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REVIEW

Emulsion stabilization by non-ionic surfactants: experiment and theory*

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Two recent texts (Becher, 1965, Sherman, 1968) have dealt in some detail with emulsions, but little attention has been given to mechanisms of emulsion stabilization by non-ionic materials, in spite of the widespread industrial use of non-ionic surfactants. Emulsions stabilized by these non-ionic compounds present interesting problems not encountered in systems which contain ionic surfactants.

At first sight it would appear that stability cannot be explained by the electrostatic repulsive forces which operate in many dispersions, but it is known that pure hydrocarbon droplets dispersed in pure water possess a net negative charge. The problems which arise in the theory of non-ionic dispersions are these. To what extent do electrostatic forces contribute to stability? How is any charge on the dispersed particles affected by the presence of non-ionic detergents or polymer molecules at the interface? What is the nature of other stabilizing forces?

Lyklema (1968) recently discussed the principles of stability of colloidal dispersions in non-aqueous media. Much of what he has written is relevant here, although we will restrict our discussion mainly to oil-in-water emulsions. The outline of the theories of stability will thus be kept to a minimum.

It is the purpose of this review to consider relevant aspects of colloid stability theory and the relation of some recent experiments to this theory, and to discuss this with regard to the use of these emulsion systems. We will deal mainly with stabilizers which are derivatives of polyoxyethylene glycols.

ASPECTS OF EMULSION STABILITY

Emulsions have been defined as heterogeneous systems of one liquid dispersed in another in the form of droplets usually exceeding 0·1 μm (Becher, 1965) in diameter. The two liquids are immiscible, chemically unreactive, and form systems characterized by a minimal thermodynamic stability.

Unless the free energy of the oil-water interface is zero, an emulsion cannot be a thermodynamically stable system, since reduction of the area of oil in contact with the water will always result from coalescence of the droplets. "Stability" is therefore a relative term; but the degree of stability can be assessed by observing the rate of change of a parameter such as interfacial area or droplet diameter. Unstabilized emulsions, referred to as oil hydrosols by King (1941), coalesce rapidly, while stabilized emulsions can retain a highly dispersed internal phase for months or years. As the free energy of the interface is the driving force for coalescence, emulsions can be stabilized by the inclusion of a surface-active substance in the

* The first of two articles, the second of which will appear on p. 233 *et seq.* and includes all the references.

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system which concentrates at the oil-water interface. The role played by the surface-active material may be threefold, depending on the chemical nature of the adsorbing material and the adsorbent. The adsorbed surfactant molecules:

(i) can decrease the free energy of the system. Although at one time this effect was considered to be largely responsible for the stability, emulsions having the same interfacial free energy may have widely differing stabilities. However, statements can still be found ascribing stability loosely to interfacial tension (e.g. Richardson, 1953);

(ii) can form a barrier delaying the coalescence of the globules. This barrier represents a combination of steric, viscous and elastic properties (Schulman & Cockbain, 1940a,b; King, 1941; Cockbain & McRoberts, 1953; and Cockbain, 1956) depending on the emulgent;

(iii) may affect the electrostatic charge of the dispersed particles.

Instability

Emulsion instability is manifested in changes in the physical properties of the dispersion such as its droplet size distribution or its rheological properties (Richardson, 1953a) or other parameters which are a consequence of the coalescence of globules or their flocculation, that is, of the alteration in the real or effective mean globule diameter respectively. *Flocculation*, which is often the precursor of coalescence can affect the appearance of both liquid and solid emulsions. It accelerates the rate of *creaming* or *settling* which in itself is regarded as a form of instability.

Inversion of emulsion type is rare although localized inversion may occur through interaction of the components of the systems with packaging materials. This type of instability is dealt with later in relation to phase volume and emulsifier type.

The problem is to prevent instability, not only to maintain the appearance of the emulsion, but so that the characteristics of the emulsion and of medicaments dissolved in the emulsion are as little changed on ageing as possible. As an example, ageing might alter the absorption of heparin from o/w emulsions where absorption of heparin appears to be directly related to the particle size and total surface area of the oil droplets (Engel & Riggi, 1969). Fat emulsions are used extensively in intravenous feeding (Geyer, 1960), but the state of the art is exemplified by the statement (Todays Drugs, 1970) that "the emulsions must be stored in a refrigerator and no antibiotics, vitamins or potassium supplements added because they may break the emulsions". Lynn (1970) reports some experiments on the addition of disodium carbenicillin and sodium cloxacillin to intravenous lipid emulsions which verify this statement.

The preservation of emulsions by lyophilization and later reconstitution (Richter & Steiger-Trippi, 1960; Lladser, Medrano & Arancibia, 1968) is not yet a sufficiently advanced technique for widespread use, hence we must consider those factors which are responsible for stability and instability in the prepared emulsion.

Macromolecules as stabilizers

The tough "skins" formed at the interface between oil and aqueous solutions of macromolecular substances have been known for many years. Qualitative experiments on emulsions stabilized by this class of emulsifier have been made by Serrallach & Jones (1931) and Serrallach, Jones & Owen (1933). These compounds, many of which are naturally-occurring, include cellulose derivatives, gums and proteins. Many synthetic polymers exhibit similar properties, including polyvinyl alcohols, ethers, pyrrolidones, salts of the alkyd resins and many others. Characterized by high molecular weights (50 000–500 000), they are frequently employed as viscosity builders

in emulsions, thereby decreasing the creaming rate and the number of collisions between the dispersed particles i.e. reducing orthokinetic flocculation). Few reports have been published on the nature of the interfacial film which presumably forms a mechanical barrier to coalescence in these emulsions (Mussellwhite, 1964; Hamill & Petersen, 1966; El'-Shimi & Izmailova, 1967; Petersen & Hamill, 1968) although thorough studies in this respect have been made by Shotton & co-workers (e.g. Shotton, Wibberley & Vaziri, 1964). Films of alginates and gelatin on emulsified liquid paraffin droplets were estimated to be about 0·25 μm thick.

In systems of light petroleum stabilized by bovine serum albumin, a direct correlation between emulsion stability and the viscoelastic properties of protein films was observed by Biswas & Hayden (1960, 1962). In a study of monolayers of bovine plasma albumin at the benzene-solution interface, Davies & Mayers (1960) were able to measure substantial interfacial viscosities. It should be pointed out, however, that in any consideration of interfacial viscosity, the magnitude of the surface rheological parameters is not a measure of the overall stability of the droplets but only an indication that some stability will be present (van der Waarden, 1958). The nature of the film is of more import than its high viscosity. For example, rigid films of hexadecanol do not produce stable emulsions of chlorobenzene (Elworthy, Florence & Rogers, 1971b). Sonntag, Netzel & Unterberger have recently confirmed this view in their statement (1970) that "in monomolecular adsorbed layers of surfactants agreement between coalescence stability on the one hand and the mechanical properties, measured parallel with the interface, on the other, would occur only in a few selected instances."

The presence of rigid interfacial films can be demonstrated by withdrawing aged pendant drops of disperse phase into a syringe. Crinkled films appear because of the reduction of surface area in contact with solution (Shotton & White, 1963; Strassner, 1968).

The questions to be posed in relation to viscous surface layers are: to what extent is the viscosity of the film itself contributing to stability and is any increased stability due to the fact that molecules of stabilizer are prevented from escape from the surface layer? It can be calculated that the concentration of the surface active components used by Schulman & Cockbain (1940) to produce their emulsions was always more than that required to form a monolayer. The excess forms, in many cases, viscous gels in the aqueous phase, hence placing doubt about the major role played by the viscosity of the interfacial film (Talman, Davies & Rowan, 1967).

Non-ionic surfactants as stabilizers

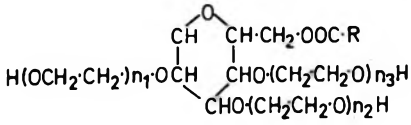
Non-ionic surfactants have many advantages over ionic surfactants as emulsifiers. In general they are less toxic and less sensitive to electrolytes and pH variations. Emulsions prepared with Triton X-400 were stable over the pH range 2·5 to 11 and exhibited stability and compatibility with many substances used in dermatology (Casadio, 1951).

In recent years the use of commercial heterogeneous and pure synthetic non-ionic detergents in surface-chemical studies has been widespread. The compounds most widely studied have been polyoxyethylene adducts of alcohols and esters. The former may be represented by the general chemical formula I.

$$Me[CH_2]_m\cdot[O\cdot CH_2\cdot CH_2]_n\cdot OH \qquad \text{I}$$

(Non-ionic surfactants having this general formula will be abbreviated to $C_{m+1}E_n$ where n is the number of ethylene oxide units in the molecule.) Synthetic homogeneous detergents of different chain lengths of the hydrocarbon portion or polar

portion may be prepared (Mulley, 1968). One widely used commercial series of non-ionic esters (Tweens or polysorbates) is represented by the chemical formula II.



$n_1 + n_2 + n_3 = n$

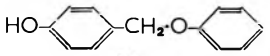
II

The great advantages of non-ionics are their lack of strong electrical charges and the ease by which their degree of hydrophilicity can be altered by increasing or decreasing the length of either the non-polar or polar chains of the molecule. The properties of non-ionic surfactants in solution have been reviewed (Shinoda, Nakagawa & others, 1963; Elworthy & Macfarlane, 1965; Schick, 1967) and their utility as emulsifying agents well documented (Becher, 1965; Sherman, 1968). However, the problem still remains of exactly how they function as emulsifying agents, and how far they are stable in the presence of electrolytes and other additives.

Polyoxyethylated non-ionics owe their solubility in water to hydrogen-bonding of solvent molecules to the ether oxygens of the hydrophilic chains. Thus, in micelles in aqueous solution and at the oil-water interface, the polyoxyethylene chains are extended outwards into the aqueous medium, while the hydrocarbon chains are orientated in the non-polar environment. It has been a matter for debate whether non-ionics make any contribution to the electrical atmosphere around dispersed emulsion globules (Becher, 1962). It was concluded from a study of the coalescence of mercury droplets stabilized with a series of non-ionic co-polymers of ethylene oxide and propylene oxide that electrostatic repulsion can be neglected in comparison with the stability imposed by the physical presence of the polymers (Watanabe, Matsumoto & Gotoh, 1965). Similar conclusions were reached from studies on water-in-benzene emulsions (Albers & Overbeek, 1959a,b). The character of the adsorbed surface film of emulsifier is regarded as being of primary importance in determining emulsion stability (Mardles & de Waele, 1951). It has been postulated that the viscosity of the interfacial film is the foremost stabilizing factor (Davies & Mayers, 1960; Gold, 1962; Sonntag & Klare, 1963; Isemura & Kimura, 1952). Some workers have suggested that film elasticity may play a role (Prins & van den Tempel, 1964; van den Tempel, Lucassen & Lucassen-Reynders, 1965). Both of these properties are dependent on the length of the polyoxyethylene chain and on the orientation of the surfactant molecule at the interface. In addition, the helical conformations of the hydrophilic chains probably function by hindering the close approach of emulsion droplets, thus preventing coalescence.

Although non-ionic surfactants are often chosen for emulsified systems because of their relatively low toxicity, several problems in formulation have arisen with their use. Most emulsions of edible fats and oils must be preserved against microbial attack. The most effective preservatives have often been the *p*-hydroxybenzoic acids. Unfortunately, these acids are inactivated by interaction with polyoxyethylene compounds in the system, and, in addition, changes in emulsion stability have occurred. The formation of complexes between phenolic compounds and ethylene oxide condensates has been frequently demonstrated (Higuchi & Lach, 1954; Guttman & Higuchi, 1956). Complex formation reduces the solubility of the polyoxyethylene non-ionic surfactants and renders them less effective as emulsifiers. Nevertheless an example has been quoted (Casadio, 1951) where the increase in viscosity caused

by the addition of a phenol (III) to emulsions stabilized by a polyoxyethylene non-ionic increased the resistance of the emulsion to separation by centrifugation.



III

Over the years, liquid emulsions have been a useful dosage form in pharmaceutical practice, because of their ability to incorporate both oil- and water-soluble components, although the emulsion has not been a modern pharmaceutical favourite. However, there have been signs of renewed interest in the possibilities they offer as dosage forms. For example, a recent communication has described the use of a w/o/w multiple emulsion as a delayed release system for chlorpromazine (Collings & Schneider, 1970). The problems associated with stability have frequently imposed restrictions on the use of emulsions as vehicles for all types of drugs.

The special problems of stabilization of aerosol emulsions and foams has been reviewed by Sanders (1970).

ASPECTS OF THE PHYSICAL CHEMISTRY OF NON-IONIC SURFACTANTS RELEVANT TO DISPERSION STABILIZATION

Non-ionic surfactants adsorb at interfaces, and above certain concentrations form micelles in aqueous solutions and in some non-aqueous solvents. The physical chemistry is discussed here briefly, with special emphasis on those properties which are likely to be of importance in emulsion stability.

Micelles of non-ionic surfactants of the polyoxyethylene ether class have a structure which is diagrammatically represented in Fig. 1. It is possible that some of the

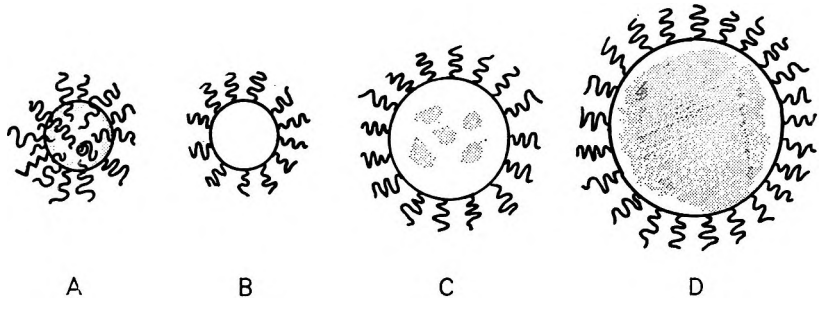


FIG. 1. A. Diagrammatic representation of a non-ionic micelle showing the inner hydrocarbon core and outer hydrated polyoxyethylated layer. B. Cross section of a micelle. C. A solubilizate laden micelle with solubilizate (stippled) in hydrocarbon region and D, a small emulsion globule

data reported on such structures can be used in the interpretation of the behaviour of non-ionics at emulsion interfaces. There is no obvious distinction between a micelle containing a large amount of solubilizate and a small globule of an emulsion (Fig. 1).

It is likely that the hydration of the surfactant at the oil-water interface is at least as high as that of the dispersed molecules because the area/molecule at the oil-water interface is greater than at the air-water interface. With regard to charge effects in dispersed systems, the presence of a net positive charge on non-ionic micelles due to oxonium ion formation has been argued by Becher (1962). Schick (1963) also

concluded from studies on surface films that the ether oxygens in the ethylene oxide adducts form oxonium ions, and Hsaio, Dunning & Lorenz (1956) also attribute the weak positive charges associated with the micelles of polyoxyethylated detergents to possible oxonium ion formation.

While adsorption of non-ionic polyethers on negatively charged particles reduces the negative charge on the particles (Glazman, 1966; Elworthy & Florence, 1967), adsorption on positively charged silver iodide sols results in charge reversal (Glazman & Kabysh, 1969). It is unlikely that positively charged chains could accomplish this sign change. However, as all oil droplets in aqueous media initially possess a *negative* charge it is reasonable to assume that the former behaviour is typical of emulsion systems.

The addition of electrolytes to non-ionic stabilized emulsions can, perhaps surprisingly, cause pronounced effects on stability (Elworthy, Florence & Rogers, 1971a). In solutions of non-ionic surfactants, generally the addition of electrolytes causes a dehydration of the ethylene oxide chains (Doscher, Myers & Atkins, 1951; Greenwald & Brown, 1954; Bailey & Callard, 1959) by disruption of hydrogen bonds (Stewart, 1943; Schick, 1962). Selected salts have been shown to exhibit interaction with polyethylene oxide ethers, reducing their solvation and producing more compact molecular conformations (Lundberg, Bailey & Callard, 1966; Hammes & Swann, 1967). The interaction appears to be more pronounced in non-aqueous solvents. The mechanism involved is unknown but has been suggested to arise from an ion-dipole interaction (Lundberg, Bailey & Callard, 1966).

The effect of salts on the interfacial tension of solutions of cetomacrogol 1000 and other detergents has been studied by Wan & Poon (1969).

Conformation of the polyoxyethylene chain

The conformation of lower molecular weight polyoxyethylene polymers has been termed "zig-zag" by Standinger (1932) and Ellis (1935), whereas higher polymers exist in the "meandering" form (synonymous with the fully extended "*trans*" form and the more condensed "*gauche*" form of Rosch (1956, 1957). Viscosity measurements led Lovell & Hibbert (1940) to the conclusion that the long polyoxyethylene chain is highly convoluted in aqueous solution. Dipole moment measurements of polyethylene glycols (PEG) in dioxan indicated a progressive increase in convolution with increase in the degree of polymerization. Since this would not infer a helical chain structure, a free rotation model of a random coil was considered (Uchida, Kurita & others, 1956), leading to the suggestion of the existence of mixtures of both the "*trans*" and "*gauche*" conformations. In aqueous solution, the "*gauche*" form probably predominates. Electrolytes reduce the configurational entropy of the ethylene oxide chains and increase the aggregation number in micelles of polyoxyethylene surfactants (Schick, Atlas & Eirich, 1962). That is, the "salting out" effect of the electrolyte causes a collapse of the chains. Such changes in conformation might have relevance in the effectiveness of non-ionics in stabilized dispersions in the presence of salts.

The conformation of polyoxyethylene chains of non-ionic detergents has been postulated from their areas per molecule calculated from surface tension measurements by application of Gibbs' equation. A series of polyoxyethylene derivatives of hexadecyl alcohol at the solution-air interface occupied areas which were much larger than the cross-sectional area of a hydrocarbon chain (0·21 nm^2) (Elworthy & Macfarlane, 1962) and the areas were less than the corresponding areas at the oil-water interface (Elworthy & Florence, 1969a). Examples of areas per molecule and micellar radii of some members of this series are listed in Table 1, the latter to show the non-extended nature of the molecules. The areas per molecule increase

Table 1. *Areas per molecule and micellar radii of polyoxyethylene glycol hexadecyl ethers.*

Oil phase	Detergent	Area/molecule (nm²) at oil-water interface	Area/molecule (nm²) at air-water interface	Micelle radius (nm)
Anisole	$C_{16}E_3$	0·45	0·31	3·2
	$C_{16}E_6$	0·56	0·38	∠·0
	$C_{16}E_9$	0·76	0·53	∠·6
	$C_{16}E_{25}$*	1·54	1·20	∠·8
Chlorobenzene ..	$C_{16}E_3$	0·48	0·31	3·2
	$C_{16}E_6$	0·80	0·38	∠·0
	$C_{16}E_9$	1·00	0·53	∠·6
	$C_{16}E_{25}$*	1·84	1·20	∠·8

* Cetomacrogol 1000. Data from Elworthy & Florence (1967, 1969a,b).

with increase in length of the hydrophilic chain, but the areas per ethylene oxide unit decrease, indicating a curled chain lying almost perpendicular to the interface. A similar conclusion was reached by Schick (1963). Thus the cross-sectional area of the solvated polyoxyethylene chain is the controlling factor in the packing of the molecules in the interface. Conditions which lead to a reduction in the cross-sectional area should lead to tighter packing of the molecules, resulting eventually in a very coherent, impervious film. However, a very close packed film with hindered hydrophilic chains would be unable to confer entropic stabilization to the system as interpenetration of the chains on neighbouring particles would then be impossible. This might partially explain the greater effect of long chain PEG compounds in stabilizing dispersions (see later).

There is a marked difference in the behaviour of non-ionic surfactants at polar and at non-polar oil-water interfaces. This can be demonstrated by the interfacial tension results obtained by Becher and shown in Fig. 2. The positions of non-ionic

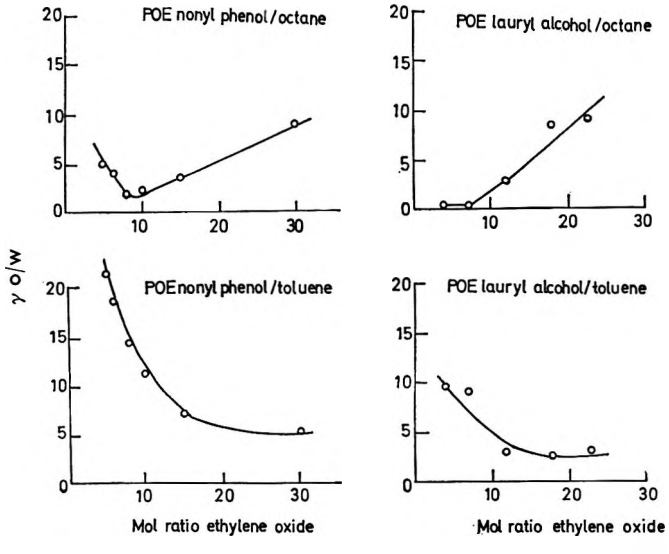


FIG. 2. Interfacial tension of lauryl alcohol ethers and nonyl phenol ethers at 0·1% concentrations against a non-polar oil (octane) and polar oil (toluene) showing the differences in behaviour as a function of ethylene oxide chain length. Reproduced from Becher (1963) by permission of Academic Press.

surface-active agents at oil-water interfaces have been estimated by Becher (1963) on consideration of the hydrogen-bonding contribution of these molcules to the net interfacial free energy. Knowledge of the effect of ethylene oxide chain length on the interfacial tension at aromatic hydrocarbon-water interfaces led to the assumption that a portion of the polyoxyethylene chain was immersed in the oil phase with the hydrocarbon chain. Fig. 3 illustrates the relative positions of the molecules. Thus,

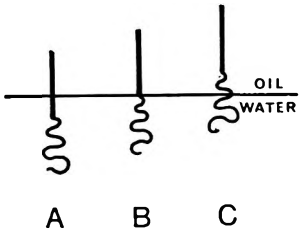


FIG. 3. Possible orientations of a non-ionic surfactant at an oil-water interface, after Becher (1963). For explanations of A, B and C see text.

a degree of hydrogen-bonding with aromatic hydrocarbons diminishes the dispersion force contribution of the oil to the interfacial free energy, resulting in higher interfacial tensions. This occurs with short ethylene oxide chains (model C in Fig. 3). With increasing ethylene oxide chain lengths, hydrogen-bonding with the aqueous phase becomes more pronounced and the molecule shifts in its direction (model B or A). With aliphatic hydrocarbons, only positions A or B would be expected. Presumably, the solvation layer surrounding emulsified oil globules would increase as the polyoxyethylene chains protrude further into the aqueous medium, and the consequences of this could explain the increase in stability of these systems with non-ionics of increasing hydrophilic chain length from E_3 to E_{25} (Elworthy & Florence, 1969a).

The importance of the hydrate layer in maintaining stability is indicated by Levi & Smirnov (1959) who showed that monoglycerides became efficient emulsifiers only when they possessed sufficient hydroxyl groups. These authors also noted that the introduction of ionizable carboxylic acid groups into the non-ionic stabilizer molecule containing many hydroxyl groups greatly increased its emulsifying properties. The introduction of ionic groups is not always beneficial, nor the explanation of their action simple, as work in this laboratory (Attwood & Florence, 1971) has shown. The addition of a sulphate group into cetomacrogol gives

$$(C_{16}H_{33}\cdot[O\cdot CH_2\cdot CH_2]_{23}\cdot OSO_3^-Na^+),$$

an emulsifier with decreased emulsifying power in chlorobenzene-in-water emulsions compared with the non-ionic parent compound, in spite of higher zeta potentials. The explanation of this behaviour is still being sought.

The differences in interfacial tension at polar and non-polar interfaces will partly explain the difference in stability of chlorobenzene and hexadecane emulsions stabilized by $C_{16}E_3$. Hexadecane in water emulsions are much more stable than chlorobenzene or anisole emulsions stabilized by the same detergent (Florence, unpublished) as shown by the results presented in Fig. 4.

Interaction forces between dispersed particles

The theory of stability of lyophobic colloids published by Derjaguin & Landau (1941) and by Verwey & Overbeek (1948) (the "DLVO theory") is still the most successful treatment of stability. The theory was developed primarily to deal with

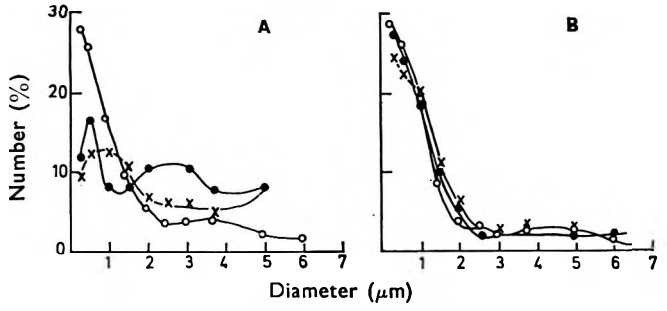


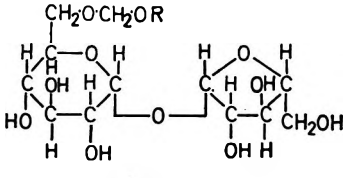
FIG. 4. Globule size distribution plots showing the greater stability of hexadecane emulsions (B) (phase volume 0·2) stabilized with 1% $C_{16}E_3$, compared with an anisole emulsion (A) of identical phasevolume with the same concentration of the same surfactant. Values at ○, 0 h; ●, 2 days; X, 15 days in both cases.

the stability of inorganic sols, which possess intrinsic electrostatic charges, but over the years, the theory and modifications of it, have been applied to suspensions of clays and to emulsions. In general, the theory predicts the energy requirements of the system which will lead to stability against flocculation or coagulation of the particles—this step being the final one for solid dispersions in a liquid medium but the initial stage of instability in emulsions, as flocculation can occur without coalescence. However, as the average proximity of the particles will be governed by the forces discussed by the theory, the barrier to flocculation should give an indication of the need for the barrier to coalescence to be an effective one.

Particles of all types possess a net electrostatic charge when suspended in a simple aqueous medium. The origin and magnitude of this charge is dependent on the nature of the medium, the nature of the particle surfaces and the presence or absence of other components in the system.

The DLVO theory of stability takes into account the interaction of two kinds of long-range forces which determine the closeness of contact of two particles approaching as a result of Brownian movement. The forces concerned are (1) the London-van der Waals' forces of attraction, and (2) the electrostatic repulsion between electrical double layers.

Since the origin of the one force is completely independent of the other, each force may be evaluated separately and the net result of their interaction obtained by summation. However, in addition to these forces of interaction, a free energy of interaction force arising from a third source: steric hindrance, involving the free energy of mixing solvated adsorbed layers, becomes of importance in systems containing non-ionic surfactants. This is particularly true for the polyoxyethylene type of non-ionic surfactants; to a lesser extent true for alkoxymethyl ethers of sucrose (IV) in which the hydrophilic chain is neither long nor very flexible.



IV

The repulsion between two particles originating in this manner has been termed entropic repulsion because of the loss of configurational entropy of the adsorbed molecules on mixing. This entropy loss is manifested as a repulsive force. This force has far greater importance in emulsion systems than in conventional lyophobic

colloids because of its short range nature, which means it is usually operative in already flocculated or coagulated systems. However, conclusions about the stability of disperse systems may be reached only if the short and the long range forces affecting the interaction of the particles are assessed. The DLVO theory can be applied without difficulty only to monodisperse particles and therefore, its application to systems having wide particle size distributions is limited. Attempts have been made by Hogg, Healy & Fuerstenau (1966) and Ho & Higuchi (1968) to assess the effect of heterodispersity.

The London-van der Waals' force of attraction

The attractive forces which exist between like molecules in a vacuum have been quantitatively identified by London (1930). The relation for a pair of equal spheres of finite particles was derived by Hamaker (1937). For values of $H/a \ll 1$ the relation for two spherical particles of radius "a" can be reduced to

$$V_A = -\frac{Aa}{12H} \qquad (1)$$

Where the distance of separation is H. A is the Hamaker constant in ergs (10^{-7} J).

When the particles "1" are not in a vacuum but are embedded in a medium of substance "2", the total interaction of two particles is dependent on the net interactions of all molecules. Hamaker deduced this interaction to be

$$A = A_{11} + A_{22} - 2A_{12} \qquad (2)$$

where $A_{22} = \pi^2 q_2^2 \beta_2$ for material "2" and $A_{12} = \pi^2 q_1 q_2 \beta_{12}$ (q is the number of molecules per cm^3 of material and β is related to the square of the polarizability) for the corresponding interaction between materials "1" and "2". If it is assumed that A_{12} can be taken as the geometric mean of A_{11} and A_{22}, then

$$A = (A_{11}^{\frac{1}{2}} - A_{22}^{\frac{1}{2}})^2 \qquad (3)$$

Thus, A is always positive. Consequently, emulsion droplets are always subject to an attraction, which has the same value for droplets in a particular emulsion whether the dispersion is o/w or w/o. Values of the Hamaker constant differ for different pairs of liquids, ranging from 1×10^{-20} J for paraffin-in-water to 2×10^{-19} J for carbon tetrachloride-water. The accuracy of estimating A is low, being often of an order of magnitude (Kitchener & Musselwhite, 1968; Gregory, 1970).

