REVIEW ARTICLE

THE MODE OF ACTION OF PENICILLIN

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Although penicillin has been used successfully in clinical medicine for some 20 years its precise mode of action has until recently been ill understood. Now, progress can be reported.

Previous reviews on its mode of action have been published by Rinderknecht (1946), Pratt and Dufrenoy (1947) and Eagle and Saz (1955), and Stenlake (1959) has recently summarised the actions of various antibiotics. Because one of the main effects of penicillin appears to be concerned with an inhibition of bacterial cell wall synthesis, the greater part of this review will deal with cell wall structure and the effect thereon of the antibiotic.

After this review was completed, a review on the mucopeptide components of bacterial cell walls was published by Work (1961) who described briefly the effect of penicillin on cell wall synthesis.

Morphological Changes Induced by Penicillin

Gardner (1940) reported that, in dilute solutions, penicillin induced a distinct lengthening in all the rod-shaped bacteria which were sensitive to the antibiotic. In a later report, Gardner (1945) extended his work to a microscopical examination of the effect of penicillin on the spores and vegetative cells of bacilli, and found that even the weakest inhibitory dose of penicillin attacked the organism in the early stages of germination. It was also shown that lytic changes in vegetative cells of *Bacillus anthracis* were less pronounced in a strong than in a weak penicillin solution.

Thomas and Levine (1945) demonstrated that penicillin in inhibitory but not completely bacteriostatic concentrations induced bizarre involution forms in Gram-negative intestinal bacteria growing in liquid or solid media. Fisher (1946) showed that the *in vitro* activity of penicillin on staphylococci caused enlargement of the bacterial cells followed by lysis. Similar effects were observed to a lesser degree on cultures of β-haemolytic streptococci and pneumococci. Fisher also made the interesting observation that the group A streptococcus was not killed in the same manner, since there was no evidence of debris to suggest that many of the bacteria had been lysed, although cultures were almost sterile after 10 hr. and completely so after 24 hr.

Duguid (1946) showed that low concentrations of penicillin induced giant forms in *Escherichia coli* and Hughes, Kramer and Fleming (1946) described the morphological changes induced by penicillin in *Proteus vulgaris*; these included (*a*) elongation up to 200 μ in length, and (*b*) production of single or multiple swellings on the rods. Often, completely spherical, actively motile forms of 6 μ or less could be observed on microscopical examination.

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The changes occurred most rapidly at 37°, and with higher concentrations of penicillin.

Later, Pulvertaft (1952) showed that at critical concentrations, penicillin, streptomycin, aureomycin, terramycin and chloramphenicol induced bacteriolysis of *E. coli* but stated that penicillin alone permitted bacterial enlargement at all concentrations followed by lysis. Pulvertaft (1952) also observed that if growth occurred after the pencillin had been neutralised, the first organisms formed were always giant forms. This is in the pattern of events found by Parker and Marsh (1946) and Eagle and Musselman (1949), who showed that the effect of the antibiotic persisted for some time after its removal.

Hughes (1955a, b; 1956) investigated the effect of penicillin on the morphology of *P. vulgaris* and demonstrated that even when the culture was derived from a single cell, variations were apparent between the individual cells; for example, some developed into long forms, some lysed, and some were relatively unaffected.

The morphological changes induced in bacterial cells by antibiotics have also been studied by Kamijo (1953a, b; 1954a, b, c, d) and by Takahashi, Sukeyuki and Kamijo (1957). The antibiotics used were penicillin, streptomycin, chloramphenicol, terramycin and erythromycin.

Liska (1959) has recently reported that penicillin causes swelling and elongation of *Streptococcus lactis*, *Streptococcus thermophilus* and *Leuconostoc dextranicum.*

Penicillin and Bacterial Growth

It was early realised that penicillin was active against dividing bacteria. Thus Hobby, Meyer and Chaffee (1942a) reported that, depending on the experimental conditions, penicillin acted as a bacteriostatic or bactericidal agent, and that it appeared to be effective only when active bacterial multiplication was taking place. This was confirmed by Chain and Duthie (1945), Hobby and others, (1942a), Lee, Foley and Epstein (1944), and Bigger (1944) offered evidence to show that penicillin exerted no bactericidal action in nutrient broth at temperatures sufficiently low to prevent the bacteria from multiplying.

Todd (1945a) showed that the most rapid bacteriolysis induced by penicillin occurred with organisms at the maximal rate of multiplication, and Knox (1945) found that young cells were particularly sensitive to the antibiotic, which was confirmed by Pratt and Dufrenoy (1947). Conditions which increased the rate of growth of haemolytic streptococci were found to increase the rate at which penicillin acted on these bacteria (Hobby and Dawson, 1944a, b), and Hahn and Ciak (1957) found that penicillin-induced lysis, as determined by "protoplast" formation, occurred only in an invironment capable of supporting bacterial growth. Pandalai and George (1947) suggested that penicillin appeared to be primarily bacteriostatic, inhibiting the growth of the organism by preventing cell division and multiplication. Duguid (1946) stated that only actively metabolising and growing cells were susceptible to penicillin and suggested that in low concentrations the antibiotic interfered with the formation of the outer

supporting wall; he also stated, however, that in high concentrations the antibiotic must act differently.

Some disagreement with the above was expressed by Schwartzman (1945), who found that the susceptibility of certain strains of *E. coli* and *Salmonella sp.* was significantly greater in synthetic medium than in meat infusion broth. Differences in penicillin activity were not caused by changes in the rate of growth in the media used.

Although penicillin is generally thought to be effective only against actively multiplying cells, it is by no means correct to state that it is without effect on resting organisms, for Garrod (1945) found penicillin to have a marked bactericidal effect on staphylococci at 10° and even at 4°, although no active cell division had occurred. Eriksen (1946) made a similar observation on the anthrax bacillus at 4°.

Garrod (1945) also showed that disinfection of *Staph. aureus* (Oxford strain H) was more rapid at 42° than at 37°, although growth at the higher temperature had ceased. With their strain of staphylococcus, however, Lee and Foley (1945) found that although the organism grew more slowly at 42° than at 37°, there was no significant difference between the rates of killing by penicillin at the two temperatures. They further found that at 50–60° growth ceased altogether and the bacteria began to die through the effect of heat, but even at these high temperatures the rate of reduction of viable cells in tubes containing penicillin was higher than that in control tubes.

Lee and others (1944), showed that high concentrations of penicillin (88 units/ml.) accelerated the death rate of staphylococci suspended in saline, and a similar result was obtained by Garrod (1945) with Ringer's solution. It must be pointed out here that Chain, Florey, Abraham and Heatley (1949) have drawn attention to the fact that Lee and others (1944) used a crude preparation (150 units/mg.) and that it was possible that the bactericidal effect demonstrated in saline was directly or indirectly caused by an impurity. It has been mentioned earlier, however, that Lee and others (1944) found that staphylococci in broth at 14° were unaffected by penicillin. If this had contained impurities, a similar bactericidal effect would have been expected to occur here.

Gunnison, Kunischige, Coleman and Jawetz (1955) investigated the effect of antibiotics *in vitro* on bacteria which were not actively multiplying, and showed that penicillin had some bactericidal effect at 37° on washed suspensions of *Staph. aureus* and *E. coli*, although high concentrations of antibiotic (500 units/ml.) were needed.

Penicillin and Lysis

Hobby, Meyer and Chaffee (1942b) found that increasing the concentration of penicillin above a certain level did not significantly influence its rate of bactericidal effect. This was confirmed by Lee and others (1944). Eagle (1948) also showed that the rate at which organisms were killed by penicillin rose to a maximum as the concentration of the drug increased, but found that with some strains of *Staph. aureus*, *Streptococcus faecalis*, and Group B β-haemolytic streptococci the organisms were killed

much more slowly when the penicillin concentration increased beyond a certain level. Kirby (1945) had earlier noticed that many more viable cells could be recovered from a staphylococcal suspension treated with 100 units/ml of penicillin than from one treated with 0·1 unit/ml., and considered this phenomenon to be a consequence of the greater retardation of the initial growth rate brought about by the larger amount of the antibiotic. Similarly, lysis of vegetative cells and spores of *B. anthracis* was more complete in concentrations near the inhibitory level of, for example, 1 unit/ml. than in higher concentrations of, for example, 100 units/ml. (Gardner 1945). Commenting on these results, Chain and others (1949) have stated: "It would appear . . . that penicillin in high concentration may have a secondary effect in suppressing some phase of the activity of certain organisms which is essential for the bactericidal action of the drug."

Eagle (1953) suggested that the accumulation of a toxic intermediate was caused by low concentrations of penicillin, whilst higher concentrations produced a secondary effect which reduced the production, or increased the destruction, of this substance.

Todd (1945b) suggested that bacteria may first be killed by penicillin and then undergo lysis through the action of autolytic enzymes; Abraham and Duthie (1946) pointed out that most of the organisms in a culture may be killed before lysis begins, which was confirmed by Gale and Taylor (1947) and more recently by Hurwitz, Reiner and Landau (1958) who found that cells of *E. coli* showed a loss of viability well before osmotic fragility became apparent.

Bonét-Maury and Pérault (1945) found that when a suspension of staphylococci lysed with small concentrations of penicillin was incubated for a further period of time, survivors which had withstood the initial bactericidal effect of the antibiotic were able to divide. This was followed, however, by a secondary lysis which occurred after 24 to 48 hr. incubation. These results have been confirmed by Abraham and Duthie (1946).

Penicillin and Amino-acid Assimilation

Gram-positive organisms, unlike several gram-negative bacteria such as *E. coli* and *Klebsiella aerogenes*, are unable to synthesise amino-acids. Certain Gram-positive bacteria, for example, staphylococci or streptococci, have acquired a mechanism for the concentration of amino-acids which compensates for this loss of synthetic ability (Gale, 1952). They possess a cell wall or membrane which enables them to actively assimilate certain amino-acids and concentrate them in the cell before metabolism or concentration into protein. Basic amino-acids, like lysine, were found by Gale (1947) to be able to diffuse through the cell wall of these Gram-positive bacteria, whilst acidic amino-acids, such as glutamic acid and aspartic acid, were unable to penetrate the wall unless an exogenous energy source was also available.

It has recently been found that Gram-negative bacteria can also effect high concentration gradients of amino-acids (Cohen and Rickenberg, 1956); there is, however, a rapid equilibration between the internal and

external medium. The technique originally employed by Gale (1947) involved extensive washing of the cells before the estimation of the internal amino-acids, so that, as pointed out by Gale (1959), the internal amino-acids are rapidly lost to the external medium.

Gale and Taylor (1947) found that, shortly after its addition to the medium, penicillin prevented growing cultures of *Staph. aureus* from accumulating glutamic acid. It had no significant effect upon the accumulation of lysine within the cells. Penicillin, 50 units/ml., inhibited the assimilation of glutamic acid by washed suspensions by only 10 per cent. The results indicated that the prevention of glutamic acid assimilation preceded the failure of respiration and the onset of general lysis, and appeared to take place with or before loss of viability. It was suggested that penicillin either combined with, or produced a reorganisation of, the cell wall such that the assimilatory mechanism was blocked.

