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REVIEW

Bacterial resistance to penicillins and cephalosporins

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In 1946, 14% of strains of *Staphylococcus aureus* isolated at the Hammersmith Hospital were resistant to benzylpenicillin; by 1947 this figure had risen to 38%, and by 1948 it had become 59% (Barber & Rozwadowska-Dowzenko, 1948). A more recent estimate (Morrison, 1961) puts the proportion of benzylpenicillin-resistant strains of hospital *Staph. aureus* as high as 80%. These figures illustrate the rapid increase in numbers of penicillin-resistant "hospital" staphylococci. It is now thought that the indiscriminate use of penicillins was a major factor responsible for this phenomenon; however, this was not known at the time. Introduction of the penicillinase-stable semisynthetic penicillins, such as methicillin and cloxacillin, to which the most of these benzylpenicillin-resistant strains are sensitive has improved the clinical situation.

The use of broad-spectrum semisynthetic β-lactam drugs, such as ampicillin, carbenicillin and cephaloridine, has made possible the treatment of infections caused by certain Gram-negative organisms which were not previously sensitive to penicillin therapy. At present, most penicillinase-producing strains of *Staph. aureus* are sensitive to the penicillinase-resistant penicillins and most coliform bacteria are sensitive to the broad spectrum β-lactam antibiotics (see Table 1). However, there inevitably exist minority populations which are resistant and, given encouragement, they will become predominant. Therefore, to avoid a repetition of the situation in which benzylpenicillin failed to control the hospital penicillinase-producing staphylococcus, the emergence of resistance must be prevented. A novel method of classification of bacterial resistance is here presented and has been used to summarize the mechanisms by which different bacteria are, or can become, resistant to penicillins and to cephalosporins, to estimate the importance of the various types of resistance, and to indicate how, with some organisms, these may be modified, or prevented. Modes of resistance to penicillins are not yet fully understood, but as many workers are actively studying this problem it is necessary to collect the available data to form a coherent picture of the facts and, more important, of the areas in which further studies would be beneficial. The genetics of penicillinase production will not be discussed in detail, except where strictly applicable to the phenotypic expression of penicillin resistance. These topics have been reviewed by Novick (1965) for staphylococci, Dubnau & Pollock (1965) for bacilli, Eriksson-Grennberg, Boman & others (1965) for escherichia, and Meynell, Meynell & Datta (1968) for R-factors.

Table 1. *Antibacterial "spectra" of several penicillins and cephalosporins*

Organisms	Benzyl-penicillin	Phenoxy-methylpenicillin Phenethicillin Propicillin	Carbenicillin	Ampicillin	Methicillin Nafcillin	Isoxazolyl-penicillins	Cephalothin Cephaloridine Cephaloglycin Cephalexin	Comment
Staph. aureus (non-penicillinase producing)	+	+	+	+	+	+	+	
Staph. aureus (penicillinase producing)					+	+++	++	A few strains resistant
Strep. pneumoniae	++	++	++	++	++	+++	++	
Strep. faecalis	++	++	++	++	++	+++	++	Many strains only marginally sensitive
Strep. pyogenes, Group A	++	+++	+++	+++++	+++	+++	+++	
Strep. viridans	++	+++	+++	+++++			+++	
N. meningitidis			++	+++++			+	
E. coli			+	+			++	Some resistant strains
K. aerogenes				+				Most strains resistant, or marginally sensitive
Pr. mirabilis (penicillinase −)			+++++	+			++	
Pr. mirabilis (penicillinase +)			+++++					
Pr. rettgeri, morgani, vulgaris			++					
Ps. pyocyanea			++					
Salmonella spp.	++			++	+	+		
H. influenzae		+		++			++	

+ Indicates that the drug is likely to be useful because concentrations attainable in the blood during therapy exceed the minimum inhibitory concentrations found *in vitro*.

PHENOTYPIC AND GENOTYPIC RESISTANCE

The sensitivity of a bacterial strain to a penicillin or cephalosporin is governed by two main factors—the resistance of each individual bacterium (the intrinsic resistance), and the capacity of the culture as a whole to produce penicillinase (β-lactamase). The level of intrinsic resistance of a strain normally falls within narrow limits of antibiotic concentration and this is regarded as an indication of "homogeneity" of the culture. Some strains, however, do not conform to this, and there is a wide and non-Gaussian distribution in the intrinsic resistance of individual bacteria of such strains; these are regarded as being "heterogeneous" in their pattern of intrinsic resistance. The significance of these strains will be discussed later. The intrinsic resistance of a strain can be conveniently measured in the laboratory by using a small inoculum (10–100 organisms) by conventional techniques, either on solid or in liquid media. As well as possessing intrinsic resistance, some bacteria may make β-lactamase and these organisms will be even less susceptible to penicillins and cephalosporins than those possessing intrinsic resistance alone. Both intrinsic resistance and penicillinase production are under genetic control; for simplicity, it is convenient to assume that their determinants are simple and independent of each other, and that only two allelic forms exist, namely intrinsically sensitive (S^+)/resistant (S^-), and incapable of destroying penicillin (P^-)/capable (P^+). The ability, or its absence, to destroy penicillin is directly dependent upon the capacity of the bacterial strain in question to produce penicillinase (EC 3.5.2.6.). P^+ strains will destroy penicillin significantly in large, but not in small, inocula. Although bacteria may also produce another penicillin-destroying enzymepenicillinacylase (EC 3.5.1.11.), this enzyme probably plays no significant part in the resistance of bacteria to penicillins for the following reasons. (i) Acylases have low affinities for their substrates, thus, although rapid deacylation can occur *in vitro* at high substrate concentrations, at the much lower penicillin concentrations attainable therapeutically the activity of these enzymes will be drastically reduced. (ii) Acylase activity at physiological pH values is suboptimal. (iii) Production of acylase by bacteria is markedly less at 37° than at lower temperatures. (iv) As the reaction is reversible, deacylation does not go to completion. (v) Both products of the reaction can possess antibacterial properties (see Cole & Sutherland, 1966; Hamilton-Miller, 1966c).

There exist four combinations of alleles which are responsible for sensitivity or significant resistance to penicillins: namely, S^+P^-, S^+P^+, S^-P^- and S^-P^+. Strains

Table 2. *System of classification used*

Class	Genotype		Phenotype
	Intrinsic Sensitivity	Penicillinase Production	
I	S^+	P^-	Sensitive
II	S^+	P^+	Resistant to hydrolysable pencillins
			Sensitive to non-hydrolysable penicillins
III	S^-	P^-	Resistant
IV	S^-	P^+	Resistant

Notes: Genotype = intrinsic susceptibility to penicillin (small inoculum).
Phenotype = overall susceptibility to penicillin (large inoculum).
Penicillinase production: this merely means production of an enzyme capable of hydrolysing the β-lactam ring in benzylpenicillin. It must not necessarily be assumed that any penicillins other than benzylpenicillin and phenoxymethylpenicillin are hydrolysed by the particular enzyme (see Table 3).

possessing the first of these will clearly be phenotypically sensitive under all conditions and those with the last two will be phenotypically resistant under all conditions. The phenotype of strains belonging to the second class (genotype S$^+$P$^+$) will depend on the conditions: when only a few organisms are present (a small inoculum), the strain will be phenotypically sensitive; a large inoculum, however, will contain enough penicillinase to be capable of destroying a significant amount of penicillin, and the strain will thus be phenotypically resistant to hydrolysable penicillins. Resistance is here best defined as the ability of a bacterial strain to multiply in the presence of concentrations of a drug usually attained *in vivo* during its normal therapeutic use; e.g. a strain of *Staph. aureus* not inhibited by 5 μg/ml of methicillin would be a resistant strain, as would be an *Escherichia coli* strain which grows in the presence of 15 μg/ml ampicillin. The scheme by which bacteria can be put into four classes is illustrated in Table 2; this classification will be used as a starting point in the discussion of resistance in this review. The symbol P$^+$ merely denotes the ability of a strain to produce an enzyme capable of hydrolysing the β-lactam ring in benzylpenicillin.

INTRINSIC RESISTANCE TO β-LACTAM ANTIBIOTICS

Since the precise mode of action of β-lactam antibiotics is not yet fully proven, mechanisms of intrinsic resistance cannot be discussed in precise molecular terms; thus, the subsequent treatment of this topic must inevitably be speculative.

In this discussion it has been assumed that all penicillins and cephalosporins have essentially the same basic mode of action, namely inhibition of mucopeptide biosynthesis. The resulting faultily constructed cell wall is unable to accommodate the cytoplasmic enlargement and internal osmotic pressure of the growing bacterial cell, and partial or complete lysis of the culture, depending upon the exact conditions, follows. Rogers (1967a) calls attention to the role of mucopeptidases in bacterial lysis. In view of the known differences in mucopeptide structure among bacterial species, it seems likely that the detailed mode of action of penicillins and cephalosporins will vary slightly from one species to another, and conversely it is also possible, but less likely, that the detailed modes of action of different β-lactam drugs against a given bacterial strain may vary from one compound to another. Some penicillins appear to have individual side-effects on certain bacteria—viz. nafcillin sensitizes *Staph. aureus* to lysozyme digestion (Warren & Gray, 1967), methicillin increases the permeability of *Staph. aureus* (Rogers, 1967a), and the permeability of coliform bacteria is increased by several penicillins (Smith, 1963a; Hamilton-Miller, 1966a). All these phenomena are caused by concentrations of penicillins which result in less than complete growth inhibition and may be regarded as side-effects due to partial impairment of biosynthesis of cell wall mucopeptide.

The final stage of bacterial mucopeptide biosynthesis is a cross-linking reaction between two peptide chains of peptidoglycan polymer and is thought to be mediated by at least two enzymes, peptidoglycan transpeptidase and D-alanine carboxypeptidase (Izaki, Matsuhashi & Strominger, 1966). The carboxyl group of the D-alanyl-D-alanine moiety of mucopeptide may react with the transpeptidase (see Fig. 1, reaction B1). Subsequent hydrolysis of the peptide bond between the two D-alanine residues would then occur (Reaction B2), leading to release of the terminal D-alanine (Reaction B3) (Strominger, Izaki & others, 1967).

Although the precise site of penicillin action is unknown, two likely hypotheses have been advanced. The first envisages that penicillin acts as a substrate analogue of the
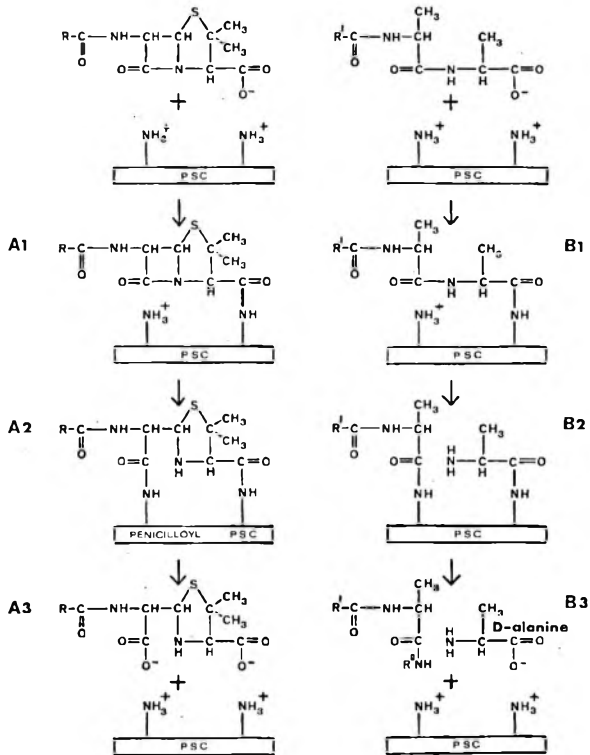
FIG. 1. A_1, A_2 and A_3 show possible reaction sequences of penicillin with the penicillin-sensitive component (PSC). B_1, B_2 and B_3 illustrate the reaction sequences whereby the terminal D-alanyl-D-alanine peptide of peptidoglycan is cross-linked. R–C = O is the acyl side-chain of penicillin. R′–C = O is the remainder of the peptide chain of peptidoglycan. R″NH is the peptide residue of another peptidoglycan chain to which the penultimate D-alanine is cross-linked.

D-alanyl-D-alanine moiety of the peptidoglycan and consequently inhibits the cross-linking reaction (Tipper & Strominger, 1965; Wise & Park 1965). The free carboxyl group of penicillin can mimic the carboxyl group of D-alanyl-D-alanine and become attached to the transpeptidase (see Fig. 1 Reaction A1). This is followed by hydrolysis of the β-lactam bond of penicillin (Reaction A2), which coincides sterically with the peptide bond of D-alanyl-D-alanine, the resulting penicilloylation of the enzyme rendering it inactive for subsequent cross-linking (Tipper & Strominger, 1965). Of the two enzymes concerned in cross-linking, the transpeptidase is the more likely candidate since penicillin inactivates it at growth inhibitory concentrations; D-alanine carboxypeptidase is also inhibited by penicillin but only reversibly and at levels inconsistent with growth inhibitory concentrations (Strominger & others, 1967).

The second hypothesis proposes that penicillin interacts with a peptidoglycan precursor binding site on the bacterial cell membrane thus preventing the enzymes necessary for completion of peptidoglycan synthesis from reaching the substrate (Rogers, 1967b). These two views may well turn out to be one and the same thing when the precise nature of the cross-linking reaction is more fully understood.

For the purposes of this discussion it is irrelevant which of these theories is correct; the component (be it enzyme or membrane) with which penicillin combines to exert its antibacterial influence will be called merely the "penicillin-sensitive component" (PSC).

The reaction between PSC and penicillin (P) may be represented thus:

$$PSC + P \rightarrow PSC-P$$
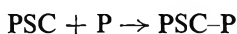
the velocity of this reaction $= k_1[P][PSC]$. The penicillins are assumed to bring about this reaction by penicilloylation (Fig 1, reaction A2), and hence the reaction is, on chemical grounds, irreversible.

For balanced growth to occur it is postulated that a certain concentration $(= [PSC]_{min})$ of free PSC must be present in the cell. The action of penicillin is to reduce the total concentration of PSC $(= [PSC]_{total})$ by sequestering PSC in the form of the inactive complex PSC-P. It can be seen that, under a given set of conditions, $[PSC]_{free} = [PSC]_{total} - [PSC-P]$; if $[PSC]_{free} > [PSC]_{min}$, growth can occur, while if $[PSC]_{free} < [PSC]_{min}$, no growth will occur. For the special case $[PSC]_{free} = [PSC]_{min}$, penicillin is present at the minimum inhibitory concentration $(= [P]_{mic})$. It follows that any process whereby $[PSC]_{free}$ can be increased will also increase $[P]_{mic}$, and thus by definition the intrinsic resistance will be increased.

It also follows that decreases in either k_1 or $[P]$ (see (2) below), for example by mutation, would reduce the rate of reaction, and thus these bacteria would possess a higher level of intrinsic resistance. Factors which could contribute towards decreases in these two parameters will be discussed below.

(1) Decrease in k_1; k_1 is a measure of the "affinity" of a penicillin for its active site, and as such has been discussed by Rolinson (1965) vis-à-vis whole cells of Staph. aureus and E. coli, and by Izaki & others (1966) for a cell-free system of PSC from E. coli. It can be seen that a slight change in the chemical nature of PSC might radically alter its affinity for a penicillin while not affecting its ability to participate in the cross-linking reaction; such a "qualitative" change in PSC could be responsible for the intrinsic resistance of the bacterium which contained it.

(2) Decrease in $[P]$. (i) Change in permeability; if the ease with which P could approach PSC were reduced, the overall effect, all other things being equal, would be to decrease $[P]$, and thus to bring about an increase in the intrinsic resistance of the strain involved. There is evidence for the existence of a substantial permeability barrier to benzylpenicillin in the intrinsically sensitive Oxford strain (Rogers, 1967b), and to methicillin in the intrinsically sensitive 524 SC strain of Staph. aureus (Rogers, 1967a). (ii) Ability of PSC-P to dissociate. Dissociation of PSC-P will clearly ease the metabolic block imposed by an inhibitory concentration of P, by releasing free sites to continue cell wall synthesis. As stated above, such a dissociation cannot be a mere reversal of reaction A2 (Fig. 1), as the cyclization of a penicilloate to a penicillin requires highly unusual chemical conditions (Sheehan & Henery-Logan, 1962); the only possible route for PSC-P to dissociate seems to be that represented by reaction A3 which is the chemical notation for

$$PSC-P \rightarrow P' + PSC,$$
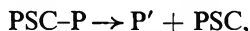
where P' represents penicilloate; the rate of dissociation is $k_2[PSC-P]$. It can thus be seen that the overall velocity of sequestration of PSC has been reduced, under these circumstances, to $k_1[P][PSC] - k_2[PSC-P]$, with a corresponding decrease in bacterial sensitivity, and thus, PSC is acting as a penicillinase (see Pollock, 1967).

There is also the following possibility:

(3) Increase in $[PSC]_{total}$; assuming that both the supply of penicillin and the amount of PSC are rate-limiting [as has been indicated by the experiments of Rogers (1967b), who showed that the reaction between P and PSC obeyed second-order

kinetics in *Staph. aureus* Oxford], any increase in the absolute amount of PSC will overcome the metabolic block imposed by penicillin. Thus, an increased synthesis of PSC will result in an increase in the intrinsic penicillin resistance on the part of a particular bacterium; such increased synthesis could be brought about by, for instance, an increased sensitivity of the relevant feedback mechanism. This type of "quantitative" alteration in PSC production could clearly take place either gradually, by adaptation, or stepwise, by mutation followed by selection of the resistant mutants.

Unfortunately, there is little or no evidence about which of these means of acquiring resistance is being used in any particular case. There is no doubt that the penicillinase mechanism (see above) is responsible for resistance to benzylpenicillin in *Staph. aureus*. There is some circumstantial evidence (Hugo & Stretton, 1966; Redai, Rethy & Vaczi, 1967; Bruns, 1967) suggesting that changes in the intrinsic resistance of *Staph. aureus* strains may be caused by changes in the lipid content of the bacteria; such changes can readily occur without any deleterious effect on growth patterns or viability (Vaczi, Redai & Rethy, 1967). It is clear that cultures composed of individuals with differing fatty acid compositions, and hence differing permeabilities to penicillins, offer a potentially rich field on which natural selection may work. It is interesting that acquisition of resistance to penicillins can bring about some radical changes in surface structures (e.g. change in phage type of *Salmonella panama*—Guinee, Scholtens & Willems, 1967), which may reflect alterations in permeability barriers.

MANIFESTATIONS OF PENICILLIN-RESISTANCE BY DIFFERENT BACTERIAL SPECIES

We have found it convenient to consider Gram-positive and Gram-negative species separately, for their resistance and sensitivity to penicillins.

(a) GRAM-POSITIVE BACTERIA

Class I. *Non-penicillinase-producing, intrinsically sensitive strains* (S+P−)

Group A streptococci (*Streptococcus pyogenes*) are typical members of this class. A survey in Czechoslovakia (Jelinkova & Jelinek, 1965) showed that the mean sensitivity of strains isolated between 1952 and 1964 has remained at about 0·015 units/ml benzylpenicillin: there was no indication of any increase in penicillin resistance over this period. Other examples of species falling into this class are non-penicillinase producing strains of *Staph. aureus* (typified by the Oxford strain), and *Listeria monocytogenes*.

Infections caused by the bacteria in this class usually respond to treatment with benzylpenicillin. Failure to respond to such treatment can usually be attributed to either accessibility difficulties (e.g. penetration of penicillin into vegetations on heart valves) or to concomitant infections by penicillinase-producing organisms (Tacking, 1954, but see also Lagerholm, Lodin & Nystrom, 1966, Quie, Pierce & Wannamaker, 1966, Markowitz, Kramer & others, 1967).

Class II. *Penicillinase-producing, intrinsically sensitive strains* (S+P+)

The typical hospital staphylococcus (Barber, 1947) is found in this class; such organisms are characteristically of phage group III, resistant to mercuric salts and to tetracycline (Richmond, Parker & others, 1964). Penicillin resistance is due to penicillinase production (Barber, 1947), and the great increase in the proportion of these strains shortly after the large-scale introduction of penicillin therapy (see Introduction) reflects the selection of penicillinase-producing strains because of their ecological

advantage over non-penicillinase-producing strains in the presence of a hydrolysable penicillin such as benzylpenicillin. There has been no case reported in which a penicillinase-producing strain of *Staph. aureus* has arisen *de novo* from a penicillinase-negative parent, i.e. a Class I to II mutation (see Fig. 2).

Penicillinase-producing *Staph. aureus* strains may have arisen from penicillinase-positive coagulase-negative strains (such as *Staph. albus*), which have gained coagulase and other factors concerned with virulence. This could occur *in vivo* either by mutation, transduction (Novick & Morse, 1967) or by a mechanism similar to exhaltation of virulence.

Organisms in this class show a typical inoculum-size effect, i.e. in small inocula they are many times more sensitive than in large inocula (Luria, 1946). That it is the intrinsic resistance of such strains that is measured by determining the minimum inhibitory concentrations for small inocula, is demonstrated by the fact that the minimum inhibitory concentration for a small inoculum of *Staph. aureus* E3T (a penicillinase-negative variant of E3—Knox, 1960; Knox & Smith, 1961) is the same as that for a small inoculum of the penicillinase-producing parent strain, E3. Moreover, Class II strains of *Staph. aureus* will, by virtue of this intrinsic sensitivity, be susceptible to β-lactam antibiotics which are not hydrolysed by staphylococcal penicillinase, such as methicillin, the isoxazolyl penicillins, quinacillin and certain cephalosporin derivatives.

Spontaneous loss of P^+ occurs *in vitro* at a rate of between 10^{-4} (Novick, 1963) and 10^{-3} (Seligman, 1966) and is therefore a Class II to I mutation (see Fig. 2). The rate of loss of P^+ is increased under "adverse conditions" such as the presence of methicillin (as in the conversion of E3 to E3T—Knox, 1960; Knox & Smith, 1961), or growth at $44°$ (May, Houghton & Perret, 1964). Gorrill & McNeil (1965) have shown that this change can also occur *in vivo*.
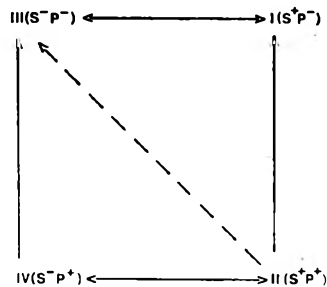
FIG. 2. Summary of changes that can occur in the intrinsic sensitivity and the capacity to produce penicillinase in *Staph. aureus* strains. P^+ = capable of producing penicillinase. P^- = not capable of producing penicillinase. S^+ = intrinsically sensitive. S^- = intrinsically resistant.

Kjellander, Klein & Finland (1963) have investigated several benzylpenicillin-resistant strains of *Staph. albus*, and found that most were resistant by a penicillinase-type mechanism; this bacterial species has been found to cause an alarmingly high mortality in cases in which it has been isolated from blood cultures (Finland, Jones & Barnes, 1959).

Recent investigations have shown that *Mycobacterium tuberculosis* produces penicillinase, and that this factor plays a not unimportant part in its resistance to penicillins (Dufour, Knight & Harris, 1966; Kasik & others, 1967). Thus, *Myco. tuberculosis* (human, bovine and avian strains) belongs in Class II; Kasik, Weber &

others (1966) have demonstrated that tuberculosis in mice may successfully be treated with a synergistic mixture of dicloxacillin and benzylpenicillin.

Class III. *Penicillinase-negative, intrinsically resistant strains* (S⁻P⁻)

Strains belonging to the Runyon Group III of "anonymous" mycobacteria, the so-called Battey strains, are resistant to penicillins and do not produce penicillinase (Dufour & others, 1966); a reason for this intrinsic resistance may be the inability of the drugs to penetrate the lipid-rich cell wall.

Strains of Group A *Strep. pyogenes* resistant to benzylpenicillin have been obtained by laboratory training techniques (Yoshioka & Kunii, 1965); such strains do not produce penicillinase, and are thus Class III strains. The resistant mutants had lost some of their virulence, and resemble some artificially produced Class III *Staph. aureus* (see below).

Group D-streptococci (enterococci) have a natural intrinsic resistance to most penicillins, a fact which often complicates the treatment of subacute bacterial endocarditis caused by this species. However, it seems that streptomycin acts synergistically with benzylpenicillin or ampicillin against certain strains of enterococci (Hewitt, Seligman, & Deigh, 1966; Simon, 1967).

Stirland & Shotts (1967) have reported that strains of *Strep. viridans* having an enhanced resistance to benzylpenicillin (Class III strains) may be selected during therapy; if such a phenomenon continues, the result will clearly be the large-scale emergence of penicillin-resistant *Strep. viridans* strains.

Pneumococci resistant to benzylpenicillin have been obtained by *in vitro* training (McKee & Houck, 1943; Eriksen, 1946; Gunnison, Fraher & others, 1968). In some cases, but not all, such strains—which had increased their resistance by from five to eighty fold—had lost mouse virulence. Hansman & Bullen (1967) isolated from a patient a type 23 pneumococcus which was relatively resistant to benzylpenicillin (MIC 0·6 μg/ml, compared with 0·03 μg/ml for typical strains) and ampicillin; this appears to be the only case of a wild pneumococcal strain showing some degree of penicillin-resistance.

