Journal of Pharmacy and Pharmacology

Published by
The Pharmaceutical Society
of Great Britain

Volume 19 No. 10
October 1967

Journal of Pharmacy and Pharmacology

Published by THE PHARMACEUTICAL SOCIETY OF GREAT BRITAIN

17 Bloomsbury Square, London, W.C.1. Telephone: 01-405 8967

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J. Pharm. Pharmac., 1967, **19**, 641–648 Received May 19, 1967

The effect of aprotic solvents on the adsorption of phenol by alumina*

B. HIGGINS AND J. H. RICHARDS

The isotherms for the adsorption of phenol onto alumina from cyclohexane, benzene, nitrobenzene, ethyl acetate and 1,4-dioxan solutions have been determined at 25°. The change in solvent in the above order causes a successive decrease in the amount of phenol adsorbed at the 'saturation level' and possible explanations of the solvent effects are discussed.

THE adsorption of non-electrolytes from solution, which is of importance in the chromatographic separation of many materials, can be affected by solute-solvent and solvent-adsorbent interactions. In the event of solute-solvent interactions, e.g. molecular complex formation, the number of free solute molecules will be reduced and the amount of adsorption will therefore decrease. It is possible that the solute-solvent complex may also be adsorbed thus resulting in a mixed surface layer of solute and solvent molecules. This possibility will depend on the nature of the complex and the adsorbent. The solvent may itself be adsorbed either at the same sites as the solute molecules, thus being in direct competition with them, or at other sites, so providing the possibility of partial block of the solute sites by steric hindrance. In either instance there will be a reduction in the degree of solute adsorption. Often the solvent may show a combination of the above effects, which involve interaction with the solute and substrate respectively.

There has been little previous work discussing in detail the effects of solvents on a particular adsorption system. Although the results for the adsorption of solutes from different solvents have been reported, no attempts have been made to find any definite correlation between the solvent properties and the effects on adsorption. For example, Kipling & Wright (1962) have shown that the amount of stearic acid adsorbed onto "Spheron 6" decreases as the solvent changes through the series cyclohexane, ethanol, carbon tetrachloride and benzene. They state that it is possible that the orientation of the molecules at the surface may vary, depending on the solvent from which they are adsorbed, although they conclude that it is more likely that the adsorbed layer is a mixed layer containing solvent as well as solute molecules. In addition, Chatterji & Singhal (1960) have shown that the amounts of butyric, propionic and acetic acids adsorbed onto alumina, decreased with solvent in the order benzene, toluene, n-propanol and water. Where n-propanol and water were used as solvents the decrease in acid adsorption was attributed to adsorption of the solvent. A mention of the possible importance of solvent-solute association was made by Venturello & Ghe

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* This work formed part of a thesis (B.H.) accepted for the degree of Ph.D. in the University of London.

(1959), who measured the adsorption of iodine onto magnesia from pentane, carbon tetrachloride, dioxan and benzene solutions. These authors found that the amount of iodine adsorbed from any solvent decreased in the above solvent order and suggested that this may be due to the formation of iodine-solvent complexes with benzene and dioxan.

In view of the small amount of available information about the effects of solvents on adsorption systems we decided to investigate a specific system in an attempt to clarify these effects.

Alumina was chosen as the adsorbent because it is a common chromatographic material and the main mechanism for the adsorption of organic non-electrolytes onto it has been reasonably well established. Phenol was chosen as the solute because its concentration in solution can be readily determined by several methods and because it is readily adsorbed by alumina (Crisp, 1956; Eric, Goode & Ibbitson, 1960). The mechanism of this adsorption has been shown to involve hydrogen bond formation between the phenolic OH group and the surface oxygen atoms of alumina (Giles, Mehta & others, 1954; Cummings, Garven & others, 1959). A series of aprotic solvents, showing a range of hydrogen-accepting ability, was chosen to provide a variation in the degree of solute-solvent interaction by intermolecular hydrogen bond formation while, at the same time, allowing the competition of the solvent for the adsorption sites of phenol to be neglected. The shifts in the hydroxyl stretching frequency ($\triangle\nu_{OH}$) of phenol in the various solvents were used as an approximate indication of the relative hydrogen accepting abilities of the solvents chosen for this investigation. These solvents were cyclohexane, benzene, nitrobenzene, ethyl acetate and 1,4-dioxan, for which the $\triangle\nu_{OH}$ values were 0, 63, 102, 161 and 307 cm^{-1} respectively, relative to the stretching frequency of the hydroxyl group of phenol in cyclohexane solution (Tsuboi, 1952; Bellamy, Hallam & Williams, 1958).

Experimental

MATERIALS

Neutral chromatographic alumina of activity grade 1 (Woelm) was stored in bulk in a well-closed container. Periodic checks were made, by measurement of the adsorption isotherm of phenol from cyclohexane solution, to ensure that there was no loss in activity. Phenol (A.R.) was fractionally distilled at atmospheric pressure (b.p. 182°), and stored in a well-closed container.

Cyclohexane (A.R.) was dried over calcium chloride and then fractionally distilled from potassium metal through a 20-plate column. The product was stored in well-closed amber containers over sodium wire. It had b.p. 80·8° at 760 mm.

Benzene (A.R.) was washed with concentrated sulphuric acid (100 ml/litre of benzene) until no further discolouration of the acid occurred. The benzene was then washed with three lots of distilled water (100 ml/litre) and dried over two successive amounts of sodium hydroxide pellets.

The dried product was distilled from potassium metal through a 20-plate column and the distillate was stored in well-closed amber containers over sodium wire. It had b.p. 80·4 at 760 mm.

1,4-Dioxan (A.R.) was refluxed over sodium until the metal remained bright and then distilled from fresh sodium through a 20 plate column. The product was stored over sodium wire in well-closed amber containers. It had b.p. 101·3° at 760 mm.

Ethyl acetate (A.R.) was dried over anhydrous sodium carbonate and then fractionally distilled through a 20-plate column. The product was stored over molecular sieve type 4A in well-closed amber containers. It had b.p. 77·1° at 760 mm.

Nitrobenzene (A.R.) was dried over calcium chloride and then fractionally distilled under reduced pressure. The product was stored in well-closed amber containers over molecular sieve type 4A. It had b.p. 211° at 760 mm.

METHOD

The adsorption of phenol by 1 g quantities of alumina from 20 ml samples of solution was measured by a batch process in glass 60 ml ampoules. The sealed ampoules were submerged in a water-bath at 25·0° and rotated at 30 rev/min for 30 min. This time had been found, from preliminary experiments, to be sufficient to allow equilibrium to be attained. After careful drying the ampoules were opened and the supernatant solutions were decanted and analysed.

The above procedure was modified for the determination of desorption isotherms. After adsorption the ampoules were opened at the tip and the supernatant solutions were decanted. The maximum amount of liquid was removed carefully without the removal of any alumina. Pure solvent (20 ml) or phenol solution of a lower concentration than that removed, was then added. The ampoules were resealed and equilibrated for a further 30 min.

A Unicam S.P. 500 spectrophotometer was used in the analysis of the phenol in solutions except when it was in nitrobenzene solution where a procedure similar to the B.P. method of assay for phenol was used. A blank determination was made using pure nitrobenzene. The accuracy of the method was tested using several solutions of known phenol concentration in nitrobenzene, and the error was less than 1%.

Results

The isotherms for the adsorption of phenol onto alumina from cyclohexane, benzene, nitrobenzene, ethyl acetate and 1,4-dioxan solutions at 25° are shown in Fig. 1.

The applicability of the Langmuir equation to these adsorption results was tested. This equation may be written as

$$\frac{c}{x_0} = \frac{1}{x_1 a} + \frac{c}{x_1} \qquad (1)$$

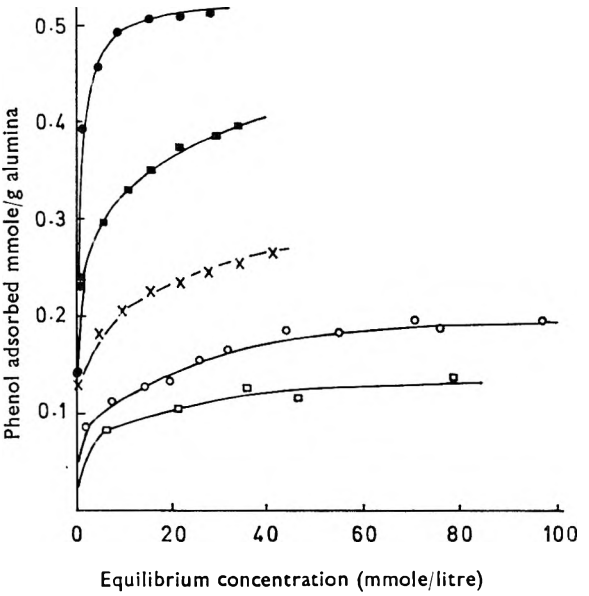

FIG. 1. Isotherms for the adsorption of phenol onto alumina from cyclohexane (●), benzene (■), nitrobenzene (×), ethyl acetate (○) and 1,4-dioxan (□) solutions at 25°.

where c is the equilibrium concentration of phenol in solution, x_0 is the amount of solute adsorbed by unit weight of the adsorbent, x_1 is the corresponding amount adsorbed at the "saturation level", i.e. the monolayer capacity of the solid, and a is constant for a particular system. Linear plots of c/x_0 against c were obtained in all cases, thus indicating the applicability of equation (1) to these results. The slopes and intercepts of these plots could be used to obtain the values of $1/x_1$ and $1/x_1a$, respectively.

Table 1 shows the values of x_1 and a obtained from a least squares treatment of the adsorption data, together with the values of the coverage factor (θ), which represents the fraction of the surface covered with phenol molecules relative to an assumed complete coverage in the cyclohexane system.

The application of the composite isotherm treatment advocated by Kipling and his co-workers (Kipling, 1951, Kipling & Peakall, 1956; Kipling & Wright, 1962, 1964) to the adsorption data obtained for the

TABLE 1. THE LANGMUIR CONSTANTS (a AND x_1) AND COVERAGE FACTORS (θ) FOR THE ADSORPTION OF PHENOL ONTO ALUMINA FROM SOLUTION AT 25°

Solvent	a	x_1 moles/g	θ
Cyclohexane ..	4·87	0·513	1·00
Benzene	0·76	0·398	0·78
Nitrobenzene ..	0·47	0·267	0·52
Ethyl acetate ..	0·12	0·214	0·42
1,4-Dioxan ..	0·09	0·154	0·30

cyclohexane and 1,4-dioxan systems produced little change in the adsorption isotherms shown in Fig. 1. This is as expected (Kipling & Wright, 1962) since only dilute solutions were involved and therefore such treatment was not used further in this investigation.

It was found that the desorption isotherms for the phenol–alumina–cyclohexane and phenol–alumina–benzene systems were identical to the corresponding adsorption isotherms.

Discussion

The identical shapes of the adsorption and desorption isotherms for the phenol–alumina–cyclohexane and phenol–alumina–benzene systems indicate that the adsorption of phenol onto alumina is completely reversible and the model of a dynamic equilibrium can therefore be envisaged. This is a factor in support of the application of the Langmuir equation to these systems. It is realized that care must be taken in the application of the Langmuir equation to solution adsorption data. However, the equation is used mainly in this investigation for the calculation of x_1, values. These calculated results show good agreement with the values obtained by extrapolation of the isotherms.

The adsorption isotherm for the phenol-alumina-cyclohexane system is similar to the 'L2' isotherm in the classification of isotherms for adsorption from solution suggested by Giles, MacEwan & others (1960). The steep slope of the first part of the adsorption isotherm indicates ease of adsorption of phenol molecules because of a large proportion of adsorption sites being initially available to the molecules. In a strictly Langmuirian system this slope decreases as the surface becomes filled with adsorbed molecules. However, the initial slope of the isotherm in Fig. 1 does not decrease as adsorption takes place, in fact the isotherm is almost coincident with the x_0 axis, and the phenol molecules are almost quantitatively adsorbed from solution giving equilibrium concentrations close to zero. This as a slight deviation from the Langmuir shape, which is not reflected in the plot of c/x_0 against c due to the low values of c and $/cx_0$. Although the adsorption isotherm is considered to be an "L2" isotherm, it is very close to the "H" type isotherm in the classification of Giles & others (1960). However, the equilibrium concentration of phenol is in fact finite in all cases and not zero, as with the "H"-type isotherm. The distinction between "H" and "L" isotherms may be somewhat artificial, since it depends on the sensitivity of the method used for the determination of equilibrium concentration.

The amount of solute adsorbed at the "saturation level" may represent the formation of a complete monolayer of adsorbate but, as pointed out by Giles & others (1960), this does not necessarily imply that there is a close-packed layer of a single species. The layer may contain solvent molecules as well as solute molecules, or it may consist of isolated clusters of solute molecules adsorbed at active sites.

Phenol molecules are believed to be adsorbed onto alumina in an end-on orientation with their hydroxyl groups directed towards the alumina

surface. Such orientation is also suggested by the "L" type of adsorption isotherms obtained in this investigation since this shape is claimed to be a characteristic of systems involving the end-on adsorption of a polar solute onto a polar substrate from a non-polar liquid (Giles & others, 1960). In addition, Giles and his co-workers (1960) state that the adsorption of a polar solute onto a polar substrate from a polar liquid usually gives an "S" type of isotherm since the solute meets with strong competition from the solvent for the adsorbent surface. Although cyclohexane, benzene and 1,4-dioxan are all non-polar, ethyl acetate and nitrobenzene are not and the isotherms for the systems involving the latter two solvents might therefore be expected to differ from those for the other systems. However the isotherms obtained for all of the systems appear to be of the "L2" type. This suggests that ethyl acetate and nitrobenzene do not compete strongly with phenol for sites on the alumina surface, and thus do not give rise to "S" type isotherms. It would appear, therefore, that interpretations of isotherms for adsorption from solution as suggested by Giles & others (1960) are of a general nature and that additional factors must sometimes be taken into account when interpreting the shape of a particular isotherm.

The initial parts of the isotherms for adsorption from cyclohexane, benzene and nitrobenzene are identical and are almost coincident with the x_0 axis. The difference between these isotherms arises at the point of breakaway from the x_0 axis these points being at 0·38, 0·23 and 0·12 mmole/g on the x_0 axis, for cyclohexane, benzene and nitrobenzene respectively. The points for the isotherms for adsorption from ethyl acetate and 1,4-dioxan solutions indicate that these isotherms may not be coincident with the x_0 axis at all and it is difficult to estimate a point of breakaway from the axis. The effect of the solvent on the initial parts of the isotherms is similar to that expected from theoretical considerations, since the varying degrees of competition of the solvents for phenol would cause a gradual increase in the equilibrium concentration of phenol required to produce a constant level of adsorption. However, it is difficult to observe a definite relationship between a constant amount of phenol adsorbed and the equilibrium concentration required to produce it in each solvent, because of the different "saturation levels" of the five isotherms. This variation in "saturation level," which is the main difference between the isotherms, is unexpected since previous work had suggested that the chosen solvents would not be adsorbed by alumina to any appreciable extent (Cummings & others, 1959; Giles & McKay, 1961). Thus, it was expected that a similar "saturation level" of phenol on alumina would be obtained in all the solvents and the equilibrium concentration at which this level would be reached would depend on the degree of phenol-solvent interaction. Although high equilibrium concentrations were used in the more active solvents no significant increases were observed in the amounts of phenol adsorbed.

The reduction of the amount of phenol adsorbed at "saturation level" from benzene, nitrobenzene, ethyl acetate and 1,4-dioxan may be due to any one of the following factors, or a combination of two or more:

(i) *Change in orientation of the adsorbed phenol molecules with change of solvent.* This may explain two levels of adsorption in a system where the molecules are adsorbed either flat or end-on at the surface. However, it is very unlikely that a change in orientation of phenol molecules could explain the five different adsorption levels observed here.

(ii) *Adsorption of a mixed monolayer of phenol and solvent molecules.* Although, as previously emphasized, the solvents should not be strongly adsorbed onto alumina, it is possible that they may be sufficiently adsorbed to affect the "saturation level" of the phenol.

(iii) *Adsorption of the phenol-solvent complex.* Phenol associates with each of the solvent species, benzene, nitrobenzene, ethyl acetate and 1, 4-dioxan, by the formation of an intermolecular hydrogen bond. It is unlikely that the phenol-solvent complex would be adsorbed, because it has no free hydrogen atom capable of bonding with oxygen atoms on the alumina surface. Adsorption will only occur if the complex hydrogen bond is broken, or if the complex is adsorbed at the surface by mechanisms other than hydrogen bonding.

(iv) *Phenol-solvent interaction.* The strength of the phenol-solvent interaction as indicated by $\triangle\upsilon_{OH}$ values (Tsuboi, 1952; Bellamy & others, 1958) increases in the inverse order to the x_1 values of the phenol-alumina-solvent systems. If the adsorbent sites on the alumina surface have a range of activity, then only those sites with a high activity are capable of adsorbing phenol molecules from a solvent such as 1,4-dioxan, in which the phenol-solvent interaction is relatively strong, since such adsorption involves the initial rupture of solute-solvent bonds. The weaker surface sites would only be of importance when the strength of the phenol-solvent bonds is reduced, as in the other solvents used in this investigation.

A consideration of the structure of alumina indicates that there will be some variation in the electronegativity of the surface oxygen atoms, depending on their position in the surface and the nature of adjacent atoms. In addition, the presence of some aluminium atoms and hydroxy groups on the alumina surface will lead to a variation in site activity, which is implicit in the proposition of different mechanisms of adsorption by alumina. Evidence for surface heterogeneity was obtained by Clark & Holm (1963), who investigated the adsorption of ammonia onto alumina and concluded that there was a broad distribution of adsorbent site energies. They stated that on adsorption the most active sites were filled first, followed by those of a lesser activity and so on. Further evidence in support of the concept of heterogeneity of the surface forces on alumina is obtained from the correlation of results for the adsorption of phenol from cyclohexane with the work of Graham (1953). This author classified adsorption systems on the basis of plots of an equilbrium function, given by $\theta/(1-\theta)c$, versus θ, which is the coverage factor, and suggested that surface heterogeneity is shown by an immediate tendency for the equilibrium function to decrease with increase in θ in these plots. A similar

treatment of the results obtained for the phenol-alumina-cyclohexane system produced this decrease in the equilibrium function.

The above evidence indicates that the adsorbing sites on the surface of alumina show a variation in activity. It would therefore seem possible to explain the difference in the "saturation levels" obtained in the various solvents on the basis of the change in the strength of the phenol-solvent interaction and the variation in the activity of the surface of alumina.

Acknowledgements. The authors would like to thank Mr. C. Gunn for the use of facilities within the School of Pharmacy, and the Principal and Governors of the College for the appointment of one of us (B.H.) as a research assistant.

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J. Pharm. Pharmac., 1967, **19**, 649–659 Received January 6, 1967

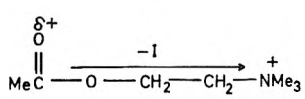
The possible role of conformational isomerism in the biological actions of acetylcholine: spectral studies

M. MARTIN-SMITH, G. A. SMAIL AND J. B. STENLAKE

Infrared studies of the NC–H stretching frequencies of selected trimethylammonium salts, including acetylcholine, were made in an attempt to obtain evidence that intramolecular NC–H - - - - O hydrogen bonding is responsible for the existence in solution of a *quasi*-ring conformation for acetylcholine. Acetylcholine and related compounds were also examined by nuclear magnetic resonance spectroscopy. The carbonyl stretching frequencies of an homologous series of acetoxy alkyl sulphones were measured in dioxan solution to gain information on the effect of electron withdrawing substituents in the absence of intermolecular hydrogen bonding. The observed high ester carbonyl absorption frequency in acetylcholine may be interpreted in terms of the inductive effect from the onium nitrogen atom but the results are not incompatible with the existence of a *quasi*-ring conformation involving C–H - - - - O hydrogen bonding between one of the *N*-methyl groups and the acetoxy oxygen atom.

RECENT publications of infrared (Fellman & Fujita, 1966) and nuclear magnetic resonance (Culvenor & Ham, 1966) spectroscopic evidence relating to the conformation of acetylcholine in various solvents prompt this report of our independent spectral studies on the neurohormone and related compounds.

