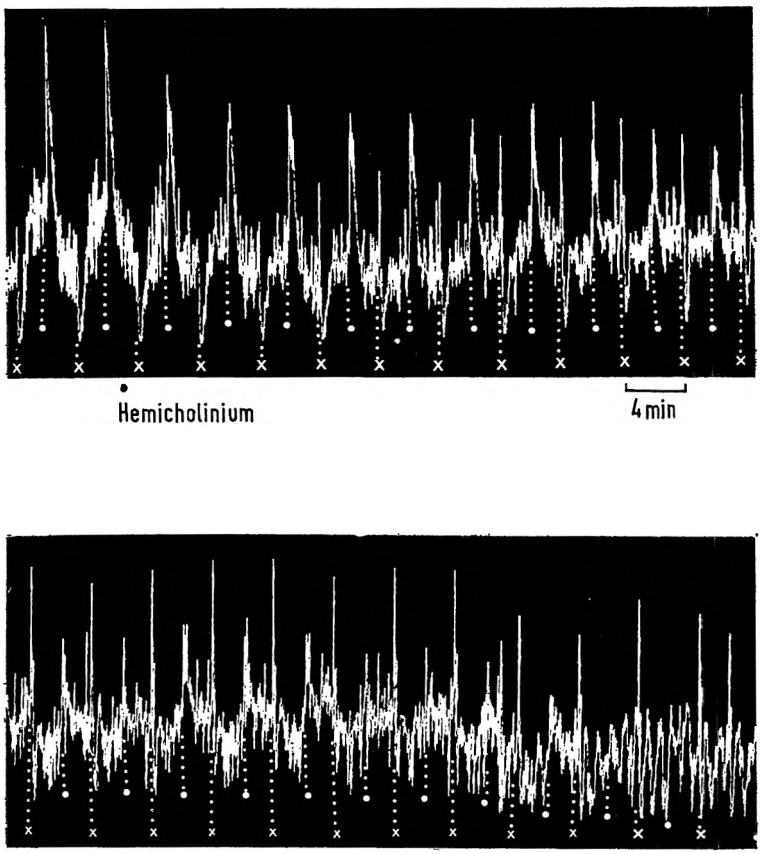

FIG. 4. Responses of guinea-pig colon to alternate stimulation of pelvic nerve (at white dots) and sympathetic nerve (at ×) using the same parameters of stimulation as in Fig. 1. The lower record is continuous with the upper. Hemicholinium (20 μg/ml) caused simultaneous failure of contractions to pelvic nerve stimulation and relaxations to sympathetic nerve stimulation: the relaxations were replaced by a motor response.

ACTIONS OF CHOLINE AFTER HEMICHOLINIUM AND TRIETHYLCHOLINE

Choline caused some degree of reversal in half of the experiments, but it was relatively ineffective where exposure to hemicholinium had been prolonged. It was more effective in restoring responses to pelvic nerve stimulation than those to sympathetic nerve stimulation, but this may be attributed to the longer exposure to hemicholinium which was required to produce failure of sympathetic responses. Fig. 5 illustrates an experiment showing a clear restoration of the inhibitory response to sympathetic stimulation by choline after failure had been produced in the presence of hemicholinium.

Choline enhanced the rate of recovery of the responses to sympathetic nerve stimulation which occurred on washing out triethylcholine.

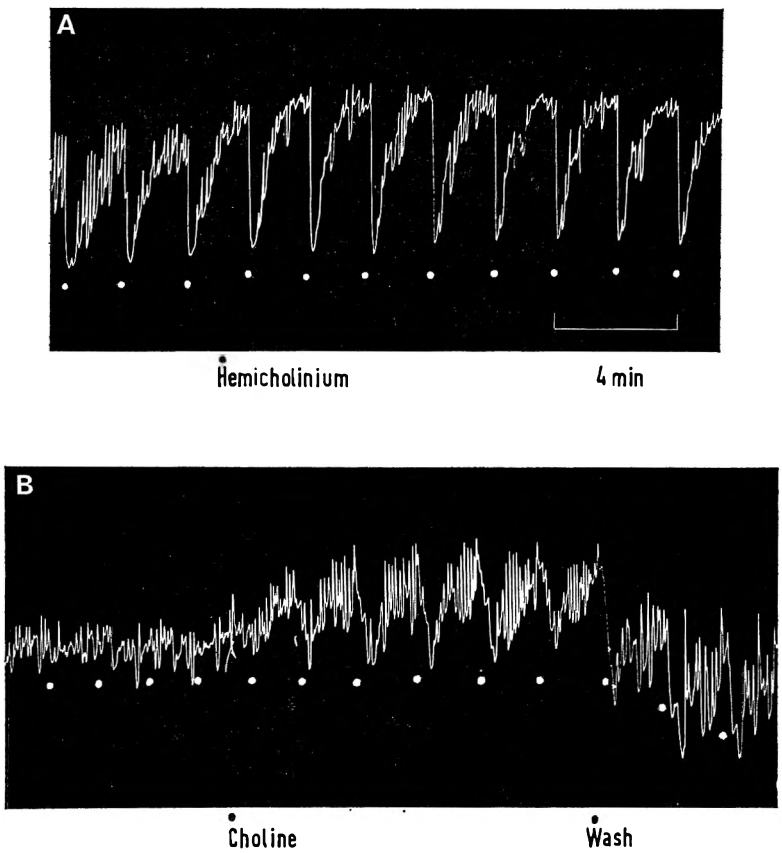

FIG. 5. Responses of guinea-pig colon to sympathetic nerve stimulation, with 2 msec pulses at 50/sec for 10 sec every 2 min at white dots. In A, hemicholinium (50 μg/ml) was added to the bath. The regime of stimulation was continuous between A and B. In B, 335 min later, the inhibitory responses were absent, then they were partly restored by choline (100 μg/ml).

ACTION OF HEMICHOLINIUM AND TRIETHYLCHOLINE ON SYMPATHETIC RESPONSES IN RABBIT COLON AND ILEUM

Chang & Rand (1960) found that hemicholinium produced a failure of the inhibitory responses to sympathetic nerve stimulation in the rabbit colon. However, Bentley (1962) reported that the inhibitory responses of rabbit ileum were not affected by 100 μg/ml of hemicholinium when the nerve was stimulated with 50 pulses/sec for 20 sec every 2·5 min for up to 45 min. It seemed possible that Bentley's finding may have been due to insufficiently vigorous stimulation. Our colleague, M. D. Day

(1963), therefore made experiments with rabbit ileum. His findings are as follows:

"Hemicholinium (50–200 μg/ml) appeared to cause a slight impairment of the responses to sympathetic nerve stimulation in 4 out of 6 preparations tested. However, in only 2 experiments did hemicholinium produce a greater impairment of nerve function than was observed in control segments taken from the same rabbits and subjected to the same amount of sympathetic nerve stimulation. The impairment of response to sympathetic nerve stimulation produced by hemicholinium was of slow onset and was increased when the nerves were stimulated either at a high frequency (50 or 100 pulses/sec) or continuously for prolonged periods (1–9 minutes). However, in no case did the impairment result in a complete abolition of the inhibitory response to sympathetic nerve stimulation even after 3 hours contact with hemicholinium."

Chang & Rand (1960) did not make any observations on the effect of choline after hemicholinium. This omission has now been remedied. Fig. 6 shows an increase in the relaxations which have become partly reduced in the presence of hemicholinium after adding choline to the bath. In the absence of hemicholinium, choline never resulting in greater relaxations, and high concentrations (200–400 μg/ml) caused reduction in responses to sympathetic nerve stimulation.

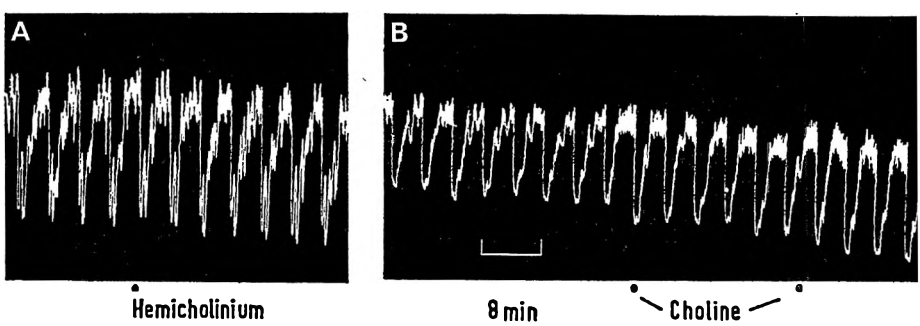

FIG. 6. Responses of rabbit colon to stimulation of sympathetic nerve with 2 msec pulses at 50/sec for 30 sec every 4 min. In A, hemicholinium (100 μg/ml) was added to the bath, and 132 min later, in B, the relaxations were less. Then choline (50 μg/ml), added at the two dots, increased the relaxations.

Triethylcholine caused a reduction in inhibitory responses to sympathetic nerve stimulation in rabbit colon and in ileum (Fig. 7).

Discussion

The responses of the guinea-pig colon to stimulation of the pelvic nerve and the sympathetic nerve closely correspond to those reported for the rabbit colon by Garry & Gillespie (1955) in all except one respect, namely the relationship between the response and the frequency of stimulation. In the rabbit colon, the threshold frequencies of stimulation were

1 every 2 sec for the pelvic and 5/sec for the sympathetic nerve; the maximal responses were obtained with 10/sec for the pelvic and 100/sec for the sympathetic nerve. However, the guinea-pig colon responded to stimulation of either nerve at a threshold frequency of 5/sec, and a frequency of 50/sec gave maximal responses. Our observations confirm Huković's findings (personal communication).

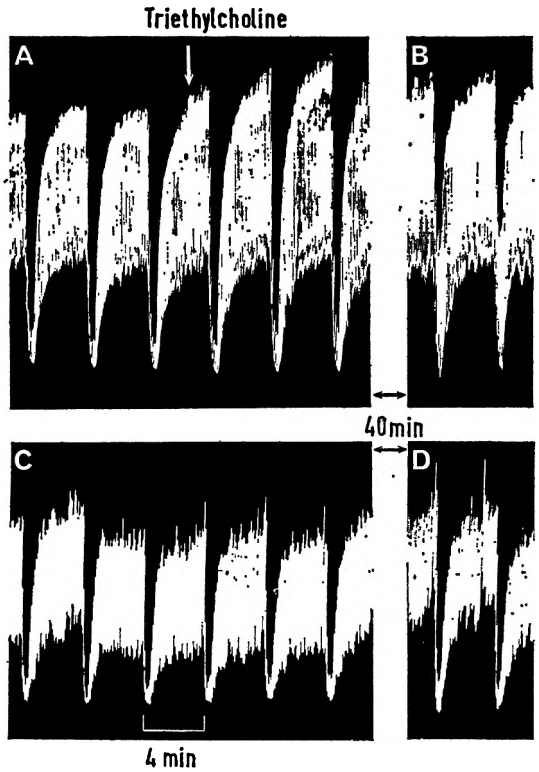

FIG. 7. Responses of rabbit ileum to sympathetic nerve stimulation with 2 msec pulses at 50/sec for 30 sec every 4 min. The records in the upper and lower rows are from two adjacent segments from the same rabbit set up at the same time in twin baths. The preparation in the upper row was treated with triethylcholine (400 μg/ml), the other preparation served as a control. Between A and B, and between C and D, 40 min elapsed during which two 12 min periods of continuous stimulation at 50/sec were given. This caused no impairment of the inhibitory response in the absence of triethylcholine, but there was a substantial reduction in response in its presence.

It was fortunate for our purposes that the guinea-pig colon responded maximally to exactly the same stimulation applied to either nerve, since it allowed a direct comparison of their relative susceptibilities to the blocking action of hemicholinium. In general, the responses to stimulation of the parasympathetic nerve are more easily and more regularly blocked than those of the sympathetic nerve. The difference in the effects of hemicholinium on response to the two nerves may be explained by the observations on the initial responses. Thus, pelvic nerve stimulation

produced a transient contraction which faded during the continued application of a train of stimuli, but sympathetic stimulation produced a response which recovered slowly after stimulation and which persisted throughout continuous stimulation of 30 min.

In some experiments a motor response was observed on stimulation of the sympathetic nerve when the relaxation had been partly or completely blocked by hemicholinium. It is unlikely that the motor response was obscuring the inhibitory response since it was so brief, and at first it was followed by inhibition. This motor response was not due to stimulation of cholinergic fibres, and differed from the motor response produced under certain circumstances by stimulation of the sympathetic nerves to the rabbit intestine (Gillespie & Mackenna, 1961; Day & Rand, 1961). Munro (1953) sometimes observed a contraction of guinea-pig intestine to periarterial nerve stimulation which was not blocked by atropine, anti-adrenaline or antihistamine drugs.

The responses of the guinea-pig colon to stimulation of the pelvic nerve involved cholinergic transmission at two junctions: the ganglionic synapse and the postganglionic endings. Transmission in the ganglionic synapses is easily blocked by hemicholinium (MacIntosh, Birks & Sastry, 1956), and it is possible that the greater sensitivity to blockade of the pelvic nerves to the colon is due to the presence of the ganglionic synapse: the facility of blockade of the responses of the guinea-pig vas deferens to hypogastric nerve stimulation has been explained in this way (Bentley & Sabine, 1963; Birmingham & Wilson, 1963). Armaly, Whinery & Long (1963) reported that there was no difference in the rate of development or the extent of the blockade of ocular response produced by stimulation of the pre- and postganglionic ciliary nerves in the cat. However, some postganglionic cholinergic nerves may be relatively resistant to hemicholinium since high doses of hemicholinium and vigorous stimulation are required to cause failure of the responses of the rat bladder to stimulation of the pelvic nerves (Huković, Rand & Vanov, 1964). Furthermore, the contractions of the guinea-pig ileum produced by Paton's method of transmural stimulation showed no signs of failure in the presence of either hemicholinium or triethylcholine (B. Hemsworth, unpublished observations), although it is believed that this stimulation excites postganglionic cholinergic nerves (Paton, 1955).

The blocking action exerted by hemicholinium on transmission is thought to be due to the depletion of acetylcholine from reserves in the nerve endings, as a result of the impaired resynthesis being inadequate to replace the amounts released by repeated stimulation (MacIntosh, Birks & Sastry, 1956; Lewartowski & Bielecki, 1963). The reversal of hemicholinium-induced blockade by choline provides good evidence that the failue is due to impaired synthesis of acetylcholine, since the inhibition of synthesis *in vitro* is prevented by choline (Gardiner, 1961), and it is believed that hemicholinium acts by competing with choline for a membrane transport system (see Schueler, 1960 and MacIntosh, 1961). Bevan & Su (1964) observed a reduction in contractions of the rabbit isolated pulmonary artery elicited by sympathetic nerve stimulation, but they

maintained that this was no evidence for a cholinergic step being involved because choline did not cause a restoration of contractions. However, Long (1961) in a brief note, gave the opinion that reversal of hemicholinium blockade of autonomic nerves with choline was less effective and required larger doses than does reversal of blockade of a somatic motor nerve. Our findings support this conclusion. We have been successful on occasions in overcoming the depression of sympathetic responses with choline, and we have occasionally been unsuccessful in overcoming depression of parasympathetic responses. There was no qualitative difference in the efficacy of choline in reversing the hemicholinium on either nerve.

An explanation that has been put forward to account for the failure of sympathetic responses caused by hemicholinium is that the sensitivity of the tissue to direct electrical stimulation is reduced at the same time (Bentley & Sabine, 1963; Bevan & Su, 1964). Our finding was that noradrenaline had the same actions after sympathetic failure as before, therefore we cannot support the explanation of reduced sensitivity in the instance of the guinea-pig ileum.