In systems of dispersed particles containing adsorbed layers of surface-active substances, the effective Hamaker constant between the particle and the medium may be radically altered. If the Hamaker constant of the solvation sheath is close to that of the dispersion medium, the sheath simply acts as a mechanical barrier preventing the close approach of the dispersed particles in the range where attractive forces become strong. This layer would then contribute to stability.

Vold (1961) has analysed the effect of adsorption on the attraction of spherical colloidal particles of radius a. In the case of a *homogeneous* adsorbed layer of thickness δ, having a Hamaker constant, A_{33}, the potential energy of attraction is given by (Ottewill, 1967, pp. 646–648):

$$V_A = -\frac{1}{12}\left[(A_{22}^{\frac{1}{2}} - A_{33}^{\frac{1}{2}})^2 \left(\frac{a+\delta}{\Delta}\right) + (A_{33}^{\frac{1}{2}} - A_{11}^{\frac{1}{2}})^2 \left(\frac{a}{\Delta + 2\delta}\right) + \frac{4a\,(A_{22}^{\frac{1}{2}} - A_{33}^{\frac{1}{2}})\,(A_{33}^{\frac{1}{2}} - A_{11}^{\frac{1}{2}})\,(a+\delta)}{(\Delta+\delta)\,(2a+\delta)}\right] \qquad (4)$$

where Δ is the distance between the surfaces of the adsorbed layers.

Using Vold's equation and estimating A_{33} from refractive index measurements, Elworthy & Florence (1969c) obtained a result which is surprising in the light of Ottewill's calculations: the adsorbed layer *increased* the attraction between chlorobenzene particles dispersed in water. Both equation and experimental derivation of A_{33} assumed that the surfactant layer is homogeneous. This is an over simplification. However, even if the magnitude of the effect is not correct, the *trend* is.

Sonntag (1968) has given equations for V_A for oil-in-water and water-in-oil emulsions based on Vold's equation, as V_A is not the same in the presence of an adsorbed layer in the two types of emulsion.

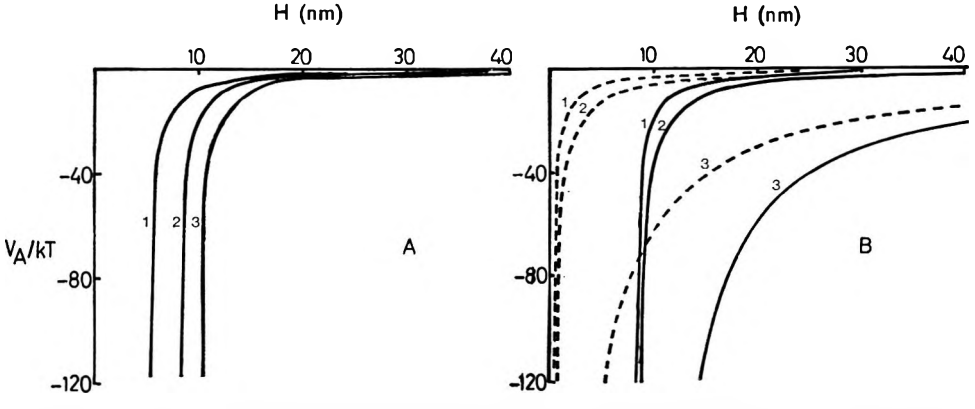


FIG. 5A. The influence of the thickness (δ) of an adsorbed layer of $C_{16}E_6$ and the attraction between two chlorobenzene droplets for a particle of size $a = 0{\cdot}5 \times 10^{-4}$ cm. 1. $\delta = 2{\cdot}5$ nm. 2. $\delta = 4{\cdot}0$ nm. 3. $\delta = 5{\cdot}0$ nm.

B. Influence of particles size "a" on the attraction between two chlorobenzene droplets: - - - uncoated; ——— coated with a layer of $C_{16}E_6$ with $\delta = 4{\cdot}0$ nm. 1. $a = 0{\cdot}5 \times 10^{-4}$ cm. 2. $a = 1 \times 10^{-4}$ cm. 3. $a = 10 \times 10^{-4}$ cm.

Fig. 5 illustrates the effect of different thicknesses of adsorbed layers of a polyoxyethylene ether $C_{16}H_{33}{\cdot}[O{\cdot}CH_2{\cdot}CH_2{\cdot}]_6OH$ ($C_{16}E_6$) on the calculated attraction energy between two chlorobenzene droplets 1 μm in diameter. As the thickness of the homogeneous layer of $C_{16}E_6$ increases, the attraction between the droplets increases. At a separation of 12·5 nm, the attraction energy is approximately doubled by increasing the layer thickness from 2·5–4·0 nm. A thickness of 5 nm results in a four-fold increase in the attractive forces. In this particular system, the Hamaker constant of the adsorbed layer was greater than that of the dispersed oil. ($A_{33} = 6{\cdot}7 \times 10^{-20}$ J, $A_{22} = 6{\cdot}3 \times 10^{-20}$ J, $A_{11} = 3{\cdot}78 \times 10^{-20}$ J.) Likewise, the attraction increases with increasing particle size for a given adsorbed layer thickness (Fig. 5) and a significant change is observed on the introduction of an adsorbed layer onto uncoated particles. It appears, therefore, that large particles will be more difficult to stabilize by adsorbed layers than small ones if only the effect on V_A is considered. In the development of the theory, Vold (1961) explains that a reduction in the interparticle attraction occurs when the density of the particle is greater than that of the medium, and the particle is coated with a weakly interacting adsorbed layer. Furthermore, it is pointed out that adsorbed layers on dispersed particles can never change the interparticle attraction into a repulsion. Derjaguin (1966) has doubted whether attenuation of attractive forces could contribute significantly to stability: this has been the conclusion of other work also (Elworthy & Florence, 1969c).

Repulsive forces arising from interaction of electrical double layers

In the DLVO theory, the distribution of counter-ions in the immediate vicinity of the charged colloidal particle is assumed to obey the Poisson-Boltzmann distribution.

The work required to bring together identical spherical particles from infinity to a distance of separation H in a liquid medium is given in an approximate form by (Derjaguin, 1939).

$$V_R = \frac{\epsilon a \psi_0^2}{2} \ln (1 + e^{-\kappa H}) \qquad (5)$$

This formula is valid only for low potentials (< 25 mV), for spheres that are large in radius compared to the thickness of the double layer (i.e. $\kappa a \geqslant 1$), and for a large separation between the spheres compared with double layer thickness (i.e. $H > 1/\kappa$). However, it can be used as an approximation for most practical systems, particularly emulsions.

In equation (5), the surface potential, ψ_0, is usually equated to ζ, the zeta potential as determined from measurements of electrophoretic mobility. The two quantities are by no means always identical, but in view of the experimental difficulties of assessing ψ_0, zeta potential may be used as a close approximation. ζ is the experimentally accessible potential difference between the bulk solution and the electrokinetic "slipping plane" or "plane of shear" which is situated in the diffuse layer close to the immobile Stern layer. (The meaning of these planes in molecular terms when a long chain hydrophilic non-ionic surfactant is adsorbed at the globule surface is not clear.)

In emulsions, as opposed to solid dispersions, the potential energy barrier to contact of two globules can increase if distortion of the globules occurs as a result of their mutual repulsion. The significance of this effect is not estimable in unstabilized emulsions. When adsorbed layers of surface-active material are present, it is less likely that distortion occurs on collision. Minute amounts of surfactant retard circulation in droplets because adsorption increases the interface viscosity. Trace amounts of surfactant cause the motion of small bubbles and drops through a liquid to resemble that of rigid bodies (Newitt, Dombrowski & Krelman, 1954).

Diffuse double layers extending into the disperse phase from the interface have been considered by Verwey (1939) but they do not appear to influence the external electrical layers to any significant extent. If the disperse phase is polar (e.g. water), then the concentration of counter-ions in the disperse phase would serve to reduce the net electrokinetic potential attributed to the particle.

The degree of stability of many dispersions cannot be explained solely on the basis of V_A and V_R. Elworthy & Florence (1969c) have treated the stability of emulsions of chlorobenzene and anisole stabilized with a series of synthetic polyoxyethylene ethers in light of colloid theory and have shown that electrical stabilization alone cannot explain the stability observed. The nature of this "other force" which is invoked to explain discrepancies between theory and experiment is not fully worked out. Nevertheless much interest has been shown in this alternative mechanism of the stabilization, which for "non-ionic" emulsions appears to play the major role (Elworthy & Florence, 1967). Results have indicated that the thickness and degree of solvation of adsorbed layers is critical (van der Waarden, 1950). Thus, the particular conformation and length of the polyoxyethylene chains of non-ionic surfactants at interfaces is likely to be an important factor in the stabilization of emulsified droplets.

The overall free energy change when the adsorbed layers on identical spherical particles mix on contact is assumed to arise from the additive contributions of several

energy changes. This free energy, being positive, results in repulsion between the particles because work is required to overcome these energy barriers.

If ΔG is the net free energy change, then

$$\Delta G = \Delta G_m + \Delta G_v + \Delta G_s + \Delta G_e^* \quad .. \quad .. \quad (6)$$

ΔG_m is the free energy change of mixing of the adsorbed layers which results in an increased concentration and density of chains in the overlap region. The increase of the chemical potential for a given polyoxyethylene chain in this region increases the polymer-solvent interactions. When the coated particles are separated by a distance less than 2δ, restrictions are imposed on the volume occupied per chain, governed by the particular conformations of the chains. This excluded volume effect is manifested as a free energy change, ΔG_v. Considerations of interacting adsorbed layers on emulsion globules must include the tendency towards desorption of the molecules at the interface as the compression due to overlapping increases. This tendency will cause an increase in the local interfacial tension which constitutes an increase of the surface free energy, ΔG_s. Collisions between emulsion particles do not always result in the formation of an aggregate or in coalescence. In fact, the dispersed droplets have been observed to exhibit a certain degree of elasticity on collision. This phenomenon may be considered as an elastic energy of repulsion, ΔG_e.

In order to assess the magnitude of each of these energy barriers to coagulation, calculations have frequently been made on models for which certain assumptions and simplifications have been introduced.

As Lyklema (1968) points out, the term "entropic" has been applied somewhat indiscriminately to the forces of repulsion arising from interaction of the adsorbed surfactant layers. This term arises because the long chains of the surfactant being restricted on contact suffer a loss of entropy and contribute to the positive free energy change, in addition to changes in solute-solvent interactions in the overlap region.

Steric or entropic repulsion

Mackor (1951), Mackor & van der Waals (1952), Clayfield & Lumb's (1966) and Meier's (1967) approaches have been discussed in some detail by Lyklema (1968). For this reason they are not treated here. Instead we concentrate on the theory of Fischer (1958) whose final equation contains parameters more amenable to substitution with experimental data than the others.

When adsorbed layers form on dispersed spherical particles, a region of high concentration of macromolecule extends outward from the particle surface into a region of lower concentration of macromolecule. The layer forms a gradient of polymer concentration which is a function of the length of the adsorbed chains, the degree of surface coverage and the conformation of the polymer chains in the environment. Consider two identical spherical particles (Fig. 6) having identical adsorbed layers of polymer chains colliding in the common medium and forming, between the particles, an overlap volume of their adsorbed layers, dV. The concentration of polymer chains is increased and one can express the excess chemical potential change of the chains in the overlap volume as the difference between the observed chemical potential change and the change expected under ideal conditions: (eqn 7). Using this approach, the stabilization lent to disperse systems by adsorbed

$$(\Delta\mu_1)_E = \Delta\mu - (\Delta\mu_1)_{ideal} \quad .. \quad .. \quad .. \quad (7)$$

* Whether these are independent quantities, and therefore additive is a debatable point.

layers was analysed by Fischer (1958). The increase in chemical potential as a result of this process generates an excess osmotic pressure arising from solvent flow to the region of high concentration. This pressure, π_E, is manifested as an energy of repulsion to mixing and its magnitude is determined by the partial molar volume of the solute, $\bar{V}_1$, the initial concentration of chains in the adsorbed layer, C, and the degree of polymer-solvent interaction, related to B, the second virial coefficient. Equation (7) may then be written

$$(\Delta\mu_1)_E = - RTB\bar{V}_1C^2 = - \pi_E\bar{V}_1 \quad .. \quad .. \quad .. \quad (8)$$

whence

$$\pi_E = RTBC^2 \quad .. \quad .. \quad .. \quad .. \quad (9)$$

The free energy of mixing in the volume element dV is related to C^2 by

$$dG_m = \frac{2dV}{\bar{V}_1} \cdot RTB\bar{V}_1C^2 = 2d\bar{V}RTBC^2 \quad .. \quad .. \quad (10)$$

for the volumes of each adsorbed layer such that $dV_1 = dV_2 = dV$, where dV_1 and dV_2 are the volume elements in the adsorbed layers of the first and second particles, respectively. Therefore,

$$\Delta G_m = \int_0^{dV} 2dVRTBC^2 = 2\int_0^{dV} \pi_E dV = 2\pi_E dV \quad .. \quad (11)$$

The total energy of repulsion over the whole overlap region between the spheres may be calculated by integrating the volume elements, dV.

The volume of a segment of a sphere is given by

$$V_s = \frac{\pi h^2}{3} 3(r_1 - h) \quad .. \quad .. \quad .. \quad .. \quad (12)$$

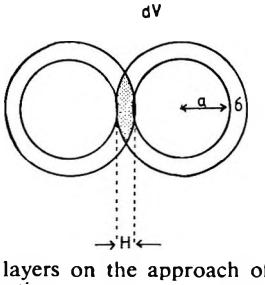


FIG. 6. The overlap of adsorbed layers on the approach of identical spherical particles: the model used to derive Fischer's equation.

In Fig. 6, $h = \delta - \frac{H}{2}$ and $r_1 = a + \delta$, hence

$$V_s = \frac{2\pi}{3}\left[\delta - \frac{H}{2}\right]^2\left[3a + 2\delta + \frac{H}{2}\right] \quad .. \quad .. \quad .. \quad (13)$$

If $V_S = V$, then from equation (11)

$$\Delta G_m = \frac{4\pi \cdot \pi_E}{3}\left[\delta - \frac{H}{2}\right]^2\left[3a + 2\delta + \frac{H}{2}\right] \quad .. \quad .. \quad (14)$$

Combining equations (9) and (14) gives

$$\frac{\Delta G_m}{kT} = \frac{B\mathcal{N}C^2 4\pi}{3}\left[\delta - \frac{H}{2}\right]^2\left[3a + 2\delta + \frac{H}{2}\right] \quad .. \quad .. \quad (15)$$

If the dispersion medium is water, the greater the hydrophilicity of the adsorbed layer, the larger is B, and the higher the free energy of mixing. ΔG_m may be readily estimated from equation (15) providing a suitable value for B can be obtained. This parameter has been evaluated for solutions of non-ionic detergents (Elworthy & McDonald, 1964) and glycols (Malcolm & Rowlinson, 1957), but discrepancies exist, depending on the method of measurement. Furthermore, data are only available for micellar solutions and the behaviour of detergents at oil-water interfaces may, or may not, be identical to that in the micelles. However, Napper (1968) found that incipient flocculation of polymer latexes occurred in dispersion media which were θ solvents for the stabilizing molecules in *free* solution as predicted by theory, viz.

$$B \propto \left(1 - \frac{\theta}{T}\right) \qquad .. \quad .. \quad .. \quad .. \quad (16)$$

where θ = Theta temperature. Thus when $T = \theta$ deviations from ideality vanish. Using the critical flocculation temperature as a criterion of stability Napper (1969) observed that aqueous dispersions stabilized by two non-ionic polymers differing by a factor of 3 in molecular weight possessed similar stability, suggesting an insensitivity of the steric stabilization to molecular size. However, with amphipathic compounds such as the $C_{16}E_x$ series of non-ionic detergents changes in molecular weight result in different adsorption properties and, hence, differing steric stabilizing powers (Elworthy & Florence, 1969b,c).

Fischer (1958) has evaluated ΔG_m for molecular overlap when the stabilizer segment density in the overlap volume is constant. However, an equation of the form given by Meier (1967) should be employed for polymeric stabilizers. Thus, for $L < H < 2L$ where L represents the contour length of the longest stabilizing moiety,

$$\Delta G_m = 2kT\,(\psi_1 - \chi_1)\,\frac{V^2}{\bar{V}_1}\,(\rho_j\rho_k)_H dV \qquad .. \quad .. \quad .. \quad (17)$$

and for $H < L$

$$\Delta G_m = 2kT\,(\psi_1 - \chi_1)\,\frac{V^2}{\bar{V}_1}\left[\int (\rho_j)_H^2 dV - \int (\rho_j)^2 dV + \int (\rho_j\rho_k)_H dV\right] \qquad .. \quad (18)$$

The relation between B, the second virial coefficient, and χ, is

$$B = RT\,\frac{(\psi_1 - \chi_1)}{\bar{V}_1\rho_2^2} \qquad .. \quad .. \quad .. \quad .. \quad (19)$$

where ρ_2 is the density of the adsorbed layer. ψ_1 is an entropy parameter which is given the ideal value of 0·5. Equation (18) represents the total change in Gibbs' free energy on bringing the particles from infinite separation to a distance, H, apart, where ρ_j and ρ_k are the segment density contributions in the volume element dV of the adsorbed chains on each particle surface.

Ottewill & Walker (1966, 1968) have used Fischer's equation (15) but substituted equation (19) for B giving

$$\frac{\Delta G_m}{kT} = \frac{4\pi\mathcal{N}C^2}{3\bar{V}_1\bar{\rho}^2_2}\left[0{\cdot}5 - \chi_1\right]\left[\delta - \frac{\bar{H}}{2}\right]^2\left[3a + 2\delta + \frac{\bar{H}}{2}\right] \qquad .. \quad (20)$$

The calculated stabilizing contribution of adsorbed layers is represented by curves of ΔG_m in kT units versus H in Fig. 7.

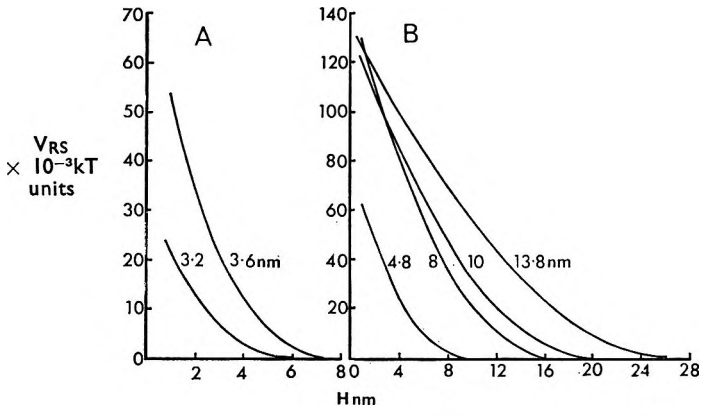


FIG. 7. The effect on V_{RS} of altering δ for (A) $C_{16}E_3$ and (B) $C_{16}E_{25}$. δ(nm) as marked on diagram.

In equations (18) and (20) it is assumed that no desorption of stabilizer can occur, which is unlikely for some non-ionics (especially in the chlorobenzene and anisole-water systems). The loss of entropy resulting from the interaction of the chains should cause some of the molecules to desorb and to gain entropy by returning to the bulk solution (Verwey, 1966). In these cases the free energy change corresponding to this process will be largely determined by the free energy of desorption. Therefore a surfactant should have a high free energy of adsorption if it is to give rise to high stability. This is a function of its solubility in the disperse and continuous phases. When the surfactant is soluble in the disperse phase (for example like $C_{16}E_3$ in chlorobenzene) it should be free to desorb at the point of collision and diffuse away into the globule. A more water-soluble detergent (e.g. $C_{16}E_{25}$) will be prevented from doing so and must remain in the interface (MacRitchie, 1967), and hence should contribute more to the stability.

Because the entropic force, however it is formulated, is a short-range force it will be more directly related to coalescence behaviour than V_A or V_R.

The interaction parameter, χ_1, may be determined in a number of ways, of which vapour pressure measurements provide the best method for concentrated solutions whereas osmotic pressure measurements are more accurate for dilute solutions. The osmotic pressure, π, is related to the concentration of a polymer of given molecular weight,

$$\frac{\pi}{C} = \frac{RT}{M} + \frac{RTC}{V_1\rho_2^2}\left(\frac{1}{2} - \chi_1\right) \quad .. \quad .. \quad .. \quad (21)$$

χ can be evaluated from plots of π/c versus c. Table 2 lists values of χ_1 from second virial coefficients on aqueous solutions of polyoxyethylene detergents and for glycols.

Since the calculated free energy of interaction is largely dependent on the value chosen for χ_1, then evidence of the effect of salts on χ_1 could lead to significant conclusions on the stability of emulsions in the presence of salts. Several reports (Schick, 1962; Hammes & Swann, 1967; Gluzman & Fridman, 1968) on solutions of non-ionic surfactants and polyethylene glycols bear out the contention that electrolytes dehydrate the ethylene oxide chains and promote their "salting out". Kuriyama (1962) has calculated χ_1 values for solutions of methoxy polyoxyethylene-(12)-dodecyl ether (MP 1–12) in the presence of sodium and calcium chloride from light-scattering results. χ_1 was observed to increase slightly with salt concentration, as expected.

Table 2. *Second virial coefficients* (B) *and* χ_1 *values for aqueous solutions of polyoxyethylene compounds.*

Compound	$B \times 10^4$* (ml mol g^{-2})	χ_1	Method	Reference
$C_{16}E_7$ (20°)	0·84	0·499	l.s.	Macfarlane (1963)
$C_{16}E_8$	2·43	0·496	l.s.	Idem
$C_{16}E_9$	3·28	0·494	l.s.	Idem
$C_{16}E_{21}$	1·40	0·497	l.s.	Idem
Methoxy-$C_{12}E_{12}$	1·90	0·497	l.s.	Kuriyama (1962)
Ethylene glycol	196·2	0·060	v.p.	Elias & Lys (1966)
Dioxyethylene glycol ..	151·2	0·160	v.p.	Idem
Trioxyethylene glycol ..	126·7	0·216	v.p.	Idem
Hexaoxyethylene glycol ..	102·2	0·271	v.p.	Idem
Nonaoxyethylene glycol	85·8	0·303	v.p.	Idem
Polyoxyethylene glycol 300 (30°)	—	0·35	v.p.	Malcom & Rowlinson (1957)

* All measurements at 25° unless otherwise specified.
v.p. = Vapour pressure. l.s. = Light scattering.

The same trend is observed when the temperature of the solutions was raised. This means that the interaction between solute and solvent decreases as temperature rises, as in the presence of salt. Calcium chloride exerted a small effect on the micellar weight and cloud point of MP 1–12 when compared with sodium chloride at the same ionic strength. But, if compared at the same molar concentration, calcium chloride is an equally (or a more) effective salt, increasing the micellar weight and decreasing the cloud point.

The second virial coefficient obtained from light-scattering measurements on micellar systems contains a term for interactions peculiar to micelles and hence of no relevance to dispersion stabilization. Negative second virial coefficients (suggesting $\chi_1 > 0{\cdot}5$) are found for a number of non-ionic systems. The change from positive to negative values with increasing temperature is coupled with decreasing stability with increasing temperature, but the negative values of B obtained for a number of glucosyl alkyl benzenes (Hutchinson, Sheaffer & Tokiwa, 1964) is probably not an indicator of stabilizing power.

A paper by Sata & Harisaki (1960) points out an interesting difference in the stabilizing power of an emulsifier of the general formula II (p. 156) in o/w and w/o emulsions. The compound stabilized drops of oil in water, but not drops of water in oil. The reason for this is that on the water side the stabilizing layer consists of three polyoxyethylene chains for every surfactant molecule, whereas on the oil side of the interface only one hydrocarbon chain protrudes and the drops of water in oil are insufficiently protected. This effect would be less marked in polyoxyethylene mono n-alkyl ethers, which might be expected to stabilize w/o emulsions equally as well as o/w emulsions given sufficient interfacial activity.

Other entropic stabilizing mechanisms

Another possible source of energy of repulsion between two dispersed particles is that arising from the elasticity of a collision, designated as ΔG_e. This aspect of the potential energy of repulsion has been dealt with by Jäckell (1964).

The importance of short-range repulsive forces in certain disperse systems cannot be entirely overlooked. After the main entropic repulsive forces have been overcome during a collision between two coated particles, surface forces in liquid lamellae between the particles may play a significant role in stabilization. A discussion of these forces is given by Kitchener & Mussellwhite (1968) in light of recent experimental findings. The role of the liquid film between globules in maintaining stability is discussed in the second part of this review (p. 233 *et seq.*).

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The protective effect of gallate antioxidants on emulsions and solutions of benzaldehyde

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In solubilized systems of benzaldehyde–water–antioxidant–cetomacrogol the ratio of benzaldehyde to gallate antioxidant is of prime importance. Provided this ratio is constant, the rate of oxidation and the induction period of the systems is independent of cetomacrogol concentration. The efficiency of the different gallates appears to be the same in L_1 micellar systems. In disperse systems the maximum oxidation rate is related to the total surface area of the droplets and to the quantity of benzaldehyde present in the disperse phase. Because changes in the relative concentration of the components of the system alter both the phase volume ratio and the total surface area a protection factor concept (ratio of the rate of oxidation of benzaldehyde in the absence of gallate: rate in the presence of gallate) was used to define the effect of gallates on the maximum rate.

Carless & Nixon (1957) and Nixon (1958) related the oxidation rate of solutions and emulsions to the relative amounts of emulsified and solubilized oil present, whilst Mitchell (1960) related the rate of oxidation to the degree of saturation of the dispersion, expressed as a saturation ratio (R). This saturation ratio was dependent upon the concentration of aldehyde required to saturate a solution. Carless & Swarbrick (1962) showed that only from a consideration of the ternary component diagram, surface active agent–benzaldehyde–water, was it possible to relate oxidation rates to the concentration and nature of the phases present.

As a preliminary to the present work the phase diagram of the system cetomacrogol 1000–benzaldehyde–water was studied and the effect of gallate antioxidants on the type and position of the phases noted (Nixon, Ul Haque & Carless, 1971). Wan & Hwang (1969), have examined a similar system but the results do not appear very conclusive and it is doubted whether they would apply to other than a small portion of the system. The present work is confined to those portions of the previously reported phase diagram which provided a dispersion fluid enough to be studied in a Warburg apparatus and in which viscosity effects did not interfere. This unfortunately precludes a study of oxidation in liquid crystal phases as, unlike the systems studied by Swarbrick (1964), these occur as viscous regions which are not amenable to study by the method available.

MATERIALS AND METHODS

Materials. The characteristics of the cetomacrogol 1000, water, benzaldehyde and gallates have been given by Nixon & others (1971).

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Methods

Measurement of oxygen uptake. The systems to be examined were prepared by dissolving the appropriate amount of gallate in the cetomacrogol with the aid of gentle heat. The amount of this homogeneous mixture required was weighed into a stoppered flask to which the water and catalyst solution was added. Benzaldehyde was weighed in and dispersed by shaking immediately before the oxidation study was made. Two ml samples were placed in the Warburg flasks and the oxygen uptake measured in the normal manner. A shaking rate of 140 strokes/min and a 150 mm sweep was used throughout. Measurements were made at 25° and a catalyst concentration of 1×10^{-5}M $CoSO_4$.

Assay of gallates. The method of Mitchell (1923) as modified by Berger, Sylvester & Hains (1960) and del Pozo & Salazar Macian (1962) was used. This method was further modified because it was found unnecessary to extract the water-insoluble gallates with butanol, as the surface-active agent solubilized them. Two ml of the solution to be tested was added to 2 ml of ferrous tartrate reagent and 10 ml of standard buffer. The volume was adjusted to 50 ml with distilled water. The absorbance of the solution was measured using an EEL Model A absorptiometer with a 604 μm filter. The colorimetric reading was the same whether the solubilized system or a butanol extract was used.