Class III strains of *Staph. aureus* fall into three categories; the first consists of an interesting type of organism—penicillinase-producing strains of *Staph. aureus* (Class II) that have been trained in the laboratory to "tolerance" to a penicillin, by means of serial sub-culture in increasing concentrations of the drug (Barber, 1961; Knox & Smith, 1961). Such organisms are resistant to the penicillin upon which they were grown (and also display cross-resistance to other penicillins—Knox, 1960—and to cephalothin—Godzeski, Brier & Pavey, 1963), solely by an intrinsic mechanism, as they have lost their capacity to produce penicillinase. It seems that this Class II to III mutation (see dotted line Fig. 2) is a summation of two separate mutational events e.g. II to I and I to III. These changes also involve loss of virulence (Barber, 1961; Knox & Smith, 1961). It thus appears, on balance, unlikely that strains of *Staph. aureus* belonging to this group can be of great clinical significance. The increased intrinsic resistance as a consequence of laboratory training techniques may be caused by a decrease in permeability of the membrane to the drug, or it may involve the use of an alternative metabolic pathway to bypass the step in cell wall synthesis which is inhibited by the penicillin. In the context it is interesting that a strain of *Pediococcus cerevisiae* has been obtained (White, 1968) which is dependent upon methicillin for growth.

The second category of Class III strains of *Staph. aureus* is comprised mainly of strains that occur naturally; Farkas-Himsley, Soeprihatin & Goldner (1961) reported having isolated from human and animal sources seven strains of *Staph. aureus* resistant to both methicillin and cloxacillin, and that did not produce penicillinase. Seligman (1966) reported that the natural rate of loss of the ability to produce penicillinase among methicillin-resistant strains of *Staph. aureus* was of the order of 10^{-3} (i.e. one organism per 1000 is no longer able to produce the enzyme) (i.e. a class IV to III mutation see Fig. 2), and thus it appears possible that the strains isolated by Farkas-Himsley and his colleagues represent such loss mutants, the mutation possibly having occurred *in vivo*.

The third category of Class III strains of *Staph. aureus* contains S^+P^- strains, for example mutants of E3T or the Oxford staphylococcus which have been obtained by selecting for intrinsic resistance in the laboratory, i.e. a Class I to III mutation, see Fig. 2. Barber (1961) reports that such strains are of decreased virulence and are often unstable in the absence of penicillin, reverting from Class III to Class I strains spontaneously (Fig. 2). Therefore it is doubtful if these Class III strains are of clinical significance.

Class IV.   *Penicillinase-producing intrinsically resistant strains* (S^-P^+)

Barber (1964) investigated 59 strains of naturally-occurring methicillin-resistant *Staph. aureus*, isolated in Britain, France and Denmark; all belonged to closely related phage types (Group III), and all produced penicillinase. It has been established that the resistance of large inocula of such strains is because they are "heterogeneous", i.e. they contain a small proportion of individuals with a high intrinsic (genotypic) resistance (Knox & Smith, 1961; Sutherland & Rolinson, 1964; Chabbert, Baudens, & others, 1965; Seligman & Hewitt, 1965). Methicillin-resistant staphylococci are also resistant to isoxazolyl penicillins (Eriksen & Erichsen, 1964) and to quinacillin (Smith, Hamilton-Miller & Knox, 1964). It has been claimed (Stewart & Holt, 1963; Eriksen & Erichsen, 1964) that the resistance of these strains is due to an increased ability on their part to destroy methicillin and isoxazolyl penicillins; however, Knox & Smith (1963) and Ayliffe & Barber (1963), using the same strains, showed that methicillin resistance was not caused by increased 'methicillinase' activity, but was due to the intrinsic mechanism. Seligman (1966) showed that penicillinase-negative mutants of 5 methicillin-resistant, penicillinase-producing *Staph. aureus* strains retained practically *in toto* their parents' resistance to methicillin. This Class IV to III mutation is shown in Fig. 2. This author, Dyke, Jevons & Parker (1966), and Hewitt & Parker (1968), suggest that a high grade resistance to methicillin (up to 500 μg/ml) is entirely due to an intrinsic mechanism.

The origin of Class IV strains of *Staph. aureus* is not known. However, laboratory training procedures can enable Class II strains to become intrinsically resistant to penicillins while retaining their P^+ characteristic as well as full virulence (Barber, 1961); an example of a Class II to IV mutation (see Fig. 2) is the change of strain 13137 to 13137/1000 (Knox & Smith, 1961). Whether this change can occur *in vivo* (e.g. during the course of treatment of a patient with a penicillin) is unknown . There is no *prima facie* reason why it should not, though it has not occurred (Stewart, 1960), even under favourable conditions (Elek & Fleming, 1960). However, since it is known that Class IV strains isolated *in vitro* are unstable in the absence of penicillins, losing their

intrinsic resistance whilst retaining their P[+] characteristic, similar conditions possibly apply *in vivo* and this would prevent their large scale emergence.

Methicillin-resistant strains generally show cross-resistance to semi-synthetic cephalosporins (Barber & Waterworth, 1964a; Hamilton-Miller & Ramsay, 1967; Hewitt & Parker, 1968). Although it has been shown (Hamilton-Miller & Ramsay, 1967) that cephaloridine is less stable to staphylococcal penicillinase than cephalothin, methicillin-resistant P[+] strains are nonetheless more sensitive to cephaloridine than to cephalothin; this is accounted for by the increased intrinsic activity of the former drug [due to its possession of a pyridinium moiety (see Hale, Newton & Abraham, 1961)], which outweighs its greater lability to penicillinase.

Methicillin-resistant strains of *Staph. aureus* maybe on the increase both in the United Kingdom and in France. Jevons (1961) stated that 3 out of 5,440 strains isolated between October and December 1960 (i.e. before the use of methicillin) were methicillin-resistant, an incidence of 0·055%; a further survey by Jevons, Coe & Parker (1963), analysed 27,479 strains isolated between October 1960 and September 1962; 102 (0·48%) were resistant to methicillin. Later, Colley, McNicol & Bracken (1965), who investigated strains isolated between September 1962 and April 1964, found an overall incidence of methicillin resistance of 4·1% and Turner & Cox (1967) have observed incidences of infection with methicillin-resistant strains of 5·4% in 1963, rising to 16·6% in 1965; however, this latter set of figures is not strictly comparable to those given earlier, as the incidence was reported in terms of total multiple-resistant strains. Chabbert, Baudens & others (1965) found that as many as 19% of strains of *Staph. aureus* isolated in Paris hospitals during 1963 were potentially resistant to methicillin and oxacillin, as they contained a small but significant proportion (0·1– 10%) of individuals with a markedly increased intrinsic resistance. A more recent survey in Paris (Bastin, Worms & Acar, 1967) shows that 36% of cases of staphylococcal septicaemia occurring between January 1963 and October 1966 were caused by methicillin-resistant strains. Breitfellner (1967), however, reports no increase, between 1960 and 1966, in the incidence of oxacillin-resistant strains isolated in Vienna. Kjellander & others (1963) reported a relatively high incidence of resistance to methicillin and to oxacillin among penicillinase-producing *Staph. albus* strains; such resistance was shown to be intrinsic, as neither penicillin was destroyed by *Staph. albus* penicillinase.

Over 300 methicillin-resistant *Staph. aureus* strains have been isolated in Great Britain (Colley & others, 1965), and further strains have been found in France (Chabbert & others, 1965), Germany (Stille & Brandis, 1965), Denmark (Eriksen & Erichsen, 1964), Sweden, Spain, Argentina and U.S.A. (Isenberg, 1965; Benner, Bennett, & others, 1965). It is interesting that relatively few strains have been isolated in U.S.A., where large scale use of methicillin and the isoxazolyl penicillins is comparable to that in Europe. Many methicillin-resistant strains belong to phage Group III; it was originally thought that methicillin resistance only occurred in this phage group, but Isenberg (1965), Benner & Morthland (1967) and Chabbert & Pillet (1967) showed that this was not so.

There have been very few serious infections in Great Britain caused by methicillin-resistant *Staph. aureus* strains—one death was reported by Stewart & Holt (1963), five by Colley & others (1965), and two serious infections by Harding (1963).

The fact that methicillin-resistance is due to the heterogeneity of bacterial cultures tends to lessen its clinical implications: a patient with an infection caused by such a

heterogeneous strain may still be treated with methicillin with a reasonable chance of success (see Chabbert & others, 1965; Bastin & others, 1967; Turner & Cox, 1967). Most of the bacteria will succumb to therapeutically attained concentrations of the drug, and the small numbers of resistant cells remaining may be adequately dealt with by the body.   However, this view is not held by Benner & Kayser (1968), who recommend vancomycin treatment.

Several workers (e.g. Barber, 1964; Sutherland & Rolinson, 1964; Seligman, 1966; Hamilton-Miller & Ramsay, 1967) have reported that methicillin-resistant strains of *Staph. aureus* contain a significant proportion of colonial variants, particularly when the bacteria are grown in the presence of methicillin.   These colonies resemble G-forms, and it seemed possible at one stage that they represent a type of L-form lacking both cell wall and sensitivity to penicillins.   If this were so, it would be a potentially dangerous situation, as such degenerative forms could act as a reservoir of staphylococci that could cause recrudescence of infection on the cessation of methicillin treatment.   However, examination of "G-like" colonies by Bulger & Bulger (1967) showed them to be composed of staphylococci with normal cell walls; and Barber (1964) had shown that the incidence of such colonial variants was greatly reduced by the incorporation of a high salt concentration into the growth medium.   This suggested that these colonies suffer from some osmotic imbalance.   Knox & Smith (1961) found that a laboratory-trained methicillin-resistant strain E3/500 (made from a Class II strain, E3) had lost its penicillinase, changed to the G-form and gained intrinsic resistance simultaneously.   It had also lost a significant amount of its mouse virulence.

It seems probable that many infections caused by methicillin-resistant staphyloccoci have been resolved without the true nature of the infecting organism becoming known; the laboratory diagnosis of methicillin-resistance cannot normally be made satisfactorily by the disc method (Kayser, 1967) and the more laborious tube dilution test requires 48 h incubation before the result can be read (Sutherland & Rolinson, 1964). Benner & Morthland (1967) recommend vancomycin for the treatment of infections with known methicillin-resistant strains of staphylococci, and Bulger (1967) favours combinations of kanamycin with either methicillin or cephalothin.   Kanamycin has also been used by French clinicians (Bastin & others, 1967).

(b) GRAM-NEGATIVE BACTERIA

The causes of penicillin resistance in Gram-negative species are not as clear as in Gram-positive species partly because intensive investigations of Gram-negative organisms did not start until the therapeutic introduction of ampicillin in 1961.   At first most workers assumed that ampicillin was hydrolysed as readily by penicillinases from Gram-negative bacteria as by staphylococcal penicillinase, and hence that all penicillinase-producing Gram-negative bacteria were considered ampicillin-resistant because they destroyed the drug (Class II strains).   However, it is now evident that the penicillinases from Gram-negative organisms often have properties very different from those of Gram-positive species, and each Gram-negative species may produce a unique penicillinase (Smith & Hamilton-Miller, 1963).   Some of the properties of the penicillinases from various species of bacteria are given in Table 3.   It can be seen, for instance, that some penicillinase-producing *E. coli* strains do not hydrolyse ampicillin (Smith, 1963a); nevertheless, it has often been assumed that such *E. coli* strains will be resistant to ampicillin by the drug inactivation mechanism.

Table 3. *Some properties of penicillinases from different bacterial species*

| | Gram-positive bacteria | | | | Gram-negative bacteria | | |
| | | | | | "Penicillinase-type" | | "Cephalosporinase-type" E. coli, Enterobacter spp., Pr. vulgaris, Pr. rettgeri, Pr. morgani, Ps. pyocyanea |
SUBSTRATE SPECIFICITY	Staph. aureus	B. cereus β-lactamase I	B. cereus β-lactamase II	B. licheniformis	Klebsiella, Proteus mirabilis	Aerobacter cloacae	
Hydrolysis of:							
Benzylpenicillin, phenoxymethyl-penicillin	Rapid	Rapid	Rapid	Rapid	Rapid	Rapid	Rapid
Phenethicillin, phenbenicillin, propicillin	Rapid	Rapid	—	Rapid	Rapid	Rapid	Very slow
6-Aminopenicillanic acid.	Slow	Slow	—	Slow	Rapid	Rapid	Very slow
Ampicillin.	Very rapid	Rapid	Rapid	Rapid	Very rapid	Very rapid	Very slow
Methicillin, isoxazolyl penicillins, quinacillin.	Very slow	Very slow	Rapid	Slow	Very slow	Rapid	Very slow
Cephalosporin C, cephaloram, cephaloridine, cephalothin.	Very slow	Very slow	Rapid	Slow	Slow	Slow	Very rapid
INDUCIBILITY	Induced at low inducer concentrations	Induced at low inducer concentrations	Inducible	Induced at low inducer concentrations	Not inducible	Not inducible	E. coli not inducible Others induced by high inducer concentrations
AFFINITY FOR COMPETITIVE INHIBITORS	Very high for semi-synthetic cephalosporins; very low for methicillin	Low for methicillin and isoxazolyl penicillins		Low for methicillin and isoxazolyl penicillins	Very high for methicillin slightly lower for cloxacillin		Very high for cloxacillin slightly lower for methicillin
MOLECULAR WEIGHT	29,600	30,700	—	23,000	Possibly similar to that of R-Factor TEM (= 16,700)		

Data are taken from Citri & Pollock (1966), and from Smith (1963a, b, and unpublished experiments), Smith & Hamilton-Miller (1963), Hamilton-Miller & Smith (1964), Hamilton-Miller (1966b, 1967 and unpublished experiments), Ayliffe (1965), Hennessey (1967), Kuwabara & Abraham (1967).

An attempt has been made to assign Gram-negative strains to four classes, as indicated on Table 2, as with Gram-positive strains.

Class I.   *Penicillinase-negative intrinsically sensitive strains* (S+P−)

Moraxella, Salmonella, Shigella, H. influenzae, N. gonorrhoeae and *N. meningitidis*, together with non-penicillinase-producing strains of *E. coli* and *Proteus mirabilis*, are normally all sensitive to ampicillin, and infections caused by them are usually cured by ampicillin therapy.   The Neisseria behave more like Gram-positive cocci being more sensitive to benzylpenicillin than ampicillin.   Failure in the treatment of infections caused by Class I Gram-negative bacteria can often be ascribed to penicillin-destroying strains (e.g. P+ strains of *Staph. aureus* or *Klebsiella aerogenes*) present at the same time —see Kjellander & Finland (1963).

Class II.   *Penicillinase-producing intrinsically sensitive strains* (S+P+)

If a bacterial strain possesses a penicillinase capable of hydrolysing ampicillin this does not necessarily mean that resistance is due to the possession of such an enzyme (see Ayliffe, 1963).   Hamilton-Miller (1965) found that penicillinase activity accounted for the observed clinical resistance of only two out of 12 penicillinase-producing *K. aerogenes* strains; the remainder were also intrinsically resistant (Class IV strains). Sutherland (1964) found, similarly, that only in some cases is penicillinase production solely responsible for resistance of penicillinase-producing Gram-negative bacteria to ampicillin.   Barber & Waterworth (1964b) have suggested that P+ strains of *Pr. mirabilis* are resistant to ampicillin by a penicillinase-type mechanism.

Increased penicillinase production in mutants selected for increased overall resistance has been assumed without further tests to be responsible for the overall resistance.   But it is essential that the intrinsic resistance (MIC with small inoculum) of the parent and resistant strains is measured before the resistance increase is so attributed.   A rise in intrinsic resistance sometimes takes place at the same time as an increase in penicillinase activity (e.g. Kabins, Sweeney & Cohen, 1966) and it is often mistakenly assumed that an inoculum size effect is proof of a penicillinase-type resistance—this is true only if the bacterial population under investigation is homogeneous.

Where ampicillin-resistance is due to penicillinase-mediated destruction of the drug, it may be possible, by inhibition of the enzyme using a competitive inhibitor such as methicillin or cloxacillin, to render such strains phenotypically sensitive to ampicillin (Hamilton-Miller, Smith & Knox, 1964; Sutherland & Batchelor, 1964).   A similar situation exists in Class II strains phenotypically resistant to the cephalosporin antibiotics: Sabath & Abraham (1964) found that cloxacillin inhibited the hydrolysis of cephalosporins by a strain of *Pseudomonas pyocyanea*, and also that the presence of this penicillin greatly increased the sensitivity of the strain to cephalosporin C, cephalothin and cephaloridine.   Results with *K. aerogenes* and *E. coli* (Hamilton-Miller, Smith & Knox, 1965) indicate that their penicillinases when hydrolysing cephaloridine are more susceptible to inhibition by methicillin, cloxacillin or quinacillin than when hydrolysing benzylpenicillin.   It is sometimes possible to overcome resistance in Class II strains by combined therapy of methicillin or cloxacillin with ampicillin or benzylpenicillin (see e.g. Sabath & others, 1967):   It is essential for the success of such combined therapy that detailed laboratory studies should precede and accompany treatment.   These should show that the bacterial strain in question is inhibited *in vitro* by the appropriate β-lactam drugs in concentrations which are actually attained in the patient being treated.   Uncontrolled trials of such combined therapy may lead to a potentially

valuable clinical tool falling into disrepute, as clinical failures will undoubtedly occur if treatment is given in an arbitrary fashion.

Class III. *Penicillinase-negative, intrinsically restraint strains* (S⁻P⁻)

Class III. *Penicillinase-negative, intrinsically restraint strains* (S$^-$P$^-$)

The list of Gram-negative bacteria falling into Class III has declined steadily owing to the consecutive introduction of ampicillin, semi-synthetic cephalosporins (cephalo-thin, cephaloridine, cephaloglycin and cephalexin) and carbenicillin into medicine. As knowledge accumulates, it seems more and more likely that those Gram-negative bacteria resistant to the newer semisynthetic β-lactam drugs produce penicillinase (again it must be stressed that this is not necessarily a cause-and-effect relationship); if this view is substantiated, very few Class III Gram-negative bacteria exist. At present, *Serratia marcescens* (Hugo & Furr, 1967) and *Acinetobacter anitratus* (Thornley, 1967), appear to be genuine members of this Class. However, for practical purposes, such strains as *Ps. pyocyanea* and the indole-producing *Proteus* species may most conveniently be included in Class III because under physiological conditions they do not normally produce penicillinase. This enzyme is only formed after induction, involving growth in the presence of high penicillin concentrations (of about 1 mg/ml, Hamilton-Miller, 1963b; Ayliffe, 1964; Jago, Migliacci & Abraham, 1963), and so these strains would be expected to produce significant amounts of penicillinase *in vivo* only when present in the urinary tract of patients treated with β-lactam drugs. Further-more, it is not clear whether all strains of these species are capable of producing penicillinase.

Sutherland, Slocombe & Rolinson (1964) found that a Class I strain of *Salm. paratyphi* B could be trained to ampicillin resistance (presumably this was a Class III resistance, although the authors did not state whether penicillinase was produced) and resistant mutants so obtained showed strong cross-resistance to chlortetracycline and chloramphenicol: this suggests a generalized decrease in permeability, limiting the access of these drugs into the bacteria. Jones (1966) made similar findings using *in vitro* trained P⁻ *E. coli* strains.

Certain bacterial species, notably *N. gonorrhoeae*, appear to have gradually built up intrinsic resistance over the years, and now seem about to become clinically resistant. Hejzlar & Vymola (1965) and Amies (1967) have indicated the alarming rise in the *in vitro* resistance of gonococci to benzylpenicillin recently, an increase paralleled by a rise in the failure rate for the single-dose treatment of gonorrhoea (Kjellander & Finland, 1963; Oller, 1967). As gonococci have not yet been shown to produce penicillinase, this increased resistance is another example of an *in vivo* selection of Class III mutants from a predominantly Class I population.

Class IV. *Penicillinase-producing intrinsically-resistant strains.* (S⁻P⁺)

Class IV. *Penicillinase-producing intrinsically-resistant strains.* (S$^-$P$^+$)

The penicillinase-producing *Ps. pyocyanea*, indole-producing *Proteus* and *Entero-bacter* spp. can be regarded as Class IV strains under certain circumstances (e.g. when they are infecting the genito-urinary tract, because ambient concentrations of penicillins or cephalosporins are then likely to be high enough to cause induction of the enzyme). The recent introduction of carbenicillin (Acred & others, 1967) has made possible the use of a penicillin to treat systemic infections by *Pr. vulgaris*, *Pr. morgani* or *Pr. rett-geri*; this penicillin is much more intrinsically active against these bacteria than any other β-lactam drug, and is claimed to be unaffected by their penicillinases.

Smith (1963a) found that, by training an intrinsically ampicillin-sensitive penicilli-nase-producing (Class II) strain of *E. coli* to ampicillin resistance *in vitro*, he obtained a

Class IV strain.   He also found that in the process of converting intrinsic sensitivity to resistance the penicillinase-producing capacity of the strain was greatly increased, even though the penicillinase did not hydrolyse ampicillin.   Similar findings have been made for *E. coli* by Percival, Brumfitt & deLouvois (1963) and by Seligman & Hewitt (1965).   In all cases, conversion of genotype S$^+$ to S$^-$ was accompanied by increased penicillinase production.   Jones (1966), however, was able to obtain P$^-$, intrinsically resistant strains (Class III) of *E. coli* by laboratory training, and Barber & Waterworth (1964a) reported similar results using both *E. coli* and a penicillinase-producing strain of *Pr. mirabilis*.   The latter authors observed increased production of penicillinase by a laboratory trained *K. aerogenes* strain.   A genetic analysis of ampicillin resistance in mutants of *E. coli* K12 has been made by Eriksson-Grennberg & others (1965).
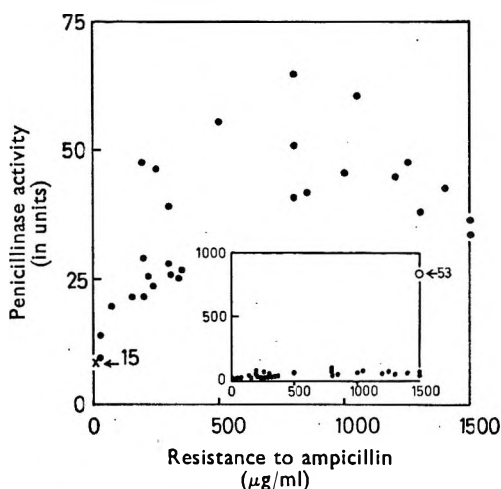

FIG. 3.   The penicillinase activities of various mutants derived *in vitro* from *A. cloacae* 15 ($\times$ on graph) are plotted against the ampicillin concentrations from which they were isolated.   Also shown is the penicillinase activity of *A. cloacae* 53 (O on graph) which was isolated *in vivo*.   Penicillinase activities are expressed as nmol of benzylpenicillin destroyed/min at pH 6·0 and and 37° C in presence of M/40 sodium phosphate buffer by a disrupted suspension of bacteria which previously contained 10⁹ viable units.

The role of penicillinase production in acquired penicillin resistance is clarified by the following observations (J. T. Smith, 1964, unpublished observations): a strain of *Aerobacter cloacae* (reference number 15), originally isolated from a patient undergoing treatment with ampicillin, was sensitive to ampicillin (MIC = 10 μg/ml), and produced a small amount of penicillinase; a further strain (reference number 53), later isolated from the same patient, was highly resistant to ampicillin (MIC = 15 mg/ml) and produced almost 100 times as much penicillinase as strain 15.   The two strains were serologically and biochemically identical, and the penicillinases produced by both also appeared identical; the action of this enzyme was inhibited by sulphydryl-blocking reagents, and it had an unusually wide substrate specificity profile (Smith, 1963b; Smith & Hamilton-Miller, 1963).   It is thought that strain 53 is a mutant of strain 15 which acquired ampicillin-resistance *in vivo*.   To determine whether it is the higher penicillinase content of strain 53 which is solely responsible for its greater resistance to ampicillin (for this penicillinase does hydrolyse ampicillin), a program of training the parent strain (15) by serial subculture on agar containing increasing concentrations of ampicillin was undertaken.   At various stages in this process, colonies were removed from the plates which contained, at that time, the highest level of ampicillin permitting

growth, and were tested to determine their level of penicillinase. The results are shown in Fig. 3. At first it seemed that the various mutants of strain 15 had gained resistance in proportion to their increase in penicillinase activity, at least in the series of mutants for which the MIC of ampicillin were up to 1 mg/ml. However, from the inset graph, it can be seen that the amount of penicillinase gained by these mutants is insignificant compared to the enormous increase in activity from the original sensitive strain 15, to the mutant strain 53 obtained *in vivo* (these are marked with X and O on the main and inset graphs, respectively). It is probable that the mutants obtained *in vitro* are resistant due to an increase in their intrinsic resistance (and therefore belong to Class IV of the present classification), and that an incidental rise in penicillinase activity has also occurred, as has been previously discussed for *E. coli*. Further substantiation of the intrinsic type of resistance is indicated by those mutants resistant to concentrations of ampicillin greater than 1 mg/ml—these mutants tend to have less penicillinase than the mutants which are less resistant to ampicillin. It is also clear that the acquisition of ampicillin resistance by strain 53 cannot have come about by a process analogous to the *in vitro* training. Seligman & Hewitt (1965) have also reported a step-wise increase in intrinsic resistance as a result of training an *E. coli* strain to ampicillin resistance; there was a concomitant rise in penicillinase production.