The comment we wish to make on the present findings is the same as that made by Chang & Rand (1960). It is possible that hemicholinium acts in one way at sympathetic nerve endings and in a different way at cholinergic nerve endings, but if a single mechanism is responsible the following argument holds. Either hemicholinium causes transmission failure by interfering with acetylcholine synthesis, in which case it provides evidence for a cholinergic link at sympathetic nerve endings, or its actions are unrelated to acetylcholine, in which case a new explanation must be found for the blockade of repeatedly stimulated motoneurones, preganglionic nerves and parasympathetic postganglionic nerves. In the light of other evidence (recently reviewed by Burn & Rand, 1964) the first alternative has much to recommend it.

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References

Armaly, M. F., Whinery, R. L. & Long, J. P. (1963). *Arch. int. Pharmacodyn.*, **145**, 89–96.
Bentley, G. A. (1962). *Brit. J. Pharmacol.*, **19**, 85–98.
Bentley, G. A. & Sabine, J. R. (1963). *Ibid.*, **21**, 190–201.
Bevan, J. A. & Su, C. (1964). *Ibid.*, **22**, 176–182.
Birmingham, A. T. & Wilson, A. B. (1963). *Ibid.*, **21**, 569–580.
Bowman, W. C. & Rand, M. J. (1961). *Ibid.*, **17**, 176–195.
Brandon, K. W. & Rand, M. J. (1961). *J. Physiol.*, **157**, 18–32.
Bull, G. & Hemsworth, B. A. (1963). *Nature, Lond.*, **199**, 487–488.
Burn, J. H. & Rand, M. J. (1960). *J. Physiol.*, **150**, 295–305.
Burn, J. H. & Rand, M. J. (1965). *Ann. Rev. Pharmacol.*, In the press.
Chang, V. & Rand, M. J. (1960). *Brit. J. Pharmacol.*, **15**, 588–600.
Day, M. D. (1963). Ph.D. Thesis. London University.
Day, M. D. & Rand, M. J. (1961). *Brit. J. Pharmacol.*, **17**, 245–260.
Gardiner, J. E. (1961). *Biochem. J.*, **81**, 297–303.
Gardiner, J. E. & Thompson, J. W. (1961). *Nature, Lond.*, **191**, 86.
Garry, R. C. & Gillespie, J. S. (1955). *J. Physiol.*, **128**, 557–576.
Gillespie, J. S. & Mackenna, B. R. (1961). *Ibid.*, **156**, 17–34.
Hukovič, S., Rand, M. J. & Vanov, S. (1965). *Brit. J. Pharmacol.* In the press.

Lewartowski, B. & Bielecki, K. (1963). *J. Pharmacol.*, **142**, 24–30.
Long, J. P. (1961). *J. med. pharm. Chem.*, **4**, 505–510.
Long, J. P. & Highgenboten, C. L. (1964). *Arch. int. Pharmacodyn.*, **149**, 385–392.
MacIntosh, F. C. (1959). *Can. J. Biochem. Physiol.*, **37**, 343–356.
MacIntosh, F. C. (1961). *Fed. Proc.*, **20**, 562–568.
MacIntosh, F. C., Birks, R. I. & Sastry, P. B. (1956). *Nature, Lond.*, **178**, 1181.
Munro, A. F. (1953). *J. Physiol.*, **120**, 41–52.
Paton, W. D. M. (1955). *Ibid.*, **127**, 40–41P.
Schueler, F. W. (1960). *Int. Rev. Neurobiol.*, **2**, 77–97.
Wilson, H. & Long, J. P. (1959). *Arch. int. Pharmacodyn.*, **120**, 343–352.
Wong, K. C. & Long, J. P. (1961). *J. Pharmacol.*, **133**, 211–215.

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Some pyrrolidines and azacycloheptanes related to reversed esters of pethidine

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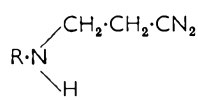
The preparation of some *N*-substituted 3-phenylpyrrolidin-3-ols and their derivatives is described, and the hot-plate analgesia activities in mice of these compounds and related azacycloheptane derivatives reported. The effect of changes in ring size upon the analgesic activities of 4-phenylpiperidine derivatives is discussed.

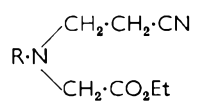
THE aim of this work was to investigate the effect of ring contraction and expansion upon the analgesic activities of derivatives of 4-phenylpiperidine. Although several five- and seven-membered ring analogues of pethidine and related compounds are known (see Table 3), the comparative pharmacology of a trio of compounds, uniform in all respects except ring size, has not previously been reported.

Chemistry

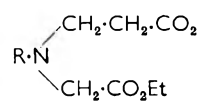
The *N*-substituted 3-pyrrolidones (IV) required for the synthesis of the pyrrolidines (VI) were prepared by a Dieckmann reaction, sodium hydride serving as the cyclisation reagent. The acyclic precursors (III) were made by alkylating the secondary amino-nitriles (I) (from benzylamine or phenethylamine and acrylonitrile) with ethyl bromoacetate followed by acid-catalysed ethanolysis of the resultant ester nitriles (II). These reactions were based on methods used in the



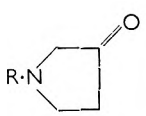
(I)



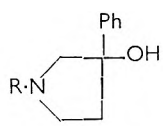
(II)



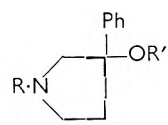
(III)



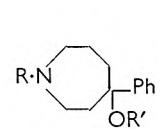
(IV)



(V)



(VI)



(VII)

R = (a) Ph·[CH_2]$_2$ (b) Ph·CH_2 (c) Ph·CO·[CH_2]$_2$

synthesis of 1-methyl-3-pyrrolidones (Cavalla, Davoll, Dean, Franklin, Temple, Wax & Winder, 1961). The pyrrolidone (IVa), with lithium phenyl, gave the tertiary alcohol (Va) which was esterified by acid anhydride-pyridine mixtures and converted to the methyl ether (VIa; R′ = Me) by hot methanol-sulphuric acid. Under the same reaction conditions corresponding 4-phenylpiperidin-4-ols formed analogous products, whereas the seven-membered-ring analogue (VIIa; R′ = H)

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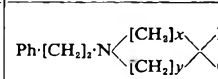
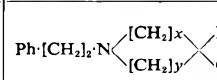
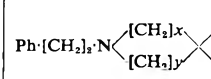
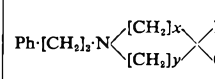
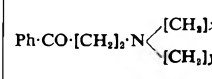
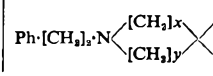
underwent elimination (Casy & Birnbaum 1964). The Mannich bases (Vc and VIIc; R′ = H) were obtained by an exchange reaction between (2-benzoylethyl)trimethylammonium iodide and the appropriate secondary base (V and VII; R = R′ = H) (formed by catalytic debenzylation of corresponding *N*-benzyl derivatives). Addition of acetic anhydride to the complex formed between the pyrrolidone (IVa) and lithium 2-furyl gave the acetoxy ester of 3-(2-furyl)-1-phenethylpyrrolidin-3-ol. This ester darkened on storage and gave a tar-like product with one mole excess of hydrogen chloride in ethanol, a reagent which converts corresponding piperidine esters to ethyl ethers (Casy, Beckett & Armstrong, 1961). The synthesis and esterification of *N*-substituted 4-phenylazacycloheptan-4-ols (VII, R′ = H) have been described elsewhere (Casy & Birnbaum, 1964).

Pharmacology

The analgesic activities of the alcohols (V and VII; R′ = H) and their derivatives were determined in mice (after subcutaneous injection), using a hot-plate method based on that described by Eddy & Leimbach (1953) (Casy, Beckett, Hall & Vallance 1961); results are given in Table 1.

TABLE 1. ANALGESIC ACTIVITIES OF AZACYCLOALKANOLS AND DERIVATIVES MEASURED BY THE HOT PLATE TEST IN MICE AFTER SUBCUTANEOUS INJECTION

Series No.	Structure	Activity		
		Pyrrolidine ($x = 1, y = 2$)	Piperidine ($x = y = 2$)	Azacycloheptane ($x = 2, y = 3$)
1		Inactive at 50 mg/kg	ED50 34 mg/kg[a] (0·8 × pethidine)	ED50 47 mg/kg (0·33 × pethidine)
2		Inactive at 100 mg/kg	ED50 4·4 mg/kg (5·7 × pethidine)	ED50 7·2 mg/kg (2·5 × pethidine)
3		Inactive at 100 mg/kg	ED50 1·5 mg/kg (17 × pethidine)	ED50 3·4 mg/kg (7 × pethidine)
4		ED50 81 mg/kg (0·25 pethidine)	Inactive at 100 mg/kg[b]	—
5		ED50 41 mg/kg (0·5 × pethidine)	—	ED50 38 mg/kg (0·5 × pethidine)
6		Inactive at 100 mg/kg	Inactive at 200 mg/kg[c]	—

a Beckett, Casy & Kirk (1959).
b Casy & Armstrong (1964).
c Casy, Beckett, Hall & Vallance (1961).

The ester (VIIa; R′ = CO·Et), the most active non-piperidine derivative tested (seven times as active as pethidine in this test), was selected for a more detailed examination. After oral administration to mice, codeine and the ester (VIIa; R′ = CO·Et) had ED50 values of 61·6 and 26·8 mg/kg respectively. After intravenous injection, the ester was 5·4 times as active as pethidine in producing a positive Straub tail response and three times more toxic (LD50 of ester 13·9 mg/kg; LD50 of pethidine 41·7 mg/kg). The ester had a Straub Index (i.v. LD50/i.v. ED50 for Straub tail effect) of 9·3, pethidine having the value 5·1. As Shemano & Wendel (1960) have claimed that this index shows some correlation with the addiction liability of analgesics, this result suggests that the ester may have addictive properties similar to, or somewhat greater than, those of pethidine. As an aid to characterising the ester as a morphine-like analgesic, the effect of nalorphine on its analgesic activity was examined. The ester was administered subcutaneously to five groups of 10 mice, four groups of which received graded doses of nalorphine at the same time. Pethidine was administered in a like manner to other groups of mice. Thirty min after injection, the presence of analgesia was determined by the hot-plate test. The results, summarised in Table 2, show that nalorphine at low dose levels antagonises the analgesic activity of the ester more effectively than it does that of pethidine.

TABLE 2. EFFECT OF NALORPHINE ON THE ANALGESIC ACTIVITY IN MICE OF 1-PHENETHYL-4-PHENYL-4-PROPIONYLOXYAZACYCLOHEPTANE AND PETHIDINE AS MEASURED IN THE HOT PLATE TEST

Compound	Dose of nalorphine mg/kg	No. of animals with analgesia
1-Phenethyl-4-phenyl-4-propionyloxy-azacycloheptane hydrochloride 15 mg/kg	—	9/10
	0·25	1/10
	0·5	0/10
	1·0	0/10
	2·0	0/10
Pethidine hydrochloride 50 mg/kg	—	8/10
	0·25	7/10
	0·5	4/10
	1·0	2/10
	2·5	1/10

The development of tolerance in mice repeatedly treated with the ester was compared with that in animals receiving pethidine. The ester was given subcutaneously to a group of 20 mice, whilst a similar group was injected with pethidine, both drugs at approximately their ED90 doses. The injections were made on 5 days a week and the presence of analgesia was determined by the hot-plate test 30 min after injection. Complete tolerance developed to pethidine after eight-days administration, whereas after 16 days only 85% of the mice receiving the ester exhibited tolerance.

In summary the evidence shows the ester (VIIa; R′ = CO·Et) to be more active than pethidine, but also more toxic and apparently possessing greater addiction liability. It is, however, active orally and tolerance develops at a slower rate than with pethidine (Fig. 1).

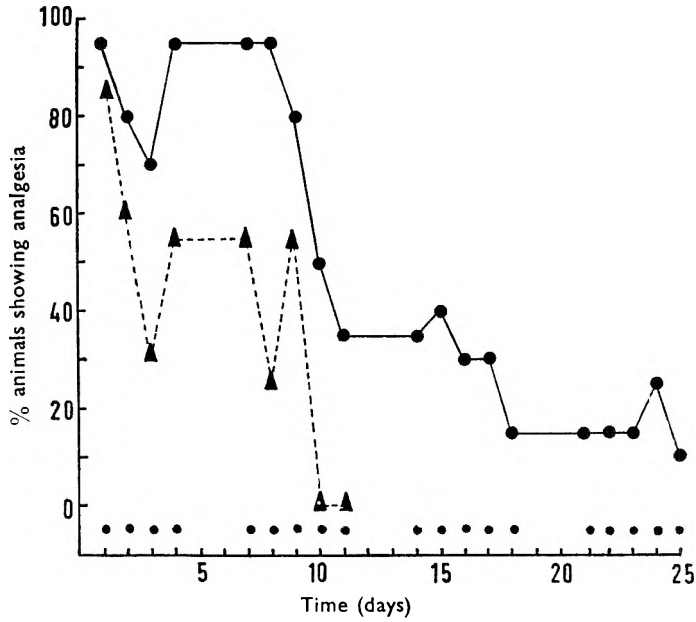
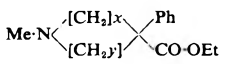
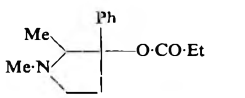
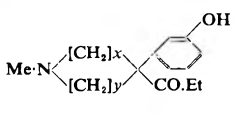
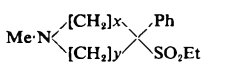
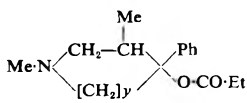


FIG. 1. The development of tolerance to the ester (VIIa, R′ = COEt), 10 mg/kg/day (●——●) and pethidine hydrochloride, 45 mg/kg/day (▲ - - ▲) injected subcutaneously on days marked ●.

Discussion

The effect of changes in ring size upon the analgesic activities of 4-phenylpiperidines is most clearly seen in the esters of series 2 and 3 (Table 1), piperidine members being potent analgesics. Ring contraction by one methylene group abolishes activity, whereas an equal degree of ring expansion produces compounds in which approximately 40% of the activity of the six-membered-ring esters is retained. Further, results with the seven-membered-ring ester (VIIa, R′ = CO·Et) indicate it to be a morphine-like analgesic; hence the nature of the retained activity is probably unaffected by ring expansion. These observations are consistent with published results from five- and seven-membered-ring compounds related to pethidine (see Table 3). All pyrrolidine derivatives reported are either inactive or nearly so. Prodilidine (No. 2, Table 3), one of the most active members of a series of esters of 1,2-dimethyl-3-phenylpyrrolidin-3-ol, is slightly less active than codeine in the antinociceptive test. Replacement of the *N*-methyl group by a phenethyl group (a change that enhances potency in 4-phenylpiperidine-type analgesics) reduces the activity of prodilidine by half (Cavalla, Selway, Wax, Scotti & Winder, 1962). The seven-membered-ring analogue of pethidine (Series 1, Table 3; ethoheptazine, Zactane) is one-third and one-fifth as active as the parent compound in rats and mice respectively in the hot-plate test. It has been used clinically with aspirin to alleviate moderate pain, has a low addiction liability and is probably not a morphine-type analgesic (see references cited by Beckett & Casy, 1962).