Distribution of gallates between the phases of emulsions. A system containing 10% w/w of cetomacrogol-gallate mixture (in a weight ratio of 2 : 1) and benzaldehyde concentrations of between 5 and 50% w/w was examined. After separation in a temperature controlled centrifuge at $25 \pm 0{\cdot}1°$ aliquot portions of the separate phases were assayed for their gallate content.

Particle size and surface area determinations. Because of dilution difficulties, which change the equilibrium conditions, particle size determinations were made by a counting technique using a projection microscope giving a 525 fold magnification. At least three different fields were counted for each dispersion. The specific surface area per unit volume was calculated from $6 \leqslant nd^2 / \leqslant nd^3$ where d is the mean diameter and n the number of droplets in each size range. The surface area per total volume of the disperse phase was also determined.

Determination of viscosity. This was determined using a Ferranti-Shirley cone and plate rotational viscometer fitted with an automatic X–Y recorder. Details of operation have been given by Nixon & Chawla (1965).

RESULTS

Solubilized systems. The oxidation of benzaldehyde when solubilized in the spherical micelles of the L_1 region shows a typical chain reaction oxidation curve in the absence of gallate antioxidants. The "induction period" shows no sudden break, but a gradual rise towards the maximum oxidation rate. There is direct proportionality between the time taken to reach a given oxygen uptake (200 ml O_2/litre of dispersion, equivalent to the end of the slow "induction period") and the cetomacrogol concentration (Table 1). This is completely contrary to the effect of cetomacrogol on the maximum oxidation rate. Here the surface-active agent has no effect on the rate attained (Table 1).

The presence of low concentrations of propyl gallate had the effect of reducing the maximum rate of oxidation and very markedly increasing both the induction

Table 1. *The "induction periods" and maximum oxygen uptake rates in L_1 systems in the absence of gallate antioxidants.*

Cetomacrogol (% w/w) in system	"Induction period" (min)	Maximum oxygen uptake rate ml/h litre^{-1}
10·0	73	412
12·5	83	418
15·0	93	421
17·5	106	413
20·0	117	420

Benzaldehyde 2·5% w/w.

period and the time taken to attain the maximum rate. The concentrations of gallate studied were in the w/w ratio benzaldehyde : propyl gallate of 6500 : 1, 2000 : 1, 1000 : 1 and 500 : 1. The benzaldehyde in the system was kept constant and the surface-active agent concentration was varied. Once again the maximum oxidation rate was found to be independent of cetomacrogol concentration (Fig. 1). A similar plot of the "induction period" appeared to show that this was also independent of the surfactant concentration (Fig. 2). However, because of the rather indefinite property being measured the scatter of points was wider than normally present.

In the systems studied one exception was found to the above generalizations. In dispersions containing 2·5% w/w benzaldehyde and 10% w/w cetomacrogol which appears to give a solubilized system, but one which is on the phase boundary, the induction period was short and the oxidation rate attained was lower than normal.

Plots of maximum uptake rates and induction periods against the molar ratio of propyl gallate to micellar benzaldehyde (i.e., the benzaldehyde solubilized as against the total concentration of benzaldehyde) show a straight line relation (Fig. 3). The line cannot be extrapolated completely to zero concentration of gallate. At some very high benzaldehyde : gallate ratio there must be a deviation from the straight line. Systems containing 10% cetomacrogol and 2·5% benzaldehyde do not follow these generalizations.

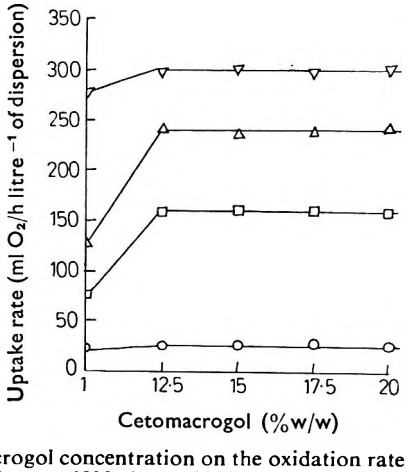

FIG. 1. Effect of cetomacrogol concentration on the oxidation rate of L_1 type solubilized systems. Benzaldehyde : gallate ratio: ▽ 6500 : 1, △ 2000 : 1, □ 1000 : 1, ○ 500 : 1. Benzaldehyde concentration 2·5% w/w.

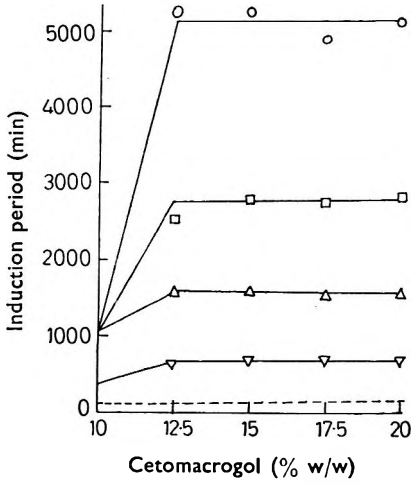

FIG. 2. Effect of cetomacrogol concentration on the induction period of L_1 type solubilized systems. Benzaldehyde : gallate ratio : ▽ 6500 : 1, △ 2000 : 1, □ 1000 : 1, ○ 500 : 1. Benzaldehyde concentration 2·5% w/w. – – – induction period in absence of gallates.

The effect of gallates other than propyl gallate is shown in Table 2. Ratios w/w of benzaldehyde : gallate of 2000 : 1 and 5000 : 1 were used at cetomacrogol concentrations of 10–20% w/w. The benzaldehyde concentration was kept constant at 2·5% w/w. Dodecyl gallate proved to be the least effective antioxidant, when considered on a weight ratio basis, although octyl gallate was only slightly better. The more water soluble and lower molecular weight ethyl gallate proved the most successful. All these gallates showed the same general shape of curve and properties as found previously with propyl gallate.

It has been shown (Heimann & von Pezold, 1957; Chipault, 1962) that high concentrations of antioxidants can exert a catalytic effect on the oxidation, but this

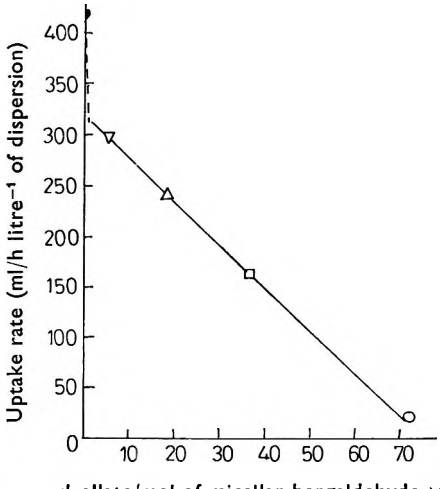

FIG. 3. Effect of low concentrations of propyl gallate on the oxidation rate of L_1 systems. Benzaldehyde : propyl gallate ratio : ▽ 6000 : 1, △ 2000 : 1, □ 1000 : 1, ○ 500 : 1. ● No gallate. Benzaldehyde concentration 2·5% w/w.

Table 2. *Maximum oxygen uptake rates (ml/h litre^{-1}) in L_1 systems containing ethyl, octyl and dodecyl gallates.*

Cetomacrogol (% w/w) in the system	Maximum uptake rates in presence of w/w benzaldehyde : gallate ratios of:					
	Ethyl gallate		Octyl gallate		Dodecyl gallate	
	5000 : 1	2000 : 1	5000 : 1	2000 : 1	5000 : 1	2000 : 1
10·0	228	120	257	182	280	200
12·5	281	225	295	261	301	270
15·0	290	220	298	267	310	280
17·5	278	230	297	264	309	275
20·0	285	237	300	265	300	276

Benzaldehyde 2·5% w/w.

has not been found with the present studies. When the amount of gallate in the system was between 0·2 and 5% w/w the oxidation showed a straight line relation which was independent of the cetomacrogol concentration and depended solely on the concentration of gallate present. The concentration of benzaldehyde used was 2·5% w/w in all dispersions (Fig. 4).

Two phase systems. The first noticeable difference in the oxidation curves of emulsified, as compared with solubilized systems, is the absence of an induction period. The rate of oxidation, in the absence of antioxidant, increased linearly for approximately 20 min to reach a maximum rate after which it slowly fell to reach a lower steady rate after about 50 min (Fig. 5).

If these maximum oxidation rates are plotted against the concentration of benzaldehyde present they exhibit a wave form having a peak at 20% w/w benzaldehyde and a trough at 50% w/w. On further increasing the concentration of benzaldehyde (80% w/w) the oxidation rate became too rapid to measure under the experimental conditions (Fig. 6).

The effect of gallate antioxidants was studied using systems of 10% w/w cetomacrogol and cetomacrogol : gallate ratios of 2000 : 1, 1000 : 1, and 500 : 1. To these systems gradually increasing amounts of benzaldehyde were added. The shape of the oxygen uptake curve was similar to that in the absence of gallates, but

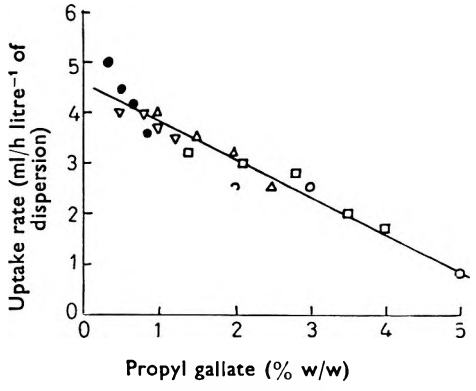

FIG. 4. Effect of high propyl gallate concentrations on the oxidation rate of L_1 phase. Benzaldehyde 2·5% w/w. Cetomacrogol: propyl gallate ratio : ○ 2 : 1, □ 4 : 1, △ 6 : 1, ▽ 9 : 1, ● 19 : 1.

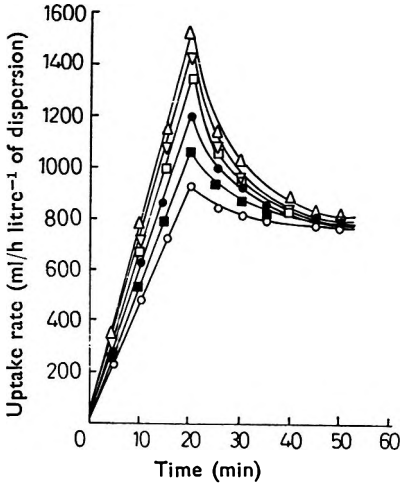

FIG. 5. Effect of benzaldehyde concentration on the oxygen uptake rates in $L_1 + L_2$ systems. Benzaldehyde % w/w, ○ 10, □ 15, △ 20, ▽ 30, ● 40, ■ 50.

considerably elongated. With ratios of 2000 and 1000:1 the maximum oxidation rate occurred in the region of 30% w/w benzaldehyde and the minimum rate at 50% w/w benzaldehyde. At a ratio of 500:1 there was neither a maximum nor minimum rate but a plateau region when the benzaldehyde exceeded 30% w/w. All systems showed a rapid increase in rate at benzaldehyde concentrations above 50% w/w.

The effect of gallates other than propyl gallate was similar (Table 3). The peak oxidation rate occurred in the region of 20–30% w/w benzaldehyde. This was followed by a trough at 50% aldehyde and a further rise as more benzaldehyde was introduced into the system. All dispersions, irrespective of the gallate they contained, commenced to oxidize too rapidly to measure accurately when a concentration of 70% aldehyde was exceeded.

The ratio of benzaldehyde to gallate changes continuously in these biphasic systems and as the disperse phase consists of benzaldehyde, in which the gallates

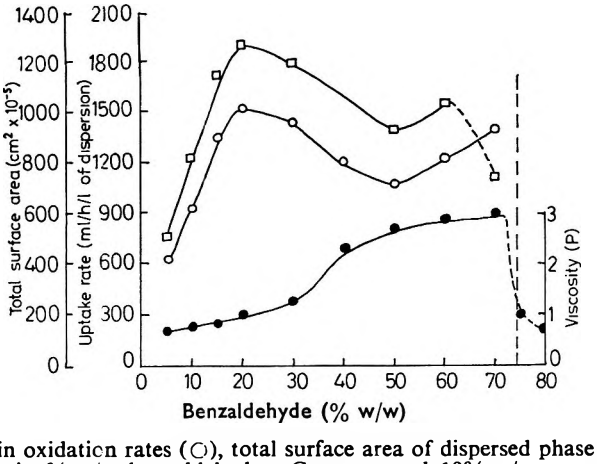

FIG. 6. Changes in oxidation rates (○), total surface area of dispersed phase (□) and viscosity (●), with increase in % w/w benzaldehyde. Cetomacrogol 10% w/w. – – – inversion from o/w to w/o dispersion.

Table 3. *Maximum oxygen uptake rates (ml/h litre^{-1}) of disperse systems in the presence of ethyl, octyl and dodecyl gallates.*

Benzaldehyde (% w/w) in the system	Maximum uptake rates in the presence of w/w cetomacrogol : gallate ratios of:				
	Ethyl gallate		Octyl gallate		Dodecyl gallate
	2000 : 1	1000 : 1	2000 : 1	1000 : 1	2000 : 1
5	110	—	270	—	335
10	380	—	404	235	423
15	475	275	650	375	643
20	700	406	801	508	825
30	670	533	900	600	920
50	625	464	742	535	840
60	705	—	936	—	—
70	865	—	1109	—	1228
75	2388	—	2780	—	2845
80	3841	—	3900	—	4035

Cetomacrogol concentration 10% w/w.

are far more soluble, it was necessary to calculate the distribution of gallate in this aldehyde disperse phase (Table 4). When 15% w/w of benzaldehyde was present the bulk of the gallate was associated with the aldehyde rather than the cetomacrogol.

The presence of discrete droplets, which over the larger part of the two phase region will contain the bulk of the benzaldehyde, distinguishes these systems from the L_1 solubilized phase. It is possible that the size or the surface area of this internal phase could influence the rate of oxidation and for this reason the systems were subjected to a droplet size analysis. As can be seen, the curve for total droplet surface area against benzaldehyde concentration followed the same shape as the rate of oxygen uptake curve, showing a maximum and minimum at the same concentrations (Fig. 6). It was also found that the total surface area/litre plotted against the oxidation rate was a straight line (Fig. 7). The relation between droplet surface area and oxidation rate is better than that between viscosity and rate, which is also shown in Fig. 6.

DISCUSSION

The oxidation of benzaldehyde in micellar dispersion may be due to the total amount of benzaldehyde present or merely to that quantity solubilized within the micelle. Mitchell & Wan (1965) have suggested that the rate of oxidation in solubilized systems is related solely to the degree of aldehyde saturation of the

Table 4. *Percentage of gallate present in the benzaldehyde droplets of biphasic systems.*

Gallate	Gallate (% w/w) present in disperse phase at the given % benzaldehyde concentrations					
	5	10	15	20	30	50
Ethyl gallate	2·8	40·4	64·1	75·9	91·9	97·3
Propyl gallate	14·9	55·2	78·9	85·7	96·1	99·0
Octyl gallate	19·6	56·2	79·5	88·5	97·1	99·1
Dodecyl gallate	21·7	62·7	79·8	89·4	98·0	100·0

System: Cetomacrogol 7·5% w/w gallate 2·5% w/w, benzaldehyde 5–50% w/w.

dispersion. The experimental conditions producing this result differed from those reported in the present paper. The shaking rate used was only 88 strokes/min whilst we found a minimum of 110 strokes/min to be necessary to ensure saturation with oxygen. The lighting and catalyst also differed. The present authors believe that because at constant benzaldehyde concentrations the amount per micelle will fall as the cetomacrogol concentration increases, the rate of oxidation, or the time taken to attain a given rate would be expected to increase. Carless & Swarbrick (1964) have suggested that oxidation in L_1 type solubilized systems depended only on the ratio of micellar benzaldehyde to micellar surface-active agent which would act as a diluent for the aldehyde. As the gallate antioxidants will be associated with the micelle, because of their bonding to cetomacrogol, they will be in intimate contact with the benzaldehyde. It is probable, as shown previously (Nixon & others, 1971), that they are in competition for the same micellar positions. In this previous work it was shown that low concentrations of gallate had no effect on the phase equilibria present.

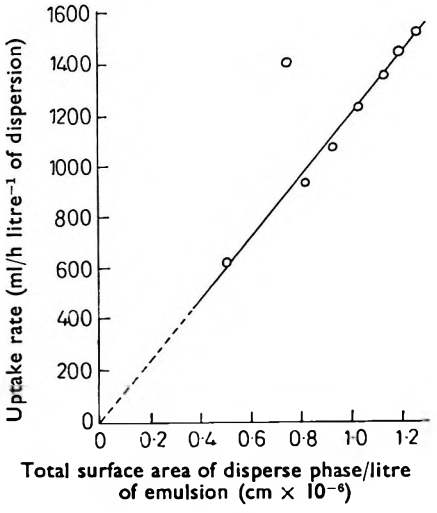

FIG. 7. Effect of total disperse phase surface area on the oxidation rate of $L_1 + L_2$ systems.

It appears that in solubilized systems the benzaldehyde:gallate ratio will be of prime importance. Provided that this ratio was constant the maximum oxidation rate of the system was found to be independent of the cetomacrogol concentration.

The oxidation in the presence of gallates followed the expression $R = {}_eR_0 - kr$. R is the oxidation rate, ${}_eR_0$ the extrapolated oxidation rate at zero gallate concentration (320 ml O_2/h litre^{-1} of dispersion), r the micellar benzaldehyde:gallate ratio and k a constant (slope). This extrapolated rate is somewhat lower than the experimental maximum rate at zero gallate concentration, which suggests that exceptionally high aldehyde:gallate ratios have little effect on the oxidation, but even so, over the range 5–80 $\times$ 10^{-5} mol gallate:mole of aldehyde, the expression is valid. The efficiency of the various gallate esters would appear to be the same in L_1 type micellar systems; the degree of protection afforded being due simply to the molar ratio of gallate to aldehyde when the maximum rate attained is considered.

The effect of cetomacrogol on the induction period (Table 1 and Fig. 2) is more indefinite. Because of the long periods of time involved and the resultant scatter

of points the "induction period", certainly in the presence of gallates, appears to be independent of cetomacrogol concentration.

The oxidation rates of disperse systems have been suggested by Swarbrick (1964) to depend on the amount of benzaldehyde in the disperse phase. However, his systems contained only small concentrations of benzaldehyde, having a maximum of 8% w/w benzaldehyde. Even with these low concentrations there was a gradual decrease in rate/g of aldehyde, commencing at a concentration of 4–5% benzaldehyde. This may have been due to the effect of the interfacial area of the droplets. The results in the present work (Figs 6 and 7) show that the maximum oxidation rate is related to the total surface area of the droplets and to the quantity of benzaldehyde present as disperse phase. Viscosity, which in these systems depended on the droplet size and phase volume ratio, was not an independent factor in the oxidation rate. This dependence of oxidation rate on the interfacial area of the droplets would suggest that the initial oxidation occurs at or near the surface of the emulsion droplet. The free radicals produced then cause the oxidation of the remaining bulk of the benzaldehyde present in the droplets. Higher surface areas could be expected to produce higher overall rates, particularly as it should be easier, because of increased diffusion, to maintain an adequate oxygen concentration within the droplets. Because of the absence of any induction period in the oxidation of bulk benzaldehyde the initial oxidation will be due to the dispersed benzaldehyde droplets. The small amount of solubilized aldehyde, because of the long induction period, played no part in determining the maximum oxidation rate of emulsions and therefore an extrapolation of Fig. 7 passes through zero.

Because changes in the relative concentrations of the components of the system will alter both the phase volume ratio and the total surface area it is necessary to make use of a protection factor concept when considering the effect of gallates on the maximum rate. This was done by determining the ratio of the rate of oxidation in the absence of gallate to the rate in the presence of gallate. A plot (Fig. 8) of this protection factor against the ratio of benzaldehyde: gallate is a straight line passing

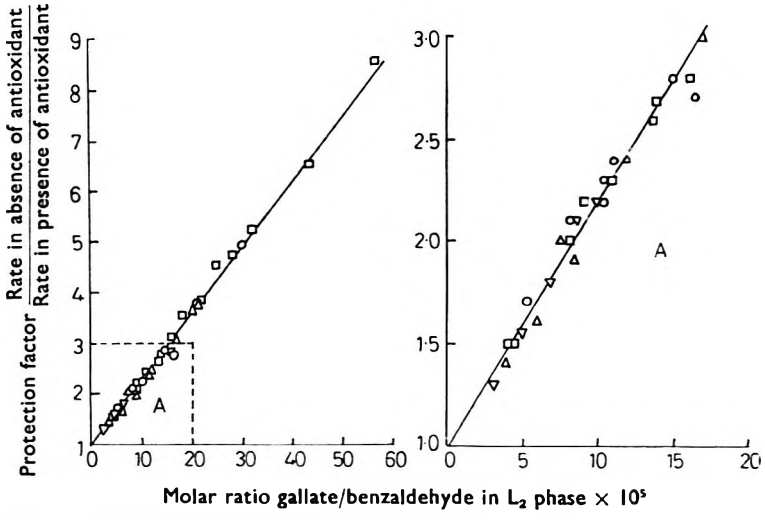

FIG. 8. Antioxidant effect produced by gallates in disperse phase ($L_1 + L_2$). Ethyl gallate ○ propyl gallate □, octyl gallate △, dodecyl gallate ▽, cetomacrogol 10% w/w.

through a protection factor of 1 when no gallate is present. Small quantities of gallates in disperse systems do not act as catalysts either on their own or complexed with metals. Wan & Hwang (1969) have found contrary indications in solubilized systems which under their experimental conditions oxidize at a far slower rate.

The efficiency of the various gallates is a function of the ratio of benzaldehyde : gallate in the disperse phase and the following expression holds in these two phase systems: $R = R_0/1 + k\, r_1$ where R_0 is the oxidation rate of the system in the absence of antioxidant, R is the oxidation rate of the system, r_1 the ratio of benzaldehyde in the disperse phase: gallate and k a constant.

The ratio of benzaldehyde: gallate appears to be the most important factor in the oxidation of benzaldehyde in the presence of gallates. The antioxidant effect is superimposed on the oxidation picture found in the absence of gallates.

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Effects of acetylsalicylic acid on serum protein binding and metabolism of tryptophan in man

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In normal persons, ingestion of aspirin causes a release of tryptophan from its binding site on serum albumin. There is a fall in bound and total serum tryptophan concentrations and a rise in free tryptophan concentrations. The urinary excretion of 3-hydroxyanthranilic acid is decreased, that of xanthurenic acid is increased and that of 3-hydroxykynurenine was increased in 4 out of 6 subjects, indicating an effect on the enzyme systems involved in the metabolism of tryptophan. Conjugates of these metabolites were shown to interfere with the method of assay of the unconjugated hydroxy acids by column chromatography. To overcome this difficulty all urine samples were first boiled in molar hydrochloric acid to hydrolyse any conjugates present. Any results obtained using non-hydrolysed urines would be misleading. This work shows that it is important to take account of the drugs used in treatment before ascribing changes in tryptophan metabolism to pathological states.

Abnormal tryptophan metabolism has been reported in a variety of human pathological conditions including rheumatoid arthritis (Price, Brown & Yess, 1965). McMillan (1960) reported that patients with rheumatoid arthritis excreted increased quantities of the tryptophan metabolite 3-hydroxyanthranilic acid. Subsequently, increased urinary excretion of kynurenine (Bett, 1962), 3-hydroxykynurenine (Pinals, 1964; Flinn, Price & others, 1964) and xanthurenic acid (Speira, 1966) has been reported. Prolonged treatment with salicylates in rheumatoid arthritis was reported to have no effect on the excretion of kynurenine, 3-hydroxykynurenine, xanthurenic acid or kynurenic acid (Bett, 1963).

L-Tryptophan is the only amino-acid bound to human serum albumin to an appreciable extent (McMenamy & Oncley, 1958). Any variation in serum albumin concentration therefore would affect that of bound L-tryptophan. In rheumatoid arthritis low serum albumin concentrations may occur (Sydenes, 1963), so it would be expected that the amount of tryptophan bound to serum albumin would be less.

Competition for the L-tryptophan binding site on serum albumin has been demonstrated with a wide variety of compounds including salicylates (McArthur & Dawkins, 1969), fatty acids (Kotake, 1964), thyroxine (Tritsch & Tritsch, 1963) and clofibrate (O'Mahony, D. R., unpublished). It is possible that other acidic compounds may also compete with tryptophan for this binding site, and consequently affect tryptophan metabolism. In addition, it is known, that adrenal corticosteroids increase the activity of tryptophan pyrrolase (Altman & Greengard, 1966) and the urinary excretion of certain tryptophan metabolites (Rose & McGinty, 1968). These factors should be considered in any studies on the metabolism of tryptophan.

Although sodium salicylate has been shown *in vitro* to compete with tryptophan for the binding site on serum albumin (McArthur & Dawkins, 1969), aspirin

(acetylsalicylic acid) was used in the present study since this is the form in which salicylates are given to patients with rheumatic diseases. Aspirin was administered to normal persons and its effect on the binding of tryptophan to serum protein, and on the excretion of tryptophan metabolites, investigated.

The excretion of tryptophan metabolites in normal urines and urines collected after a tryptophan load has also been examined. The importance of hydrolysing any conjugates of 3-hydroxyanthranilic acid and 3-hydroxykynurenine in urine samples before assay of these metabolites has been investigated.

MATERIALS AND METHODS

Materials

Visking dialysis tubing (0·65 cm inflated diameter) was obtained from the Scientific Instrument Centre.

3-Hydroxy-DL-kynurenine and xanthurenic acid were obtained from Koch-Light Laboratories Ltd., Colnbrook, and L-tryptophan from BDH Chemicals Ltd., Poole, Dorset. 3-Hydroxyanthranilic acid and Dowex-50W x 12 (200–400 mesh) ion exchange resin were supplied by Sigma Chemical Company, St. Louis.

All other chemicals were of analytical grade and distilled water was used throughout.

Collection of samples

Eight healthy adult student volunteers (6 male, 2 female, aged 21–23 years) were studied. Blood samples (50 ml) were taken at 10 a.m., after a light breakfast of coffee or tea and toast. On the same day, 1800 mg of acetylsalicylic acid (6 tablets of aspirin) were ingested between 9 and 11 p.m. and a further 1800 mg between 8.30 and 9.30 a.m. on the following morning; 50 ml blood samples were taken at 10 a.m., allowed to clot at room temperature and then centrifuged at 3000 rev/min for 5 min. The serum was removed and samples taken for determination of tryptophan (free + total) and salicylate. Normal urine, and urine after administration of aspirin were collected for the 14 h from 11 p.m. on day 1 until 1 p.m. on day 2.

Tryptophan loading

L-Tryptophan (5 g) was administered orally as a suspension in yoghurt at 10 a.m. to six student volunteers. Blood samples (20 ml) were taken immediately after to obtain zero time levels of serum tryptophan and then at hourly intervals until 4 p.m., and the sera collected. On a subsequent occasion aspirin was administered to the same students as described above, and 5 g L-tryptophan ingested before collection of the first blood sample at 10 a.m. Blood samples (20 ml) were then taken at hourly intervals until 4 p.m. Urine was collected for 6 h after ingestion of the tryptophan load, acidified with hydrochloric acid and stored at −20°.

Dialysis of serum

Visking dialysis tubing was soaked in distilled water for 4 h before use. Sacs of the Visking tubing, containing 1 ml of distilled water, were immersed in 8 ml serum and dialysis allowed to proceed for 20 h at room temperature (McArthur & Dawkins, 1969). Samples of the dialysate were then taken for estimation of free tryptophan.

Determination of free and total tryptophan in serum

Tryptophan (free and total) was estimated by its conversion to the norharman derivative according to the method of Hess & Udenfriend (1959) and its fluorescence measured spectrofluorometrically.

Free tryptophan. Samples (0·5 ml) of dialysate were added to water (1·65 ml) and 20% w/v trichloroacetic acid (TCA) (0·85 ml); formaldehyde (0·2 ml 20% w/v) was then added and the solutions heated in a boiling water bath for 25 min, before addition of hydrogen peroxide (0·2 ml, 6% w/v). The solutions were then boiled for a further 25 min. After cooling, the fluorescence of the solutions was measured on an Aminco Bowman spectrofluorometer, activation 365 nm, emission 440 nm.

Total tryptophan. Samples (1 ml) of serum were deproteinized by addition of water (4 ml) and ice-cold TCA (2 ml 20% w/v). After centrifugation at 3000 rev/min for 5 min, 3 ml samples of the supernatant were taken for formation of the norharman derivative of tryptophan.

Bound tryptophan. The amount of tryptophan bound to serum protein was calculated by subtraction of the free from the total concentration.

Determination of serum salicylate

The salicylate levels (total and free) in serum were assayed using the method of Trinder (1954).