A further example of Class IV resistance is given by Benner, Micklewait & others (1965) who observed that in certain strains of Aerobacter, resistance to cephalothin and to cephaloridine was not necessarily based on production of "cephalosporinase".

A characteristic feature of Gram-negative penicillinase-producing bacteria is their permeability barrier, which limits the access of certain penicillins (notably benzyl-penicillin and ampicillin) into the cell (Smith, 1963a; Smith & Hamilton-Miller, 1963; Hamilton-Miller, 1965; Ayliffe, 1965; Datta & Kontomichalou, 1965; Sabath, Jago & Abraham, 1965). This barrier seems to have some connection with resistance of *K. aerogenes* strains to penicillins, as is evidenced by the following findings: (i) in *K. aerogenes* 418 there was a relation between antibacterial activity and ease of permeation for seven penicillins (Hamilton-Miller, 1963a); (ii) 6-aminopenicillanic acid and cephaloridine, both of which obtain free access into many coliform organisms, are consistently among the most active of the β-lactam antibiotics against *K. aerogenes* (Smith & Hamilton-Miller, 1963; Hamilton-Miller, 1963a, 1965; Hamilton-Miller & others, 1965); (iii) in twelve *Klebsiella* strains a significant relation was found between intrinsic resistance and lack of permeability to benzylpenicillin (Hamilton-Miller, 1965). Izaki & others (1966) showed that a cell-free system synthesizing mucopeptide was almost as sensitive to methicillin as to benzylpenicillin whereas the intact cells were insensitive to methicillin. They concluded that these results supported the hypothesis that impermeability can play a significant role in intrinsic resistance.

There are good precedents for a lack of permeability being responsible, at least in part, for bacterial resistance to other antimicrobial agents; resistance to tetracycline (Izaki & Arima, 1963), chloramphenicol (Okamoto & Mizuno, 1964) and actinomycin D (Leive, 1965) has been explained in these terms though further investigations into the chloramphenicol-resistant bacteria revealed that resistance was due to drug destruction in some cases (Okamoto & Suzuki, 1965).

The increased incidence of hospital-acquired infections of Gram-negative origin (Kessner & Lepper, 1967) is disturbing, because such infections (notably Pseudomonas) are notoriously difficult to eradicate. The recently observed worldwide increase in the frequency of isolation of members of the Enterobacteriaceae which possess an R-factor

(R$^+$ strains), and thus display multiple and transferable drug resistance (see Watanabe, 1963, 1967) has aggravated the situation. Thus, Mitsuhashi, Hashimoto & others (1967) observed that most *Shigella, E. coli, Klebsiella* and *Proteus* strains isolated from clinical sources in Japan during 1965 were R$^+$, and H. W. Smith (1966) has demonstrated a striking increase in the isolation rate of R$^+$ *E. coli* strains from diseased animals, especially pigs, over the first half of the present decade.

The means by which R-factors confer resistance to β-lactam drugs are not yet clear, although there are several significant points. It is interesting to note the superficial similarity between R$^+$ strains and the strains with multiple resistance selected by *in vitro* training to resistance to a single drug by Sutherland & others (1964). Strains carrying an R-factor which confers resistance to ampicillin (i) produce a penicillinase which is characteristic of the R-factor, and not of the host, and (ii) possess permeability barriers to benzylpenicillin and ampicillin (Datta & Kontomichalou, 1965; Datta, 1965). Hamilton-Miller (1965, unpublished observations) has investigated the extent to which the resistance to β-lactam antibiotics shown by R$^+$ strains is due to penicillinase, and to what extent it is connected with manifestations of impermeability. The four strains used (very kindly given by Dr. Naomi Datta of the Postgraduate Medical School), were host *E. coli* K12 (F$^-$met$^-$), and this host carrying, singly, the three R-factors R_{1818}, R_{7268} and R_{TEM} (the subscript reference numbers signify the host strains from which the particular R-factors were isolated). Some relevant details of these strains, as determined for this experiment, are given in Table 4. The presence of an R-factor can be seen not to affect the permeability of any strain to cephaloridine,

Table 4. *Hydrolytic activities and permeability of* E. coli *K12 (host), and the same strains carrying 3 different "R" factors, toward benzylpenicillin, ampicillin and cephaloridine*

			Benzylpenicillin		Ampicillin		Cephaloridine	
			*Activity	†PF	*Activity	†PF	*Activity	†PF
E. coli K12			1·5	1	0·15	1	2·7	1
Host + R_{1818}	..	..	18·4	3	36·8	3	10·3	1
Host + R_{7268}	..	..	200	8	380	8	158	1
Host + R_{TEM}	..	..	1422	9	2262	11	1280	1

* Activity = Rate of hydrolysis (nmol substrate destroyed/min) at pH 7·4 and 37° C in presence of M/40 sodium phosphate buffer, by a suspension containing 10^9 bacteria/ml, after disruption.

† PF = Permeability Factor = $\dfrac{\text{rate of hydrolysis by disrupted suspension}}{\text{rate of hydrolysis by same suspension, intact}}$

See Hamilton-Miller (1965) for full experimental details.

Results for *E. coli* K12 (host) have been published already (Hamilton-Miller & others, 1965).

Table 5. *Minimum inhibitory concentrations ($\mu g/ml$) of 3-β-lactam antibiotics against large (10^6) and small (10) inocula of* E. coli *K12 (host), and the same strain carrying 3 different "R" factors*

				Benzylpenicillin		Ampicillin		Cephaloridine	
				10^6	10	10^6	10	10^6	10
					MIC		MIC		MIC
E. coli	..	..	..	62	31 (2)	20	2·5 (8)	16	2 (8)
host + R_{1818}	..	..		500	31 (16)	1000	31 (32)	31	2 (16)
host + R_{7268}	..	..		4000	125 (32)	4000	250 (16)	125	4 (32)
host + R_{TEM}	..	..		8000	1000 (8)	8000	1000 (8)	500	8 (64)

Figures in brackets, MIC for large inoculum ÷ MIC for small inoculum, represent amount of "penicillinase"-type resistance.

whereas with the two penicillins, the higher the penicillinase activity, the larger the permeability factor. Minimum inhibitory concentrations of the three antibiotics against large and small inocula of the four strains were then measured, by the tube dilution technique. Table 5 shows that, with cephaloridine, there is little or no increase in intrinsic resistance conferred by the presence of an R-factor, and that rises in hydrolytic activity go along with increases in "penicillinase-type" resistance. For the two penicillins, on the other hand, there are definite rises in intrinsic resistance, and no relation exists between increases in "penicillinase-type" resistance and hydrolytic activity. Thus it seems likely that, while an increase in penicillinase activity can account for the increased resistance to cephaloridine, alone, it cannot explain the much greater increase in resistance to benzylpenicillin and ampicillin. These latter increases are paralleled closely by rises in permeability factors, and it may well be that the fact that R$^+$ strains are not freely permeable to benzylpenicillin and ampicillin at least partly explains the increase in resistance to these two compounds. It seems, on the evidence at present available, that R$^+$ strains are best classified in Class IV.

Studies at the Pasteur Institute (Chabbert & Baudens, 1966; Baudens & Chabbert, 1967) have shown that there is no relation between the level of resistance to penicillins and the amount of penicillinase activity, in naturally-occurring R$^+$ salmonellae; such a relation would be expected if there were a "cause and effect" between resistance and penicillinase (Hamilton-Miller, 1965). Decreases in cellular permeability to drugs have also been proposed by other workers to account for R-factor mediated resistance (D. H. Smith, 1966; Unowsky & Rachmeler, 1966). Of particular interest in this context are the findings of Guinee & others (1967) who found that infection of *Salm. panama* with an R-factor results in a change in phage-type of the infected strain. This may be indicative of a radical alteration in the surface structure of the cells, which could alter permeability.

Transferable multiple resistance poses a threat of extreme potential danger; outbreaks of infections caused by R$^+$ strains can spread quickly and are often difficult to treat (Lewis, 1967; Anderson, 1968). Yoshikawa & Sevag (1967), however, report that R$^+$ strains of *E. coli* are sensitive to mepacrine, while R$^-$ strains are not; they deduce that R-factors may confer sensitivity to mepacrine; it is conceivable that this finding may be of clinical significance.

CONCLUSIONS

Bacterial resistance to β-lactam antibiotics can result from two separate mechanisms, drug inactivation and drug tolerance, either or both of which may be responsible for the overall resistance exhibited by the organism in question. As a result, three classes of bacterial resistance can be observed.

Although laboratory studies of bacteria selected for increased resistance to β-lactam drugs usually result in the isolation of mutants which are resistant due to drug tolerance the validity of such investigations is doubtful as it is rare for this to be the sole mechanism of resistance noted clinically. Two species, however, do show this type of resistance *in vivo*, the gonococcus and *Strep. viridans*. With bacteria like these it would seem that *in vitro* studies have some meaning. Thus drug tolerance can be a danger since it is relatively easy to select increasingly resistant organisms during serial subculture in increasing concentration of β-lactam drugs.

The most important organism causing serious clinical resistance by β-lactam drug inactivation has been the penicillinase-producing *Staph. aureus*. It is significant that

the penicillinase of this species is an inducible enzyme and thus cultures are resistant because much higher levels of penicillinase are stimulated by the presence of a β-lactam drug. Fortunately, single cocci are little more resistant than members of the same species which lack penicillinase, probably because they succumb to penicillins before enzyme induction can occur. As a consequence, when the penicillinase-stable penicillins were introduced they had and are continuing to have success in overcoming the resistance of the penicillinase-producing *Staph. aureus*.

The same approach towards resistance by drug inactivation cannot be taken with those Gram-negative bacteria where penicillinase production is constitutive (i.e. non-inducible). If the cells produce sufficient enzyme, individual bacteria may be resistant owing to the penicillinase they possess, but if the level of production per cell is low, single cells may not be significantly resistant. However, large numbers of such cells may exhibit therapeutic resistance which may be of a low degree since a modest increase in penicillin concentration may inhibit growth; nevertheless this increase may be outside the scope of therapeutic blood levels.

Generally these two cases may themselves be oversimplified since inherent resistance is additionally often present in penicillinase-producing Gram-negative bacteria. In many cases such organisms are sufficiently resistant by the non-destructive mechanism that penicillinase production appears superfluous. Another similar example is the production of penicillinase by type I *E. coli*. When mutants more highly resistant to ampicillin are selected these contain more penicillinase than the parent strain and yet the extra penicillinase produced seems of little avail since it does not hydrolyse ampicillin significantly.

This raises the question of the real function of penicillinase (Pollock, 1967). It is uncertain whether any of the β-lactam antibiotics are the substrate or the analogues of a hitherto undiscovered substrate, the enzymic alteration of which is useful for bacterial metabolism rather than primarily a defensive mechanism against antibiotic attack.

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Application of differential scanning calorimetry to the study of sulphathiazole crystal forms

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Methods of preparation and characterization of two crystal forms of sulphathiazole using differential scanning calorimetry and infrared spectroscopy are described. Heats of transition and fusion calculated from thermal analysis are 1778 ± 68 Cal mol^{-1} and 6615 ± 235 Cal mol^{-1} respectively. The transition temperature depends on the rate of heating and the sample source. Interconversion of the crystal forms under different conditions such as heating and suspension in water is described. An assay procedure based on the area of the thermal transition peak of Form I was devised and results of analysis of synthetic mixtures of Forms I and II are given.

The existence of sulphathiazole in different polymorphic forms was first reported by Grove & Keenan (1941) who described methods of preparation and characterization of two crystal forms. Later, Miyazaki (1947) added a third form which melted at 173–175° without polymorphic transformation. Mesley & Houghton (1967) reported the existence of three crystalline forms, which they correlated with Miyazaki's forms, and added also a fourth amorphous modification. Solubility, dissolution rate and thermal behaviour of sulphathiazole have been studied by Brandstaetter-Kuhnert & Martinek (1965); Milosovich (1964); Inoue & Saito (1961) and Guillory (1967). In most of these publications, methods of preparation and characterization of only two crystalline modifications are described.

A re-examination of this problem using differential scanning calorimetry is described below. The number of forms is of direct relevance to the B.P.C. infrared identification test and the preparation of stable aqueous suspensions of sulphathiazole.

EXPERIMENTAL AND RESULTS

Apparatus

A Perkin-Elmer DSC-1 differential scanning calorimeter fitted with effluent analyser was used. Samples of pure indium were used to calibrate the instrument. Dry nitrogen at 30 ml/min was used as carrier gas. Various rates of heating were employed ranging from 0·5° to 64°/min. Samples usually varied from 1–10 mg and were weighed with an electromicrobalance (EMB-1 Research and Industrial Instruments Co.).

Infrared spectra of Nujol mulls were recorded on a Unicam SP200 double beam spectrometer fitted with a sodium chloride prism (Fig. 1). Wavelength calibration with a polystyrene film showed an accuracy of ± 3 wavenumbers.

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Material

Two batches of sulphathiazole were used; a B.P.C. authentic specimen and a micro-milled material (May and Baker Ltd.) of B.P.C. quality. Both were of Form I.

Preparation of the crystal forms

Form I was prepared by crystallization of sulphathiazole from either boiling water, acetone-chloroform mixture, dilute ammonia solution or 95% aqueous ethanol. The crystals were dried by passing a current of air through them on the filter funnel. Crystal crops were stored in closed containers in a cool place. Different solvents produced crystals of varying shapes and sizes but all were placed in one category since they possessed similar properties (see below).

Form II was obtained by crystallization from s-butanol or n-amyl alcohol; it was also prepared by heating Form I to 180°. Neither infrared spectroscopy nor differential scanning calorimetry showed the presence of solvents in any of the samples.

Characterization of the crystal forms

The infrared spectra of Forms I and II in Nujol mulls (Fig. 1) showed differences which correspond to the Forms I and II described by Guillory (1967).
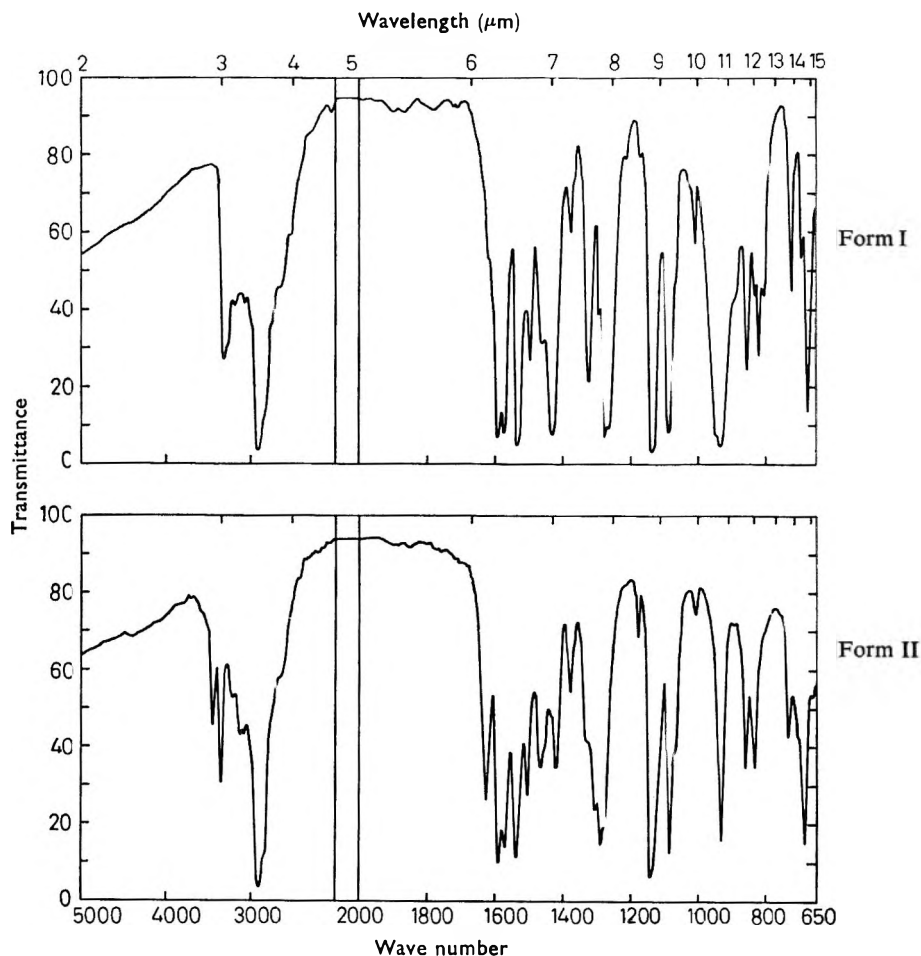

FIG. 1. Infrared spectra of sulphathiazole crystal forms in Nujol mulls.

While with differential scanning calorimetry Form II showed only one endothermic transition corresponding to melting at *ca* 205°, Form I showed two endothermic transitions. The first corresponds to polymorphic transformation of Form I to II and the second to melting. In no case has melting been observed at temperatures below 200° (cf. Miyazaki, 1947). The temperature of both transitions, but especially the first, varied with the rate of heating, shape and size of crystals and crystals prepared under different conditions for (different solvents and different rates of crystallization—see Table 1).

Table 1. *Effect of sample source and rate of heating on transition temperatures of sulphathiazole*

Sample	Temp. at peak max of 1st transition (°C)		Temp. at peak max of 2nd transition (°C)	
	16°/min	32°/min	16°/min	32°/min
B.P.C. specimen	174	—	207	—
Micromilled preparation ..	174	180	207	213
Form I—boiling water ..	158·5	—	207	—
Form I—acetone/chloroform ..	163	169	208	212
Form I—dil. ammonia ..	176	181	208	214
Form I—95% ethanol	165, 176·5	175, 181	206	213
Form I—susp. of II in water ..	139	—	206	—
Form II—s-butanol	—	—	—	213
Form II—amyl alcohol ..	—	—	200·5	206

Guillory (1967) and Grove & Keenan (1941) have previously drawn attention to the effect of rate of heating on the transition temperature. However, the first transition was observed for all samples of Form I in the temperature range 140–180°. Fig. 2 shows typical behaviour of some forms and mixtures of them. In some, e.g. Form I prepared by crystallization from 95% aqueous ethanol, two transitions have been observed corresponding to the transformation to Form II. Two species of Form I are
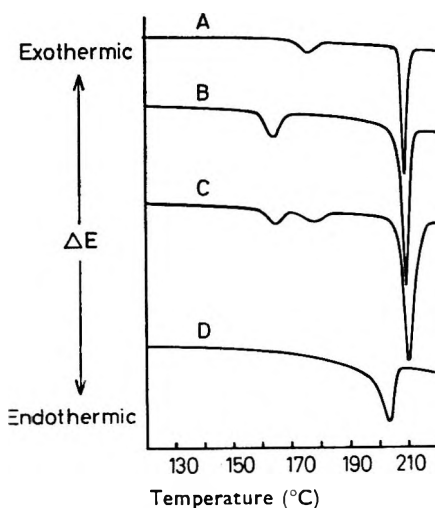

Fig. 2. Thermal analysis curves of sulphathiazole crystal forms ($\triangle$E is proportional to milli-calories/s rate of heating = 16°/min). A. Form I. B.P.C. B. Form I, crystallized from acetone–chloroform. C. Mixture of Form I crystallized from acetone–chloroform and Form I crystallized from dilute ammonia. D. Form II, crystallized from amyl alcohol.

apparently produced in different proportions from this solvent. No effluent vapour was detected for any of the samples studied. Rate of heating had no effect on peak areas in the range of 8–32°/min, and consequently heats of transition are constant. Different species of Form I have the same heat of transition.

The heats of transition and fusion were calculated from peak areas (measured by planimeter), using the calibration curve of Carless, Moustafa & Rapson (1966). These were 1778 ± 68 Cal mol^{-1} ($P = 0.95$) for the first transition in the authentic B.P.C. specimen and 6615 ± 235 Cal/mol^{-1} ($P = 0.95$) for melting of Form I crystallized from 95% aqueous ethanol.

Interconversion of the crystal forms

(a) *Crystallization.* Forms I and II may be interconverted by crystallization from the appropriate solvent (see above). Only two forms were encountered. The change of Form II to I after suspension in water was detected by filtering off the solid which was dried in a current of air, and then examined by infrared spectroscopy and differential scanning calorimetry.

(b) *Heating.* Form I changes to Form II on heating to 180°. This is in agreement with results of previous workers (Milosovich, 1964; Brandstaetter-Kuhnert & Martinek 1965, among others). Although the change was rapid above the transition temperature, prolonged heating below this had no effect on the crystal form. Transformation from Form I to Form II was independent of the rate of heating.

(c) *Suspension in water.* It has been reported (Sanchez, 1962) that the metastable form of sulphathiazole is readily transformed into the stable form in the presence of moisture. In the present study, Form II changed to Form I upon suspension in water—the time necessary to achieve this transformation varied according to the source of the crystals and their size. For Form II prepared by crystallization from s-butanol, this was about two weeks at room temperature.

(d) *Grinding.* Dry grinding of either crystal form by hand or in a vibration mill for periods up to 5 min had no detectable effect on the infrared spectra of the crystals. Vibration milling of Form I crystallized from 95% ethanol resulted in the transition temperature being lowered by about 5°.

Estimation of Form I in mixtures of crystal forms

Since only Form I shows an endothermic transition in the temperature range 125–180°, use has been made of this in the estimation of this form. A calibration curve (Fig. 3) was established relating the peak-area of this transition to the sample weight using the authentic B.P.C. material. Using this curve, a series of mixtures of Forms I and II and of Form I from different sources were analysed. In the latter case the total area of any peaks in the temperature range 125–180° was measured. Results of these assays are shown in Table 2.

DISCUSSION

Few papers have appeared in the literature in which three or more forms have been reported (Miyazaki, 1947; Mesley & Houghton, 1967). In the present investigation only two crystal forms were identified, none of which melts at 175° as suggested by these authors.

Reported transition temperatures for Form I to Form II show wide variation. Using solubility data, Milosovich (1964), reported a low transition temperature of
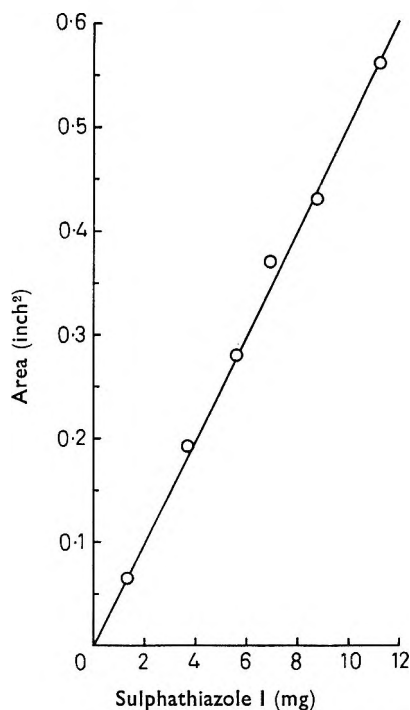
FIG. 3. Calibration curve for the first thermal transition in sulphathiazole Form I. (Rate of heating = 16°/min, range 4, chart speed = 0·75 inch/min).

Table 2. *Determination of Form I in mixtures of other crystal forms, by differential scanning calorimetry*

Mixture		Form I found mg	Error
Form I (BPC authentic specimen) mg	Other materials mg		
2·174	5·948 Form II	1·83	—16%
5·572	5·914 Form II	4·67	—16%
8·814	1·940 Form II	8·12	—8%
8·825	3·335 Form II	7·90	—10%
5·260	3·396 Form II	5·07	—4%
2·302	5·214 Form II	2·01	—13%
8·546	4·550 *Form I	11·97	—9%
4·560	6·226 †Form I	10·55	—2%

N.B. *Form I—crystallized from dilute ammonia.
    † Form I—crystallized from acetone chloroform mixture.
    All Form II was crystallized from n-amyl alcohol.

94·5° ± 2·7°, whilst using differential thermal analysis transition temperatures as high as 161° have been reported by Guillory (1967). Intermediate values are given by Inoue & Saito (1961). The present investigation has shown variation between different samples of Form I. The rate of heating, method of preparation, and particle size, affect the transition temperature (Table 1). The shape and size of the crystals will affect the thermal conductivity and hence transition temperature. However, further investigation is necessary to determine whether Form II crystallized from amyl alcohol differs from the other forms, or whether its lower melting point is due solely to

particle size effects.   In spite of variations in transition temperatures, the constancy of the energy uptake appears to offer a method for the estimation of Form I in a mixture of the two forms.