In contrast to other comparative infrared studies (Fellman & Fujita, 1962, 1963, 1965, 1966) which were primarily concerned with establishing the influence of the inductive effect from the quaternary ammonium group (*1*), our studies were undertaken in an attempt to establish whether or not the cyclic conformation (*2*) of acetylcholine owed its stability to intramolecular $\overset{+}{N}C–H$ - - - - O hydrogen bonding as suggested by Sutor (1962, 1963) and Whittaker (1963). The evidence leading to the conclusion that the cyclic conformation *2*, or the similar conformation *3*,



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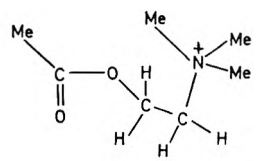

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for acetylcholine may exist in solution as well as in the solid state (Canepa, Pauling & Sörum, 1966; Culvenor & Ham, 1966) has been summarized in our earlier paper (Martin-Smith, Smail & Stenlake, 1967) together with speculations about the possible role of these conformations as the "muscarinic" conformer of acetylcholine.

Although the inductive effect of the quaternary ammonium group as shown in (*1*) would explain (compare Jones & Sandorfy, 1956) the observed high carbonyl absorption frequency in the infrared spectum of acetylcholine (Fellman & Fujita, 1962, 1963, 1965, 1966) as well as the

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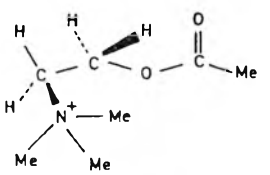
high electrophilicity of the ester carbonyl carbon atom as evidenced by kinetic studies (Butterworth, Eley & Stone, 1953; Fellman & Fujita, 1962) [despite challenge of this interpretation by Canepa & Mooney (1965)], it is also possible that the kinetic results and the infrared carbonyl frequency are the result of electron withdrawal from the carbonyl group *via* the acyloxy oxygen atom due to hydrogen bonding (*4*). This would serve to depress the permanent polarization of the carbonyl group with consequent rise in its absorption frequency (Henbest & Lovell, 1957; West, Korst & Johnson, 1960; Bruice & Fife, 1962; Biggins, Cairns & others, 1963).

Hydrogen bonding involving C–H groups as proton donors is not without parallel and it has been concluded that intramolecular C–H - - - - O bonds exist in the crystal structures of a number of compounds (Sutor, 1962, 1963; Canepa & others, 1966). Hydrogen bonding involving the C–H group has also been suspected in other instances (Arshid, Giles & Jain, 1956; Cummings, Garven & others, 1959; Pinchas, 1963) and the various arguments have been well reviewed (Pimentel & Maclellan, 1960). Allerhand & Schleyer (1963) have shown that the ability of C–H groups to act as proton donors in intermolecular hydrogen



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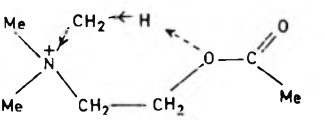

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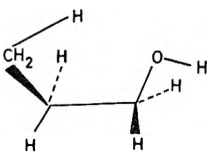
bonds depends upon the hybridization of the carbon atom ($sp > sp^2 > sp^3$) and increases with the number of electron withdrawing groups. They further concluded that the C–H group of an sp^3-hybridized carbon atom will only display proton donor propensities in those instances where that carbon atom is attached to at least two strongly activating groups. At variance with these generalizations and more pertinent to the present situation is the recent infrared study of Krueger & Mettee (1964). This has been interpreted as showing the conformational heterogeneity of propan-1-ol (*5*) in dilute carbon tetrachloride solution and evidence is presented for the existence of a weak intramolecular C–H - - - - O hydrogen bond by interaction of the C–H of the methyl group with one of the oxygen lone-pair orbitals. The favourable geometry of the n-propanol molecule is considered to be responsible for the existence of the C–H - - - - O hydrogen bond. In the *quasi*-ring conformation of acetylcholine (*2*) analogous features pertain in the favourable disposition for C-H - - - - O hydrogen bonding of one of the *N*-methyl groups and the acyloxy oxygen atom (Canepa & others, 1966; Culvenor & Ham, 1966). This, together with the reinforcing effect of an electron-withdrawing substituent, *viz.* the quaternary nitrogen atom, would rationalize the stabilization of *2* by the proposed intramolecular mechanism as in *4*.

The further application of infrared spectroscopy appeared to offer a promising method of establishing whether or not conformation *2* of acetylcholine existed in solution. In addition to the effect of C–H - - - - O hydrogen bonding on the carbonyl stretching frequency, the effect of such bonding should also be reflected in the nature of the C–H stretching and deformation frequencies of the *N*-methyl groups of acetylcholine. Accordingly an infrared study of these absorptions in acetylcholine and several other selected trimethylammonium salts was undertaken. Acetylcholine and related compounds were also examined by nuclear magnetic



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resonance spectroscopy. The carbonyl stretching frequencies of the sulphones $MeCH_2SO_2[CH_2]_nOCOMe$ (where n = 1–3) were measured in dioxan solution to gain information on the effect of electron-withdrawing substituents on these absorptions in a situation uncomplicated by intramolecular hydrogen bond formation. Fellman & Fujita (1966) made their measurements of the carbonyl stretching frequencies of the series $Me_3\overset{+}{N}[CH_2]_nOCOMe$ (where n = 1–4) in ethanolic solution and in dimethyl sulphoxide solution. In the former solvent, intermolecular hydrogen bonding between solvent and solute must be presumed to occur and indeed the split carbonyl absorptions observed by these workers (Fellman & Fujita, 1962) could well have their origin in such hydrogen bonding. Although similar intermolecular effects are not possible with dimethyl sulphoxide, the marked hygroscopic properties of this solvent make it far from ideal. Since all of our quaternary ammonium salts were insoluble in solvents incapable of acting as hydrogen donors, the series of sulphones (which can be regarded as analogues of the ω-acetoxyalkyltrimethylammonium series in which the sulphone grouping replaces the onium function as an electron withdrawing substituent) were employed as suitable model compounds.

Experimental

INSTRUMENTS

Infrared spectra were measured in rigorously dried dioxan solution, liquid film and solid state (KCl disc) employing a Unicam SP 100 double beam spectrophotometer equipped with an SP 130 sodium chloride prism-grating double monochromator operated under vacuum conditions. Nuclear magnetic resonance spectra were recorded in deuterium oxide solution employing a Perkin–Elmer R 10 spectrometer operating at 40 Mc/sec and using 3-trimethylsilylpropionsulphonic acid as internal standard.

TERTIARY BASES (Table 2)

Dimethyloctylamine and dimethylaminoacetone were prepared by the addition of octyl bromide and monochloroacetone respectively to an ethereal solution of anhydrous dimethylamine. 2-Dimethylaminoethanol was redistilled from the commercial product through a 9 inch Vigreux column, b.p. 134·5° at 760 mm.

QUATERNARY SALTS (Table 1)

Acetylcholine chloride, (±)-acetyl-β-methylcholine chloride and succinylcholine chloride were obtained by repeated recrystallization of commercial samples from ethanol-acetone-ether followed by drying *in vacuo* over phosphorus pentoxide. Tetramethylammonium iodide, octyltrimethylammonium iodide, choline iodide and acetonyltrimethylammonium iodide were all prepared by the addition of iodomethane to an ethanolic solution of the appropriate amine followed by repeated recrystallizations from aqueous ethanol, ethanol, ethanol-acetone or ethanol-acetone-ether.

SULPHONES (Table 4)

Acetoxymethyl ethyl sulphone. Acetoxymethyl ethyl sulphide (Böhme, Fischer & Frank, 1949), prepared from chloromethyl ethyl sulphide (Böhme, 1936) and anhydrous sodium acetate, was oxidized with an ethereal solution of monoperphthalic acid. The solution was evaporated to dryness under reduced pressure at room temperature and the residue extracted with dry chloroform. Distillation yielded the product, b.p. 98–100° at 0·5 mm. [Böhme & others (1949) record b.p. 107° at 0·7 mm] (Calc. for $C_5H_{10}O_4S$: C, 36·1; H, 6·1; S, 19·3%. Found: C, 36·4; H, 6·0; S, 19·1%.)

2-*Acetoxyethyl ethyl sulphone.* Ethyl 2-hydroxyethyl sulphone (Exner, 1954) (6·9 g), acetic anhydride (12·5 ml) and concentrated sulphuric acid (3 ml) were heated for 1 hr at 100°. The reaction mixture was cooled, crushed ice (6 g) added and the whole extracted with chloroform (2 × 100 ml, 2 × 50 ml). The combined extracts were dried (Na_2SO_4), the chloroform removed and the residue distilled to give 2-acetoxyethyl ethyl sulphone (1·5 g) as an almost colourless oil, b.p. 126–127° at 0·2 mm. (Found: C, 40·2; H, 6·8; S, 17·5%. $C_6H_{12}O_4S$ requires C, 40·0; H, 6·7; S, 17·8%.)

3-*Acetoxypropyl ethyl sulphone.* Ethyl 3-hydroxypropyl sulphide (Rothstein, 1937) (10 g) in glacial acetic acid (25 ml) was cautiously treated with aqueous hydrogen peroxide (30% w/v, 18 ml) at such a rate that the temperature did not exceed 40°, and the mixture was then left overnight. The solvent was removed *in vacuo* and the residue distilled to give crude ethyl 3-hydroxypropyl sulphone (7 g), b.p. 161–163° at 0·7 mm, m.p. 36–37° (from chloroform-carbon tetrachloride and cooling to −10°). A mixture of the sulphone (7 g), acetic anhydride (14 ml) and concentrated sulphuric acid (3 ml) treated as described in the preparation of 2-acetoxyethyl ethyl sulphone afforded 3-acetoxypropyl ethyl sulphone (2 g), b.p. 147–149° at 0·2 mm. (Found: C, 43·5; H, 7·3%. $C_7H_{14}O_4S$ requires C, 43·3; H, 7·3%.)

Results

The absorption frequencies in the 3,100–2,700 cm^{-1} region (ν_{CH}) of representative quaternary ammonium salts in the solid state (KCl disc) and of some of the corresponding tertiary bases as thin liquid films are shown in Tables 1 and 2, respectively. The absorption frequencies in the region 1,500–1,390 cm^{-1} (δ_{CH}) measured from the solid state spectra of the quaternary salts are given in Table 3. Table 4 shows the infrared data ($\nu_{c=o}$) obtained from solution spectra (dioxan) of the sulphones formally related to acetylcholine and its higher homologues.

Discussion

EXAMINATION OF CH ABSORPTIONS

For the unambiguous interpretation of the ν_{CH} and δ_{CH} absorptions in solution the selection of solvent is necessarily restricted to one incapable of accepting a hydrogen bridge proton in order to eliminate the possibility of hydrogen bonding between solute and solvent molecules. Since no such solvent also capable of dissolving the quaternary salts could be found, a comparison was made between the infrared spectrum of a given trimethylammonium salt in the solid state and its corresponding tertiary base in the liquid state. Any conclusions would be complicated by possible intermolecular hydrogen bonding and differences in solid and liquid state spectra but such a comparison might indicate any influence of the positively charged nitrogen atom on the *N*-methyl CH stretching and deformation frequencies. Also, a comparison of the spectra of quaternary salts theoretically capable of forming C–H - - - - O hydrogen bonded quasi-rings with those unable to do so might indicate whether hydrogen bonding was indeed occurring.

CH stretching frequencies. No effect unequivocally attributable to the influence of C–H - - - - O bonding on ν_{CH} is apparent from Tables 1 and 2 but nevertheless the data are not incompatible with such bonding. The spectra of both the quaternary salts and the tertiary bases exhibit a multiplicity of peaks in the 3,100–2,700 cm^{-1} region making detailed interpretation difficult. Simplification of the spectra by deletion of overtones of lower frequency absorption modes did not prove feasible. Ebsworth & Sheppard (1959) have made the assignments ν_{CH_3} asymm. 3,005 cm^{-1} and ν_{CH_3} sym. 2,925 cm^{-1} for the tetramethylammonium ion and indeed in all the quaternary salts (Table 1) strong absorption is observed at 3,010–3,000 cm^{-1} (3,019 cm^{-1} for succinylcholine chloride) corresponding to ν_{CH_3} asymm. No similar consistency is observed in the 2,925 cm^{-1} region but there does appear to be a regularity in the absorptions observed at 2,952–2,940 cm^{-1} (2,957 cm^{-1} in succinylcholine chloride). Fellman & Fujita (1966) recorded absorption at 3,010–3,008 cm^{-1} for the CH stretching mode in acetylcholine which would thus appear to be a normal ν_{CH} of trimethylammonium compounds. The C–H stretching absorption in *N*-methyl compounds occurs at lower frequency than in *C*-methyl compounds (Cross, 1961) due to the greater

TABLE 1. SOLID STATE SPECTRA (KCl DISC)

Compound	Absorption frequencies in 3100–2700 cm^{-1} region cm^{-1}										Carbonyl stretching frequency cm^{-1}
1. Tetramethyl-ammonium iodide	3100 3054	3010	3005sh			2949	2928 2905	2880	2830	2780 2730	—
2. Trimethyloctyl-ammonium iodide	3024	3010				2940	2917	2867 2851		2781	—
3. Choline iodide	3026		3005	2985		2950	2930	2881	2820	2770	—
4. Acetylcholine chloride	3023	3010	3000sh	2977	2960	2946	2920	2852	2825	2807 2790	1737
5. Acetyl-β-methylcholine chloride	3043	3010sh	3000	2982	2965	2938	2920			2796	1738
6. Succinyl-choline chloride	3048 3024sh 3019			2973	2957	2933					1740
Acetonyl-trimethyl-ammonium iodide	3055 3046	3009		2952			2915			2806 2776 2740	1734

sh denotes shoulder.

TABLE 2. THIN LIQUID FILM SPECTRA

Compound	Absorption frequencies in 3100–2700 cm^{-1} region cm^{-1}							Carbonyl stretching frequency cm^{-1}
8. Dimethyloctylamine	2952	2925	2868	2853	2810	2778	2760	—
9. 2-Dimethylaminoethanol ..	2967sh	2944		2855	2820	2777		—
10. Dimethylaminoacetone ..	2970	2944	2873		2823	2776		1725sh 1715

sh denotes shoulder

electronegativity of the nitrogen atom, whereas in quaternary ammonium compounds the C–H stretching absorption occurs at higher frequency than in tertiary amines (Braunholtz, Ebsworth & others, 1958) despite the still greater inductive effect of the positively charged nitrogen atom. The weak bands in the region 2,800–2,400 cm^{-1} of the spectrum of the tetramethylammonium ion have been interpreted as combination frequencies involving probably both infrared- and Raman-active fundamentals (Ebsworth & Sheppard, 1959). Compounds 3–7 (Table 1), in which C–H - - - - O hydrogen bonding is possible, are characterized by absorptions between 3,000 and 2,950 cm^{-1} (cf. compounds 1 and 2). These peaks in compounds 3–7 could represent absorption frequencies corresponding to those just above 3,000 cm^{-1} (ν_{CH_3} asymm.) in compounds 1 and 2 lowered due to hydrogen bonding, although absorption at *ca* 2,985 cm^{-1}, common to the solid state spectra of all choline esters examined, has been tentatively ascribed to superposition of C–H stretching from the acyl group on weak C–H stretching originating in the choline

moiety (Whittaker, 1963). Whereas choline, acetylcholine, (±)-acetyl-β-methylcholine and succinylcholine show well-defined bands at 2,985, 2,977, 2,982 and 2,973 cm^{-1} respectively, the methiodide of dimethylaminoacetone shows absorption at 2,952 cm^{-1} which would not be inconsistent with NC–H - - - - O hydrogen bonding because the greater inherent proton-accepting propensity of a carbonyl oxygen, as compared with an hydroxylic or acyloxy oxygen (Jaffé, 1953; Stewart & Yates, 1960), would be expected to give a greater decrease in the bond order of the C–H bond concerned with consequent lowering of the absorption frequency.

The fundamental C–H stretching absorptions of the dimethylamino-compounds (Table 2) are observed at 2,823–2,810 cm^{-1} and 2,778–2,766 cm^{-1}. These values are thus in close agreement with the literature assignments of 2,825–2,810 cm^{-1} and 2,775–2,765 cm^{-1} for the $-NMe_2$ group in amines (Braunholtz & others, 1958; Hill & Meakins, 1958). Once again the complexity of the spectra prevents simple correlation in the terms sought.

CH deformation frequencies. If C-H - - - - O hydrogen bonding is involved in the stabilization of the proposed quasi-ring conformation (*4*) then the effect of such bonding might also be expected to affect the CH deformation frequencies. The frequency shift should be towards higher wave numbers due to an increase in the restoring force tending to keep the C–H bond directed towards the oxygen atom (Hallam, 1963). The relative upward shift is, however, smaller than the relative downward shift in the corresponding stretching modes (Hallam, 1963) although in

TABLE 3. SOLID STATE SPECTRA (KCl DISC)

Compound	Absorption frequencies in 1500–1390 cm^{-1} region cm^{-1}										
1. Tetramethyl-ammonium iodide		1484								1403	1396
2. Octyltrimethyl-ammonium iodide		1484	1478sh 1470sh	1462sh			1433			1408	1395
3. Choline iodide		1485		1466sh	1455			1423	1410		
4. Acetylcholine chloride	1492sh	1487	1475		1455	1445	1435	1425	1412		
5. Acetyl-β-methyl-choline chloride	1495sh	1483		1465		1442				1409	1390sh
6. Succinylcholine chloride	1497	1483			1455	1448		1429 1421sh	1415		
7. Acetonyltrimethyl-ammonium iodide		1485	1470			1449	1435		1416	1401	

sh denotes shoulder.

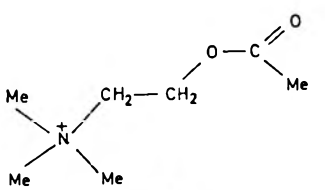
only a few instances have the effects of H-bonding on deformation modes been well substantiated. Ebsworth & Sheppard (1959) made the assignments δ_{CH_3} asymm. 1,483 cm^{-1} and δ_{CH_3} sym. 1,403 + 1,397 cm^{-1} for the tetramethylammonium ion.

The solid state spectral data of acetylcholine, (±)-acetyl-β-methylcholine and succinylcholine in the 1,500–1,390 cm^{-1} region are compared

with a number of quaternary ammonium salts in Table 3. Thus tetramethylammonium iodide shows three well defined peaks at 1,484, 1,403 and 1,396 cm^{-1} in complete agreement with the assignments of Ebsworth & Sheppard (1959). Octyltrimethylammonium iodide shows well-defined absorptions at comparable frequencies, although in this instance considerable broadening coupled with a series of shoulders on the low frequency side of the 1,484 cm^{-1} peak (a manifestation of the eight carbon atom chain) can be seen. In the remaining compounds of Table 3 the δ_{CH_3} sym. modes cannot be identified with certainty but there is a well-defined band at 1,485 $\pm$ 2 cm^{-1} corresponding to δ_{CH_3} asymm. There is broadening in succinylcholine, which shows a second well-resolved peak at 1,497 cm^{-1}, also in acetylcholine and (±)-acetyl-β-methylcholine which show shoulders at 1,492 and 1,495 cm^{-1} respectively. As evidence for C–H - - - - O hydrogen bonding this is equivocal in view of the poor documentation of this region. Moreover deformation modes frequently couple with other vibrational modes in the lower "finger print" region of the spectrum (Hallam, 1963). Choline iodide (Table 3, No. 3) and acetonyltrimethylammonium iodide (No. 7), although theoretically capable of C–H - - - - O hydrogen bonding, do not show similar characteristics.

EXAMINATION OF CARBONYL ABSORPTIONS OF ACETYLCHOLINE AND (±)-ACETYL-β-METHYLCHOLINE

Since liquids containing hydrogen atoms capable of hydrogen bond formation could not be employed as solvents and insolubility of the compounds limited the selection of solvent, spectra were determined in dry dioxan, in which acetylcholine and (±)-acetyl-β-methylcholine proved sparingly soluble. Both showed split carbonyl absorptions at



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1,753 cm^{-1} and 1,732 cm^{-1} possibly due to the co-existence of conformations akin to *4* and *6*. However, low intensity absorption at *ca* 1,720 cm^{-1}. (indicative of traces of free acetic acid despite rigorous precautions to exclude moisture) prevented firm assignment of these dual ester carbonyl absorptions to conformation effects. Ethyl acetate and diethyl succinate used as standards of reference showed single $\nu_{C=O}$ in dioxan solution at 1,743 cm^{-1} and 1,737 cm^{-1} respectively.