Hancock (1953) showed that the addition of M sucrose or M sodium chloride (but not glycerol) to the medium protected the cells from the inhibition of amino-acid transport by penicillin; the inhibitions which occurred in the absence of stabilising agent were thus secondary effects (Gale, 1960).

Schwartzman (1946) showed that the resistance of Gram-negative organisms to penicillin was increased by the presence of glutamic and aspartic acids, and Wyss (1951) suggested that Gram-negative bacteria were resistant to the antibiotic because they were able to synthesise their own supply of amino-acids. It was also suggested that Gram-positive bacteria became resistant to penicillin because mutants recovered the ability to synthesise their own amino-acids.

Bondi, Kornblum, and De St. Phalle (1954) investigated the amino-acid requirements of penicillinase-producing (PP) and non penicillinase-producing (NPP) strains of *Micrococcus pyogenes*. The only major difference was found to be an impairment of the ability of NPP strains to synthesise all the amino-acids required for growth. These results are in contrast to those of Gale and Rodwell (1949) who found that artificially-resistant strains showed marked differences in their amino-acid requirements from the parent strains. Cugurra and Savora (1958), however, found that a strain of *Staph. aureus* and the same strain after being made resistant to penicillin showed the same amino-acid composition.

Hotchkiss (1950) suggested that penicillin interfered with the bacterial synthesis of amino-acids, and Simmonds and Fruton (1950) stated that the bacteriostatic action of penicillin was due to interference in the incorporation of glycine into peptide.

Gale (1958, 1959) and Gale and Folkes (1953a, b, c) have shown that penicillin has an effect on protein synthesis only in concentrations much higher than those required to inhibit growth or cell wall synthesis. Hancock and Park (1958) have been able to confirm directly that penicillin inhibits the incorporation of amino-acids into the peptide portion of the wall substance but not into the protein fraction of the cells.

Mandelstam and Rogers (1959) have also found that the incorporation of glutamic acid, glycine and alanine could take place directly into the

cell wall substance of staphylococci, that this process could occur in the absence of protein synthesis, and that it was inhibited by penicillin, but not by chloroamphenicol.

The Uptake of Penicillin by Bacteria

Cooper and Rowley (1949) found that radioactive penicillin was taken up by bacteria in amounts which increased with penicillin concentration in the external environment, and showed that there was a direct relation between the sensitivity of an organism and the amount of penicillin attached to it. This uptake was greatly increased when growth occurred in the presence of penicillin, which was confirmed by Maas and Johnson (1949a). Eagle (1953), however, was unable to confirm this, and found that cell-free bacterial extracts were able to bind penicillin, and to approximately the same degree per unit weight as intact cells. Maas and Johnson (1949b) showed that yeast cells did not bind penicillin, nor did the antibiotic penetrate the cell wall.

These workers (1949a, b) suggested that the antibiotic was bound by a component which was present in the cells in extremely small amounts. This trace component was termed the penicillin-binding component (PBC) by Cooper (1956).

By shaking cells of *Staph. aureus* with small glass beads, Cooper, Rowley and Dawson (1949) obtained a cell wall fraction and a cytoplasmic fraction, and showed that the radioactive penicillin was concentrated in the cytoplasm of the cell of this organism.

PBC was found to occur in penicillin-sensitive strains, but resistant strains (previously selected as being non penicillinase-producing strains) were either without PBC or sheltered it from penicillin at low concentrations. This was not the experience of Maas and Johnson (1949a) but by increasing the penicillin concentration to equally effective (LD99·9) levels, Eagle (1954) found that the amount bound by naturally-occurring resistant strains was relatively constant despite wide variations in their sensitivity to penicillin.

Cooper (1955) found that penicillin was bound to a lipid-containing fraction close to the cell wall. Mitchell and Moyle (1951; 1956) showed that the cytoplasmic membrane was a complex lipoprotein, and it thus seems likely that the particles which bound penicillin were originally cytoplasmic membrane.

Maas and Johnson (1949a, b) found that the bulk of the penicillin was not excreted when renewed growth and multiplication occurred, but remained in the daughter cells. This could explain the reports (Parke and Marsh, 1946; Eagle and Musselman, 1949) that, after transfer to penicillin-free medium, the first cells formed were always abnormal.

Cell Wall Structure and Penicillin Action

Before present-day knowledge of the mode of action of penicillin is reviewed, it is necessary to describe in some detail the chemical constitution of the cell wall.

Bacteria possess a rigid cell wall external to the cytoplasmic (cell, protoplasmic) membrane and cytoplasm. This wall is responsible for the shape and morphological integrity of the bacterial cell.

Dawson (1949) showed that it was possible to isolate cell walls by shaking bacteria with small glass beads in a sonic oscillator. This method can also, of course, be used for studying intracellular enzymes (Hugo, 1954).

Salton and Horne (1951) used this method to prepare cell walls of *E. coli*, *Str. faecalis*, and *Salmonella pullorum*. By measuring the ultraviolet absorption spectra of walls of the first two organisms, they were able to show that only traces of nucleic acid or purine- or pyrimidine-containing compounds were present in pure wall preparations. In a short communication, Salton (1952a) showed that the cell walls of Gram-negative bacteria contained a far higher lipid content and a more complete range of amino-acids than those from Gram-positive organisms. Salton (1952b) subsequently showed that the cell wall of *Str. faecalis* was essentially a mucopolysaccharide. The sugar components of the polysaccharide were identified as glucose, galactose and rhamnose. Pentose was absent, which substantiated the earlier finding (Salton and Horne, 1951) that no significant amounts of nucleic acid were present in the cell wall. A hexosamine was identified as glucosamine, and the predominating amino-acids were found to be alanine, glutamic acid and lysine. It was later shown that the walls of Gram-negative bacteria contained far less hexosamine than Gram-positive cell walls (Salton, 1953).

Cummins and Harris (1955; 1956) prepared cell wall suspensions of various Gram-positive species by Mickle disruption, and found that a very high proportion of the amino-acid moiety of the cell wall could in each case be accounted for in terms of three or four of glycine, alanine, lysine, glutamic acid, aspartic acid and diaminopimelic acid (DAP). The last-named was first detected in the cell walls of various bacteria by Work (1951).

Work and Dewey (1953) made a systematic investigation of the distribution of DAP among micro-organisms, and showed it to be present in nearly all the bacteria examined. The most widely distributed form was found to be the mesoform (Hoare and Work, 1957) which was present in, for example, the cell walls of *E. coli* and *Rhodospirillum rubrum* (Salton, 1957), although the L-form may also be found, for example, in the-cell walls of *Clostridium welchii* (Salton, 1957).

Cummins and Harris (1956) pointed out that the cell walls of the Gram-positive bacteria which they examined contained either DAP or lysine as a major component but not both in similar quantities, which suggested that they had similar structural functions. DAP could be decarboxylated to lysine; it might thus have been expected that the DAP decarboxylase would be found in those cases in which lysine and not DAP was a major cell wall component, but no such simple relationship existed. Work (1959) showed that DAP was decarboxylated to lysine, and that DAP was a major, but not the only, source of lysine in *E. coli*.

A hitherto unknown hexosamine, first found in bacterial spores (Strange and Powell, 1954; Strange and Dark, 1956) was provisionally characterised

as 3-O-α-carboxyethylhexosamine by Strange (1956) and termed "muramic acid". This formula was confirmed by Kent (1957).

Glucosamine and muramic acid have been found to be universal constituents of cell walls of Gram-positive bacteria (Cummins, 1956), and have also been shown to be present in the walls of several Gram-negative organisms, for example, *E. coli* and *Salmonella gallinarum* (Salton, 1957). Galactosamine may sometimes be present, as in *Clostridium welchii* (Salton and Ghuysen, 1957).

Work (1957) has emphasised that the walls of Gram-positive bacteria possess a common basal structure in which alanine; glutamic acid, muramic acid and glucosamine, and frequently DAP, appear as monomeric building blocks.

TABLE I

THE CHEMICAL COMPOSITION OF BACTERIAL CELL WALLS

Constituent	Cell walls of	
	Gram-positive bacteria	Gram-negative bacteria
Lipid	Low, about 2 per cent	High, about 20 per cent
Amino-sugar	Usually high, about 15 per cent or more	Usually low, about 2–4 per cent
Polysaccharide	Usually higher in Gram-positive organisms	
Amino-acids	4–5 main ones, only	Almost a complete range
DAP	Found in all bacterial cell walls so far tested, except staphylococci and related species.	

Reviews concerning bacterial cell walls have been published by Salton (1956; 1959), Cummins (1956), Work (1957) and Zilliken (1960). Work (1961) has recently reviewed the literature pertaining to the chemistry of the mucopeptide components of cell walls.

A summary of the chemical constitution of the cell walls of Gram-positive and Gram-negative organisms is given in Table I.

Park and Johnson (1949) described the uptake of labile phosphate in penicillin-treated *Staphylococcus aureus*, and Park (1952a, b, c) succeeded in isolating three uridine nucleotides which accumulated. These contained uridine-5′-pyrophosphate linked to an unidentified *N*-acetylamino sugar and (i) a peptide of D-glutamic acid, L-lysine and alanine in ratio 1 : 1 : 3 (Fig. 1), or (ii) a peptide of L-alanine or (iii) no peptide.

These nucleotides were found to account for a considerable fraction of the total cellular phosphate. Hotchkiss (1950) suggested that penicillin interfered with the bacterial synthesis of protein from amino-acids, the process being blocked at such a point that peptide intermediates accumulated instead.

The amino-sugar was later identified as muramic acid and Park and Strominger (1957) showed it to be present in the nucleotide which accumulated in *Staph. aureus* treated with penicillin, and that the ratio of amino sugar: D-glutamic acid: L-lysine: alanine in the cell walls of this organism was 1 : 1 : 1 : 3, which was the same as that in the nucleotide. This suggested (Park and Strominger, 1957; Strominger, Park and Thompson, 1959) that the uridine-5′-pyrophosphate *N*-acetylmuramic acid peptide was a precursor of the bacterial cell wall, and that the mechanism of action and selective toxicity of penicillin were related to the inhibition of biosynthesis of the bacterial cell wall.

Strominger (1957a, b) has suggested that the inhibition of ribonucleic acid (RNA) synthesis by penicillin was due to the side-tracking and trapping of uridine by the Park nucleotides.

Strominger and Threnn (1959) showed that the alanine of the peptide portion of the nucleotide, and of the cell wall, was composed of 1/3 L-isomer and 2/3 D-isomer. Strominger and Ito (1959) inferred that, since incomplete peptides were also induced, the peptide portion was synthesised by stepwise addition of amino-acids, and succeeded in separating each of the enzymes catalysing the stepwise addition of (1) L-alanine, (2) D-glutamic acid, (3) L-lysine, and (4) D-alanine-D-alanine. Each of these reactions required adenine triphosphate and a divalent cation.