Different methods of preparation of sulphathiazole crystal forms has led to some confusion.   Mesley & Houghton (1967) obtained Form C, which corresponded to Miyazaki's (1947) Form α', by crystallization from dilute ammonia solution. Mesley & Houghton also reported that the infrared spectrum of this form was in fact very similar to that of Form A.   In the present study, recrystallization from dilute ammonia produced Form I which should be identical with Mesley & Houghton's Forms A or C. In spite of the fact that this had a somewhat different transition temperature from other varieties of Form I, no melting was observed at 175° when it was heated on the DSC-1 or in a Gallenkamp melting point apparatus [cf. Miyazaki's (1947 form α', m.p. 175°]. This form therefore appears to be another variety of Form I since it has the same infrared spectrum and heat of transition and it is not justifiable to call it a new form. Grove & Keenan (1941) Milosovich (1964) and Brandstaetter-Kuhnert & Martinek (1965) all quoted a melting point of 175° for Form I which could be due to heating the sample at a rapid rate when melting occurs at 175° without polymorphic transition, as noted by Guillory (1967).   Variation of the transition temperature of I and II could be interpreted on the basis of the factors discussed earlier.   Recrystallization from ethanol produced Form I, contrary to the findings of Mesley & Houghton (1967) who reported that it produced substantially form B.

The heat of transition calculated in the present investigation (1778 ± 68 Cal mol^{-1}) shows good agreement with the value of 1744 Cal mol^{-1} calculated by Milosovich (1964) from solubility measurements of the two crystal forms.   However, Guillory (1967) using differential thermal analysis reported a heat of transition of 1420 ± 40 Cal mol^{-1}.   The heat of fusion found in the present work (6615 ± 235 Cal mol^{-1}) is also slightly but consistently higher than that reported by Guillory (1967): viz. 5960 ± 210 Cal mol^{-1}.

The use of differential scanning calorimetry for the estimation of Form I of sulpha-thiazole has been shown to be feasible though not highly accurate (Table 2).   Experimental values are always less than the theoretical, and this may be due to the mixing of samples of different particle shapes and sizes.   The heterogeneity of the sample mixture leads to a slight decrease in the area of the transition peak.   This effect is non-specific and has been observed for mixtures of Forms I and II and also for mixtures of samples of Form I from different sources (see Table 2).   No reasonable correction factor relating the transition peak area at the fusion peak area could be found because admixture of a second sample was found to affect both peaks to different degrees. The errors were smaller when one variety of Form I was the major component in a mixture, interference being in this case at a minimum.   This method however, is useful in giving an approximate measure of the proportion of various forms of mixture, Form II being calculated by difference from the total.   This assay procedure is obviously useful when rates of transformation in aqueous suspensions are being studied.

Previous suggestions (Carless & Foster, 1966) that the B.P.C. authentic specimen was a mixture of two forms must be modified since the present study suggests that it consists mainly of Form I alone.   Thermal behaviour and infrared spectrum are typical of this form.   For the purposes of the identification test, it is suggested that any sample of sulphathiazole should be heated to a temperature of 180°, then its infrared spectrum determined in Nujol mull.   The infrared spectrum of Form II should then

be reproduced in all cases.   This is a simpler procedure than the alternative of Mesley & Houghton (1967) which involves recrystallization from n-propanol.

Accelerated crystal growth in aqueous suspensions of sulphathiazole by temperature cycling has been studied by Carless & Foster (1966).   No polymorphic changes were detected in these systems which consisted of Form I suspensions (Foster, personal communication).   It would be of interest to study the particle size distribution and polymorphic changes of Form II in aqueous suspension as this may be analogous to the cortisone acetate suspensions where crystal growth is initiated mainly by polymorphic transformation (Moustafa, 1967).   If this was so then the use of Form I of sulphathiazole would be necessary for the preparation of stable aqueous suspensions.

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Central muscle relaxant properties of 2,6-dimethylphenethylurea and related compounds

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Twenty-eight phenylalkylureas with alkyl, hydroxy, methoxy or chloro-substituents in the aryl ring have been synthesized and tested for central depressant and muscle relaxant properties. In this series, the dimethylphenethylureas with at least one *ortho*-methyl group show unexpectedly selective muscle relaxant activity. 2,6-Dimethyl-phenethylurea inhibits polysynaptic reflexes more readily than mono-synaptic ones, but also depresses muscle contractions by an action independent of its effect on interneuronal transmission. It is a weak anticonvulsant, suppresses rage episodes in "fighting" mice, and has no selective blocking action on conditioned responses. It thus has a pharmacological profile resembling those of mephenesin and mepro-bamate, but distinct from either, and differing still more from those of phenobarbitone, chlordiazepoxide or chlorpromazine. However, tolerance to its action develops readily.

Urea derivatives have long been known to possess central depressant properties (see review by Wheeler, 1963). De Beer, Buck, Hjort and their co-workers (1934, 1935, 1937) found that the fairly widespread hypnotic activity of aryl, alkyl and mixed aryl alkyl ureas correlated tolerably well with some of their physical properties. The range of compounds included in their broad survey was restricted by the need for moderate water solubility, since the compounds were administered intraperitoneally, and no quantitative assessment was made of the dose required to produce actions other than hypnosis or death. Depression of motor activity, ataxia and loss of right-ing reflex are frequently seen on oral administration of aralkylureas to mice. The pinnal reflex is often abolished at a dose much lower than that required to abolish the corneal reflex, suggesting a mephenesin-like muscle-relaxant action (Goodsell, Toman, & others, 1954).

This paper describes some aralkylureas with one or more ring substituents (I), which show selective depressant effects of this kind. More detailed pharmacological results

R—⟨benzene ring⟩—(CH₂)ₙ·NH·CO·NH₂   R = H, Me, Et, Cl, OMe
I   n = 0–3
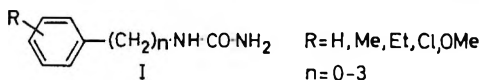
are given for 2,6-dimethylphenethylurea (cpd 16), one of the more interesting of these compounds as a potentially useful sedative and muscle relaxant (Green & Willey, 1967).

CHEMISTRY

o-, *m*- and *p*-Methylphenethylurea (*o*-, *m*- and *p*-tolylethylurea; compounds 4–6) were supplied by Smith Kline & French Laboratories, Philadelphia. The other com-pounds were synthesized by conventional methods. The unsubstituted aralkylureas

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(cpds 1–3), and 2,4-dimethylbenzylurea (cpd 8) (Hinrichsen, 1889), 3,5-dimethyl-benzylurea (cpd 12) (Landau, 1892), 2,5- and 3,4-dimethylbenzylurea (cpds 9, 11) (Trivedi & Trivedi, 1958), 2,4- and 3,4-dimethoxyphenethylurea (cpds 22, 23) (Buck, 1934), 3,4,5-trimethoxyphenethylurea (cpd 25) (Jansen, 1931), 2,6-xylylurea (cpd 7) (Dahlbom & Österberg, 1955), β-hydroxyphenethylurea (cpd 28) (Mannich & Thiele, 1915) and *p*-hydroxyphenethylurea (cpd 27) (Cloetta & Wünsche, 1923) have been described previously. Analytical data and melting points for the new ureas are given in Table 1.

Table 1. *Substituted aralkylureas*

Compound

R—⟨benzene ring⟩—(CH$_2$)$_n$·NH·CO·NH$_2$

No.	R	n	Empirical formula	m.p. °C (un-corrected)	Required (%) C	H	N	Found (%) C	H	N
10	2,6-Me$_2$	1	C$_{10}$H$_{14}$N$_2$O	240 (dec.)	67·4	7·9	15·7	67·6	8·0	15·6
13	2,3-Me$_2$	2	C$_{11}$H$_{16}$N$_2$O	141–2	68·7	8·4	14·6	68·6	8·4	14·4
14	2,4-Me$_2$	2	C$_{11}$H$_{16}$N$_2$O	129	68·7	8·4	14·6	68·6	8·35	14·5
15	2,5-Me$_2$	2	C$_{11}$H$_{16}$N$_2$O	133–5	68·7	8·4	14·6	68·65	8·4	14·3
16	2,6-Me$_2$	2	C$_{11}$H$_{16}$N$_2$O	173–4	68·7	8·4	14·6	68·4	8·4	14·7
17	3,4-Me$_2$	2	C$_{11}$H$_{16}$N$_2$O	121–3	68·7	8·4	14·6	68·7	8·2	14·7
18	3,5-Me$_2$	2	C$_{11}$H$_{16}$N$_2$O	141–2	68·7	8·4	14·6	68·3	8·5	14·4
20	2,4-Cl$_2$	2	C$_9$H$_{10}$Cl$_2$N$_2$O	174	46·4	4·3	12·0	46·4	4·2	11·8
21	2,6-Cl$_2$	2	C$_9$H$_{10}$Cl$_2$N$_2$O	165	46·4	4·3	12·0	46·5	4·4	11·8
19	2,6-Et$_2$	2	C$_{13}$H$_{20}$N$_2$O	145–6	70·9	9·15	12·7	70·95	9·0	12·65
24	2,4,6-Me$_3$	2	C$_{12}$H$_{18}$N$_2$O	205 (dec.)	69·9	8·8	13·6	69·9	8·8	13·4
26	2,6-Me$_2$	3	C$_{12}$H$_{18}$N$_2$O	154	69·9	8·8	13·6	69·8	8·8	13·55

All the ureas were obtained by heating the appropriate aralkylamine salt with aqueous sodium cyanate solutions (Kehm & Whitehead, 1963; Green & Willey, 1967). 2,4- 2,5- and 3,4-Dimethylbenzylamine were available commercially (Koch-Light Laboratories Ltd.); 2,6-dimethylbenzylamine was obtained by reduction of 2,6-dimethylbenzonitrile with lithium aluminium hydride (Herr, Enkoji & Dailey, 1957) and 3,5-dimethylbenzylamine from α-bromomesitylene (Aldrich Chemical Co. Inc.) with hexamine (Galat & Elion, 1939). The substituted phenethylamines were synthesized by one of two methods: (a) treatment of an arylmagnesium halide with ethylene oxide to give a phenethyl alcohol, which with hydrogen bromide gave the phenethyl bromide (Cagniant, Jecko & Cagniant, 1960), which was converted into the amine by the Gabriel reaction with potassium phthalimide followed by hydrazinolysis (Sheehan & Bolhofer, 1950); or b) by treatment of a benzyl halide with an alkali cyanide to give the corresponding phenylacetonitrile which was then reduced with lithium aluminium hydride (Benington, Morin & Clark, 1960). 2,6-Xylylpropylamine was likewise obtained from 2,6-dimethylphenethyl bromide by conversion into the nitrile and subsequent reduction.

PHARMACOLOGY

Methods

General. Unless otherwise stated, drugs were suspended in 0·5% gum tragacanth (w/v) and administered orally. Only 2,6-dimethylphenethylurea (cpd 16) was studied in all the tests, but other compounds were given for comparison in most tests, and all the compounds (listed in Table 2) were examined for their effects in conscious mice.

Dose-range studies. Drugs were given in a volume of 10 ml/kg to groups of 3 mice

(weight range 16–24 g) at doses spaced in geometric progression (dose ratio = 2). Each mouse was placed in a separate plastic beaker and observed for motor stimulation or depression, ataxia and loss of pinnal, corneal and righting reflexes. The effects were maximal usually within 5 min to 1 h. The dose producing a particular effect in 50% of the mice at the time of maximum activity (ED50) was calculated using the method of moving averages (Weil, 1952). A rough estimate of the LD50 was made from the number of mice dying up to 24 h after administration of the drug.

Anticonvulsant activity. At various times after administration of the drug, seizures were induced in groups of 5 mice by intravenous leptazol (56 mg/kg), intravenous strychnine hydrochloride (0·63 mg/kg), or an electroshock (25 mA, 50 Hz for 0·18 s) applied through Spiegal corneal electrodes, using the apparatus and technique described by Swinyard (1949) and by Woodbury & Davenport (1952). These chemical or electrical stimuli were just sufficient to induce tonic extensor spasms in the hind legs of 99% of the mice. The ED50 was estimated as the dose of drug required to prevent these tonic seizures in half the mice.

Effect on locomotor activity in mice (after Dews, 1953). Four groups of 5 mice per "light" box were used at each dose of drug. Control groups given the tragacanth suspension, but no drug, were tested at the same time to obviate errors due to spontaneous diurnal variation in the activity. The mice were placed in the boxes immediately after being given the drug and the total numbers of interruptions of the light beam every 15 min were integrated for each set of 4 boxes and recorded by a print-out device.

Effect on fighting behaviour in mice (Tedeschi, Tedeschi & others, 1959). Fighting behaviour in mice was produced by exposing pairs to a mild electric foot shock in an enclosed space. Ten pairs were used at each dose of drug. At various times after the drug, the number of rage episodes (i.e. periods when the mice stood on their hind legs and sparred) was recorded during a 3 min exposure to the electroshock. The ED50 was calculated as the dose of drug required to prevent 5 of the 10 pairs of mice from exhibiting more than 3 rage episodes in 3 min.

Effect on conditioned avoidance responses in rats (after Cook & Weidley, 1957). Rats were trained to escape from a box with an electrified grid floor by jumping into a small elevated side chamber, and to associate the shock with a buzzer. With further training, some rats learned to jump into the side chamber as soon as they were placed on the grid, without waiting for either buzzer (conditioned response) or shock (unconditioned response). This effect is denoted as the secondary conditioned response, and only rats which had learned this response were used in the drug tests. Drugs were given in a volume of 2 ml/kg to groups of 8 rats. Estimates were then made of the dose of drug required to suppress each type of response in half the rats at various times after drug administration.

Other behavioural studies. Compound 16 was given orally to conscious dogs or squirrel monkeys, which were then observed for behavioural changes and loss of reflexes.

Effect on reflexes in the anaesthetized cat. Cats anaesthetized with chloralose (80–100 mg/kg, i.v.) were used. Flexor contractions of the left tibialis anterior muscle were elicited by stimulating the central end of the severed ipsilateral tibial nerve with single rectangular pulses of 0·5 ms duration at a strength about 10V. In some experiments, contractions were also elicited by applying 1 ms pulses through electrodes placed directly on the muscle. Each flexor contraction was recorded alternately with a contraction of the right quadriceps femoris muscle, elicited by tapping the patellar tendon.

RESULTS

Dose-range studies. Table 2 summarizes the dose range studies in mice for 28 aralkylureas. Phenobarbitone, chlorpromazine, chlordiazepoxide, meprobamate and mephenesin are included for comparison.

Table 2. *Toxicity and CNS depressant properties of aralkylureas*

Compound

$$R\!-\!\!\langle\!=\!\rangle\!-\!(CH_2)_n\!\cdot\!NH\!\cdot\!CO\!\cdot\!NH_2$$

			ED50 (g/kg) for					
No.	R	n	Loss of pinnal reflex	Loss of corneal reflex	Motor de-pression	Ataxia	Loss of righting reflex	Approx. LD50 (g/kg)
1	H	1	0·2	1·4	0·06	0·1	0·6	0·6
2	H	2	0·45	1·1	0·1	0·4	1·1	2·5
3	H	3	1·6	3·2	1	1·5	2·3	>3
4	2-Me	2	0·4	0·6	0·07	0·2	0·6	1·5
5	3-Me	2	0·4	0·9	0·07	0·2	0·9	1·5
6	4-Me	2	0·6	1·8	0·2	0·9	1·4	>1·5
7	2,6-Me₂	0	2·2	>3·2	1	1	>3·2	>3
8	2,4-Me₂	1	2·5	>3·2	0·1	1·4	>3·2	>3
9	2,5-Me₂	1	1	>3·2	0·4	0·7	>3·2	>3
10	2,6-Me₂	1	1	>3·2	0·15	0·9	>3·2	>3
11	3,4-Me₂	1	1	>3·2	0·2	1·4	>3·2	>3
12	3,5-Me₂	1	1·5	>3·2	0·1	1·0	>3·2	>3
13	2,3-Me₂	2	0·1	0·45	0·05	0·22	0·6	1·2
14	2,4-Me₂	2	0·12	0·6	0·06	0·15	0·7	1·5
15	2,5-Me₂	2	0·2	0·6	0·07	0·15	0·7	2·3
16	2,6-Me₂	2	0·1	0·6	0·06	0·2	0·3	1·5
17	3,4-Me₂	2	0·5	2	0·25	0·6	3	3
18	3,5-Me₂	2	0·7	1·5	0·06	0·9	1·1	>1·5
19	2,6-Et₂	2	0·6	>3·2	0·05	0·5	1·5	>3
20	2,4-Cl₂	2	0·25	0·9	0·07	0·4	1·1	>3
21	2,6-Cl₂	2	0·02	0·07	0·005	0·15	0·08	0·1
22	2,4-(OMe)₂	2	2	>3·2	0·7	2	>3·2	>3
23	3,4-(OMe)₂	2	1	>3·2	0·15	1	>3·2	>3
24	2,4,6-Me₃	2	2	>3·2	0·7	3	>3·2	>3
25	3,4,5-(OMe)₃	2	3	>3·2	0·35	>3·2	>3·2	>3
26	2,6-Me₂	3	0·3	>3·2	0·1	0·2	0·9	>3
27	4-OH	2	2	>3·2	0·6	>3·2	>3·2	>3
28	C₆H₅·CH(OH)·CH₂·NH·CO·NH₂		>3·2	>3·2	0·6	>3·2	>3·2	>3
	Chlordiazepoxide		0·5	1·5	0·1	0·1	3	3
	Meprobamate		0·2	0·45	0·1	0·15	0·7	1
	Mephenesin		0·1	0·6	0·1	0·1	0·7	2·5
	Chlorpromazine		0·05	0·1	0·002	0·05	2	>2
	Phenobarbitone		0·07	0·2	0·04	0·08	0·15	0·3

Table 3. *Anticonvulsant activity of 2,6-dimethylphenethylurea and other central and spinal depressants*

Drug	Time after oral administration (h)	ED50 (mg/kg) against		
		Electro-shock	Leptazol	Strychnine
2,6-Dimethylphenethylurea	0·5	300	300	600
Mephenesin	0·25	250	250	700
Meprobamate	1	140	50	300
Chlordiazepoxide	1	30	5	40
Phenobarbitone	3	15	5	40
Chlorpromazine	1	200	300	>300

Comparative studies with 2,6-dimethylphenethylurea. In Table 3, the ability of 2,6-dimethylphenethylurea (cpd 16) to prevent convulsions induced by electroshock, leptazol or strychnine is compared with that of a variety of other central or spinal

depressants.   The anticonvulsant action of mephenesin and, to a lesser extent, of 2,6-dimethylphenethylurea is more transient than that of the other drugs, and these two compounds were consequently tested at shorter times after administration.

Potency of these short-acting drugs as depressants of locomotor activity in the "light" box was determined from the dose required to halve the number of counts in the period 15–30 min after injection of the drug.   Under these conditions the ED50 for 2,6-dimethylphenethylurea was 150 mg/kg compared with 300, 400, 100 and 200 mg/kg for meprobamate, mephenesin, 2,6-diethylphenethylurea (cpd 19) and 2,6-xylylpropylurea (cpd 26) respectively.

Potency as antagonists of rage episodes in 'fighting' mice was assessed at the time of maximum activity, namely 15 min after administration of 2,6-dimethylphenethylurea or mephenesin, or 1 h after the other drugs tested.   ED50 values were 150, 100, 30 and 10 mg/kg respectively for 2,6-dimethylphenethylurea, meprobamate, chlorpromazine and chlordiazepoxide.   Mephenesin failed to suppress rage episodes in half the mice at non-prostrating doses.

2,6-Dimethylphenethylurea has little selectivity in blocking conditioned reflexes in rats.   The secondary conditioned response was lost in half the rats at about 200 mg/kg while both the conditioned and unconditioned responses were lost at about 400 mg/kg. Similar values were obtained for meprobamate.   In contrast, chlorpromazine blocked the conditioned responses at much lower doses than the unconditioned response, ED50 values being about 5 and 15 mg/kg for the secondary conditioned response and the conditioned response, and > 30 mg/kg for the unconditioned response.

In male squirrel monkeys, oral administration of 50 mg/kg of 2,6-dimethylphenethylurea caused pronounced depression of motor activity lasting about 5 h, accompanied by ataxia and weakening of the corneal, myotatic and grasping reflexes.   At 12·5 mg/kg there was still some depression, but this was much less marked than at the higher dose.

In conscious female dogs (beagles), 100 or 200 mg/kg orally of 2,6-dimethylphenethylurea caused ataxia and abolition or weakening of the flexor, extensor, placing, extensor postural thrust and righting reflexes.   However, the knee jerk reflex was unaffected.   Male dogs showed an essentially similar pattern of effects but the drug was less active.

In mice, rats and dogs, there is a fairly rapid development of tolerance and a second dose of 2,6-dimethylphenethylurea 24 h after the first generally produced less effect.

Simultaneous recording of both the flexor and patellar reflexes in anaesthetized cats showed that 2,6-dimethylphenethylurea suppressed the polysynaptic flexor reflex more than the monosynaptic patellar reflex.   However, as shown in Fig. 1, the differentiation between mono- and poly-synaptic reflexes was much less sharp than with mephenesin, which had no effect on the patellar reflex in doses producing at least 50% inhibition of the flexor reflex.

Besides its inhibitory action on interneuronal transmission, 2,6-dimethylphenethylurea also has a direct depressant effect on the muscles themselves.   A dose of 20 mg/kg intravenously reduced the contractions of the tibialis muscle in response to direct stimulation, or to stimulation of the peroneal nerve leading to the muscle, by up to 40%.   This direct depressant effect renders the results obtained in experiments such as that shown in Fig. 1 liable to misinterpretation.   A clear differentiation between the various effects of 2,6-dimethylphenethylurea was brought out in an anaesthetized cat given a large dose (200 mg/kg) orally.   In this cat, the patellar reflex was reduced
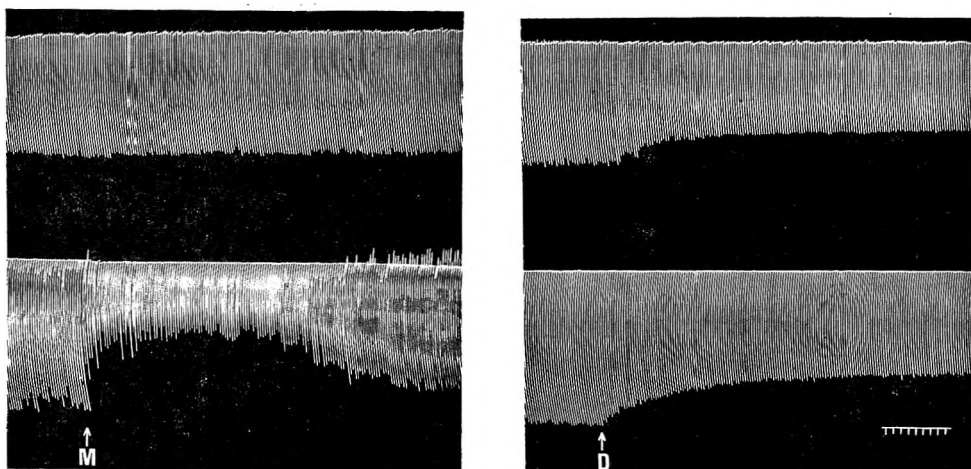
FIG. 1.   Effect of mephenesin and 2,6-dimethylphenethylurea on patellar and flexor reflexes. Cat anaesthetized with chloralose (80 mg/kg) and injected intravenously at M with mephenesin (20 mg/kg), and after 90 min, at D, with 2,6-dimethylphenethylurea (10 mg/kg).   Upper panels —patellar reflex, lower panels—flexor reflex.   Single stimuli (10V, 0·5 ms) at 15 s intervals.   Time 1 min.

within 10 min to 60% of normal, at which level it remained for a further 4 h.   The flexor reflex declined more slowly, taking 30 min to drop to 50% of normal, but it then continued to fall, disappearing completely after about 3 h.   The contractions of the tibialis muscle in response to direct stimulation fell to about 35% of normal in 1 h, but remained at this level for a further 3 h.   At this time, similar responses were elicited by stimulation of the peroneal nerve, showing the absence of neuromuscular block.

DISCUSSION

In view of the small number of animals used in the dose range studies, no significance should be placed on minor differences in activity.   Nevertheless, several clear-cut qualitative structure-activity relations emerge from the results in Table 2.   If a high ratio ED50 (corneal reflex)/ED50 (pinnal reflex) coupled with a low ED50 for the pinnal reflex relative to the LD50 is used as a criterion of selective muscle relaxant potency (Goodsell & others, 1954), the dimethylphenethylureas (cpds 13–16) containing at least one methyl group in the *ortho*-position stand out clearly.   The dimethyl-benzylureas (cpds 8–12), 3,4- and 3,5-dimethylphenethylurea (cpds 17, 18) and 2,6-xylylurea (cpd 7) are much less active, as are the monomethylphenethylureas (cpds 4–6) and phenethylurea itself (cpd 2).   Addition of a third methyl group to give 2,4,6-trimethylphenethylurea (cpd 24) also results in a tenfold fall in activity.   The di- or tri-methoxy-compounds (cpds 22, 23, 25) likewise have only low activity.   2,4-Dichlorophenethylurea (cpd 20) is about half as active as the corresponding dimethyl compound (cpd 14), but 2,6-dichlorophenethylurea (cpd 21) is exceptional in being both appreciably more active and much more toxic than the 2,6-dimethyl compound (cpd 16).   Replacement of the two methyl groups in positions 2 and 6 by ethyl groups to give 2,6-diethylphenethylurea (cpd 19) leads to a large rise in ED50 for abolition of both pinnal and corneal reflexes, but to no change in ED50 for motor depression.   In the "light" box experiments, this compound was slightly more active as a depressant than the corresponding dimethyl compound.   A similar change in

activity profile occurs when the methylene chain is lengthened to give 2,6-xylyl-propylurea (cpd 26).