EXAMINATION OF CARBONYL ABSORPTIONS OF THE SULPHONES

Table 4 shows the carbonyl stretching frequencies of the series of sulphones $EtSO_2[CH_2]_nOCOMe$ (n = 1–3) in dry dioxan solution. These compounds may be regarded as analogues of the ω-acetoxyalkyl-

trimethylammonium compounds in which the sulphone group replaces the onium function as an electron withdrawing substituent. But in contrast, being soluble in solvents incapable of acting as hydrogen donors, they were expected to yield information on the effect of –I substituents on the carbonyl stretching frequency in a situation uncomplicated by intermolecular hydrogen bonding between solvent and solute. All show a higher ester carbonyl stretching frequency than ethyl acetate or diethyl-succinate (Table 4), and only acetoxymethyl ethyl sulphone (n = 1)

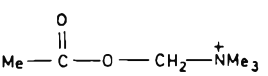
TABLE 4. CARBONYL STRETCHING FREQUENCIES OF SULPHONES IN DRY DIOXAN SOLUTION

Sulphone $EtSO_2(CH_2)nOCOMe$	Carbonyl stretching frequency cm^{-1}	
n = 1	1775sh	1764
n = 2		1751
n = 3		1745
Ethyl acetate		1743
Diethyl succinate		1737

sh denotes shoulder.

shows any indication of a split carbonyl absorption—there being a shoulder on the high frequency side at 1,775 cm^{-1}. The progressive decrease in the ester carbonyl stretching frequency as n increases from 1 to 3 agrees with the expected decrease in the influence of the inductive effect of the sulphone group and supports the conclusions of Fellman & Fujita (1963, 1966) in the series $Me_3\overset{+}{N}[CH_2]_nOCOMe$, where n = 1–4. It also provides an analogy for the observations of Nakamoto, Morimoto & Martell (1962) who showed that protonation of the nitrogen atom of the iminodiacetic acid di-anion produced a shift to higher frequency of the carboxylate carbonyl absorption.

The arguments of Fellman & Fujita (1963) to account for the higher frequency of the carbonyl group in acetylcholine in terms of the inductive effect of the quaternary nitrogen atom have been stated by Canepa & Mooney (1965) to be unsatisfactory. From earlier infrared studies (Gerrard, Mooney & Willis, 1961) of substitution patterns in mono-substituted benzene derivatives, Canepa & Mooney (1965) argue that the contribution from (*1*) is not expected to be important and they show by attenuated total reflection and transmission measurements that the



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carbonyl frequency in acetylcholine is of the order expected for aliphatic esters. The high frequency carbonyl absorptions observed by Fellman & Fujita (1963) [and omitted by Canepa & Mooney (1965) in their criticism] at *ca* 1,780 cm^{-1} for trimethylammonium-methyl acetate (*7*) and observed at 1,775 cm^{-1} and 1,764 cm^{-1} for acetoxymethyl ethyl sulphone (Table 4) are difficult to rationalize if one accepts the conclusions of Canepa & Mooney (1965) that the mesomeric and/or inductomeric interaction between alkoxy and acyl groups are negligible.

NUCLEAR MAGNETIC RESONANCE STUDIES

A comparison of the nmr spectra of tetramethylammonium iodide, octyltrimethylammonium iodide, acetylcholine chloride and (±)-acetyl-β-methylcholine chloride in deuterium oxide solution failed to reveal any evidence of C–H - - - -O hydrogen bonding (compare Fellman & Fujita, 1966). The tetramethylammonium ion showed a 1 : 1 : 1 triplet structure similar to that observed in the tetraethylammonium ion (Bullock, Tuck & Woodhouse, 1963) due to coupling of the protons to the ^{14}N nucleus ($I = 1$). The other three compounds all showed unresolved *N*-methyl proton absorptions at 6·88 τ, 6·76 τ and 6·81 τ respectively. Culvenor & Ham (1966) deduced that in deuterium oxide solution acetylcholine appears to exist largely in one conformation (*3*). These spectra were recorded at 60 Mc/sec whereas those in the present study being recorded at 40 Mc/sec were insufficiently resolved to be of the same diagnostic value. Even at 60 Mc/sec the *N*-methyl proton absorption of acetylcholine at 6·76 τ (Culvenor & Ham, 1966) remains unresolved. Thus if C–H - - - - O hydrogen bonding is present in acetylcholine in deuterium oxide solution then interchange between hydrogen bonded and non-hydrogen bonded species is probably too fast at room temperature to be detectable by nmr spectroscopy (Pople, Schneider & Bertstein, 1962).

CONCLUSIONS

From the above discussion it is apparent that the results are not incompatible with the existence of intramolecularly hydrogen bonded forms such as the quasi-ring structures (*2* and *3*) which exist in the crystalline state (Canepa & others, 1966) and in aqueous solution (Canepa, 1965; Culvenor & Ham, 1966), respectively. The high ester carbonyl stretching frequency of acetylcholine although perhaps best interpreted in terms of an inductive effect from the onium nitrogen atom can also be interpreted in terms of C–H - - - - O hydrogen bonding between one of the *N*-methyl groups and the acetoxy oxygen atom. Thus, while the studies involving the sulphones indicate that inductive effects exert a big influence on the ester carbonyl absorptions, the absorptions occurring between 3,000 and 2,950 cm^{-1} in the selected choline esters may represent ν_{CH} absorptions lowered in frequency as a result of C–H - - - - O hydrogen bonding. The δ_{CH} absorptions in the choline esters also provide circumstantial evidence for C–H - - - - O hydrogen bonding but unequivocal interpretation of these absorptions must await better documentation of this spectral region.

Acknowledgments. The authors gratefully acknowledge the help of Dr. G. Eglinton and Mrs. F. Lawrie of the Department of Chemistry, University of Glasgow, who prepared the infrared spectra and of Dr. P. Bladon of the Department of Pure and Applied Chemistry of this University who recorded the nuclear magnetic resonance spectra.

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J. Pharm. Pharmac., 1967, **19**, 660–666 Received March 17, 1967

The autoxidation of sodium salicylate in aqueous solution

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The induction period of the auto-oxidation of aqueous alkaline solutions of sodium salicylate has been examined by following the oxygen concentrations polarographically. The reaction is photochemical, and is catalysed by copper, iron and manganese, although the increase in rate is less than 150% of the rate of the uncatalysed reaction. Bicarbonate ions are not necessary for reaction, which is first order with respect to light intensity and oxygen and salicylate concentrations.

THE darkening of aqueous alkaline solutions of sodium salicylate is due to oxidation. Changes in extinction of aerated sodium salicylate solutions with time have been measured and the process shown to be dependent on the aeration rate (Beynon & James, 1964). The form of the plot of extinction against time suggested autoxidation, but the procedure used had the disadvantages that the reaction was heterogeneous, and extinction could not be linked directly with reactant concentration. We have further investigated oxygenated solutions of salicylate and determined their oxygen concentrations polarographically.

Experimental

Determination of oxygen. The polarographic determination of oxygen has been summarized in several reviews (for example, Page, 1952). There are two reduction steps: at $E_{0\cdot5} = -0\cdot1$ V (vs. standard calomel electrode) representing reduction to hydrogen peroxide, and at $E_{0\cdot5} = -0\cdot9$ V due to reduction of hydrogen peroxide. Determinations were made at $-0\cdot1$ V using a Cambridge recording polarograph with mercury anode and direct current. A complete reduction step was traced in each determination to ensure that measurements were made on the limiting current plateau. Preliminary experiments showed that the diffusion current was not affected by the presence of sodium bicarbonate, sodium salicylate or changes in pH. Sodium chloride (0·1 M) was used as indifferent electrolyte in most determinations; when the molar concentration was varied between 0·08 and 0·13 M, or sodium chloride substituted in part by sodium bicarbonate or sodium hydroxide, the diffusion current remained constant. No polarographic maxima were seen. The calibration curve of diffusion current against oxygen concentration, using Krogh's (1935) modification of Winkler's method, was a straight line passing through the origin.

Rate determinations. Three ml of a solution containing all the components required for the reaction except sodium salicylate was placed in a polarograph cell, protected from the atmosphere by a mercury seal, and suspended in a constant temperature bath at 50°. Oxygen was bubbled

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through to a predetermined concentration, indicated by the diffusion current, and if exceeded was reduced with a current of nitrogen. Concentrations were arranged to give the required composition when the reaction was started by adding 3 ml of a salicylate solution to the polarograph cell. Blank determinations were made. Initial rates of disappearance of oxygen were obtained from the tangent of the plot of oxygen concentration against time at zero time. Initial rates were used to formulate the rate equations in order to avoid complications occurring later in the autoxidation chain. The kinetic curves over the complete 80 min runs followed a similar pattern.

Effect of ultraviolet irradiation. The constant temperature bath was coated with black paint and covered with a black cardboard lid. One glass side of the bath was left unpainted and faced onto a light-tight tunnel normal to it. A rail along the axis of the tunnel carried a Hanovia medium pressure arc in quartz with a spectrum range from 1850 Å to the near infrared. The source was mounted on a trolley which could be moved along the rail, and its distance from the mixture measured by means of a rule attached to the track. The polarograph cell was placed near to and at a fixed distance from the irradiated side.

To examine the effect of the irradiation, rates were measured with the light source at varying distances from the reaction. Quantum output was measured using potassium ferrioxalate (Hatchard & Parker, 1956). The radiation was confined to the 3025 and 3125 Å wavebands. A solution of nickel chloride, placed in the beam, absorbed the longer wavebands and the glass side of the tank absorbed the shorter wavebands All other determinations were made with the lamp 30 cm from the polarograph cell, and without the nickel chloride filter.

Effect of metallic ions. Neither sodium edetate nor sodium cyanide had any effect on reaction rate.

Measured quantities of manganic sulphate were added to the indifferent electrolyte solution before passing oxygen, and the concentration of manganese confirmed from the diffusion current at −1·51 V (vs. standard calomel electrode). The salicylate solution was then added and the rate of uptake of oxygen determined as before. A similar procedure was carried out with ferric and cupric ions. The effects of these are shown in Fig. 1.

Effect of hydroxyl and bicarbonate ion concentrations. Rate determinations were made at a series of different pH values. The ionic strength was kept constant with sodium chloride, and the pH varied by adding different quantities of sodium hydroxide. pH values were measured on an E.I.L. pH meter using glass and calomel electrodes. In similar experiments, sodium bicarbonate concentration was varied and pH and ionic strength kept constant. Substituted salicylic acids were examined in the same way.

Absorptiometer measurements. This procedure has been described previously (Beynon & James, 1964).

Results and discussion

The rapid darkening of sodium salicylate—sodium bicarbonate solutions has been attributed to traces of metal impurity in the sodium bicarbonate. Mesnard & Marzar (1950) proposed iron as the catalyst, Zwikker & Weber (1940) manganese, and Liberelli (1935) iron and copper. Fig. 1 shows that all these catalysed the uptake of oxygen, rapidly reaching a limiting value less than 50% greater than the uncatalysed rate. The polarograms suggested that metallic impurity in the reactants could not be greater than 0·3 parts per million, and the failure of sodium edetate or sodium cyanide (for copper) to retard the reaction shows that these ions, if present as impurity below this level, have no effect under the conditions described. These results do not necessarily contradict the opinions of the above authors, in fact, absorptiometer measurements with more

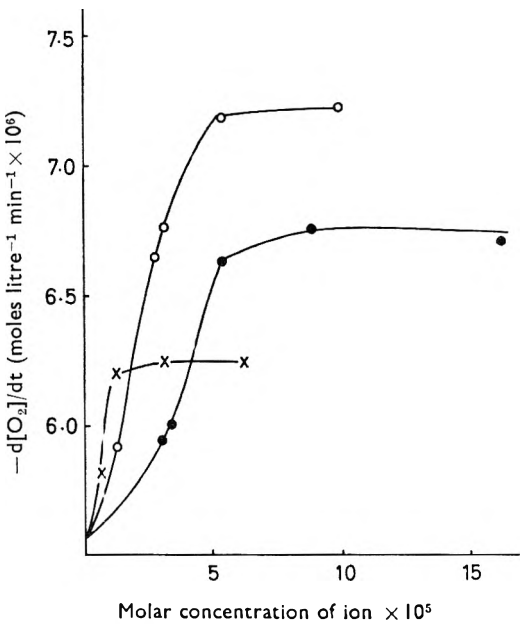

FIG. 1. Effect of metallic ions on reaction rate. $[C_7H_5O_3Na]_0 = 1{\cdot}5 \times 10^{-3}$. $[O_2]_0 = 7{\cdot}35 \times 10^{-4}$. ●, Mn^{2+}; ×, Cu^{2+}; ○, Fe^{3+}.

concentrated mixtures have indicated that sodium edetate has a marked effect on retarding discolouration (Beynon & James, 1964). In that work the rate of increase in extinction was shown to pass through three stages typical of auto-oxidation processes—an induction period with very little colour development, followed by a rapid increase in extinction which subsequently slowed down. The induction period lasted for 100 min, hence the observations in this communication, which were made for 80 min, belong to this period. Metal ion catalysis of auto-oxidation usually affects the second stage (Ingold, 1961), and it is probable that it is this stage of the salicylate oxidation which is markedly influenced by metal ions.

Hilton & Bailey (1938) have stated that light has very little effect on the reaction, but most workers (for example Grill, 1932; Ernst & Menashi, 1963) agree that it is light sensitive, although no attempt has been made to relate rate with light intensity. This we did by observing the effect of ultraviolet light on the extinction of 0·3M sodium salicylate solutions. Under these conditions the length of the induction period was proportional to, and the rate inversely proportional to the distance of the energy source from the reaction mixture. In determining the order of the reaction with respect to the radiation intensity, we found it was important to ensure that the mixture was uniformly illuminated throughout. The 3025 and 3125 Å emission bands of mercury were used because wavelengths lower than 3025 Å are absorbed by glass, and wavebands higher than 3125 Å are not absorbed by salicylic acid. Extinction measurements at the two emission wavelengths revealed that the concentrations used in the preliminary experiments absorbed most of the radiation in less than the first mm. Since this would result in a reaction with diffusion as a complicating factor, subsequent polarographic determinations were made with $2{\cdot}0 \times 10^{-5}$ M solutions of sodium salicylate, which absorbed less than 10% of the incident radiation. Order with respect to radiation intensity was calculated by plotting log intensity against log initial rate. A straight line of slope 1·00 was obtained (Fig. 2). Similar graphs (Fig. 3) gave slopes of 0·90 for salicylate and 0·92 for oxygen, indicating that the rate equation for the main reaction at constant pH is probably,

$$\frac{-d[O_2]}{dt} \propto I_0[O_2] \quad \ldots \quad (1)$$

where I_0 is incident radiation intensity, at constant salicylate concentration, and

$$\frac{-d[O_2]}{dt} \propto [O_2]\,[C_7H_5O_3Na] \quad \ldots \quad (2)$$

at constant radiation intensity.

The variation in reaction rate with pH, shown in Fig. 4 supports the observations of Greenish & Beesely (1915) and of Hilton & Bailey (1938) that the rate reaches a maximum around pH 10·3, but the fact that mixtures containing sodium bicarbonate follow the same pattern as those without sodium bicarbonate is contrary to the supposition of these authors that the bicarbonate ion is necessary for the reaction to proceed. The broken lines in Fig. 4 represent the effect of pH on the rates of oxidation of two substituted salicylic acids and indicate that there is a connection between the pH at which the maximum rate occurs and the pK_a value of the phenol group. This suggests that the reacting species is the ion R(OH)COO′, and that the rise and fall in the reaction rate with pH are due to the equilibria,

$$R(OH)COOH \overset{K_1}{\rightleftharpoons} R(OH)COO' + H^+ \quad \ldots \quad (3)$$

$$R(OH)COO' \overset{K_2}{\rightleftharpoons} R(O')COO' + H^+ \quad \ldots \quad (4)$$

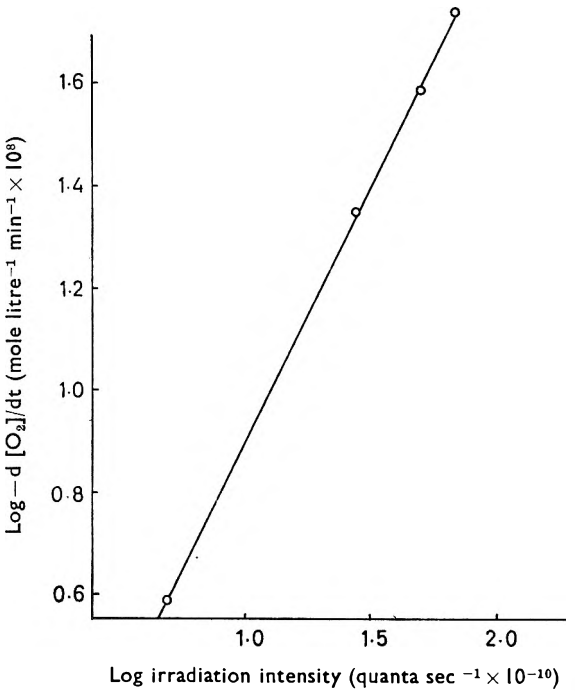

FIG. 2. Effect of irradiation intensity on reaction rate. $[C_7H_5O_3Na]_o = 2{\cdot}0 \times 10^{-5}$. $[O_2]_o = 8{\cdot}0 \times 10^{-5}$.

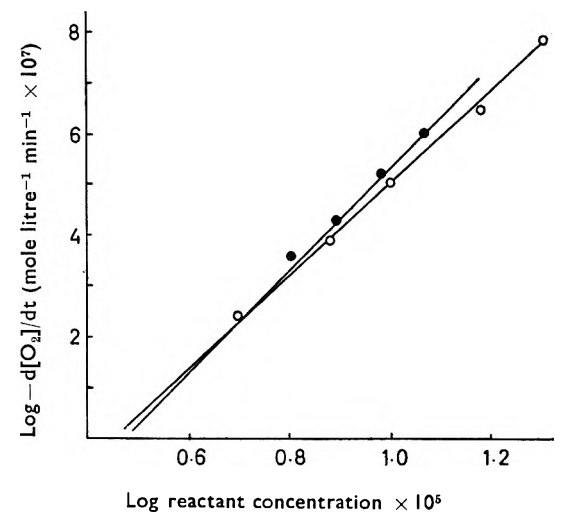

FIG. 3. Effect of reactant concentration on reactant rate. ○ Salicylate ($[O_2]_o = 1{\cdot}02 \times 10^{-4}$); ● oxygen ($[C_7H_5O_3Na]_o = 1{\cdot}25 \times 10^{-4}$).

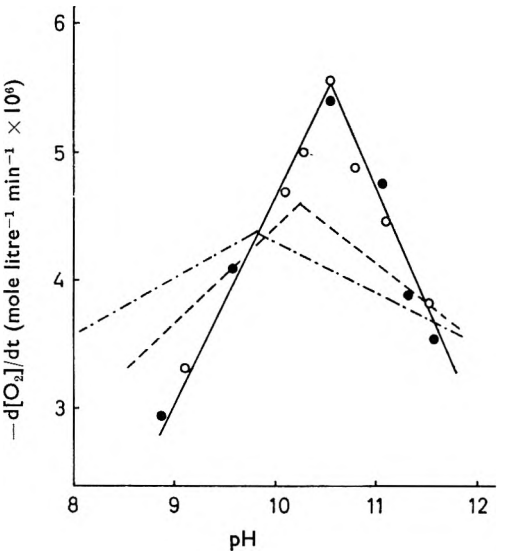

FIG. 4. The effect of pH on reaction rate. — Sodium salicylate, pK_{a_2} 2·56 $\times$ 10^{-14} at 25°: ○ with HCO_3'; ● without HCO_3'. —·— Sodium 5-nitrosalicylate, pK_{a_2} 4·58 $\times$ 10^{-13} at 25°. – – – Sodium 5-bromosalicylate, pK_{a_2} 1·46 $\times$ 10^{-13} at 25°. $[O_2]_o$ = 2·35 $\times$ 10^{-4}. $[Salicylate]_o$ = 1·5 $\times$ 10^{-3}.

The pH independent rate equation would then be,

$$\frac{-d[O_2]}{dt} = k[O_2]\,[R(OH)COO'] \quad .. \quad .. \quad .. \quad (5)$$

at constant irradiation intensity.

It can be calculated that,

$$[\text{Total phenolic acid}] = [R(OH)COO']\left\{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}\right\} \quad .. \quad (6)$$

and substitution for $[R(OH)COO']$ from (5) gives,

$$[\text{Total phenolic acid}] = \frac{-d[O_2]}{dt}\left\{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}\right\}\Big/ k[O_2] \quad .. \quad (7)$$

Table 1 shows the initial rates of oxygen consumption at various pH values. Substitution of these in equation (7) gave 6 simultaneous equations, which on least squares analysis, using an Elliot 803 electronic computer, yielded the values,

$$K_1 = 2{\cdot}04 \times 10^{-10}; \quad K_2 = 8{\cdot}36 \times 10^{-14} \quad .. \quad ..$$

Table 1 shows the second order constants obtained by substituting these dissociation constants in equation (7). They are reasonably constant in comparison with the $-d[O_2]/dt$ results and do not appear to pass through a maximum.