TABLE 3. ANALGESIC ACTIVITIES OF SOME PHENYLAZACYCLOALKANES

Series No.	Structure	Activity		
		Pyrrolidine ($x = 1, y = 2$)	Piperidine ($x = y = 2$)	Azacycloheptane ($x = 2, y = 3$)
1		Inactive in mice at 200 mg/kg[a]	(Pethidine) ED50 9·9 mg/kg in mice[b] ED50 11·2 mg/kg in rats[c]	(Ethoheptazine) ED50 42·6 mg/kg in mice[b] ED50 33·5 mg/kg in rats[c]
2		(Prodilidine) 0·8 × codeine in rats[d]	—	—
3		—	(Ketobemidone) ED50 1·6 mg/kg in mice[b]	ED50 16·5 mg/kg in rats (0·7 × pethidine)[c]
4		—	1 × pethidine in mice[e]	ED50 32 mg/kg in rats (0·3 × pethidine)[c]
5		—	(Alphaprodine) ED50 1·9 mg/kg (Betaprodine) ED50 0·7 mg/kg in mice[b]	(Proheptazine) ED50 1·0 mg/kg in mice[b]

a Macdonald & others (1946); *b* Eddy, Halbach & Braenden (1956); *c* Braenden, Eddy & Halbach (1955); *d* Cavalla & others (1961); *e* Buchi & others (1952).

The azacycloheptane derivatives of series 3, 4 and 5 (Table 3) similarly retain some of the activity of their piperidine analogues. Proheptazine (series 5, Table 3) is a potent analgesic probably of the morphine-type [it produces physical dependence when administered in relatively high doses (Eddy, Halbach & Braenden 1956)]. Its activity cannot be compared directly with that of a piperidine congener since its stereochemistry has not been established.

The results show, in general, that the analgesic property measured is retained (although in reduced degree) in seven-membered-ring analogues of active piperidine derivatives but is absent or weak in five-membered congeners. This generalisation may be interpreted in terms of differences in the relative orientation of, and distance between, the basic centre and the aromatic group in such compounds. These structural parameters, considered of importance in the association between drug molecules and the analgesic receptor (Beckett & Casy 1965), appear to be optimal in a six-membered ring. Although the relationship of the two features must be modified when an additional methylene group is included in the nitrogen-containing ring, the molecule is free to adopt a wide range of conformations (Eliel 1962), some of which may satisfy (in part) requirements for drug-receptor association. In contrast, the distance between

the nitrogen atom and the aromatic group in pyrrolidine derivatives must of necessity be less than that obtaining in piperidine analogues; further, the orientation of the two features is restricted to narrower limits through the more rigid, planar nature of the 5-membered ring (Eliel 1962).

In most instances, the pK_a values of pyrrolidine derivatives are approximately one unit lower than those of piperidine and azacycloheptane analogues (Birnbaum 1964). It is difficult, however, to assess the influence of reduced basic character upon the activities of pyrrolidine derivatives, because, while causing the proportion of ionised to non-ionised molecules in the vicinity of the receptor to be lower than that obtaining in piperidine and azacycloheptane congeners (drug-receptor association is considered to involve protonated molecules), it would also be expected to facilitate transport of drug molecules to the active site [lipid barriers are more easily penetrated by unionised molecules (Brodie & Hogben 1957)].

Experimental

Melting-points are uncorrected. Analyses by Drs. Weiler and Strauss, Oxford. Equivalent weights of bases and salts were determined by titration with 0·02N perchloric acid in glacial acetic acid using Oracet Blue B as indicator. Salts were crystallised from ethanol-ether unless otherwise stated. Free bases were recovered from acidic reaction products by treatment with aqueous ammonia and ether extraction.

N-(2-*Cyanoethyl*)*phenethylamine* (Ia). Acrylonitrile (53 g, 1 mole) was added to phenethylamine (121 g, 1 mol) at a rate such that the temperature of the mixture did not rise above 30°. The product was left at room temperature for 14 days and then distilled to give the secondary amine (Ia) (164 g), b.p. 122°/0·4 mm. (Found: equiv. wt 175. Calc. for $C_{11}H_{14}N_2$: 174). It gave a *hydrobromide*, m.p. 166° from ethanol. Found: C, 52·7; H, 5·95; N, 10·65; equiv. wt 258. $C_{11}H_{15}BrN_2$ requires C, 51·8; H, 5·9; N, 11·0%; equiv. wt 255. *N*-(2-Cyanoethyl)benzylamine (Ib), b.p. 131–134°/0·65 mm (Martin, Pecher, Peeters & Van Malder, 1958, report b.p. 148–150°/1 mm), was prepared similarly from acrylonitrile and benzylamine. It gave a hydrobromide, m.p. 184°. (Martin & others, 1958, report m.p. 176°). Found: C, 49·6; H, 5·6; N, 11·9; equiv. wt 243. $C_{10}H_{13}BrN_2$ requires C, 49·8; H, 5·4; N, 11·6%; equiv. wt 241.

N-(2-*Cyanoethyl*)-N-*ethoxycarbonylmethylphenethylamine* (IIa). Ethyl bromoacetate (83·5 g, 0·5 mole) was added over a period of 2 hr to a stirred mixture of the secondary base (Ia) (87 g, 0·5 mol), anhydrous potassium carbonate (69 g, 0·5 mole) and ethyl methyl ketone (200 ml) maintained at the reflux temperature. When addition was complete the mixture was heated for a further 6 hr under reflux, filtered and the filtrate evaporated. The residue was distilled to give the *tertiary base* (IIa) (122 g), b.p. 172–174°/1·1 mm. Found: C, 69·05; H, 7·8; N, 10·5; equiv. wt 261. $C_{15}H_{20}N_2O_2$ requires C, 69·2; H, 7·7; N, 10·8%; equiv. wt 260.

N-(2-*Cyanoethyl*)-N-*ethoxycarbonylmethylbenzylamine* (IIb), b.p. 160°/0·9 mm, was similarly prepared from the secondary amine (Ib). (Found: equiv. wt 244. Calc. for $C_{14}H_{18}N_2O_2$: 246). It gave a *hydrobromide*, m.p. 148°. Found: C, 51·4; H, 5·6; N, 8·45; equiv. wt 328. $C_{14}H_{19}BrN_2O_2$ requires C, 51·4; H, 5·8; N, 8·6%; equiv. wt 327.

N-(2-*Ethoxycarbonylethyl*)-N-*ethoxycarbonylmethylphenethylamine* (IIIa). *Method* 1. Dry hydrogen chloride was passed into a solution of the cyano-ester (IIa) (26 g) in ethanol (100 ml) for 5·5 hr, ammonium chloride separating after the gas had been passed for 2 hr. The mixture was then heated under reflux for 3 hr, left at room temperature overnight, and then filtered. The filtrate (and ethanol washings) was evaporated, and the free base, liberated from the residual oil, was distilled to give the diester (IIIa) (25·8 g, 82%) b.p. 148–152°/0·3 mm. (Found: equiv. wt 302. Calc. for $C_{17}H_{25}NO_4$: 307). It had an intense absorption peak at 1735 cm^{-1} (ester carbonyl); nitrile absorption bands near 2250 cm^{-1} were absent.

Method 2. A mixture of the cyano-ester (IIa) (78 g), concentrated sulphuric acid (120 g) and ethanol (180 ml) was heated under reflux for 18 hr, cooled, and diluted with water (350 ml). The free base, liberated by means of aqueous potassium carbonate solution (20%) was distilled to give the diester (IIIa) (68 g, 74%), b.p. 154–158°/0·7 mm. Its infrared spectrum was identical with that of the compound obtained by method 1. Attempts to prepare a crystalline derivative of the diester (IIIa) failed. N-(2-*Ethoxycarbonylethyl*)-N-*ethoxycarbonylmethylbenzylamine* (IIIb), b.p. 144°/0·75 mm, was prepared from the cyano-ester (IIb) by Method 1. Found: C, 65·8; H, 8·1; equiv. wt 294. $C_{16}H_{23}NO_4$ requires C, 65·5; H, 7·85%; equiv. wt 293.

1-*Phenethyl*-3-*pyrrolidone* (IVa). The diester (IIIa) (15·4 g, 0·05 mol) was added to a stirred suspension of sodium hydride (50% suspension in mineral oil, 2·4 g, 0·05 mole) in xylene (60 ml) maintained at 60°. A vigorous initial reaction, subsequently controlled by the rate of addition, ensued; when the addition was complete the mixture was heated under reflux for 3 hr, cooled and treated with water to decompose any excess of sodium hydride. The product was extracted with hydrochloric acid (6N, 80 ml) (one drop of this extract gave a blood-red colour with aqueous $FeCl_3$), and the extract heated under reflux for 3 hr (the product gave no coloration with aqueous $FeCl_3$). The solution was evaporated to small bulk and made alkaline with a concentrated aqueous solution of sodium hydroxide. The oil (7·5 g) which separated was isolated and distilled to give the pyrrolidone (IVa) (4·7 g, 50%) b.p. 108–110°/0·5 mm. (Found: equiv. wt 192. Calc. for $C_{12}H_{15}NO$: 189), as a colourless oil which rapidly darkened on exposure to the atmosphere. It gave a *hydrochloride*, m.p. 153°. Found: C, 64·3; H, 6·9; N, 6·1; equiv. wt 227. $C_{12}H_{16}ClNO$ requires C, 63·9; H, 7·1; N, 6·2%; equiv. wt 226. 1-*Benzyl*-3-*pyrrolidone* (IVb), b.p. 109°/1·1 mm, was similarly prepared from the diester (IIIb). It gave a *hydrochloride*, m.p. 192°. Found: C, 62·6;

H, 6·5; N, 6·6; equiv. wt 216. $C_{11}H_{14}ClNO$ requires C, 62·4; H, 6·6; N, 6·6%; equiv. wt 212.

1-*Phenethyl*-3-*phenylpyrrolidin*-3-*ol* (Va) *and derivatives.* Freshly prepared 1-phenethyl-3-pyrrolidone (14·3 g, 0·075 mol) in ether (75 ml) was added to a stirred, ice-cooled solution of lithium phenyl in ether (75 ml), prepared from lithium (1·3 g, 0·19 mole) and bromobenzene (14·3 g, 0·09 mol). The product, after being stirred for 1 hr at room temperature, was decomposed with ice and glacial acetic acid (12 ml); the solid which separated after storage at 5° was collected and washed with ether. The free base (17·5 g) derived from this solid was treated with ethanolic HBr to give 1-*phenethyl*-3-*phenylpyrrolidin*-3-*ol* (Va) *hydrobromide*, m.p. 121°. Found: C, 62·2; H, 6·2; N, 4·3; equiv. wt 351. $C_{18}H_{22}BrNO$ requires C, 62·1; H, 6·3; N, 4·0%; equiv. wt 348. A mixture of the pyrrolidinol (Va) (8 g), acetic anhydride (12 ml) and pyridine (12 ml) was heated under reflux for 3 hr, xylene added and the solvents evaporated under reduced pressure. The residue, with a slight excess of ethanolic HCl, gave 3-*acetoxy*-1-*phenethyl*-3-*phenylpyrrolidine* (VIa, R′ = CO·Me) *hydrochloride* (6 g), m.p. 163°. Found: C, 69·4; H, 7·2; equiv. wt 349. $C_{20}H_{24}ClNO_2$ requires C, 69·5; H, 6·95%; equiv. wt 346. The corresponding 3-*propionyloxypyrrolidine* (VIa, R′ = CO·Et) *hydrochloride*, m.p. 171°, was similarly prepared using propionic anhydride (no pyridine). Found: C, 70·15; H, 7·2; N, 4·0; equiv. wt 362. $C_{21}H_{26}ClNO_2$ requires C, 70·1; H, 7·2; N, 3·9%; equiv. wt 360. A mixture of the 3-acetoxypyrrolidine (VIa, R′ = CO·Me) hydrochloride (2 g), concentrated sulphuric acid (10 ml) and dry methanol (50 ml) was heated under reflux for 6 hr. Excess of aqueous ammonia was added, and the precipitated NH_4Cl removed by filtration. The filtrate (and methanol washings) was concentrated and then diluted with water. The oil (1·4 g) which separated was isolated and treated with methanolic HCl to give 3-*methoxy*-1-*phenethyl*-3-*phenylpyrrolidine* (VIa, R′ = Me) *hydrochloride*, m.p. 194° from methanol-ether. Found: C, 71·95; H, 7·8; N, 4·3; equiv. wt 319. $C_{19}H_{24}ClNO$ requires C, 71·8; H, 7·6; N, 4·4%; equiv. wt 318. It had an absorption peak at 1095 cm^{-1} (characteristic of the C–O stretching frequency in related 4-alkoxypiperidines, Casy, Beckett & Armstrong, 1961.

3-*Acetoxy*-3-(2-*furyl*)-1-*phenethylpyrrolidine.* The pyrrolidone (IVa) (33 g) in ether was added to a stirred, ice-cooled solution of lithium 2-furyl in ether [prepared from lithium (2·8 g), bromobenzene (31·4 g) and freshly distilled furan (13·6 g)]. Acetic anhydride (34 ml) in ether was then added, the mixture stirred at room temperature for 1 hr and poured onto crushed ice and glacial acetic acid (34 ml). Basic material (37·3 g), isolated from the solid which separated, was purified by chromatographing on alumina and eluting with light petroleum (b.p. 40–60°): benzene (9:1) to give the impure 3-acetoxypyrrolidine (19·5 g) as a yellow oil. This oil, with slightly less than an equimolar quantity of ethanolic HCl gave 3-*acetoxy*-3-(2-*furyl*)-1-*phenethylpyrrolidine hydrochloride*, m.p. 127° decomp. from ethanol. Found: C, 64·2; H, 6·5;

N, 4·4; equiv. wt 339. $C_{18}H_{22}ClNO_3$ requires C, 64·4; H, 6·6; N, 4·2%; equiv. wt 336. The salt decomposed on exposure to the atmosphere and when treated with acid reagents.

1-Benzyl-3-phenylpyrrolidin-3-ol (Vb) *and related compounds.* 1-Benzyl-3-pyrrolidone (IVb) (44 g) was treated with lithium phenyl [prepared from lithium (3·9 g) and bromobenzene (43 g)] in the usual manner to give the *pyrrolidinol* (Vb) *hydrochloride* (18 g), m.p. 156°. Found: C, 71·4; H, 6·8; N, 4·6; equiv. wt 285. $C_{17}H_{20}ClNO$ requires C, 70·5; H, 6·9; N, 4·6%; equiv. wt 290. This salt (15·2 g) in ethanol (300 ml) was shaken with hydrogen at room temperature and pressure in the presence of palladised charcoal (10%, 1·5 g); the uptake of hydrogen (1600 ml) required 24 hr. The mixture was filtered, the filtrate evaporated and the residue (9·9 g) crystallised from ethanol-ether to give *3-phenylpyrrolidin-3-ol* (V, R = H) *hydrochloride*, m.p. 148°. Found: C, 59·55; H, 6·9; N, 7·1; equiv. wt 197. $C_{10}H_{14}ClNO$ requires C, 60·15; H, 7·0; N, 7·0%; equiv. wt 200. Nitrogen was passed for 8 hr through a mixture of the pyrrolidinol (V, R = H) (1·8 g), (2-benzoylethyl)trimethylammonium iodide (3·8 g), anhydrous Na_2CO_3 (2·4 g) and dimethylformamide (35 ml). Water (200 ml) was added and the cloudy product stored at 5° whereupon the impure 1-(2-benzoylethyl)-3-phenylpyrrolidin-3-ol (Vc) (2·5 g), m.p. 76° separated. It gave a hydrochloride, m.p. 156°. Found: C, 68·05; H, 6·8; N, 4·5; equiv. wt 329. $C_{19}H_{22}ClNO_2$ requires C, 68·8; H, 6·6; N, 4·2; equiv. wt 332.

1-(2-Benzoylethyl)-4-phenyl-1-azacycloheptan-4-ol) was similarly prepared from 4-phenyl-1-azacycloheptan-4-ol (Casy & Birnbaum, 1964). It gave a *hydrochloride*, m.p. 148–150°. Found: C, 69·8; H, 7·1; N, 4·4; equiv. wt 353. $C_{21}H_{26}ClNO_2$ requires C, 70·1; H, 7·2; N, 3·9%; equiv. wt 360.