Two possible routes for metabolism of phenethylurea (cpd 2) are ring or side-chain hydroxylation to give *p*-hydroxyphenethylurea (cpd 27) or β-hydroxyphenethylurea (cpd 28), both of which are almost inactive. If such metabolism occurred, this might account for the short duration of the activity displayed by many of the compounds.

The activity profile for 2,6-dimethylphenethylurea (cpd 16) in these dose-range experiments thus lies somewhere between those for meprobamate and mephenesin. 2,6-Diethylphenethylurea (cpd 19) and 2,6-xylylpropylurea (cpd 26) resemble chlordiaz-epoxide in having considerably greater potency as motor depressants than in depress-ing corneal and pinna reflexes, but they differ from chlordiazepoxide in not causing ataxia so readily.

From this group of compounds, 2,6-dimethylphenethylurea was chosen for investi-gation in more detail. It has only weak anticonvulsant activity and in this respect it closely resembles mephenesin, and differs from meprobamate, which is moderately active against leptazol. As a motor depressant in the "light" box, it is slightly more active then either meprobamate or mephenesin and differs from phenobarbitone which depresses motor activity only at doses high enough to cause almost complete prostra-tion. Tedeschi & others (1959) have shown that a distinctive feature of meprobamate is its ability to suppress rage episodes in "fighting" mice at a lower dose than is required to depress motor activity or to prevent electrically-induced convulsions. Although 2,6-dimethylphenethylurea, unlike mephenesin, will also suppress these rage episodes, the ratio ED50 (motor depression)/ED50 (rage episodes) is appreciably less than for meprobamate (1 instead of 3). Like meprobamate, and in contrast to chlorpromazine, 2,6-dimethylphenethylurea displays little selectivity in suppressing conditioned responses in rats.

In both conscious dogs and anaesthetized cats, 2,6-dimethylphenethylurea inhibits polysynaptic reflexes more readily than the patellar reflex, which is usually regarded as monosynaptic. However, it is less selective in this respect than is mephenesin, and it also has a depressant effect on the muscle contractions independent of its depressant effect on interneuronal transmission.

Thus, 2,6-dimethylphenethylurea has an activity profile resembling those of mephen-esin and meprobamate, but distinct from either, and differing still more from those of phenobarbitone, chlordiazepoxide and chlorpromazine.

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A screening method for vasodilator drugs

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The use of the isolated perfused central artery of the rabbit ear for screening vasodilator drugs is described. The effects of these drugs are shown by reduction of vasoconstrictor responses to intermittent sympathetic stimulation or to injections of noradrenaline or histamine or by reduction of vascular spasms produced by continuous sympathetic stimulation or infusion of 5-hydroxytryptamine. The relative potencies of the vasodilators used against intermittent sympathetic stimulation, in order are: adenosine = sodium nitroprusside = CIBA 31,531-Ba [5-amino-1-(1-methylpiperid-4-yl)- 3-(pyrid-4-yl) pyrazole] > glyceryl trinitrate > papaverine = amino-phylline > dipyridamole ≫ sodium nitrite = hydrallazine.

The isolated perfused central artery of the rabbit ear provides a vascular preparation with a post-ganglionic sympathetic innervation that is suitable for testing the effects of drugs directly on blood vessels. The artery segment constricts when its sympathetic periarterial nerves are stimulated or when noradrenaline, histamine or 5-hydroxytryptamine are injected into the perfusion fluid (de la Lande & Rand, 1965).

Since the smooth muscle in this preparation is already fully relaxed, vasodilator actions of drugs cannot be observed directly. However, with the tone raised by continuous sympathetic stimulation, vasodilatation has been observed with acetylcholine (de la Lande & Rand, 1965) and bradykinin (Starr & West, 1966).

An anomalous observation with this preparation was made by Gay, Rand & Wilson (1967) who showed that isoprenaline, generally a potent vasodilator drug, did not dilate the artery even when the tone was raised. Instead, it caused constriction, though its potency was several thousandfold weaker than that of noradrenaline. The explanation was that the preparation lacks β-adrenoreceptors and isoprenaline weakly stimulates α-adrenoreceptors.

This paper deals with the use of the isolated perfused rabbit ear artery preparation for screening vasodilator drugs.

EXPERIMENTAL

A segment of the central artery of the rabbit ear was set up as described by de la Lande & Rand (1965). The preparation was perfused with McEwen solution at a constant flow rate of 6 ml/min using a Watson-Marlow flow inducer. The perfusion fluid was bubbled with 5% carbon dioxide in oxygen, and was maintained at a constant temperature of 37°.

Stimulation of the periarterial sympathetic nerves or the injection of noradrenaline, histamine or 5-hydroxytryptamine (5-HT) produced constriction of the artery segment. These responses were measured as changes in perfusion pressure with a Statham pressure transducer and were recorded with an Offner Dynograph pen recorder. The sympathetic nerves were stimulated by means of bipolar platinum ring electrodes placed around the proximal end of the artery using 1 ms square wave pulses at rates of 10 to 20 pulses/s and supramaximal voltage. Injections and infusions of drugs were

given into the rubber connection near the arterial cannula. All the drugs tested were freshly prepared in McEwen solution and were injected in volumes ranging from 0·04 to 0·20 ml. Infusions were made by means of a Palmer slow injection apparatus at rates of 0·05 to 0·20 ml/min.

The drugs used were acetylcholine chloride, adenosine, aminophylline, CIBA 31,531-Ba [5-amino-2-(1-methylpiperid-4-yl)-3-(pyrid-4-yl)pyrazole hydrochloride], dipyrida-mole, glyceryl trinitrate, histamine hydrochloride, 5-hydroxytryptamine creatine sulphate, hydrallazine, noradrenaline hydrochloride, papaverine hydrochloride, sodium nitrite, and sodium nitroprusside. The doses and concentrations of the drugs are expressed in terms of the compounds described above.

RESULTS

Reduction of intermittent vasoconstrictor responses produced by bursts of sympathetic stimulation or injections of noradrenaline or histamine

Infusions of the vasodilator drugs tested reduced the responses of the artery to sympathetic nerve stimulaiton. Representative records are shown in Fig. 1 with papaverine, dipyridamole and CIBA 31,531-Ba.
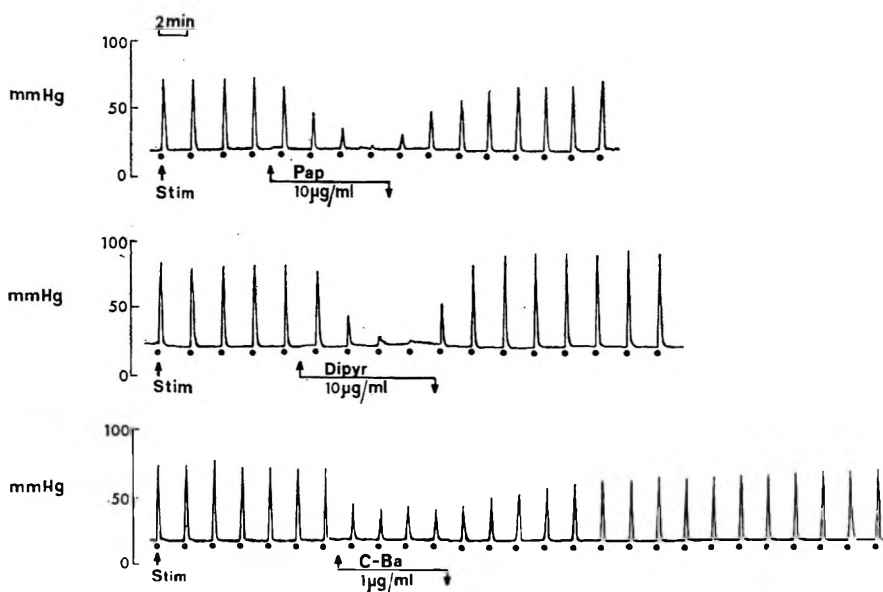
FIG. 1. Records of 3 separate experiments. In each, the periarterial sympathetic nerves were stimulated as indicated by the black dots for 10 s at 2 min intervals with 1 ms pulses at a frequency (10 to 20 pulses/s) sufficient to cause an increase in perfusion pressure of approximately 50 mm Hg. Infusions of papaverine (Pap), dipyridamole (Dipyr) and CIBA 31,531-Ba (C-Ba) were given for the periods and in the concentrations indicated beneath the records.

All of the drugs also reduced vasoconstrictor responses to injections of noradrenaline. Fig. 2 (top record) shows the effect of an infusion of sodium nitroprusside (1 μg/ml).

The potencies of the vasodilator drugs were compared by finding the minimal concentration of each drug which produced unequivocally a detectable reduction in the responses to sympathetic stimulation. The reproducibility of control responses was such that a reduction of 5 to 10% could generally be clearly recognized. Threshold concentrations were also found for the reduction of responses to injected noradrenaline

Table 1. *Threshold concentration of drugs in reducing vasoconstrictor responses to sympathetic stimulation and to injections of noradrenaline*

		Threshold concentration (μg/ml)		Relative molar potency Adenosine = 100%	
Vasodilator drug		Sympathetic stimulation	Noradrenaline injection	Sympathetic stimulation	Noradrenaline injection
Adenosine		0·5	0·01	100	100
Sodium nitroprusside ..	..	0·5	0·05	112	22
CIBA 31,531-Ba	..	0·5	0·05	112	22
Glyceryl trinitrate ..	..	1·0	0·20	43	4
Papaverine	..	2·0	0·50	36	3
Aminophylline	..	2·0	0·20	43	8
Dipyridamole	..	5·0	1·00	19	2
Sodium nitrite	..	50·0	1·00	0·3	0·3
Hydrallazine	..	50·0	0·20	0·6	3

(Table 1). In all instances, the threshold concentration to reduce the response to injected noradrenaline was lower than a concentration required to reduce the response to sympathetic stimulation. Threshold concentrations were chosen to compare potencies quantitatively because they gave more consistent results than were obtained using concentrations causing 50% depression of responses; this was probably because log dose-response lines for the substances were not always parallel. Determination of pA_2 was not used as it is not applicable to sympathetic nerve stimulation.

All of the vasodilator drugs reduced vasoconstrictor responses to injections of histamine to about the same extent as they reduced responses to noradrenaline. Examples with infusions of dipyridamole (10 μg/ml) and CIBA 31,531-Ba (1 μg/ml) are illustrated in the lower two records of Fig. 2. These are from experiments in which histamine and noradrenaline were injected alternately in doses that produced approximately equal vasoconstrictor responses.
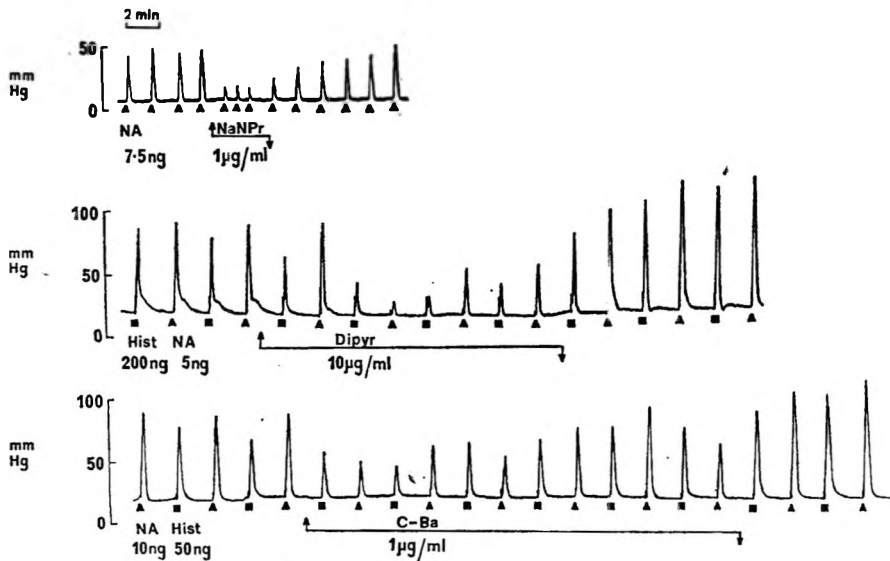
FIG. 2. Records of 3 separate experiments. Vasoconstrictor responses were induced by injections of noradrenaline (NA) as indicated by ▲ or by injections of histamine as indicated by ■ in the doses indicated beneath each record. Infusions of sodium nitroprusside (NaNPr), dipyridamole (Dipyr) and CIBA 31,531-Ba (C-Ba) were given for the periods and in the concentrations indicated beneath the records.

Reduction of vascular spasm induced by continuous sympathetic stimulation or infusion of vasoconstrictor drugs

Vasodilator actions in the perfused ear artery can also be demonstrated by a fall in perfusion pressure caused by injections or infusions of vasodilator drugs during a sustained constrictor response.

The vasodilator action of an injection of acetylcholine during sympathetically induced vascular spasm, an effect previously demonstrated by de la Lande & Rand (1965), is shown in Fig. 3. This also illustrates that an infusion of 100 μg/ml of
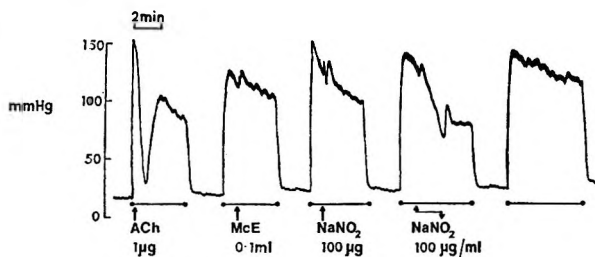
FIG. 3. Periods of sustained vasoconstriction were obtained in the preparation by stimulation with 1 ms pulses at 10 pulses/s as indicated by each pair of black dots joined by a horizontal line beneath the records. From left to right, the records show the following: The vasodilator response to 1 μg of acetylcholine (ACh) in a volume of 0·1 ml; a control injection of 0·1 ml of McEwen solution (McE); injection of 100 μg of sodium nitrite; infusion of 100 μg/ml of sodium nitrite; and a control period of stimulation.

sodium nitrite reduced the sustained vasoconstrictor response to sympathetic stimulation, but a single injection of 100 μg did not. Sodium nitrite is the weakest of the vasodilator drugs studied. With the others, vasoconstrictor spasm was reduced by injection of doses ranging from 1 to 100 μg.

Sodium nitroprusside was amongst the most potent of the vasodilator drugs studied; in a dose of 1 μg it produced marked inhibition of the sustained vasoconstriction produced either by infusion of 5-HT or by sympathetic stimulation (Fig. 4).
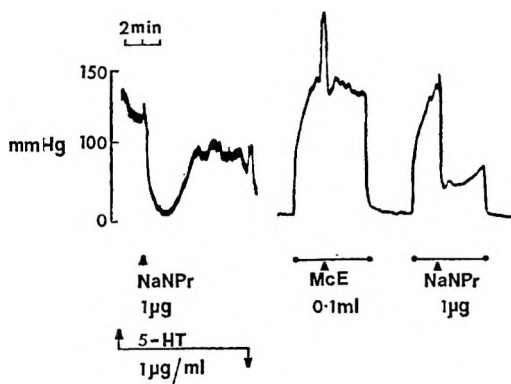
FIG. 4. Vasodilator effects of injections of 1 μg of sodium nitroprusside (NaNPr) during vascular spasms produced by infusion of 1 μg/ml of 5-hydroxytryptamine (5-HT) and sympathetic stimulation. A control injection of 0·1 ml of McEwen solution (McE) produced an artifact of an increase in perfusion pressure.

DISCUSSION

The results indicate that the isolated perfused artery from the rabbit ear provides a useful preparation for screening drugs having vasodilator activity. The preparation has the advantages that it is robust, economical and simple to set up. Furthermore, results are obtained more rapidly than with spiral strips of blood vessels and vascular spasms may be produced not only with vasoconstrictor drugs but also with sympathetic stimulation.

The preparation can be used to investigate the mode of action of vasodilator drugs. The vasodilator drugs tested reduced the vasoconstrictor responses to injected noradrenaline and histamine, to sympathetic stimulation and to infusion of 5-HT which suggests that their main effect is exerted directly on arterial smooth muscle. Relatively specific blocking drugs such as phenoxybenzamine and phentolamine produced a marked reduction in the vasoconstrictor responses to nerve stimulation and to injected noradrenaline but not to injected histamine (Gay & others, 1967). Each of the vasodilator drugs used was more effective in reducing the vasoconstrictor responses to noradrenaline than those to sympathetic stimulation. The ratios of threshold concentrations of the drugs in producing these two effects ranged as follows: papaverine, glyceryl trinitrate, dipyridamole, 1:5; sodium nitroprusside, aminophylline, CIBA 31,531-Ba, 1:10; adenosine, sodium nitrite, 1:50; hydralazine, 1:250. The fact that these drugs were effective in lower concentrations in counteracting the effect of injected noradrenaline than the effect of noradrenaline released by nerve stimulation is in accord with general experience. Possible explanations for the wide divergence in ratios are that drugs with a low ratio may reduce the release of noradrenaline or that drugs with a high ratio may facilitate the release.

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The use of the *in vivo* trachea preparation of the guinea-pig to assess drug action on lung

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A method for recording the effects of drugs on an isolated *in vivo* segment of trachea and on lung pressure of a deeply anaesthetized animal is described. Acetylcholine, angiotensin, histamine, 5-hydroxytryptamine, prostaglandin (PG) $F_{2\alpha}$ and slow-reacting substance of anaphylaxis, but not bradykinin, caused an increase in tracheal segment pressure and an increase in lung pressure. Bradykinin caused an increase in lung pressure but a fall in tracheal segment pressure. It was concluded that bradykinin acts mainly on the smaller airways and $PGF_{2\alpha}$ mainly on the larger airways. The tracheal segment responded to adrenaline, aminophylline, ephedrine, isoprenaline, papaverine, PGE_1 and PGE_2. Propranolol reduced or abolished responses to all these bronchodilators except PGE_1 and PGE_2.

For many years the preparation of Konzett-Rössler (1940) has been used to assess the effects of drugs on lung (Collier, Holgate, & others, 1960; Bhoola, Collier & others, 1962; Collier & Shorley, 1963; Berry & Collier, 1964; Collier, James & Schneider, 1966; James, 1967). Although the method has been modified (Collier & others, 1960) and made more sensitive (Rosenthale & Dervinis, 1968), since it estimates changes in lung volume, it remains more sensitive to changes in lung compliance (Widdicombe, 1963) than to changes in airway resistance. The Konzett-Rössler preparation does not respond directly to the effects of bronchodilator drugs. To assess these effects a bronchoconstrictor agent is normally used (James, 1967). A more accurate assessment of bronchodilator action can be made by measuring airway resistance (Diamond, 1967; Familiar, Wardell & Greene, 1967).

In the experiments now reported, measurements were made of lung pressure and of pressure inside a segment of trachea *in vivo* (Kahn, 1907; Green & Widdicombe, 1966), using artificially ventilated guinea-pigs. An assessment of drug effect on lung compliance and airway resistance could thus be made. The effects of bronchoconstrictor and bronchodilator drugs on this preparation have been investigated and compared.

EXPERIMENTAL

Materials

The following were used as bronchoconstrictor agents: acetylcholine bromide, angiotensin, bradykinin (synthesized by Nicolaides & De Wald, 1961), histamine acid phosphate, 5-hydroxytryptamine creatinine sulphate (5-HT), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Dr. J. E. Pike of the Upjohn Co.)

The following were used as bronchodilator agents: adrenaline tartrate, aminophylline, ephedrine hydrochloride, isoprenaline sulphate, papaverine sulphate and prostaglandins E_1 and E_2 (PGE_1 and PGE_2) (Dr. J. E. Pike of the Upjohn Co.). As antagonists, atropine sulphate, meclofenamic acid [*N*-(2,6-dichloro-*m*-tolyl)anthranilic acid] (Winder, Wax & Welford, 1965) as the sodium salt, mepyramine maleate, methysergide bimaleate, propranolol hydrochloride and tolazoline hydrochloride were used.

Procedure

Guinea-pigs (Duncan-Hartley strain), 600–1000 g, were anaesthetized with 60 mg/kg of pentobarbitone sodium given intraperitoneally. A cannula (ventilation cannula, Fig. 1) was inserted into the lower trachea as near as was possible to the thorax. The
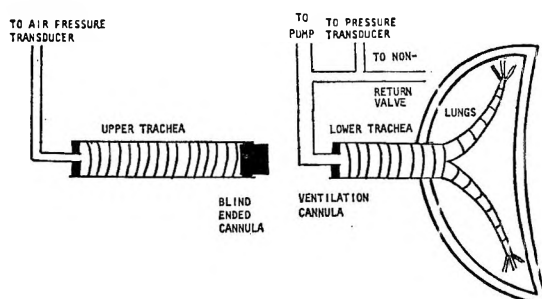

FIG. 1. Diagram to show the arrangement of the apparatus. For description see Experimental Procedure.

guinea-pig was ventilated through this cannula with a Starling miniature respiratory pump of 8 ml stroke volume and at a rate of 72 strokes/min. The side arm of the cannula was connected to a non-return water valve set for a pressure of 10 cm of water. Intrapulmonary pressure (lung pressure) was measured by a differential air pressure transducer attached to the cannula and recordings were made on a multi-channel electronic recorder. A second cannula (blind ended) was inserted into the trachea directed towards the larynx (see Fig. 1). A third cannula was inserted into the upper trachea as far as was possible from the lungs and was connected to a very sensitive air pressure transducer. Great care was taken when inserting the cannulae, to avoid complete interruption of the nerve or blood supply to the "isolated" segment of trachea. Measurements of pressure within the segment of trachea (tracheal segment pressure) were made to assess the "tone" of the segment and tracings were made on the multi-channel recorder. For intravenous administration of drugs, the jugular vein was cannulated. For intra-arterial administration, a cannula was inserted into the aortic arch via the carotid artery. All drugs were given intravenously except where specified.

RESULTS

Anaesthetic. Animals were anaesthetized with 60 mg/kg intraperitoneally of pentobarbitone sodium. At this level of anaesthesia, bronchoconstrictor agents such as histamine produced an increase followed by a decrease of pressure in the tracheal segment. Increasing the depth of anaesthesia with intravenous doses of pentobarbitone sodium did not materially alter quantitatively or qualitatively the response obtained (Fig. 2).

Bronchoconstrictor agents

Acetylcholine. 1–10 µg caused a slight increase in lung pressure and a small increase followed by a decrease of tracheal segment pressure. Both increases in pressure were abolished by 1 mg/kg of atropine. The decrease in pressure in the trachea after atropine and acetylcholine was still present but much reduced.

Angiotensin. 2–5 µg caused an increase in lung pressure and an increase followed by a decrease of tracheal segment pressure. Neither response was antagonized by meclofenamate.
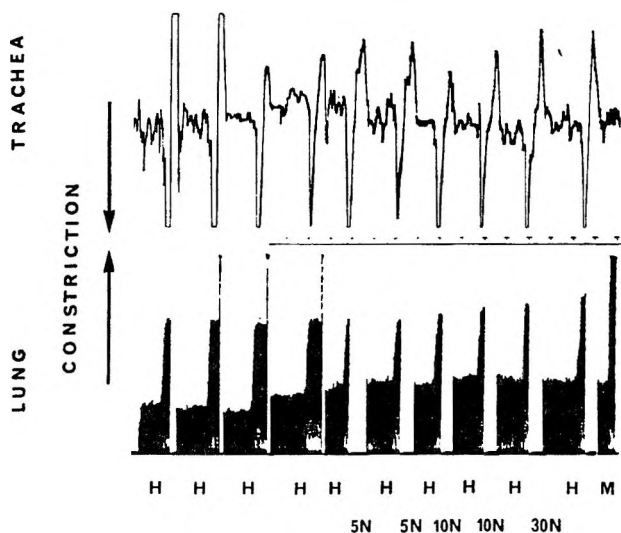
FIG. 2. Effect of anaesthetic on responses to histamine on lung pressure and tracheal segment pressure in the guinea-pig. Upper tracing—tracheal pressure, lower tracing—lung pressure. Guinea-pig of weight 800 g anaesthetized with pentobarbitone sodium 60 mg/kg i.p. and artificially ventilated. All doses were given intravenously. H, 4 μg histamine; N, dose of pentobarbitone sodium in mg/kg; M, maximum response of lung pressure obtained by clamping the trachea. All doses were given at 10 min intervals ↑ direction of increased pressure; time 1 min. The breaks in the lung pressure tracing are due to movement of the recording paper when no record was being made.