TABLE 1. THE EFFECT OF HYDROGEN ION CONCENTRATION ON REACTION RATE

$[O_2]_o = 8{\cdot}0 \times 10^{-5}$ $[C_7H_5O_3Na]_o = 2{\cdot}0 \times 10^{-5}$

	pH					
	10·1	10·6	10·8	11·1	11·6	12·6
$-d[O_2]/dt \times 10^7$ (mole litres^{-1} min^{-1})	4·19	4·54	5·89	6·66	5·45	4·40
k (calc. from eqn 7) litres mole^{-1} min^{-1}	377	325	398	437	356	370

The first dissociation constant of salicylic acid of $1{\cdot}06 \times 10^{-3}$ is much higher than K_1 ($2{\cdot}04 \times 10^{-10}$). Hermans, Leach & Scheraga (1963) have reported a second pK_a of 12·48 at 45° and an enthalpy of ionization of 10·5 kcal/mole. Substitution of these values in the van't Hoff isochore gave a dissociation constant of $4{\cdot}32 \times 10^{-13}$ at 50°, which does not agree with the second dissociation constant (K_2) calculated above. Weller (1961) has reported that the excited states of organic acids have vastly different pK_a values from the ground states. In general, phenols are more dissociated in the excited state, and carboxy acids more dissociated in the ground state. There is no information on the behaviour of molecules in which phenol and carboxy are conjugated, but it is possible that the dissociation constants calculated above are those of the excited state of salicylic acid, and that in this reaction the most reactive form is either $C_6H_4(OH)COO'^*$ or $C_6H_4(COOH)O'^*$, where * denotes activated state.

Acknowledgements. We are grateful to Dr. E. Whittle of the Department of Chemistry, University College, Cardiff, for his advice and interest and to Mr. E. Stuart, of the Department of Mathematics, Welsh College of Advanced Technology, for the computer results.

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J. Pharm. Pharmac., 1967, **19**, 667–673 Received April 10, 1967

Effect of desipramine on the depolarized isolated renal artery

P. HRDINA* AND S. GARATTINI

Depolarization of the isolated renal artery of the rat by a potassium-rich solution caused a triphasic response. The artery contracted submaximally then relaxed partially, this relaxation being followed by a slowly developing contracture. Desipramine reduced the height of the initial contraction, increased the relaxation and reduced the contracture. The latter action was antagonized by increasing the calcium ion concentration of the depolarizing solution. Calcium caused a contraction of the depolarized artery and the contraction was reduced by the drug. The results suggest that the relaxation of the depolarized artery produced by desipramine may be due to interference with the action of calcium in initiating and maintaining a contraction of the arterial smooth muscle.

IMIPRAMINE-like drugs are known to produce a dual effect on the adrenergic system. At low doses they increase the pharmacological response to exogenous or endogenous noradrenaline (Sigg, 1959; Thoenen, Hürlimann & Haefely, 1964; Ursillo & Jacobson, 1965; Bonaccorsi & Garattini, 1966; Bonaccorsi & Hrdina, 1966). At relatively high doses, however, imipramine and its congeners decrease adrenergic responses. This inhibition has also been observed on the isolated renal artery (Hrdina & Garattini, 1966).

To obtain information about the effect of desipramine on mechanisms affecting membrane permeability it was decided to investigate its effect on the depolarized vascular smooth muscle.

Experimental

METHODS

A constant flow technique, as previously described (Hrdina & Garattini, 1966; Hrdina, Bonaccorsi & Garattini, 1967) was used to perfuse the isolated renal artery of the rat. Contraction of the artery caused an increase in perfusion pressure and relaxation of the artery a fall in perfusion pressure.

Solutions of the following constitution (mmoles/litre) were used for perfusion. *Krebs-bicarbonate solution:* NaCl 118·0; KCl 5·6; $CaCl_2$ 2·5; $MgSO_4$ 0·55; $NaHCO_3$ 15·0; KH_2PO_4 0·9; glucose 5·5; final pH 7·4. *K_2SO_4 depolarizing solution:* K_2SO_4 126·0; KCl 5·6; $CaCl_2$ 2·5; $KHCO_3$ 3·6; glucose 5·5 with the final pH adjusted to 7·4 with KOH.

The perfusion solutions were gassed continuously with a mixture of oxygen 95% and carbon dioxide 5%. The isolated renal artery preparation was first perfused with the Krebs-bicarbonate solution. After 60 min equilibration, the basic perfusion fluid was exchanged for the K_2SO_4

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depolarizing solution. Noradrenaline was injected in a volume of 0·1 ml proximal to the artery. Other drugs were added to the perfusion solutions or introduced by microinfusion using a Braun motor-driven syringe, delivering 0·1 ml/min of drug solution. Each drug concentration was tested on at least four, but generally six, different renal arteries.

Drugs used were: (−)-noradrenaline bitartrate (Recordati); cocaine hydrochloride (C. Erba); desipramine hydrochloride (Geigy, S. A.); chlorpromazine sulphate (Farmitalia); phenoxybenzamine hydrochloride (Smith Kline & French); phentolamine hydrochloride (Regitin amp., Ciba). Drug concentrations are expressed as the molar concentration of the salt, except noradrenaline, which is expressed as base.

Results

The effect of the depolarizing solution on the isolated renal artery. Depolarization of the isolated renal artery of the rat by a potassium-rich solution caused a triphasic response. The artery contracted submaximally then relaxed partially, this relaxation being followed by a slowly developing contracture (Fig. 1). In the depolarized artery noradrenaline is still able to produce a contraction, but its effect is reduced slightly as shown by the shift of the dose-response line to the right (Fig. 2).

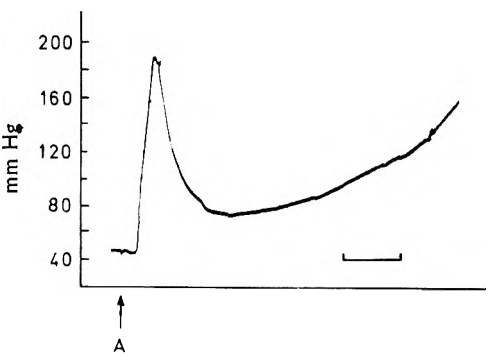

FIG. 1. Isolated perfused renal artery of the rat. At A, perfusion with ordinary Krebs bicarbonate solution was stopped and perfusion with K_2SO_4 depolarizing solution started. The response of the depolarizing solution has three phases (see text). Contraction of the artery is indicated by a rise in the perfusion pressure. Time scale: 10 min.

THE EFFECT OF DESIPRAMINE ON THE DEPOLARIZED ARTERY

The response of the artery to depolarization by K_2SO_4 Krebs solution was altered by desipramine which in a concentration of $6{\cdot}6 \times 10^{-6}$M reduced the initial contractile response to depolarization and increased the subsequent relaxation (Fig. 3). The reduction by desipramine ($6{\cdot}6 \times 10^{-8} - 6{\cdot}6 \times 10^{-6}$M) of the slow developing contracture to depolarization is also shown in Fig. 4.

The relaxant effect of desipramine on the slowly developing contracture following depolarization was quantitated by expressing the relaxation as

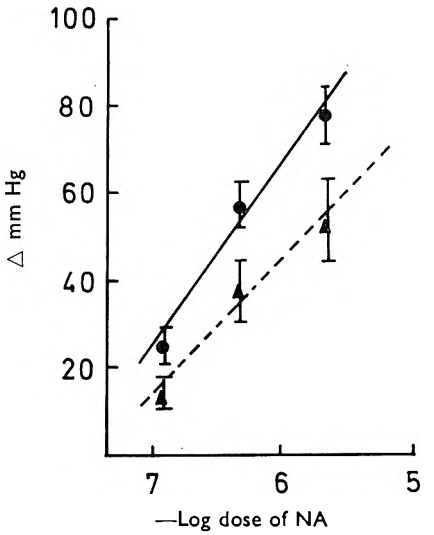

FIG. 2. The effect of K_2SO_4 depolarizing solution on the responses of the isolated renal artery to noradrenaline. Responses in Krebs-bicarbonate solution are shown by the continuous line and responses in the presence of K_2SO_4 depolarizing solution by the broken line. Each point with standard errors represents the mean of 4 experiments.

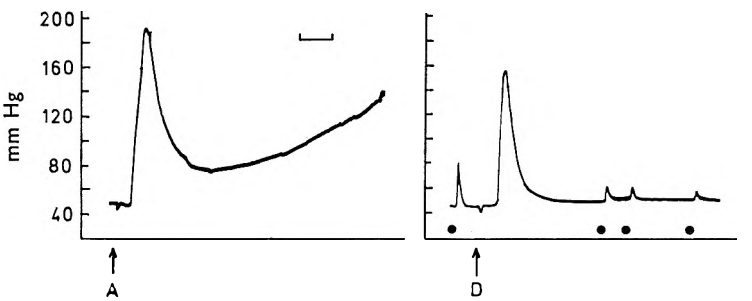

FIG. 3. Effect of desipramine on the response of isolated artery to K_2SO_4 depolarizing solution. Note the inhibition of the initial contraction and the following almost complete relaxation of muscle. The effect of a subsequent dose of noradrenaline (0·5 μg at ●) is diminished due to the adrenolytic action of desipramine at the concentration used. At A, K_2SO_4 solution; at D, K_2SO_4 solution + desipramine $5{\cdot}6 \times 10^{-6}$M. Time scale: 10 min.

a percentage of the maximal response of the artery to depolarization in the absence of the drug.

Desipramine in concentrations of $6{\cdot}6 \times 10^{-8}$, $1{\cdot}7 \times 10^{-6}$ and $6{\cdot}6 \times 10^{-6}$M produced a dose-dependent relaxation of the contracture response; these results are summarized in Table 1.

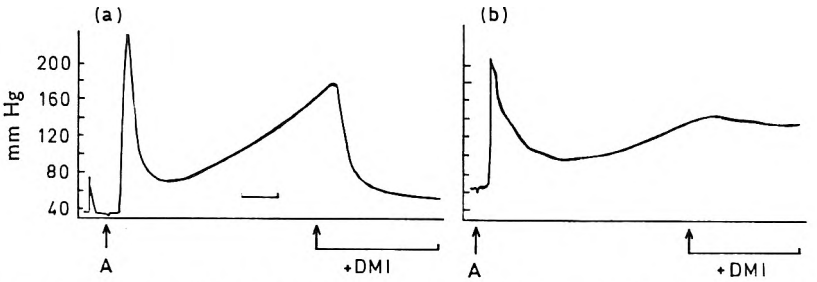

FIG. 4. Relaxing effect of two concentrations of desipramine (DMI) $6{\cdot}6 \times 10^{-6}$M in (a); $6{\cdot}6 \times 10^{-5}$M in (b) in the phase of the sustained contracture of the vessel. At A, K_2SO_4 solution alone. Time scale: 10 min.

THE EFFECT OF CHLORPROMAZINE, PHENTOLAMINE, PHENOXYBENZAMINE AND COCAINE

These drugs were tested on the contracture phase of the response to depolarization and the results were calculated as for desipramine above. Chlorpromazine ($5{\cdot}6 \times 10^{-6}$M) produced relaxations. Phenoxybenzamine ($5{\cdot}9 \times 10^{-6}$M) and phentolamine ($6{\cdot}25 \times 10^{-6}$M) had no effect. These results are summarized in Table 1. Cocaine (10^{-5}M) also had no effect on the contracture caused by depolarization however it induced relaxation at the high concentration of 4×10^{-4}M.

THE EFFECT OF DESIPRAMINE ON THE CONTRACTIONS CAUSED BY CALCIUM IN THE DEPOLARIZED ARTERY

Calcium ($6{\cdot}8 \times 10^{-3}$M) caused a contraction of the depolarized artery. The contraction was reduced by desipramine ($1{\cdot}7 \times 10^{-6}$M) (see Fig. 5). The reduction of the calcium induced contraction was proportional to the concentration of desipramine (Fig. 6). The depolarized artery contracted by potassium in the presence of calcium 2·5 mM was relaxed by desipramine ($6{\cdot}6 \times 10^{-6}$M) and this relaxation is converted to a contraction by increasing the concentration of calcium to 12·5 mM (Fig. 7).

Discussion

Several smooth muscle preparations when immersed in potassium-rich solutions respond by a characteristic contraction. Depolarized prepara-

TABLE 1. THE EFFECT OF DRUGS ON THE CONTRACTURE RESPONSE OF THE RENAL ARTERY TO DEPOLARIZATION BY K_2SO_4-KREBS SOLUTION

Drug	No. of experiments	Concn (M)	Relaxation* ±s.e. %
Desipramine	6	$6{\cdot}6 \times 10^{-8}$	2·38 ± 4·3
"	7	$1{\cdot}7 \times 10^{-6}$	36·1 ± 8·6
"	11	$6{\cdot}6 \times 10^{-6}$	51·8 ± 5·9
Chlorpromazine	5	$5{\cdot}6 \times 10^{-6}$	59·2 ± 14·9
Phentolamine	5	$6{\cdot}25 \times 10^{-6}$	8·1 ± 3·6
Phenoxybenzamine	4	$5{\cdot}9 \times 10^{-6}$	no effect

*Relaxation is calculated as the % of the maximal response that the tissue can produce in response to depolarization in the absence of the drug.

tions, including those of vascular muscle, still respond to various stimulatory or inhibitory drugs (Robertson, 1960; Durbin & Jenkinson, 1961; Edman & Schild, 1961; Briggs, 1962; Waugh, 1962a,b; Hinke & Wilson, 1962). However, there is a marked difference in the pattern of response to potassium-rich solutions of strips from a large conducting vessel (i.e. aorta) and from resistance vessels (i.e. artery of mesoappendix) (Bohr & Goulet, 1961). The contractile response of resistance vessels is rapid and is followed by a partial relaxation, while the response of aortic tissue is slower and sustained. The isolated perfused renal artery contracts in a similar manner to the resistance vessels, being a "muscular" type of

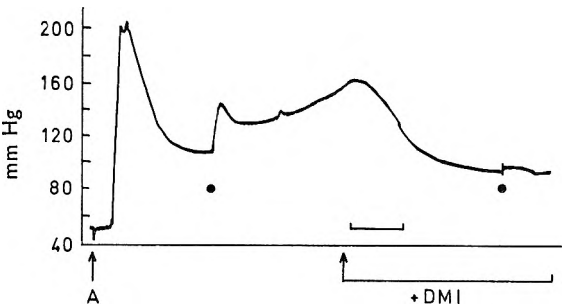

FIG. 5. The effect of calcium ions and desipramine on the depolarized renal artery. The artery was depolarized with K_2SO_4 at A. $CaCl_2$ ($6{\cdot}8 \times 10^{-5}$M at ●) caused a contraction which was reduced by desipramine ($1{\cdot}7 \times 10^{-6}$M). Time scale: 10 min.

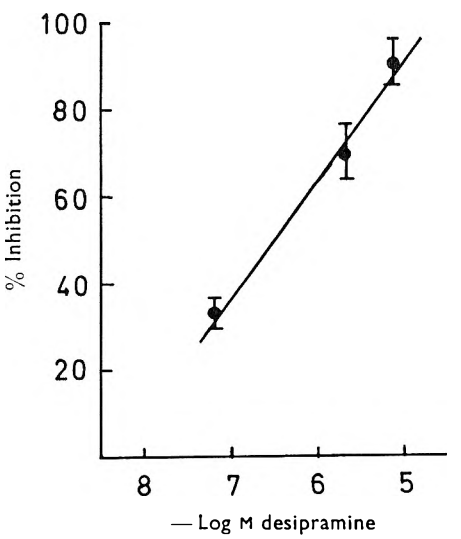

FIG. 6. The effect of desipramine on the contraction of the depolarized artery produced by calcium chloride ($6{\cdot}8 \times 10^{-5}$M). The reduction of the contraction by desipramine is expressed as a % of the initial contraction. Each point is the mean ± standard error of 4 experiments.

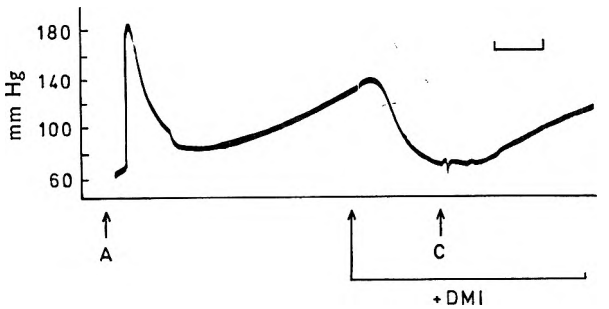

FIG. 7. The interaction of desipramine and calcium ions on the depolarized renal artery. The artery was depolarized with K_2SO_4 at A. At the second arrow desipramine $6{\cdot}6 \times 10^{-6}$M was added to the perfusate. At C, the calcium concentration, as $CaCl_2$, was raised from the normal level of 2·5 to 12·5 mM. Time scale: 10 min.

artery with an important function in regulating the blood flow into the kidney.

Noradrenaline still contracted the depolarized artery but its effect was slightly decreased. This is in accord with observations made by Cuthbert & Sutter (1965) and Jenkinson & Morton (1965) in other preparations. However, Hinke & Wilson (1962) did not observe any change in the effect of noradrenaline in the depolarized ventral tail artery of the rat.

Our results support the view based on previous findings (Haedings & Rondell, 1961; Waugh, 1962b; Cuthbert & Sutter, 1965; Kovalčik, Smyk & Blaškova, 1965), that depolarization of the membrane is not an essential step in the vasoconstriction induced by noradrenaline. Desipramine caused a decrease of the initial contractile response of vascular smooth muscle to the potassium-rich solution and also a dose-dependent relaxation of the muscle tone in the phase of the sustained contracture. It must be emphasized that even large doses of desipramine (10^{-5}M) do not change the muscle tone in a normal polarized vessel preparation.

Desipramine is known to have an adrenolytic effect (Haefely, Hürlimann & Thoenen, 1964; Ursillo & Jacobson, 1965) which has also been shown in preparations similar to those used in the present experiments (Bonaccorsi & Hrdina, 1966). However the drug was effective in relaxing the depolarized smooth muscle in a dose which did not exert an adrenolytic effect, but potentiated the response to noradrenaline. Phenoxybenzamine even in a high concentration ($5{\cdot}9 \times 10^{-6}$M) did not relax the depolarized preparation and phentolamine had much less effect than desipramine in a similar concentration. It does not seem likely that the reduction of the smooth muscle tone in depolarized artery induced by desipramine can be due to adrenolytic action. Furthermore there is no conclusive evidence that the contractile response of the smooth muscle to a potassium-rich depolarizing solution is due to stimulation of α-adrenergic receptors or to release of adrenergic transmitter, although such an effect has been suggested by Cervoni (1966).

The contractile response of smooth muscle to potassium-rich solution is attributed to an increased calcium influx due to changed membrane

permeability caused by potassium. The contraction was shown to be proportional to the concentration of calcium ions in the perfusate (Briggs, 1962; Waugh, 1962b; Hinke, Wilson & Burnham, 1964). Calcium itself is effective in causing contraction of depolarized smooth muscle, in contrast to its lack of action in normal preparations. The contraction of the depolarized renal artery caused by calcium was antagonized by desipramine to a degree proportional to the dose. On the other hand, an increase of calcium concentration in the perfusate increased the tone of relaxed muscle even in the presence of desipramine.

The drug and calcium can be considered therefore as functional antagonists, perhaps because desipramine, by stabilizing the membrane, inhibits the influx of calcium ions needed to trigger the initial contraction. The drug may also block the release inward of ionized calcium from the membrane. It is postulated that this release of calcium is responsible for the maintenance of the sustained contracture (Briggs, 1962; Hurwitz, Battle & Weiss, 1962); cocaine is similarly antagonized by calcium on the longitudinal muscle of the guinea-pig ileum (Hurwitz & others, 1962; Hagen & Hurwitz, 1963; Weiss, Coalson & Hurwitz, 1961).

In our experiments cocaine was able to reduce the tone of the depolarized artery but only in a concentration several hundred times higher than the effective concentration of desipramine. Chlorpromazine resembles desipramine in causing relaxation of the depolarized renal arteries. Chlorpromazine and desipramine are in this respect different from the potent adrenolytics phentolamine and phenoxybenzamine.

Acknowledgement. This work was partially supported by a grant from J. R. Geigy, S.A., Basel, Switzerland.