Determination of tryptophan metabolites in urine

Xanthurenic acid was determined according to Satoh & Price (1958). Separation of the tryptophan metabolites, 3-hydroxykynurenine and 3-hydroxyanthranilic acid was by ion-exchange chromatography using modifications of the methods of Brown & Price (1956) and Heeley (1965). The specificity of this method has been confirmed by comparison with gas-liquid chromatography (Rose & Toseland, 1967). Columns (3·5 × 1·2 cm) of Dowex-50W resin, H^+ form, were equilibrated with 0·5M HCl in glass chromatography columns (10 × 1·2 cm). They were operated under gravity. Since tryptophan metabolites are known to be conjugated in the body, the presence of conjugates of 3-hydroxyanthranilic acid and 3-hydroxykynurenine in the urine samples was studied as follows. Urine was collected for 6 h after oral administration of L-tryptophan (5 g) to one normal subject and to two patients with rheumatoid arthritis. Samples of these urines before and after acid hydrolysis were chromatographed. To hydrolyse any conjugates present, the urines were boiled for 1 h in 1M HCl, cooled and diluted to 0·5M HCl before application to the columns.

The urine samples collected from the students were acid hydrolysed before column chromatography. The hydrolysates were diluted with distilled water to 0·5M HCl and samples containing less than 2 mg of each metabolite applied to the columns. The columns were washed with 40 ml of 0·5M HCl and the eluates discarded. The columns were then eluted with 2M HCl followed by 5M HCl; 80 and 20 ml fractions were collected in each case and analysed separately to ascertain separation of the two metabolites. 3-Hydroxyanthranilic acid was contained in the 2M HCl fraction and 3-hydroxykynurenine in the 5M HCl fraction. Standard solutions of both metabolites were boiled in M HCl and chromatographed under the same conditions. Mean recoveries of these metabolites were 95–98%. These metabolites were only

determined in urines collected after ingestion of a tryptophan load since the method was insufficiently sensitive to detect the low levels present in normal urines.

Standard solutions of 3-hydroxyanthranilic acid and 3-hydroxykynurenine were prepared in 2 and 5M HCl respectively. Samples (3 ml) of these solutions containing 0–100 μg/ml were diazotized by treatment with 0·2 ml 0·25% w/v sodium nitrite. After 3 min, ammonium sulphamate (0·2 ml 10% w/v) was added and the absorbance of the solutions read immediately at 370 nm (Brown, 1957) against a water blank. Aliquots (3 ml) of eluates were analysed similarly. In addition, the absorbance of the eluates (3 ml + 0·4 ml 10% ammonium sulphamate) were measured and subtracted from the diazotized values.

Thin-layer chromatography of the 2 and 5M HCl eluates was carried out to ascertain complete separation of 3-hydroxyanthranilic acid and 3-hydroxykynurenine. The 2 and 5M HCl eluates obtained from the non-hydrolysed urines of a normal subject and patients with rheumatoid arthritis were chromatographed before and after boiling for 1 h in M HCl, and the results compared with those of the hydrolysed urines. Thin-layer chromatography was carried out using 10 cm plates, the adsorbent being cellulose powder MN 300. The solvent system was sodium acetate buffer M, pH 5·4. The metabolites and reference compounds were detected by their fluorescence in ultraviolet light (254 nm).

RESULTS

Effect of aspirin on serum tryptophan concentration in normal subjects

After ingestion of aspirin, the mean free tryptophan concentration in the serum of eight subjects rose from 4·0 to 6·0 μM and that of tryptophan bound to serum protein decreased from 32·0 to 17·0 μM (Table 1). The total (bound + free) tryptophan in serum decreased from 36·0 to 23·0 μM in the presence of salicylates. These results show that salicylates significantly reduce the binding capacity of serum protein for tryptophan with a consequent increase in the free tryptophan concentration. The serum concentrations of salicylate were 100–175 μg/ml. The concentration of tryptophan determined in the dialysates (free tryptophan) has been multiplied by

Table 1. *Tryptophan concentrations in serum of eight normal subjects before and after ingestion of aspirin.*

	Tryptophan concentration in serum (μM)					
	Total		Free		Bound	
Subjects	Normal level	After aspirin	Normal level	After aspirin	Normal level	After aspirin
I	32·7	17·2	3·3	4·1	29·4	13·1
II	25·8	21·8	3·7	4·7	22·1	17·1
III	32·1	21·8	3·3	7·8	28·8	14·0
IV	34·3	25·2	2·9	4·7	31·4	20·5
V	48·3	26·8	5·4	7·2	42·9	19·6
VI	32·3	23·3	4·3	6·3	28·0	16·9
VII	32·1	13·5	3·2	4·9	28·9	8·5
VIII	52·9	35·4	6·2	7·8	46·7	27·6
Mean μM ± s.d.	36 ± 9	23 ± 6	4 ± 1	6 ± 1	32 ± 8	17 ± 5
% Change in mean after aspirin		−36%		+50%		−47%
P Value		0·01		0·01		0·01

1·125 to correct for the dilution during dialysis. This correction factor does not, however, account for any small change in the equilibrium of bound to free tryptophan which may occur on dilution of the serum. The results have been analysed by the Wilcoxon Paired Difference test, the minimal acceptable level of significance being taken as $P = 0{\cdot}10$.

Effect of aspirin on serum tryptophan concentration after a loading dose of tryptophan

Total, free and bound serum tryptophan concentrations rose to a maximum between 1 and 2 h after ingestion of a load of L-tryptophan, the results showing a 10–12 fold increase in bound and total and a 30-fold increase of free tryptophan (Table 2). Similar results were obtained after ingestion of tryptophan and aspirin. The tryptophan concentrations at 6 h however, were markedly lower in the presence of salicylates and approached normal.

Table 2. *Tryptophan concentrations in serum of six normal subjects after ingestion of* (a) 5*g* L-*tryptophan and* (b) 1·8 *g aspirin and* 5 *g* L-*tryptophan.*

Time after ingestion of tryptophan (h)	Tryptophan concentrations in serum (μM ± s.d.) Total		Free		Bound	
	Without aspirin	After aspirin	Without aspirin	After aspirin	Without aspirin	After aspirin
0	34 ± 7	22 ± 3	4 ± 1	6 ± 1	30 ± 6	16 ± 3
1	413 ± 166	365 ± 44	109 ± 38	123 ± 42	304 ± 138	242 ± 24
2	435 ± 102	396 ± 25	148 ± 49	120 ± 23	287 ± 71	276 ± 34
3	324 ± 94	309 ± 53	95 ± 53	75 ± 32	233 ± 79	234 ± 32
4	200 ± 32	196 ± 53	46 ± 18	52 ± 9	154 ± 15	144 ± 55
6	126 ± 62	67 ± 8	18 ± 7	22 ± 11	108 ± 61	45 ± 5

In the aspirin experiments, the total (free + bound) salicylate concentration in serum from six subjects was determined at hourly intervals for 6 h after ingestion of tryptophan. The total salicylate values were in the range 100–175 μg/ml serum. Free salicylate in serum was calculated by estimation of free salicylate in the dialysates. Free salicylate varied between 65 and 90% of the total concentration in normal sera and in sera after a tryptophan load.

Excretion of tryptophan metabolites

The excretion of the tryptophan metabolites 3-hydroxykynurenine, 3-hydroxyanthranilic acid and xanthurenic acid was determined in urines before and after the ingestion of aspirin (Table 3).

In each subject studied there was a significant decrease in the excretion of 3-hydroxyanthranilic acid and an increase in xanthurenic acid in the urines collected after ingestion of aspirin. An increase in the excretion of 3-hydroxykynurenine was observed in 4 out of the 6 subjects studied. Aspirin produced a large increase in the ratio 3-hydroxykynurenine : 3-hydroxyanthranilic acid and a decrease in the ratio 3-hydroxykynurenine : xanthurenic acid and in the ratio 3-hydroxyanthranilic acid : xanthurenic acid.

The effect of aspirin on the normal excretion of xanthurenic acid without prior administration of tryptophan was studied. In the presence of salicylates the excretion

Table 3. *Excretion of tryptophan metabolites,* 3OH-kynurenine, 3OH-anthranilic acid and xanthurenic acid were measured in urine collected for 6 h from 6 normal subjects after oral administration of (*a*) 5 g L-tryptophan and (*b*) 1·8 g aspirin + 5 g L-tryptophan. Each value represents the mean (μM/6 h $\pm$ standard deviation).

Metabolite	After 5 g L-tryptophan μM $\pm$ s.d.	After 5 g L-tryptophan + 1·8 g aspirin μM $\pm$ s.d.	% Increase or decrease after aspirin	*P* Value
3OH-Kynurenine ..	176 $\pm$ 60	211 $\pm$ 99	+20%	N.S.*
3OH-Anthranilic acid	97 $\pm$ 32	62 $\pm$ 33	−36%	0·10
Xanthurenic acid ..	50 $\pm$ 22	96 $\pm$ 48	+92%	0·05
$\frac{\text{3OH-KYN}}{\text{3OH-ANT}}$	1·81	3·40		0·05
$\frac{\text{3OH-KYN}}{\text{XA}}$	3·52	2·19		0·05
$\frac{\text{3OH-ANT}}{\text{XA}}$	1·94	0·65		0·05

* Not significant.

of xanthurenic acid increased by 45% from 12·9 $\pm$ 6·9 to 18·7 $\pm$ 8·9 μM (*P* 0·05) without a tryptophan load compared with 92% after a tryptophan load (Table 3).

Table 4 gives the results obtained for the estimation of 3-hydroxyanthranilic acid, 3-hydroxykynurenine and any conjugates of these compounds in the 2 and 5M HCl eluates from the column chromatography of urines before and after acid hydrolysis. In each subject there appears to be more 3-hydroxyanthranilic acid excreted than 3-hydroxykynurenine in the eluates obtained from non-hydrolysed urines. This is reversed if these urines are hydrolysed before column chromatography.

Thin-layer chromatography of the 2 and 5M HCl eluates, from hydrolysed urines revealed complete separation of 3-hydroxyanthranilic acid from 3-hydroxykynurenine, i.e. 3-hydroxyanthranilic acid in the 2M HCl eluate and 3-hydroxykynurenine in the

Table 4. *Column chromatography of urine, before and after acid hydrolysis.* Excretion of 3OH-anthranilic acid and 3OH-kynurenine after administration of 5 g L-tryptophan to one normal subject (*a*) and to patients with rheumatoid arthritis (*b*) and (*c*).

		3OH-anthranilic acid (2M eluate) mg/6 h	3OH-kynurenine (5M eluate) mg/6 h	$\frac{\text{3OH-KYN}}{\text{3OH-ANT}}$
Non-hydrolysed urine	(a)	18*	14	0·78
	(b)	220*	104	0·47
	(c)	91*	65	0·71
Hydrolysed urine	(a)	14	30	2·14
	(b)	57	374	6·50
	(c)	20	148	7·40

* 2M eluate of non-hydrolysed urine contained 3OH-anthranilic acid and a conjugate of 3OH-kynurenine.

5M HCl eluate. The fluorescence and R_F values of two spots observed corresponded to the authentic reference compounds. Thin-layer chromatography of the 2M HCl eluates from the non-hydrolysed urines however, revealed several spots. One spot gave the fluorescence as 3-hydroxykynurenine and another similar to 3-hydroxyanthranilic acid but with different R_F values to these compounds. A third spot corresponded to 3-hydroxyanthranilic acid.

Thin-layer chromatography of these 2M HCl eluates after boiling resulted in the appearance of a spot identical with 3-hydroxykynurenine. The 5M HCl eluates from the non-hydrolysed urines revealed one spot identical with 3-hydroxykynurenine.

DISCUSSION

Salicylates and tryptophan binding to serum protein

The results presented indicate competition of salicylate with tryptophan for the binding site on serum protein *in vivo* since bound tryptophan was reduced by about 50% after ingestion of aspirin. A similar results has previously been demonstrated *in vitro* after adding sodium salicylate to pooled human serum (McArthur & Dawkins, 1969). Free tryptophan, however, *in vivo* did not rise to the concentrations obtained *in vitro*, and the total serum tryptophan (free + bound) was much reduced in the presence of salicylates. Presumably, *in vivo* the tryptophan freed from its binding site diffuses into tissues and becomes available for metabolism or excretion, hence the observed reduction in total serum tryptophan.

In the presence of salicylates, there was no significant difference in the concentration of bound tryptophan 2–4 h after the 5 g tryptophan load; the normal bound serum tryptophan concentrations before loading and that 6 h after the tryptophan load, were significantly reduced (Table 2). However, the values obtained between 2 and 4 h were difficult to interpret since the time curves and concentrations obtained for total serum tryptophan varied considerably during this time in three of the six subjects studied. These differences may have been due to variation in the rate of absorption of tryptophan from the small intestine. After a load of tryptophan, the very high serum concentrations occurring between 2 and 4 h appear to result in preferential binding of tryptophan to serum albumin. However, at the lower physiological concentration of tryptophan, salicylates are able to compete with the binding sites giving rise to the observed reduction of bound tryptophan. At zero time (i.e. before loading) the ratio total salicylate : tryptophan is 30 : 1; at 6 h after ingestion of the tryptophan load the ratio is 8 : 1. At these concentrations the displacement of tryptophan by salicylates is marked. During the 2–4 h after the tryptophan load however, the ratio is between 2 : 1 and 5 : 1. From these ratios it would seem that tryptophan is more firmly bound to serum albumin than salicylates, since relatively high concentrations of salicylates are required to show a marked displacement of tryptophan.

The carboxyl group of tryptophan is one of the stereospecific requirements for its binding to serum albumin (McMenamy & Oncley, 1958). These workers concluded that the *N*-terminal group of albumin is the ionizable group involved at the protein-binding site. Similar results were obtained in studies of the binding of L-thyroxine to human serum albumin; the α-amino-group of the amino-terminal aspartic acid residue of the protein being a vital part of the strong binding site (Tritsch & Tritsch, 1963). At pH 7·35 tryptophan is a competitive inhibitor for thyroxine binding but

not at pH 8·70. Tritsch & Tritsch concluded that "other groups of presently unknown identity are involved in both sites, and that the binding sites of tryptophan and thyroxine are not identical but overlap at least around the region of the amino-group of the aspartic acid residue". Acidic drugs containing carboxyl groups, such as salicylates, may bind to albumin by competition with tryptophan for the carboxyl group binding site. Clofibrate (chlorphenoxyisobutyric acid), another acidic drug, was reported to combine with plasma proteins and to displace albumin-bound tryptophan in rats (O'Mahony, D. R., unpublished). In addition, a fall in the serum tryptophan was observed. Competitive displacement of tryptophan and other naturally occurring metabolic substrates, like hormones and fatty acids, may be a factor in the mode of action of such drugs.

Urinary excretion of tryptophan metabolites

The procedure used for assay of 3-hydroxyanthranilic acid and 3-hydroxykynurenine will also detect conjugates of these compounds. Any such conjugates present in the urine will therefore also be determined. The results obtained (Table 4) indicate the presence of conjugates of these metabolites in non-hydrolysed urines. This was confirmed by thin-layer chromatography in which a conjugate of 3-hydroxykynurenine was detected in the 2M HCl eluate of non-hydrolysed urine. This conjugate of 3-hydroxykynurenine, possessing different physical and chemical properties to the free hydroxy acid, is eluted in column chromatography by 2M HCl rather than by 5M HCl which elutes the free hydroxy acid. This finding explains the decrease in 3-hydroxyanthranilic acid and increase in 3-hydroxykynurenine in the eluates from the chromatography of hydrolysed urine compared with non-hydrolysed urine in all the subjects. The figures obtained for the total excretion of 3-hydroxyanthranilic acid and 3-hydroxykynurenine in the 2 and 5M HCl eluates are less with non-hydrolysed urine than with hydrolysed urine. It is possible that other conjugates of both compounds were lost from the columns in the initial washings with 0·5M HCl. In the present work it was not necessary to identify the conjugates of these metabolites since all were hydrolysed by boiling in M HCl to give the free hydroxy acids. To obtain complete separation of these metabolites it is therefore necessary to hydrolyse any conjugates which may be present in the urine before column chromatography. Since the degree of conjugation clearly differs from subject to subject, any results obtained using non-hydrolysed urines will be misleading.

Overall effects of salicylates on tryptophan metabolism

In the presence of salicylates the rate of metabolism of tryptophan and the excretion of its metabolites may be affected by the associated changes in serum tryptophan concentrations and by the effect of salicylates on the enzymes involved in the metabolism of tryptophan. Since free serum tryptophan is increased in the presence of salicylates it is possible that more tryptophan would enter the kynurenine pathway and this could perhaps account for the observed increase in xanthurenic acid, but not the decrease in 3-hydroxyanthranilic acid. Tryptophan, itself at very high levels, is known to activate the first enzyme of this pathway namely tryptophan pyrrolase in various species (Knox & Mehler, 1950). However, in man, given a test load of 2 g L-tryptophan there was no increase in the activity of tryptophan pyrrolase (Altman & Greengard, 1966). It is therefore unlikely in the present work that this enzyme

is activated by the small increase in free tryptophan observed *in vivo* after aspirin, without a tryptophan load.

It has recently been reported that salicylates at a concentration of 1 mM inhibit the enzyme tryptophan pyrrolase *in vitro* by 74% (Satoh & Moroi, 1969). Since this was the mean serum salicylate level determined *in vivo*, it is more likely that the tryptophan pyrrolase activity is inhibited by salicylates rather than activated by the small increase in free tryptophan. Any increase in the excretion of tryptophan metabolites may simply be due to the presence of more tryptophan available for entrance to the pathway. Salicylates, *in vitro* inhibit the enzyme 3-hydroxyanthranilic acid oxidase (Vescia & di Presco, 1962). One would therefore expect a block in the pathway at this point with an increase in the excretion of 3-hydroxyanthranilic acid. Since this is not observed it seems probable that salicylates may either be exerting an inhibitory effect on the kynureninase enzyme responsible for the conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid or activation of the amino-transferase enzyme involved in the formation of xanthurenic acid (Fig. 1). Salicylates are known to be generally inhibitory with regard to transaminases (Gould & Smith, 1965). However, salicylates have been found to activate L-tryptophan-α-oxoglutarate aminotransferase (Gould & Smith, 1965).

The present work shows that it is important to take account of the effects of drugs used in treatment before ascribing changes in tryptophan metabolism to pathological states. Patients with rheumatoid arthritis frequently take aspirin. This may influence tryptophan metabolism by increasing the level of free tryptophan in the blood and by changing the pattern of the urinary excretion of its metabolites.

In conclusion, the presence *in vivo* of salicylates in blood results in the competitive

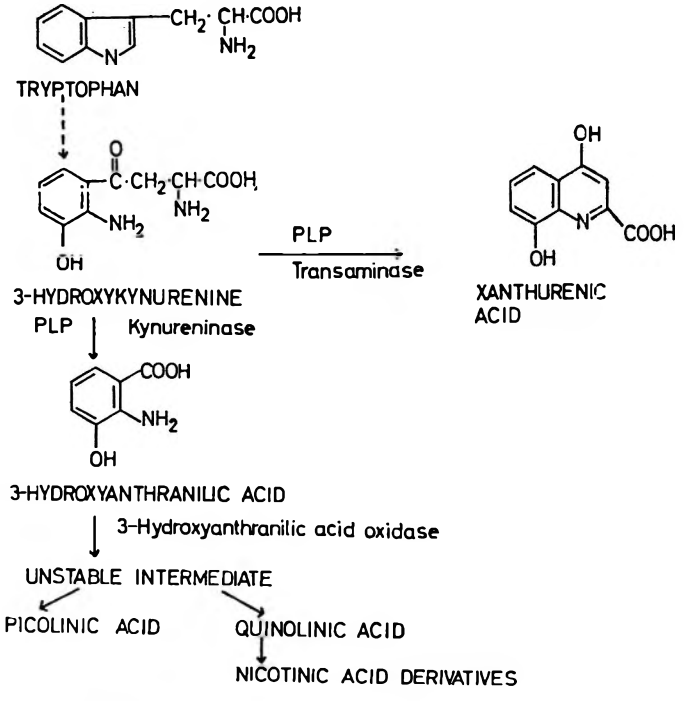

FIG. 1. Pathways of tryptophan metabolism. PLP = Pyridoxal phosphate.

displacement of tryptophan bound to serum albumin and a lowering of the total tryptophan in serum, and to an alteration in the pattern of excretion of at least two of the tryptophan metabolites in the kynurenine pathway, indicating an effect on the enzyme systems involved in the metabolism of tryptophan. The presence in the urines of conjugates of the metabolites studied clearly indicates the necessity to hydrolyse all urine samples before using column chromatography to estimate these compounds.

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The identification, isolation, and preservation of Δ^9-tetrahydrocannabinol (Δ^9-THC)*†

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(−)-*trans*-Δ^9-Tetrahydrocannabinol (Δ^9-THC) was isolated from marihuana plant extract, by adsorptive column and glc. The adsorptive column chromatography method consisted of chromatographing marihuana extract on a column packed with a mixture of silica gel (gas chromatography grade (100/120 mesh), silver nitrate and calcium sulphate ($CaSO_4 \cdot H_2O$) (3 : 1 : 0·5) with benzene as the eluting solvent. The glc method consisted of chromatographing the extract on a 3 ft silanized glass column ($\frac{3}{8}$ inch o.d.) packed with 1·5 ft of 2% QF-1 and 1·5 ft of 2% OV-17 on chromosorb W, AW 30–60 mesh, prep grade. A purity of 99% for the isolated Δ^9-THC was confirmed by infrared spectroscopy, nuclear magnetic resonance, mass spectroscopy. The effects of storage conditions on Δ^9-THC stability, monitored by glc, indicated the best method for preserving Δ^9-THC was at 0°, protected from light, stored under nitrogen.

Mechoulam & Gaoni (1967), Turk, Forney & others (1969) and most recently Mechoulam (1970) have reviewed adsorptive column chromatography techniques for the isolation of Δ-9-tetrahydrocannabinol (Δ^9-THC) and other cannabinoids from marihuana plant material.

Kingston & Kirk (1961), Farmilo & Davis (1961), Heaysman, Walker & Lewis (1967), Caddy, Fish & Wilson (1967), Lerner & Zeffert (1968), and Turk (1970) have reported methods for the separation and identification of marihuana components by gas liquid chromatography. However, methods for the separation and isolation of Δ^9-THC and other cannabinoids by preparative glc have not been found in the literature.

This paper reports two chromatographic methods for isolating Δ^9-THC and its stability under various storage conditions.

MATERIALS AND METHODS

Extraction of marihuana. Thailand marihuana was sieved through a 20 mesh screen and extracted in a Soxhlet apparatus with light petroleum (65–75°) for 3 h or until the glc analysis indicated the extraction of Δ^9-THC to be complete. The light petroleum extract was then washed with an aliquot of 4% NaOH in 50% ethanol (3 × 10 ml). The basic ethanolic fraction containing the cannabinoids was

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acidified to approximately pH 4 with 6N HCl and re-extracted with light petroleum (3 × 10 ml). This extract was flash evaporated under reduced pressure. The extracted resin was then dissolved in benzene for adsorptive column chromatography (3·2–3·5 g extract/5 ml benzene) or in chloroform for preparative glc.

Analytical assay of cannabinoids by gas liquid chromatography. The instrument was an F and M (Hewlett-Packard) Scientific 402 High Efficiency Gas Chromatograph, with a flame ionization detector and an L and N Speedomax W (0–1 mv) recorder having a chart speed of 30 inches/h. A single "U" shaped silanized glass column (4 ft by $\frac{1}{4}$ inch o.d.) was used; the first 1·5 ft were packed with 10% QF-1, the second 1·5 ft with a mixture of 1% OV-1, 1% OV-17 and the last 10 inches (nearest the detector side) with 2% OV-17. Each of these polymers were coated on chromosorb G, AW-DMCS, 100/120 mesh. The operating conditions were: injector port 285°; oven temp. 240°; flame detector 285°; carrier gas (He) flow 60 ml/min; hydrogen and oxygen flow rates 35 and 260 ml/min, respectively.

Isolation of Δ^9*-THC by adsorptive column chromatography.* Silica gel (gas chrom. grade 100/120 mesh), silver nitrate and calcium sulphate ($CaSO_4 \cdot H_2O$) (250 g in a ratio of 3 : 1 : 0·5) (all materials were from Matheson, Coleman and Bell); were ground for 12 h, dried (85–90°) for approximately 2 h and 500 g of the hot adsorbent slurried with approximately 1 litre of benzene. The slurry was shaken until free of clumps and then transferred to a glass column (4·8 × 60 cm), which settled to a bed of approximately 4·8 × 45 cm. Benzene was passed through the column for 6 h, and then 3·2 to 3·5 g of marihuana resin per 5 ml benzene was placed on the column and eluted with benzene. Fractions (10 ml) were collected, concentrated by flash evaporation and the concentrate analysed by glc. All fractions containing Δ^9-THC, 99% pure, were pooled, washed three times with 0·2N HCl to remove the $AgNO_3$ and then stored under nitrogen at 0° protected from light.

Preparative glc isolation of Δ^9*-THC.* A Varian Aerograph Autoprep Model 712, equipped with an effluent splitter, collection system, and flame ionization detector was used. A single ovoid silanized glass column (3 ft by $\frac{3}{8}$ inch o.d.) was packed with 1·5 ft of 2% QF-1 and 1·5 ft of 2% OV-17 on chromosorb W, AW-DMCS 30–60 mesh. The operating conditions were: oven 230°; injector/detector 285°; exit tip 250°; carrier gas (He) flow 182 ml/min; hydrogen flow 33 ml/min; air flow 220 ml/min; and split ratio 10 to 1. Fig. 1 is a typical chromatogram produced by this system.

Decomposition of Δ^9*-THC stored under various conditions.* Δ^9-THC (pure by glc) was divided into six fractions. Each fraction was placed in a clear vial and stored under a different condition. The content of each vial was analysed periodically by glc, over five months, to determine the extent of decomposition. The storage conditions are listed in Table 2.

RESULTS AND DISCUSSION

Thailand marihuana after NaOH-ethanolic washing produced the gas chromatogram seen in Fig. 2. For an efficient separation of the cannabinoids, the ratio of Δ^9-THC to CBN must be at least 2:1. The marihuana used had a dry weight content of 4–6%, Δ^9-THC. Marihuana, having a Δ^9-THC content of 1% or less, does not yield pure Δ^9-THC in sufficient quantities to justify using this method.

Glc was primarily used to determine the components present in the eluted

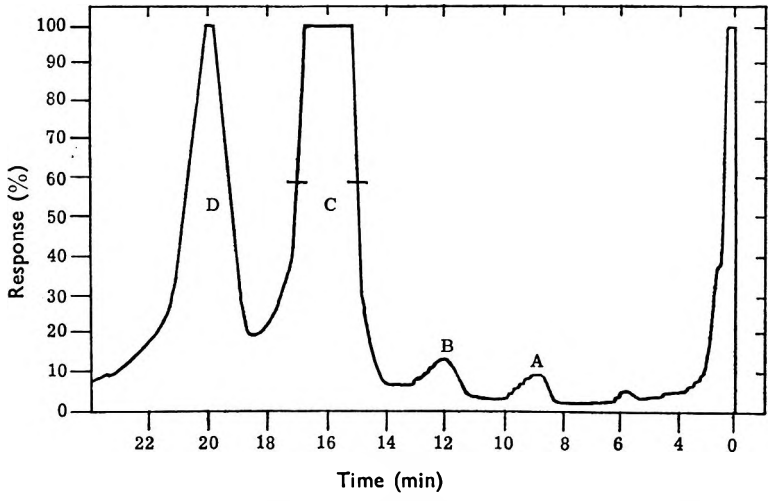

FIG. 1. Preparative gas chromatogram of marihuana extract and retention time of the cannabinoids. Column: 2% QF-1, 2% OV-17 on chromosorb W, AW 30/60 mesh. A. Cannabidiol. B. Δ^8-THC. C. Δ^9-THC. D. Cannabinol.