Bradykinin. Given intravenously, bradykinin (2–20 μg) increased the lung pressure and induced a small decrease in the tracheal segment pressure. Meclofenamate (2 mg/kg) antagonized the response to bradykinin on the lung pressure (Fig. 3a). Propranolol pretreatment increased the response to bradykinin on the lung pressure and abolished the decrease of the tracheal segment pressure (Fig. 3b left). Given intra-arterially, bradkyinin induced a decrease in the tracheal pressure, but did not affect the lung pressure (Fig. 3b right).

Histamine. 2–4 μg caused an increase in lung pressure and an increase followed by a decrease in tracheal segment pressure (Fig. 2). Both responses were antagonized by 2 mg/kg mepyramine, but not by 2 mg/kg meclofenamate.

5-Hydroxytryptamine, 2–4 μg gave similar responses to those obtained with histamine and could be antagonized by pretreatment with 1 mg/kg of methysergide.

Prostaglandin $F_{2\alpha}(PGF_{2\alpha})$. 20–50 μg in the absence of propranolol caused a small pressure increase in the trachea, with very little effect on the lung pressure. In the presence of propranolol, $PGF_{2\alpha}$ increased the lung pressure and caused a large increase in tracheal segment pressure. Neither response was antagonized by 2 mg/kg meclofenamate (Fig. 4).

Slow-reacting substance in anaphylaxis (SRS-A). 0·5–4 mg of partially purified material caused an increase in lung pressure and tracheal segment pressure. These responses were antagonized by 2 mg/kg of meclofenamate (Fig. 5). In this figure a small decrease in tracheal pressure after meclofenamate may be observed.

Bronchodilator agents

Adrenaline. 0·05–0·2 μg produced a small increase followed by a large decrease of the tracheal segment pressure. The decrease was antagonized by pretreatment with

propranolol and the increase by pretreatment with tolazoline.   Adrenaline did not produce a detectable change in the lung pressure (Fig. 6a).
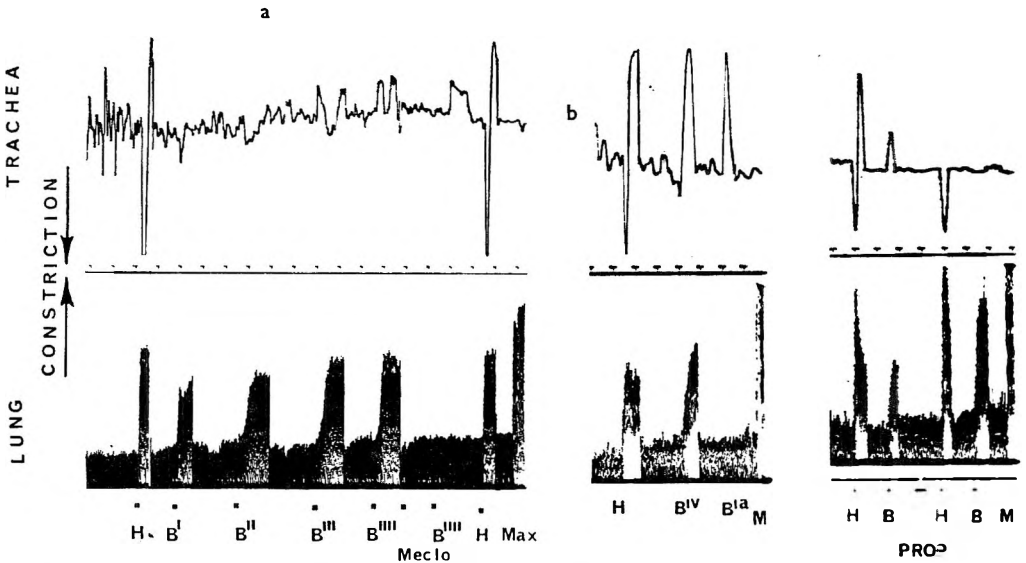

FIG. 3.   a.   Effect and antagonism of bradykinin on lung pressure and tracheal segment pressure in a guinea-pig, 710 g.   H, 2µg histamine; B′, 2 µg bradykinin; B″, 4µg bradykinin; B‴, 8 µ bradykinin; B‴′, 20 µg bradykinin; Meclo, 2 mg/kg meclofenamate.

b.   Effect of propranolol on the response to intravenous bradykinin and the effect of brady-kinin given intra-arterially on lung pressure and tracheal segment pressure in the guinea-pig. Right-hand panel: Guinea-pig, 920 g.   H, 3 µg histamine; B, 2 µg bradykinin; PROP, 5 mg/kg of propranolol.   All substances were given intravenously.   Left-hand panel: Guinea-pig 850 g. H, 3 µg histamine; B, i.v. 5 µg bradykinin given intravenously; B i.a., 5 µg bradykinin given intra-arterially.

Other details as for Fig. 2.
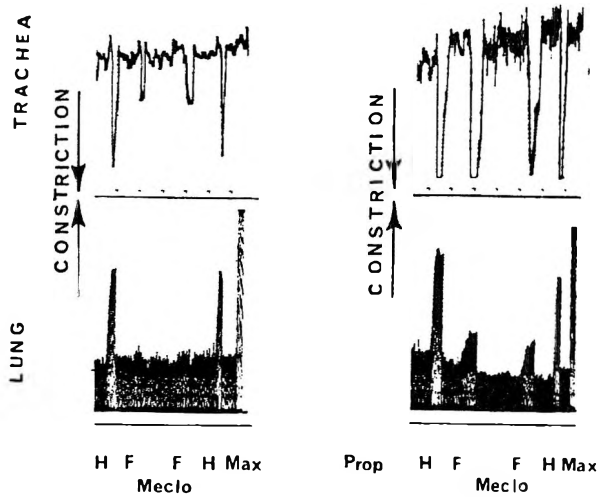
FIG. 4.   Effect of prostaglandin $F_2\alpha$ (PGF$_2\alpha$) on lung pressure and tracheal segment pressure in the guinea-pig.   Left-hand tracing: Guinea-pig, 840 g.   H, 2 µg histamine; F, 50 µg PGF$_2\alpha$; Meclo, 2 mg/kg meclofenamate sodium.   Right-hand tracing: Guinea-pig, 750 g, pretreated with propranolol (Prop), 5 mg/kg intravenously.

Other details as for Fig. 2.

Aminophylline. Doses of 1–3 mg caused a decrease in tracheal segment pressure but did not alter the lung pressure. After pretreatment with propranolol, the change in tracheal segment pressure due to aminophylline was reduced.

Ephedrine. 0·5–1 mg caused a decrease in the tracheal segment pressure but no recordable effect on lung pressure. The tracheal response was antagonized by propranolol.
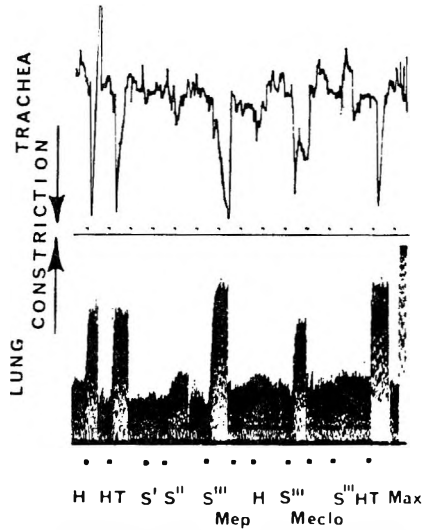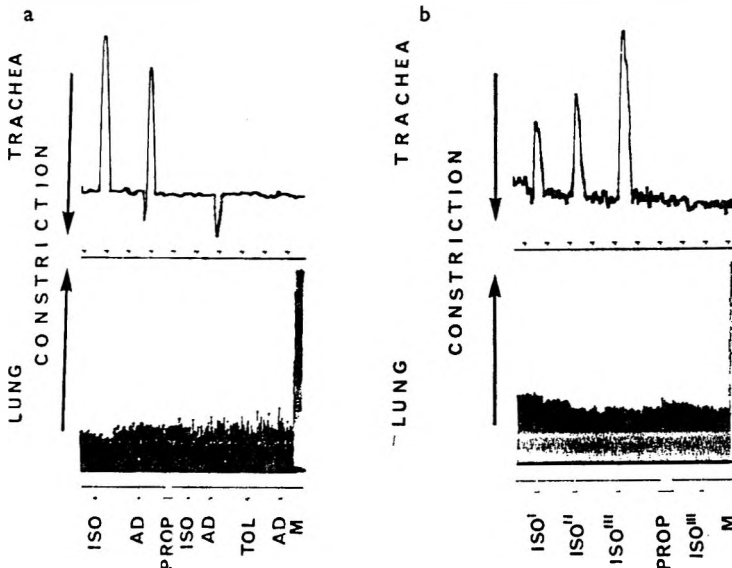

FIG. 5. Effect and antagonism of slow-reacting substance in anaphylaxis (SRS-A) on lung pressure and tracheal segment pressure in the guinea-pig. Guinea-pig, 860 g. H, 3 μg histamine; HT, 2 μg 5-hydroxytryptamine; S', 0·5 mg SRS-A; S'', 1 mg SRS-A; S''', 3 mg SRS-A; Mep, 2 mg/kg mepyramine; Meclo, 2 mg/kg meclofenamate sodium. Other details as for Fig. 2.

FIG. 6. a. Effect and antagonism of adrenaline on lung pressure and tracheal segment pressure in a guinea-pig, 920 g. ISO, 0·01 μg isoprenaline; AD, 0·1 μg adrenaline; PROP, 5 mg/kg propranolol; TOL, 5 mg/kg tolazoline.

b. Effect and antagonism of isoprenaline on lung pressure and tracheal segment pressure in a guinea-pig, 870 g. ISO', 0·002 μg isoprenaline; ISO'', 0·004 μg isoprenaline; ISO''', 0·01 μg isoprenaline; PROP, 5 mg/kg propranolol.

Other details as for Fig. 2.

Isoprenaline. 2–10 ng caused a dose-related pressure fall in the trachea, but no response on the lung pressure. The tracheal response was antagonized by propranolol (Fig. 6b).

Papaverine. 0·5–3 mg gave similar results to those obtained with aminophylline. The pressure fall in the trachea was only partially antagonized by propranolol.

Prostaglandin E_1 and E_2 (PGE$_1$ and PGE$_2$). Both prostaglandins caused a decrease in tracheal segment pressure with no recordable effect on lung pressure. The responses were not antagonized by 5 mg/kg of propranolol. PGE$_1$ was approximately twice as potent as PGE$_2$ in this test (Fig. 7).

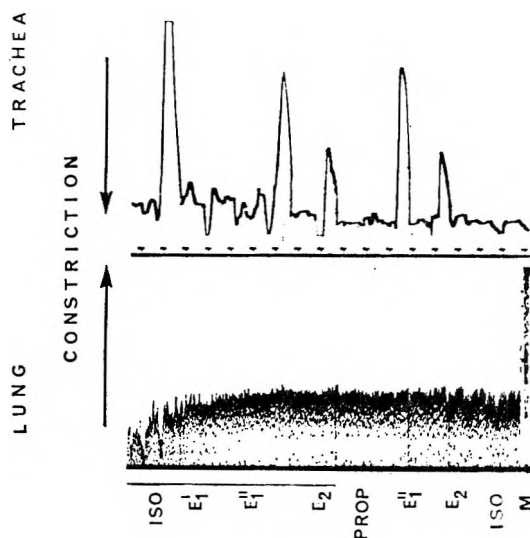The results obtained with the above drugs are summarized in Table 1.

FIG. 7. Effects of prostaglandins E_1 and E_2 on lung pressure and tracheal segment pressure in a guinea-pig, 840 g. ISO, 0·05 μg isoprenaline; E_1', 5 μg prostaglandin E_1; E_1'', 10 μg prostaglandin E_1; E_2, 10 μg prostaglandin E_2; PROP, 5 mg/kg propranolol. Other details as for Fig. 2.

DISCUSSION

With the exception of the prostaglandins, the effects of the drugs used in these experiments on the Konzett-Rössler preparation have been well documented (Collier & others, 1960; Holgate & Warner, 1960; Berry & Collier, 1964). These effects are similar to those on lung pressure described above. This discussion will therefore be mainly confined to the effects of drugs on the tracheal preparation. The pressure rise in the trachea caused by acetylcholine, angiotensin, histamine and 5-HT as well as their effects on lung pressure indicate that these drugs act on both large and small airways. These results for acetylcholine, histamine and 5-HT confirm those obtained by Cole-batch, Olsen & Nadel (1966) in the cat. The fall in tracheal segment pressure following the increase due to these drugs could be abolished by propranolol. Most preparations responded biphasically.

Fig. 3 shows that bradykinin did not increase tracheal segment pressure after large doses, although changes in lung pressure were obtained and could be antagonized. Because bradykinin may have been inactivated before reaching the trachea (Ferreira & Vane, 1967) it was given intra-arterially; but still it produced no pressure increase. To check that the lack of response was not due to a physiological antagonism between

Table 1. *Effect of some drugs on lung pressure and tracheal segment pressure in the guinea-pig.* Each result was derived from at least 3 experiments All drugs were given intravenously except for bradykinin, i.v., intravenous; i.a., intra-arterial; + pressure increase; − pressure fall; +−increase followed by a decrease in pressure; 0, no effect

| Drug | Effect on | | Antagonist | Effect of drug after antagonist on | |
	Trachea	Lung		Trachea	Lung
Acetylcholine	+−	+	Atropine	−	0
Angiotensin	+−	+	Meclofenamate	+−	+
Bradykinin i.v.	−	+	Meclofenamate	−	0
Bradykinin i.v.	−	+	Propranolol	0	+
Bradykinin i.a.	−	0	—		
Histamine	+−	+	Mepyramine	−	0
5-HT	+−	+	Methysergide	−	0
$PGF_{2\alpha}$	+	+	Meclofenamate	+	+
SRS-A	+	+	Meclofenamate	0	0
Adrenaline	+−	0	Propranolol	+	0
Adrenaline	+−	0	Propranolol + tolazoline	0	0
Aminophylline	−	0	Propranolol	− (reduced)	0
Ephedrine	−	0	Propranolol	0	0
Isoprenaline	−	0	Propranolol	0	0
Papaverine	−	0	Propranolol	− (reduced)	0
PGE_1	−	0	Propranolol	−	0
PGE_2	−	0	Propranolol	−	0

the effect of bradykinin and the effect of subsequently released catecholamines, propranolol was given before a dose of bradykinin. Even under these conditions, bradykinin did not induce a rise in pressure in the trachea. Bradykinin appears therefore to exert its effect mainly on the smaller airways. This conclusion confirms the finding of Jankala & Virtama (1963) using bronchography. The pressure fall in the trachea after the administration of bradykinin was probably due to catecholamine discharge (Collier, James & Piper, 1965; Piper, Collier & Vane, 1967) and could be abolished by propranolol pretreatment.

The large increase in pressure in the trachea due to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) confirms previous observations of the effect of this compound in guinea-pig lung (Änggård & Bergström, 1963) and for human lung (Sweatman & Collier, 1968). The response was not antagonized by meclofenamate, indicating a difference between human isolated bronchial muscle (Sweatman & Collier, 1968) and this *in vivo* preparation of guinea-pig lung. The greater effect of $PGF_{2\alpha}$ on the trachea than on the lung indicates that $PGF_{2\alpha}$ acts mainly on the larger airways. This may explain why Änggård & Bergström (1963) failed to show an effect on cat isolated bronchial chain, but obtained a response on cat isolated tracheal chain.

Slow-reacting substance of anaphylaxis (SRS-A) caused a pressure increase in both trachea and lung and in this respect differed from the responses obtained with bradykinin. These responses were antagonized by meclofenamate, thus confirming the findings of Berry & Collier (1964) with the Konzett-Rössler preparation of guinea-pig lungs *in vivo*.

The slight fall in the tracheal segment pressure following intravenous administration of meclofenamate confirms similar results obtained in this laboratory using human isolated bronchial muscle preparations.

The action of bronchodilator drugs could clearly be demonstrated on the tracheal preparation. Their action and antagonism on this preparation confirms and extends

those previously reported on the Konzett-Rössler preparation (James, 1967). A real advantage of the present technique over previous *in vivo* ones in demonstrating these effects is that no bronchoconstrictor agent need be used. The tracheal preparation responds repeatedly and in a dose-dependent way to bronchodilator substances.

The fall in tracheal segment pressure after administration of prostaglandins E_1 and E_2 (PGE_1 and PGE_2) confirms previous observations (Main, 1964; Khairallah, Page & Türker, 1967; Sheard 1968; Sweatman & Collier, 1968). In the present preparation, PGE_1 was approximately twice as potent as PGE_2. Neither response was antagonized by propranolol.

The response of the trachea to adrenaline showed the presence of a dilator component that was antagonized by propranolol and of a constrictor component that was antagonized by tolazoline. This result confirms those previously published (Nagasaka, Bouckaert & others 1964; James, 1967).

This preparation was found useful to determine roughly the site of action of bronchoconstrictor drugs on the airway system of the guinea-pig and to determine directly the mode of action and potency of bronchodilator drugs.

Acknowledgements

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Effect of diazepam and chlordiazepoxide on the metabolism of other drugs

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Diazepam and chlordiazepoxide, even at high single doses, do not inhibit the metabolic transformation of *p*-nitroanisol, aniline and aminopyrine by the 9,000 *g* fraction of rat liver. Although diazepam and chlordiazepoxide increase the sleeping time induced by pento-barbitone, this effect is not explained by an increase in the brain level of pentobarbitone. Repeated administrations of the two benzo-diazepines increase the metabolism of *p*-nitroanisol, aniline, amido-pyrine and shorten the sleeping time induced by pentobarbitone in rats. There was a concomitant reduction of the concentration of brain pentobarbitone.

In recent years, increasing evidence has been obtained about the possibility that drugs may interfere with the activity of drug-metabolizing enzymes in liver micro-somes. More than two hundred compounds have been found to stimulate drug metabolism and many of these also reduce drug metabolism in given experimental conditions. Chlordiazepoxide was included among the "inducers" (Conney, 1967). Hoogland, Miya & Bousquet (1966) suggested that tolerance to the skeletal muscle relaxant effects of chlordiazepoxide could be explained on the basis of a stimulation of liver microsomes responsible for chlordiazepoxide metabolism. The purpose of this investigation was to compare the activity of chlordiazepoxide and diazepam, *in vivo* and *in vitro*.

EXPERIMENTAL

Materials and methods

Female Sprague-Dawley rats, 150 ± 10 g maintained on food and water *ad libitum*, were used. Diazepam and chlordiazepoxide were dissolved in carboxymethylcellulose (0·5% v/v) and were given by oral intubation. Experiments were always made in the afternoon.

In vitro *experiment.* Pretreated animals were killed by decapitation, livers were quickly removed, placed in dry-ice, weighed and homogenized in 1·15% KCl solution. This homogenate was centrifuged at 4° at 9,000 *g* and the supernatant fraction used (Kato & Takanaka, 1967).

Substrates were: *p*-Nitrophenol 2 (μmol), aniline (5 μmol) and aminopyrine (5 μmol). At the end of the incubation period (30 min) aliquots of the incubation medium were removed and analysed for metabolites, *p*-nitrophenol, *p*-aminophenol and 4-amino-antipyrine respectively (Gilbert & Golberg, 1965).

In vivo *experiment.* Chlordiazepoxide- or diazepam- pretreated rats were given pentobarbitone (30 mg/kg i.p.). Sleeping time was evaluated as that time elapsing between loss and regaining of the righting reflex.

Pentobarbitone was measured in the whole brain 90 min after the administration (Brodie, Burns & others, 1953). The weight of the liver and the liver proteins were not affected by the repeated treatments with diazepam.

<div align="center">RESULTS</div>

Effect of acute pretreatment on microsomal enzyme activity in vitro. Oral pretreatment with diazepan (50 and 100 mg/kg) or chlordiazepoxide (100 mg/kg) in a single dose, does not inhibit the liver microsomal enzyme systems responsible for the metabolism of aniline, *p*-nitrophenol and aminopyrine (Table 1).

Table 1. *Effect of diazepam and chlordiazepoxide on liver metabolic activity of other compounds*

Treatment	mg/kg oral	*p*-Nitrophenol nmol ± s.e.	4-Aminoantipyrine nmol ± s.e.	*p*-Aminophenol nmol ± s.e.
Saline	—	(15) 440 ± 30	(4) 64 ± 10	(4) 520 ± 50
Diazepam ..	50	(15) 400 ± 30	(4) 66 ± 6	(4) 597 ± 70
Diazepam ..	100	(4) 300 ± 30	(4) 59 ± 10	(4) 590 ± 60
Chlordiazepoxide	100	(15) 440 ± 20	(4) 49 ± 4	(4) 530 ± 50

Chlordiazepoxide, diazepam or saline were given 2 h before the assay.
Enzyme activity is shown as nmol of metabolites formed per g liver/h.
Figures in brackets refer to the number of experiments.

Effect of acute pretreatment on pentobarbitone response. Pretreatment with diazepam (25 mg/kg, orally) or chlordiazepoxide (50 mg/kg, orally) results in a significant increase in pentobarbitone-induced sleeping time when intervals between benzodiazepines and barbiturate treatment were 1, 2 and 4 h. The results in Table 2 show also that the pretreatment caused non appreciable change in brain pentobarbitone levels measured 90 min after its injection.

Experiments made with higher doses of diazepam or chlordiazepoxide were not conclusive because of the increased toxicity of the combined treatments.

Table 2. *Effect of diazepam and chlordiazepoxide on pentobarbitone brain level and sleeping time in rats*

No. of rats	Treatment	mg/kg oral	Hours between treatment and pentobarbitone	Sleeping time min ± s.e.	Pentobarbitone concentration μg/g brain ± s.e.
15	Saline ..	—	—	42 ± 4	15·25 ± 1
6	Diazepam ..	25	1	65 ± 5	15·66 ± 2 8
8	Diazepam ..	25	2	68 ± 5*	13·19 ± 1 3
6	Diazepam ..	25	4	72 ± 10*	15·8 ± 1 3
5	Chlordiazepoxide	50	2	80 ± 3*	16·3 ± 1 5
5	Chlordiazepoxide	50	4	84 ± 1*	18·6 ± 0 9

* $P < 0.01$.
Pentobarbitone concentration in brain was measured 90 min after its administration (30 mg/kg i.p.).

Effect of repeated treatments on enzyme activity in vitro. The results in Table 3 indicate that when treatments with diazepam or chlordiazepoxide were repeated for at least 3 days, 24 h after the last treatment, the activity of liver enzymes present in the 9,000 *g* supernatant fraction is significantly increased.

Diazepam and chlordiazepoxide show a similar type of activity.

Table 3. *Effect of repeated treatments with diazepam or chlordiazepoxide on liver metabolic activity of other compounds*

Treatment	mg/kg oral	p-Nitrophenol nmol ± s.e.	4-Aminoantipyrine nmol ± s.e.	p-Aminophenol nmol ± s.e.
Saline	—	(13) 320 ± 30	(8) 40 ± 7	(8) 500 ± 30
Diazepam	50	(13) 370 ± 40	(8) 60 ± 7*	(8) 680 ± 70*
Diazepam	100	(9) 510 ± 30*	(9) 120 ± 10*	(9) 860 ± 80*
Diazepam	10 × 3†	(5) 410 ± 50	(5) 80 ± 10*	(5) 720 ± 100*
Chlordiazepoxide	100	(13) 630 ± 30*	(10) 100 ± 9*	(10) 890 ± 80*

Treatments were repeated on 3 consecutive days and determinations were made 24 h after the last administration.
Enzyme activity is indicated as nmol of metabolites formed per g liver per h.
Figures in brackets refer to the number of experiments.
* $P < 0.01$ in respect to saline.
† Diazepam was given three times a day at 8 a.m., 2 p.m. and 8 p.m.

Effect of repeated pretreatment on pentobarbitone response. The results in Table 4 indicate that repeated treatment with diazepam or chlordiazepoxide reduced the sleeping time to pentobarbitone when it was given intraperitoneally 24 h after the final dose of the benzodiazepines. The corresponding pentobarbitone concentrations in brain measured 90 min after its administration, were significantly lower in benzodiazepine-pretreated rats than in controls.

Table 4. *Effect of repeated treatments with diazepam or chlordiazepoxide on brain pentobarbitone level and sleeping time in rats*

No. of rats	Treatment	mg/kg oral	No. of days	Pentobarbitone effect Sleeping rats (%)	Pentobarbitone effect Sleeping time (min ± s.e.)	Pentobarbitone concentration μg/g brain ± s.e.
24	Saline	—	–	100	60 ± 2	15·2 ± 0·5
8	Diazepam	25	1	100	68 ± 5	14 ± 0·4
16	Diazepam	50	1	100	48 ± 3	15 ± 0·6
5	Diazepam	6·25	3	100	47 ± 4	16·9 ± 0·5
10	Diazepam	12·5	3	90	49 ± 4	12·9 ± 1·3
10	Diazepam	25	3	100	38 ± 5†	15 ± 1·3
15	Diazepam	50	3	94	43 ± 2*	12·2 ± 0·9†
14	Diazepam	100	3	72	31 ± 4*	12 ± 0·7†
10	Chlordiazepoxide	12·5	3	90	29 ± 2*	14·3 ± 1·5
10	Chlordiazepoxide	25	3	80	32 ± 4*	10·6 ± 1·2†
5	Chlordiazepoxide	50	3	80	29 ± 7†	8·4 ± 1·8*
16	Chlordiazepoxide	100	3	69	30 ± 3*	7·7 ± 1·0*

Pentobarbitone (30 mg/kg i.p.) was given 24 h after the last treatment and 90 min before the determination of brain levels.
P versus saline: * <0.01.
† < 0.05.