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J. Pharm. Pharmac., 1967, **19**, 674–681 Received March 21, 1967

The use of the rabbit ear artery in the bioassay of catecholamines in urine

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Urine causes transient constriction of the isolated central artery of the rabbit ear. The substance responsible is qualitatively indistinguishable from noradrenaline when assessed by α-adrenergic blockade, sensitivity to oxidation by ferricyanide, behaviour on alumina, and comparison of dose response curves. The artery is equally sensitive to noradrenaline and adrenaline and approximately one hundred times less sensitive to dopamine. The results of bioassay, and chemical assay using the trihydroxyindole method, indicate that the constrictor response of the artery provides a useful guide to the urinary content of noradrenaline plus adrenaline.

A method for the bioassay of catecholamines on the isolated artery of the rabbit ear was described by de la Lande & Harvey (1965). The following experiments were made to assess the application of this method to the assay of catecholamines in urine.

Experimental

METHODS

Bioassay. The only modifications to the method of perfusing the artery described by de la Lande & Harvey (1965) were: (i) the use of short arterial segments (1 to 4 cm) which were cleaned of adhering tissue, and (ii) cannulation and mounting of the artery in the organ bath so that the direction of flow was upwards (de la Lande, Cannell & Waterson, 1966). The cannula must be of sufficient diameter to contribute little if any resistance to the perfusion. The sensitivity is reduced and the responses become erratic if the cannula itself increases the perfusion pressure by more than 10 to 20 mm Hg.

After perfusion of the artery was commenced (approximately 8 ml/min) test doses of noradrenaline were injected at 2–5 min intervals for approximately 1 hr, after which 5-hydroxytryptamine (5-HT), 10–20 μg/litre was added to the perfusion fluid. When there was no further change in sensitivity to noradrenaline, mepyramine 10 μg/litre was added to the perfusion fluid. Control solutions of noradrenaline bitartrate (10 ng/ml) were prepared in 0·9% saline containing ascorbic acid 0·005% (ascorbic saline). Urine was filtered, adjusted to a pH of between 5·5 and 7·0, and diluted with ascorbic saline. Solutions for assay (0·1–0·3 ml) were injected into the intraluminal perfusion medium at 2 min intervals and their vasoconstrictor actions recorded by the increases in perfusion pressure.

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Chemical assay. The noradrenaline and adrenaline contents of the urine were measured by the trihydroxyindole procedure.

A 25 ml sample of filtered urine at pH 4·0 containing ascorbic acid (0·01%) and sodium edetate 1·0 g (2%) was agitated with 1–1·5 g of activated alumina by means of nitrogen bubbles while the pH was brought to, and maintained at 8·4 (±0·1) with M sodium hydroxide. After 2½ min the alumina was separated by centrifugation at 4°, and washed twice by shaking and subsequent centrifugation, with 10 ml lots of deionized water. Elution was by 0·2M acetic acid, the alumina being shaken for 4 min periods with two 10 ml portions of the acid and the supernatants collected after centrifugation. After the addition of 2 ml of 3% sodium edetate, 10 ml of the combined supernatant was adjusted to pH 6·0 with 0·5M Na_2HPO_4, centrifuged, and diluted to 20 ml (referred to subsequently as eluate).

For oxidation, 0·1 ml of $K_3Fe(CN_6)$ (0·25%) was added to a solution of 1·0 ml of eluate, 0·5 ml of sodium phosphate buffer (0·2M) at pH 6·5, and 2·0 ml of distilled water. Oxidation was terminated after 3 min by the addition of 1·0 ml of a solution of ascorbic acid, sodium hydroxide and β-thiopropionic acid. (This solution was prepared 15 sec earlier by adding 0·4 ml of 10% β-thiopropionic acid to 2·0 ml of a solution of 0·2% ascorbic acid in 5M sodium hydroxide prepared 5 min previously.) Fluorescence was measured after 10 and 15 min using filter sets I and II respectively on a Turner model 110 fluorometer fitted with the high sensitivity kit and blue lamp (Palmer, 1964). Blanks were prepared by reversing the order of addition of the alkali-ascorbic-thiopropionic mixture, and potassium ferricyanide (Price & Price, 1957). Each assay was made in duplicate with internal controls for noradrenaline and adrenaline.

The above method is that of Euler & Floding (1955) modified by (i) the use of a batch instead of a column procedure for alumina adsorption (Crout, 1961); (ii) the differential wavelength procedure for discriminating between noradrenaline and adrenaline (Bertler, Carlsson & Rosengren, 1959); (iii) the use of β-thiopropionic acid to stabilize the lutines (Palmer, 1964). The filter set combinations were those described in the "Manual of Clinical Laboratory Procedures" by G. K. Turner Associates. Filter set I gives excitation at 405 mμ and fluorescence at 510 mμ, and yields nearly equal readings for noradrenaline and adrenaline. Filter set II gives peak excitation at 436 mμ and fluorescence at and above 520 mμ, and yields approximately four times greater fluorescence for adrenaline than for noradrenaline. The fluorescent spectra of urine samples were checked periodically on a Farrand spectrophotofluorimeter.

In 16 urine samples assayed by the above method, 15 parallel assays were made on the same urine samples containing added noradrenaline 100 ng/ml (12 samples) or adrenaline 100 ng/ml (three samples). The mean recovery of added noradrenaline was 84·5%. The recovery was low in three samples (40, 53 and 58%), and in the remainder recoveries ranged between 77 and 123%. Recoveries of added adrenaline were 73, 82 and 87%.

Results

An injection of urine into the artery always caused a transient rise in perfusion pressure qualitatively similar to that produced by noradrenaline or histamine (Fig. 1). The following tests indicated that the response was probably due to catecholamines.

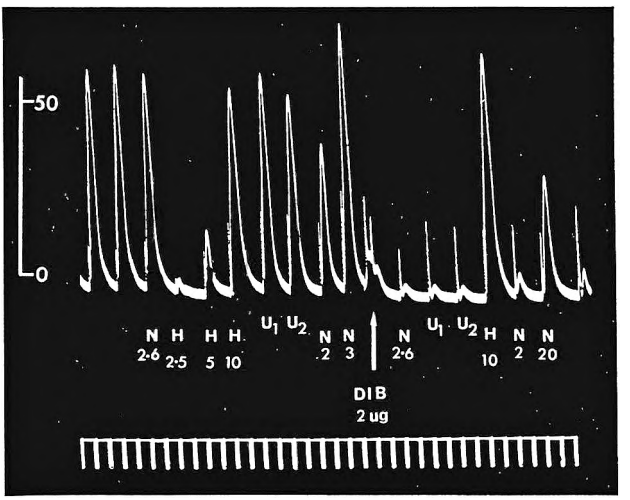

FIG. 1. Constrictor responses of an artery, in mm Hg, to injections of noradrenaline (N), histamine (H), and two diluted urine samples (U_1 and U_2), before and after phenoxybenzamine HCl (DIB) in the dose shown. The doses of N are in ng, of H are in μg; the volumes of U_1 and U_2 are each 0·2 ml, corresponding to 0·04 ml of the undiluted urines. Mepyramine (10 ng/ml) and 5-HT (20 ng/ml) present throughout. Time scale in min. Ordinate, increase in perfusion pressure in mm Hg.

Phenoxybenzamine abolished the response to urine and to noradrenaline (17 urine samples). The response to histamine was either unaffected or enhanced to a small extent, indicating that the effect of phenoxybenzamine on the urine response was not due to non-specific depression of the artery. A typical result is included in Fig. 1.

The responses to 16 urine samples were unaffected or changed in magnitude by less than 20% by mepyramine in concentrations (10 μg/litre) which abolished equivalent responses to histamine. However, the response to one urine sample was abolished by mepyramine. The sample was from a patient with suspected sympathetic nerve degeneration; subsequent analysis on the guinea-pig ileum revealed that this urine contained an abnormally large amount of histamine (28 mg/24 hr) compared with the normal daily output of 17–90 μg (Gowenlock & Platt, 1963).

In each of 10 urine samples the response was abolished by pretreating the urine with potassium ferricyanide at pH 6·0. Potassium ferricyanide oxidizes both noradrenaline and adrenaline at this pH (Euler & Floding, 1955). Potassium ferricyanide pretreatment was as follows. The pH

of the urine was adjusted to approximately 6·5, and 0·5 ml of 0·2M phosphate buffer (pH 6·5) added to a 5 ml sample. To this was added 1·0 ml of 0·25% zinc sulphate followed by 2·0 ml of 0·25% potassium ferricyanide. After at least 3 min the latter was converted to ferrocyanide with 0·5 ml of 2% ascorbic acid, and the sample brought to the same dilution with ascorbic saline as the untreated urine. Injections of 0·05–0·4 ml of this "oxidized urine" produced no response and caused little alteration in the sensitivity of the preparation to noradrenaline given simultaneously or subsequently.

The constrictor material in the urine was adsorbed onto alumina, and eluted from alumina in an identical fashion to catecholamines. The experimental conditions of adsorption and elution were those employed for chemical assay (see methods), with the minor modification that 0·2M hydrochloric acid was used for elution instead of acetic acid. The activity remaining in a 10 ml sample of urine after shaking with 1 g of alumina, and the activity in the (neutralized) hydrochloric acid eluate of the alumina, were estimated by the volume required to elicit the same contractile activity as the untreated urine. In each of 5 urines, the treated urine contained less than 10% of the activity of the untreated control. The activities recovered in the acid eluates were between 70 and 80% of that originally present in three urines. In two urines the activities recovered were approximately 124 and 140% of that originally present.

On the basis of the above tests, it was assumed that the constrictor activity of the urine was due to catecholamines.

Quantitative estimations of the catecholamine content of the urine sample were made by comparing the responses of an artery to each of the following: (1) a urine sample; (2) the urine sample plus noradrenaline; (3) noradrenaline added to the urine sample after the constrictor action of the urine had been abolished with ferricyanide; (4) noradrenaline added to ascorbic saline. Each solution was tested in duplicate or triplicate at three dose levels. An example of the curves obtained on one artery for one of the urine samples (No. 6, Table 1) is shown in Fig. 2. The main feature is that the shapes of the curves are similar and departures in slope and curvature are relatively minor. The results of eight experiments are summarized in Table 1. It will be noted that the estimates of noradrenaline content based on the two types of noradrenaline control solutions (ascorbic saline, and oxidized urine) showed reasonable agreement, with the exception of experiment 3 where the separate estimates were 105 and 67 ng/ml. In this experiment there was also a large discrepancy between the measured and calculated contents of noradrenaline in the urine to which noradrenaline was added (the recovery of added noradrenaline being 152% instead of 100%). The difference between the noradrenaline assays probably reflected the presence of sensitizing substances in the urine. A number of urine samples were screened during this study by the less rigorous procedures of dose matching or comparison at two dose levels with noradrenaline in ascorbic saline. The mean noradrenaline-equivalent content of the total of 48 urines from 31 subjects (including those in Table 1) was 56·3 $\pm$ 29·3 ng/ml (s.d.). In Table 2

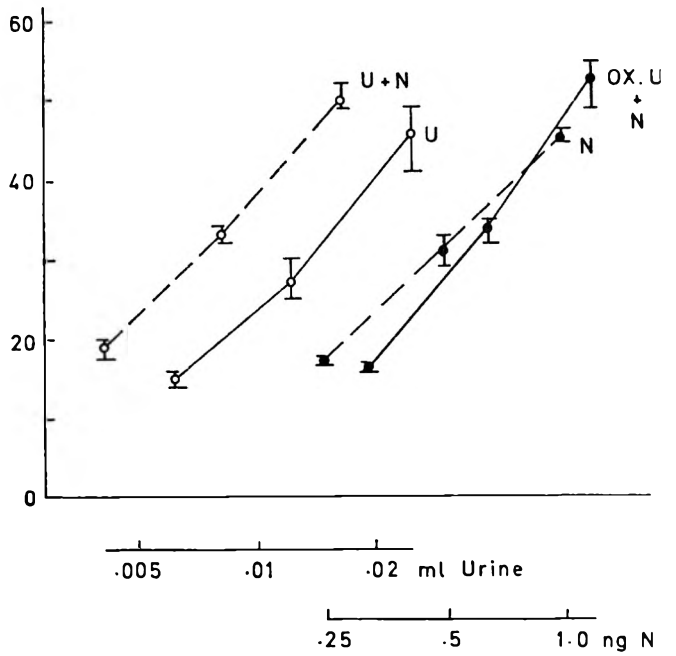

FIG. 2. Dose-response curves of an artery to urine (U), urine + noradrenaline 40 ng/ml (U + N), noradrenaline in ascorbic saline (N), and urine whose contractile activity had been abolished with ferricyanide and to which noradrenaline 40 ng/ml was subsequently added (OX.U + N). The extent of the variation of the responses to repeated doses is shown by a vertical bar. Ordinate: increase in perfusion pressure in mm Hg. The abscissa, for U and U + N is ml of urine, and for OX.U + N, and N, is ng of noradrenaline. The urine sample is No. 6 of Table 1.

TABLE 1. NORADRENALINE-EQUIVALENT CONTENTS OF URINE SAMPLES

Sample No.	Compared with noradrenaline in ascorbic saline			Compared with noradrenaline in oxidized urine		
	Mean (ng/ml)	Range (ng/ml)	% recovery of added noradrenaline	Mean (ng/ml)	Range (ng/ml)	% recovery of added noradrenaline
1	38	35–41	81	47	42–50	88
2	54	48–65	105	54	48–58	101
3	105	93–120	152	67	57–79	103
4	72	60–85	—	87	72–101	—
5	46	43–49	93	54	52–55	111
6	42	38–44	81	44	40–51	79
7	51	49–54	107	53	52–55	109
8	44	40–51	122	59	52–70	148
Means	56		105	58		105

The noradrenaline-equivalent contents of the urine samples were calculated from the dose response curves at the lowest, middle, and highest levels of the responses common to noradrenaline and to the urine. Hence the mean in each case is based on three estimates of which the minimum and maximum values are included under "range".

TABLE 2. NORADRENALINE-EQUIVALENT CONTENTS OF URINE SAMPLES

Origin of sample	Noradrenaline equivalent ng/ml	No. of samples	% recovery of added noradrenaline	No. of samples	Ratio* of activity of adrenaline to noradrenaline	No. of samples	Comments
31 subjects 3 to 24 hr samples	56·3 ± 29·3 (s.d.)	48	112 ± 47 (s.d.)	24	0·9 ± 0·06 (s.d.)	12	The recoveries of noradrenaline in 21 of the samples were between 63 and 133%. However, in three samples the recoveries were 200, 224 and 250%.
V	(i) 275 (ii) 12·5	1 1	100 100	1 1	1·0 0·6	1 1	Samples (i) and (ii) taken in each case before, and after, removal of adrenal tumours.
M	(i) 800 (ii) 85	1 1	Not est'd. ,,		1·0	1 1	
P	(i) 790 (ii) 417	1 1	130 Not est'd.	1 1	1·2	1	Samples (i) and (ii) taken at an interval of 3 months from a patient with tumours of extra-adrenal chromaffin tissue.

* Estimated by dose matching

this level is compared with those of two patients with phaeochromocytoma of the adrenal medulla, and one with recurrent phaeochromocytomas of extra-adrenal chromaffin tissue. The method revealed high levels of noradrenaline in the urine of each of the patients, and low levels after the removal of the medullary tumours. Complete removal of tumours was not possible in the third patient.

CHEMICAL ASSAY

The results of bioassay were compared with those of chemical assay on 12 urines, comprising 4–24 hr samples from 11 colleagues or patients. The bioassay was based on comparison with noradrenaline in ascorbic saline, using two dose levels of each. The results are shown in Table 3. The bioassay was within ±25% of the chemical assay in nine of the

TABLE 3. COMPARISON OF BIOASSAY AND CHEMICAL ASSAY FOR NORADRENALINE AND ADRENALINE

Subject	Chemical assay (ng/ml)		Bioassay (ng/ml)	Ratio of results $\frac{\text{Bioassay}}{\text{Chemical assay}}$
	Noradrenaline	Noradrenaline + Adrenaline	Noradr. equiv. (= noradr. + adren.)	
P	740	740	790	1·07
V	124	239	275	1·15
RH (ii)	164	164	125	0·76
NP	57	77	42, 73	0·58, 0·95
AB	35·5	59	60	1·02
JW	48	53	40	0·76
JMc	21	50	41, 50	0·82, 1·0
ID	40	46	46, 48	1·0, 1·04
MT	30	45	43	0·96
DF	33·2	44	27–40 (51)	0·77
RH (i)	17·8	36	66	1·83
JM	24	34	17–27 (28)	0·66

Note (1) The chemical assay data are corrected for a mean recovery of noradrenaline + adrenaline of 85%.

(2) The mean ratio $\frac{\text{bioassay}}{\text{chemical assay}}$ is 0·95.

comparisons, but gave values which were 0·67 and 1·82 times those obtained by chemical assay in two samples (JM and RH (i) respectively). The cause of discrepancy between chemical and bioassay was not analysed systematically, but in three of the comparisons it may be attributed largely to the bioassay. Thus the dose response curves to urine samples JM and DF were much flatter than those to their noradrenaline control solutions and for this reason ranges rather than mean values are quoted in Table 3. The difference between the curves was consistent with an inhibitory effect of the urine on the sensitivity of the artery to noradrenaline, and this was confirmed by the finding that after treating the urines with alumina, the noradrenaline-equivalent contents of the hydrochloric acid eluates of the alumina (shown in brackets in Table 3) were greater than those of the untreated urines. An inhibitory effect of urine may also have accounted for an unusual discrepancy in sample NP. This sample, when assayed a second time on the same artery after an interval of approximately 2 hr, yielded a noradrenaline-equivalent content of 73 ng/ml compared with the initial value of 42 ng/ml.

ADRENALINE AND DOPAMINE

The ratio of the sensitivity of the artery to adrenaline compared with that of noradrenaline in 12 arteries was 0·9 ± 0·06 (s.d.). The corresponding mean ratio for dopamine was approximately 0·01. This value is based on ratios of 0·01, 0·01, 0·01, 0·004 and 0·0025 observed in the present study, and ratios of 0·03, 0·03, 0·015 and 0·01 described previously (de la Lande & Harvey, 1965).

Discussion

The vasoconstrictor substance(s) in urine, and noradrenaline behaved in a similar fashion when assessed by (i) sensitivity to blockade by phenoxybenzamine; (ii) sensitivity to oxidation by ferricyanide; (iii) adsorption onto and elution from alumina; (iv) comparison of dose response curves. Each of these tests is not conclusive in itself, but together they provide strong evidence that catecholamines are solely responsible for the constrictor activity on the artery of most urine samples. The catecholamines may be assumed to be mainly noradrenaline and adrenaline, since the constrictor potencies of these amines are approximately equal and are 30 to 300 times greater than that of dopamine. The normal urinary output of dopamine is 100–200 μg/day (Udenfriend, 1962), i.e. approximately two to six times that of noradrenaline plus adrenaline, assuming mean normal daily outputs of the latter amines of either 36 μg (Crout, 1961) or 68 μg (Jacobs, Sobel & Henry, 1961).

The comparisons between biological and chemical assay indicate that the noradrenaline-equivalence of the constrictor response to urine corresponds reasonably well with the content of noradrenaline plus adrenaline. The agreement is of a similar order to that obtained by Euler & Floding (1955) on urine samples assayed by the trihydroxyindole method, and by biological assay on the cat blood pressure, and rat uterus.

If it is assumed that the discrepancy between chemical and bioassay is due largely to error in the bioassay, the error appears to be somewhat less than $\pm 40\%$ for most urines. An error of this magnitude is probably unimportant in phaeochromocytoma, where one of the contributory aids to diagnosis is a urinary catecholamine output of 200 μg/day or more, i.e. at least twice the upper limit of the normal range (Udenfriend, 1962).

Although the method cannot be used as a quantitative substitute for the chemical method particularly where urinary levels are in the lower range of normal, by providing a semi-quantitative guide to these levels it should prove useful in screening programmes to aid the selection of urines for subsequent chemical assay. Its major advantage over other bioassay methods including the aortic strip method of Helmer (1961) is that it combines the virtues of high sensitivity to noradrenaline and to adrenaline, with speed of assay, and ease of preparation of the artery and the urine.