The infrared absorption spectra of all compounds described were consistent with assigned structures and were recorded on a Unicam S.P. 200 spectrophotometer.

References

Beckett, A. H. & Casy, A. F. (1962). *Progress in Medicinal Chemistry.* Vol. 2, p. 71. Edited by Ellis, G. P. & West, G. B. London: Butterworths.

Beckett, A. H. & Casy, A. F. (1965). *Ibid.*, Vol. 4 in press.

Beckett, A. H., Casy, A. F. & Kirk, G. (1959). *J. med. pharm. Chem.*, **1**, 37–58.

Birnbaum, H. (1964). Thesis. University of London.

Braenden, O. J., Eddy, N. B. & Halbach, H. (1955). *Bull. World Hlth Org.*, **13**, 937–998.

Brodie, B. B. & Hogben, C. A. M. (1957). *J. Pharm. Pharmacol.*, **9**, 345–379.

Buchi, J., Prost, M., Eichenberger, H. & Lieberherr, R. (1952). *Helv. Chim. acta*, **35**, 1527–1536.

Casy, A. F., Beckett, A. H. & Armstrong, N. A. (1961). *Tetrahedron*, **16**, 85–93.

Casy, A. F., Beckett, A. H., Hall, G. H. & Vallance, D. K. (1961). *J. med. pharm. Chem.*, **4**, 535–551.

Casy, A. F. & Armstrong, N. A. (1965). *J. med. Chem.*, **8**, 57–61.

Casy, A. F. & Birnbaum, H. (1964). *J. chem. Soc.*, 5130–5134.

Cavalla, J. F., Davoll, J., Dean, M. J., Franklin, C. S., Temple, D. M., Wax, J. & Winder, C. V. (1961). *J. med. pharm. Chem.*, **4**, 1–19.

Cavalla, J. F., Selway, R. A., Wax, J., Scotti, L. & Winder, C. V. (1962). *Ibid.*, **5**, 441–451.

Diamond, J., Bruce, W. F. & Tyson, F. T. (1964). *J. org. Chem.*, **7**, 57–60.
Eddy, N. B., Halbach, H. & Braenden, O. J. (1956). *Bull. World Hlth. Org.*, **14**, 353–402.
Eliel, E. L. (1962). *Stereochemistry of carbon compounds*, p. 248. New York: McGraw-Hill.
Macdonald, A. D., Woolfe, G., Bergel, F., Morrison, A. L. & Rinderknecht, H. (1946). *Brit. J. Pharmacol.*, **1**, 4–14.
Martin, R. H., Pecher, J., Peeters, J. & Van Malder, C. (1958). *Bull. soc. chim. Belg.*, **67**, 256–269.
Shemano, I. & Wendel, H. (1960). *The Pharmacologist*, **2**, No. 2, 97.

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Nitroethylenes and related compounds as trichomonacides and candidacides

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Several of the compounds examined, particularly certain β-nitrostyrenes and di(2-vinyl)benzenes, were highly active *in vitro* against both *Trichomonas vaginalis* and *Candida albicans*, which are commonly found together in vaginitis. The urinary recovery of the compounds after oral administration, however, suggested that the drugs had not gained access to the circulation in amounts sufficient to ensure a worthwhile effect against the organisms and, moreover, they proved irritant to vaginal mucosa.

LOCAL drug treatment of *Trichomonas vaginalis* infection in women, where the condition is overt, has met with indifferent success and such treatment is inapplicable to men who are often symptomless carriers of the pathogen. The introduction of metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole (Cosar & Julou, 1959) marked a great advance in therapy for it was effective orally.

In a search for alternative drugs we were prompted by the well-known antibacterial activity of β-nitrostyrenes to examine the general class of nitroethylenes against *T. vaginalis*. Bocobo, Curtis, Block & Harrell (1954) have observed that certain nitrostyrenes were active against *Candida albicans*. This is of particular interest, since mixed infection by this pathogen and *T. vaginalis* is common.

We report both *in vitro* and *in vivo* investigations made with a number of nitroethylenes variously substituted with aromatic, heterocyclic and aliphatic groups.

Methods

CHEMISTRY

The method of preparation of the new compound is given in Table 1. The remaining compounds were made by published methods.

Method A. 2N Potassium hydroxide solution (5 ml) was added dropwise over 15 min to a stirred solution of the aldehyde (10 mmol) in nitromethane (640 mg) and ethanol (10 ml) kept at 0–2°. After a further 5 min 2N hydrochloric acid (25 ml) was added and the precipitated nitroethylene was collected by filtration, washed with water, dried *in vacuo* and purified as indicated.

Method B. A mixture of the aldehyde (10 mmol) and the equivalent amount of the appropriate nitroparaffin in absolute ethanol (5 ml) and butylamine (1 mmol) was refluxed for 6 hr. Next morning the precipitate was collected by filtration, washed with ethanol, dried *in vacuo* and purified.

Method C. (a) *Nitrocarbinol.* Methanolic potassium hydroxide solution (2·3 mmol) was added dropwise to a suspension of the aldehyde (2 mmol)

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TABLE 1. PREPARATION, PHYSICAL CONSTANTS AND ANALYSES OF NEW COMPOUNDS

Compound	Method	Yield %	M.p. or (b.p.)	Analyses					
				Carbon %		Hydrogen %		Nitrogen %	
				Calculated	Found	Calculated	Found	Calculated	Found
m-Fluoro-β-nitrostyrene	A[1]	56	44·5–46·5°	57·5	57·6	3·6	3·6	8·4	8·6
p-Fluoro-β-nitrostyrene	A[1]	55	99·5–101°	57·5	58·3	3·6	3·8	8·4	8·5
p-(2-Nitrovinyl)benzoic acid	A[3]	23	285° (dec.)	56·0	56·4	3·65	3·9	7·25	7·2
p-(2-Nitrovinyl)cinnamic acid	A[4]	60	286–287° (dec.)	60·3	60·2	4·1	4·3	6·4	6·2
β-(*p*-2-Nitrovinylphenyl)propionic acid	A[3]	36	188–190°	59·7	59·9	5·0	5·2	6·3	6·2
o-(2-Nitrovinyl)phenoxyacetic acid	A[3]	—	173–175°	53·8	54·0	4·1	4·2	6·3	6·4
2,5-Di(2-nitrovinyl)furan	A[4]	30	225° (dec.)	45·7	46·5	2·9	3·0	—	—
4-(2-Nitrovinyl)-1-phenylpyrazole	A[3]	67	146–148°	61·4	61·75	4·2	3·7	19·5	19·5
1-Methyl-4-(2-nitrovinyl)pyrazole	A[3]	45	129–129·5°	47·05	47·3	4·6	5·05	27·4	27·05
2-Methylthio-4-(2-nitrovinyl)-1-*p*-tolylimidazole	A[3]	22	189–190°	56·7	57·1	4·8	4·8	15·3	14·3
m-Di(2-nitroprop-1-enyl)benzene	B[3]	16	104°	58·1	58·2	4·9	4·5	11·3	11·2
1,3,5-Tri(2-nitroprop-1-enyl)benzene	B[3]	12	201°	54·05	54·8	4·5	4·4	12·6	12·5
o-Methoxy-β-nitrostyrene	B[1]	8	34·5–35·5°	60·3	60·5	5·1	5·2	7·8	8·1
1-Methyl-2-(2-nitrovinyl)pyrrole	B[3,5]	24	[4] 101–102°	55·25	55·6	5·3	5·55	18·4	18·1
4-(2-Nitrovinyl)quinoline	B[3,5,6]	27	166·5–167·5° (dec.)	—	—	—	—	14·0	13·6
1-Methyl-5-(2-nitrovinyl)imidazole	B[3,5,7]	28	157–158°	47·05	46·8	4·6	4·6	27·4	27·55
4-(2-Nitrovinyl)thiazole	B[3,5]	9	108–111°	38·5	38·5	2·6	2·85	17·95	17·8
5-(2-Nitrovinyl)thiazole	B[3,5]	23	176·5–179°	38·5	38·35	2·6	2·5	17·95	17·2
4-(2-Nitrovinyl)-2-phenyl-2*H*-1,2,3-triazole	B[3,5,7]	34	128–130·5°	55·55	55·9	3·7	4·2	25·9	25·5
4-(2-Nitrovinyl)-1-propyl-1*H*-1,2,3-triazole	B[3,5,7]	9	115–116°	46·15	47·0	5·5	5·7	30·8	29·9
4-(1-Hydroxy-2-nitroethyl)-1-*p*-tolylimidazole	C(a)[1]	73	151–152·5°	58·3	58·85	5·3	5·6	—	—
4-(2-Nitrovinyl)-1-*p*-tolylimidazole	C(b)[3]	98	154–155° (dec.)	62·9	62·8	4·8	4·8	18·3	18·35
4-(1-Hydroxy-2-nitroethyl)-2-methanesulphonyl-1-*p*-tolylimidazole	C(a)[1]	70	165–168°	48·0	48·1	4·65	4·6	12·9	12·8
2-Methanesulphonyl-4-(2-nitrovinyl)-1-*p*-tolylimidazole	C(b)[3]	80	150–151°	50·8	50·8	4·3	4·1	13·7	13·4
1-*p*-Cyanophenyl-2-nitroethanol	C(a)[1]	24	96–98°	56·25	56·3	4·2	4·1	14·6	14·6
1-Methyl-2-nitroethyl chloroacetate	D	72	112–115·5°/ 3·0 mm	33·1	32·8	4·4	4·5	7·7	7·6
1-(Nitromethyl)propyl chloroacetate	D	60	102–118°/ 3·8 mm	36·8	37·5	5·1	5·3	7·2	6·3
1-(Nitromethyl)butyl chloroacetate	D	69	(128–137°/ 0·3 mm)	40·1	40·3	5·8	5·9	6·7	6·8
1-Methyl-2-nitroethyl hemisuccinate	E	4	70–71°	41·0	41·1	5·4	5·6	6·8	6·9
1-(Nitromethyl)propyl hemisuccinate	E	10	76–77°	43·8	44·0	6·0	6·0	6·4	6·4
1-(Nitromethyl)butyl hemisuccinate	E	5	71–72°	46·35	46·55	6·5	6·6	6·0	6·0

[1] Purified from sublimation. Recrystallised from: [2] light petroleum (60–80°): [3] ethanol: [4] ethanol - dimethylformamide. [5] Reaction conducted at room temperature.
[6] Methylamine used as a catalyst in place of butylamine. [7] Propylamine used as a catalyst in place of butylamine.

in ethanol (9 ml) and nitromethane (183 mg) at 0°. The mixture was brought to pH 8 with dilute hydrochloric acid and the precipitate was collected by filtration, washed with water, dried *in vacuo* and purified.

(b) *Dehydration.* A mixture of the nitrocarbinol (1 mmol), anhydrous sodium acetate (330 mg) and acetic anhydride (1·5 g) was heated to 100° for 20 min and then poured into water (50 ml). Dilute sodium hydroxide solution was added until pH 8 was reached and the product was isolated as above.

Method D. A mixture of the nitrocarbinol and a molar equivalent of chloroacetyl chloride was kept at room temperature for 6 days and then fractionally distilled.

Method E. A mixture of the nitrocarbinol (5 mmol), succinic anhydride (600 mg) and concentrated sulphuric acid (2 drops) was stirred for 2 hr at 40°, then treated with benzene, and the whole was poured onto ice. Succinic acid was filtered off and the separated benzene layer was extracted with ice-cold sodium bicarbonate solution. Acidification of the aqueous extract liberated the hemisuccinate, which was extracted in benzene. After evaporation of the solvent, the product was crystallised from isopropyl ether/light petroleum (60–80°).

MICROBIOLOGY

Trichomonacidal assay. Essentially the conditions described by Trussell (1947) for the comparative evaluation of trichomonacides were followed. A sterile stock solution of each of the compounds was prepared in a liver medium (Feinberg & Whittington, 1957) from which two-fold dilutions were taken to give a range from 500 to 1 μg/ml. Compounds insoluble in water were first dissolved in a minimum volume of dimethylformamide. Control studies showed that this solvent had no effect on the organisms at the dilutions employed. The final pH of all solutions was adjusted to 5·8. Two ml volumes of each concentration were transferred to sterile tubes and inoculated with 0·1 ml of a culture of *T. vaginalis* (Strain W3). The organisms were maintained in Feinberg's medium and subcultured daily so that the maximum number of actively multiplying organisms was achieved in a 24-hr culture. It is known that the minimal lethal concentration of some trichomonacidal drugs varies with the size of the inoculum, hence throughout the screening the inoculum was kept to a standard of approximately 250,000 organisms per tube. All the experiments were duplicated. The tubes were incubated at 37° for 24 hr. If the organisms were non-motile microscopically and failed to grow within nine days of being subcultured into the same medium, they were presumed to be dead.

Candidacidal assay. A range of dilutions of each compound was prepared as above. Tubes containing 2 ml of Sabouraud fluid medium (Oxoid), which was used for all cultures, were inoculated with 0·02 ml of a suspension of *C. albicans* equivalent to approximately 20,000 cells per inoculum. After incubation at 37° for 24 hr the tubes were subcultured by plating an aliquot onto Sabouraud agar; failure of the subcultures to grow within five days indicated death.

ANIMAL STUDIES

Urinary excretion. The test compounds were administered orally to rats at a dose of 1/10th of their LD50 or 200 mg/kg if the LD50 was in excess of 2 g/kg.

The rats were placed in stainless steel metabolism cages and the urine was collected in glass vessels containing approximately 10 mg of streptomycin and 10 mg of benzylpenicillin potassium to prevent the heavy bacterial overgrowth which would otherwise interfere with the microbiological assay. Urine was collected from 0–6 hr and from 6–24 hr after dosing and its trichomonacidal activity determined as described above.

Irritancy. Doses (12·5–100 mg) of the test compounds incorporated into 0·1 ml of a polyethyleneglycol base were administered intravaginally to rats. Two days later 0·5 ml of an aqueous suspension (4 mg/ml) of azovan blue was injected intravenously and after 2 hr the rats were killed. The vaginae were opened and examined for signs of dye-leakage; stained sections were prepared in the normal way and examined microscopically for signs of inflammation or damage.

Results and discussion

The trichomonacidal and candidacidal activities of a collection of nitroethylenes variously substituted with aromatic, heterocyclic and aliphatic groups are shown in Table 2. For comparison, metronidazole killed *T. vaginalis* at 2 μg/ml and nystatin killed *C. albicans* at 32 μg/ml. Neither drug was active against both organisms.

In the aromatic series generally, although differences are neither marked nor wholly consistent, substituents on the ring, whether electro-positive or -negative, tend to enhance activity against both pathogens. Salt-forming groups which enhance water solubility have a variable effect. A dimethylamino-group weakens, and a quaternary ammonium group totally abolishes, the activity. A side chain bearing a carboxyl group reduces candidacidal activity but has a less predictable effect on trichomonacidal activity. Polysubstitution of the aromatic ring, particularly with additional nitrovinyl groups, increases the activity against *Trichomonas* but, with two exceptions, weakens it against *Candida.*

Replacement of the benzene ring with various heterocyclic rings did not lead to any compounds with outstanding activity. They were usually inferior to the nitrostyrenes. In all instances examined, quaternisation of a ring nitrogen atom rendered the compound inactive.

Aliphatic nitro-olefins had weak activity and the carbinols from which they were prepared were even less active—similar effects, denoted with an asterisk in Table 2, were observed in the aromatic and heterocyclic series—but their esters were as active, or more so, than the related olefins.