DISCUSSION

Measurements of *in vitro* liver enzymatic activity after acute pretreatment with diazepam or chlordiazepoxide show no significant difference from the controls. Similar results were obtained when the levels of brain pentobarbitone were measured *in vivo* although the sleeping time was increased. This evidence suggests that the two benzodiazepines should not be considered to be inhibitors of liver microsomal enzymes in rats, probably also because they are rapidly metabolized (Zbinden & Randall, 1967; Marcucci, Guaitani & others, 1968).

Increased activity of liver microsomal metabolizing enzymes was demonstrated after administration of diazepam and chlordiazepoxide in experiments *in vivo* and

in vitro. Our results show that diazepam's "inducing" activity is similar to that exerted by chlordiazepoxide. It should be remembered, however, that diazepam is more potent than chlordiazepoxide (Zbinden & Randall, 1967) and that the doses active in our experiments exceeded the minimal doses exerting specific pharmacodynamic responses (Zbinden & Randall, 1967; Marcucci & others, 1968).

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Effect of nialamide and methyldopa on the analgesic action of morphine in rats and mice

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Methyldopa and nialamide enhance the analgesic action of morphine when administered separately in the rat. However, the association of both drugs in the same animal diminishes the effect of the analgesic. Results observed in mice differ from those found in rats, whereas methyldopa induces a slight increase in morphine analgesic effect, nialamide is ineffective. The administration of both drugs in combination increases morphine effect in a way similar to that obtained when methyldopa and morphine are administered together.

Compounds that induce a lowering of catecholamine levels in the central nervous system generally produce a decrease of the analgesic effect of morphine (Schneider, 1954; Sigg, Caprio & Schneider, 1958; Witkin, Maggio & others 1960; Medaković & Banić, 1964; Takagi, Takashima & Kimura, 1964). This antagonistic action is not shared by methyldopa. On the contrary, methyldopa induces a synergistic action when injected either a few or 24 h before the analgesic (Contreras & Tamayo, 1966; Contreras, Quijada & Tamayo, 1967). Since monoamine oxidase inhibitors cause an increase in brain monoamines, an examination of the influence of nialamide on the synergistic action of methyldopa was thought of interest.

EXPERIMENTAL

Groups of 8 adult male rats weighing between 180–230 g were employed in every experiment. Analgesia was tested by the method of Macht & Macht (1940) as modified in our laboratory (Contreras & Tamayo, 1966). Results are expressed as the mean area calculated according to Winter & Flataker (1950).

In adult male mice, weighing 25–30 g, the analgesia was assessed by the method of Woolfe & MacDonald (1944). Mice were placed on a hot plate heated at $54° \pm 0.5°$ at 30 min intervals. The reaction time was measured from the moment the animal was placed on the plate until it reacted by licking its fore paws or by a sudden jump.

In rats the statistical significance was assessed by the *t*-test. In mice an ED50 of morphine was calculated by the usual method of probit analysis, counting as affected those animals in which post-injection reaction time exceeded the upper confidence limit $(P, 0.001)$ of the initial reaction time. The χ^2 test was applied to the results.

Solutions in distilled water were prepared in concentrations such that each rat received a maximum of 0·4 ml/100 g. For mice the solutions were prepared for a maximum of 1 ml/100 g. All drugs were injected intraperitoneally. The drugs employed were α-methyldopa, nialamide and morphine. The scheme of their administration is described in results.

RESULTS

Effect of methyldopa and nialamide either alone or in combination on the analgesic action of morphine in rats (Table 1). The administration of methyldopa and nialamide

Table 1. *Effect of methyldopa and nialamide on the reaction threshold and on the analgesic action of morphine in rats*

Drug, mg/kg	Time before analgesic test	Area ± s.e.*
Saline		40 ± 24
Morphine, 10	30 min	480 ± 52
Methyldopa, 125	24 h	78 ± 43
Nialamide, 100	5 h	28 ± 25
Methyldopa, 125	24 h	
Nialamide, 100	5 h	65 ± 29
Methyldopa, 125	24 h	
Nialamide, 100	19 h	72 ± 26
Methyldopa, 125	24 h	
Morphine, 10	30 min	1340 ± 80†
Nialamide, 100	5 h	
Morphine, 10	30 min	744 ± 81‡
Nialamide, 100	19 h	
Morphine, 10	30 min	732 ± 98‡
Methyldopa, 125	24 h	
Nialamide, 100	5 h	
Morphine, 10	30 min	65 ± 45§
Methyldopa, 125	24 h	
Nialamide, 100	19 h	
Morphine, 10	30 min	406 ± 62§

* Analgesic effect estimated by the electrical stimulation method.
† Significantly increased from morphine alone ($P < 0.001$).
‡ Significantly increased from morphine alone ($P < 0.02$).
§ Significantly decreased from morphine plus methyldopa ($P < 0.001$).

to control animals did not significantly alter the reaction threshold in rats. A synergistic action was observed in rats treated with the monoamine oxidase inhibitor or when methyldopa was administered on the morphine analgesic action. However, when both nialamide and methyldopa were administered to the same animals the effect of morphine was partially or totally counteracted. The antagonism was more evident in groups receiving the injection of nialamide 5 h before the analgesic.

Effect of methyldopa and nialamide either alone or in combination on the analgesic action of morphine in mice (Table 2). An ED50 of morphine was injected in every case. In contrast to that observed in rats, no additional analgesic activity was obtained at 30 and 60 min controls in animals treated with methyldopa, but a slight increment was observed at the 90 min control ($P < 0.05$).

Effects induced by methyldopa on morphine analgesia did not change by the concomitant administration of nialamide. The administration of the monoamine oxidase inhibitor alone did not significantly modify the effect of the alkaloid.

Although not shown in Table 2, methyldopa (500 mg/kg) by itself increased the reaction time when measured at 4 to 6 h after its administration.

DISCUSSION

The effect of the decarboxylase inhibitor, methyldopa, on the analgesic action of morphine in rats shows some peculiarities.

The methyldopa by itself increases the reaction threshold 4 to 6 h after its administration, but if a 24 h period is allowed to elapse the reaction threshold is unaltered and the effect of morphine is markedly increased. This synergistic effect is not consistent with the fact that those drugs which produce a depletion of catecholamines also induce an antagonism of morphine analgesia. Furthermore, the antagonism by reserpine of morphine analgesia is prevented by methyldopa (Contreras & Tamayo, 1966).

Table 2. *Effect of methyldopa and nialamide on the analgesic action of morphine in mice*

Drug, mg/kg	Time before analgesic test	N*	% of mice showing analgesia Time (min)		
			30	60	90
Saline		60	0·0	0·0	0·0
Morphine, 3	30 min	61	49·1	34·4	4·9
Methyldopa, 125	24 h				
Morphine, 3	30 min	21	57·1	38·0	19·0†
Methyldopa, 500	24 h				
Morphine, 3	30 min	20	50·0	45·0	20·0†
Methyldopa, 500	72 h				
Morphine, 3	30 min	21	52·3	23·8	4·7
Nialamide, 100	5 h				
Morphine, 3	30 min	23	65·2	47·8	17·3
Methyldopa, 125	24 h				
Nialamide, 100	5 h				
Morphine, 3	30 min	22	54·5	50·0	31·0‡
Methyldopa, 500	24 h				
Nialamide, 100	5 h				
Morphine, 3	30 min	20§	44·4	50·0	27·7‡

* Number of mice.
† Statistically significant difference from morphine alone. $P < 0.05$.
‡ Statistically significant difference from morphine alone $P < 0.01$.
§ Two animals died.

The successive administration of methyldopa, nialamide and morphine also produced unexpected results. What might have been expected, at most, was a similar or greater effect than that obtained when morphine was injected after either methyldopa or nialamide alone. The possibilities that could account for the reduction of the analgesic effect could be: (a) catecholamine liberation being in part responsible for morphine analgesia, (b) the synergistic action of methyldopa being exerted through the accumulation of methylcatecholamines resulting from its biotransformation, and (c) the monoamine oxidase inhibitor opposing the liberation of methyl derivatives. This last explanation is offered only for methylcatecholamines present in the central nervous system, since nialamide in a single dose does not antagonize the action of morphine alone in rats. Nevertheless, chronic treatment with monoamine oxidase inhibitors induces a reduction of morphine effect (Timsit, 1965) which could be due to a minor liberation of catecholamines by morphine in that experimental situation.

The different mechanisms implicated in the responses to thermal stimulation by mice and electrical stimulus by rats might account for the dissimilar results observed although a different sensitivity to the drugs might also exist.

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LETTERS TO THE EDITOR

Resistance to tetrodotoxin in toad sympathetic nerves

During an investigation of the innervation of the gut of the toad (*Bufo marinus*), it was found that the excitatory responses of the isolated small intestine to splanchnic nerve stimulation were resistant to blockade by a wide variety of autonomic blocking drugs, including the local anaesthetic drugs procaine and cinchocaine. To check whether this response was due to a direct spread of the stimulating current to the muscle, the drug tetrodotoxin was applied. Tetrodotoxin is known to prevent the specific sodium conductance change during the action potential in nerve fibres (Narahashi, Moore & Scott, 1964; Nakamura, Nakajima & Grundfest, 1965; Kao, 1966; Takata, Moore & others, 1966), and on the other hand it has only minimal effects on action potentials in vertebrate smooth muscles (Bülbring & Tomita, 1966; Kuriyama, Osa & Toida, 1966). Because of these properties it has been possible to use tetrodotoxin to give an effective "denervation" of vertebrate smooth muscle preparations (Gershon, 1966, 1967; Bülbring & Tomita, 1966; Bell, 1968), a concentration of 5×10^{-7} g/ml sufficing to do this. However, in the present experiments it was found that tetrodotoxin in doses of up to 5×10^{-6} g/ml failed to cause any reduction of the contraction of the toad intestine caused by splanchnic nerve stimulation (Fig. 1). On the other hand, the response of the intestine was abolished by cutting the splanchnic nerves between the stimulating electrodes and the muscle.

TTX
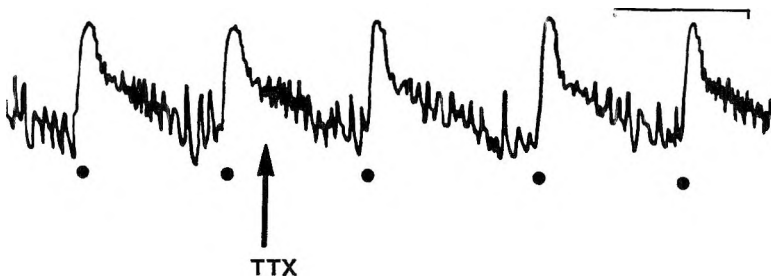
FIG. 1. Responses of isolated toad duodenum to splanchnic nerve stimulation. Tetrodotoxin (10^{-6} g/ml), added to the bath at the arrow, does not affect the responses. Stimuli (10/s, 1 ms for 30 s) at black dots. Time marker = 5 min.

These results strongly suggest that the fibres of the toad splanchnic nerve are resistant to tetrodotoxin. To investigate this further, conventional extracellular recordings were made of action potentials from the isolated splanchnic nerve, using silver wire electrodes; simultaneously, sciatic nerve action potentials were recorded in the same bath. The stimulus was adjusted to elicit an action potential peak propagating at 35 m/s (A-fibres) in the sciatic nerve and at 5·5 m/s (B-fibres) in the splanchnic nerve (Fig. 2A). Splanchnic B-fibre action potentials were studied because Bishop & O'Leary (1938) suggested that the B-fibres mediate excitatory responses of the intestine in the bullfrog. The application of tetrodotoxin (10^{-7} g/ml) rapidly abolished the A-fibre spike of the sciatic nerve, but the B-fibre spike of the splanchnic was not affected by tetrodotoxin, even when the concentration was raised to 5×10^{-6} g/ml (Fig. 2B). Thus conduction in the splanchnic B-fibres was found to be at least relatively resistant to tetrodotoxin.
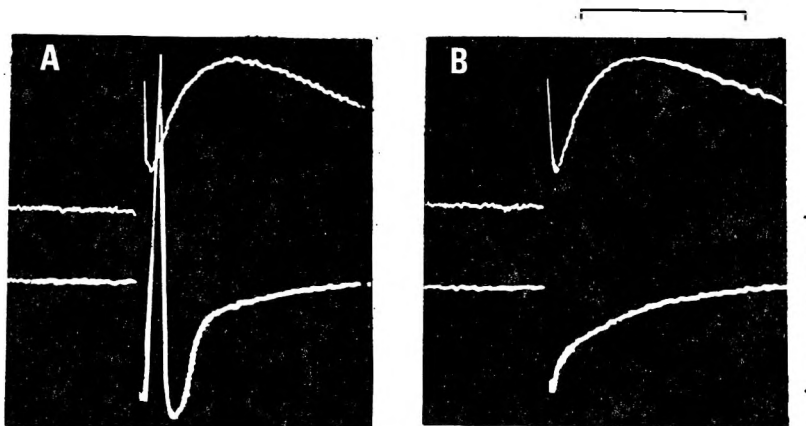
FIG. 2. Extracellular action potentials recorded from toad sciatic and splanchnic nerves. Normal action potentials shown in (A). In (B), 15 min after the addition of tetrodotoxin (5×10^{-6} g/ml), the splanchnic B-fibre action potential (upper trace) is unaffected, but the sciatic A-fibre action potential (lower trace) is abolished. Vertical calibration = 5 mV, horizontal calibration = ms.

The relative resistance of the splanchnic B-fibres to tetrodotoxin may indicate that calcium ions are carrying at least part of the inward current of the action potential. Koketsu & Nishi (1968) have reported that calcium action potentials can be sustained by frog sympathetic ganglion cells, and that these action potentials persist in the presence of tetrodotoxin. This would be consistent with the lack of effect of procaine on the intestinal responses to splanchnic nerve stimulation reported here, since this drug does not act on the specific calcium conductance mechanism of the action potential in barnacle giant muscle fibres (Hagiwara & Nakajima, 1966). But whatever the reason for the resistance, it is clear that tetrodotoxin cannot be used blindly to achieve chemical "denervation" of vertebrate smooth muscle. It must first be shown that the drug does in fact prevent the initiation of action potentials in the nerves supplying the organ under study.

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Non-spherical emulsion particles

In recent years two explanations have been proposed to account for the occurrence of non-spherical disperse phase particles in oil-in-water emulsions stabilized by an ionic emulsifier combined with a long-chain alcohol. Such emulsions are usually prepared by dissolving the emulsifying agents at 60–70° in the phase in which they are soluble, and mixing whilst hot. When such a mixture is stirred until cold, a stable emulsion results. It has been proposed that emulsions of liquid paraffin in water stabilized by cetrimide and cetostearyl alcohol contain non-spherical particles due to localized close-packing of the particles during preparation of the emulsion (Groves & Scarlett, 1965, Groves & Freshwater, 1967). It was considered that this would lead to deformation of the particles which would be retained on cooling, due to the rigidity of the interfacial film. Where the cetostearyl alcohol content was high, it was thought that these polyhedral emulsion particles were formed by solidification of the disperse phase.

An alternative explanation is that the alcohol is deposited as discrete crystals in the oil phase as the emulsion cools, and that these crystals deform the particles (Axon, 1957, Barry, 1968a, b, Talman & Rowan, 1968). This phenomenon has been observed with emulsions stabilized by cetyl alcohol and sodium lauryl sulphate, and cetostearyl alcohol and cetrimide. The extent of crystallization has been reported to be affected by the diluent used in mounting the emulsion for microscopy (Barry, 1968a).

Schulman & Cockbain (1940) have reported non-spherical particles in water-in-oil emulsions, prepared using emulsifiers such as calcium oleate, calcium elaidate, heptadecylamine in the presence of sodium sulphate, and digitonin with cholesterol. Except for the last, all the above emulsions were prepared by phase inversion. Those containing calcium oleate or elaidate were first formulated as oil-in-water emulsions stabilized by the sodium soap, and inverted by addition of a calcium salt solution. The resulting calcium soap acted as a water-in-oil emulsifier. It was proposed that addition of the calcium salt solution would allow the oil particles to flocculate and coalesce, entrapping continuous phase as discrete irregular 'sacks', which would retain their shape due to the rigidity of the interfacial film.

Several water-in-oil emulsions have been examined in this laboratory, including Oily Cream B.P., White Liniment B.P.C., dispersions of distilled water in liquid paraffin stabilized with sorbitan esters, and also formulations similar to those of Schulman & Cockbain, containing calcium oleate or elaidate. The emulsions stabilized by the calcium soaps were prepared by inversion, and also directly, that is, by dispersing the calcium oleate or elaidate in liquid paraffin at 50°, adding distilled water at 50° and stirring vigorously until cold.

Each emulsion was examined microscopically after dilution with liquid paraffin. When the dilution was mounted, it was found that disperse phase particles adhered to the slide and coverslip, and in the process became non-spherical. When the microscope was focused into the bulk of the liquid, spherical particles were seen. The method of preparation of emulsions stabilized by calcium soaps was found not to influence the phenomenon of adhesion and wetting.

When the slides and coverslips were siliconized to prevent wetting, using a 2% v/v solution of dimethyldichlorosilane in carbon tetrachloride (Repelcote, Hopkin and Williams Ltd.), the disperse phase particles were spherical in every case.

Typical results are shown in Fig. 1a and b, which are photomicrographs of a directly prepared water-in-oil emulsion stabilized with calcium elaidate. The preparation was diluted as described, and photographed at a magnification of 600×, using a Wild M20 microscope fitted with a phase contrast optical system. Deformation of the particles on the non-siliconized slide is plainly visible, whereas particles on the siliconized slide are spherical.

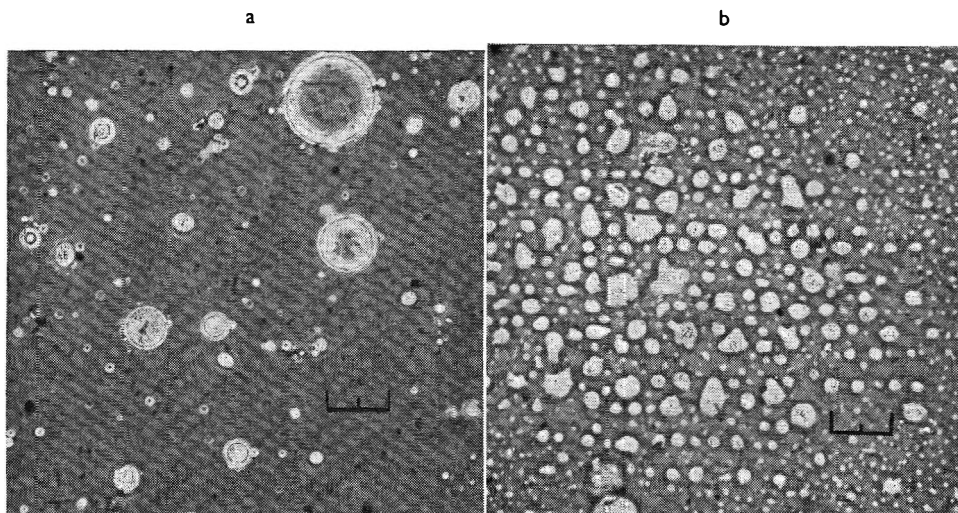a   b

FIG. 1. Photomicrographs, in phase contrast, of a directly prepared water-in-oil emulsion stabilized with calcium elaidate. (a) Siliconized slide; (b) Non-siliconized slide. One division = 10 μm.

We propose, therefore, that when non-spherical globules are seen in a microscopic mount of a sample selected from the bulk of a freshly prepared water-in-oil emulsion, this is probably due to adhesion of the particles to the microscope slide and coverslip. This causes rupture of the interfacial film, with consequent wetting of the glass surfaces by the water. This adhesion and wetting is thought to be due to the slightly polar nature of the glass, which is a preferable environment for the water than the non-polar continuous phase. This phenomenon could affect the stability of water-in-oil emulsions stored in glass containers, as it would tend to promote coalescence of the disperse phase at the container surface. In special circumstances, for example when the emulsion has been aged, or subjected to large stresses such as those imposed during transportation or large range temperature cycling, it is possible that some globules may deform in the bulk of the emulsion. If the emulsifier forms a solid, condensed film (particularly if this is a multilayer), then these particles may be stabilized in the deformed state.

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On the cumulation of digitoxin and digitoxigenin in isolated heart muscle preparations

In recent investigations it could be demonstrated that after presaturation of isolated atria with $[^3H]$-labelled cardenolides the total tissue concentration of these drugs declines very slowly during wash-out (Kuschinsky, Lüllmann & Zwieten, 1968a,b). The positive inotropic action of these drugs, however, disappears far more rapidly on wash-out than might be expected from the decline in tissue concentration (Lüllmann, Weber & Zwieten, 1969). It seemed to us of interest to know whether in isolated heart muscle preparations cardenolides showed cumulative effects when, after wash-out of an initial effect, the same drug was applied a second time in the same concentration. The second dose was administered after wash-out in such manner that approximately half of the initial tissue concentration was left, although at that moment the positive inotropic action had already disappeared. Although digitoxin is known to accumulate *in vivo* as a result of slow renal elimination, its cumulative effect in isolated heart muscle of mammalian species has not yet been studied (Kuschinsky, 1968). Isolated atria, dissected from guinea-pigs of either sex (weight 250–350 g) (Hoditz & Lüllmann, 1964) were suspended in oxygenated Tyrode solution, containing 1·2 m-equiv Ca^{2+}/litre. The volume of the organ bath was 20 ml, its temperature was kept at 30°. The atria were stimulated electrically by means of rectangular pulses (duration 3 ms, frequency 180/min). The contractions were recorded continuously via a strain gauge and a Hellige type 86HE-t recorder. Digitoxin or digitoxigenin were injected into the organ bath after 30 min of equilibration. The final concentration was 1×10^{-7} g/ml for both drugs (i.e. digitoxin $1·3 \times 10^{-7}$M and digitoxigenin $2·7 \times 10^{-7}$M). The increase in contractile force was established and expressed as percentage of the initial value. The maximal effect was reached about 15–30 min after drug administration. Subsequently, 60 min after application of the cardenolide the medium was rapidly replaced by cardenolide-free Tyrode solution. After a wash-out period of 30 min, a second dose of digitoxin or digitoxigenin was administered in such manner that the same final bath concentration was reached as after the first application. Again, the inotropic effect was determined and compared with the effect of the first dose. In separate control experiments, the effects of digitoxin and its aglycone were studied in atria which had been equilibrated for 120 min, i.e. the same period as required until in the first series of organs the second amount was administered. These control experiments were designed to establish whether after a longer period of equilibration (120 instead of 30 min) the inotropic effect of the cardenolides would be different, since the contractile force of isolated atria usually decreases upon prolonged incubation.
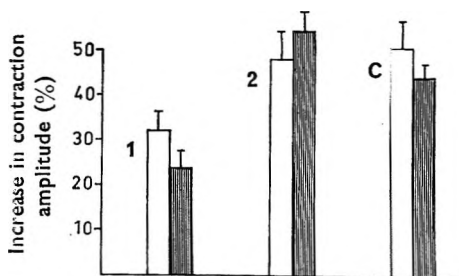

FIG. 1. Positive inotropic effect of digitoxin (open columns) and digitoxigenin (hatched columns) after 30 min of equilibration (first application) and after wash-out of the first dose for 30 min (second application). C = effects of both cardenolides (1×10^{-7} g/ml) after 120 min of equilibration (control experiments).

The results of our experiments are shown in Fig. 1. After an equilibration period of 30 min digitoxin and its genin caused positive inotropic effects of 32·3 ± 3·8% (n = 23) and 24·0 ± 3·8% (n = 13), respectively (mean ±s.e.). After the second application of digitoxin and its genin in the same organ after wash-out as described above, the contraction amplitude increased by 48·3 ± 6% (n = 17) and 54·3 ± 4·5% (n = 11) respectively. For both drugs, these effects are significantly larger than those of the first dose (P <0·01, Student's t-test). If, however, the first amount of digitoxin or its genin was administered after an equilibration period of 120 min, as in the control experiments, the positive inotropic effects were 50·6 ± 5·7% (n = 14) and 48·3 ± 6·0% (n = 17). For both drugs this increase in contractile force was significantly higher (P <0·01) than that observed after an equilibration period of 30 min. On the other hand, no difference existed (P >0·05) between the effect of a second application (120 min after the beginning of the experiments) and that of the cardenolides administered after 120 min without a first amount, as in the control experiments. Obviously, the mere fact that the equilibration period has been extended from 30 until 120 min causes an increased inotropic response to digitoxin or its aglycone. Therefore, no cumulative effect occurs if two subsequent doses separated by an interval of 30 min of wash-out are applied. The increased response to the second application is only due to the prolonged incubation of the isolated organ. As would be expected, the contraction amplitude was lower after 120 min of incubation than after 30 min. Accordingly, the relative increase will be larger after 120 min than after 30 min of incubation. Concomitantly, there exists no evidence whatsoever for a cumulative effect of either digitoxin or its aglycone in isolated heart muscle under the given experimental conditions. After wash-out for 30 min the tissue concentration of digitoxin is still about 60% of the initial value, whereas for digitoxigenin 53% is left in the isolated atrial tissue (Kuschinsky & others, 1968a,b). In spite of the quite considerable concentration of cardenolide left in the tissue, the newly added amount of either drug did not give rise to an increased pharmacological effect.