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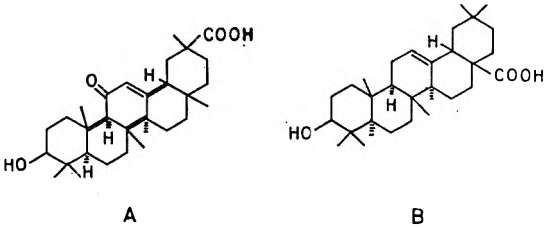
J. Pharm. Pharmac., 1967, **19**, 682-689 Received March 22, 1967

The preparation of some derivatives of glycyrrhetic acid and oleanolic acid

P. D. G. DEAN*, T. G. HALSALL AND M. W. WHITEHOUSE†

Some new derivatives of glycyrrhetic acid and oleanolic acid were prepared and characterized. Various combinations of the modified and normal functional groups were synthesized as required for a separate study of the uncoupling activities of glycyrrhetic acid and oleanolic acid. The hydroxyl functions of these two acids were modified to β-carboxypropionyl, acetyl and β-methoxycarbonylpropionyl esters. The carboxyl function of glycyrrhetic acid was converted to amide, *p*-amidobenzoic acid, *o*-amidobenzoic acid and the glycine conjugate of the acid. 11-Deoxy (glycyrrhetic acid) and 9,11-dehydro-11-deoxo (from both acids) analogues were prepared. 11-Oxo analogues of oleanolic acid were synthesized as well as the methyl esters.

THE acid, 18β-glycyrrhetic acid (glycyrrhetinic acid, 3β-hydroxy-11-oxo-5α,18β-olean-12-en-30-oic acid; I) is the aglycone of glycyrrhizin (glycyrrhizic acid), a diglucuronide present in liquorice. This pentacyclic triterpenoid acid possesses some striking pharmacological properties, inhibiting diuresis (so-called "mineralcorticoid" activity) and when administered as the 3-*O*-hemisuccinate ester to man, promoting the healing of stomach ulcers (Parke & Williams, 1962; Doll, Hill & others, 1962).



A B

FIG. 1. Structure of: A. Glycyrrhetic acid. B. Oleanolic acid.

It also exhibits anti-inflammatory activity in laboratory animals (Finney & Somers, 1958; Kraus, 1960; Aleshinskaya, Aleshkina & others, 1964; Tangri, Seth & others, 1965) and, in common with many other anti-inflammatory drugs, uncouples oxidative phosphorylation in liver mitochondria and in extrahepatic tissues such as cartilage (Whitehouse & Haslam, 1962).

The relation between the chemical structure and uncoupling activity of glycyrrhetic acid has been analysed by Whitehouse, Dean & Halsall (1967). For this purpose, some novel derivatives of glycyrrhetic acid and oleanolic acid (3β-hydroxyolean-12-en-28-oic acid; II) were required and their preparation and characterization are now described.

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Experimental

MATERIALS

Oleanolic acid, and purified 18α- and 18β-glycyrrhetic acid were kindly made available by Dr. S. Gottfried and the late Professor E. E. Turner, F.R.S. (Biorex Laboratories, London, E.C.1). A commercial sample of ammonium glycyrrhizate was obtained from L. Light & Co., Colnbrook, Bucks. Other derivatives of the glycyrrhetic acids and derivatives of oleanolic acid were synthesized as described below. Silica gel for column chromatography (type M.F.C.) was obtained from Hopkin and Williams, Chadwell Heath, Essex. Alumina (type H, P. Spence, Widnes, Lancs.) was deactivated with 10% of 10% (v/v) aqueous acetic acid.

METHODS

The purity of all compounds tested for uncoupling activity was checked by thin-layer chromatography on silica gel (Merck, Darmstadt; Grade H "nach Stahl") usually with ethyl acetate or ethyl acetate-light petroleum b.p. 60–80° (1 : 1 v/v) as the developing solvent. Compounds were visualized on the developed chromatoplates by one or more of the following procedures: spraying with water, exposure to iodine vapour; spraying with a 10% (w/v) solution of antimony trichloride in chloroform or glacial acetic acid, followed by heating at 105°; spraying with a 5% (w/v) solution of dodecamolybdophosphoric acid in ethanol and then heating at 80°. Ionophoresis on paper in aqueous or aqueous ethanolic buffers (containing up to 40% v/v ethanol) was also used to characterize triterpenoid acids; these were visualized by any of the above procedures or by inspection of dried ionophoretic strips under ultraviolet light.

Preparation of derivatives of glycyrrhetic acid (see Table 1 of Whitehouse, Dean & Halsall, 1967)

Methyl 3-O-(*β-carboxypropionyl*)-18α-*glycyrrhetate* (comp. 9 in the paper of Whitehouse & others 1967). A solution of 18α-glycyrrhetic acid (1 g) in methanol was treated with an excess of ethereal diazomethane solution. The mixture was acidified with acetic acid (0·5 ml) after 15 min and the ether evaporated off under reduced pressure. The white solid, methyl 18α-glycyrrhetate (1 g), which remained was dissolved in dry pyridine (20 ml) and the solution was treated with succinic anhydride (3 g). The mixture was heated under reflux for 8 hr.

The reaction mixture was cooled, poured into water (500 ml) and the precipitate filtered off, washed first with dilute hydrochloric acid, then with water, and then dried. Recrystallization from acetic acid-water gave methyl 3-*O*-(β-carboxypropionyl)-18α-glycyrrhetate as needles, m.p. 237–9° (Rf, 0·11; Rf, for methyl 18α-glycyrrhetate, 0·8) (Found: C, 71·15; H, 9·1; $C_{35}H_{52}O_7 + H_2O$ requires C, 70·8; H, 9·0%).

Methyl 3-O-(*β-carboxypropionyl*)-18*β-glycyrrhetate* (comp. 10). 18β-Glycyrrhetic acid was esterified with diazomethane as for 18α-acid and the resulting ester recrystallized from ethanol to yield plates, m.p. 253–4°.

A solution of the methyl 18β-glycyrrhetate (1 g) in pyridine (20 ml) was treated with succinic anhydride (0·5 g) and the mixture heated under

reflux for 12 hr. The reaction mixture was cooled and poured into water (20 ml). The suspension was extracted with ether (3 × 100 ml) and the ethereal extracts dried over magnesium sulphate. The solvents were evaporated leaving a white solid. Recrystallization from ethanol-water gave methyl 3-*O*-(β-carboxypropionyl)-18β-glycyrrhetate as plates, m.p. 265–7°; $[\alpha]_D$ + 70 ± 1° (*c*, 1·05 in chloroform) (Found: C, 69·8; H, 8·9; $C_{35}H_{52}O_7 + H_2O$ requires C, 69·2; H, 9·0%).

3-O-*Acetyl*-18β-*glycyrrhetamide* (comp. 11). 18β-Glycyrrhetic acid was acetylated with acetic anhydride and pyridine. The product, 3-*O*-acetylglycyrrhetic acid, was recrystallized from ether to yield the acetate, m.p. 289–90°.

A solution of the acetylglycyrrhetic acid (1·6 g) in redistilled thionyl chloride (10 ml) was heated under reflux for 4 hr, while protected from atmospheric moisture with a calcium chloride tube. The excess of thionyl chloride was evaporated under reduced pressure and the remaining solid (acetylglycyrrhetyl chloride) was cooled and treated with strong ammonia solution (sp.gr. 0·88; 10 ml); a vigorous reaction took place and a white solid separated from the solution. The ammonia was evaporated and the aqueous suspension extracted with ether (3 × 50 ml). The combined ethereal extracts were dried over magnesium sulphate and the ether was evaporated. The product (1·6 g) was examined by thin-layer chromatography. Two spots were detected, Rf 0·63 and 0·25 (Rf, for acetylglycyrrhetic acid, 0·25). Recrystallization from ethanol gave 3-O-*acetyl*-18β-*glycyrrhetamide* as needles, m.p. 302–5° (Found: C, 74·6; H, 9·25; N, 2·4. $C_{32}H_{49}O_4N + H_2O$ requires C, 73·8; H, 9·7; N, 2·7%).

o-(3-O-*Acetyl*-18β-*glycyrrhetamido*)*benzoic* acid (comp. 22). 3-*O*-Acetyl-18β-glycyrrhetyl chloride (0·4 g) was added to a solution of anthranilic acid (0·3 g) in 10% sodium hydroxide solution (10 ml). The suspension was shaken at room temperature for ½ hr, the mixture acidified to litmus with dilute hydrochloric acid and extracted with ether. The ethereal extracts were dried over magnesium sulphate and the ether evaporated to give a brown solid (0·7 g). Recrystallization from methanol (twice) (charcoal) gave o-(3-O-*acetyl*-18β-*glycyrrhetamidobenzoic acid* as needles, m.p. 269° (decomp.). Rf, 0·05 (Rf for acetylglycyrrhetic acid, 0·25).

p-(3-O-*Acetyl*-18β-*glycyrrhetamido*)*benzoic acid* (comp. 23). 3-*O*-Acetyl-18β-glycyrrhetyl chloride (0·4 g) was added to a solution of *p*-aminobenzoic acid (0·3 g) in 10% sodium hydroxide solution (10 ml). The resulting suspension was acidified to litmus with dilute hydrochloric acid and extracted with ether-methanol (3:1 v/v) (3 × 100 ml). The extracts were combined and dried over magnesium sulphate. The solvents were evaporated to give a pale orange solid. Recrystallization from methanol gave *p*-(3-*O*-acetyl-18β-glycyrrhetamido)benzoic acid as needles, m.p. 260° (decomp.); Rf, 0·06 (Rf for acetylglycyrrhetic acid, 0·25).

3-O-(β-*Methoxycarbonylpropionyl*)*glycyrrhetic acid.* Succinic anhydride was converted into β-methoxycarbonylpropionyl chloride (Cason, 1955). It was obtained as a colourless liquid, b.p. 64–64·5°/1·7 mm.

A solution of glycyrrhetic acid (5·1 g) in pyridine (20 ml) was treated

with a solution of the β-methoxycarbonylpropionyl chloride (3 ml) in pyridine (50 ml). The reaction mixture was allowed to stand for 4 days. The solution was poured into water (100 ml) and extracted with ether (3 × 250 ml). The ethereal extracts were combined and dried over magnesium sulphate. The solvents were evaporated under reduced pressure to give a brown gum. 12-Fold fractional recrystallization from methanol-water gave 3-O-(*β-methoxycarbonylpropionyl)glycyrrhetic acid* as needles, m.p. 246–50° $[\alpha]_D$ + 74° ± 2° (*c*, 0·6 in chloroform) (Found: C, 71·6; H, 9·0; $C_{35}H_{52}O_7$ requires C, 71·9; H, 8·95%).

3-O-(*β-Methoxycarbonylpropionyl)glycyrrhetamide.* A solution of the 3-*O*-(β-methoxycarbonylpropionyl)glycyrrhetic acid (240 mg) in redistilled thionyl chloride (2 ml) was heated under reflux for 5 hr. Atmospheric moisture was excluded by using a calcium chloride tube. The excess of thionyl chloride was evaporated under reduced pressure, leaving a pale yellow solid. The solid was cooled and treated with strong ammonia solution (4 ml sp.gr. 0·88) and the reaction mixture was shaken for ½ hr at room temperature. The excess of ammonia was evaporated and the aqueous suspension extracted with ether (3 × 5 ml). The ethereal extracts were combined and dried over magnesium sulphate. The ether was evaporated leaving a pale yellow solid (200 mg). Recrystallization from methanol-acetone gave needles, m.p. 210–6°. The product was examined by thin-layer chromatography; two spots were detected, as having an Rf of 0·48 (principal component) and the other an Rf of 0·65 (Rf for 3-*O*-(β-methoxycarbonylpropionyl)glycyrrhetic acid, 0·66). Further recrystallization from methanol-acetone gave needles, m.p. 216–8°.

Hydrolysis of 3-O-(*β-methoxycarbonylpropionyl)glycyrrhetamide.* A solution of the ester (109 mg) in dimethylformamide (15 ml) was treated with anhydrous lithium iodide (1 g) and the solution heated under reflux for 12 hr (Dean, 1965). The reaction mixture was cooled and poured into water. The precipitate which formed was filtered off, washed with water and dried. Recrystallization from chloroform gave 3-*O*-(β-carboxypropionyl)glycyrrhetamide as plates, m.p. 247–50° (single spot on thin-layer chromatography).

9,11-Dehydro-11-deoxo-18β-glycyrrhetic acid (comp. 14). A solution of 18β-glycyrrhetic acid (3·9 g) in ethanol (100 ml) was treated with a solution of potassium borohydride (2 g) in water (10 ml). The mixture was heated under reflux for 1 hr, when a further quantity of potassium borohydride (2 g) was added to the reaction mixture, and the heating continued for 1 hr. The resulting solution was cooled and poured into dilute hydrochloric acid (about 100 ml). The suspension was extracted with ether (3 × 100 ml) and the ethereal extracts combined, washed with water and dried over magnesium sulphate. The ether was evaporated leaving a white solid, m.p. 284–6° (3·5 g). Examination by thin-layer chromatography showed the presence of three compounds with Rf 0·45, 0·35, 0·23 and with ultraviolet absorption maximum at 283 mμ, absent and 250 mμ respectively. The reaction product was adsorbed on silica gel (400 g) and successively eluted with light petroleum–benzene–ether. Elution with 20% ether–benzene gave crystalline fractions which showed

one spot when examined by thin-layer chromatography: Rf, 0·45, λ_{max} 283 mμ (ϵ, 10,000). Later fractions gave mixtures of two compounds with Rf, 0·45 and 0·23.

The yield of the combined diene fractions (λ_{max} 283 mμ) was 3 g. When this was recrystallized from methanol, 9,11-dehydro-11-deoxo-18β-glycyrrhetic acid was obtained as needles, m.p. 293–5°; λ_{max} 283 mμ (ϵ, 11,500) (Kurono (1938) gives m.p. 287° for this compound, prepared by sodium and ethanol reduction of glycyrrhetic acid).

3-O-(*β-Carboxypropionyl*)-9,11-*dehydro*-11-*deoxo*-18*β-glycyrrhetic acid* (comp. 15). A solution of 9,11-dehydro-11-deoxo-18β-glycyrrhetic acid (300 mg) in pyridine (10 ml) was treated with succinic anhydride (500 mg) and the mixture heated under reflux for 8 hr. The dark solution was cooled and poured into ice-dilute hydrochloric acid (approx. 30 ml). The product was extracted with ether (3 × 50 ml) and the ethereal extracts combined, washed with water (2 × 25 ml) and dried. Evaporation of the ether gave a solid (250 mg), which was recrystallized from acetic acid-ethanol-water (9 : 2 : 1 v/v) to give 3-O-(*β-carboxypropionyl*)-9,11-*dehydro*-11-*deoxo glycyrrhetic acid* as octahedra, m.p. 264–7°; $[\alpha]_D$ + 136 ± 2° (*c*, 0·4 in chloroform); Rf, 0·1 (Rf, of starting material, 0·45).

11-*Deoxo*-18*β-glycyrrhetic acid* (comp. 12). A solution of 18β-glycyrrhetic acid (1·5 g) in glacial acetic acid (50 ml) was shaken with platinum oxide catalyst (0·5 g) for 7 hr under hydrogen at a pressure of 15 lb/in.^{2} The metallic suspension was filtered off and the filtrate evaporated to 30 ml under reduced pressure. 11-Deoxo-18β-glycyrrhetic acid crystallized as needles, m.p. 328–9°. The product had no ultraviolet absorption at 250 mμ and moved as a single spot on thin-layer chromatography; Rf, 0·6 (Rf for glycyrrhetic acid, 0·43; Rf of an authentic sample donated by the late Professor E. E. Turner, 0·6).

3-O-(*β-Carboxypropionyl*)-11-*deoxo*-18*β-glycyrrhetic acid* (comp. 13). A solution of 3-*O*-(β-carboxypropionyl)-18β-glycyrrhetic acid (455 mg) in acetic acid (18 ml) was shaken with platinum oxide catalyst (25 mg) in a hydrogen atmosphere as described. When the filtrate was evaporated to 5 ml, 3-O-(*β-carboxypropionyl*)-11-*deoxo*-18*β-glycyrrhetic acid* crystallized spontaneously as needles, m.p. 284–6° (Found: C, 72·3; H, 9·4. $C_{34}H_{52}O_6$ requires C, 73·2; H, 9·4%); $[\alpha]_D$ + 55° ± 2° (*c*, 1·0 in ethanol). Only one component was detected by thin-layer chromatography Rf, 0·32 (Rf, for the starting material, 0·25). A solution of the product in ethanol had no ultraviolet absorption maximum at 250 mμ.

11-*Deoxo*-18*α-glycyrrhetic acid.* A solution of 18α-glycyrrhetic acid (383 mg) in dry dioxan (20 ml) was treated with platinum oxide catalyst (278 mg) suspended in acetic acid (20 ml). The hydrogenation was made as described above for 48 hr. The suspension was filtered and the filtrate was evaporated to dryness. The product was recrystallized from acetic acid to give impure 11-deoxo-18α-glycyrrhetic acid as needles, m.p. 288–92°. The ultraviolet absorption at 250 mμ of this product indicated that it also contained starting material (approx. 20% of which could not be separated by fractional recrystallization from acetic acid. [This mixture was examined for uncoupling activity].

N-(18β-*Glycyrrhetyl*)*glycine* ("Glycyrrhetinuric acid") (comp. 19). A solution of 18β-glycyrrhetic acid (4·7 g), freshly prepared ethyl glycine ester (1·0 ml) and dicyclohexylcarbodi-imide (2·06 g) in chloroform-methylene dichloride (1 : 1) (75 ml) was stirred at 25–30° for $\frac{1}{2}$ hr and at 20° for 48 hr. Acetic acid (5 drops) was added and the reaction mixture was filtered through sintered glass. The filtrate was diluted with chloroform and ether (total volume 100 ml) and passed down a column of alumina (B.D.H.). Elution with ether-chloroform (1 : 1) 500 ml gave two components, which were not glycyrrhetic acid, with Rf, 0·51 and 0·64 on thin-layer chromatography. (Rf for glycyrrhetic acid, 0·24 on the same plate).

The above solution was evaporated to dryness, the resulting solid was taken up in methanol (20 ml) and potassium hydroxide (0·6 g) was added. The solution was stirred at room temperature. After 2 hr, the solution was acidified with dilute hydrochloric acid and extracted with ethyl acetate (3 × 50 ml). The combined ethyl acetate extracts were washed with water, dried over magnesium sulphate and the solvent evaporated.

The remaining solid was dissolved in ether and adsorbed on silica gel; elution with ether and ethyl acetate-ether 1 : 1 gave a single component when examined by thin-layer chromatography (Rf, 0·01) (Rf for glycyrrhetic acid, 0·20).

Evaporation of the solvents gave a colourless gum which was extracted with water, light petroleum (b.p. 60–80°) and ethyl acetate. About 80% was insoluble in water and light petroleum, but soluble in ethyl acetate. This portion was recrystallized from ethyl acetate-light petroleum to give needles, m.p. 264–4°. Recrystallization did not change the melting point. ν_{max} 2·92, 3·04, 5·87, 6·06, 6·21 μ (Nujol mull); (Found: C, 70·8; H, 9·1; N, 2·8. $C_{32}H_{49}O_5N + \frac{1}{2} H_2O$ requires C, 70·4; H, 9·4; N, 2·6%).

Preparation of derivatives of oleanolic acid

Methyl 3-O-*acetyl*-11-*oxo*-18β-*oleanolate*. This compound was prepared according to the method described by Bilham, Kon & Ross (1942). Rf, 0·79 (Rf for starting material, 0·85). The product was recrystallized from methanol-acetone to give methyl 3-*O*-acetyl-11-oxo-18β-oleanolate as needles, m.p. 240–1°; λ_{max} 250 mμ (ϵ, 10,960). [Bilham & others (1942) give 241–41·5°; λ_{max} 250 mμ (ϵ, 11,000)].

Hydrolysis of methyl 3-O-*acetyl*-11-*oxo*-18β-*oleanolate*. A solution of this ester (3·4 g) was partially hydrolysed with lithium iodide in 2,4,6-collidine (Elsinger, Schreiber & Eschenmoser, 1942). The product was recrystallized (charcoal) from methanol-chloroform to give 3-*O*-acetyl-11-oxo-18β-oleanolic acid as plates, m.p. 263–4° (the above authors give 264–5°).

The 3-*O*-acetyl group resisted (attempted) hydrolysis with 5% ethanolic potassium hydroxide solution under conditions which did not isomerize the D/E *cis*-ring junction.

11-*Oxo*-18α-oleanolic acid (comp. 29). A solution of 3-*O*-acetyl-11-oxo-18β-oleanolic acid (1 g) in ethanol (10 ml) was treated with potassium hydroxide (0·5 g). The mixture under nitrogen in the dark was heated under reflux for 3 hr and allowed to cool. The reaction mixture was

acidified with dilute sulphuric acid and extracted with chloroform (3 × 5 ml). The combined chloroform extracts were washed with water and dried over magnesium sulphate. The chloroform was then evaporated off and the colourless gum which remained was recrystallized from aqueous acetic acid to give 11-oxo-18α-oleanolic acid as plates m.p. 266–8° [Ruzicka & Cohen (1937) give 267–271°].