The acute oral toxicity of the more active compounds in mice was low, ranging from 500 to 2000 mg/kg, but the recovery from urine, at best only 1–2% of that of metronidazole, suggested that absorption was poor and an adequate serum concentration of the drugs was unlikely to have been achieved. (A reliable assay of these drugs in blood or serum is

lacking). To assess the potentiality of the compounds as topical candidacides—there is no satisfactory systemic treatment for this condition—they were administered intravaginally to rats in an ointment base. Subsequent examination of the vaginal tissues revealed greater damage than was observed with nystatin.

TABLE 2. ACTIVITY OF SOME NITRO-ETHYLENES AGAINST *Trichomonas vaginalis* AND *Candida albicans*

Results are mean values of at least 2 estimations

Compound	Minimal lethal concentration (μg/ml)	
	T. vaginalis	*C. albicans*
Aromatic		
β-Nitrostyrene	8	10
2-Nitroprop-1-enylbenzene	8	8
o-Fluoro-β-nitrostyrene	4	8
m-Fluoro-β-nitrostyrene	4	2
p-Fluoro-β-nitrostyrene	4	8
o-Chloro-β-nitrostyrene	8	4
m-Chloro-β-nitrostyrene	16	8
p-Chloro-β-nitrostyrene	8	2
p-Bromo-β-nitrostyrene	4	1
p-Iodo-β-nitrostyrene	8	2
o,β-Dinitrostyrene	4	4
m,β-Dinitrostyrene	4	2
p,β-Dinitrostyrene	4	16
p-Cyano-β-nitrostyrene	8	8
*1-*p*-Cyanophenyl-2-nitroethanol	64	64
o-Hydroxy-β-nitrostyrene	4	16
m-Hydroxy-β-nitrostyrene	2	4
p-Hydroxy-β-nitrostyrene	8	16
o-Methoxy-β-nitrostyrene	8	8
m-Methoxy-β-nitrostyrene	8	16
p-Methoxy-β-nitrostyrene	4	4
p-(2-Nitrovinyl)benzoic acid	8	125
p-(2-Nitrovinyl)cinnamic acid	4	125
β-(*p*-2-Nitrovinylphenyl)propionic acid	4	64
o-2-Nitrovinylphenoxyacetic acid	32	250
p-Dimethylamino-β-nitrostyrene	16	125
5-Fluoro-2,β-dinitrostyrene	4	500
4-Chloro-3,β-dinitrostyrene	4	32
m-Di(2-nitrovinyl)benzene	2	8
m-Di(2-nitroprop-1-enyl)benzene	2	64
p-Di(2-nitrovinyl)benzene	1	1
p-Di(2-nitroprop-1-enyl)benzene	1	16
1,3,5-Tri(2-nitrovinyl)benzene	4	500
1,3,5-Tri(2-nitroprop-1-enyl)benzene	1	500
Heterocyclic		
2-(2-Nitrovinyl)furan	16	32
5-Nitro-2-(2-nitrovinyl)furan	32	16
2,5-Di(2-nitrovinyl)furan	4	8
2-(2-Nitrovinyl)thiophen	16	16
1-Methyl-2-(2-nitrovinyl)pyrrole	16	64
3-(2-Nitrovinyl)indole	16	125
3-(2-Nitrovinyl)pyridine	8	16
3-(2-Nitroprop-1-enyl)pyridine	4	8
4-(2-Nitrovinyl)quinoline	8	32
4-(2-Nitrovinyl)-2-phenyloxazole	4	>500
5-(2-Nitrovinyl)-3-phenylisoxazole	4	>500
4-(2-Nitrovinyl)-1-phenylpyrazole	16	16
1-Methyl-4-(2-nitrovinyl)pyrazole	125	32
1-Methyl-5-(2-nitrovinyl)imidazole	16	125
4-(2-Nitrovinyl)-1-*p*-tolylimidazole	8	32
*4-(1-Hydroxy-2-nitroethyl)-1-*p*-tolylimidazole	8	64
2-Methylthio-4-(2-nitrovinyl)-1-*p*-tolylimidazole	4	>500
2-Methanesulphonyl-4-(2-nitrovinyl)-1-*p*-tolylimidazole	16	250
*4-(1-Hydroxy-2-nitroethyl)-2-methanesulphonyl-1-*p*-tolylimidazole	125	500
4-(2-Nitrovinyl)thiazole	16	4
4-(2-Nitroprop-1-enyl)thiazole	8	8
5-(2-Nitrovinyl)thiazole	32	8
5-(2-Nitroprop-1-enyl)thiazole	8	16
4-(2-Nitrovinyl)-2-phenyl-2*H*-1,2,3-triazole	2	8
4-(2-Nitrovinyl)-1-propyl-1*H*-1,2,3-triazole	16	250

TABLE 2—*continued*

Compound	Minimal lethal concentration (μg/ml)	
	T. vaginalis	*C. albicans*
Aliphatic		
1-Nitropropene	16	—
1-Nitrobut-1-ene	64	64
1-Nitropent-1-ene	250	8
1-Nitropropan-2-ol	250	125
1-Nitrobutan-2-ol	125	125
1-(Nitromethyl)butyl acetate	4	16
1-(Nitromethyl)propyl acetate	64	32
1-Methyl-2-nitroethyl acetate	32	32
1-Methyl-2-nitroethyl chloroacetate	32	64
1-(Nitromethyl)propyl chloroacetate	64	32
1-(Nitromethyl)butyl chloroacetate	32	16
1-Methyl-2-nitroethyl hemisuccinate	32	125
1-(Nitromethyl)propyl hemisuccinate	64	64
1-(Nitromethyl)butyl hemisuccinate	64	32

Acknowledgements. The *Trichomonas vaginalis* strain W3 was kindly supplied by Dr. J. G. Feinberg.

References

Bocobo, F. C., Curtis, A. C., Block, W. D. & Harrell, E. R. (1954). *Proc. Soc. exp. Biol. N.Y.*, **85**, 220–222.

Catterall, R. D. & Nicol, C. S. (1957). *Brit. med. J.*, **2**, 29–31.

Cazemier, C., Goslings, W. R. O., Houwert, K. A. F., van Leeuwen, D. P., Lubbers, G. J. & Kok, P. C. (1959). *Antibiotic Med. Clin. Ther.*, **6**, 601–605.

Cosar, C. & Julou, L. (1959). *Ann. Inst. Pasteur.*, **96**, 238–341.

Feinberg, J. G. & Whittington, M. S. (1957). *J. chem. Path.*, **10**, 327–329.

Trussell, R. E. (1947). *Trichomonas vaginalis and Trichomoniasis*, 1st ed., p. 54. Illinois: Charles C. Thomas.

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Note on the estimation of ibufenac in serum

S. S. ADAMS AND E. E. CLIFFE

A method for the estimation of ibufenac (4-isobutylphenylacetic acid) in serum is described. The acidified serum is extracted with ether and the drug estimated by quantitative paper chromatography.

THE use of ibufenac (4-isobutylphenylacetic acid) in the treatment of rheumatoid arthritis has recently been described by Thompson, Stephenson & Percy (1963) and Chalmers (1963); and Adams, Cliffe, Lessel & Nicholson (1963) have reported some of its pharmacological properties. The drug is metabolised but the metabolites are inactive.

A means of estimating the concentration of the drug in serum was required and the physical properties of the drug were such that it seemed possible a method similar to that reported for the determination of fatty acids by Lederer & Lederer (1957), could be applied. We report the method which we have developed, which estimates the drug but not its metabolites.

Experimental

MATERIALS

Bromocresol purple reagent. Bromocresol purple (0·1 g) was dissolved in warm ethanol (20 ml) and made up to approximately 80 ml with water. The colour was adjusted to a reddish purple with 0·1N sodium hydroxide solution (about 10 ml required) and the volume made up to 100 ml with water.

Serum. Human serum was used. It can be stored at −15 to −40° for up to one week before the estimation.

EXTRACTION PROCEDURE

Serum (2 to 5 ml) containing the drug, n-octanol (0·025 ml), diethyl-ether (100 ml) and N hydrochloric acid (0·5 or 1 ml) were measured into a 250 ml separatory funnel and shaken gently for 20 min. The inclusion of octanol usually prevented emulsion formation but occasionally with certain sera an emulsion formed; then the mixture was centrifuged in a stoppered tube. An aliquot (approximately 80 ml) from the ether layer was evaporated to dryness in a round-bottomed tube (125 mm × 38 mm) on a steam-bath. The size of the tube is important since smaller tubes give a low recovery of drug. The residue was transferred to a 15 ml conical centrifugal tube in three lots of 90% ethanol (2, 1, 0·5 ml). This solution was evaporated in a vacuum dessicator over P_2O_5 and the residue was taken up in 90% ethanol (0·2 to 0·4 ml).

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CHROMATOGRAPHY

Whatman No. 1 paper was used and single 5 μl spots of standard solutions of ibufenac in 90% ethanol (12, 6, 3 and 1·5 mg/ml) were run on every sheet. The alcoholic serum extracts were concentrated on the paper by applying 25 to 100 μl in 5 μl volumes to the same position. The paper was dried under a draught of warm air between each application. The solvent system was n-butanol (AR):ammonia (AR; wt/ml 0·88): water, in the proportions 30:5:15 by volume. The papers were run for 16 hr by descending chromatography at 22°, then thoroughly dried and sprayed with a solution of 0·1% (w/v) bromocresol purple solution.

Ibufenac appeared as a yellow spot on a blue background (Rf 0·66) and the colour remained for 2 to 5 min. During this time an assessment of the amount of the compound in the unknown spots was made by comparing their area and intensity with that of the standards. At least two observers made each assessment, and each unknown sample was measured on two or three separate occasions.

RECOVERY OF IBUFENAC ADDED TO HUMAN SERUM

A solution of the sodium salt of ibufenac not exceeding 0·5 ml was added to 5 ml serum. The serum concentrations so produced covered the range of those found in rheumatoid patients receiving ibufenac treatment which was from 1 to 4 mg/100 ml.

Results and discussion

As shown in Table 1, on one occasion a control sample of serum gave an apparent concentration of 0·5 mg/100 ml (25 μg in 5 ml). In the total of 13 determinations the mean recovery was 99% with a standard error

TABLE 1. RECOVERY OF IBUFENAC FROM HUMAN SERUM

Five ml serum was used in each instance

Amount added (μg)	Concentration in /serum (mg100 ml)	Serum batch	Amount found (μg)	Recovery (%)	Mean recovery (± s.e.)
0	0	1	25	—	—
		2	0	—	
		2	0	—	
		3	0	—	
		6	0	—	
50	1	2	63	126	113 (±15)
		2	65	130	
		3	42	84	
100	2	1	113	113	95 (±9)
		2	100	100	
		3	83	83	
200	4	1	220	110	97 (±6)
		1	180	90	
		3	152	76	
		4	195	98	
		4	220	110	
400	8	3	284	71	—
500	10	5	500	100	—

of 5% and the values fell within the range 71 to 130%; the mean recovery did not vary significantly with the ibufenac concentration in the serum.

Light petroleum (b.p. 40–60°) was not satisfactory when used in place of diethyl-ether because emulsions formed more readily and low values were obtained when estimations were made after adding known amounts of ibufenac to serum. The time of shaking was also varied; extraction was virtually complete after 10 min but we have preferred to use 20 min to ensure complete extraction.

Phenylmercuric nitrate added to serum at a final concentration of 1 in 5000 was ineffective as a preservative when the serum was stored at room temperature for two days, although the estimation of added drug was not significantly affected. Samples awaiting estimation were therefore stored and transported at -15 to $-40°$.

The estimation of compounds by quantitative paper chromatography is now widely used and various claims have been made of the accuracy of the determinations. Reid & Lederer (1950) measured fatty acids with an accuracy of 2 to 5% by a method based on determinations of spot area. In our experience the latter measurements are too time-consuming to warrant the extra accuracy. Douglas, Ludwig, Ginsberg & Berger (1950) were able to measure the metabolites of mebutamate by a visual comparison of spots on paper chromatograms with a precision of $\pm 15\%$. The present results show that ibufenac in serum can be estimated with a similar accuracy.

The estimation described needs to be made under carefully controlled conditions. For instance, an acid or alkaline atmosphere causes serious interference with colour contrast on spraying the papers. The chief disadvantage with this method is that although the bench time is reasonably short, an estimation requires a minimum of 48 hr for completion.

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References

Adams, S. S., Cliffe, E. E., Lessel, B. & Nicholson, J. S. (1963), *Nature Lond.*, **200**, 271–272.

Chalmers, T. M. (1963). *Ann. rheum. Dis.*, **22**, 358–362.

Douglas, J. F., Ludwig, B. J., Ginsberg, T. & Berger, F. M. (1962). *J. Pharmacol.*, **136**, 5–9.

Lederer, E. & Lederer, M. (1957). *Chromatography*, 2nd ed., p. 184, Amsterdam: Elsevier.

Reid, R. L. & Lederer, M. (1952). *Biochem. J.*, **50**, 60–67.

Thompson, M., Stephenson, P. & Percy, J. S. (1963). *Ann. rheum. Dis.*, **23**, 397–404.

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An automatic apparatus for repeated stimulation of isolated organs by agonist drugs

A. B. WILSON

A simple commutator for attachment to a standard laboratory kymograph has been designed to operate automatically an apparatus for producing repeated contractions of isolated organs by agonist drugs.

THE advantages of an automatic apparatus are now well-established for quantitative and repetitive experiments on isolated organs. For example, in estimates of drug antagonisms (Schild, 1947), or in biological assays (Gaddum & Lembeck, 1949; Godfrey, Mogey & Taylor, 1950; Boura, Mongar & Schild, 1954), an increase in accuracy is achieved because of the greater uniformity of time intervals and fluid volumes; also the experimenter is able to work two or more organ baths concurrently.

This paper describes an automatic apparatus for producing uniform contractions of isolated organs by the repeated injection of agonist drugs. The apparatus has been used in experiments to investigate the inhibitory actions of spasmolytic drugs, using the uniform contractions of the organs as a reference level of excitatory activity. The apparatus is an improved design of that demonstrated to the British Physiological Society (Wilson, 1957), and has proved to be reliable during several years' use in this department. It differs in two ways from the apparatus described by other workers (Schild, 1946, 1947; Gaddum & Lembeck, 1949; Godfrey, Mogey & Taylor, 1950; Boura, Mongar & Schild, 1954). Firstly, it uses a simple mechanical switching system for operating a series of the electromagnetic valves described by Schild (1947). Secondly, instead of producing contractions by replacing the organ bath contents with the final dilution of an agonist drug (Gaddum & Lembeck, 1949; Boura, Mongar & Schild, 1954), it uses an automatic syringe for injecting small volumes of a concentrated solution of the drug. Lock (1961) has described some of the benefits of an automatic syringe; in the present experiments the method has the additional advantage that unlike the apparatus used by Gaddum & Lembeck (1949), an inhibitory drug added to the organ bath during the rest period of the preparation is not drained out before the subsequent challenge with the agonist solution.

EXPERIMENTAL METHOD

This is shown in Fig. 1 which is a kymograph record of a typical experiment on a preparation of guinea-pig small intestine. The automatic apparatus was used to produce a series of uniform contractions at each of two dose levels of histamine. The contractions caused by the larger dose of histamine were used as a reference level of excitatory activity from which was plotted a dose-response relation of the inhibition of histamine contractions by methylamphetamine.