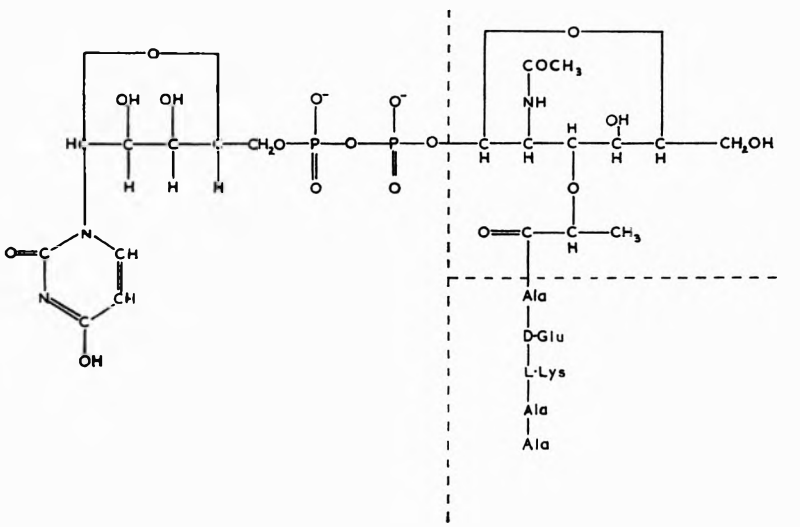

FIG. 1. 5′-Uridine pyrophosphate *N*-acetylmuramic acid peptide (Park nucleotide).

Nathensen and Strominger (1959) showed that pencillin inhibited DAP incorporation and cell wall synthesis in *E. coli* in a manner analogous to the inhibition of lysine incorporation and cell wall synthesis in *Staph. aureus*.

It has previously been stated that Cooper (1955) found that penicillin was bound at the cytoplasmic membrane. Thus, a hypothetical transglycosidase is strategically located to transfer the *N*-acetylamino sugar peptide from uridine pyrophosphate, which is inside the membrane, to an acceptor (cell wall site) outside the membrane (Park and Strominger, 1957).

Other antibacterial agents can also induce nucleotide accumulation. Thus, Gale and Folkes (1953b, c) found that bacitracin had essentially the same effects on *Staph. aureus* as penicillin, and Park (1958) reported that, in addition to penicillin, cycloserine, glycine and bacitracin caused marked accumulation of uridine 5′-pyrophosphate *N*-acetylamino sugar

derivatives in *Staph. aureus*. Chlortetracycline in high concentrations had a similar effect; in low concentrations, however, it inhibited protein synthesis. Accumulation of these nucleotides has also been shown by Abraham (cited in Park, 1958) for bacitracin, and by Strominger, Threnn and Scott (1959) for novobiocin, cycloserine and gentian violet.

Gentian violet was found (Strominger, Threnn and Nathensen, 1958) to produce a block at an earlier point in the metabolic sequence of biosynthesis of the cell wall than penicillin, inducing the accumulation of uridine nucleotides not containing amino-acids. Cytidine nucleotides have also been found to accumulate in a strain of *Staph. aureus* inhibited by this substance (Armstrong, Baddiley, Buchanan and Carss, 1958; Strominger, 1959).

Strominger, Scott and Threnn (1959) found that a DAP-requiring mutant of *E. coli* contained a high steady state concentration of a uridine nucleotide in which the peptide portion was represented as L-alanine-D-glutamic acid-meso-DAP-D-alanine-D-alanine. This compound was the analogue of (I). When the *E. coli* mutant was deprived of DAP, the DAP-containing nucleotide disappeared, and a uridine nucleotide identical to one accumulating in *Staph. aureus* deprived of lysine accumulated. The peptide portion of this was represented as L-alanine-D-glutamic acid only.

After Weibull's discovery (1953a, b) that lysozyme-induced protoplasts of *Bacillus megaterium* could be stabilised with 0·1–0·2M sucrose, came reports of "protoplast" formation induced by penicillin in *P. vulgaris* (Liebermeister and Kellenberger, 1956) in *E. coli* and *Salmonella typhimurium* (Lederberg, 1956; 1957), and in *E. coli* (Hahn and Ciak, 1957). These results were confirmed by McQuillen (1958a), and Hugo (1958) showed that the method was applicable to a wide range of Gram-negative bacteria.

Gebicki and James (1958; 1960) used this method to obtain spheres of *Aerobacter aerogenes* and Lark (1958a, b) showed that penicillin induced "globular forms" and "crescents" in *Alcaligenes faecalis*. Sphere formation has also been induced by penicillin in *Xanthomonas phaseoli* (Nozzolillo and Hochster, 1959).

Salton and Shafa (1958) carried out a chemical analysis of the "walls" of penicillin-induced spheres of *Salmonella gallinarum* and *Vibrio metchnikovii*, and showed them to contain the same amounts of lipid and polysaccharide (determined as reducing sugar) as did cell walls. On the other hand, there was a 30–50 per cent decrease of hexosamine and DAP in the sphere "walls" and McQuillen (1958a) found that penicillin-induced spheres of *E. coli* contained much less DAP in their trichloroacetic acid-precipitable fraction.

The use of the word protoplast for the spherical forms induced by penicillin was criticised by Brenner and others (1958) on the grounds that they probably had cell wall constituents attached to them (Salton and Shafa, 1958). Hurwitz and others (1958) have suggested that the term spheroplast be used to differentiate these spheres from the round forms (protoplasts) induced in Gram-positive bacteria by lysozyme. This term will be used hereafter.

Lark and Lark (1959) showed that the phenol-insoluble fraction of *Alcaligenes faecalis*, in which alanine, glutamic acid, lysine and DAP were present, was responsible for the rigidity of the cell wall of this organism. Weidel, Frank and Martin (1960) showed the cell wall of *E. coli* strain B to be composed of three layers (as had earlier been proposed

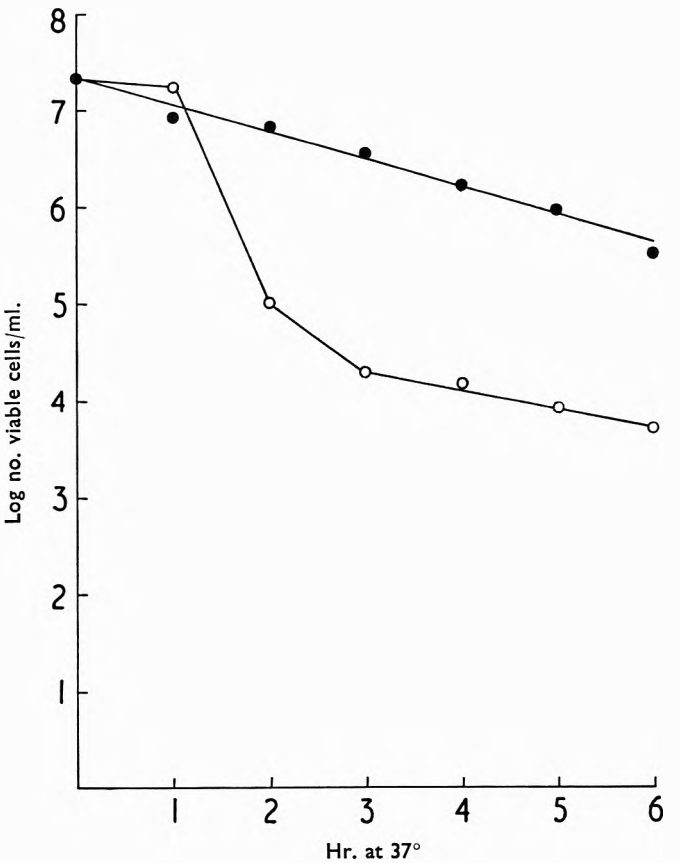

FIG. 2. Effect of incubation at 37° on the ability of spheroplasts of *E. coli* induced by 100 u/ml. penicillin to revert to the rod form.
●——● Colonies formed by spheroplasts.
○——○ Colonies formed by rods.

by Kellenberger and Ryter, 1958). These were: (i) Rigid (R) layer: innermost, rigid. (ii) Lipopolysaccharide layer: intermediate, soft. (iii) Lipoprotein layer: outermost, soft.

The R-layer was found to be composed of glutamic acid, alanine, DAP, glucosamine and muramic acid.

Thus, the accumulation of cell wall precursors (Park nucleotides), the decreased content of some constituents in the rigid layer of the cell wall and the morphological changes induced in bacteria can all be explained by the loss of integrity of the cell wall after the interruption of wall synthesis.

An interesting additional fact is that by growing certain bacterial species unable to synthesise DAP in media containing limiting amounts of this substance, morphological changes similar to those found with penicillin were observed (Meadow, Hoare and Work, 1957; McQuillen, 1958a, b).

Lederberg (1956) and Lederberg and St. Clair (1958) described the reversion of the penicillin-induced spheroplasts of *E. coli* to the rod form

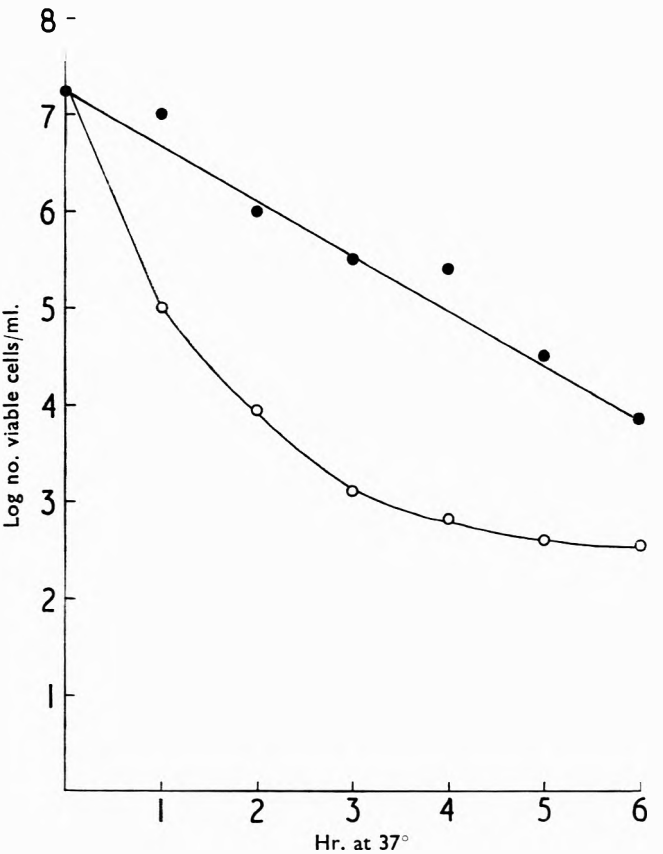

FIG. 3. Effect of incubation at 37° on the ability of spheroplasts of *E. coli* induced by 5000 u/ml. to revert to the rod form.

●——● Colonies formed by spheroplasts.
○——○ Colonies formed by rods.

on dilution into protective medium lacking penicillin, and Landman, Altenbern and Ginoza (1958) found that each intact spheroplast was capable of giving rise to either an L-form or a rod form. These observations on reversion to rods suggested that the spheroplasts were able to resynthesise a new rigid wall after removal of penicillin, an observation which had not been seen with lysozyme-induced protoplasts of Gram-positive bacteria.