Methyl 3-O-(*β-methoxycarbonylpropionyl*)*oleanolate.* A solution of methyl oleanolate (5 g) in pyridine (50 ml) was treated with a solution of β-methoxycarbonylpropionyl chloride (3 ml) in pyridine (25 ml). The solution was shaken at room temperature for 48 hr. The reaction mixture was poured into water (100 ml) and extracted with ether (3 × 100 ml). The ethereal extracts were evaporated to dryness and the residue dried over concentrated sulphuric acid *in vacuo* for 48 hr. Recrystallization of the product (5·6 g) from methanol-ethyl acetate gave needles, m.p. 164–7°. The product gave a single spot on thin-layer chromatography: Rf, 0·74 (Rf, for methyl oleanolate, 0·65). Further recrystallizations gave *methyl* 3-O-(*β-methoxycarbonylpropionyl*)*oleanolate* as needles, m.p. 166–7°; $[\alpha]_D$ + 57° ± 1° (*c*, 1·04 in chloroform) (Found: C, 73·7; H, 9·65; $C_{36}H_{56}O_6$ requires C, 74·0; H, 9·6%).

Methyl 3-O-(*β-methoxycarbonylpropionyl*)-11-*oxo*-18*β-oleanolate.* A solution of chromium trioxide (2·5 g) in 90% acetic acid (35 ml) was added dropwise over ½ hr to a solution of methyl 3-*O*-(β-methoxycarbonylpropionyl)-18β-oleanolate (2·6 g) in glacial acetic acid (23 ml). The mixture was then heated under reflux for 1·5 hr. Water (10 ml) was added and the solution left to cool. The white precipitate which formed was filtered off, washed with water and dried (2·1 g). The product was adsorbed on deactivated alumina (150 g) and eluted with light petroleum (b.p. 60–80°) –benzene–ether. The fractions eluted with benzene and ether showed one spot when examined by thin-layer chromatography; Rf, 0·63 (Rf for starting material, 0·74); λ_{max} 250 mμ; ν_{max} 1650cm^{-1}; the starting material did not absorb radiation at these frequencies. Recrystallization (three times) from methanol-water gave *methyl* 3-O-(*β-methoxycarbonylpropionyl*)-11-*oxo*-18*β-oleanolate* as needles, m.p. 193–193·5°; $[\alpha]_D$ + 66° ± 2° (*c*, 0·87 in chloroform); λ_{max} 250 mμ, (ε, 11,000) (Found: C, 71·9; H, 8·9; $C_{36}H_{54}O_7$ requires C, 72·2; H, 9·0%). This compound gave no reaction with a Zimmermann reagent (2% ethanolic solution of *m*-dinitrobenzene mixed with 3·5N potassium hydroxide (1:1 v/v) (Corker, Norymbeski & Thow, 1962).

Hydrolysis of methyl 3-O-(*β-methoxycarbonylpropionyl*)-11-*oxo*-18*β-oleanolate.* This diester was hydrolysed by the following method of Dean (1965). A solution of the ester (212 mg) in dimethylformamide (10 ml) was treated with lithium iodide (*ca.* 1 g) and the solution heated under reflux for 22 hr. The reaction mixture was cooled and poured into water. The precipitate which was formed was filtered off, washed with water and dried. Recrystallization from acetic acid gave 3-O-(*β-carboxypropionyl*)-11-*oxo*-18*β-oleanolic acid* (comp. 31) as plates, m.p. 257–8° (one spot when analysed by thin-layer chromatography).

3-O-(*β-Carboxypropionyl*)-9,11-*dehydro*-18*β-oleanolic acid* (comp. 32).

A solution of 3-*O*-(β-carboxypropionyl)-18β-oleanolic acid (1·7 g) in carbon tetrachloride (30 ml) and light petroleum (b.p. 60–80°) (3·6 ml) was treated with *N*-bromosuccinimide (0·84 g). The reaction mixture was irradiated with a 250 W lamp for 7 min. During this period the solution was allowed to reflux. The reaction mixture was rapidly cooled and the unchanged succinimide was filtered off. Xylene (30 ml) and pyridine (10 ml) were added to the filtrate and the solution was evaporated at 90° to remove low-boiling solvents. A further quantity of pyridine (5 ml) was added. The solution was heated under reflux for 20 min and then filtered. Evaporation of the solvents under reduced pressure gave a yellow solid. Recrystallization from aqueous ethanol gave plates, m.p. 238–244°; recrystallization from acetic acid-water gave needles, m.p. 256–7°, λ_{max} 274 mμ (ε, 11,000).

Discussion

In instances where acid chlorides were required as intermediates in a synthetic sequence and where the ultimate product would probably have been water-insoluble, the protection of the 3β-hydroxyl group was achieved using the methylated half esters of succinic acid; the methyl ester could be selectively hydrolysed using lithium iodide in dimethylformamide without loss of the resulting hemisuccinate esters. The latter had a far greater degree of water solubility than the parent alcohol.

This procedure was also used before the oxidation of oleanolic acid.

Acknowledgements. We are very grateful to Dr. S. Gottfried, Biorex Laboratories, for supplying glycyrrhetic and oleanolic acids as starting materials; also to Exeter College, Oxford and the city of Oxford Education Committee for personal support. This work was also supported by grants to the Department of Biochemistry from the Rockerfeller Foundation, New York and the Nuffield Foundation, London.

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J. Pharm. Pharmac., 1967, **19**, 690–693 Received June 6, 1967

The effect of α-methyldopa on excretion of noradrenaline metabolites

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The effect of α-methyldopa on the rate of release of noradrenaline metabolites in patients with essential hypertension has been examined. The excretion of normetadrenaline increases during the first few hr after a dose of α-methyldopa while α-methylnormetadrenaline can be detected in the urine 8–12 hr after the initial dose. The results support the idea that α-methyldopa displaces noradrenaline from storage sites, the noradrenaline in turn being replaced by α-methylnoradrenaline.

THE blood pressure of hypertensive patients may be lowered by (−)-α-methyldopa (Oates, Gillespie & others, 1960; Sjoerdsma & Udenfriend, 1961). Suggestions that have been put forward to explain its action are: (1) that it blocks the synthesis of noradrenaline; (2) that it depletes body stores of noradrenaline; (3) that it replaces noradrenaline at its storage sites with α-methylnoradrenaline.

The first suggestion was made by Sourkes (1954), who showed that large amounts of α-methyldopa inhibited dopa decarboxylase. This prevented the formation of noradrenaline by blocking the penultimate stage in its synthesis. Gillespie, Oates & others (1962) and Pletscher (1963) suggested that α-methyldopa has a reserpine-like action and depletes body stores of noradrenaline. Day & Rand (1963) proposed the third mechanism. They suggested that α-methylnoradrenaline, which is formed from α-methyldopa (Carlsson & Lindqvist, 1962), displaced noradrenaline from its storage sites at sympathetic nerve endings. Once held at the nerve endings it acted as a "false transmitter substance". When the nerves were stimulated they released α-methylnoradrenaline instead of the true transmitter, noradrenaline, but since α-methylnoradrenaline had a much smaller pressor activity than noradrenaline, the blood pressure fell.

It seemed likely that some information might be obtained about the action of α-methyldopa by examining its effect on the rate of release of noradrenaline. The rate of noradrenaline secretion cannot be measured by a simple direct method but it can be measured indirectly by measuring the output of its metabolites in the urine. The most abundant of these is 4-hydroxy-3-methoxymandelic acid. Unfortunately, this metabolite is also a metabolite of adrenaline. We therefore measured normetadrenaline, the 3-*O*-methyl derivative of noradrenaline.

We now report the effect of α-methyldopa on the rate of excretion of normetadrenaline in patients with essential hypertension.

Methods

The method (Stott & Robinson, 1966) measures total normetadrenaline (i.e. free and conjugated) with an error of ±10%. Our results are not corrected for recovery, which ranged from 60–75%.

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The subjects were 10 patients (4 men, 6 women) with essential hypertension, kept in bed for the duration of the experiments. Their bladders were first emptied and the urine discarded. Then a control specimen of urine was collected over a period of 2 hr. The patients then took 500 mg of (±)-α-methyldopa by mouth and were told to drink liberally. Further urine specimens were collected at successive 2 hr intervals for 6 to 12 hr after the drug was given.

A similar experiment was made on one healthy normal subject, to whom 250 mg of (±)-α-methyldopa was given intravenously.

The normetadrenaline content of all the urine specimens was measured. Some of the specimens collected after the drug was taken were examined by two-dimensional paper chromatography for phenolic acids and amines (Robinson, Ratcliffe & Smith, 1959; Robinson & Smith, 1962).

Blood pressures were measured every 15 min throughout the experiments.

Results

The patients' output of normetadrenaline consistently rose in the first few hr after taking α-methyldopa. The output reached a peak between 4 and 6 hr and usually fell to the control values between 8 and 12 hr after they had taken the drug. α-Methylnormetadrenaline appeared in the urine 8–12 hr after the drug had been given, the level varying between 0·04–0·6% of the dose.

Fig. 1 shows the normetadrenaline output in μg/min of one patient. Table 1 gives normetadrenaline values before and at intervals after a dose of α-methyldopa.

The blood pressures of most of the patients fell slightly during the first hr and then rose to a peak between 1 and 1¾ hr after the drug was taken.

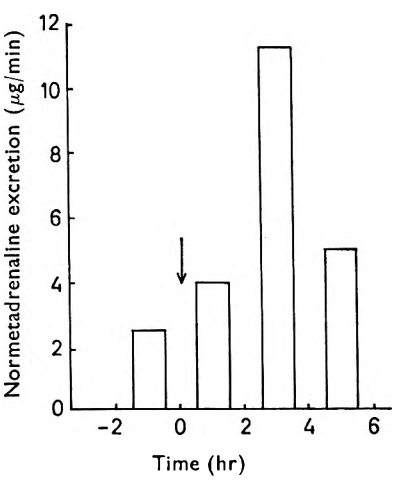

FIG. 1. Normetadrenaline excretion before and after taking 500 mg (±)-α-methyldopa. The drug was taken at the point marked by the arrow.

TABLE 1. NORMETADRENALINE VALUES (μG/MIN) BEFORE AND AFTER A DOSE OF α-METHYLDOPA

Patient No.	Before treatment	After treatment					
		2 hr	4 hr	6 hr	8 hr	10 hr	12 hr
1	0·48	1·1	9·9	0·96	1·4	0·8	—
2	1·1	3·7	4·5	3·2	3·2	3·1	3·2
3	0·49	1·1	2·3	2·4	1·0	—	—
4	0·96	2·0	2·2	0·8	1·7	—	—
5	1·3	1·0	1·6	0·6	—	1·0	0·3
6	2·7	1·9	2·9	4·2	4·2	3·5	—
7	1·6	—	2·7	0·9	1·4	—	—
8	2·2	4·5	11·7	6·7	3·1	—	—
*9	5·3	6·3	5·8	4·4	4·2	—	—
*10	5·0	7·6	4·5	5·9	5·0	—	—
Normal subject	1·4	2·2	2·9	4·3	2·1	—	—

* Patients Nos 9 and 10 subsequently were shown to have phaeochromocytomas.

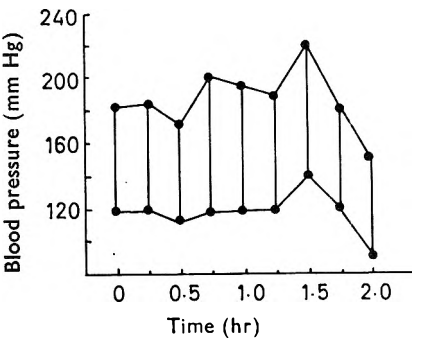

FIG. 2. Blood pressure changes after taking 500 mg (±)-α-methyldopa.

There was then a fall and 2 hr after the drug had been taken the blood pressures were lower than the control value (Fig. 2).

In the normal subject, the blood pressure did not change appreciably. Nevertheless, the output of normetadrenaline rose, reached a peak at 6 hr and then fell.

The output of phenolic acids was examined in one patient. The excretion of homovanillic acid fell strikingly. Two hr after taking the drug this metabolite formed about 50% of the control value and at 9 hr it was undetectable on the paper chromatogram. The urinary vanillic acid excretion was increased tenfold 9 hr after the patient had taken α-methyldopa.

Discussion

Our main finding was the rise in normetadrenaline excretion that occurred in the first few hr after α-methyldopa had been taken. If the main action of the drug is to inhibit dopa decarboxylase, a fall would have been expected rather than a rise. The finding that α-methyldopa causes an increased excretion of normetadrenaline could be explained in terms of a reserpine-like action. In addition, it is evident that the decarboxylation of dopa to dopamine was inhibited since the excretion of the dopamine metabolite, homovanillic acid, fell promptly and markedly.

It seems likely that this inhibition of the decarboxylation step is the result of substrate competition—the overwhelmingly greater amounts of α-methyldopa competing more successfully for the available dopa decarboxylase than the much smaller amounts of dopa. The prompt fall in homovanillic acid excretion suggests that α-methyldopamine and α-methylnoradrenaline are being formed equally promptly.

If the view of Day & Rand about the action of the drug is correct, it would be expected that the excretion of normetadrenaline would rise shortly after the drug was given, as noradrenaline was being displaced from its storage sites. Only after noradrenaline had been displaced would α-methylnoradrenaline start to be released.

Our results agree with this. The excretion of normetadrenaline rose in the first few hrs after the drug, but it was not until after this initial rise had subsided that α-methylnormetadrenaline began to appear in the urine.

During the period when the excretion of normetadrenaline was high, the patients' blood pressures were raised. This is consistent with there being a slightly increased circulating blood level of noradrenaline. It is interesting that during the same period the normal subject's blood pressure did not rise, though his excretion of normetadrenaline did. A possible explanation is that the blood pressure homeostatic mechanisms are more effective in normal persons than in hypertensive patients.

Gjessing (1964a,b; 1965) studied patients with periodic catatonia and found pronounced overactivity of the sympathetic nervous system during the psychotic stupor phase. He found that after treatment with α-methyldopa the excretion of α-methylnormetadrenaline followed the course of the disease in the same manner as did the excretion of normetadrenaline. Gjessing's results strongly suggest that after treatment with α-methyldopa, α-methylnoradrenaline is released when sympathetic nerves are stimulated.

Acknowledgements. We wish to thank the Research Sub-Committee of the Birmingham Regional Hospital Board for a grant which enabled this work to be done. We should also like to thank Dr. S. R. F. Whittaker for kindly allowing us to study patients under his care.

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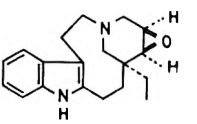
The isolation and identification of conoflorin from *Tabernaemontana chippii* Stapf. H. Huber

SIR,—We have isolated and identified an alkaloid from *T. chippii* (family Apocynaceae). The plant was collected from the Bibani district of Ashanti, Ghana, the leaves dried below 50° and the alkaloid extracted by percolation with ethanol. The extract was reduced to a small volume under reduced pressure and acidified with glacial acetic acid. The acid solution was then poured slowly into a large volume of very dilute acetic acid with vigorous stirring. The aqueous acidic layer was separated from the tars and the total bases precipitated by addition of ammonia in the presence of ice. Thin-layer and paper chromatography using a number of different solvent systems indicated that only one alkaloid was present. The pure alkaloid was obtained by chromatography on alumina using chloroform as eluant; recrystallization from ethanol (80%) gave colourless needles, m.p. 168–169°, $[\alpha]_D^{20} = +24°$ (in chloroform). Elemental analysis gave an empirical formula $C_{19}H_{24}N_2O$ which was confirmed and shown to be the molecular formula by mass spectrometry. The ultraviolet spectrum in absolute ethanol gave typical indolic-type absorption at λ_{max} 230 mμ (ϵ = 34,000), λ_{max} 285 mμ (ϵ = 9,300) and λ_{max} 293 mμ (ϵ = 7,120) and the infrared spectrum in Nujol showed a strong band at 3,340 cm^{-1} (indolic N – H stretching) but no bands indicating the presence of O–H or C=O groups, which suggests that the oxygen atom is present in an ether linkage. The mass spectrum of the alkaloid indicated a molecular ion at m/e 296, a base peak at m/e 140 and other significant peaks at m/e 267, 249, 211, 157, 156, 144 and 143.

The above data are in good agreement with those reported for the alkaloid conoflorin (I) whose isolation from *Conopharyngia longiflora* (Stapf.) and



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structural elucidation has recently been reported by Dugan, Hesse & others (1967). The proton magnetic resonance spectrum of conoflorin was subsequently found to be identical with that of the above alkaloid, therefore confirming its identity with conoflorin.

Acknowledgements. We are grateful to Dr. M. Hesse, University of Zurich, for supplying the proton magnetic resonance spectrum of conoflorin and to Mr. F. N. Hepper, Royal Botanic Garden, Kew, for botanical verification of plant material.

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Contraction of rat vas deferens by cocaine

SIR,—During studies on isolated tissues, designed to examine the effect of drugs which inhibit uptake mechanisms on the potency of sympathomimetic amines, we have attempted to use cocaine on the isolated rat vas deferens.

Vasa deferentia from young adult rats were split longitudinally, to facilitate washing of drugs from the tissue, and were suspended in a 10 ml bath of Krebs solution (containing 200 μg/ml ascorbic acid) aerated with oxygen 95% and carbon dioxide 5% at 29°. Isotonic contractions were recorded using a modified Statham 10B strain gauge.

In 8 experiments in which 10^{-5}M cocaine was left in the bath for 30 min before testing its effect on noradrenaline, adrenaline and phenylephrine, a small contraction to cocaine was observed. In another 7 experiments 10^{-4}M cocaine caused a pronounced contraction of the tissue. After doses of cocaine, spontaneous activity of the preparation made it difficult to obtain quantitative results. In some preparations spontaneous activity was troublesome with high doses of noradrenaline or adrenaline before cocaine was added, and was intensified by the addition of cocaine. Tachyphylaxis to the cocaine contraction occurred even up to 60 min after the addition of the first dose. If responses to noradrenaline were obtained between the doses of cocaine the contraction to cocaine was restored. The restoration or maintenance of the cocaine contraction was dependent on the dose of noradrenaline used.

Cocaine, 10^{-4}M, caused no initial contraction of 6 vasa taken from rats pretreated with reserpine (2 mg/kg on each of 2 days before the experiment). If responses to large doses of noradrenaline were then obtained, a contraction on the addition of cocaine was now observed. The size of the restored response depended on the amount of noradrenaline used. Spontaneous activity in all preparations from reserpinized animals was pronounced on the addition of cocaine. In both normal and reserpinized preparations, piperoxan (10^{-4}M), left in contact with the tissue for 15 min before the addition of cocaine, caused a complete block of the cocaine contraction.

The use of cocaine to inhibit uptake of amines into the adrenergic nerve terminals of the isolated rat vas deferens has not proved satisfactory since cocaine causes a contraction and initiates or exaggerates spontaneous activity. The contraction of the rat vas deferens by cocaine is probably due to an indirect sympathomimetic effect causing a release of noradrenaline. The contraction shows tachyphylaxis, does not occur in reserpinized preparations and is blocked by the α-adrenergic blocking drug, piperoxan. Sympathomimetic effects of cocaine have been reported by workers on other tissues (Teeters, Koppanyi & Cowan, 1963; Maengwyn-Davies & Koppanyi, 1966).

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In vitro assessment of anti-inflammatory activity

SIR,—Whitehouse & Skidmore (1965) have reported that many acidic anti-inflammatory drugs inhibit histidine decarboxylases *in vitro*, presumably by displacing the pyridoxal phosphate coenzyme from the apoenzyme, while analogues of these compounds with little or no anti-inflammatory activity do not inhibit these enzymes. At the same time, it was reported (Skidmore & Whitehouse, 1965) that these histidine decarboxylase inhibitors displaced pyridoxal phosphate from some of its binding sites on bovine plasma albumin, in a manner essentially parallel to their effect on histidine decarboxylases. These authors further found that the same drugs inhibited the binding of 2,4,6-trinitrobenzaldehyde to bovine plasma albumin. In view of the good correlation between clinical antirheumatic activity and inhibition of trinitrobenzaldehyde binding to albumin, it was suggested that the system might serve as an *in vitro* model for the assay of potential anti-inflammatory activity. The purpose of this report is to present findings based on extensive use of the system which suggest a poor correlation between *in vitro* and *in vivo* anti-inflammatory activity.