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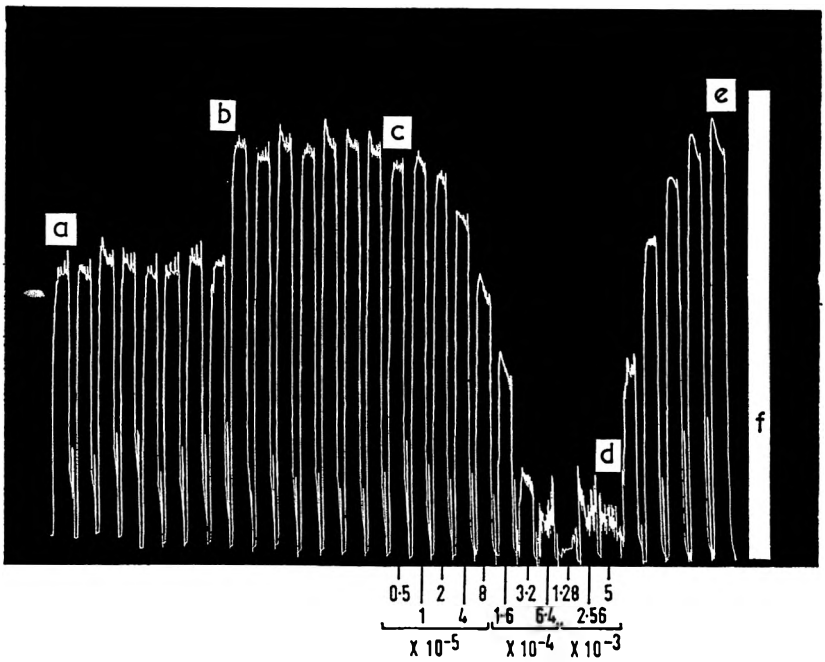

FIG. 1. Uniform longitudinal contractions of the guinea-pig isolated ileum to repeated injections of histamine at each of two dose levels, and a sequential dose-response record of the inhibition of the contractions by methylamphetamine. Doses of histamine were injected automatically at 4 min intervals and allowed to act for 1 min; the ileum was then washed twice with fresh Krebs solution. a–b = final bath concentrations of 4×10^{-8} histamine; b–e = final bath concentrations of 8×10^{-8} histamine. c–d = inhibition caused by graded doses of methylamphetamine, added to the organ bath by hand 2 min before a subsequent automatic injection of histamine; final bath concentrations of methylamphetamine are given on the molar scale. d–e = recovery during 4×10^{-8} histamine. f = maximal contraction of the preparation to histamine.

The spasmolytic drugs used in the experiments are not injected automatically, but are manually injected into the organ bath directly. This method allows the dose-inhibitory response relations of the spasmolytic drugs to be established by randomised or sequential consecutive graded doses (Fig. 1). It also avoids adding the spasmolytic drugs to the reservoir of physiological saline solution, a technique which gives satisfactory results with competitive antagonists (Schild, 1947; Godfrey, Mogey & Taylor, 1950) but which can cause errors in experiments using low concentrations of unstable drugs, such as the sympathomimetic amines.

AUTOMATIC OPERATION OF THE ORGAN BATH

For the automatic working of one organ bath, five electromagnetic valves (Schild, 1947) are used to compress polyvinyl or latex tubing; they control the measurement of Krebs solution, the filling and emptying of the organ bath, and the measurement and injection of the agonist drug solution (Fig. 2). Table 1, first column, lists the sequence of operations which, in the experiment shown in Fig. 1, were performed automatically

to elicit and record each histamine contraction and to return the preparation to rest; the sequence was repeated every 4 min. As judged by the uniform contractions to repeated doses of the agonist drugs (Fig. 1), the precision of the automatic measurements is high.

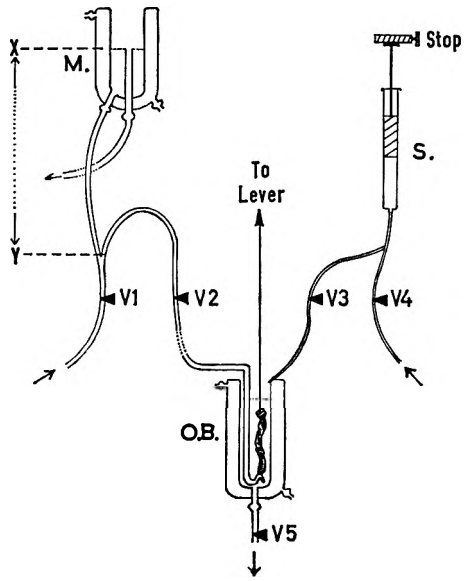

FIG. 2. The arrangement of the apparatus and electromagnetic valves (V1 to V5) used for the automatic operation of one organ bath (O.B.). V4 controls the filling of an automatic syringe (S) from a reservoir of agonist drug solution pressurised to 180 mm Hg; with V3 alone open, the weight of the syringe piston forces the measured dose of agonist into the organ bath. Sequential operation of V1 and V2 controls the filling of an overflow measuring chamber (M) with pre-warmed Krebs solution from a reservoir above the apparatus, and the flow of the measured volume of solution (X—Y) into the organ bath. V5 empties the organ bath to waste. Polyvinyl tubing of 3 mm outside diameter and 2 mm inside diameter was used for V3 and V4. Latex thyroid drain tubing, $\frac{1}{4}$ inch diameter was used for V1, V2 and V5.

SWITCHING SYSTEM AND TIMER

The switching system is a commutator of 100 separate stud contacts, which can energise the electromagnetic valves with current taken from a moving contact wiper. The timer is a standard laboratory kymograph, the main spindle being used to drive the contact wiper (Fig. 3).

The commutator has a flat circular base-plate on which there are one continuous ring contact and two outer concentric circles each of 50 separate stud contacts (Fig. 3). The base-plate is rigidly mounted on the body of the timing kymograph; the contact wiper clamps to the spindle and has three downward-facing brushes which rotate on the upward-facing ring or stud contacts of the base-plate (Fig. 5). The single brush of the wiper receives direct current from the ring, and the other two brushes transfer the current, in sequence, to each stud in the two circles; the backs of the studs are wired to an equal number of separate terminals on a wiring panel.

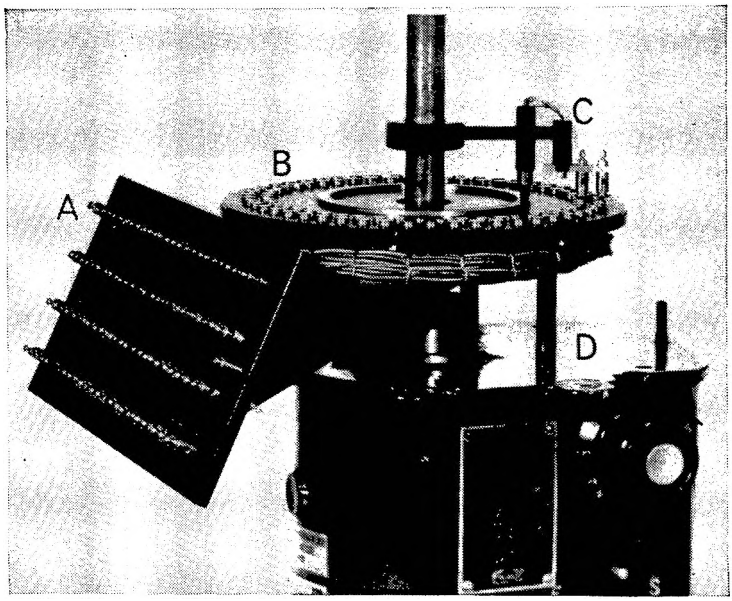

FIG. 3. The switching system and timer. A = wiring panel. B = commutator base-plate. C = contact wiper. D = timing kymograph.

The timer drives the contact wiper and its speed governs the frequency of operation of the electromagnetic valves. A Palmer "Electric Twelve" laboratory recording kymograph has been used, and makes a readily available timing unit with a speed range to suit all types of isolated organs: the spindle speed (and hence contact wiper speed and valve cycle) is almost continuously variable from one revolution in 0·8 sec to one revolution in 13 hr.

TABLE 1. CONNECTIONS OF THE ELECTROMAGNETIC VALVES (V1 TO V5), TWIN-CONTACT RELAYS (RK AND R1 TO R5) AND COMMUTATOR STUD CONTACTS (1 TO 100) USED FOR THE AUTOMATIC WORKING OF THE ORGAN BATH IN THE EXPERIMENT SHOWN IN FIG. 1.

Sequence of automatic operations	Electromagnetic valve	Twin contact relay	Commutator contacts
Fill automatic syringe with agonist drug solution ..	V4	R4	1–4
Recording kymograph on	—	RK	5–7
Inject agonist drug solution; recording kymograph running	V3	R3	8–12
Recording kymograph running	—	RK	13–27
Measure Krebs solution; recording kymograph running	V1	R1	28–31
Recording kymograph running	—	RK	32–34
Empty Organ Bath	V5	R5	35–37
Fill organ bath with Krebs solution	V2	R2	38–41
Measure Krebs solution	V1	R1	46–49
Empty Organ bath	V5	R5	53–55
Fill organ bath with Krebs solution	V2	R2	56–59

Column one gives the sequence of operations which was repeated at intervals of 4 min. to stimulate and record one contraction of the ileum and to return the preparation to rest.

WIRING CIRCUIT

Connections of the electromagnetic valves to the stud contacts of the commutator are made through the wiring panel and are determined by the particular requirements of each experiment. Table 1, columns two and four, lists the connections used in the experiment shown in Fig. 1.

The contact wiper can be provided with current (100 V, D.C.) to energise the electromagnetic valves directly, but because heavy arcing burns the commutator contacts, an indirect system is preferred. In the indirect system the commutator is used in a low voltage circuit (9 V, D.C.) to operate a series of 100 ohm Post Office telephone relays (Table 1, column 3): the twin contact sets of these relays carry 100 V, D.C. to the electromagnetic valves, or 240 V, A.C. to the kymograph on which the contractions of the preparation are recorded.

Fig. 4 gives the circuit for the simultaneous automatic operation of two organ baths; in this instance the apparatus shown in Fig. 2 is duplicated.

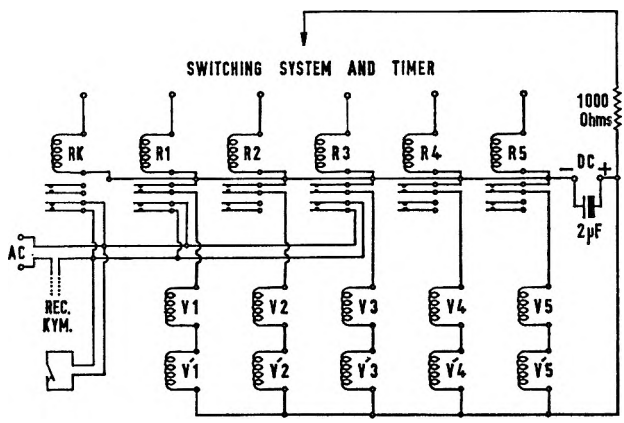

FIG. 4. The circuit for the simultaneous automatic operation of two organ baths. V1 to V5 are the electromagnetic valves whose use is illustrated in Fig. 2; V'1 to V'5 are the equivalent electromagnetic valves of a duplicate apparatus. R1 to R5, and RK are relays whose twin contact sets carry 100 V, D.C. to the electromagnetic valves, or 240 V, A.C. to the recording kymograph (REC.KYM.) on which the contractions of the preparations are recorded. The resistance of each relay and electromagnetic valve is 100 ohms.

CONSTRUCTION OF COMMUTATOR

The base-plate of the commutator is an 8-inch diameter disc of $\frac{1}{4}$-inch "Ebonite" or similar material. One hundred 4 BA hexagonal-head brass screws are turned to form the separate stud contacts and are mounted in two concentric circles of fifty each, 3 inches and $3\frac{3}{8}$ inches respectively from the centre of the base-plate; the stud contacts in the outer circle alternate with those in the inner at intervals of 3° 36′. A brass ring (4 inches outside diameter, $\frac{1}{4}$ inch wide and $\frac{3}{16}$ inch thick), slotted into the base-plate concentrically with the studs, forms the continuous ring contact. All the contacts are turned smooth to a level about $\frac{1}{8}$ inch proud of the upper surface of the base-plate.

A lip on the circumference of the base-plate takes a push fit dust cover with a perspex top, the centre of which, like that of the base-plate itself, is drilled to pass and to clear the $\frac{3}{4}$-inch diameter main spindle of the timing kymograph. The base-plate is rigidly mounted on three brass rods which are screwed into the top of the timing kymograph.

The wiring panel is a sheet of $\frac{1}{4}$-inch "Ebonite", 11 inches by $4\frac{1}{2}$ inches. This is fixed to the under surface of the commutator base-plate, at an angle of about 45 degrees, by two "Ebonite" arms each approximately 8 inches long. The panel is drilled to take four rows of 25 separate screw terminals; the back of each terminal is permanently wired to the back of a stud contact on the commutator base-plate, in the sequence in which the moving wiper makes contact with the studs (that is, adjacent screw terminals are wired to adjacent stud contacts on the inner and outer circles alternately). An additional screw terminal on the panel is wired to the ring contact on the base-plate to carry current to the contact wiper.

The contact wiper has a split collar of brass which clamps to the main spindle of the timing kymograph (Fig. 3), and an arm of $\frac{1}{4}$-inch diameter brass rod which carries three spring-loaded brushes (Fig. 5). The wiper

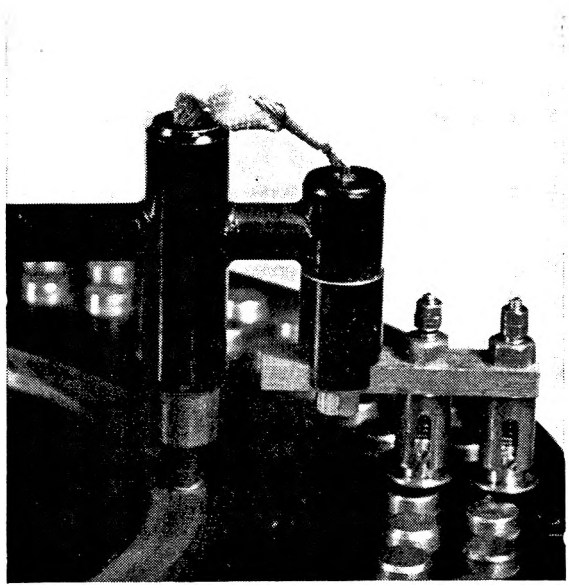

FIG. 5. The three downward-facing brushes of the contact wiper, which rotate on the ring or stud contacts of the commutator base-plate.

is insulated from the spindle of the timing kymograph by a "Tufnol" insert in the split collar. The inner brush on the wiper arm is a spring-loaded copper rod which transfers current from the ring contact on the commutator to the two outer brushes: each of these has a domed foot of brass which runs on one of the two circles of stud contacts. The feet must offer minimal mechanical resistance to the motor of the timing

kymograph, and to avoid intermittent function of the electromagnetic valves, must provide electrical continuity between successive stud contacts in the switching sequence. These requirements are met, firstly, by having the angle of the domed feet greater at the leading than at the trailing edges, and secondly, by making the domes of a size and shape (overall diameter about $\frac{5}{16}$ inch, with short trailing edges) which prevents them from bridging adjacent contacts in their own circles, whilst at the same time maintaining continuity between adjacent contacts of the inner and outer circles. The orientation of the domed feet is maintained by a locating pin on their stems.

USE OF APPARATUS

The apparatus was designed for use in experiments to investigate the spasmolytic actions of physiological or "independent" (Gaddum, 1957) antagonists of stimulant drugs, but it is also suitable for use in experiments with competitive antagonists, for example, in estimating pA_2 (Schild, 1947) by the method of Lockett & Bartlet (1956). The commutator switching system and the kymograph timing system may also find application in other automatic procedures, as a flexible alternative to the more usual combination of telephone uniselectors and a timing device (Schild, 1946, 1947; Gaddum & Lembeck, 1949; Godfrey, Mogey & Taylor, 1950; Boura, Mongar & Schild, 1954).