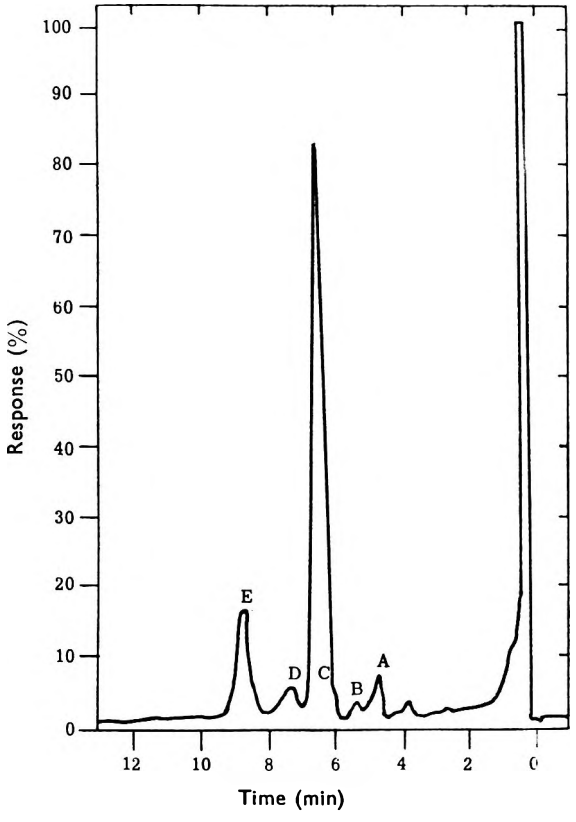

FIG. 2. Gas chromatogram of Thailand marihuana after NaOH-ethanolic washing. Column: 4 ft silanized glass column packed with 1·5 ft of 10% QF-1, 1·5 ft of a mixture of 1% OV-17, 1% OV-1, and the last 10 inches (nearest the detector side) with 2% OV-17. All polymers coated on chromosorb G, AW-DMCS, 100/120 mesh. A. Cannabidiol. B. Δ^8-THC. C. Δ^9-THC. D. Unknown. E. Cannabinol.

adsorptive column fractions. Δ^9-THC and Δ^8-THC can be separated using this analytical system (Fig. 3).

Pure cannabinol starts to be eluted at approximately 600 ml and ceases to be eluted after 800 ml; for Δ^9-THC the volumes are 900 ml to 1200 ml.

Adsorptive column chromatography. Purity of the Δ^9-THC isolated by this method was established by comparison of the infrared spectrum of Δ^9-THC with that for Δ^9-THC reported by Mechoulam & Gaoni (1967), by comparison of the nuclear magnetic resonance spectrum of the material with the known structure of Δ^9-THC

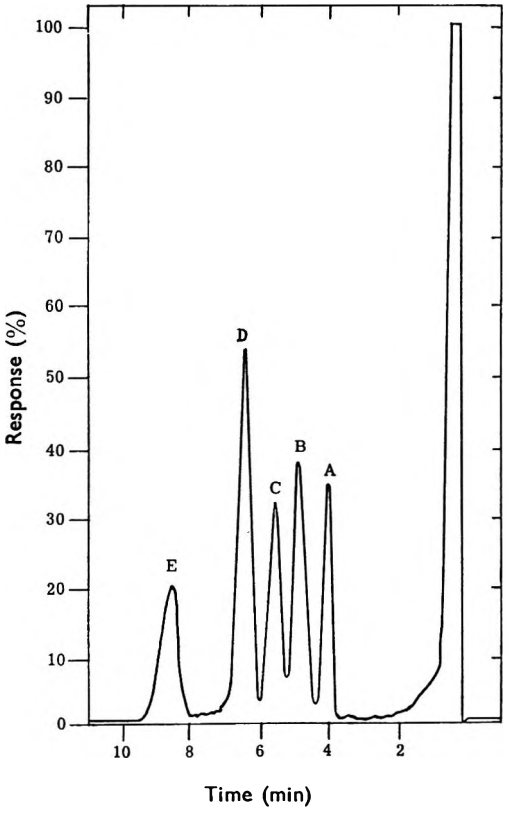

FIG. 3. Cannabinoids which can be separated, and detected in marihuana by gas liquid chromatography analysis. A. Cannabicyclol. B. Cannabidiol. C. Δ^8-THC. D. Δ^9-THC. E. Cannabinol.

(Mechoulam & Gaoni, 1967) and by mass spectrometry of the material which showed the isolated component was compatible with the spectrum reported for Δ^9-THC by Claussen, Fehlhaber & Korte (1969).

Pure cannabinol was also isolated by the adsorptive column chromatography method.

The efficiency of the method can be seen in Table 1. The Thailand marihuana used contained 4% (w/w) of Δ^9-THC. Recovery was 55%.

The amount of carboxylic precursors of THC isomers in the sample was not determined.

Table 1. *Recovery (%) of Δ⁹-Tetrahydrocannabinol (Δ⁹-THC)*

Sample	Weight of △⁹-THC in sample (g)	%△⁹-THC in sample (w/w)	% of Step I
Manicured Thailand marihuana (60 g) ..	2·40	4·0	—
Step I Solvent extraction (6·4 g Resin)	2·40	37·5	100
Step II NaOH-ethanolic washing (3·2 g Resin) ..	2·20	68·7	91·7
Step III Yield of △⁹-THC after adsorptive column chromatography (1·325 g△⁹-THC) ..	1·32	99·0	55

Preparative glc isolation of Δ⁹-THC. The advantage of preparative glc purification is that a compound can be isolated, collected, and stored under inert gas in one operation. In addition, more than one component can be collected if sufficient is present (see Fig. 1).

Purity of the isolated Δ⁹-THC was confirmed by infrared spectroscopy. The yield was 9 mg/24 h.

The Δ⁹-THC obtained by column chromatography was of equivalent purity but the amount of Δ⁹-THC that can be purified by the two methods differs widely. In 48 h (the time taken to elute Δ⁹-THC from the absorptive column) the average yield of 99% Δ⁹-THC from an adsorptive column was 1·2 g, whereas, with glc the amount was 18 mg.

Decomposition of purified Δ⁹-THC stored under various conditions. Table 2 shows the effects of storage conditions on the stability of Δ⁹-THC from marihuana. The findings were that the major decomposition product was cannabinol. Δ⁹-THC stored in acetone at room conditions decomposed at a more rapid rate than did the material stored under N_2 or exposed to air at room conditions. Decomposition was significantly inhibited by storage under nitrogen, air, or in acetone at 0° in the dark.

Table 2. *Decomposition (%) of purified Δ⁹-THC stored under various conditions*

		Room temperature, exposed to light			Refrigerated (0°), in dark		
Time analysed		Under N_2	Under air	In acetone	Under N_2	Under air	In acetone
Initial analysis	THC	100	100	100	100	100	100
	CBN	—	—	—	—	—	—
19th day	THC	100	100	96·7	100	100	100
	CBN	—	—	3·3	—	—	—
2nd month	THC	95·1	97·4	91·4	100	100	100
	CBN	4·9	2·6	8·6	—	—	—
4th month	THC	64·1	71·4	11·1	100	96·9	100
	CBN	35·9	28·6	88·9	—	3·1	—
5th month	THC	28·4	5·9	2·4	97·9	97·1	98·0
	CBN	71·6	94·1	97·6	2·0	2·9	2·0

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Mass spectrometry of fluorinated corticosteroidal 1,4-dien-3-ones of the British Pharmacopoeia

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The mass spectrum of each of the B.P. 1968 fluorinated corticosteroidal 1,4-dien-3-ones is shown to be sufficiently characteristic to be of value as a means of identification. Mechanisms for the principal fragmentations are discussed.

Mass spectrometry has received significant recognition in the pharmaceutical literature as a powerful analytical tool (Coutts, 1968; Coutts and Locock, 1968, 1969; Dreyfuss, Cohen & Hess, 1968; Shipchandler & Soine, 1968; Lee & Soine, 1969). It is our experience that the mass spectrum of a corticosteroid is characteristic for any particular compound, and can serve as an unequivocal means of identification. One particular advantage of this technique is that the molecular weight of the compound is indicated.

Although in a recent communication (Lodge & Toft, 1970) we described a fundamental difference between the mass spectra of betamethasone and dexamethasone, a comparative study of all the fluorinated corticosteroidal 1,4-dien-3-ones of the B.P. has not been reported. Reference standards are provided in the B.P. for betamethasone (9α-fluoro-11β,17α,21-trihydroxy-16β-methylpregna-1,4-dien-3,20-dione), betamethasone 17-valerate, betamethasone 21-valerate, dexamethasone (9α-fluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-dien-3,20-dione), fluocinolone acetonide (6α,9α-difluoro-11β,21-dihydroxy-16α,17α-isopropylidenedioxypregna-1,4-dien-3,20-dione) and triamcinolone acetonide (9α-fluoro-11β,21-dihydroxy-16α,17α-isopropylidenedioxypregna-1,4-dien-3,20-dione). In addition, we have included triamcinolone acetonide 21-t-butylacetate for comparison purposes with the other acetonides.

Significant differences in the mass spectra of isomeric pairs of compounds can often be seen, as, for example, with betamethasone (Fig. 1a) and dexamethasone (Fig. 1b), which are configurational isomers at C-16 (Lodge & Toft, 1970). The isomeric pair of esters betamethasone 17-valerate (Fig. 1c) (the ester of a tertiary alcohol) and betamethasone 21-valerate (Fig. 1d) (the ester of a primary alcohol) show several differences in a comparison of their respective mass spectra. The 17-valerate produces a relatively large amount of a fragment of m/e 315, apparently due to loss of one molecule of valeric acid plus carbons 20 and 21 with their attendant groups. The molecular ion (m/e 476) is hardly seen at all. The 21-valerate has a much more significant molecular ion, and relatively much less of the m/e 315 fragment. The base peak in the spectrum of the 21-valerate is at m/e 122, arising from cleavage of the 6–7 and 9–10 bonds and transfer of two hydrogens to the charged fragment (the A-ring plus C-6 and C-10); there is rather less of the fragment of m/e

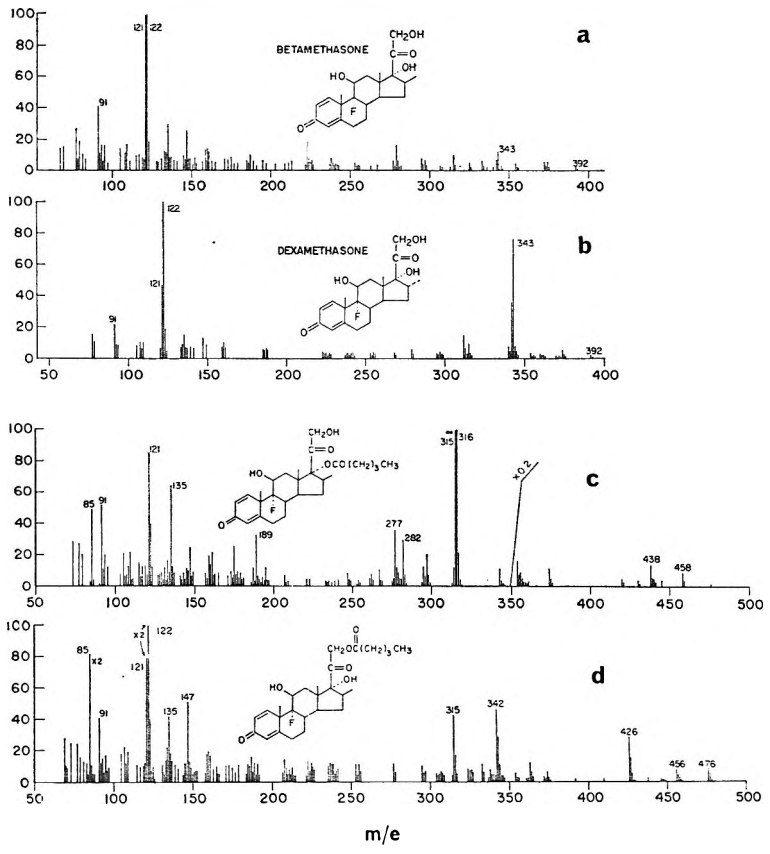
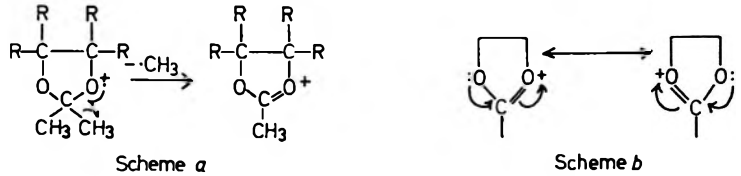


FIG. 1a. Betamethasone—9α-fluoro-11β,17α,21-trihydroxy-16β-methylpregna-1,4-dien-3,20-dione. b. Dexamethasone—9α-fluoro-11β,17α,21-trihydroxy-16α-methylpregna-1, 4-dien-3,20-dione. c. Betamethasone 17-valerate. d. Betamethasone 21-valerate.

121, formed from the same cleavage but with the transfer of only one hydrogen. With the corresponding peaks in the spectrum of the 17-valerate, the situation is reversed, the m/e 121 fragment predominating.

It has been reported (Budzikiewicz, Djerassi & Williams, 1967) that molecular ion peaks are not detectable in the mass spectra of acetonides, and that the peak of highest mass is the M–15 species, which arises as shown in scheme *a*. The ion formed is stabilized by resonance of the positive charge between the two oxygen atoms (scheme *b*). In the case of the three corticosteroid acetonides a molecular ion is seen. This difference is probably due to the fact that the earlier spectra were obtained on instruments with indirect inlet systems which involved heating the sample before entering the ion chamber. In our work the samples were vaporized directly in the



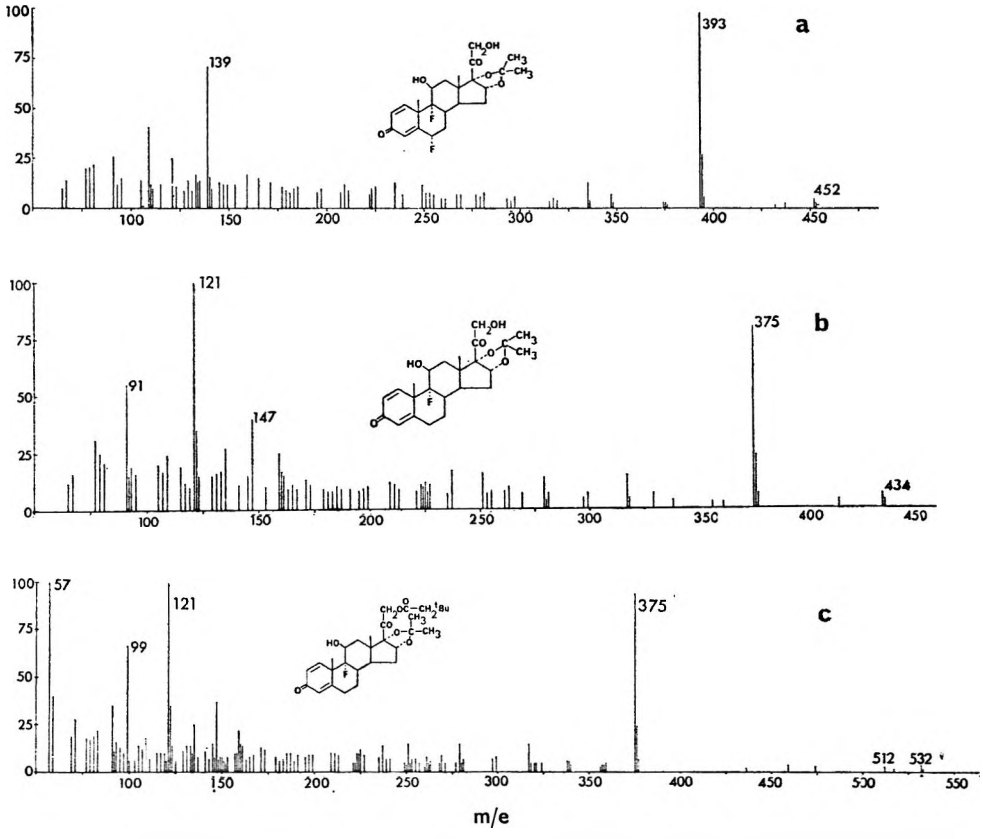
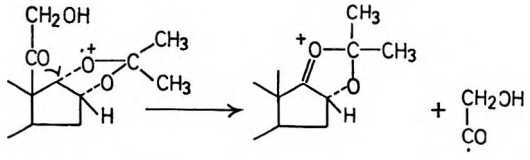


FIG. 2. a. Fluocinolone acetonide—6α,9α-difluoro-11β,21-dihydroxy-16α,17α-isopropylidenedioxypregna-1,4-dien-3,20-dione. b. Triamcinolone acetonide—9α-fluoro-11β,21-dihydroxy-16α,17α-isopropylidenedioxypregna-1,4-dien-3,20-dione. c. Triamcinolone acetonide 21-t-butylacetate.

ion chamber thus minimizing thermal decomposition before ionization. (For identification purposes it is preferable to compare the spectrum of an unknown with the spectrum of the standard obtained under identical conditions and on the same instrument.) In two of the spectra a small peak (Fig. 2a and c) is seen at M–15 (m/e 517 for triamcinolone acetonide 21-t-butylacetate and m/e 437 for fluocinolone acetonide). This is presumably due to loss of methyl from the acetonide by scheme *a*. Such a fragment from triamcinolone acetonide was not significant enough to be measured on this scale.

There is apparently an alternative competing fragmentation (scheme *c*) which allows the formation of a different stable ion. Loss of the two carbon side chain



Scheme *c*

from C–17 is probably preferred because the fragment O=C–CH_2OH is relatively much more stable than CH_3.

Triamcinolone acetonide (Fig. 2b) and its 21-t-butylacetate (Fig. 2c) both show fragments at m/e 375, corresponding to cleavage of the 17–20 bond. Fluocinolone acetonide (Fig. 2a), being in effect 6-fluoro-triamcinolone acetonide, shows a corresponding fragment at m/e 393, the additional fluorine having, and indeed expected to have, no effect on this fragmentation.

The other principal fragmentation is again cleavage of the 6–7 and 9–10 bonds, with the transfer of one hydrogen to the charged fragment. In triamcinolone acetonide and its ester this causes a fragment at m/e 121; in the fragmentation of fluocinolone acetonide the 6α-fluorine atom remains with the charged fragment, which thus appears at m/e 139. The fluorine does not appear to affect the hydrogen-transfer process.

All three acetonides show a small fragment at M–20 (loss of HF) and this is common to all the compounds under consideration (except betamethasone 17-valerate which shows a peak at (M–18)–20 but not at M–20.

The spectrum of triamcinolone acetonide 21-t-butylacetate has two large peaks which do not appear in the spectra of the other two acetonides; both may be attributed to the ester moiety. The fragment of m/e 99 represents cleavage of the O–CO bond, and that of m/e 57 cleavage of the CH—$C(CH_3)_3$ bond, forming a relatively stable t-butyl ion. Betamethasone 21-valerate undergoes a similar cleavage of the O–CO ester bond to form a comparable fragment of m/e 85.

Conclusion

Mass spectrometry affords a means of distinguishing between corticosteroids which are closely related chemically. In our experience, the mass spectrum of each of these compounds is unique, and thus the technique is a powerful analytical tool.

METHOD

Mass spectra were recorded on a Hitachi-Perkin-Elmer Model RMU-6D or an AEI model MS-12 mass spectrometer with an ionization voltage of 70 eV. The compounds were introduced directly into the ion chamber, and the temperature of the probe was raised until a sufficient vapour pressure of compound was obtained that the spectrum could be measured. Probe temperatures were in the range 180–250°.

The compounds are part of the Food and Drug Directorate collection of reference steroids.

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The role of cyclic AMP in isoprenaline-induced cardiac necroses in the rat*

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Single administrations of isoprenaline chloride (6–36 μg/kg, s.c.) resulted in the appearance of acute myocardial necroses in the rat within 24 h. The percentage of animals with lesions was directly related to the dose of isoprenaline. Dibutyryl cyclic AMP (1–50 mg/kg, i.p.) induced myocardial lesions of the same type, however the percentage of animals affected was not dose-dependent. Pretreatment with theophylline (75 mg/kg, s.c.) administered to inhibit cardiac phosphodiesterase activity, induced a 48-fold reduction in the ED50 of isoprenaline and potentiated the nucleotide-induced cardiac lesions which became dose-dependent. Thus, a mediatory role for cyclic AMP in the isoprenaline-induced cardiac necroses in the rat is suggested.

It is well known that isoprenaline and other catecholamines are capable of inducing myocardial necroses in the rat (Rona, Chappel & others, 1959; Chappel, Rona & others, 1959; Wexler & Kittinger, 1963; Ferrans, Hibbs & others, 1964; Kahn, Rona & Chappel, 1969). β-Adrenergic blocking agents were found to prevent competitively the appearance of these lesions (Mehes, Rajkovits & Papp, 1966; Dorigotti, Gaetani & others, 1969). Since Robison, Butcher & Sutherland (1967) suggested an association of the β-receptors with adenyl cyclase, this could indicate involvement of the adenyl cyclase-cyclic AMP system in the production of these lesions. This possibility was further strengthened by the finding that a rise in the heart levels of cyclic AMP occurred in the rat after catecholamine administration (Robison, Butcher & others, 1965; Namm & Mayer, 1968). Thus, if the cardiac lesions induced by the catecholamine isoprenaline were mediated through cyclic AMP, the phosphodiesterase inhibitor theophylline, by preventing the breakdown of cyclic AMP (Butcher & Sutherland, 1962) should potentiate the isoprenaline-induced lesions. Furthermore, the administration of exogenous cyclic AMP alone or in the presence of theophylline should induce similar pathological changes. This report deals with the results obtained in testing these two concepts.

METHODS

Male Wistar rats in the weight range of 250–270 g were used. The animals were divided into six groups as indicated in Table 1. Group I served as control and was injected with vehicle (normal saline). Groups II, III and V received isoprenaline, theophylline, or dibutyryl cyclic AMP respectively, at various doses. Groups IV and VI were pretreated with theophylline, followed in approximately 20 min by isoprenaline or dibutyryl cyclic AMP, respectively.

Twenty-four h after drug administration all animals were killed by decapitation.

* A preliminary report of some of these results was presented at the Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics, August 1970, Palo Alto, California.

The hearts were removed, fixed in a solution of 5% formal saline dehydrated in ethanols (70 to 90%) followed by amyl acetate (100%), cleared in toluene and embedded under vacuum in paraffin. Four longitudinal sections (6 μm) of each heart were made and stained with haematoxylin eosin. The hearts were considered lesioned when, under microscopical examination, at least one focus of acute myocardial necrosis was observed (Dorigotti & others, 1969). When applicable, drug ED50's and 95% confidence limits for the production of myocardial necroses were calculated by the methods of Litchfield & Wilcoxon (1949).

All drugs were freshly dissolved in normal saline and injected in volumes of 0·2 ml/100 g subcutaneously, with the exception of dibutyryl cyclic AMP which was injected intraperitoneally. Doses used are expressed as salts, with the exception of theophylline which is expressed as base.

The following drugs were used: theophylline ethylenediamine (aminophylline, BDH); dibutyryl cyclic AMP (N^6–$O^{2'}$-dibutyryl adenosine-3′-5′-cyclic phosphate monosodium,$5H_2O$, Calbiochem); (±)-isoprenaline hydrochloride [(±)-isopropylnoradrenaline hydrochloride, K & K Labs].

RESULTS

At macroscopic examination, no gross pathological changes were seen in rat hearts of any group 24 h after drug administration.

Histologically, foci of acute myocardial necrosis were seen in the cardiac ventricles of a certain percentage of rats in all drug-treated groups. The areas most frequently affected were the papillary muscles and the apices; the ventricular walls, however, were also sometimes affected. The lesions appeared as typical, acute, focal necroses consisting of fragmentation and vacuolization of the myocardial fibres, and mononuclear inflammatory cell infiltrates with a few scattered polymorphonuclear leucocytes. Organized, chronic cardiac lesions were seen in approximately 2% of rats used and were considered to be endemic in the rat population. In all groups, only those animals showing the acute type of lesions (acute myocardial necroses) were considered in the results.

As indicated in Table 1, no acute myocardial lesions were seen in the saline-treated rats (Group I). After isoprenaline (Group II) the number of animals showing acute focal myocardial necroses was found to be dose-dependent, the ED50 being 13 μg/kg. Theophylline (Group III) caused acute focal cardiac necroses in only 1/10 rats at 75 mg/kg and in 3/10 rats at 150 mg/kg whereas higher doses were lethal. On the other hand, pretreatment with the lower dose of theophylline (75 mg/kg) resulted in a 48 fold reduction in the ED50 of isoprenaline, it now being 0·27 μg/kg (Group IV).

The occurrence of lesions with dibutyryl cyclic AMP (Group V) were not dose-dependent, 1/10 rats presenting acute focal cardiac necroses at 1 (Fig. 1A), 10 and 50 mg/kg. Once again, however, pretreatment with theophylline (75 mg/kg) markedly facilitated the production of cardiac lesions resulting from the administration of dibutyryl cyclic AMP (Group VI), which now appeared to be dose-dependent, the ED50 was 0·55 mg/kg.

Thus, pretreatment with theophylline markedly increased the number of animals exhibiting acute focal myocardial necroses after administration of either isoprenaline or dibutyryl cyclic AMP. Additionally, in some affected animals the histological picture changed from that of an isolated focus to that of disseminated focal necroses (Fig. 1B).

Table 1. *Number of rats showing acute myocardial necroses.**

Group	Treatment	Dose/kg	Route	No with lesions/ No tested	ED50/kg	95% Confidence Limits
I	Normal saline	—	s.c.	0/30	—	—
II	Isoprenaline	6 μg	s.c.	1/10		
		14 μg	s.c.	6/10	13·00 μg	(8·10–20·80)
		36 μg	s.c.	9/10		
III	Theophylline	75 mg	s.c.	1/10	>150·00 mg	—
		150 mg	s.c.	3/10		
IV	Theophylline† + isoprenaline	0·1 μg	s.c.	4/10		
		0·3 μg	s.c.	5/10	0·27 μg	(0·09–0·81)
		1·0 μg	s.c.	8/10		
V	Dibutyryl cyclic AMP	1 mg	i.p.	1/10		
		10 mg	i.p.	1/10	>50·00 mg	—
		50 mg	i.p.	1/10		
VI	Theophylline† + dibutyryl cyclic AMP	0·25 mg	i.p.	4/10		
		0·50 mg	i.p.	4/10	0·55 mg	(0·23–1·26)
		1·00 mg	i.p.	7/10		

* Hearts were examined 24 h following treatment.
† Theophylline (75 mg/kg, s.c.) was administered 20 min before isoprenaline or dibutyryl cyclic AMP. Doses listed refer to the latter two compounds.

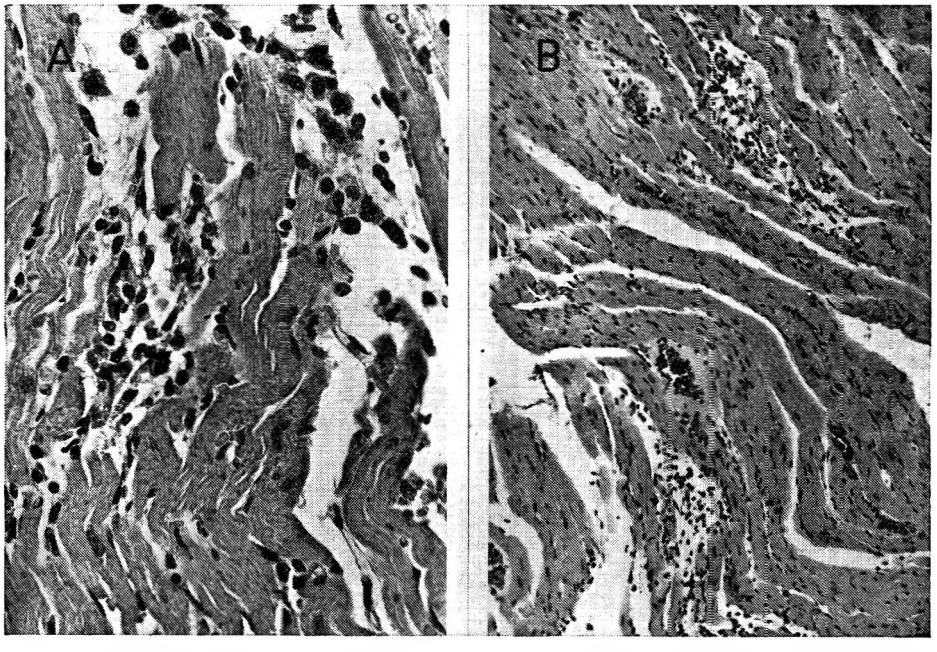

FIG. 1.A. Photomicrograph of a section of a heart of a rat injected with dibutyryl cyclic AMP (1 mg/kg, i.p.) and killed at 24 h. A necrotic focus is shown. H.E. × 630.
B. Photomicrograph of a section of a heart of a rat injected with theophylline ethylenediamine (75 mg/mg, s.c.) and dibutyryl cyclic AMP (1 mg/kg, i.p.) and killed at 24 h. Disseminated foci of necrosis are shown. H.E. × 196.