In vitro anti-inflammatory activity was determined by the trinitrobenzaldehyde assay described by Skidmore & Whitehouse (1965). *In vivo* anti-inflammatory activity was assayed using the carrageenan foot oedema method (Winter, Risley & Nuss, 1962) or the azovan blue-carrageenan pleural effusion method (Sancilio & Rodriguez, 1965) or both, at doses ranging from 75 to 316 mg/kg. Of 57 diverse organic acids screened *in vitro*, 42 (74%) gave results equivalent to or greater than acetylsalicylic acid. In most instances comparable activity could not be demonstrated *in vivo*; some representative results are summarized in Table 1.

TABLE 1. EFFECT OF VARIOUS ACIDIC COMPOUNDS IN *in vitro* AND *in vivo* ANTI-INFLAMMATORY ASSAYS

Compound	Anti-inflammatory assay		
		in vivo†	
	in vitro % inhibition*	Foot oedema	Pleural effusion
α,5-Diphenyl-2H-tetrazole-2-acetic acid	58·0	0	
m-[(α-Phenylphenacyl)amino]-benzoic acid	49·8	0	
m-[3-(2,4-Dioxo-1H,3H-quinazolinyl)]-benzoic acid	21·4	0	
5,5′-Selenobis-salicylic acid	76·7	0	0
3-Octylsalicylic acid	74·9		0
N-Acetyl-*p*-aminophenol	86·4	0‡	
3-*O*-(β-Carboxypropionyl)-11-oxo-18β-olean-12-en-30-oic acid	59·1		0
Acetylsalicylic acid	19·1	+	+

* Measured at 430 mμ.
† 0—inactive, +—active.
‡ Marginal activity observed at 400 mg/kg.

These results indicate that with a variety of acidic structures (including tetrazole alkanoic, substituted aminobenzoic, substituted benzoic, substituted salicylic, and triterpene acids, and a phenolic compound) significant *in vitro* anti-inflammatory activity does not correlate with *in vivo* anti-inflammatory activity. Thus, while the method may be useful in the preliminary evaluation of anti-inflammatory activity, since clinically active compounds are detected, many false positive results will arise from such a screen. The fact that clinically active compounds do interfere with trinitrobenzaldehyde binding to serum albumin, while certain closely related but clinically inactive compounds do not,

has been interpreted as suggesting a possible relation to the mechanism of anti-inflammation; however, these results suggest that any such relation is not specific.

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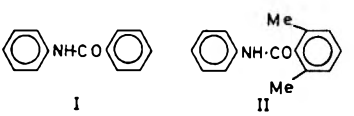
The influence of the stability of the amide link on the formation of methaemoglobin by anilides

SIR,—It is considered that the reactions involved in the formation of methaemoglobin by aromatic amides are (*a*) hydrolysis of the amide, (*b*) metabolism of the amine produced to the appropriate species, and (*c*) oxidation of the haemoglobin by the amine metabolite (McLean, Murphy & others, 1967). The stability of the amide link in anilides may be modified by (*a*) substitution on the aromatic ring, and (*b*) substitution on the acyl group. McLean & others (1967) examined a wide range of derivatives of acetanilide which were substituted on both the acyl and aromatic moieties and found no correlation between the stability of the amide group and the ability of the compounds to induce the formation of methaemoglobin in cats. They came to the conclusion that the nature of the amine formed by hydrolysis of the aromatic amide was of prime importance in determining the amount of methaemoglobin formed by the compounds. We wish to report an example in which the hydrolysis of an anilide is the rate determining step in the formation of methaemoglobin. It is well known that disubstitution in the 2,6-positions of an aromatic amide or ester confers considerable stability on the amide or ester group because of the so-called "*ortho*-effect". McLean & others (1967) attempted to determine the importance of the "*ortho*-effect" in controlling the ability of an aromatic amide to form methaemoglobin by examining a series of 2,6-dimethylanilides. These amides formed much less methaemoglobin than the corresponding unsubstituted anilides but the influence of the "*ortho*-effect" could not be assessed because 2,6-dimethylaniline was also a poor former of methaemoglobin. To obtain evidence that the rate of hydrolysis of an anilide can be the rate determining step in the formation of methaemoglobin, benzanilide (I) and 2′, 6′-dimethyl-



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benzanilide (II) have now been examined. The compounds were prepared by condensing the appropriate acid chloride with aniline. 2,6-Dimethylbenzoic acid was prepared by the method described by Thomas & Canty (1962) and converted to 2,6-dimethylbenzoyl chloride by treatment with thionyl chloride. Benzanilide was recrystallized from benzene-ethanol as white crystals, m.p.

163–164° (found: C, 78·8; H, 5·6; N, 7·0. $C_{13}H_{11}NO$ requires C, 79·1; H, 5·6; N, 7·1%). 2′,6′-Dimethylbenzanilide was recrystallized from ethyl acetate-ligroin as white needles m.p. 138·5° (found: C, 79·9; H, 6·4; N, 6·2. $C_{15}H_{15}NO$ requires C, 80·0; H, 6·7; N, 6·2%). The ability of the compounds to form methaemoglobin in cats was determined by the method described by McLean & others (1967). The results obtained, together with the results which had previously been obtained for acetanilide and aniline are given in Table 1.

TABLE 1. METHAEMOGLOBIN (% TOTAL HAEM PIGMENTS) FORMED IN CATS AFTER ADMINISTRATION OF ANILINE AND SOME ANILIDES

Compound	Dose	No. of cats	Mean % Met-Hb formed						Mean % Met-Hb formed over total time
			Time after admin. of compound (hr)						
			1	2	3	4	5	6	
Acetanilide	0·5 mmole/kg oral	5	38·0	66·9	73·5	78·1	76·6	78·5	55·6
Benzanilide	1 mmole/kg oral	5	17·8	41·4	43·1	33·4	27·1	20·5	30·6
2′,6′-Dimethylbenzanilide	1 mmole/kg oral	2	1·8	1·2	1·2	0·5	2·3	0·7	1·1
Aniline	0·25 mmole/kg i.v.	5	70·6	70·0	66·6	64·4	55·6	—	65·4

It can be seen that aniline (0·25 mmole/kg i.v.) and acetanilide (0·5 mmole/kg, oral) formed approximately the same amount of methaemoglobin, while benzanilide (1 mmole/kg, oral) formed about half as much, and 2′,6′-dimethylbenzanilide (1 mmole/kg, oral) formed virtually none. Since with all these compounds the methaemoglobin produced is related ultimately to the concentration of aniline present in body fluids, the conclusion to be drawn is that the slow rate of hydrolysis of 2′,6′-dimethylbenzanilide *in vivo*, and to a lesser extent of benzanilide, is the overall rate determining step in the formation of methaemoglobin by these compounds. The conclusion from these results considered along with the results of McLean & others (1967) is that it is possible to modify the ability of an aromatic amide to form methaemoglobin by retarding hydrolysis of the amide group but this is only manifest when profound changes are made in the reactivity of the amide link.

Acknowledgements. One of us (S.M.) wishes to thank the Pharmaceutical Society of N.S.W. S. E. Wright Research Trust for a research scholarship.

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Anaphylactic shock and the blood sugar level

SIR,—Dhar, Sanyal & West (1967) reported that the severity of anaphylactic shock in rats is much altered by varying the level of glucose in the blood. When hypoglycaemia has been induced before challenge, systemic shock is potentiated, whereas shock is delayed when hyperglycaemia is present. We have now found that the severity of systemic anaphylactic shock under these conditions does not always correspond with that of local cutaneous anaphylactic shock.

Groups of 8 Wistar albino rats, 100–150 g, were sensitized by an intraperitoneal injection of horse serum (1 ml) and challenged by an intravenous dose of the same amount of antigen 12 days later (Sanyal & West, 1958). In most of the experiments, *Bordetella pertussis* vaccine (0·25 ml of 80 × 10^9 organisms/ml) was also injected with the sensitizing dose of antigen. Hypoglycaemia was induced by injecting insulin (5 I.U./kg) intraperitoneally 30 min before challenge whilst experimental diabetes was produced by injecting alloxan (300 mg/kg) subcutaneously into fasted animals 4 days before challenge. Glucose hyperglycaemia was produced by intraperitoneal injections of 5 ml of a 25% (w/v) solution of glucose in water. Mortalities were recorded at 2 and 24 hr respectively after challenge. Other groups of sensitized rats were used to determine the severity at 3 hr after challenge either of intestinal haemorrhage or of the local cutaneous reaction resulting from the subcutaneous injection of antigen (0·1 ml) into one of the paws of rats sensitized 12 days previously and then injected with azovan blue dye (12 mg/kg, intravenously) 30 min before challenge. The intensities of both reactions were recorded on relative scales from 0 to +++. Blood sugar levels were determined using the Folin-Wu method.

The results are in Table 1. Anaphylactic shock without the aid of adjuvant

TABLE 1. EFFECT OF DIFFERENT PRETREATMENTS ON THE MORTALITY OF RATS AND THE EXTENT OF INTESTINAL HAEMORRHAGE AND LOCAL ANAPHYLACTIC REACTION (RECORDED ON A RELATIVE SCALE FROM 0 TO +++) AS A RESULT OF ANAPHYLACTIC SHOCK 12 DAYS AFTER SENSITIZATION WITH HORSE SERUM.
The blood sugar values (mg/100 ml ± s.e.) before challenge are also shown.

Bordetella pertussis vaccine	Pretreatment	Blood sugar	Mortality (out of 8 animals)		Intestinal haemorrhage	Local anaphylactic reaction
			2 hr	24 hr		
Absent	None	98 ± 4·6	0	0	0	++
Present	None	68 ± 10·4	8	8	+++	+++
Absent	Insulin	57 ± 11·0	8	8	+++	++
Present	Alloxan	283 ± 32·6	0	2	+	+++
Present	Glucose	409 ± 31·0	1	8	++	+++
Present	Alloxan + Insulin	101 ± 6·0	0	4	+	+++

was minimal and there were no deaths and no intestinal haemorrhage; nevertheless, the local cutaneous reaction (as measured by the extent and degree of blueing of the paw injected with the specific antigen) was marked. When adjuvant was present, the blood sugar level was statistically significantly reduced and anaphylactic shock was so severe that all 8 animals died within 2 hr with typical extensive haemorrhagic lesions in the intestines; the local response was also intense and greater than that recorded when *B. pertussis* vaccine was absent. Whereas there were no deaths in the group of 8 animals after anaphylactic shock when no vaccine was present, all 8 died within 2 hr when insulin was injected 30 min before challenge; blood sugar levels were about the same as those found

when the vaccine (but no insulin) was injected with the sensitizing dose, and they were significantly lower than the value of non-sensitized control rats (97 ± 6·8 mg per 100 ml). Typical extensive haemorrhagic lesions were found in the intestines of all of these animals although the intensity of the cutaneous reaction was not different from that found in control animals subjected to anaphylactic shock.

In experimental diabetes, the mean blood sugar level at the time of challenge was over 280 mg/100 ml and only mild anaphylactic shock developed; there were no deaths at 2 hr, but 2 out of 8 animals died overnight with minimal intestinal haemorrhage. Nevertheless, the local cutaneous anaphylactic reaction was intense, the raised blood sugar level failing to modify the severity of the reaction. Similarly, high doses of glucose delayed the time of death (although all 8 animals in the group died within 24 hr), reduced the severity of the haemorrhagic reaction in the intestines, and failed to reduce the local cutaneous response. When insulin was injected into animals made diabetic with alloxan and the blood sugar levels were not statistically significantly different from animals sensitized to the antigen without the aid of adjuvant, there were no deaths 2 hr after challenge and only half of the animals died by 24 hr. Intestinal haemorrhage was minimal and yet the local cutaneous anaphylactic reaction in the paw was intense.

The results show that, during anaphylactic shock in rats, hypoglycaemia greatly increases the severity of intestinal haemorrhage but does not always increase the intensity of the local cutaneous reaction; also, hyperglycaemia markedly reduces the systemic reaction and yet the local cutaneous reaction is not modified. Finally, when insulin is used to reduce the blood sugar level of diabetic rats to control values, systemic anaphylactic shock is reduced but the local cutaneous reaction remains at a maximum.

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An insertable orifice tube for *in situ* contamination counts of solutions in opened ampoules and vials using the Coulter Counter

SIR,—Attention has recently been focussed upon contamination by particles in injection solutions (Garvan & Gunner, 1963, 1964; Groves & Major, 1964; Groves, 1965, 1966; Vessey, Kendall & Peters, 1966; Vessey & Kendall, 1966). The Australian Department of Health has now (1966) issued a draft standard for the measurement of particulate matter in such solutions, using a Coulter Counter. To date little emphasis has been placed on the problems associated with the detection of contamination in glass ampoules and vials containing small volume solutions.

Large volume solutions can be transferred readily into the counting chamber by pouring, after adequate washing and rinsing of the chamber, with no increase in contamination level. The average count above 3·5 μ, and standard deviation, of twelve saline samples from a 1 litre Polyfusor container was 108 $\pm$ 11.

One well cleaned and dried 2 ml ampoule was sealed and opened in the normal way, and the fracture surface touched onto 6 ml of the "counted" saline; particles above 3·5 μ numbered 1460. Then the fracture surfaces from six cleaned ampoules were added to a fresh 6 ml of the saline when the count was 4690.

It is easily calculated that the surface of the one ampoule contributed approximately 8,100 particles above 3·5 μ and the six gave an average of about 4,850 each. Thus transfer of solution from ampoules by pouring or shaking into the counting chamber, or both, must increase contamination levels.

Transfer by a hypodermic syringe would be expected to prevent this. Commercially available disposable sterile syringes and well cleaned re-useable syringes were obtained, and filled to 2 ml 6 times from a supply of the counted Polyfusor saline. Each time the syringe was emptied into a well-cleaned and rinsed vial and the new contamination levels determined from the sum of two 0·5 ml sample counts above 3·5 μ. The numbers of particles above 3·5 μ for successive fillings for both types of syringe were 1043, 2073, 846, 723, 302, 741 for the reusable syringe and 270, 212, 209, 372, 370, 895 for the disposable syringe. Thus further filling and dispensing did not quickly reduce the new counts to the original "clean" level showing that additional contamination was continually coming from within the syringes, presumably by friction of the plunger and barrel.

To count small volumes of solutions of this sort, which cannot be easily transferred, a narrow version of the standard orifice tube which fits any Coulter Counter has been developed. This may be inserted directly into the ampoule itself. The present specification requires that the tube, with external electrode wrapped closely around it, should conveniently pass a 4·5 mm aperture. This is adequate for most British ampoules of 2 ml volume or more, and most American ampoules down to 1 ml or sometimes 0·5 ml. It has been verified experimentally that these narrow orifice tubes do not contribute to an increased noise level, and thus artificial counts, and do not reduce the performance of the Coulter Counter in any way. Care is necessary in using these tubes to avoid artificial counts from air bubbles; the tubes themselves are less robust than standard tubes. They have been manufactured with orifices of 50, 70 and 100 μ diameter to cover the needs in this field of study, that with a orifice of 100 μ being used in this present work.

The effect of transfer by a well cleaned re-useable syringe was again observed with solutions of drugs in 0·7% sodium chloride, from 2 ml and 5 ml all-glass ampoules, and compared with counts by the "direct" technique using the new orifice: transfer by syringe 710 $\pm$ 47; counted *in situ* 166 $\pm$ 10. The syringed

samples were again more contaminated. The direct technique gives a more meaningful result and seems therefore a significant advance in measuring true contamination levels in solutions in small ampoules. It is evident also that no major contamination is created by a glass shower entering the ampoule on fracture; such glass fragments as are present, however, would also be removed by the syringe.

All counts were made with a Coulter Counter Model B. This instrument has the advantage that changes in conductivity of the counting solution (e.g. the dissolved salt concentration) do not affect calibration over a wide range. Calibration was with Dow polyvinyltoluene latex (Dow Chemical Company, Midland, Michigan, U.S.A.) with a quoted mean diameter of 3·49 μ. Cleaning of equipment was by soaking for 24 hr in Decon 75 detergent solution (R. W. Jennings, Ltd., Nottingham), rinsing and ultrasonically vibrating in filtered de-ionized water (triply passed through 0·45 μ Millipore membranes) and rinsing in filtered saline (Polyfusor, with 108 ± 11 particles/ml above 3·5 μ). The equipment was shaken in clean air to remove surplus moisture, then used immediately. Drying, where necessary, was aided by rinsing in filtered acetone.

Acknowledgements. I thank Mr. D. Hoskins for the special orifice tubes, Mr. B Plumb for his assistance with the many particle counts, and Dr. M. J. Groves (Boots Pure Drug Co., Ltd.) for helpful comment and the provision of special ampoules.

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High Street South,
Dunstable, Beds.
June 8, 1967

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Book Review

THE UPTAKE AND STORAGE OF NORADRENALINE IN SYMPATHETIC NERVES. By Leslie L. Iversen. Pp. xiv + 253 (including Index). Cambridge University Press, London, 1967. U.K. 57s. 6d.; U.S.A. $11.00.

In the last decade, the application of new technical methods has uncovered much information about autonomic pharmacology. The use of electron microscopy and the widespread exploitation of histochemical methods, especially the catecholamine fluorescence method, has given a much clearer picture of the structures at nerve-effector cell junctions. Examination of sub-cellular components, separated by sucrose density-gradient centrifugation, has provided information on the biochemical properties of some of the structures revealed by the microscope. The more recent availability of radioactive-labelled drugs of high specific activity has enabled the techniques of autoradiography and scintillation counting to be profitably exploited in studying drug localization and distribution. The almost frenzied application of these new methods to the investigation of adrenergic mechanisms is responsible for the exciting new developments in the understanding of what happens during neuro-effector cell transmission in the sympathetic nervous system.

While enzymatic destruction of acetylcholine is still thought to be an important part of the process for terminating the action of the mediator of cholinergic nerves, in recent years it has been realized that enzymatic destruction of the mediator is not the major process terminating action at adrenergic nerve terminals. It is currently believed that removal of the noradrenaline from the synaptic biophase is effected by an active process, residing in the membrane of the nerve terminal, by which means the noradrenaline is taken up again into the intraneuronal storage pools.

New information has been accumulating rapidly and the timely appearance of this concise book, which summarizes existing methods and results and assesses critically the conclusions which may be drawn from them, is particularly welcome. Dr Leslie Iversen was invited by the Cambridge University Press to expand his PhD thesis into a monograph. The title belies the scope of the book; the author deals with more than just the uptake and storage of noradrenaline and in fact covers a good deal of what is currently known of adrenergic mechanisms (including the adrenal medulla). This is a bonus to the general reader and ensures that the new information can be appreciated in the context of less recent discoveries.

The book starts with brief descriptions of the methods which have been used to obtain the results referred to later. The methods for extraction and isolation of catecholamines from tissue and fluids, chromatographic separation, fluorimetric assay, histochemical techniques and the use of radioactive-labelled materials are all dealt with in turn and there is a useful list of references at the end of this chapter. In the next three chapters, Dr Iversen describes the discovery of the sympathetic neurotransmitter and the distribution, storage and metabolism of the catecholamines (he emphasizes the non-specificity of the enzyme normally known as dopa decarboxylase by indicating his preference for the term aromatic L-amino acid decarboxylase). There follows a chapter on the release of noradrenaline from adrenergic nerves which leads logically to Dr Iversen's main concern, and the aspect of the problems of adrenergic mechanisms to which his own researches have made fundamental contributions, namely the fate of the transmitter after release. Chapters 7 and 8 consider firstly the uptake and storage of catecholamines and then the mechanisms of action of drugs which interfere with or

modify these processes. Great care is taken to distinguish between storage and uptake and it is pointed out that the two terms are often, wrongly, used synonymously. In these chapters the author is on familiar ground and is largely describing his own discoveries and those of his collaborators, both here and in the United States.

It is apparent that, with few exceptions, most of the evidence has been obtained from isolated hearts of rats or rabbits (no doubt because of the ease with which these organs can be isolated and perfused through their blood vessels). It would now seem timely to discover whether the hypothesis is of general application by enquiring whether the findings apply to a wider range of sympathetically innervated organs.

The morphological and functional subdivisions of the distribution of noradrenaline within adrenergic nerves is one of the more speculative aspects of adrenergic mechanisms: here the interpretations are based much more on inference than on direct measurement and an honest re-appraisal of the situation, including the author's own scheme for noradrenaline storage pools, is presented in Chapter 9. The last chapter is the least satisfactory, being devoted to catecholamines in the central nervous system, a topic which could more profitably be presented in a monograph of its own, than in the space of twenty pages.

Dr Iversen's book is easy to read and is of a convenient size. It should now be in the possession of all research workers in the field, even those who followed the developments by reading the original papers. Others to whom the book can be recommended are students reading for degrees in pharmacology and physiology; they are fortunate to have such a concise monograph available to them.

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Journal of Pharmacy and Pharmacology

OCTOBER 1967 VOL. 19 No. 10

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Letters to the Editor

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