Acknowledgement. I am grateful to all my colleagues who have helped in the design and construction of the apparatus, and in the preparation of this paper.

References

Boura, A., Mongar, J. L. & Schild, H. O. (1954). *Brit. J. Pharmacol.*, **9**, 24–30.
Gaddum, J. H. (1957). *Pharmacol. Rev.*, **9**, 211–218.
Gaddum, J. H. & Lembeck, F. (1949). *Brit. J. Pharmacol.*, **4**, 401–408.
Godfrey, E. I., Mogey, G. A. & Taylor, D. L. (1950). *Ibid.*, **5**, 381–388.
Lock, J. A. (1961). *J. Pharm. Pharmacol.*, **13**, 378–379.
Lockett, M. F. & Bartlet, A. L. (1956). *Ibid.*, **8**, 18–26.
Schild, H. O. (1946). *Brit. J. Pharmacol.*, **1**, 135–138.
Schild, H. O. (1947). *Ibid.*, **2**, 189–206.
Wilson, A. B. (1957). *J. Physiol.*, **136**, 6P.

Letters to the Editor

Influence of ascorbic acid on the sensitivity of guinea-pig ileum

SIR,—The response of guinea-pig ileum to spasmogenic drugs varies widely at different times of the year. Munro (1951) reported that there was also a variation of response along the length of the ileum. A possible factor involved in these variations may be the ascorbic acid content of the diet.

Guinea-pigs weighing between 300 and 500 g were maintained on a diet of Rank SG1 pellets for three weeks, one half of the animals receiving daily 50 mg of ascorbic acid per animal in the drinking water. They were then killed and segments of the ileum, cleared of mesentery, were set up in 10 ml isolated organ baths, bathed in aerated Tyrode ringer at 32°, contractions being recorded on a smoked drum with an isotonic lever system. The ileum from the ascorbic acid-supplemented group was at least ten times more sensitive to acetylcholine and to histamine than that of animals not receiving ascorbic acid. This suggests that ascorbic acid plays a role in sensitivity of the ileum to spasmogens. In the ascorbic acid-supplemented group the preparations of terminal ileum were very sensitive, the threshold dose was as low as 100 pg/ml of acetylcholine and 200 pg/ml of histamine in the bath fluid.

It is of interest that Blaber & Cuthbert (1961) used large guinea-pigs for the assay of small amounts of acetylcholine, the ileum from smaller animals being insensitive. We have noted that guinea-pigs over 700 g in weight are less susceptible to scurvy than are smaller animals. The insensitivity of the ileum of smaller guinea-pigs noted by Blaber & Cuthbert and ourselves may therefore be due to ascorbic acid deficiency. We have also observed that neutralised ascorbic acid in a concentration of 5 mg/ml in the bath fluid increased the sensitivity of the ileum to acetylcholine and histamine, even when tissues from the ascorbic acid supplemented animals were used. This may therefore be a useful method of increasing tissue sensitivity to spasmogens.

We feel that these observations may be relevant to student exercises using guinea-pig ileum. An investigation of the ascorbic acid content of the diet of guinea-pigs in the different laboratories may be relevant.

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References

Blaber, L. C. & Cuthbert, A. W. (1961). *J. Pharm. Pharmacol.*, **13**, 445–446.
Munro, A. F. (1951). *J. Physiol. (Lond.)*, **112**, 84–94.

Suppression of an "adjuvant arthritis" in alloxan-diabetic rats

SIR,—Several reports suggest a relationship between carbohydrate metabolism, inflammation and allergy. In general, hyperglycaemia reduces, and hypoglycaemia increases, inflammatory and allergic phenomena. Alloxan-diabetic rats are more resistant to anaphylactoid reactions (Goth, Nash, Nagler & Holman, 1957), and produce less granulation tissue around subcutaneously-implanted foreign bodies than do normal animals (Nagy, Redei & Karady, 1961). Diabetogenic doses of alloxan, or high doses of glucose, greatly reduce the anaphylactic reaction in rats sensitised to horse serum (Thompson, 1961). Similarly, resistance to sensitisation with *Bordetella pertussis* is increased in alloxan-diabetic mice (Ganley, 1962).

The pathogenesis of rheumatoid arthritis has been the subject of controversy for many years, and many experimental models have been suggested and, on the whole, rejected (see Gardner, 1960). The possible involvement of an auto-immune response in this disease is now being widely considered, and an experimental model in rats which resembles in some respects, the human disease, is also being investigated (Pearson, 1964). This model, a so-called "adjuvant arthritis" is produced by injecting Freund's adjuvant, or a variety of acid fast bacilli, into rats. About 14 days after this injection a polyarthritis develops that affects the peripheral joints, ears and tail.

In the present study we have investigated the development of a polyarthritis in alloxan-diabetic rats. Female rats (130–150 g) were given 150 mg/kg of alloxan intraperitoneally and those that had blood sugar levels of over 350 mg% one week later, were used. Groups of normal and alloxan-diabetic rats were given a subcutaneous injection of 0·05 ml of a 2·5 mg/ml suspension of dead tubercle bacilli in liquid paraffin into the plantar surface of the left hind paw. The bacilli were derived from human stains PN, DT and C. The diameter of the foot originally injected was measured before and at intervals after injection, using the method described by Newbould (1963). The rats were carefully observed for the development of secondary lesions of the paws, ears and tail. The blood sugar levels of the alloxan-diabetic rats were checked weekly. The result of 28 days observation are summarised in Table 1.

In the alloxan-diabetic rats, when compared with normal animals, there was a significant reduction in the primary inflammatory response to injection of dead bacilli into the paw. The appearance of secondary lesions was delayed in the

TABLE 1. SUPPRESSION OF AN "ADJUVANT ARTHRITIS" IN ALLOXAN-DIABETIC RATS

	Normal					Alloxan-diabetic			
Days after adjuvant injection	Mean increase in foot thickness mm ± s.e.	Distribution of secondary lesions Foot	Tail	Ear	Mean number of secondary lesions/rat	Mean increase in foot thickness mm ± s.e.	Distribution of secondary lesions Foot (Ear, nil)	Tail	Mean number of secondary lesions/rat
3	4·46 ± 0·12	0	0	0	0 (18)	2·59 ± 0·21	0	0	0 (12)
5	5·02 ± 0·14	0	0	0	0 (18)	3·22 ± 0·33	0	0	0 (12)
7	5·20 ± 0·17	0	0	0	0 (18)	3·04 ± 0·27	0	0	0 (11)
10	5·10 ± 0·24	3	0	0	0·17 (18)	2·69 ± 0·23	0	0	0 (11)
12	5·12 ± 0·24	3	0	0	0·18 (17)	2·29 ± 0·18	0	0	0 (11)
14	5·07 ± 0·21	11	4	0	0·88 (17)	2·68 ± 0·18	1	0	0·09 (11)
17	5·42 ± 0·21	19	4	6	1·70 (17)	3·70 ± 0·17	3	1	0·36 (11)
19	4·93 ± 0·18	20	8	6	2·00 (17)	3·58 ± 0·22	3	6	0·82 (11)
21	4·96 ± 0·22	20	8	6	2·00 (17)	3·50 ± 0·23	3	7	0·91 (11)
24	4·95 ± 0·21	20	8	6	2·00 (17)	4·00 ± 0·28	3	7	0·91 (11)
26	4·72 ± 0·16	20	8	6	2·00 (17)	3·80 ± 0·23	2	6	0·73 (11)
28	4·77 ± 0·18	20	8	6	2·00 (17)	3·78 ± 0·25	2	6	0·73 (11)

Figures in parentheses indicate number of animals.

hyperglycaemic rats and the average number of new lesions produced was reduced from 2·0 to 0·91 per rat. The distribution of the new lesions appeared to be different in the two groups. In normal animals the feet were predominantly affected and in hyperglycaemic animals the tails.

The mechanism of this suppressive effect of hyperglycaemia in allergic and inflammatory conditions is unknown. It has been suggested that hyperglycaemia inhibits, and hypoglycaemia potentiates the antigen-antibody reaction if this reaction involves a carbohydrate moiety (Adamkiewicz, 1963). An example is the tuberculin reaction which high blood sugar levels decrease and low levels increase (Cornforth & Long, 1953). Polysaccharide antigens are known to be involved in this reaction. However, this hypothesis does not take account of how hyperglycaemia inhibits anaphylactoid reactions and suppresses the formation of granulation tissue, processes which do not involve antigen-antibody combination.

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References

Adamkiewicz, V. W. (1963). *Canad. Med. Ass. J.*, **88**, 806–811.
Cornforth, J. W. & Long, D. A. (1953). *Lancet*, **1**, 160–164.
Ganley, O. H. (1962). *Canad. J. Biochem.*, **40**, 1179–1183.
Gardner, D. L. (1960). *Ann. rheum. Dis.*, **19**, 297–317.
Goth, A., Nash, W. L., Nagler, M. & Holman, J. (1957). *Amer. J. Physiol.*, **191**, 25–28.
Nagy, S., Redei, A. & Karady, S. (1961). *J. Endocrin.*, **22**, 143–146.
Newbould, B. B. (1963). *Brit. J. Pharmacol.*, **21**, 127–136.
Pearson, C. M. (1964). *Arthritis Rheum.*, **7**, 80–86.
Thompson, G. E. (1961). *Nature, Lond.*, **190**, 822.

Validity of ptosis as a measure of the central depressant action of reserpine

SIR,—Ptosis is a characteristic feature of the action of reserpine in many animal species and has been used for the bioassay of reserpine-like alkaloids (Rubin, Malone, Waugh & Burke, 1957). It is usually regarded as a sign of the central action of reserpine, and the ability of drugs to prevent reserpine-induced ptosis has been proposed as a test for antidepressants (Chen, 1964). However, ptosis is also produced by adrenergic-neurone blocking agents of the quaternary ammonium or guanidine types (Costa, Kuntzman, Gessa & Brodie, 1962; Fielden & Green, 1965), which do not enter the brain in significant amounts (Boura, Copp, Duncombe, Green & McCoubrey, 1960). Since reserpine causes profound noradrenaline depletion in peripheral adrenergically-innervated tissues, with consequent loss of sympathetic function (Carlsson, Rosengren, Bertler & Nilsson, 1957), it is pertinent to inquire whether reserpine-induced ptosis may not also occur as a result of peripheral sympathetic blockade.

In Table 1, the extent of ptosis, scored on a 0–8 scale (Rubin & others, 1957), is compared with the depletion of heart and brain noradrenaline, or brain 5-hydroxytryptamine (5-HT) 4 hr after subcutaneous injection of various doses of

TABLE 1. EXTENT OF PTOSIS AND DEPLETION OF NORADRENALINE AND 5-HT IN MICE TREATED WITH RESERPINE (R) OR RESERPINE PLUS BRETYLIUM (R + B)

Dose of reserpine (mg/kg)	Ptosis		Noradrenaline depletion (%)				5-HT depletion (%)	
			Heart		Brain		Brain	
	R	R + B	R	R + B	R	R + B	R	R + B
0·025	0	—	40	—	<10	—	—	—
0·05	0	—	65	—	10	—	—	—
0·1	2·0	—	85	—	15	—	—	—
0·3	4·8	0·3	>95	40	35	25	25	30
0·5	5·5	2·0	>95	70	60	65	50	50
1·0	6·3	3·5	>95	85	80	90	70	75

reserpine (Serpasil, Ciba), or reserpine plus bretylium tosylate (20 mg/kg), into groups of 6 mice. The hearts from all 6 mice were pooled, as were the brains from 2 mice in each group. The noradrenaline and 5-HT were extracted with butanol and assayed fluorimetrically (Mead & Finger, 1961; Fielden & Green, 1965). The minimal dose of reserpine producing ptosis was 0·1 mg/kg, which lowered heart noradrenaline by 85% but brain noradrenaline by only 15%. Reserpine is known to deplete noradrenaline more readily from peripheral tissues than from brain (Carlsson & others, 1957), and loss of cardiac responses to sympathetic stimulation has been shown to be detectable after depletion of 85–90% of heart noradrenaline (Gaffney, Chidsey & Braunwald, 1963). More striking evidence that reserpine-induced ptosis is caused primarily by peripheral noradrenaline depletion is the protection afforded by bretylium, a quaternary ammonium compound which does not readily penetrate the brain (Boura & others, 1960). Bretylium alone does not significantly affect mouse-heart noradrenaline although it does produce ptosis which, however, disappears within 4 hr. When it is injected together with reserpine, the reserpine-induced ptosis is markedly decreased as is the fall in heart noradrenaline, but the fall in brain noradrenaline or 5-HT is not. The decrease in ptosis is roughly correlated with the prevention of heart-noradrenaline depletion. This effect is essentially a delay in onset of ptosis, not a reversal, since ptosis was as marked 24 hr after reserpine plus bretylium as after reserpine alone; and if bretylium was given 4 hr after reserpine no diminution in ptosis was seen. Bretylium has previously been shown to protect rats against heart-noradrenaline depletion after giving reserpine (Callingham & Cass, 1962).

Brodie, Spector & Shore (1959) attempted to differentiate between passive eyelid closure due to sympathetic blockade on the one hand, and active eyelid closure due to central parasympathetic stimulation on the other. We have been unable to observe such a distinction, but our experiments do not exclude the possibility that a centrally-produced active eyelid closure may occur with doses of reserpine above 1 mg/kg. It nevertheless seems clear from our results that ptosis after low doses of reserpine is produced by a peripheral rather than a central mechanism. This being so, prevention of reserpine-induced ptosis should be looked at with some caution before it is accepted as a sign of a central antidepressant action.

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References

Boura, A. L. A., Copp, F. C., Duncombe, W. G., Green, A. F. & McCoubrey, A. (1960). *Brit. J. Pharmacol.*, **15**, 265–270.
Brodie, B. B., Spector, S. & Shore, P. A. (1959). *Pharmacol. Rev.*, **11**, 548–564.
Callingham, B. A. & Cass, R. (1962). *J. Pharm. Pharmacol.*, **19**, 385–389.
Carlsson, A. F., Rosengren, E., Bertler, A. & Nilsson, J. (1957). In *Psychotropic Drugs*, Editors, Garattini, S. & Ghetti, V., p. 363–372. Amsterdam: Elsevier.
Chen, G. (1964). In *Evaluation of Drug Activities: Pharmacometrics*, Editors, Laurence, D. R. & Bacharach, A. L. Vol. 1, p. 239–260. London: Academic Press.
Costa, E., Kuntzman, R., Gessa, G. L. & Brodie, B. B. (1962). *Life Sciences*, **1**, 75–80.
Fielden, R. & Green, A. L. (1965). *Brit. J. Pharmacol.* (in the press).
Gaffney, T. E., Chidsey, C. A. & Braunwald, E. (1963). *Circulation Res.*, **12**, 264–268.
Mead, J. A. R. & Finger, K. F. (1961). *Biochem. Pharmacol.*, **6**, 52–53.
Rubin, B., Malone, M. H., Waugh, M. H. & Burke, J. C. (1957). *J. Pharmacol.*, **120**, 125–136.

Seasonal variation in the resistance of rats

SIR,—For the past three years, the sensitivity of rats to anaphylactic shock has been found to show seasonal variation. It was first thought that the antigen or the adjuvant might have been modified in the summer months but this possibility was finally ruled out by our obtaining similar results with different antigens and different adjuvants. It has since been found that the resistance of the animals varies with the season, as illustrated in Table 1.