DISCUSSION

Robison & others (1965) and Namm & Mayer (1968) showed that a rise in the myocardial level of cyclic AMP occurred in the rat after catecholamine administration. This increase in cardiac cyclic AMP levels appearently results from stimulation of β-adrenergic receptors, which are thought to be associated with adenyl cyclase (Robison & others, 1967). Thus, the finding that β-adrenergic blocking agents competitively prevent the appearance of catecholamine-induced myocardial necroses in this species (Mehes, Rajkovits & Papp, 1966; Dorigotti & others, 1969) suggested an involvement of the adenyl cyclase-cyclic AMP system.

In the present experiments, support for the involvement of this system in catecholamine-induced myocardial necroses was provided by the use of theophylline, a phosphodiesterase inhibitor which prevents the breakdown, and thus, inactivation of cyclic AMP (Butcher & Sutherland, 1962). Indeed it was found that even minimal doses of theophylline markedly potentiated isoprenaline, a β-adrenergic receptor stimulant, in its ability to induce acute myocardial necroses.

On the other hand, administration of dibutyryl cyclic AMP alone failed to mimic the isoprenaline-induced lesions on the rat heart. However, Robison & others (1965) also did not succeed in mimicking the myocardial effects of catecholamines in the isolated perfused rat heart with either cyclic AMP or the dibutyryl derivative. Apparently, these authors found that the rat heart exhibits a low permeability toward the nucleotides, coupled with a high phosphodiesterase activity. This latter aspect may be the more important one, since as shown in the present experiments, pretreatment with theophylline markedly potentiated the dibutyryl cyclic AMP-induced acute myocardial lesions, which became dose-dependent. This finding provides additional support for the involvement of cyclic AMP in catecholamine-induced cardiac lesions.

Consequently, in rats a mediatory role for the adenyl cyclase-cyclic AMP system may explain the pathological changes induced in the myocardium by the administration of β-adrenergic receptor stimulants such as isoprenaline.

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The effect of thymoxamine on peripheral blood vessels as monitored by the ^{133}Xe clearance technique

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The rate of clearance of intra-articularly administred ^{133}Xe provides an indirect method for the measurement of synovial tissue perfusion. The effect of an intra-articular injection of thymoxamine, and of isoprenaline was to increase the clearance rate whereas the converse was seen with noradrenaline. The prior administration of thymoxamine did not affect the isoprenaline response. In suitable doses, thymoxamine both prevented, and reversed the noradrenaline effect.

The specific α-adrenergic blocking agent, thymoxamine which acts reversibly by competitive inhibition (Birmingham & Szolcsányi, 1965; Birmingham, Ernest & Newcombe, 1969), affords the opportunity of selectively studying α-receptor function. We have used this compound to examine adrenergic control of the peripheral blood vessels in canine synovium using a radio-isotope technique.

The clearance rate of radioactive xenon (^{133}Xe) from the diarthrodial joint cavity has been shown to be reproducible (Dick, Whaley & others, 1970a), and to provide an indirect measure of synovial tissue perfusion (Dick, St. Onge & others, 1970b).

In the present work the rate of clearance of intra-articularly injected ^{133}Xe from the canine stifle joint was used to monitor the effects of the active compounds on synovial perfusion. The effect of thymoxamine alone was studied, and, to further characterize the result, the effects of isoprenaline and noradrenaline alone and in combination with thymoxamine were examined.

MATERIALS AND METHODS

Both stifle joints of adult greyhounds, 20 to 28 kg, were examined. Anaesthesia was induced with sodium thiopentone (20 mg/kg) and maintained with a 5:3 mixture of nitrous oxide and oxygen and additional (1%) trilene. A catheter in the brachial artery permitted continuous recording of blood pressure and pulse rate.

Injection technique

A 15/16 gauge needle was inserted into the joint cavity by a medial infra-patellar approach and its position confirmed by aspiration of clear synovial fluid. If the aspirate was blood stained, the joint was not used. The radioactive gas xenon (^{133}Xe) (Radiochemical Centre, Amersham) was dissolved in sterile 0·9% sodium chloride and an amount less than 0·5 ml injected into the joint cavity, the needle remaining *in situ* and being closed by a syringe containing 0·9% sodium chloride. Each time an active compound was administered, $< 0{\cdot}25$ ml of ^{133}Xe solution was injected with it. At no time did the total volume injected exceed by more than 50% the amount of synovial fluid aspirated initially.

Counting technique

A lightly collimated detector (Ekco N559 D) incorporating a thallium activated sodium iodide scintillation crystal and photomultiplier was positioned 2 inches from, and directly above the patella. Pulses were fed through a pulse height analyser and ratemeter (Ekco 1750) and recorded on a Rikadenki chart recorder (B24) at a paper speed of 20 mm/min.

The graphs were sampled at 1 min intervals and the absolute count rate was plotted onto semilogarithmic graph paper as a function of time, monitoring being continued throughout each experiment. Baseline values were obtained from the period 1 to 15 min after ^{133}Xe injection, $T\frac{1}{2}$ values (min) being readily obtained from the semilogarithmic plots. As the clearance rate became slower, the semilogarithmic plots approached the horizontal and the errors of calculating the $T\frac{1}{2}$ values were magnified. Accordingly all values in excess of 150 min have been recorded simply as $T\frac{1}{2} = > 150$ min.

The effects of thymoxamine, isoprenaline and noradrenaline alone were studied in groups 1, 2 and 3. Isoprenaline was given after thymoxamine to group 4. Increasing doses of noradrenaline were given after a fixed dose of thymoxamine to group 5, and increasing doses of thymoxamine were given before a fixed dose of noradrenaline to group 6. Increasing doses of thymoxamine were also given after a fixed dose of noradrenaline to Group 7.

Drugs used were: thymoxamine (Warner), (±)-1-noradrenaline acid tartrate (Bayer) and isoprenaline sulphate in normal saline (Boots). These were diluted in sterile 0·9% sodium chloride solution.

RESULTS

The results are shown in Figs 1–3 and Table 1.

In eleven of the twelve studies in Group I (thymoxamine alone, 5, 10, 20 , 30 or 40 μg) the clearance rate after the drug greatly exceeded the baseline value (Table 1).

Table 1. *The effect of thymoxamine, isoprenaline and noradrenaline on the ^{133}Xe clearance rate.*

	Thymoxamine (Group 1)			Isoprenaline 2·5 μg (Group 2)		Noradrenaline 2·5 μg (Group 3)	
	$T\frac{1}{2}$ (min)		$T\frac{1}{2}$ (min)	$T\frac{1}{2}$ (min)		$T\frac{1}{2}$ (min)	
	Before	Dose (μg)	After	Before	After	Before	After
	42	40	26	58	16	66	89
	54	40	28	39	6	54	101
	23	40	17	44	21	38	120
	80	30	38	46	19	47	98
	48	30	38	64	28	42	86
	53	30	31	73	31	49	125
	89	20	42				
	68	20	30				
	44	10	26				
	56	10	38				
	*34	5	30*				
	48	5	16				
Mean	53		30	54	20	49	103
Range	23–89		16–38	39–73	6–31	38–66	86–125
No. showing change		11/ 12 (1 equivocal)		6/6		6/6	

* = equivocal response.

There was no clear relation between effect and the dose used. The remaining result was equivocal. In Groups 2 and 3 the results were consistent: in the six studies with isoprenaline (2·5 μg) the clearance rate after the drug exceeded the baseline values, and in the six studies with noradrenaline (2·5 μg) the clearance rate was reduced after injection (Table 1). Change in clearance rate was abrupt and marked in every instance. When isoprenaline (2·5 μg) was administered after thymoxamine (40 or 200 μg) (Group 4) a marked increase in clearance rate ensued [mean $T\frac{1}{2}$ after thymoxamine (with range) 37 (28–46) at 40 μg; 34 (31–38) at 200 μg, and after isoprenaline 19 (9–26).

The results obtained when increasing doses of noradrenaline (0·5, 1, 2·5, 10 μg) were given after a fixed dose (60 μg) of thymoxamine (Group 5) are in Fig. 1; a dose related response was obtained. When increasing doses of thymoxamine (10, 20, 40, 60, 80, 100 μg) were administered before to a fixed dose (2·5 μg) (Group 6) of noradrenaline, again a dose-related response was obtained (Fig. 2). At a 20 μg dose of thymoxamine, the clearance rate was subsequently abruptly and markedly reduced by noradrenaline. But with thymoxamine 80 or 100 μg no such response to noradrenaline was obtained. Variable responses were noted when the dose of thymoxamine was 40 or 60 μg.

Increasing doses of thymoxamine after noradrenaline (1·0 μg) (Group 7) gave no response until the dose reached 200 μg. Then the clearance rate was abruptly and markedly increased after injection of the thymoxamine (Fig. 3).

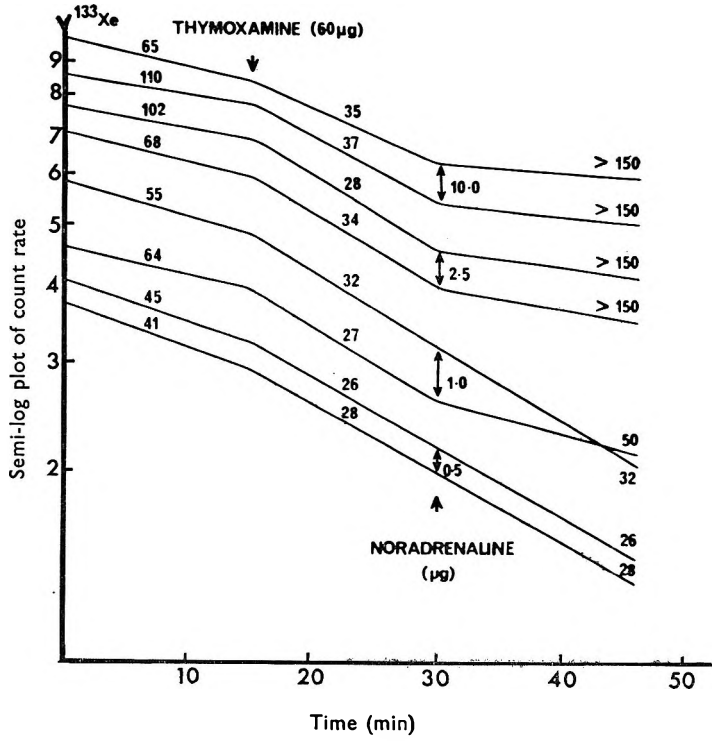

FIG. 1. The effect of increasing doses of noradrenaline after a fixed dose of thymoxamine on the ^{133}Xe clearance rate (Group 5). The $T\frac{1}{2}$ values before and after thymoxamine (60 μg) and after doses of noradrenaline from 0·5–10 μg are shown.

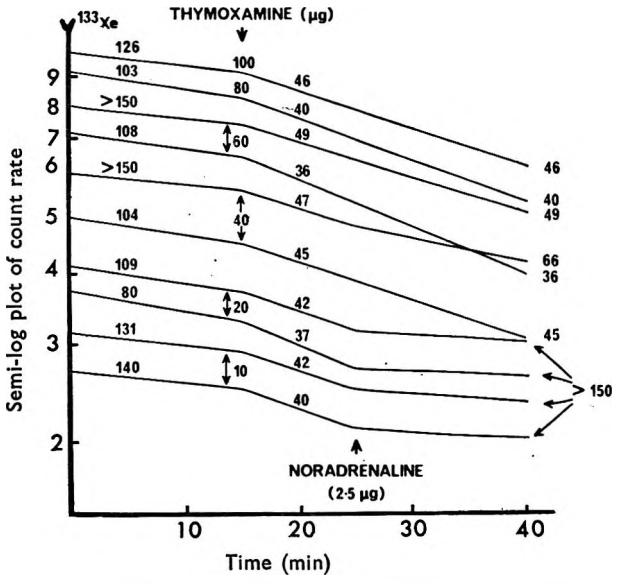

FIG. 2. Effect of increasing doses of thymoxamine before a fixed dose of noradrenaline on the ^{133}Xe clearance rate (Group 6). The $T_{\frac{1}{2}}$ values before and after thymoxamine (10–100 μg) and after noradrenaline (2·5 μg) are shown.

DISCUSSION

The monitoring system used is reproducible (Dick & others, 1970a) and provides an indirect quantitative measure of perfusion (Dick & others 1970b). The term perfusion is appropriate since blood flow through non-nutritive shunts will not be detected (Friedman, 1968). Although the confidence with which the results can be expressed in absolute units of perfusion cannot be complete because the method is

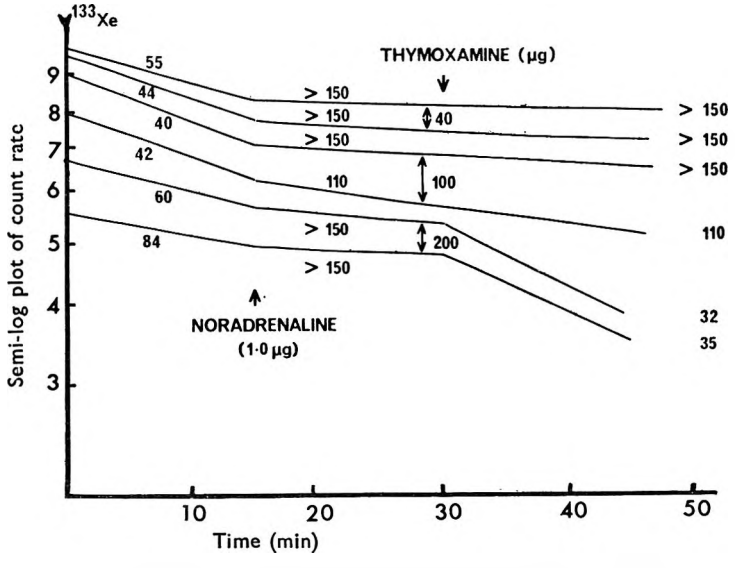

FIG. 3. Effect of increasing doses of thymoxamine after a fixed dose of noradrenaline on the ^{133}Xe clearance rate (Group 7). The $T_{\frac{1}{2}}$ values before and after noradrenaline (1 μg) and after thymoxamine (40–200 μg) are shown.

indirect and no other method is available for confirmation, the large changes in clearance rate associated with drug administration may be attributed to pharmacologically induced changes in perfusion. The results may be interpreted to indicate that whereas noradrenaline reduces synovial perfusion, intra-articular isoprenaline and thymoxamine increase clearance rate and therefore synovial perfusion rate. The results with noradrenaline, and with isoprenaline, are explicable in terms of their known effects on other vascular beds (Goodman & Gilman, 1965), but interpretation of the effect of thymoxamine is less obvious. Increase in clearance rate, and in perfusion rate may be the result of a direct effect of the drug on small blood vessels. But the evidence for this is inconclusive. A further possibility supported by the conclusions of Birmingham, Akubue & Szolcsányi (1967) and Turner, Harrison & Schoenfeldt (1969) is that the compound releases these peripheral vessels from a basal α-adrenergically mediated vasoconstrictor tone, which has been demonstrated previously in canine joints (Cobbold & Lewis, 1956).

The results also demonstrate that whereas thymoxamine has no influence on β-adrenergically mediated isoprenaline response, the drug will abolish the α-mediated effect of noradrenaline. The effect is dose related and can be modified by either increasing the dose of the agonist or reducing the dose of the blocking agent. Furthermore, the noradrenaline effect can be reversed by a large dose of thymoxamine. These results support the views of Birmingham & Szolcsányi (1965) that the mode of action of thymoxamine is by competitive antagonism. The dose ratio at which the modifying effect of thymoxamine was seen varied. When thymoxamine was given first (Groups 5 and 6) the dose ratio was about 60 (thymoxamine) to 1 (noradrenaline). However, when noradrenaline was given first the corresponding ratio was 200:1. Variation in affinity of the compounds for the receptor binding sites may explain this discrepancy.

It would seem from the results that the ^{133}Xe clearance technique provides a suitable model for the *in vivo* study of pharmacologically induced changes in perfusion. The method is particularly attractive since it involves only minimal interference with the physiological state of the animal.

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Mechanism of MJ 1999-induced blockade of neuromuscular transmission

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MJ 1999 [4-(isopropylamino-1-hydroxyethyl)methanesulphonanilide hydrochloride] produced a dose-related blockade of indirectly evoked twitches of the rat diaphragm, without materially altering the effects of direct stimulation. The magnitude of blockade was comparable whether the indirect stimulation was at 1/s or 1/20 s. The blockade was less pronounced at 25° than at 34°. The blockade was antagonized by neostigmine, potassium chloride, succinylcholine, tetraethylammonium, adrenaline, isoprenaline and, to some extent, by noradrenaline. Blockade by MJ 1999 was additive to that due to tubocurarine; large concentrations of yohimbine, tolazoline, phenoxybenzamine and dihydroergotamine quickly antagonized MJ 1999 blockade. Small concentrations of adrenaline, isoprenaline, tolazoline or yohimbine prevented MJ 1999 blockade. MJ 1999 had no effect on nerve conduction. It is proposed that MJ 1999 has a two-fold mode of action at the neuromuscular junction; a curare-like effect and an action on β-receptors.

Sympathomimetic amines and the stimulation of sympathetic nerves can affect the skeletal neuromuscular system in many ways (see Bowman & Nott, 1969). There is strong evidence that β-adrenoreceptors are involved in the direct actions of adrenaline on the mammalian muscle. However, the effects of β-adrenoreceptor blocking agents on neuromuscular transmission have not been extensively examined. Pronethalol and propranolol, *in vivo* and *in vitro*, have yielded variable results (Morales-Aguilerá & Vaughan Williams, 1965; Türker & Kiran, 1965; Standaert, Levitt & Roberts, 1966; Wislicki & Rosenblum, 1967; Usubiaga, 1968). The neuromuscular blockade observed was ascribed to a local anaesthetic action of these agents, to depression of motor nerve terminals or to reduced output of the transmitter.

The β-adrenoreceptor blocker, MJ 1999 (Lish, Weikel & Dungan, 1965) has little local anaesthetic activity either in specific tests (Lish & others, 1965; Schmid & Calvin, 1967) or in neuronally evoked responses of smooth muscle (Bartlet & Hassan, 1969). It seemed worthwhile therefore to investigate this agent further for reported neuromuscular blocking effects (Standaert & Roberts, 1967).

METHODS AND MATERIALS

The rat phrenic nerve diaphragm preparation

The preparations were obtained from male Norwegian rats, 200 to 300 g. The bathing medium was Tyrode solution, g/litre: NaCl 8·0, KCl 0·2, $CaCl_2$ 0·2, $MgCl_2$ 0·1, Na_2HPO_4 0·05, $NaHCO_3$ 1·0 and dextrose, 2·0, maintained at 34° ± 1° and

gassed with 5% carbon dioxide in oxygen; pH after equilibration was 7·6. Stimulation was carried out alternately every 10 s by supramaximal pulses applied directly to the muscle (pulse width 5·0 ms) or to the nerve (pulse width 0·2 ms).

The effect of blocking drugs was examined by determining the latency period, $T\frac{1}{2}$ (the time to half-decay of twitch tension), and also the magnitude of blockade. These parameters were computed from the plot of percentage blockade (semi-logarithmic scale) against time (min) as described by Freeman (1968).

Method for local anaesthetic activity

In 5 experiments about 2·5 cm of phrenic nerve along with the stimulating electrodes was bathed in a chamber separated from the one which lodged the hemidiaphragm and the remaining portion of the nerve (Matthews & Quilliam, 1964).

Drugs. The drugs used were (+)-tubocurarine chloride, neostigmine methylsulphate, choline chloride, succinylcholine chloride, tetraethylammonium bromide, yohimbine hydrochloride, tolazoline hydrochloride, phenoxybenzamine hydrochloride, dihydroergotamine methane-sulphonate, (−)-adrenaline (Sigma Chemical Co.), (±)-noradrenaline hydrochloride (Sigma Chemical Co.), (±)-isoprenaline hydrochloride (Fluka AG) and MJ 1999 [4-(isopropylamino-1-hydroxyethyl) methanesulphonanilide hydrochloride, Mead Johnson & Co.]. Doses of adrenaline, noradrenaline and isoprenaline refer to the base and of the other compounds to the salts.

RESULTS

Effect of MJ 1999 on the responses to electrical stimulation

MJ 1999 (6·25 to 25 μg/ml) reduced indirectly evoked twitches of the rat diaphragm; reduction of direct twitches was moderate and seen only in higher concentrations (Table 1). The regression of mean maximal blockade of indirect twitches on log dose of MJ 1999 was linear and highly significant ($P < 0·001$). With each concentration of MJ 1999 the decline of twitch height was exponential. MJ 1999 (30·0 μg/ml) produced a mean blockade of 90%; the mean latency period and $T\frac{1}{2}$ were 2·3 and 5·2 min, respectively (n = 16, Table 1). Using this as test concentration it was found

Table 1. *Rat phrenic nerve diaphragm preparation. Effect of MJ 1999 on electrical stimulation.*

Experimental conditions*	MJ 1999 (μg/ml)	n =	Indirect twitches. Mean ± s.e. Latency period (min)	% Blockade	Time to half-decay (min)	Direct twitches. Mean % blockade ± s.e.
1. Nil (control)	6·25	5	3·4 ± 0·3	27·0 ± 2·1	—	7·0 ± 5·0
	12·5	5	2·6 ± 0·2	54·5 ± 3·0	—	15·5 ± 3·5
	25·0	8	2·5 ± 0·2	80·0 ± 1·3	5·3 ± 0·6	18·5 ± 4·2
	30·0	16	2·3 ± 0·3	90·0 ± 3·2	5·2 ± 0·7	20·0 ± 2·5
2. Indirect stimulation at 1/s	30·0	5	3·1 ± 0·4	85·5 ± 3·8	5·8 ± 1·1	—
3. Bath temperature 25°	30·0	5	4·4 ± 0·9†	65·0 ± 5·0†	11·1 ± 1·2†	17·0 ± 1·5
4. In presence of						
adrenaline (60 ng/ml)	30·0	4	—	0·0†	—	0·0
isoprenaline (60 ng/ml)	30·0	3	—	0·0†	—	0·0
tolazoline (7 μg/ml)	30·0	4	10·2 ± 3·0†	10·0 ± 2·0†	—	0·0
yohimbine (5 μg/ml)	30·0	3	8·3 ± 3·6†	7·0 ± 4·0†	—	0·0

* Tyrode solution at 34° C, gassed with 5% carbon dioxide in oxygen, pH 7·6; stimulation, supramaximal pulses, direct (width 5·0 ms) and indirect (width 0·2 ms) alternated at 10 s intervals.
† Differs significantly ($P < 0·05$) from the effect of 30 μg/ml MJ 1999.

that consecutive exposures to MJ 1999, separated by 30 min wash periods, gave reproducible data with up to 5 exposures without tachyphylaxis. The blocking activity of MJ 1999 was similar whether stimulation was at 1/s or at 1/20 s (Table 1) and was markedly reduced at a bath temperature of 25°.

Effects of various drugs on MJ 1999 blockade

The blocking activity of MJ 1999 (30 μg/ml), like that of tubocurarine (1·0 μg/ml), was antagonized by neostigmine, KCl, succinylcholine, choline and by tetraethylammonium (Table 2). Also, MJ 1999 and tubocurarine had an additive action (mean block due to MJ 1999, 6·26 μg/ml with tubocurarine, 0·3 μg/ml was 43%; mean block due to tubocurarine alone, 22%; n = 4).

Table 2. *Antagonism of MJ 1999- and tubocurarine-induced blockade of indirectly induced contractions of rat diaphragm by various drugs.* The test dose of MJ 1999 (30·0 μg/ml) and of tubocurarine (1·0 μg/ml) was such that it produced over 90% blockade of indirect twitches. Values are means from a minimum of 4 experiments.

Drug	Concentration (μg/ml)	Mean % antagnoism of blockade MJ 1999	Tubocurarine
Neostigmine	2·0	62	68
KCl	5·0	48	56
Succinylcholine	5·0	45	35
Choline	100·0	51	60
Tetraethylammonium ..	200·0	70	78
Isoprenaline..	0·3	80	0
Adrenaline	0·3	72	50
Noradrenaline	0·6	40	35
Yohimbine	30·0	100	0
Tolazoline	30·0	80	0
Phenoxybenzamine.. ..	30·0	74	0
Dihydroergotamine ..	30·0	68	0

In view of the similarities of blockade by MJ 1999 and tubocurarine, it was of interest to examine if the two agents further resembled one another in their interaction with sympathomimetic amines. Table 2 shows that tubocurarine blockade was antagonized by adrenaline and to a lesser degree, by noradrenaline; isoprenaline was ineffective. Furthermore, the anticurare action of adrenaline was blocked by tolazoline (5·0 μg/ml) added 2–4 min before addition of adrenaline (n = 4; Fig. 1B). On the other hand, MJ 1999 blockade was quickly antagonized by adrenaline as well as by isoprenaline; noradrenaline was partly effective (Table 2). In the presence of small concentrations of adrenaline or isoprenaline, MJ 1999 did not exhibit any blocking activity (Table 1). The antagonistic action of adrenaline to MJ 1999 was not significantly affected by tolazoline (n = 4; Fig. 1A).

In experiments in which the effect of tolazoline on the antagonistic action of adrenaline to MJ 1999 was examined, it was found that if the concentration of tolazoline was increased, there was a reversal of MJ 1999 blockade even before the addition of adrenaline. Further observations (Table 2) showed that all the four α-adrenoreceptor blockers studied, yohimbine, tolazoline, phenoxybenzamine and dihydroergotamine, produced a quick and near complete reversal of MJ 1999 blockade whereas they had no effect on the blockade produced by tubocurarine.

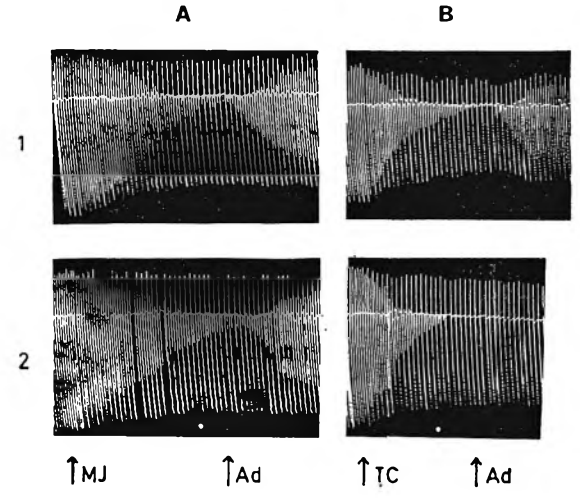

FIG. 1. Rat phrenic nerve diaphragm preparation. Tyrode solution, 34°, gassed with 5% CO_2 in O_2. Alternate direct (pulse width, 5·0 ms) and indirect (pulse width, 0·2 ms) supramaximal stimulation at 10 s intervals. MJ 1999 (30 μg/ml, at MJ) and tubocurarine (1·0 μg/ml, at TC) blocked indirect twitches (in A and B, respectively). Adrenaline (0·3 μg/ml, at Ad) antagonized the blockade (A–1 and B–1). Tolazoline (5·0 μg/ml, at white dot) added before adrenaline did not counteract the anti-MJ 1999 effect of adrenaline (A–2) but it abolished anti-curare effect of this drug (B–2).

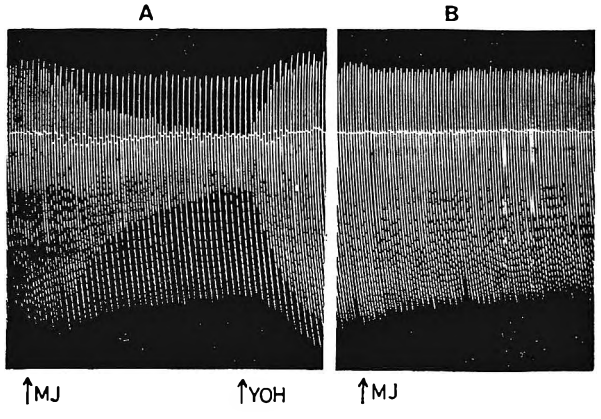

FIG. 2. Rat phrenic nerve-diaphragm preparation (see Fig. 1 for experimental conditions). A. Blockade of indirect twitches by MJ 1999 (30 μg/ml, at MJ) and its reversal by yohimbine (30 μg/ml, at YOH). B. MJ1999 had no blocking effect when bath fluid contained yohimbine (5·0 μg/ml).