These results are in accordance with those of Lullmann & others (1969) which demonstrated that the positive inotropic effect of digitoxin disappeared after 15–25 min of wash-out, whereas for digitoxigenin only 5 min were required. Obviously, no cumulative effect occurs in isolated atria on wash-out because the positive inotropic effect of the cardenolides declines far more rapidly than the total tissue concentration of the drug. The present experiments once more confirm that most of the digitoxin (or its aglycone) accumulated by heart muscle is bound in a non-specific manner, and is of no importance to the inotropic effect.

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Potentiation of the actions of pentobarbitone by nikethamide

The analeptic drug nikethamide is still used as a respiratory stimulant in intoxication with central depressants (Goodman & Gilman, 1965). We have observed that nikethamide does not antagonize the action of pentobarbitone in mice, but in fact significantly prolongs the pentobarbitone-induced sleeping time.

Male mice (15–25 g) were injected with sodium pentobarbitone (45 mg/kg, i.p.). Sleeping time was measured as the time between loss and return of righting reflex. In a second group of animals, nikethamide (25 mg/kg) was injected intravenously 10 min after the barbiturate. Sleeping times are recorded in Table 1. Nikethamide significantly prolonged the sleeping time. There are two possible mechanisms for the potentiation of the action of sodium pentobarbitone by nikethamide. The analeptic or its metabolites could act as a depressant in mice, or nikethamide could inhibit the metabolism of the barbiturate.

The former proposition does not seem likely since nikethamide produced no signs of depression when injected into mice of the same strain, rather it increased spontaneous movement. To test the second possibility, mice were pretreated with SKF 525A (diethylaminoethyl diphenylpropylacetate). This drug inhibits microsomal enzymes which metabolize barbiturate drugs (Brodie, 1956). Mice pretreated with 3 mg/kg SKF 525A slept five times longer than animals given pentobarbitone alone. The dose of sodium pentobarbitone was reduced in order to induce a sleeping time approximately equivalent to that of the previous experiments. The results of this experiment are also recorded in Table 1. Nikethamide significantly reduced sleeping time when microsomal enzymes were inhibited. If a single dose of nikethamide was injected into normal mice 24 h before an experiment, sleeping time was shortened, this was presumably due to the known action of nikethamide in causing enzyme induction (Kato & Chiesara, 1962).

Table 1. *Prolongation of pentobarbitone sleeping time by nikethamide in mice untreated and treated with SKF 525 A (3 mg/kg orally)*

Treatment	Sleeping time to pentobarbitone 45 mg/kg, i.p. alone	Sleeping time to pentobarbitone 30 mg/kg, i.p. after SKF 525 A
Saline 0·1 ml/10 g	12·75 ± 2·2 (10)*	13·0 ± 1·7 (10)*
Nikethamide 25 mg/kg	22·6 ± 2·4 (10)	2·32 ± 0·54 (10)
	(*P* = 0·02)	(*P* = 0·001)
Saline 0·1 ml/10 g	10·8 ± 1·0 (10)	21·9 ± 2·5 (10)
Nikethamide 25 mg/kg	17·6 ± 2·1 (10)	4·0 ± 0·08 (10)
	(*P* = 0·02)	(*P* = 0·001)

* No. of observations.

We believe these experiments demonstrate that nikethamide prolongs barbiturate sleeping time by inhibition of those microsomal enzymes that in normal conditions metabolise barbiturates. In the presence of SKF 525 A nikethamide produces its well known stimulant action and shortens barbiturate sleeping time.

These experiments bear out that observations of Kato, Chiesara & Vassanelli (1963) that nikethamide inhibits microsomal enzymes *in vitro*, and also strongly suggest that nikethamide is contraindicated in cases of respiratory depression due to barbiturate intoxication.

It is of note that textbooks of pharmacology still report that nikethamide is used for barbiturate induced respiratory failure and commercial literature still recommends this drug as a stimulant in overdosage with sedative drugs. We suggest that in cases of barbiturate overdosage nikethamide may well delay recovery.

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Inhibition of nucleic acid polymerases
by salicylate *in vitro*

The incorporation of labelled amino-acids into the proteins of rat isolated diaphragm and of microsomal preparations from rat liver is inhibited by salicylate in concentrations of 0·3 mM and above (Dawkins, Gould & Smith, 1966). The initiation and maintenance of protein synthesis requires RNA and DNA and we have therefore studied the effects of salicylate on the activities of nucleic acid polymerases prepared from rat liver.

Liver nuclei were isolated from male rats (300–450 g) of the Wistar strain by the method of Widnell & Tata (1964) with the following modifications. A 10% (w/v) homogenate was centrifuged at 700 g for 10 min. The pellet was suspended in 5 vol of 2·4 M sucrose containing 1 mM MgCl$_2$, centrifuged at 50,000 g for 1 h and the purified nuclear pellet washed twice in 0·25 M sucrose containing 1 mM MgCl$_2$, before final suspension. RNA polymerase activity was estimated at 17°, to minimize interference from ribonuclease, by measuring the incorporation of radioactivity from ATP-α^{32}P into RNA in a Beckman LS 200B liquid scintillation system, using glass fibre discs. The RNA product from the mixtures incubated at 17° and 37° had a DNA-like base composition [(G + C)/(A + U) = 0·75]. DNA polymerase was purified up to the ammonium sulphate fractionation stage (Mantsavinos, 1964) and desalted by dialysis. Calf thymus DNA was used as a primer and the activity measured by the incorporation of radioactivity from dATP- ^{3}H into DNA.

Salicylate, in concentrations of 3 mM and above, significantly decreased the incorporation of radioactive ATP into RNA in the rat liver preparation (Table 1). DNA polymerase activity is inhibited by salicylate concentrations of 1 mM and above (Table 2).

These preliminary observations suggest that salicylates may interfere with the biosynthesis of nucleic acids and hence of proteins. A further implication of the present results is that an inhibitory action of salicylates on nucleic acid biosynthesis may be one of the factors concerned in the teratogenic effects of the drugs. It has been reported that the injection of the drugs in pregnant rats and mice not only produces premature birth and foetal death (Eriksson & Larsson, 1968) but also several congenital malformations of the litters carried to full term (Warkany & Takacs, 1959; Larsson, Bostrom & Ericson, 1963).

Table 1. *Effect of salicylate on the nuclear* RNA *polymerase*

Salicylate (mM)	No. of expts	pmol nucleotide incorporated per mg DNA	P
0	8	2001 ± 288	
0·3	7	1807 ± 153	0·2
0·6	7	1717 ± 259	0·1
1·0	8	1699 ± 273	0·05
2·0	6	1692 ± 141	0·05
3·0	7	1638 ± 112	0·005
6·0	8	1613 ± 199	0·005
10·0	6	1503 ± 243	0·005
15·0	7	1355 ± 254	0·001
20·0	7	1212 ± 267	0·001

Incubation mixtures contained in a total volume of 0·5 ml; GTP, CTP and UTP, 0·5 μmol each; ATP α-^{32}P, 0·5 μmol (specific activity 2·5 μCi/μmol); MnCl$_2$, 2 μmol; saturated (NH$_4$)$_2$SO$_4$, pH 7·5, 0·05 ml; Tris-HCl Buffer, pH 7·5, 50 μmol; 0·1 ml of nuclear preparation in 0·25M Sucrose containing 200 μg of DNA. Incubation is for 1 h at 17°; the reaction stopped by the addition of 3 ml of cold N HClO$_4$ containing 0·02 M Na$_4$P$_2$O$_7$. After standing at 0° for 30 min, the precipitate was collected by centrifugation, washed twice with 0·2 N HClO$_4$ containing 0·02 M Na$_4$P$_2$O$_7$, once with cold ethanol and twice with cold ethanol : ether mixture (3 : 1). RNA was extracted according to the directions of Widnell & Tata (1964). The results, given as means and standard deviations, have been analysed by the Students t-test; the minimal acceptable level of significance being taken as $P = 0·005$.

Table 2. *Effect of salicylate on the incorporation of* dATP-^{3}H *into* DNA *in vitro.*

Salicylate (mM)	No. of expts	Specific activity	P
0	5	63·2 ± 9·7	
0·3	5	57·4 ± 3·2	0·3
0·6	5	59·5 ± 8·7	0·6
1·0	5	41·6 ± 4·4	0·005
2·0	5	38·3 ± 5·5	0·001
3·0	5	33·6 ± 3·9	0·001
6·0	5	27·7 ± 9·8	0·001
10·0	5	25·5 ± 7·5	0·001
15·0	5	24·2 ± 5·8	0·001
20·0	5	18·5 ± 1·7	0·001

Incubation mixture contained in a total volume of 0·5 ml: 50 nmol of dCTP, dGTP and TTP; dATP-^{3}H, 5 nmol (specific activity 70 μCi/μmol), 4·0 μmol, MgCl$_2$; 0·5 μmol,2-mercaptoethanol; 50 μg calf-thymus DNA; 50 μmol, Tris-HCl buffer; pH 8·0, sodium salicylate, 0–10 μmol, 0·1 ml enzyme solution containing 2·95 mg/ml of protein. The specific activities, given as means and standard deviations, are expressed as pmol of dAMP-^{3}H incorporated into DNA per mg of enzyme.

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Evidence for a cholinergic mechanism in brain involved in the tetrabenazine reversal by thymoleptic drugs

The antagonism of the reserpine-like syndrome elicited in rats by tetrabenazine and other synthetic benzoquinolizines is used as a screening procedure for imipramine-like tricyclic antidepressants. The interest has been particularly focused on the antagonism of the reserpine-like behavioural depression (sedation, catalepsy) and its conversion into a "compulsive" locomotor hyperactivity (Bickel & Brodie, 1964; Matussek & Rüther, 1965; Sulser & Soroko, 1965; Sulser, Owens & others, 1968).

A similarity seems to exist between all the behavioural features shown by rats treated with thymoleptic agents of the imipramine group and tetrabenazine and rats treated with a single large dose of reserpine. In previous papers from this laboratory a "paradoxical" stereotyped behaviour consisting of locomotion, sniffing and rearing has been described in reserpinized rats (Schiørring & Randrup, 1968; Scheel-Krüger & Randrup, 1968). This behaviour, which occurred in several rats as periodic bursts of hyperactivity about 4–6½ h after reserpine, has been shown to be dependent on a cholinergic mechanism in brain (Scheel-Krüger & Randrup, 1968; Scheel-Krüger, in preparation).

However, in contrast the behaviour shown by the rats receiving a thymoleptic and tetrabenazine was observed to be more pronounced and more continuous.

The present experiments were made to establish whether a cholinergic mechanism in brain had any influence on the compulsive locomotor activity provoked by thymoleptics and tetrabenazine.

Male Wistar rats, 225–275 g, were kept in individual cages (floor area 21 × 27 cm and height 16 cm). Drug injections were given subcutaneously. In preliminary experiments 14 rats received 20 mg/kg of desipramine 2 h before 100 mg/kg of tetrabenazine. Another 14 rats received 40 mg/kg of nortriptyline 1 h before 100 mg/kg of tetrabenazine.

Eleven rats from the desipramine-tetrabenazine treatment and another 11 rats from the nortriptyline-tetrabenazine treatment showed characteristic compulsive activity, which began 1–3 h after the tetrabenazine injection in the desipramine group and 2–4 h after tetrabenazine in the nortriptyline group. The rearing (standing-up on hind-legs) occurred most often during the locomotion when the rat reached a corner of the cage.

Hyperactivity of this kind has been observed, in previous experiments for periods of more than 6 h at a time. When the activity had gone on for at least 1 h the rats were given 10 mg/kg of hyoscine. The locomotion was immediately continued when the rats were returned to the cages. However, from 5–10 min after hyoscine the locomotion, sniffing, rearing, and exophthalmus were completely inhibited for more than 1 h. The rats showed the characteristic hunched-back "bison" posture with ptosis. Catalepsy, tested for on a vertical wire netting, was present.

The inhibition was shown by all rats from the desipramine group (11/11) and all but one rat of the nortriptyline group (10/11). The 3 rats from each treatment group which did not show any reversal with hyperactivity even 3½ h after tetrabenazine, received 0·2 mg/kg physostigmine. From 5–15 min after this injection the catalepsy and ptosis were antagonized and all 6 rats began the compulsive locomotor activity with sniffing and rearing.

A quantitative evaluation of these behavioural activities was made in the following experiments (Table 1).

Table 1. *A cholinergic mechanism involved in the tetrabenazine-thymoleptic reversal of locomotion, sniffing and rearing.* All these rats from the desipramine-tetrabenazine groups showed continuous behavioural activities for at least 1 h before the injection of saline or hyoscine given $3\frac{1}{2}$ h after tetrabenazine. The rats from the imipramine-tetrabenazine groups did not show any behavioural activities before the injections of physostigmine or saline given $2\frac{1}{2}$ h after tetrabenazine. 5 min after the last drug injections (hyoscine, physostigmine or saline) the locomotion (L), sniffing (S) and rearing (R) of each rat were counted for the following hour. Maximal count is 30.

Desipramine-tetrabenazine						Imipramine-tetrabenazine					
Saline Behavioural counts for each rat			Hyoscine Behavioural counts for each rat			Saline Behavioural counts for each rat			Physostigmine Behavioural counts for each rat		
L	S	R	L	S	R	L	S	R	L	S	R
24	30	7	0	0	0	0	0	0	10	7	6
30	30	10	0	0	0	0	0	0	11	9	2
30	30	12	0	0	0	0	0	0	8	29	3
30	30	14	0	0	0	0	0	0	12	13	6
30	30	18	0	0	0	0	0	0	20	17	11
30	30	28	0	0	0	0	0	0	23	17	2
30	30	29	2	2	0	0	0	0	25	24	9
30	30	30	13	13	11	0	0	0	27	25	11
30	30	30	15	15	5	9	10	7	30	27	1
30	30	30	23	23	21	18	15	15	30	30	8
						30	30	30			
			* <0·01, <0·01, <0·01						* <0·05, <0·05, >0·05		
			† ≤0·01, ≤0·01, ≤0·005						† ≤0·005, ≤0·005, ≤0·005		

* Values of *P* for the effect of hyoscine and physostigmine on the behavioural activities compared with the saline controls (The Rank test, Snedecor, 1956).

† Values of *P* for the corresponding comparison of the number of rats giving responses (Fischer's exact probability test, Siegel, 1956).

Compulsive hyperactivity was provoked in the rats by desipramine (20 mg/kg) given 2 h before tetrabenazine (100 mg/kg). Hyoscine (10 mg/kg) was injected $3\frac{1}{2}$ h after tetrabenazine into 10 rats and another 10 rats received saline. Only rats previously showing continuous behavioural activities for at least 1 h before the injections of either hyoscine or saline were used.

Ten rats received imipramine (50 mg/kg) 2 h before tetrabenazine (100 mg/kg). Physostigmine (0·2 mg/kg) was injected $2\frac{1}{2}$ h after tetrabenazine. Eleven rats received the same drug treatment but received saline instead of physostigmine. The rats from this imipramine treatment did not show any behavioural activities during $2\frac{1}{2}$ h after the tetrabenazine injections. Preliminary experiments had shown that the reversal of the tetrabenazine sedation with imipramine was a very weak effect which only occurred in a few rats and usually about 4–5 h after tetrabenazine.

The locomotion, sniffing and rearing were counted by visual observation. After every 2 min period the presence of each of these behavioural features was recorded separately for each rat with 1 point for a positive response. The behavioural countings were made 5 min after the last drug injections (hyoscine, physostigmine or saline) and were continued for the following hour. The maximal count is thus 30 for each behavioural feature.

The results obtained for each of these 41 rats and comparisons of the drug treatments made by the Rank test (Snedecor, 1956) are given in Table 1.

The present experiments imply that a cholinergic mechanism in brain is involved in the tetrabenazine-reversal of locomotion, sniffing and rearing provoked by thymoleptic agents of the imipramine group. A close similarity thus seems to exist between this reversal and the "paradoxical" stereotyped behaviour shown by reserpinized rats. However, the metabolism of the catecholamines is probably implicated in the reversal of the tetrabenazine-sedation by thymoleptic agents into a locomotor hyperactivity since two conditions for the reversal have been previously considered necessary: first, a marked and rapid depletion of the catecholamine level in the brain (tetrabenazine action), and second, blockade of the amine-uptake mechanism at the level of the cell membrane (thymoleptic action) (Sulser, Bickel & Brodie, 1964; Matussek & Rüther, 1965; Sulser & Soroko, 1965; Sulser & others, 1968).

In addition, it seems to us that the possibility now exists that this reversal is closely connected with the depression of the central noradrenaline neurotransmission leaving a central cholinergic predominance. Thus, the reversal does not occur when the release rate of noradrenaline is greatest but when the level of noradrenaline seems most depressed (Sulser & others, 1964; Bartonicek, 1965; Sulser & Soroko, 1965; Matussek & Rüther, 1965; Häggendal, 1968).

The noradrenaline biosynthesis is strongly affected by reserpine, perhaps because reserpine (and tetrabenazine) inhibits the uptake of dopamine into the amine granules to which the enzyme dopamine-β-oxidase is bound (Dahlström, Fuxe & Hillarp, 1965; Kaufman & Friedman, 1965; Stjärne, 1966; Udenfriend, 1966; Rutledge & Weiner, 1967).

Further, the possibility seems to exist that thymoleptic drugs, by blocking the re-uptake of extraneuronal-released catecholamines, increase the depletion induced by reserpine or tetrabenazine of a functionally imporant amine pool. Carlsson & Waldeck (1965) and Hamberger & Malmfors (1967) reported the increased disappearance of [^{3}H]metaraminol and α-methylnoradrenaline by combined treatment with protriptyline and reserpine. High doses of thymoleptics decreased the endogenous levels of the catecholamines (Hamberger, 1967).

Carlsson, Fuxe & others (1966) found that both desipramine and protriptyline reduced the accumulation of noradrenaline after dopa in both central and peripheral noradrenaline neurons in rats pretreated with reserpine and nialamide.

A preliminary result of ours showed that a high dose of aceperone (10 mg/kg) which is an extremely potent noradrenaline antagonist with central effect (Janssen, Niemegeers & others, 1967) did not inhibit the compulsive locomotion, sniffing and rearing produced by desipramine and tetrabenazine (10 rats).

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A model for evaluation of thiazide-induced hypotension

The clinical efficacy of thiazide treatment alone or in combination with hypotensive drugs has long been recognized. Patients respond to chronic thiazide administration with a gradual decrease in blood pressure and a maximum response within 3–4 weeks after initial treatment. When the treatment is terminated an elevated pressure is resumed within 1–2 weeks. The hypotensive activity of the thiazides has been postulated to reside in the apparent decrease in body sodium and plasma volume (Dustan, Cummings & others, 1959; Friedman, Nakashima & Friedman, 1960). It is interesting to note, however, that thiazide treatment does not result in blood pressure lowering in rats with reno-vascular occlusive hypertension, which has been shown to be accompanied by a larger than normal amount of tissue sodium (Tobian & Coffee, 1964; Redleaf & Tobian, 1958). Hypertension induced by deoxycortone acetate and NaCl is also accompanied by an elevated tissue sodium (Tobian & Redleaf, 1957). I have made experiments to determine if production and maintenance of steroid and salt hypertension can be inhibited with chronic thiazide administration. These results have been compared with the clinical situation.

Hypertension was produced in two groups (A and B) of weanling (21 days) Sprague-Dawley rats by implanting two 25 mg pellets of deoxycortone acetate subcutaneously in the dorsal neck region. The animals were fed a Purina Laboratory diet containing 8% NaCl for the following 10 weeks and then regular Purina Laboratory diet was resumed. Animals in Group A received a placebo suspension subcutaneously for 16 weeks and animals in Group B a 10 mg/kg cyclothiazide suspension.

A sham operation was made on Group C and cyclothiazide was administered in an acacia suspension (10 mg/kg, s.c.) daily for the following 16 weeks. These animals were not fed the salt diet.

Group D was made hypertensive in a manner similar to A and B, however thiazide treatment (10 mg/kg, s.c.) was not started until 13 weeks after initial induction of hypertension. Thiazide administration was continued for 14 weeks and the animals were maintained throughout on the 8% NaCl diet.

Blood pressures were measured by an indirect method each week (Willard, Powell & Henderson, 1964). Where applicable Student's t-values were accepted as significant at $P < 0.05$.

As in previous experiments with this technique, hypertension developed after 3–6 weeks treatment with the steroid and salt (Fig. 1). The concomitant treatment with cyclothiazide did not alter the time for production or degree of hypertension. Since there is no difference in blood pressure between Groups A and B during the production of hypertension, it may be that the elevation in pressure is due to a "sodium load",

which the dose of cyclothiazide fails to reduce sufficiently.   When the animals were returned to normal diet (14 weeks) the blood pressure fell in both groups of rats, but the fall was greater in animals treated with cyclothiazide (Fig. 1).   At 16 weeks a significant difference (40 mm Hg, P <0·05) between Groups A and B to thiazide treatment became evident.
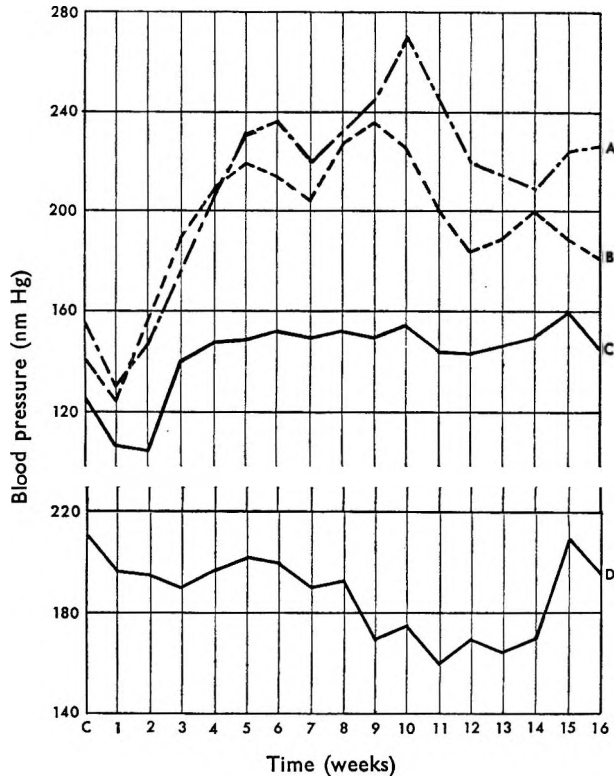

FIG. 1.   Deoxycortone acetate-hypertensive rats in Groups A and B.   Normotensive animals in Group C.   Group A was the hypertensive control and Groups B and C received cyclothiazide 10 mg/kg daily for 16 weeks.
   Group D was composed of animals with established deoxycortone acetate hypertension and was treated with cyclothiazide for the first 14 weeks.

The delayed response in Groups A and B with cyclothiazide suggested that long-term administration of drug might be necessary to produce hypotension.   In other studies involving rats with reno-vascular occlusion (Tobian & Coffee, 1964; Tobian, Janacek & others, 1962; Renzi, Chart & Gaunt, 1959), the thiazide was probably administered for too short a duration to produce blood pressure lowering.   Group D had been hypertensive for 13 weeks when the thiazide treatment was started.   The 14 week treatment resulted in a gradual fall in blood pressure (Fig. 1).   After 14 weeks when the treatment was stopped the blood pressure resumed the original hypertensive level.   The contrast between the slow hypotensive response and relative fast recovery of hypertension corresponds to results obtained clinically.   Since the animals were maintained continuously on 8% NaCl during the entire 16 weeks, the factor affected by cyclothiazide, possibly body sodium, is slow to respond.   However, orthodox knowledge implies that the decrease in sodium and in expanded extracellular fluid should change within a few days after the start of thiazide treatment.

It is known that normotensive animals respond to thiazide treatment with NaCl excretion and diuresis (Renzi & others, 1959). However, the thiazide-treated normotensive animals in Group C did not have a blood pressure lower than has been observed in previous studies which employed the indirect technique for blood pressure measurement (Willard & others, 1964). Thiazide treatment also has little blood pressure lowering activity in normotensive patients.

These experiments demonstrate a similarity between clinical results and the animal model.

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