Russell (1961) investigated the reconversion to rods of spheroplasts of *E. coli*, induced by 100 or 5,000 units/ml. penicillin, by diluting the

spheroplasts into penicillin-free protective media (A) or water (B) and plating into sucrose-Mg^{++} agar. Subtraction of colonies of B from those of A then gave the number of spheroplasts which were capable of engendering typical bacillary colonies. The results of such a determination (Figs. 2, 3) indicated that reversion of spheroplasts to rods was dependent on two factors; (i) penicillin concentration, and (ii) length of incubation.

Lederberg (1956, 1957) and Lederberg and St. Clair (1958) showed that there was a one-for-one conversion of rods into spheroplasts. Landman and others (1958), however, made the interesting point that only about 50 per cent of the original rods could survive as spheroplasts. By using synchronously-dividing cultures of *Alcaligenes faecalis*, Lark (1958b) showed that penicillin-induced crescent formation was dependent on the stage of growth in the synchronous cycle.

Hugo and Russell (1960a; 1961) investigated the effects of penicillin in hypertonic medium on *E. coli* and on an active penicillinase producer, *Cloaca cloacae*, and concluded that penicillin in high concentrations killed the cells by a mechanism other than that involving cell wall synthesis (as measured by spheroplast induction). In this connection, it is of interest to note that Smith, Payne and Watson (1960) were unable to induce spheroplasts by penicillin treatment in *Aerobacter* (*Cloaca*) *cloacae*. Spheroplasts were, however, induced by a modification of the lysozyme method of Zinder and Arndt (1956).

Hurwitz and others (1958) suggested that penicillin did not act solely by making the cells susceptible to its lytic action, and Prestidge and Pardee (1957) stated that the formation of spheroplasts could be objected to on the grounds that it was highly dependent on the external environment, and that the mechanism leading to sphere formation in hypertonic medium was not necessarily the same as that leading to death of the organism in a hypotonic medium. They further suggested that penicillin caused the formation of an enzyme which attacked the cell membrane and allowed the cell contents to escape.

Hugo and Russell (1960a; 1961) found that some bacteria were able to survive the action of high concentrations of penicillin, and showed that surviving cells of *E. coli* could not be classified as mutants. The presence of survivors was also shown by Landman and others (1958), but Hurwitz and others (1958) and Nozzolillo and Hochster (1959) reported the complete absence of persisting viable organisms, although the method adopted by the latter in this respect is open to criticism.

Spheroplast formation induced by penicillin has also been observed in Gram-positive bacteria, for example, in staphylococci (Murray, Francombe and Mayall, 1959) and in *Bacillus cereus* and *B. anthracis* (Foldes and Meretey, 1960).

It is of interest to note that penicillin-induced spheroplasts retain at least some of the biochemical capabilities of the rods from which they were derived (Sheinen and McQuillen, 1959; Russell, 1961).

Sedlaczek, Czerniawski and Zablocki (1958) found that the lipopolysaccharide-protein complex of *E. coli* had a specific protective effect on Gram-positive organisms against penicillin.

This leads to the concept that the two outer layers of the cell wall of *E. coli* prevent the antibiotic from reaching its site of action. In this context it is of interest to note the work of English, McBride and Huang (1960) who found that the resistance to penicillin of some bacteria was due to (*a*) penicillinase production or (*b*) penicillin acylase activity. A third group, however, were resistant to similar penicillin concentrations but possessed neither enzyme.

Baddiley and his colleagues (Baddiley, Buchanan, Carss, Mathias and Sanderson, 1956) isolated two nucleotides from *Lactobacillus arabinosus*. These were identified as cytidine diphosphate glycerol (Baddiley, Buchanan,

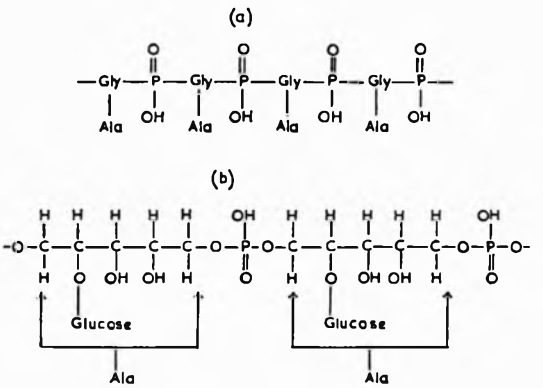

FIG. 4. (a) Teichoic acid containing glycerol
(b) Teichoic acid containing ribitol (the precise point of linkage of the alanine molecules is as yet undetermined).
Gly represents glycerol
Ala represents alanine.

Mathias and Sanderson, 1956) and cytidine diphosphate ribitol (Baddiley, Buchanan, Carss and Mathias, 1956; Baddiley, Buchanan and Carss, 1957). A compound containing ribitol phosphate was isolated from the cell wall of this organism (Baddiley, Buchanan and Greenberg, 1957) and ribitol phosphate was also shown to be present in large amounts in the cell walls of *B. subtilis* and *Staph. aureus* H, but not in *E. coli* and *Micrococcus lysodeikticus* (Baddiley, Buchanan and Carss, 1958).

Armstrong and others (1958) suggested the term "teichoic acids" (from the Greek "teichos" meaning "a wall") for these ribitol phosphate polymers.

It was found that cytidine diphosphate ribitol (but not cytidine diphosphate glycerol) accumulated in *Staph. aureus* treated with penicillin or crystal violet, which may again be thought of as an effect of penicillin in interfering with wall synthesis, and (this time) with teichoic acid production. Armstrong and others (1958) suggested that cytidine diphosphate glycerol was concerned in the synthesis of the protoplast membrane, since this has been shown (Mitchell and Moyle, 1956) to contain glycerophosphate.

In a recent investigation, however, Baddiley and Davison (1961) have shown that the cell walls of certain strains of lactobacilli contained a

glycerol teichoic acid, whilst a ribitol teichoic acid was found in the walls of other bacteria of this species.

Structure a (Fig. 4) is a compound containing glycerol and is the repeating unit of a teichoic acid from the walls of *B. subtilis* (Baddiley, 1960).

No mention has been made of the induction of L-forms by penicillin and other agents. The interested reader is referred to the recent review by Klieneberger-Nobel (1959).

Although the present review deals with the mode of action of penicillin, it is not out of place to mention the recent isolation of the penicillin "nucleus", 6-aminopenicillanic acid (6-APA) (Batchelor, Doyle, Nayler and Rolinson, 1959) from which it is hoped to prepare a wide range of semi-synthetic penicillins. It is of interest to note that both 6-APA and penicillins other than benzylpenicillin can also induce spheroplast formation in Gram-negative bacteria (Hugo and Russell, 1960b, c).

The many reported cytological changes of cell swelling, elongation, lysis, bacteriostasis and death, and the biochemical findings of the many workers in this complex field are, after twenty years of study, beginning to form a coherent pattern. The suppression of lysis by allowing penicillin to act on dividing cells in hypertonic medium was a vivid cytological demonstration of the action of the drug. Cell wall analyses and the detection of cell wall precursors in media in which penicillin was acting were of equal importance in the elucidation of the antibacterial mechanism.

As yet, the secondary action of penicillin has to be clearly elucidated, although possible mechanisms have been postulated.

In the field of structure-action relationships, it is of great interest that the parent amine, 6-aminopenicillanic acid, is itself capable of inducing effects on cell wall formation similar to those induced by benzylpenicillin.

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RESEARCH PAPERS

POLYMORPHISM OF CORTISONE ACETATE

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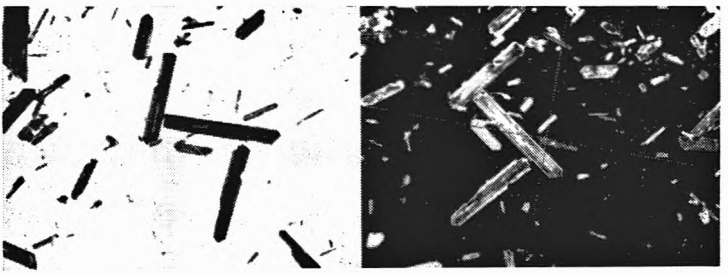
Evidence is presented that cortisone acetate occurs in at least five distinct crystalline forms. Four of these forms are unstable in presence of water and change to the stable Form I. Physical data, including spectroscopic and X-ray crystallographic constants, are recorded, and the application of these physical measurements to the recognition of the different forms, and the value of doing this with pharmaceutical preparations are discussed.

It has been reported that cortisone acetate may exist in different crystalline forms, but description of these in the scientific literature is scanty and incomplete. Accurate knowledge is important in the first place because infra-red absorption measurements on paraffin mulls or potassium halide disks prepared from crystalline cortisone acetate may be used for purposes of identification, and the curves obtained differ with the crystalline form. Garratt and Marshall (1954) deduced from their infra-red measurements that five different crystalline forms existed but their paper, which was a general one dealing with the application of infra-red spectroscopy to pharmaceutical analysis, did not give details of the characterisation of these forms. The second reason for the importance of distinguishing crystalline form finds expression in the patent literature that is concerned with processes of preparing stable, non-caking suspensions of cortisone acetate in a form suitable for intramuscular injection. The first patent application, U.S.P. (1954), B.P. (1951), claimed the discovery that three out of four, or possibly five, crystalline forms of cortisone acetate were unstable in presence of an aqueous medium and were converted into "Form 5" in the presence of water. Forms 1, 2, 3 and 5 were described and their X-ray diffraction patterns recorded. The second patent application, U.S.P. (1958), claimed the preparation of Forms A, B, and C, all, in contrast to Forms 1, 2, or 3, stable in aqueous vehicles. Forms B and C gave X-ray diffraction patterns similar to Forms 1 and 3, respectively. Form A, stable only in presence of water, differed crystallographically from any other form previously described. X-ray diffraction patterns for one form were also measured by Beher, Parsons and Baker (1955, 1958) but apparently in both these publications the figures for cortisone and for cortisone acetate were transposed. Apart from other considerations, the state of confusion of the crystallographic data appeared to us to justify a fresh examination, and it was hoped that a combination of spectroscopic and X-ray diffraction methods would provide a way of uniquely characterising and identifying these compounds.