Moreover, previous administration of small concentrations of either yohimbine or tolazoline could prevent blockade due to MJ 1999 (Table 1). Fig. 2 shows the interaction between MJ 1999 and yohimbine.

Effect of MJ 1999 *on nerve-conduction*

MJ 1999 (50 μg/ml) added to the "nerve bath" had no effect on indirectly induced contractions of the diaphragm over at least 40 min.

DISCUSSION

The blockade of indirect twitches due to MJ 1999 does not appear to be due to reduced efficiency of the neurosecretory process. MJ 1999 had no effect on axonal conduction and, though this in itself does not rule out reduced impulse traffic at the terminal nerve endings, such an effect is unlikely as MJ 1999 is free of membrane stabilizing activity. Moreover, the agent, unlike pronethalol or propranolol, has no effect on succinylcholine-induced repetitive firing, fasciculation or augmented twitch tension (Usubiaga, 1968). However, the nature of the blocking activity, as well as the antagonistic effect of various drugs which either increase the efficiency of the neurosecretory process or increase the effect of released transmitter, suggests that MJ 1999 has a tubocurarine-like mode of action.

Our results also suggest that MJ 1999 has a second component of action on β-adrenoreceptors. Thus, the rank order of potency of sympathomimetic amines as antagonists of MJ 1999 and tubocurarine was different. Isoprenaline antagonized MJ 1999 blockade. Furthermore, the antagonistic action of adrenaline to MJ 1999, unlike the anticurare activity of this agent, was not influenced by tolazoline and hence does not seem to be mediated through α-adrenoreceptors. The antagonism of MJ 1999, but not of tubocurarine, by larger concentrations of α-adrenoreceptor blocking agents is a further point of interest. The antagonism of α-adrenoreceptor blocking agents by β-adrenoreceptor blockers at the smooth muscle effector is well documented (Hull, Elthrington & Horita, 1960; Olivers, Smith & Anorow, 1967; Yamamura & Horita, 1968) and is generally believed to be due to a phenomenon of displacement at the receptor level. The reverse type of antagonism, that is, of β- by the α-adrenoreceptor blockers has not yet been reported but a similar displacement at receptor level could well be implicated.

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LETTERS TO THE EDITOR

A note on the assay of atropine sulphate injections

During a study on the stability of some batches of atropine sulphate injections, the U.S.P. XVII and B.P. 68 methods of assay were compared. Samples of three commercial batches of atropine sulphate injections (U.S.P. XVII), having pH values between 5·2 and 6·0, were subjected to autoclaving for 1 to 6 h. These injections were then assayed by the two methods (Table 1). The B.P. method gave, for all the samples examined, higher results compared with the U.S.P. method. Batch C, which showed by the U.S.P. method about 50% decomposition, gave about 36% decomposition by the B.P. method (Table 1). This particular batch revealed, on using a thin-layer chromatography system,* two spots corresponding to atropine and tropine; the former gave an orange colour and the latter a deep violet colour with dilute potassium iodobismuthate solution. It was concluded, therefore, that the hydrolytic product tropine may interfere in the B.P. method. Tropine, chromatographically pure, was analysed following the U.S.P. and B.P. methods of assay of atropine sulphate injections. The results (Table 2) showed that tropine interferes in the B.P. method; the plot is linear and has a slope of 1·52 indicating the constant contribution due to tropine. The latter in a concentration of 20·3 mg% (corresponding to 100% hydrolysis of atropine sulphate) gave by the B.P. method the equivalent of 30·6 mg% of atropine sulphate. In the U.S.P. method, however, the effect due to

Table 1. *Results of assay of atropine sulphate injections, autoclaved for various time intervals, by the U.S.P. XVII, B.P. 68 and the modified B.P. methods.*

Batch No.	Autoclaving time (h)	Results of assay*		
		U.S.P.	B.P.	Modified B.P.
A	1	101·76	106·02	100·80
B	4	92·64	94·36	91·41
C	6	50·06	63·91	48·88

* Average of four replicates (±1·5%).

Table 2. *Results of assay of tropine by the U.S.P. XVII and B.P. 68 methods of assay of atropine sulphate injections and by the modified B.P. method.*

Tropine added mg%	Corresponding % hydrolysis*	Results of assay in terms of mg% atropine sulphate†		
		U.S.P.	B.P.	Modified B.P.
2·03	10	3·01	3·20	
4·06	20	3·63	6·70	The results
8·12	40	3·88	11·45	obtained ranged
12·18	60	4·01	21·55	between 1 and
16·24	80	4·95	24·65	1·8%
20·30	100	5·83	30·61	

* Calculated on the basis that 1 mg atropine sulphate yields on complete hydrolysis 0·203 mg tropine.

† Average of four replicates (±1·5%).

* Dimethylformamide–ammonia–ethanol–ethyl acetate(1:1:6:12) using Silica Gel G as the adsorbent.

Table 3. *Analyses of mixtures of atropine sulphate and tropine, corresponding to various degrees of hydrolysis, by the U.S.P. XVII, B.P. 68 and modified B.P. methods.*

Mixture No.	Composition (mg%) Atropine sulphate	Tropine	Hydrolysis %	Results of assay* U.S.P.	B.P.	Modified B.P.
1	5·0	19·29	95	9·93	33·82	5·07
2	20·0	16·24	80	22·75	44·50	20·20
3	50·0	10·15	50	52·01	64·34	50·91
4	70·0	6·09	30	74·74	79·11	71·03
5	80·0	4·06	20	83·80	84·00	79·71
6	85·0	3·05	15	88·06	86·91	84·54
7	90·0	2·03	10	92·77	91·83	90·31
8	100·0	0	0	99·10	100·64	100·30

* Average of four replicates (±1·5%).

tropine was less pronounced being between 3·01 and 5·83% for 10 and 100% hydrolysis respectively (Table 2). Mixtures containing varying amounts of atropine sulphate and tropine, corresponding to different degrees of hydrolysis, were prepared and assayed by the two methods. The results obtained (Table 3) showed the non-selectivity of the B.P. method due to tropine interference. In mixtures showing 30 to 95% hydrolysis the contribution due to tropine was similar to that found in Table 2 for tropine solutions. In other mixtures, with lower percentages of hydrolysis, the effect of tropine was less pronounced (Table 3). Analysis of the mixtures by the U.S.P. method revealed that the method is more selective especially for mixtures of low percentages of hydrolysis (Table 3). The low contribution of tropine in the U.S.P. method is probably due to its loss during extraction as the result of partitioning (being more water soluble). Partial volatilization of tropine also occurs during the evaporation of the chloroform–ethanol mixture. The percentage loss of tropine during the U.S.P. assay was estimated to be 92·3%.

Trials were made to improve the B.P. method. We found that tropine did not interfere when dilute sulphuric acid was substituted for the acetate buffer of pH 2·8. This was shown in Tables 2 and 3, where no interference due to tropine was observed in mixtures showing up to 95% hydrolysis.

It should be pointed out that both official methods are satisfactory for the control analysis of the injections since the interference due to tropine lies within the official limits specified in the monographs. However, for stability studies the suggested modified B.P. method is more selective than both official methods beside being simpler and less time consuming than the U.S.P. procedure.

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The preferred conformation of noradrenaline

The structural theory of the adrenergic receptor and transmitters and their complexes is currently in a primitive, though active, state. A recent review (Korolkovas, 1970) summarizes various structural postulates. Rapid advances in computer and mathematical techniques have recently made it possible to consider fairly elaborate quantum mechanical studies on some of the "intermediate" sized biologically important molecules. For instance, the simple but powerful extended Hückel method (Hoffmann, 1963) and the more advanced CNDO/INDO methods (Pople, Beveridge & Dobash, 1967) have been used to study a variety of problems of interest in quantum chemistry in the past several years. Kier (1969) has recently systematically examined the structural properties of neural compounds including noradrenaline with the extended Hückel method. He found that the calculated lowest energy conformation was consistent with his postulates on the structure of the α-receptor site. By examining rotation about the $-HC(OH)CH_2$ bond he found the most stable conformation to be that with the catechol rotated 180° from the $-NH_3^+$. Because of the important question involved here it seemed reasonable to examine the same problem with more sophisticated, yet still tenable, INDO theory suggested by Pople & others. In addition, a study of the dependence of the conformation of the α-receptor portion of the molecule (the $-NH_3^+$) upon chelation of the β-receptor portion of the molecule (the catechol functional groups) was examined.

The INDO method is an approximate molecular orbital method which explicitly treats all valence electrons in the same framework; the σ–π separability approximation of earlier theories is not made, This theory (Pople & others, 1967) along with its predecessor, the CNDO method, have proved successful in predicting geometries, electron paramagnetic resonance hyperfine coupling constants, nuclear spin coupling constants and barriers to internal rotation. The essential approximation made is to assume zero all integrals for which the product $\phi_\nu(1)\phi_\mu(1)$ has ν and μ on different atoms. In particular, the electronic wave function is an antisymmetrized product of molecular orbitals $\psi_i = \Sigma C_{i\nu}\phi_\nu$, composed of Slater atomic orbitals. The self consistent field equations are iterated allowing the α-spin and β-spin molecular orbitals to vary independently until some external criterion on self-consistency (for instance non-changing total energy upon reiteration) is met. This method has proved to be successful in predicting barriers to internal rotation; the barriers of CH_3CH_3, CH_3NH_2 and CH_3OH are found to be in the correct 1:2:3 ratio.

The co-ordinates for the calculation were evaluated using the parameters suggested in the crystallographic study on the noradrenaline HCl derivative (Carlstrom & Bergin, 1967). For the chelated catechol study, a hydrogen was replaced by Li and the Li then placed 0·2 nm from one oxygen and 0·22 nm from the other. Two waters of hydration were then placed in a tetrahedral arrangement (one above, one below the plane of the catechol ring). The Li–H_2O distance was taken to be 0·23 nm (Li–O) and the two water oxygens were taken to be 0·29 nm apart. The total charge on the chelated molecule was then held at +1 as the protonated form.

Fig. 1 displays the dependence of the total energy on the rotation about the $-HC(OH)CH_2$ bond. For noradrenaline two conformations, the 60° and 180° structures, have the same energy with a small barrier of 6·276 kJ (1·5 kcal) between them at 120° rotation. The 180° degree structure agrees with Kier's extended Hückel calculation (Kier, 1968); however, he found this structure to be substantially lower in energy than two other minima which appeared in his results: one at 60° and one at 300°. The 300° minima (60° in the notation of Kier) does not occur in the INDO calculation. The largest barrier (29·288 kJ; 7 kcal) in our calculation occurs at the 240° conformation.

When the $-Li(H_2O)_2$ chelate is substituted for one of the hydrogens, the rotational

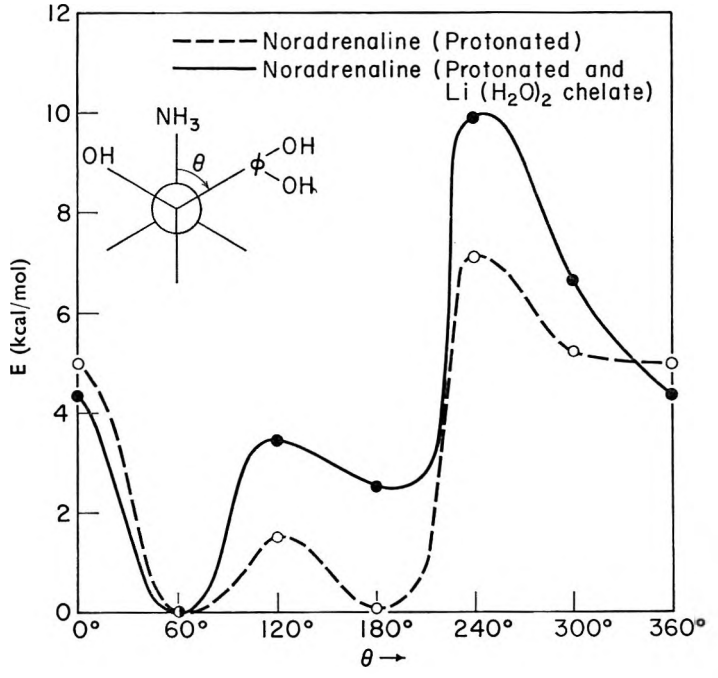

FIG. 1. Energy versus $-CH(OH)-CH_2-$ rotation angle.

minimum at 180° essentially disappears and is replaced by a slight minimum about 10·46 kJ (2·5 kcal) above the 60° minimum which remains. Thus, a change in environment at the β-receptor site can have an important effect on the α-receptor site conformation. The largest barrier for the chelated compound still occurs at the 240° rotation and is increased over the non-chelated case to 41·84 kJ (10 kcal).

The origin of the barrier seems to be a delicate balance of several factors. Examination of the nuclear repulsion term shows that the 180° conformation is most favoured. The attraction between the NH_3^+ group and the alkyl OH will be a maximum at 120° rotation but this is an "eclipsed" configuration. It is therefore not unreasonable that the minimum occurs at either 60° or 180° with a small hump between resulting from eclipsed repulsions. The 240° configuration is the least stable probably because it is an eclipsed configuration and the (NH_3^+)—OH distance (attractive) is quite large.

We conclude that the likely conformation in solution will be the 60° configuration. This assumes a strong interaction through a metal at the β-receptor site and assumes that the actual binding will not be substantially different from that of the "gaseous" molecule we have studied. The 180° well is only 10·46 kJ (2·5 kcal) above the 60° well; thus, a receptor favoring 180° over 60° could, by increased bonding opportunity for the 180° conformation, overcome this relatively small difference. It may be of some interest to point out that Li^+ and Mg^{2+} have almost the same ionic radii and that Mg is thought to be one of the metals binding noradrenaline to ATP. Li has a maximum co-ordination of four whereas Mg has a maximum co-ordination of six. Thus, it may be that Li^+, when given in the treatment of manias, may temporarily increase but eventually decrease the free noradrenaline in the brain by replacing Mg^{2+} and thereby weakening the bonding to the noradrenaline storage region.

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A simple method for the quantitative extraction of dye extravasated into the skin

Increased vascular permeability is usually demonstrated by the leakage of certain dyes injected intravenously. Though the intensity of this reaction has been measured in various ways (Jori, Bentivoglio & Garattini, 1961; Parratt & West, 1958; Frigeni, Gazzanica & Bonanomi, 1970), the best way is to extract and determine the extravasated dye quantitatively. Of several methods for achieving this some do not give satisfactory recovery (Jancsó-Gábor, Szolcsányi & Jancsó, 1967), while others are laborious (Nitta, Hayashi & Norimatsu, 1963; Judah & Willoughby, 1962). The dye can be determined quantitatively by relatively simple procedures (Ankier & Whiteside, 1969; Udaka, Takeuchi & Movat, 1970), but this takes a long time at relatively high temperatures. We previously proposed a method in which the chopped skin is violently agitated in a mixed solvent of acetone and a commercial detergent, Emal, in a homoblendor for 15 min (Harada, Takeuchi & Katagiri, 1966). This method, however, had two demerits; (1) requirement of volume adjustment after the homoblendor process and (2) the use of a detergent that is a mixture of various compounds. Both defects have since been improved as follows. (1) Volume change due to volatilization of acetone was easily prevented by replacing the homoblendor procedure by a 24 h incubation at room temperature with occasional shaking. (2) Each ingredient of the detergent was examined individually and the component effective in the extraction was identified as sodium sulphate. The following experiment demonstrates the usefulness of this improved method.

First, the recovery of dye injected intradermally was examined. Various amounts of azovan (Evans) blue were injected into the skin of rats. After 30 min, each blue area, which was about 10–15 mm in diameter, was erased, cut into about 10 pieces with scissors and mixed with a medium composed of 14 ml of acetone and 6 ml of a 0·5% aqueous solution of sodium sulphate in a test tube. The tube was closely firmly with parafilm and left to stand for 24 h at room temperature (20°) with occasional mild shaking. Each preparation was then centrifuged for 10 min at 300 rev/min and the supernatant separated. Percentage recovery of the dye was calculated by comparing the absorbance of the supernatant at 620 nm with that of a standard sample prepared by mixing the corresponding amount of azovan blue and normal skin pieces in the same medium *in vitro*. The method gave a recovery of over

95% in all cases where the quantity of the dye injected was less than 200 μg, and a plot of the absorbance of the supernatant against the amount of the dye injected was linear. For quantitative recovery of the dye a sodium sulphate solution of 0·5% or more was necessary.

Changing the volume ratio of acetone to sodium sulphate solution greatly influenced the yield, the optimal ratio being estimated as 7:3. Raising the temperature to 37° together with continuous mild shaking had no significant effect on the extraction efficiency. Replacement of acetone by alcoholic solvents such as methanol, ethanol or butanol gave very low recovery of the dye. Pontamine sky blue injected intradermally was similarly quantitatively extracted from the rat skin. When trypan blue was used, however, the yields were lower than 90% throughout the dose range up to 200μg. The technique was also applicable to the extration of injected azovan blue and pontamine sky blue from mouse, guinea-pig, and rabbit skin.

This improved method could be applied to the extraction of dye which was exuded from the capillaries into the skin. Two blue spots were produced on the back of a rat by intradermal injection of 5-hydroxytryptamine at two sites, together with intravenous injection of azovan blue. One of the spots was extracted with the mixture of sodium sulphate and acetone, while the other was extracted with a mixture of acetone and the commercial detergent. The two extracts had almost the same absorbance.

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The inducement of tone and its inhibition in isolated tracheal muscle

We have recently described a preparation of the isolated intact trachea of the guinea-pig which can be used to assess the activity of bronchodilator drugs by determining their abilities to antagonize the temporary rise in intraluminal pressure induced by electrical stimulation (Farmer & Coleman, 1970). Subsequently we investigated the effect of various drugs on the resting intraluminal pressure in the non-electrically stimulated intact trachea preparation. The preparation set up, as described by Farmer & Coleman (1970), has an intraluminal pressure equal to or slightly above atmospheric pressure. Acetylcholine (0·1–30 μg/ml) and histamine (1–100 μg/ml) caused graded increases in intraluminal pressure of 1–25 mm Hg, and typical dose-response curves were obtained for these spasmogens. pA_2 values for atropine against acetylcholine and mepyramine against histamine were determined by the method of Arunlakshana & Schild (1959). Increasing doses of atropine caused successive shifts to the right of the acetylcholine dose-response curve and a pA_2 (30 min) of 8·46 $\pm$ 0·768 (n = 3) was obtained. A pA_2 value for mepyramine could not be obtained as the shifts in the histamine dose-response curves were not parallel. This is surprising as the antagonism of histamine by mepyramine on the guinea-pig ileum was shown by Arunlakshana & Schild (1959) to be competitive.

The small residual intraluminal pressure of the intact trachea preparation is reduced by β-adrenoceptor stimulants and doses producing maximal responses lowered the intraluminal pressure to just below atmospheric pressure. On washing, the intraluminal pressure rose to its previous level in about 10 min. The responses to β-stimulants were reproducible but pressure changes involved were too small to allow satisfactory quantitative evaluation. These results are similar to those previously described by Jamieson (1962), Wellens (1966) and Guirgis (1969). However, we have found that a suitably high intraluminal pressure could be developed if the tracheal lumen was momentarily exposed to atmospheric pressure immediately after the β-stimulant was washed from the bath. The tone then recovered quickly but to a level higher than before.

Repetition of this procedure resulted in successive increases in the level of the intraluminal pressure until equilibrium was reached usually after the fourth or fifth cycle. At this time the intraluminal pressure varied from 14–22 mm Hg and sometimes exceeded 30 mm Hg. Very occasionally the intraluminal pressure failed to

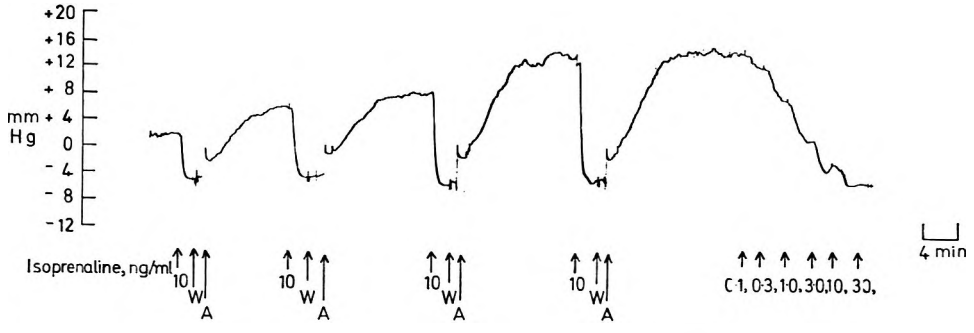

FIG. 1. The development of intraluminal pressure in the trachea by repeated dosing with isoprenaline and exposure to atmospheric pressure. The effects of graded doses of isoprenaline on the developed pressure are also illustrated. (W) wash, (A) exposure to atmospheric pressure.

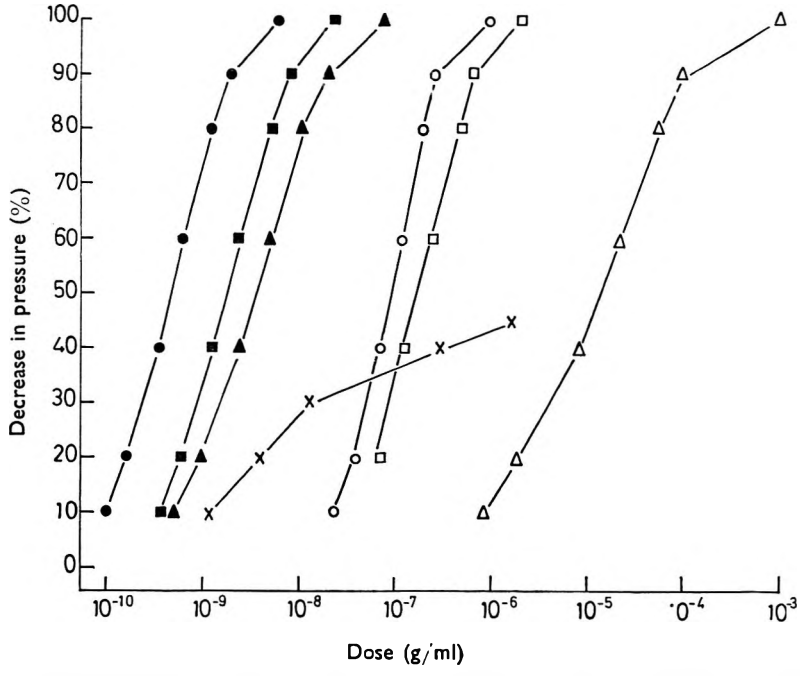

FIG. 2. Cumulative dose-response curves for a variety of agents with spasmolytic activity (●—●) isoprenaline, (■—■) salbutamol, (▲—▲) adrenaline, (○—○) noradrenaline, (□—□ papaverine, (△—△) choline theophyllinate and (×—×) atropine.

develop above 10 mm Hg and the tissue was discarded. The effects of various antagonists on the developed intraluminal pressure were studied in order to characterize this response. Atropine (1–3 μg/ml) partially reversed the developed intraluminal pressure (45–50%) while mepyramine (10 μg/ml) and BOL (10 μg/ml) had no significant effect. The developed pressure is thus due to both cholinergic and other unidentified components. Reduction of the developed intraluminal pressure now provided a suitable means for assessing the actions of bronchodilator drugs. The development of high intraluminal pressure following exposure to isoprenaline is illustrated in Fig. 1.

Cumulative dose-response curves for the spasmolytic actions of isoprenaline, adrenaline, noradrenaline, salbutamol, choline theophyllinate, papaverine and atropine are illustrated in Fig. 2. Order of potency was isoprenaline > salbutamol > adrenaline >> noradrenaline > papavarine >> choline theophyllinate; atropine caused only partial reversal of intraluminal pressure and could not be included.

The technique of inducing high intraluminal pressure in the intact trachea preparation and its subsequent reduction by spasmolytic agents offers a major advantage over all previously described intact trachea preparations. Furthermore, the procedure used did not alter the sensitivity of the preparation to spasmolytic agents when compared to those of the isolated tracheal chain and spiral preparations. The advantages of the intact trachea preparation over tracheal chain or spiral preparations are two fold. Firstly the intact trachea preparation is far easier and quicker to set up and the response to, and recovery from, the actions of spasmolytic agents is much shorter than those found using the chain or spiral preparations. It is concluded that the preparation described offers a simple, quick and sensitive means for detecting and evaluating the spasmolytic actions of bronchodilator drugs.

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Atropine-resistance of the urinary bladder innervation

The vertebrate urinary bladder is provided with a parasympathetic excitatory innervation. However, while the excitatory effects of acetylcholine on the bladder muscle are strongly antagonized by atropine or hyoscine, the nerve-mediated responses persist with only slight reduction in amplitude (Langley & Anderson, 1895). This evidence has led Henderson & Roepke (1934) and Ambache & Zar (1970) to argue that the excitatory innervation of the bladder is, at least in part, not cholinergic. Other workers have maintained that the innervation is solely cholinergic and have put forward apparently credible theories to explain the inability of muscarinic antagonists to prevent neuromuscular transmission. There are two primary conditions under which muscarinic antagonists such as atropine would not prevent cholinergic transmission to the bladder muscle. First, the receptors specifically occupied by acetylcholine released from nerves could be physically inaccessible to atropine. Second, atropine may reach the receptors but be unable to prevent acetylcholine from occupying the receptors.

There is no evidence to support the suggestion of Carpenter & Rand (1965) that the acetylcholine receptors in the neuromuscular junctions of the bladder are inaccessible to atropine.

Electron microscopic studies have not revealed the existence of any barriers isolating nerve-muscle complexes from the remaining extracellular space (Caesar, Edwards & Ruska, 1957; Thaemert, 1963; Nagasawa & Mito, 1967). In fact the relation between axons and smooth muscle cells in the bladder is similar to the arrangement found in the adrenergically-innervated vas deferens (Merrillees, 1968), yet neuromuscular transmission in the vas deferens is susceptible to blockade by competitive antagonists of α-adrenergic actions (e.g. Boyd, Chang & Rand, 1960). There is therefore no reason to believe that atropine cannot similarly reach all cholinergic receptors in the urinary bladder.

Since atropine is evidently able to penetrate into the neuromuscular junction, its inability to prevent acetylcholine from occupying the receptors indicates that either atropine is displaced from the receptors competitively by high local concentrations of acetylcholine (Huković, Rand & Vanov, 1965) or atropine cannot occupy the cholinergic receptors, i.e. they are not muscarinic. The suggestion that acetylcholine displaces atropine from muscarinic receptors competitively requires that either the amount of acetylcholine released is greater or the width of the synaptic cleft is smaller

in the bladder than in other, atropine-sensitive cholinergic neuromuscular junctions. This theory was tested, using preparations of rat and guinea-pig urinary bladders, bisected in the sagittal plane and suspended in McEwen solution (McEwen, 1956) at 35°. The nerves in the bladder wall were stimulated at 1–6 Hz with pulses of 0·2–1 ms for periods of 10 s via platinum ring electrodes placed around the preparations. The excitatory responses to electrical stimulation were abolished by tetrodotoxin (5×10^{-7} g/ml), showing that there was no direct stimulation of the muscle.

The effects of hyoscine were tested on responses to nerve stimulation and to acetylcholine in preparations treated with pentolinium (5×10^{-5} g/ml) to prevent nicotinic actions of acetylcholine. Hyoscine (3×10^{-7} g/ml) reduced nerve-mediated responses by from 17 to 41%. However, the concentration of hyoscine could be raised to 10^{-3} g/ml without reducing the responses to nerve stimulation by more than 43% (Fig. 1); single stimuli were still effective in causing contractions. Hyoscine (10^{-3} g/ml) abolished responses to acetylcholine at 5×10^{-3} g/ml, and, even at 10^{-2} g/ml, acetylcholine caused only a slight contraction. If the transmitter substance released by the excitatory nerves is acetylcholine, to be consistent with these results the concentration of acetylcholine reaching the receptors would have to exceed 5×10^{-3} g/ml.