TABLE 1. CHANGES IN THE MORTALITY RATE OF WISTAR RATS SUBJECTED TO ANAPHYLACTIC SHOCK AT DIFFERENT TIMES OF THE YEAR 1964

Month	No. of rats tested	No. of deaths	Mortality rate (%)
Jan.–Feb.	45	40	89
April–May	28	20	71
July–Aug.	28	4	14
Nov.–Dec.	36	33	92

Male Wistar albino rats (body weight 150–200 g) obtained from A.R.C., Compton, were sensitised using horse serum (0·5 ml) and *Bordetella pertussis* vaccine (0·25 ml of 80,000 $\times$ 10^6 organisms per ml) intraperitoneally. Ten to twelve days later, they were challenged intravenously with horse serum (1 ml) and deaths were recorded over 24 hr. During the period from June to September, they were relatively insensitive to anaphylactic shock, whereas at other times high mortality rates were obtained. It was possible to reduce the challenging dose to 0·05 ml in the winter and obtain similar high mortality rates. This observation may be of importance to those who are studying the mechanism of anaphylactic shock *in vivo* and that of the antigen-antibody reaction using isolated mast cells.

A similar change in the sensitivity of rats has also been noted after experimental traumatic or tourniquet shock. To produce traumatic shock, anaesthetised male Wistar albino rats were rotated in a revolving drum (40 rotations/min) so that at each rotation they fell 18 inches (the Noble–Collip technique). They were then removed from the drum and their mortality rates were recorded over 24 hr. The results shown in Table 2 indicate that rats in June and July were much more resistant than those in November and needed at least twice as long in the revolving drum to produce similar mortality rates.

To produce tourniquet shock, male Wistar albino rats were restrained and rubber tourniquets were placed high up on the hindlimbs for 4, 5 or 6 hr. They

TABLE 2. CHANGES IN THE MORTALITY RATE OF WISTAR RATS SUBJECTED TO EXPERIMENTAL TRAUMATIC SHOCK AT DIFFERENT TIMES OF THE YEAR 1964

Month	No. of rats tested	Mortality rate (%) after various rotations				
		200	400	600	1,000	1,600
June–July ..	45	0	0	0	50	100
Sept.–Oct. ..	25	0	0	50	100	—
Nov.–Dec. ..	33	0	50	100	—	—

were then returned to their cages and mortality rates were recorded over 24 hr. The results shown in Table 3 indicate that rats in June were more resistant than those in December.

TABLE 3. CHANGES IN THE MORTALITY RATE OF WISTAR RATS SUBJECTED TO EXPERIMENTAL TOURNIQUET SHOCK AT DIFFERENT TIMES OF THE YEAR 1964

Month	No. of rats tested	Mortality rate (%) after various times of application		
		4 hr	5 hr	6 hr
June–July	40	0	0	90
Nov.–Dec.	30	10	90	100

The dextran anaphylactoid reaction does not occur in diabetic rats or in rats in which the blood sugar levels have been markedly raised by injections of glucose or other monosaccharides. The mechanism of this inhibitory action has not yet been fully elucidated and it may be that the rate of entry of dextran, a polymer of glucose, into cells is modified by glucose or that the breakdown of dextran into glucose is accelerated. When the inhibitory effect of glucose was tested at different times of the year using a standard amount of the monosaccharide, less inhibition was found in November than in June. Male Wistar albino rats were given 2 doses of glucose, each of 1·5 g/kg intraperitoneally, the first, 30 min before the dextran (240 mg/kg intraperitoneally), and the second at the same time as the dextran. The anaphylactoid reaction was then recorded over the next 4 hr using an arbitrary visual scoring system. Table 4 shows that

TABLE 4. CHANGES IN THE INHIBITION OF THE DEXTRAN ANAPHYLACTOID REACTION IN WISTAR RATS BY GLUCOSE AT DIFFERENT TIMES OF THE YEAR 1964

Month	No. of rats tested	% inhibition
June–July	20	74
Sept.–Oct.	20	24
Nov.–Dec.	28	18

rats probably utilised glucose easier in the summer and marked inhibition of the reaction occurred. The absorption of dextran may also have been more efficiently delayed by glucose in the summer, as doubling of the dose of glucose in the winter resulted in marked inhibition of the reaction. Similar results were obtained with galactose as the inhibitory agent and dextrin (Astra) as the effective stimulatory agent.

The reason for these seasonal changes in resistance has still to be found.

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Lack of the effect of melatonin on the frog spermatogenic reaction

SIR,—Parenterally injected homogenised tissues from cow pineal glands diminish the spermatogenic response of the male frog to chorionic gonadotrophin injection (Juszkiewicz & Rakalska, 1963). Recently, Wurtman, Axelrod & Chu (1963) reported that some of the effects of the pineal gland on gonad function might be mediated by melatonin. Daily injections of microgram amounts of melatonin to rats decreased the incidence of oestrus and reduced ovarian weight. It was found that pinealectomy in rats was followed by an increase in the incidence of oestrus; this increase was inhibited by melatonin treatment (Chu, Wurtman & Axelrod, 1964).

However, there is some discrepancy in the reported effects of melatonin on the functioning of gonads. De la Lastra & Croxatto (1964) found that the whole human brain contains a substance producing depletion of the ovarian ascorbic acid, but they failed to obtain similar results in rats injected with melatonin. Moreover, Kappers (1962) reported that melatonin did not affect spermatogenesis in full grown rats.

It was of interest to find whether melatonin affected the spermatogenic response of the male edible frog, *Rana esculenta* L., to chorionic gonadotrophin injection. In the course of preliminary investigations, with the conditions we used we found that the most suitable dose was 20 I.U. of chorionic gonadotrophin (Gonadotrophine chorionique I.S.H., Paris) in 0·5 ml of saline solution; the best response was attained while examining the urine 3 and 6 hr after the injection of the hormone. Solutions of melatonin in ethanol were prepared freshly for injection with a subsequent 1:100 dilution with water. They were so adjusted that 0·5 ml contained 10, 100 or 500 μg of the substance. The preparations were injected into the dorsal lymph sac of the frogs. A saline solution of homogenised cow pineal glands (0·5 ml/100 mg of fresh tissue) was given once, 6 hr, and melatonin solutions twice, 12 hr and 30 min, before the injection of chorionic gonadotrophin. The experiment was made simultaneously on 242 male frogs in 6 groups.

TABLE 1. EFFECTS OF PARENTERALLY INJECTED MELATONIN AND HOMOGENIZED TISSUES FROM COW PINEAL GLANDS ON THE SPERMATOGENIC RESPONSE OF THE MALE FROG, *RANA ESCULENTA*, TO CHORIONIC GONADOTROPHIN INJECTION

Treatment		Spermatogenic response		
	No. of frogs	No. of frogs	Relative %	P*
Control group; chorionic gonadotrophin 20 I.U. ..	70	44	100	—
Melatonin 500 μg	35	1	4	0·001
Melatonin 10 μg; chorionic gonadotrophin 20 I.U. ..	35	21	95	0·9
Melatonin 100 μg; chorionic gonadotrophin 20 I.U. ..	34	19	89	0·5
Melatonin 500 μg; chorionic gonadotrophin 20 I.U. ..	34	22	103	0·8
Cow pineal 0·1 g; chorionic gonadotrophin 20 I.U. ..	34	5	23	0·001

* Compared with the control group by the χ^2 method with Yates' correction.

The results are in Table 1. The number of frogs which gave a positive spermatogenic response in a control group was taken as 100% (relative per cent). In relation to that figure the percentage of frogs reacting in the experimental groups was calculated.

We have been unable to find any action of injected melatonin on the frog spermatogenic reaction, whereas homogenised tissues from cow pineal glands

exhibited very significant inhibition, facts which support our previous experience (Juszkiewicz & Rakalska, 1963).

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References

Chu, E. W., Wurtman, R. J. & Axelrod, J. (1964). *Endocrinol.*, **75**, 238–242.
Juszkiewicz, T. & Rakalska, Z. (1963). *Nature, Lond.*, **200**, 1329–1330.
Kappers, J. A. (1962). *Gen. comp. Endocrin.*, **2**, 610–611.
de la Lastra, M. & Croxatto, H. (1964). *Nature, Lond.*, **204**, 583–584.
Snedecor, G. W. (1956). *Statistical Methods*, 5th ed., pp. 212–225. Ames, Iowa: Iowa State University Press.
Wurtman, R. J., Axelrod, J. & Chu, E. W. (1963). *Science*, **141**, 277–278.
Wurtman, R. J., Axelrod, J. & Potter, L. T. (1964). *J. Pharmacol.*, **143**, 314–318.

Mathematical treatment for oral sustained release drug formulations

SIR,—Recently a detailed mathematical treatment of drug release from oral sustained release dosage forms was presented by Rowland & Beckett (1964), who made a direct criticism of an equation presented by me to calculate the maintenance dose for sustained release (Nelson, 1957). These authors argued that the earlier presentation did not take into account the amount of drug that might be released from the maintenance portion of the dose during the time the initial dose was being absorbed. It should be pointed out that sustained release forms do exist from which only insignificant amounts of the maintenance portion of drug are released until the initial dose is absorbed (for example, Spansule and repeat action tablets). Therefore, the treatment presented by Rowland & Beckett (1964) applies only to the special case where release from the "free form" and "maintenance form" begins at the same time.

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References

Nelson, E. (1957). *J. Amer. pharm. Ass., Sci. Ed.*, **46**, 572–573.
Rowland, M. & Beckett, A. H. (1964). *J. Pharm. Pharmacol.*, **16**, *Suppl.* 156*T*–162*T*.

Amphetamine and stores of noradrenaline

SIR,—Tyramine is known to exert at least part of its pressor activity through the release of noradrenaline from its storage sites in postganglionic adrenergic nerve endings (Burn & Rand, 1958; Lockett & Eakins, 1961; Hertting, Axelrod & Patrick, 1961; Bhagat, 1963; 1964). Schumann & Philippu (1962) have further shown that tyramine and other indirectly acting sympathomimetic amines displace noradrenaline in almost stoichiometric proportions (mole by mole) from the isolated granules of the adrenal medulla. If this occurs also with noradrenaline in sympathetic postganglionic nerve endings then each injection of tyramine would decrease the noradrenaline content of the heart. But tyramine is also a good substrate for monoamine oxidase and is removed immediately from its stores probably mostly by oxidase action. If a sufficient time is allowed between successive injections of tyramine, the rate of replenishment of noradrenaline keeps pace with its release and consequently no tachyphylaxis develops. The other indirectly acting sympathomimetic amines, for example, amphetamine, which are not substrates for monoamine oxidase, may remain bound with the store, may prevent the entry of subsequent doses of indirectly acting amine (tyramine), and also the replenishment by more noradrenaline. Therefore, tachyphylaxis to these amines would be rapid and of a more permanent nature. This is consistent with the views of Blaschko (1962) that binding of amine at the site of storage may be responsible for the phenomenon of tachyphylaxis.

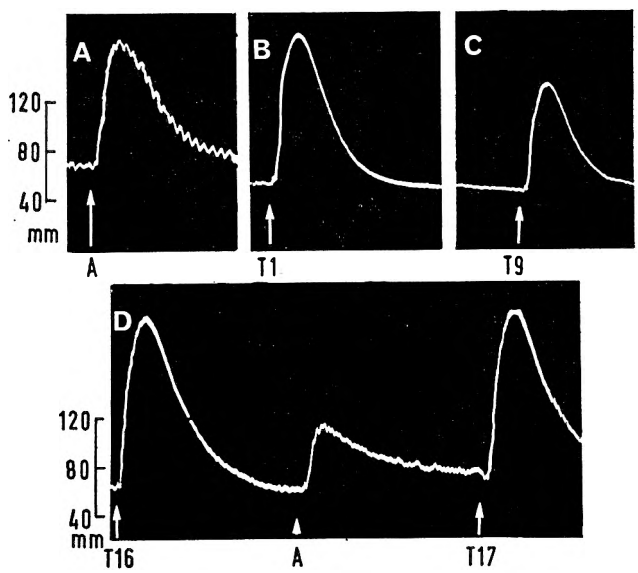

FIG. 1. A. Pressor response of a spinal cat (3·0 kg) to 200 μg/kg of amphetamine (A). This result is typical of four experiments.
B, C, D. Pressor responses of a spinal cat (3·2 kg). Tyramine (800 μg/kg) was injected intravenously at 15 min intervals. The numerical sequence of tyramine doses (T) is indicated. After the 16th dose 200 μg/kg amphetamine (A) was injected. This result is typical of four experiments.

Previous work from this laboratory (Bhagat, Kopin, Gordon & Booker, 1964; Bhagat, Gordon & Kopin, 1965) has shown that responses to repeated doses of

tyramine (800 μg/kg), administered intravenously to spinal cats, at first progressively diminish in size, that is, tachyphylaxis develops; with continued tyramine administration the response gradually returns to normal, that is, an escape from tyramine tachyphylaxis occurs. When the response to 800 μg/kg of tyramine has returned to normal, the response to exogenous noradrenaline is not significantly enhanced. The catecholamine content of the heart is diminished when tachyphylaxis is demonstrable (after the 9th dose), but is even further diminished when the response to tyramine has returned to normal (after the 16th dose). Biochemical evidence has been presented which indicates that tyramine administration accelerates noradrenaline synthesis, since tyramine is itself a precursor of noradrenaline (Chidsey, Kaiser & Lehr, 1964). This increased rate of synthesis appears to replenish an easily released noradrenaline store since escape from tachyphylaxis occurs, even though the total catecholamine content continues to diminish.

It is known that there is a cross tachyphylaxis between individual indirectly acting sympathomimetic amines. Presumably these amines act on the same store or pool of noradrenaline. It was therefore of interest to see whether the response to amphetamine returned to normal at a time when the response to tyramine had returned to normal (after 16th dose).

Cats of 2 to 3·5 kg body weight and of either sex were anaesthesised with ether and spinal preparations were set-up as described by Burn (1952). The arterial blood pressure was recorded from a carotid artery with a mercury manometer. All drugs were injected into a cannula tied into the femoral vein unless otherwise stated, and flushed in with 0·5 ml of normal saline.

The results indicated that at the 16th dose, the response to tyramine returned to normal (Fig. 1). Intravenous administration of 200 μg/kg of amphetamine showed a reduced response which was about 40 [±3·5 (4)] % of the normal. The response to tyramine after amphetamine was unaltered.

These results suggest that the mechanism by which amphetamine releases catecholamines is different from that of tyramine and may not be by an action on the same stores or pools of noradrenaline.

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References

Bhagat, B. (1963). *J. Pharm. Pharmacol.*, **15**, 152.
Bhagat, B. (1964). *Arch. int. Pharmacodyn*, **147**, 26–35.
Bhagat, B., Kopin, I. J., Gordon, E. & Booker, W. M. (1964). *Pharmacologist*, **6**, 206.
Bhagat, B., Gordon, E. & Kopin, I. J. (1965). *J. Pharmacol.* (in press).
Blaschko, H. (1962). *In Hypertension Recent Advances.* Editor, Brest, A. N. & Moyer, J. H. Pp. 321–329, London: Kimpton.
Burn, J. H. (1952). *Practical Pharmacology*, Blackwell, Oxford.
Burn, J. H. & Rand, M. J. (1958). *Brit. J. Pharmacol.*, **13**, 471–479.
Chidsey, C. A., Kaiser, G. A. & Lehr, B. (1964). *J. Pharmacol.*, **144**, 393–398.
Hertting, G., Axelrod, J. & Patrick, R. W. (1961). *Biochemical Pharmacol.*, **8**, 246–247.
Lockett, M. F. & Eakins, K. E. (1960). *J. Pharm. & Pharmacol.*, **12**, 720–725.
Schumann, H. J. & Philippu, A. (1962). *Nature* (*Lond.*), **193**, 890–891.

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Letters to the Editor

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