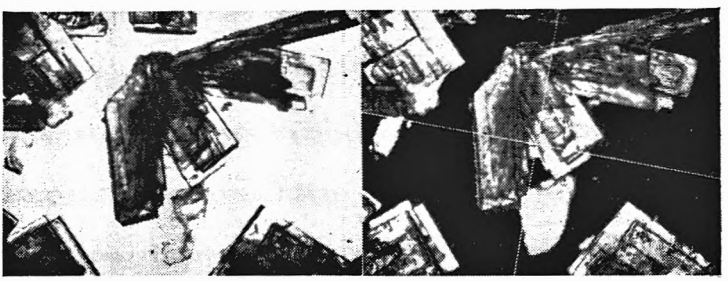
Methods

X-Ray Diffraction

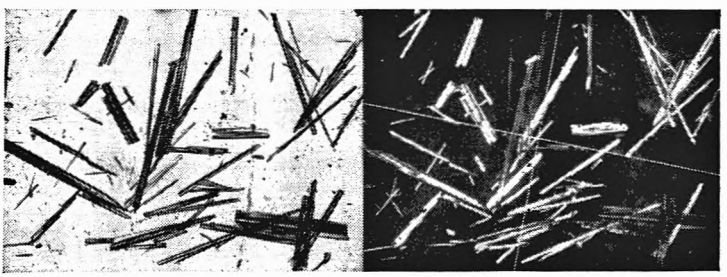
The X-ray diffraction patterns of single crystals were recorded using a Phillips X-ray tube with filtered copper radiation ($\lambda = 1{\cdot}5418$ Å) and



Roussel sample; Form I. (a) × 21



From chloroform; Form II. (b) × 5·75

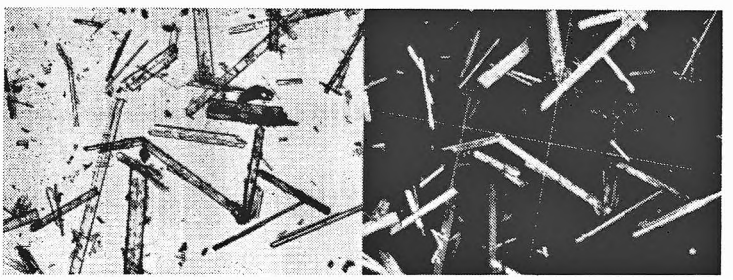


Needles from benzene; Form II. (c) × 9

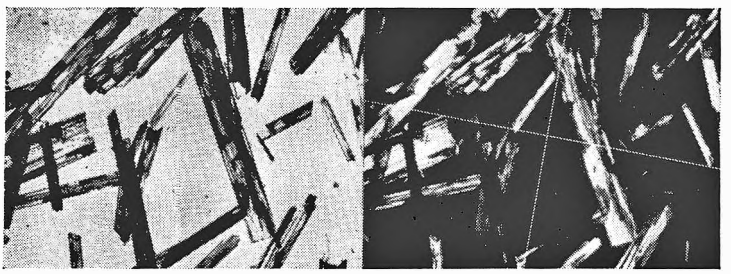
Prisms from benzene; Form III. (d) × 9

PLATE I. Photomicrographs of various forms of cortisone acetate by transmitted and polarised light. Photographs of cortisone (alcohol) crystals are included for comparison.

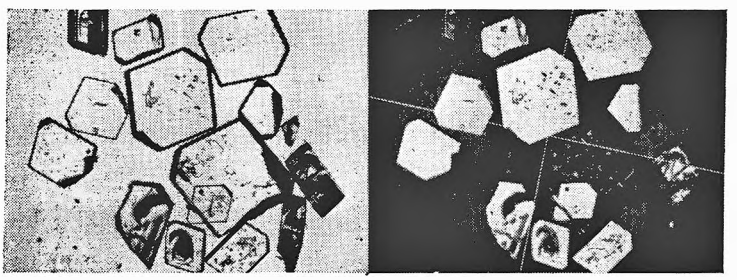
CORTISONE ACETATE—SOLVATED FORMS



From CCl_4/MeOH; Form IV. (e) × 18



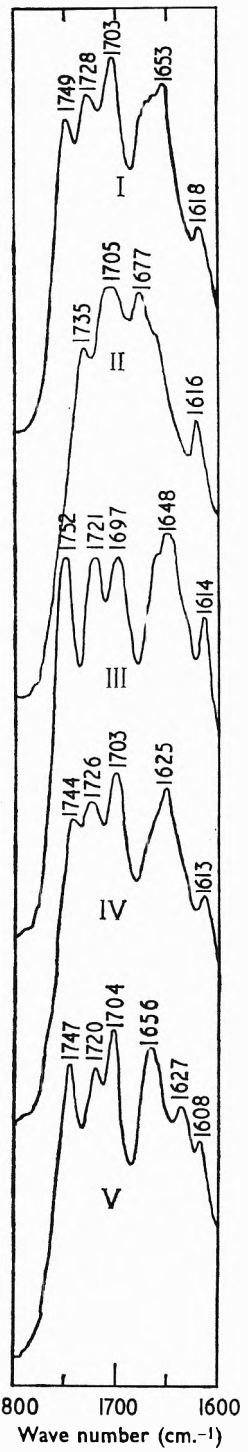
From ethanol; Form V. (f) × 9



Free cortisone × 5·75

conventional X-ray stationary and moving-film goniometers. The bulk-density of the crystals was measured in continuous gradient density columns (Low and Richards, 1952, and Linderstrom-Lang, 1937, 1938). The column containing the crystals was centrifuged and the density of the crystal layer subsequently measured by calibration with non-miscible liquids of known density. Using this method densities accurate to 0·002 g./ml. could be estimated. A combination of the X-ray and density measurements enabled the calculation of molecular weights to an accuracy of about 5 per cent.

The diffraction patterns of powdered samples were initially recorded on a 3 cm. X-ray camera. Subsequently the patterns were remeasured



more accurately from diffraction photographs taken with filtered chromium radiation on a Phillips 114·6 mm. powder-camera. Intensities from these photographs were estimated photometrically and we would like to thank Professor A. J. C. Wilson and his colleagues for all the powder-data quoted in this paper.

Melting Points

These were observed between crossed Polaroid plates in a Kofler apparatus.

Infra-red Absorption

These measurements were carried out on compressed disks of substance dispersed in potassium chloride. The material (1–2 mg.) was ground with dry potassium chloride (300 mg.) prepared by the method of Hales and Kynaston (1954) and the mixture compressed to a disk of 12·5 mm. diameter. The instrument used was a Perkin-Elmer double-beam Model 21, with a sodium chloride prism, recording on a wave-number scale. A check of wave-number calibration points, including the water-vapour peak at 1,700 cm.$^{-1}$, was made with each measurement.

Results

I. The Crystalline Forms of Cortisone Acetate

One crystalline form, I, was from a commercial preparation and was not encountered in our own experiments except as a microcrystalline material produced by alteration of the other forms. Four other distinct forms, II-V, were isolated by crystallising cortisone acetate from various solvents or solvent mixtures.

Form I

Source. The single crystals, m.p. 241–245°, used in the investigation were part of a sample given by Laboratoires Francais de Chimiotherapie (Roussel) to the Medical Research Council Steroid Reference Collection. This form is stable in the presence of water.

Optics. The crystals were in the form of spear-shaped opaque needles, elongated parallel to the [b] crystallographic axis. They extinguished

FIG. 1. Infra-red absorption curves of crystalline modifications (Forms I-V) of cortisone acetate in potassium chloride disks.

about 3° from this axis. A typical field of these crystals in both transmitted and polarised light is illustrated in Plate I, (a).

Crystallographic data. The crystals were monoclinic with a = 15·68 Å, b = 7·52 Å, c = 26·58 Å, $\beta = 97°$. The observed density was 1·25 g./ml. for the single crystals, which gave a calculated molecular weight of 387·98. Since the molecular weight of cortisone acetate, $C_{23}H_{30}O_6$, is 402·47, Form I is not a hydrated one. The only observed absences were $0k0$ when k is odd leading to a space group of $P2_1$. There are six molecules in the unit cell distributed in three crystallographically independent groups.

Infra-red absorption. The five peaks and the shoulder in the 1,800–1,600 cm.$^{-1}$ region are characteristic (see Fig. 1, Curve I). The hydroxyl band is at a high wave number (3430 cm.$^{-1}$) and there is no peak at about 850 cm.$^{-1}$ although in other respects the "finger-print" region below 1,500 cm.$^{-1}$ is practically indistinguishable from that of the other forms except as mentioned below in Form II.

Form II

Source. Cortisone acetate that had been crystallised from aqueous ethanol was dissolved in the minimum amount of boiling benzene (8 g. in 1 l.) and a little solvent was distilled to remove water. The solution, cooled overnight, deposited needles (Form II), m.p. 235–238°, and a few prisms (Form III), m.p. 251–253°. Evaporation of a chloroform solution of cortisone acetate to a concentration of about 20 to 25 per cent yielded large crystals m.p. 241–246°, which gave X-ray and infra-red absorption measurements identical with those of form II. The crystals were chunky, transparent prisms Plate I, (b) elongated parallel to the [a] axis. Extinction was inclined 7° to the edge.

Optics. The appearance of this form is illustrated in Plate I (b), (c). Though the square-ended needles that crystallised from benzene and the large flat plates from chloroform appeared superficially different, they gave identical infra-red and X-ray spectra. Both types of crystal were elongated parallel to the [b] axis and extinguished about 6° from the edge.

Crystallographic data. The crystals were orthorhombic a = 11·21 Å, b = 27·14 Å, c = 7·11 Å. The measured density of the single crystals was 1·21 g./ml. corresponding to four molecules in the unit cell. The density of the powdered samples extracted from various tablets was less homogeneous and extended between 1·20–1·21 g./ml. The calculated molecular weight was 393·01 and the space group $P2_12_12_1$ from absences.

Infra-red absorption. The curve in the 1,800–1,600 cm.$^{-1}$ region had a characteristic shape with four peaks (see Fig. 1, Curve II). In the "finger-print" region there is a prominent peak at 1,275 cm.$^{-1}$ slightly higher than the peak at 1,230 cm.$^{-1}$, much less marked in the absorption spectra of other forms. The hydroxyl peak is at 3,380 cm.$^{-1}$. As this form is the one constantly obtained by evaporation of a chloroform solution of cortisone acetate to dryness and is therefore the form into which any other can be transformed readily, it is considered worth while to reproduce the

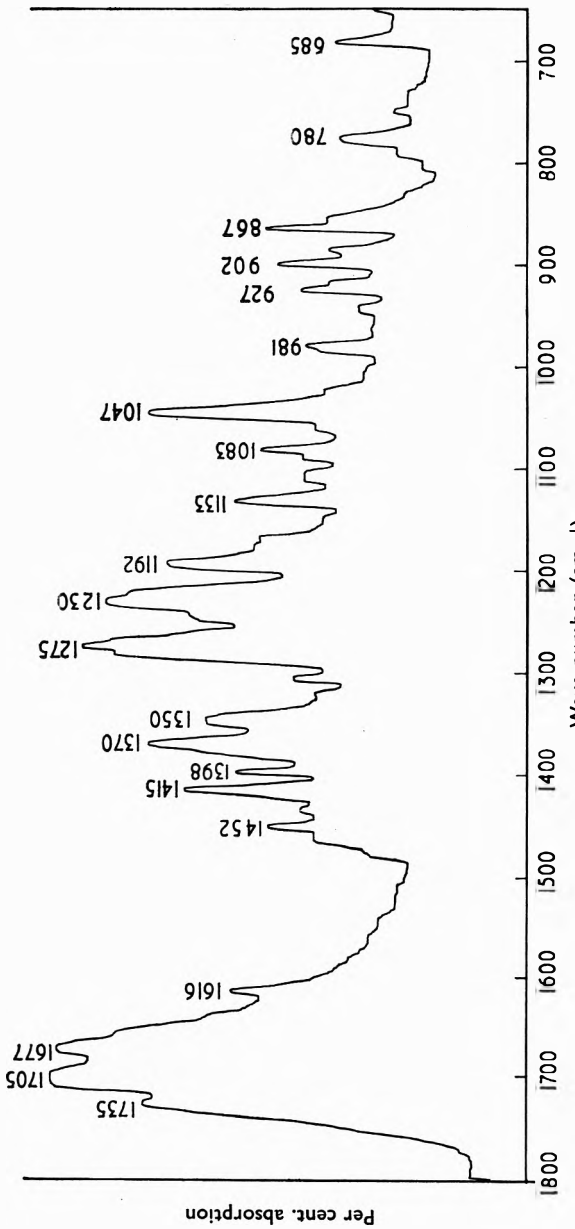


FIG. 2. Infra-red absorption of cortisone acetate (Form II) in a potassium chloride disk.

complete curve from 1,800 cm.$^{-1}$ to 650 cm.$^{-1}$, which has not been published before (Fig. 2). The curve in chloroform solution has been reproduced by Dobriner, Katzenellenbogen and Jones (1953) and by Roberts, Gallagher and Jones (1958).