The concentration of acetylcholine reaching the receptors could be estimated if both the total amount released by stimulation and the "synaptic volume" into which it is released were known. Two studies have been made of the acetylcholine output from stimulated bladders (Carpenter & Rand, 1965; Chesher, 1967). The highest output per pulse reported, for rat bladders stimulated at 1 Hz, is $6{\cdot}3 \times 10^{-10}$ g per g tissue (Carpenter & Rand, 1965), assuming a bladder weight of 100 mg (Chesher, 1967). For comparison, the output from guinea-pig ileum per pulse during stimulation at 1 Hz is 8×10^{-10} g per g tissue (Paton & Zar, 1968), indicating that acetylcholine is

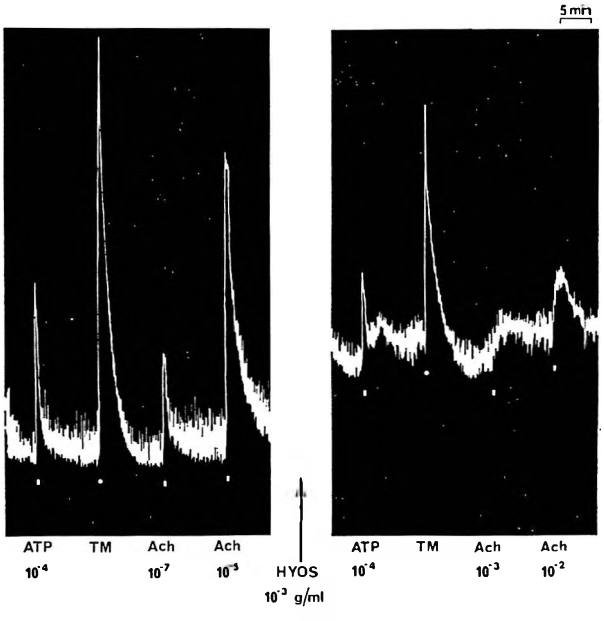

FIG. 1. The effect of a high concentration of hyoscine on excitatory responses of the isolated guinea-pig bladder. Preparation treated with pentolinium, (5×10^{-5} g/ml). The first panel shows contractions produced by adenosine triphosphate (ATP, 10^{-4} g/ml for 30s), transmural stimulation (TM) at 5 Hz for 10 s and acetylcholine (Ach, 10^{-7} and 10^{-5} g/ml for 30 s). In the second panel, 90 min after the addition of hyoscine (HYOS, 10^{-3} g/ml), contractions produced by adenosine triphosphate and transmural stimulation are present, though reduced. Note that acetylcholine (10^{-3} g/ml) is without effect, whereas 10^{-2} g/ml causes contractions. Time marker, 5 min.

released from bladder tissue in normal quantities. It is probably released, as proposed for adrenergic autonomic nerves (Malmfors, 1965), from the varicosities of the terminal axons. To achieve the most favourable conditions for displacement of atropine to occur, this quantity of acetylcholine must be released into the smallest possible synaptic volume. A minimal estimate of synaptic volume can be arrived at as follows.

1. The closest approximation of axon varicosities to bladder muscle cells is 20 nm (Caesar & others, 1957; Thaemert, 1963). Varicosity diameter is about 1μm. The 'unit synaptic volume', defined as the discoid space between a varicosity and a closely apposed muscle cell is therefore no less than 0·016 μm^3.

2. As a least estimate, there is one 20 nm neuromuscular apposition per muscle cell in the bladder (Caesar & others, 1957; Nagasawa & Mito, 1967). The least number of muscle cells in a gram of tissue can be calculated, from the greatest measured volume of single cells (3500 μm^3 in guinea-pig vas deferens) (Merrillees, 1968) and the smallest measured intracellular space of smooth muscle tissues (about 60% of the tissue volume) (Burnstock, 1970), to be $1{\cdot}7 \times 10^8$ cells/g (assuming a specific gravity of 1). The least total number of 'unit synaptic volumes' is $1{\cdot}7 \times 10^8$ per g tissue.

3. From 1 and 2, the least estimate of 'total synaptic volume' is $2{\cdot}7 \times 10^6$ μm^3/g tissue.

To achieve a maximal estimate of the concentration of acetylcholine reached at the muscle cells, it is assumed that the total measured output is released into the 'synaptic volume' as defined above, i.e. there is no release from varicosities further than 20 nm from a muscle cell. If this is assumed, the concentration of acetylcholine at the muscle cells is $2{\cdot}3 \times 10^{-4}$ g/ml. It can be seen that this concentration is at least 20-fold smaller than the least concentration shown above to excite the bladder in the presence of hyoscine (10^{-3} g/ml). If more realistic assumptions, especially about the mode of release of acetylcholine, are made, the concentration achieved will be even smaller. It seems clear that the atropine-resistance of the excitatory nerves cannot be explained in terms of displacement of atropine from receptors by acetylcholine.

The alternative explanation for the inability of atropine to prevent occupation of receptors by acetylcholine is that the receptors are not muscarinic. Whether these receptors are postulated to be nicotinic or of any other cholinergic type, the fact remains, as shown above, that acetylcholine would have to reach the bladder muscle in a concentration greater than 5×10^{-3} g/ml, a concentration which cannot be achieved physiologically. Clearly this theory does not explain the atropine-resistant transmission.

The partial blockade of nerve-mediated responses by low concentrations of muscarinic antagonists (Ursillo, 1961) and the enhancement of responses by anticholinesterase drugs (Edge, 1955) show that part of the bladder innervation is cholinergic. The only direct evidence that the innervation is wholly cholinergic is the observation that hemicholinium-3 and botulinus type D toxin severely reduce excitatory transmission to the bladder (Hukovic & others, 1965; Carpenter, 1967). While this blockade might be due to the action of these agents in preventing acetylcholine release, both are known to have additional actions, at least against adrenergic transmission (Chang & Rand, 1960; Rand & Whaler, 1965). The blockade produced by both agents, although consistent with the theory that the nerves are entirely cholinergic, clearly does not prove the case. However, since from the arguments here advanced the theories explaining atropine-resistance in terms of cholinergic transmission would appear not to be feasible, the opinion of Henderson & Roepke (1934) that the innervation also contains a non-cholinergic excitatory component would seem to hold.

If there is a non-cholinergic excitatory innervation of the bladder, it must be asked what transmitter substance is released. Evidence has already beeen presented against transmitter actions of catecholamines, 5-hydroxytryptamine, bradykinin, and histamine (Huković & others, 1965; Edvardson, 1968; Gyermek, 1962; Ambache & Zar, 1970). Another possibility is that the transmitter substance is adenosine triphosphate (ATP), which causes contraction of canine bladder muscle (Matsumura, Taira & Hashimoto, 1968) and which has been suggested as a transmitter substance released by autonomic nerves in the gut (Burnstock, Campbell & others, 1971) and in the portal vein (Hughes & Vane, 1967). A strong contraction of the guinea-pig bladder was obtained with ATP (10^{-4} g/ml) in the present experiments (Fig.1). The contraction was not affected by tetrodotoxin (5×10^{-7} g/ml) and therefore appears to be due to a direct action on the muscle. Hyoscine (3×10^{-7} g/ml) did not reduce the ATP response; even at 10^{3} g/ml, hyoscine did not prevent ATP contractions (Fig. 1). It can be seen that this experiment provides better evidence, however scant, for ATP than for acetylcholine as the transmitter substance released by the atropine-resistant nerves.

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Effect of aspirin on the fate of bishydroxycoumarin in the rat

Coldwell & Zawidzka (1968) showed that the oral administration of a single dose of aspirin (100 mg/kg) to male rats on a regimen of the anticoagulant drug, bishydroxycoumarin, decreased the one-stage prothrombin time of blood collected 20 h after administration of the analgesic. Subsequently, this action of aspirin was observed in female and male rats after chronic dosing and was produced even more intensely by an equivalent amount of sodium salicylate, while several other analgesics were without any effect (Zawidzka & Coldwell, 1970). Analysis of the serum samples obtained from some of these animals for bishydroxycoumarin, using the spectrophotometric method of Nagashmia, Levy & Nelson (1968), suggested that aspirin might be affecting the serum bishydroxycoumarin concentration. This lead to the possible mechanism of the observed anti-bishydroxycoumarin effect of aspirin and sodium salicylate has now been investigated further.

Male albino rats of the Wistar strain, 175–200 g, acclimatized to the laboratory environment for at least one week, were dosed intraperitoneally with bishydroxycoumarin daily for 3 days at 2·0, 1·5 and 1·5 mg/100 g, respectively. The dose administered on day 3 included 171·4 μg/100 g of [^{14}C]bishydroxycoumarin having a specific activity of 2·58 mCi/mmol. Simultaneously with the administration of bishydroxycoumarin on day 3, aspirin (100 mg/kg)was administered orally to 6 of the 11 animals. The animals were then placed immediately in metabolism cages with free access to water; food was made available for a short period 24 h after the final drug administration. Tail blood specimens (10 μl) were taken at 0·5, 1, 2, 4, 7, 12, and 24 h and urine and faeces were each collected over the periods from 0 to 24 h and 24 to 48 h after the final drug administration. The samples were analysed for radioactivity using an accepted liquid scintillation counting procedure (radioactivity is expressed as unmetabolized bishydroxycoumarin).

The blood decay profiles for bishydroxycoumarin in the presence and absence

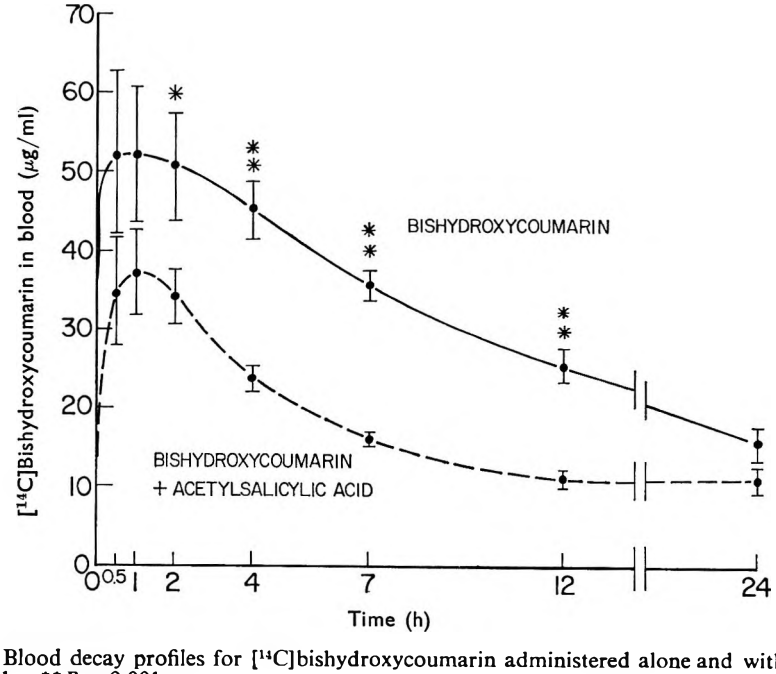

FIG. 1. Blood decay profiles for [^{14}C]bishydroxycoumarin administered alone and with aspirin. $^{*}P < 0{\cdot}01$. $^{**}P < 0{\cdot}001$.

of aspirin are illustrated in Fig. 1. The concentrations in the animals given bishydroxycoumarin alone were significantly higher than those in the animals that received aspirin with the anticoagulant in the period from 2 to 12 h after the final drug administration, the differences being highly significant ($P < 0.001$) at the 4, 7, and 12 h intervals. After 24 h the blood bishydroxycoumarin concentrations were similar in each group. The high standard errors for the mean levels at 0·5 and 1 h indicate the variability in the rate of absorption of this drug when given intraperitoneally.

The half-life of bishydroxycoumarin in the blood, during the period of relatively rapid disappearance, in the presence and absence of aspirin, was 4·5 and 9·9 h, respectively. During these periods, which were 1 to 10 h in the former group and 1 to 12 h in the latter, the rate of disappearance of bishydroxycoumarin from the blood followed apparent first-order kinetics. Nagashima, Levy & Back (1968) reported plasma $T_{\frac{1}{2}}$ values for bishydroxycoumarin from 4·6 to 5·6 h in male, Sprague-Dawley rats of 430–470 g after single intravenous doses of the drug ranging from 2 to 20 mg/kg.

Christensen (1964) obtained a value of 9·2 h in white female rats of about 190 g that were injected intravenously with 5·2 mg of the anticoagulant. Differences in $T_{\frac{1}{2}}$ values of bishydroxycoumarin no doubt reflect differences in the species, strain and weight of animals used and also the dose and route of administration.

The amounts of bishydroxycoumarin recovered in the urine and faeces from the two groups of animals during the 48 h after the final administration of the drugs were not significantly different at either the 24 h or 48 h collection periods. Approximately 66% of the dose of bishydroxycoumarin administered on day 3 was recovered during the subsequent 48 h. Elimination of a significant amount of bishydroxycoumarin in the faeces (43–45%) was confirmed (Christensen, 1965).

We are not aware of any previous reports indicating that the administration of a therapeutic dose of aspirin decreases the circulating blood levels of bishydroxycoumarin during a time when there were significant salicylate levels in the blood. It has been suggested that salicylates may compete with coumarin anticoagulants for protein binding sites (Formiller & Cohon, 1969) which could result in more rapid excretion of the unbound drug. Such transient effects might be obscured in the analysis of the 24 h urine and faeces collections. The slight change in physiologic pH resulting from the concomitant administration of aspirin might be a factor affecting the distribution of bishydroxycoumarin since its binding to albumin is known to vary markedly with changes in the acidity of the environment (Nagashima & others, 1968).

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Further studies on the mechanism of the central hypotensive effect of L-dopa, DL-*m*-tyrosine and L-α-methyldopa

Injection of L-dopa and DL-*m*-tyrosine after inhibition of peripheral decarboxylase produce a hypotensive response via their catabolites in the central nervous system (Henning & Rubenson, 1970a, 1970b; Rubenson, 1971). There is evidence that this action is brought about through activation of inhibitory mechanisms possibly of noradrenergic nature. With DL-*m*-tyrosine the hypotensive effect appeared to be elicited via displacement of endogenous amines, presumably noradrenaline (Rubenson, 1971).

The structurally related amino-acid L-α-methyldopa (α-MD) lowers arterial blood pressure in animals and man through mechanisms which are not yet completely understood (for review see Henning, 1969) but there is evidence that decarboxylation of α-MD within the central nervous system is necessary. Henning & Rubenson (1971) recently found that pretreatment with a dopamine-β-hydroxylase inhibitor (FLA-63) blocked the formation of α-methylnoradrenaline, further, the hypotensive response of α-MD was abolished.

The present investigation was designed to clarify further the role of direct and indirect mechanisms in the hypotensive effects seen after L-dopa, DL-*m*-tyrosine and α-MD.

Male Sprague-Dawley rats, 200–300 g, were used. Mean arterial blood pressure was recorded in conscious unrestrained animals through indwelling arterial catheters connected to Statham P 23 DC pressure transducers writing on a Grass polygraph (Henning, 1969). The blood pressure values are averages of recordings for 10 min periods immediately before the administration of the drugs, except the values of the maximal hypotensive response seen after L-dopa and DL-*m*-tyrosine which are the average of recordings 15–20 min after injections of L-dopa and 5–10 min after injection of DL-*m*-tyrosine.

Noradrenaline was determined by the method of Bertler, Carlsson & Rosengren (1958), dopamine as described by Carlsson & Lindqvist (1962). Each analysis was generally performed on pooled organs from two animals of similar weight.

The drugs used were L-3,4-dihydroxyphenylalanine (L-dopa), DL-3-hydroxyphenylalanine (DL-*m*-tyrosine), DL-α-methyl-3-hydroxyphenylalanine (DL-α-methyl-*m*-tyrosine, α-MMT), DL-α-methyl-*p*-tyrosine-methylester (H 44/68), L-α-hydrazino-α-methyl-(3,4-dihydroxyphenylpropionic acid (MK 486). All drugs were administered intraperitoneally. For doses and time intervals see Fig. 1. In blood pressure experiments, tests of significance were by analysis of variance, with two independent criteria of classification, followed by *t*-test.

Injection of MK 486 (50 mg/kg) did not influence the mean arterial blood pressure significantly within 30 min ($P > 0{\cdot}1$, see Fig. 1A). Thirty min after pretreatment with MK 486, injection of L-dopa (200 mg/kg) resulted in a significant lowering of blood pressure, the maximum effect being reached after 15–20 min ($P < 0{\cdot}001$; $n = 4$). Thirty min after the dopa injection the animals were given another injection of MK 486 (50 mg/kg) and 30 min later, when blood pressure had returned to the baseline, another injection of L-dopa (200 mg/kg) had no significant influence on the blood pressure (Fig. 1A). After both dopa injections the animals showed an aggressive behaviour and increased spontaneous motility with a maximum at about 30 min.

Responses to DL-*m*-tyrosine (400 mg/kg) given under the same conditions closely followed those for L-dopa (Fig. 1A), the blood pressure fall 5–7 min after the first

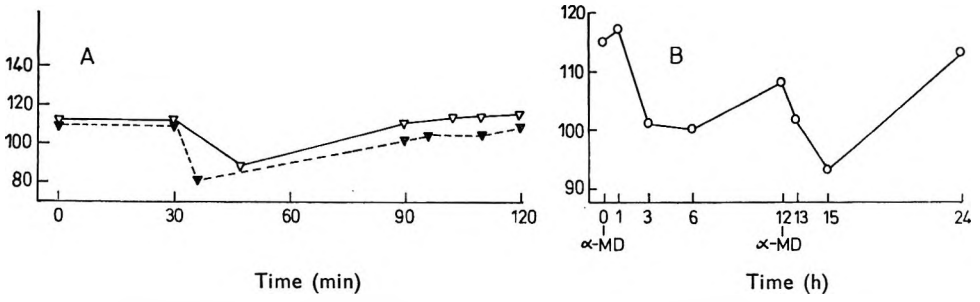

FIG. 1. Mean arterial blood pressure in conscious normotensive rats after i.p. injection of drugs as indicated. The blood pressure values represent averages of recordings during ten-minute periods before and after the drugs indicated except the values immediately after L-dopa and DL-*m*-tyrosine which represent the average of the recording 15–20 min after dopa and 5–7 min after *m*-tyrosine; s.e. were calculated by analysis of variance. Ordinate: mean arterial blood pressure (mm Hg).

A. Injections of MK 486 (50 mg/kg) at 0 and 60 min and of L-dopa (200 mg/kg) or DL-*m*-tyrosine (400 mg/kg) at 30 and 90 min: s.e. for L-dopa experiments = 3·59, 8 exp. (open symbols) and for DL-*m*-tyrosine experiments = 2·74, 6 exp. (solid symbols).

B. Injections of L-α-methyldopa (400 mg/kg) at 0 and at 12 h: s.e. = 3·73, 8 exp.

injection being significant at $P < 0{\cdot}001$ (n = 4). Both injections increased spontaneous motility.

α-MD (400 mg/kg) lowered mean arterial blood pressure significantly after 3 and 6 h ($P < 0{\cdot}001$; n = 8). Another dose given 12 h later caused a significant lowering of mean arterial blood pressure after 3 h which was not significantly different ($P > 0{\cdot}1$; *t*-test, process of pairing) from that seen 3 h after the first injection (Fig.1B).

The effect of L-dopa (200 mg/kg) on blood pressure was studied after pretreatment with α-MMT (three doses of 400 + 400 + 200 mg/kg, 27, 15 and 3 h before L-dopa) and the tyrosine hydroxylase inhibitor H 44/68 (250 mg/kg, 60 min before L-dopa) and MK 486 (100 mg/kg, 30 min before L-dopa). The α-MMT pretreatment has no significant influence on mean arterial blood pressure (Rubenson, 1971). No significant difference existed between the blood pressure values recorded (between 100–120 mm Hg), i.e., the hypotensive action of L-dopa was blocked ($P > 0{\cdot}1$).

In animals pretreated with α-MMT + H 44/68 + MK 486 as in the blood pressure experiments, brain dopamine and noradrenaline were markedly lowered [the mean values, μg/g ± s.e., with no pretreatment were: dopamine 0·69 ± 0·055 (n = 3), noradrenaline 0·38 ± 0·055 (n = 3); after treatment with α-MMT + H44/68 + MK486 these were: 0·13 ± 0·023 (n = 2), 0·01 ± 0·003 (n = 2)]. To test the possible influence of a dopa decarboxylase inhibitory effect of α-MMT (Hess, Connamacher & others, 1961; Porter, Totaro & Leiby, 1961) the accumulation of brain dopamine and noradrenaline after injection of L-dopa (200 mg/kg) was studied after α-MMT + H 44/68 + MK 486 as used in the blood pressure experiments. The values (μg/g) [dopamine 3·21 ± 0·175 (n = 3), noradrenaline 0·12 ± 0·005 (n = 3)] were compared with those obtained after MK 486 + L-dopa only [dopamine 2·90 ± 0·114 (n = 3), noradrenaline 0·36 ± 0·006 (n = 3)]. There was no decrease in formation of dopamine ($P > 0{\cdot}1$).

The rapid hypotensive response to injections of L-dopa and *m*-tyrosine after peripheral dopa decarboxylase inhibition agrees with previous findings (Henning & Rubenson, 1970a, 1970b; Rubenson, 1971). But the lack of effect of the second injection of dopa or-*m*-tyrosine might be due to a decreased availability of central monoamines displaced after the first injection of the amino-acids.

Further, the hypotensive effect of L-dopa was abolished after pretreatment with

α-MMT in combination with tyrosine hydroxylase inhibition. Similar results have been obtained with *m*-tyrosine (Rubenson, 1971). The present study has revealed that the pretreatment with α-MMT and inhibition of tyrosine hydroxylase caused a marked depletion of brain dopamine and noradrenaline. The dopa decarboxylase inhibition by α-MMT was of minor importance. Taken together these findings make it probable that endogenous stores of dopamine and noradrenaline are of great importance for the mechanism by which the hypotensive effect of L-dopa and DL-*m*-tyrosine is elicited.

Henning & Rubenson (1971) reported the hypotensive effect of α-MD to be unchanged after a combined pretreatment with α-MMT + H 44/68 + MK 486 similar to that used in the present study. However, they found the hypotensive effect was abolished after a dopamine-β-hydroxylase inhibitor. Their experiments point to the importance of a direct action of the α-MD catabolites, presumably α-methyl-noradrenaline. This view is compatible with the present results that α-MD produces the same hypotensive response after a second injection (see Fig. 1b).

In conclusion, L-dopa and DL-*m*-tyrosine cause a rapid lowering of blood pressure after dopa decarboxylase inhibition which can be blocked by central catecholamine depletion and is abolished after a second injection. On the other hand the slower developing hypotensive effect of L-α-MD remains under these conditions. L-Dopa and DL-*m*-tyrosine are rapidly decarboxylated, whereas the accumulation of α-methylated amines from α-methyldopa has a slower time course and results in a more sustained depletion of endogenous amines (for ref. see review of Muscholl, 1966).

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Metabolic degradation of prostaglandin E_1 in the rat plasma and in rat brain, heart, lung, kidney and testicle homogenates

Änggård & Samuelsson (1964, 1966) showed that prostaglandins are metabolized by oxidation of the secondary alcohol group at C-15 in swine lungs. This reaction is catalysed by NAD^+-dependent 15-hydroxy-prostaglandin dehydrogenase (Änggård & Samuelsson, 1966). Recently Nakano (Nakano, 1970a, b, c) showed that prostaglandin E_1 (PGE_1) was converted to less polar metabolites in dog isolated kidneys. Little information is available on the distribution of this enzyme or on the rate of the prostaglandin degradation in tissues other than lungs and kidneys. The present study was made to compare the rate of metabolism of PGE_1 in rat plasma, brain, heart, kidney and testicle with that in rat lung.

Male Holzman rats (200–250 g) were fed freely with Purina rat chow. They were killed by cervical dislocation and the lungs, kidneys, heart, brain and testicles were removed immediately from several rats and pooled. The tissues were homogenized at 4° in 4 volumes of Bücher medium (20 mM KH_2PO_4, 72 mM K_2HPO_4, 27·6 mM nicotinamide, 3·6 mM $MgCl_2$, pH 7·4 with a Potter-Elvehjem tissue grinder. The homogenates were centrifuged at 10 000 *g* for 20 min and protein in the supernatant was determined (Lowry, Rosenbrough & others, 1951); concentrations usually ranged from 10 to 15 mg/ml. The supernatant was shaken with 0·1 μCi/ml of ^{3}H-PGE_1 (28 Ci/mmol), 50 ng/ml of PGE_1 and 2 mmol of NAD^+ at 37·5°. Before and 2, 5, 10, 20, 40 and 60 min after the incubation was started, an aliquot (4 ml) of the samples was pipetted into tubes containing 0·5 ml of N HCl solution to terminate the reaction and to acidify to pH 3·0. ^{3}H-PGE_1 and its metabolites were extracted twice with ethyl acetate. The extract was separated with discontinuous silicic acid column chromatography using different ratios of ethyl acetate–toluene as described previously (Nakano, 1970a, b). PGE_1 was eluted with 70% ethyl acetate in toluene, whereas a less polar metabolite, thought to be 15-keto PGE_1, was eluted with 40% ethyl acetate in toluene (Nakano, 1970a, b). An aliquot (4 ml) of each chromatography fraction was pipetted into a counting vial, 15 ml of the scintillation fluid added, and the radioactivity of each sample was counted.

Crystalline PGE_1 was donated by Dr. J. E. Pike, Upjohn Co. Ltd, Kalamazoo, U.S.A. and ^{3}H-PGE_1 was obtained from New England Nuclear Corp. 11-α-Hydroxy-9,15-diketo-13-prostanoic acid (15-keto-PGE_1) was synthesized by MnO_2 oxidation of PGE_1 according to the method described by Attenburrow, Cameron & others (1952). The purity of the PGE_1, and 15-keto-PGE_1 was ascertained by thin-layer chromatography (Gréen & Samuelsson, 1964).

The silicic acid chromatography of the extracted rat plasma before incubation revealed a single peak of ^{3}H-PGE_1, which was eluted in fractions 11–14 (solvent system; ethyl acetate–toluene, 70:30). The silicic acid chromatography of the extract of the rat plasma which was incubated at 37° for 1 h showed an almost identical pattern with a single peak corresponding to ^{3}H-PGE_1. Thus practically no metabolic degradation of PGE_1 was detected in the rat plasma. This agrees with the findings of Vane (1969) that the biological activity of PGE_1 is not altered by incubation with rat plasma. In contrast, the silicic acid chromatography of the extract of the rat lung homogenate showed that after 20 min incubation the ^{3}H-PGE_1 peak in fractions 11–14 had practically disappeared. Instead, a prominent peak appeared in fractions 6–9 which was apparently due to a less polar PGE_1 metabolite (which we called Metabolite 1). Thin-layer chromatography showed that the R_F value, 0·63, of Metabolite 1 was identical with that of 15-keto-PGE_1 (Änggård & Samuelsson, 1964).

Very slow metabolic degradation of ^{3}H-PGE_1 was observed in the rat plasma and in the brain and heart homogenates. In contrast, the metabolic degradation of

^{3}H-PGE_1 occurred in the rat testicle, kidney and lung. The kidney and lung homogenates metabolized 95% of ^{3}H-PGE_1 within 20 min, whereas the rat testicle homogenate converted 80% of ^{3}H-PGE_1 within 20 min.

From the present study, it is evident that little metabolism of any released or injected PGE_1 occurs in rat plasma or in homogenized brain or heart. In contrast, rat homogenized lung, kidney and testicle metabolize PGE_1 quite quickly as do guinea-pig, cat, rabbit and dog lung, cat liver and dog kidney (Änggård & Samuelsson, 1964; Ferreira & Vane, 1967; Nakano, 1970a, b). PGE_1 is converted into 15-keto-PGE_1 by the oxidation of the secondary alcohol group at 15-C in swine lung and this appears to be the mechanism in rat lung. PGE_1 in guinea-pig lung is converted into dihydro-PGE_1 and 15-keto-dihydro-PGE_1 by the reduction of the Δ^{13}-double bond. The enzyme that catalyses the oxidation of PGE_1 has been identified as NAD^+-dependent 15-hydroxy-prostaglandin dehydrogenase which is specific for PG (Änggård & Samuelsson, 1966; Nakano, Änggård & Samuelsson, 1969). Further evidence for the inactivation of PG in rat, dog and human lung is that the hypotensive effect of PGE_1 or $PGF_{2\alpha}$ injected intra-arterially or into the left atrium is greater than with intravenous injection (Bennett, Eley & Scholes, 1968; Bergström, Carlson & others, 1965; Nakano & Cole, 1969; Nakano & Kessinger, unpublished data). It is not known whether this is due to uptake of PG by the lung, but if the metabolites are present in the blood they presumably have little effect; the vasodilator activities of the PGE_1 metabolites, 15-keto-PGE_1 and 15-keto-dihydro-PGE_1 are approximately 1/100 to 1/150 of that of PGE_1 in the dog hind-limb preparation (Nakano & Kessinger, unpublished data). In summary, rat homogenized lung, liver and testicle inactivate PGE_1. The product formed by the lung appears to be 15-keto PGE_1. The plasma and homogenized brain or heart have little ability to metabolize PGE_1.

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