Form III

Source. This form, m.p. 251–253°, crystallised from benzene at the same time as Form II and was separated by hand. Conditions for the exclusive separation of this form from benzene could not be found.

Crystallographic data. The crystals were orthorhombic a = 12·50 Å, b = 20·95 Å, c = 7·96 Å. The observed density of the single crystals was 1·24 g./ml. but that of the powdered sample was only 1·200 g./ml.—probably because of contamination. These crystals were also free of solvent of crystallisation since the calculated molecular weight was 383·00. There are four molecules in the unit cell and the space group is $P2_12_12_1$ from absences.

Infra-red absorption. The five peaks and shoulder in the 1,800–1,600 cm.$^{-1}$ region are characteristic (see Fig. 1, Curve III) differing from peaks of Form I in wave number and, especially, relative intensity. The hydroxyl band is a broad one at 3,350 cm.$^{-1}$.

Form IV

Source. A solution of 10 g. of cortisone acetate in about 300 ml. of boiling, moist alcohol deposited platy crystals on cooling. These melted at 245–247° after becoming opaque at 111–116°. Analysis gave C, 63·3; H, 7·1 per cent. Loss at 140°/15 mm. 10·25 per cent. Calc. for $C_{23}H_{30}O_6$ 2·5 H_2O: C, 62·8; H, 8·0 per cent. Loss, 10·1 per cent. Calc. for $C_{23}H_{30}O_6$, $2H_2O$. C, 63·1, H, 7·8, Loss 8·2 per cent. The material after removal of solvent gave the infra-red absorption of Form III.

Optics. Columnar crystals with spear-shaped ends. Elongated parallel to the [c] axis, which is also a cleavage direction Plate I, (e). The crystals often appeared powdery because of efflorescence. Extinction position was about 6° from the needle edge.

Crystallographic data. The crystals were orthorhombic with cell-dimensions a = 9·76 Å, b = 30·59 Å, c = 7·58 Å. The observed density was 1·26 g./ml. Density calculated for four molecules of cortisone acetate only in the unit cell was 1·18 g./ml. The difference between the observed and calculated values of the density could be accounted for by assuming the presence of two molecules of water of crystallisation per molecule of cortisone acetate.

Infra-red absorption. There is an ill-defined triple hump (3,420, 3,320 and 3,230 cm.$^{-1}$) in the hydroxyl region, and the shape of the curve in the 1,800–1,600 cm.$^{-1}$ region is characteristic (see Fig. 1, Curve IV).

Form V

Source. Cortisone acetate (1 g.) was recrystallised from a boiling mixture of carbon tetrachloride (15 ml.) and methanol (5 ml.). Needles separated on cooling, having m.p. 238–242° after becoming opaque at

105–110°. This form rapidly loses solvent of crystallisation in air and changes to Form II. X-ray diffraction patterns could only be obtained from freshly prepared material.

Optics. Large striated needles, elongated parallel to the [b] axis. Only the transparent needles were of this form, an opaque deposit indicating change to Form II. Extinction angle was about 4° from the edge.

Crystallographic data. The crystals were monoclinic with a = 9·65 Å, b = 7·46 Å, c = 16·4 Å, $\beta = 98°$. Space group was $P2_1$ from absences. The observed density of single crystals was 1·25 g./ml. which together with the unit cell measurements gave a calculated molecular weight of 438·2, indicating the presence of two molecules of water of crystallisation per molecule of cortisone acetate. Density calculated for this degree of hydration is 1·251 g./ml.

Infra-red absorption. There are three definite peaks 3,530, 3,400 and 3,280 cm.$^{-1}$ in the hydroxyl region, and in the range 1,800–1,600 cm.$^{-1}$ the curve is characteristic, with six peaks (see Fig. 1, Curve V).

II. Interconversions

Forms II, III, IV and V, as intact crystals, are stable for some time in presence of water, but when shaken or ground transformation to Form I takes place rapidly. In typical experiments it was observed that crystals of Form II were apparently unchanged in water after 126 hr., whereas in a shaking machine a change was seen after 20 hr. and was complete after 60 hr. The transformation was followed by withdrawing samples at intervals and taking an X-ray photograph of the powder directly. The time to first appreciable transformation could be decreased to 4 hr. by grinding the crystals to a fine powder before contact with water, but, probably because of caking of the suspensions, complete transformation still took about 56 hr. Continuous grinding under water brought about complete transformation within 45 min.

III. Commercial Preparations

Two specimens of cortisone acetate injection available to us gave, after separation of the powder from the suspension, the infra-red absorption and X-ray measurements characteristic of Form I—as might be expected from the systematic investigations reported above.

Five specimens of tablets of cortisone acetate from different manufacturers were available to us. All showed a moderately intense absorption band in the infra-red at 1,650 cm.$^{-1}$, which was due to the excipient, and prevented any certain conclusion about the crystalline form of the cortisone acetate.

To ascertain this the tablets were shaken for a short time with water and the insoluble material examined without delay. Of the four tablets from different manufacturers examined by infra-red absorption and X-ray diffraction after this treatment, two were found to contain Form I and two to contain Form II. After longer shaking (several hr. or overnight) all yielded Form I.

Discussion

Cortisone acetate crystallises in a variety of forms both hydrated and anhydrous which can be characterised by a combination of X-ray and infra-red spectroscopic methods. The data obtained by these methods

TABLE I

CRYSTALLOGRAPHIC CONSTANTS FOR SINGLE CRYSTALS OF CORTISONE ACETATE

Cortisone acetate $C_{23}H_{30}O_6$. M.Wt. = 402·47

	Form I	Form II	Form III	Form IV	Form V
a	15·68	11·21	12·50	9·76	9·65
b	7·52	27·14	20·95	30·59	7·46
c	26·58	7·11	7·96	7·58	16·40
β	97·0				98·0
V	3112·04	2165·58	2084·52	2263·07	1163·76
Z	6	4	4	4	2
Sp	$P2_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_1$
D_{obs}	1·250	1·210	1·250	1·260	1·250
anhyd. $D_{cal.}$	1·288	1·234	1·282	1·181	1·148
hydr. $D_{cal.}$				1·280	1·251

a, b, c = cell constants in Å. β = cell angle in degrees. V = cell volume in Å³. Z = number of molecules in unit cell. Sp = space-group. D_{obs} = density observed g./ml. anhydr. $D_{cal.}$ = density calculated without hydration. hydr. $D_{cal.}$ = density calculated assuming two molecules of water per molecule of cortisone acetate.

TABLE II

INDEXED X-RAY DIFFRACTION POWDER DATA FOR VARIOUS FORMS OF CORTISONE ACETATE

d = spacing in Ångstrom units, I/I_0 relative intensities (photometrical). hkl = Miller index of reflecting planes

FORM I

d(Å)	I/I_0	hkl	d(Å)	I/I_0	hkl
13·19	10	002	5·30 (2)	70	005, 204
10·73	30	102	4·93	30	014
8·79	30	003	4·69 (3)	60	303
7·78	20	200	4·49	10	213 (?)
7·23	10	011 (?)	4·33	60	304
6·596	10b	004	3·74	10	020
6·14 (1)	100	112	3·629	10	121
5·78	50b	104, 013	2·920	20	009
5·48	30	203	2·850	10	026

FORM II

d(Å)	I/I_0	hkl	d(Å)	I/I_0	hkl
13·57	60	020	2·759	30	420, 091
8·64	5	120	2·674	10	430
6·79	40	040	2·612	40	072
5·835 (1)	100	111	2·561	20	312, 302
5·491 (2)	90	210	2·525	20	322
5·09	50	220, 131	2·463	30	332
4·89	40	041	2·395	5	182, 342
4·77	50	230	2·360	20	272
4·484	60	141	2·322	40	352
4·322 (3)	70	240	2·280	10	1.11.1, 033
4·177	30	221	2·247	30	192
3·900	40	250	2·201	20	3.10.0
3·665	10	170	2·145	20	223
3·530	5	002	2·112	10	521, 1.10.2
3·410	50	251	2·084	5	531, 063
3·247	40	180	2·047	10	382
3·142	40	261, 042	2·002	20	
3·063	40	081	1·965	20	
2·961	5	052	1·909	20	
2·919	5	222	1·869	10b	
2·860	40	152	1·833	20	

FORM III

d(Å)	I/I_0	hkl	d(Å)	I/I_0	hkl
10·47 (5)	60	020	3·036	5	232
8·03	50	120	2·891	20	052 (spotty line)
7·44 (2)	80	011	2·805	20b	071, 421
6·71	20	101	2·688	5	440, 431
6·34	30	021	2·587	20	113
5·99	50	210	2·533	20	342
5·65	40	121	2·494	10	081
5·24 (1)	100	040, 031	2·445	30	412, 181, 203
4·83 (3)	70	140	2·384	30	223
4·45	30	221	2·328	10	521, 143
4·13	30	141	2·240	20	303
3·98	60	002	2·126	5	452
3·73	40	112	2·094	20	0.10.0
3·570	50	122	2·065	5	282
3·486	20	321	2·010	10	462
3·340	20	132	1·980	5	014, 621
3·192	30	251	1·943	10	433
3·104	20	161			

FORM IV

d(Å)	I/I_0	hkl	d(Å)	I/I_0	hkl
15·29 (3)	70	020	3·517	30	112
9·30	trace	110	3·420	30	081
8·23	70	120	3·271	40	270 (?)
7·65	30	040	3·208	60	142
6·79	10	021	2·997	50	340
6·04	30	031, 140	2·960	10	101
5·875 (1)	100	111	2·900	50	162
5·575	40	121	2·808	40	281
5·384	10	041	2·741	40	360
5·184	40	150	2·698	10	082
4·88	70	200	2·630	10	291
4·714 (2)	80	141 (?)	2·467	20	371
4·279	40	151	2·431	30	410
4·23	40	061	2·358	50	282
4·10	50	201	2·253	40	0.13.1
3·995	40	170	2·194	40	233
3·89	40	161	2·137	40	173
3·83	40	080	1·999	30	0.13.2
3·77	trace	012	1·934	20	283
3·654	50	?			

FORM V

d(Å)	I/I_0	hkl	d(Å)	I/I_0	hkl
16·24 (3)	70	001	3·195	40	300
8·79	50	101	2·984	60	015
8·12	60	002	2·918	50	204
6·66	30	102	2·766	30	024
5·88 (1)	100	110	2·693	10	
5·69	50	111	2·629	30	
5·49	70	012	2·438	10	
4·97	40	112	2·329	40	
4·77 (2)	80	201, 200	2·280	30	
4·42	40	201	2·235	20	
4·11	60b	113, 004	2·203	10	
3·939	trace	104	2·162	10	
3·859	trace	203	2·140	10	
3·75	50	020	1·991	10	
3·664	30	021	1·964	20	
3·587	trace	014	1·932	30	
3·516	20	114	1·895	10	
3·428	10	213	1·870	10	
3·253	30	122			

are summarised in Tables I–II. Plate I illustrates the appearance of the various forms for microscopic identification.

In general the most reliable X-ray diffraction data for the identification of complex organic substances are obtained from single crystals. Powder diffraction patterns are, however, technically quicker and simpler to obtain and their value in the pharmaceutical field has been demonstrated

by recent work on some barbiturates by Huang (1953) and by Williams (1959) and on antibiotics by Kennard, Cornforth, Humphrey and Lightbown (1955). The powder patterns of various forms of cortisone acetate (Table II) are sufficiently distinctive for diagnostic use.

All forms of cortisone acetate examined by us changed to the stable Form I on prolonged contact with water. The mechanism of this transformation is obscure since the stable form is not a hydrated one and represents the most complex crystallographic arrangement with three pairs of independent molecules in each cell. The transformation is usually accompanied by appreciable caking of the suspension containing the crystals and for this reason commercial preparations of cortisone-acetate are converted to Form I before the preparation of aqueous suspensions for parenteral administration. A similar procedure would appear to be possibly advantageous in the preparation of tablets of cortisone acetate for oral administration.

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ANALGESIC ACTION OF CHLORPROMAZINE AND RESERPINE IN RELATION TO THAT OF MORPHINE

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An analgesic action of reserpine is reported in mice. This effect, detected by the hot-plate method, is of little, or only of moderate intensity, in the first 2 hr. after injection; maximal intensity is obtained 48–72 hr. after the injection of the drug, and then decreases gradually; after 144 hr. injected animals behave again like controls. The analgesic effect of reserpine seems to differ from that shown by morphine or that shown by chlorpromazine which is quicker in onset and disappears in a few hours. Reserpine potentiates the analgesic action of morphine. This potentiation can be observed 2 hr. after the injection of reserpine. The early potentiating period is followed by a later period of summation of effects. Since a short interval after the injection, reserpine does not exert any analgesic effect, but is able to potentiate morphine analgesia. this action has been regarded as a direct one; the later period of analgesia and summation of effects with morphine has been interpreted as an indirect action of the drug.

The importance of the study of analgesic actions of tranquillising drugs has been emphasised in a recent symposium at the New York Academy of Sciences (Annals, 1960). Of special interest are the relations existing between the analgesic actions of tranquillising drugs and those of analgesics of the morphine group, in view of the possibility that a better knowledge of the mechanism of action of either group may be attained. On the other hand, the measurement of such a complex phenomenon as pain, by the common algesimetric methods, might indicate differences in the mode of action of drugs, some exerting their effect through a central action, as those of the morphine group, and others by a local action, interfering in the intimate mechanism of production of the painful stimuli in the skin or in the peripheral nervous endings.

Recent experiments indicate the presence in the body of pain producing factors like acetylcholine, histamine, 5-hydroxytryptamine, (5-HT) and polypeptides (Armstrong, Jepson, Keele, and Stewart, 1957), the release of which might contribute to the stimulation of the peripheral nervous endings and therefore to the genesis of painful stimuli. Heating, especially, has been shown to release certain of these factors (bradykinin), at temperatures as low as 45°. Reserpine, but not chlorpromazine, appears to interfere with the skin reaction at that temperature (Rocha e Silva and Antonio, 1960). At 55° histamine and 5-HT might also be released and participate in the reaction.

It seems established that chlorpromazine, besides having an analgesic action of its own, exerts a potentiating action on morphine, when both drugs are simultaneously injected in laboratory animals, as has been described by Courvoisier, Fournel, Ducrot, Kolsky and Koetschet (1953),

Schneider (1954), Frcmmel and Fleury (1959), though Kopera and Armitage (1954) were unable to demonstrate such an action using 1·5 mg./kg. morphine sulphate and 10 mg./kg. chlorpromazine.

The analgesic action of reserpine has not been admitted (Schneider, 1954; Bein, 1953; Bein, Gross, Tripod and Meier, 1953; Bein, 1956). An antagonism between reserpine and the analgesic action of morphine has been described (Schneider, 1954); on the other hand Tripod and Gross (1957) observed the analgesic action of morphine to be potentiated by reserpine in mice.

A comparative and detailed study of the analgesic actions of these groups of drugs as done in the foregoing paper, might contribute to a clarification of some still obscure points.

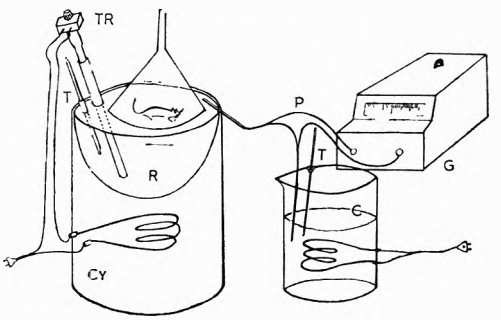

FIG. 1. Apparatus used—Cy: cylinder; R: copper receiver; TR: thermo-regulator; T: thermometers; P: thermocouple; C: cylinder; G: galvanometer.

MATERIAL AND METHODS

The experiments were carried out on male mice weighing between 10 and 20 g.

The analgesic action was measured by the hot-plate method. The apparatus (Fig. 1) consisted of a metal cylinder to which a copper semi-spherical receiver filled with water was adapted. The system was closed with a metal plate of 20 cm. diameter, on which the animals were placed. The temperature is adjusted by a thermo-regulator which corrected wide variations of temperature. A finer regulation was obtained using a thermo-couple. One of the poles of the couple was fixed to the plate by an adhesive strip and the other immersed in a cylinder of water, gradually heated by an electric resistance. The thermocouple was connected to the poles of a galvanometer. The temperature on the plate was accurately determined as being equal to that of the water bath when the galvanometer read zero.

The reaction time (RT) was measured from the moment the animal was placed on the plate until it presented any sign of discomfort as licking of both front paws simultaneously or rubbing the nose with both paws. Sometimes, the end point was indicated by a sudden jump.

The animal was restrained under a glass funnel.

The reaction time at 45° for a group of 20 mice, each used only once had a mean ± its standard error of 65·5 ± 7·1 sec.; at 50° for 50 mice it was 13·5 ± 0·5; at 55° for 75 mice it was 5·5 ± 0·2 and at 60° for 75 mice 5·7 ± 0·2 (Fig. 2). We have selected 55° for all assays since the error arising from small variations of temperature is small.

Drugs used. Reserpine: Serpasol Ciba (crystallised pure alkaloid of rauwolfia), injectable (2·5 mg./ml.). Chlorpromazine: Amplictil Rhodia (chloro-3-dimethylamine-3-propyl-10-phenothiazine chlorhydrate), injectable (25 mg./ml.). Morphine: morphine hydrochloride. Enila S.A.

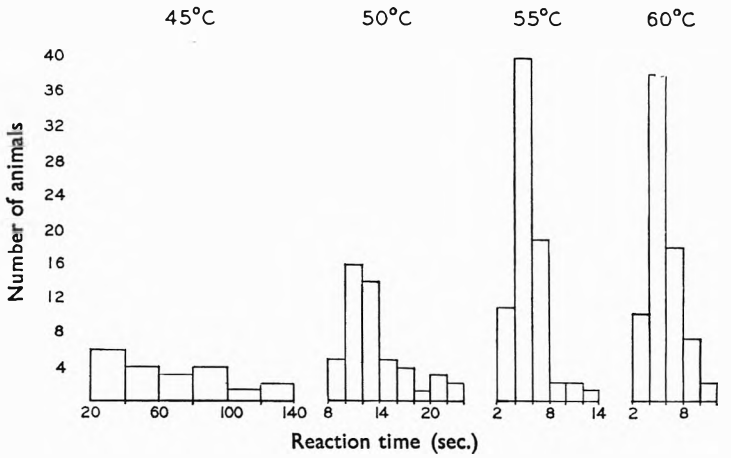

FIG. 2. Distribution of Normal Animals at 45, 50, 55, and 60° C. (temperatures of the plate).

Animals were injected subcutaneously and solutions were prepared in such concentrations that each animal received a maximum of 1 ml. Dilutions were made in distilled water.

Mice treated with reserpine received a single dose of the drug and the RT was measured 2, 24, 48, 72, 96, 120 and 144 hr. after the injection. Animals treated with chlorpromazine received a single dose of the drug and the RT was measured 1, 2, 4 and 24 hr. after the injection. Animals treated with reserpine and morphine received a single dose of reserpine 2 hr. before and one of morphine 30 min. before the measurement of the RT; 24 and 48 hr. after the injection of reserpine 2 further doses of morphine were given and 30 min. thereafter the RT was again measured.

Controls for this group received one dose of morphine daily, 30 min. before the determinations of the RT.

A second control group did not receive any treatment and the RT was measured in a sequence similar to that for the specified groups. That is, in the controls for the group treated with reserpine, the RT measurements were done at zero hour and at 24, 48, 72, 96, 120 and 144 hr. In the controls for the group treated with chlorpromazine the RT measurements were done at zero hour and 1, 4 and 24 hr. as with the animals submitted to the drug.

RESULTS

Effect of Chlorpromazine on RT *at* 55°

A group of 50 mice treated with 5 mg./kg. of chlorpromazine (0·1 mg./ml.) show an increase in RT (Table I, Fig. 3), 1 hr. after the injection of the drug and this is maximal around 2 hr. No significant difference

TABLE I

REACTION TIME (RT) VALUES OF A GROUP OF ANIMALS INJECTED WITH 5 MG./KG. CHLORPROMAZINE. COMPARISON WITH VALUES OF CONTROL GROUP

Dose	RT (Mean ± SE) at 55°				Number of animals
	1 hr. after inj.	2 hr. after inj.	4 hr. after inj.	24 hr. after inj.	
5 mg /kg.	49·6 ± 7·1	90·7 ± 6·0	71·9 ± 7·3	9·8 ± 0·5	50
Controls	6·3 ± 0·3	9·6 ± 0·7	9·6 ± 0·5	9·9 ± 0·5	30

is seen between treated (50) and control animals (30) 24 hr. after the drug has been given.

There were no deaths.

Effect of Reserpine on RT *at* 55°

Animals (100) injected with 5 mg./kg. reserpine (0·1 mg./ml.) presented a gradual increase in RT (Table II) with a maximum around 48 hr. after the injection. The RT values decreased thereafter falling to those of the controls (50 mice) around 144 hr. after administration. Of the 100 test

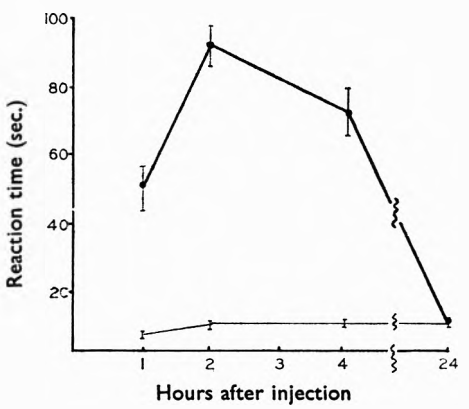

FIG. 3. Effect of chlorpromazine (●—●) on reaction time of mice submitted to the hot plate test (55° C.). Comparison with a control group (—).

animals, 24 died: 1 between 24 and 48 hr. after the administration of the drug, 8 between 48 and 72 hr., 11 between 72 and 96 hr., 3 between 96 and 120 hr., 1 between 120 and 144 hr.

Animals (50) treated with 1 mg./kg. reserpine (0·02 mg./ml.), were distributed in a similar curve with lower mean RT values. Maximum RT

in this group was observed around 72 hr. after the injection of the drug. No difference from the control group (50 mice) is seen 120 hr. after the drug (Table II). No deaths were observed in this group.

TABLE II

REACTION TIME (RT) VALUES OF A GROUP OF ANIMALS INJECTED WITH 1 AND 5 MG./KG. RESERPINE, COMPARED WITH A CONTROL GROUP

	RT (Mean ± SE) at 55°							
Dose	2 hr. after inj.	24 hr. after inj.	48 hr. after inj.	72 hr. after inj.	96 hr. after inj.	120 hr. after inj.	144 hr. after inj.	Number of animals
1 mg./kg.	5·6 ± 0·4	10·2 ± 0·6	14·7 ± 0·7	15·5 ± 0·8	13·2 ± 0·8	8·4 ± 0·4	8·2 ± 0·4	50
5 mg./kg.	9·9 ± 1·9	21·0 ± 1·5	28·7 ± 1·8	23·9 ± 1·7	17·7 ± 0·8	14·1 ± 0·7	10·4 ± 0·6	100
Controls	5·8 ± 0·3	11·3 ± 0·7	10·6 ± 0·5	11·1 ± 0·4	11·0 ± 0·5	9·8 ± 0·4	8·6 ± 0·4	50

Effect of Reserpine and Morphine on RT *at* 55°

The combined effects of reserpine and morphine were observed in two assays. In the first, threshold doses of 1 mg./kg. (0·02 mg./ml.) of both substances were injected 2 hr. and 30 min. before the measurement of the RT. Those values were compared with control animals separately injected with each of the two drugs.

In the group of 20 animals which received 1 mg./kg. morphine only, an increase in the RT from 5·5 ± 0·2 sec. (normal values) to 7·8 ± 0·4 sec.

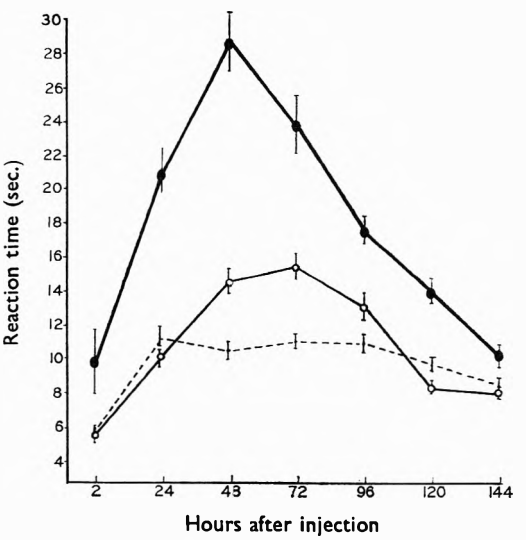

FIG. 4. Effect of reserpine (●—●, 5 mg.; ○—○, 1 mg./kg.) on reaction time of mice submitted to the hot plate test (55° C.), compared with a control group (- - -).

was observed. In the group of 50 animals which received 1 mg./kg. reserpine only, the increase in the RT was not significant. In the group which received both drugs (40 mice) a significant increase in the RT was seen, to 14·1 ± 1·0 sec.

It seems clear that a potentiation of the analgesic effect of morphine has occurred. Table III.

TABLE III

REACTION TIME (RT) VALUES OF ANIMALS TREATED WITH THRESHOLD DOSES OF RESERPINE AND MORPHINE

Treatment	RT (Mean ± SE)	Number of animals
Reserpine (1 mg./kg.) + Morphine (1 mg./kg.)	14·1 ± 1·0	40
Reserpine (1 mg./kg.)*	5·6 ± 0·4	50
Morphine (1 mg./kg.)	7·8 ± 0·4	20

*Data from Table II.

TABLE IV

REACTION TIME (RT) VALUES IN THE GROUP SUBMITTED TO COMBINED TREATMENT: RESERPINE + MORPHINE. COMPARISON WITH THE GROUP SUBMITTED ONLY TO THE ACTION OF RESERPINE AND TO THE GROUP SUBMITTED ONLY TO THE ACTION OF MORPHINE

Treatment	RT (Mean ± SE) at 55°			Number of animals
	2 hr. after reserpine inj. 30 min. after 1st dose of morphine	24 hr. after reserpine inj. 30 min. after 2nd dose of morphine	48 hr. after reserpine inj. 30 min. after 3rd dose of morphine	
Reserpine 5 mg./kg. (single dose) + Morphine 5 mg./kg. (3 doses)	67·3 ± 3·6	44·5 ± 3·5	54·4 ± 3·6	100
	2 hr. after reserpine inj.	24 hr. after reserpine inj.	48 hr. after reserpine inj.	
Reserpine* 5 mg./kg. (single dose)	9·9 ± 1·9	21·0 ± 1·5	28·7 ± 1·8	100
	30 min. after 1st dose	30 min. after 2nd dose	30 min. after 3rd dose	
Morphine 5 mg./kg. (3 doses)	22·5 ± 2·1	21·5 ± 1·7	21·4 ± 1·9	45

*Data from Table II.

In the second assay both drugs were injected in a dose of 5 mg./kg., reserpine 2 hr. and morphine, 30 min. before the assay.

In a group of 100 animals, new determinations of RT were made 24 and 48 hr. after the injection of reserpine and 30 min. after a new dose of morphine. The results can be seen in Table IV and Figs. 5 and 6.

In the animals which received only morphine (45 animals) 30 min. before the assay and whose RT were determined three times at intervals of 24 hr., the values were 22·5 ± 2·1; 21·5 ± 1·7 and 21·4 ± 1·9 respectively (Table IV).

The 100 animals which received only reserpine showed a small increase (9·9 ± 1·9 sec.) in their RT compared with normal values, 2 hr. after and a remarkable increase 24 and 48 hr. after the injection of the drug, respectively to 21·0 ± 1·5 and 28·7 ± 1·8 sec. (Table IV).

Of interest is the fact that animals which received both drugs in the first day presented a remarkable increase in the RT to 67·3 ± 3·6 sec., which might indicate a clear potentiating effect of reserpine towards

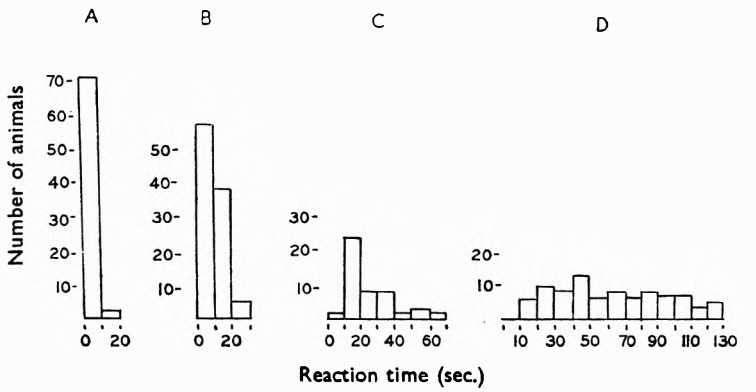

FIG. 5. Distribution of animals submitted to the combined treatment reserpine + morphine (2 hr. after reserpine and 30 min. after 1st dose of morphine). Comparison with reserpine treated, morphine treated and control animals.

A: controls (no treatment)—75 animals.

B: 2 hr. after reserpine (5 mg./kg.)—100 mice.

C: 30 min. after 1st dose of morphine (5 mg./kg.)—45 animals.

D: combined treatment: 2 hr. after reserpine (5 mg./kg.) and 30 min. after 1st dose of morphine (5 mg./kg.)—100 mice (10 mice over 130 sec.).

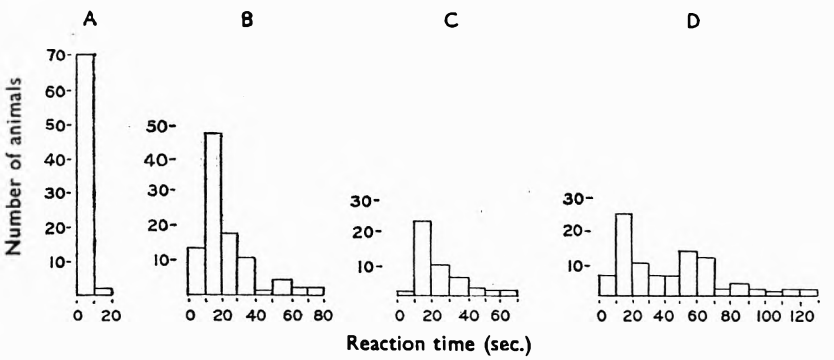

FIG. 6. The same as Fig. 5, 24 hr. after reserpine and 30 min. after 2nd dose of morphine.

A: controls (no treatment)—75 mice.

B: 24 hr. after reserpine (5 mg./kg.)—100 mice.

C: 30 min. after 2nd dose of morphine (5 mg./kg.)—45 mice.

D: combined treatment: 24 hr. after reserpine (5 mg./kg.) and 30 min. after 2nd dose of morphine (5 mg./kg.)—100 mice (5 mice for 130 sec.).

morphine. In subsequent days also, an increase in the RT was observed but this more as if a summation of the effects of reserpine and morphine had occurred, since both produce an increase in the RT as seen in Table IV.

In the period between 2 and 24 hr. after reserpine, 3 animals died in this group.

Conclusions and Discussion

Our results suggest that in mice there is an analgesic action due to reserpine, that can be clearly detected after 24 hr. and which remains for some days.

In the first 2 hr. after the injection of reserpine the effect is still undetectable or of moderate intensity; the maximum effect is obtained in 48–72 hr. and then decreases gradually. After 144 hr. the effect disappears and the injected animals behave like the controls.

The analgesic effect of reserpine seems to differ from that of morphine or chlorpromazine. That of chlorpromazine is of quick onset and is transient.

Between 48 and 72 hr. after the injection of reserpine the analgesic effect is more or as intense as that observed 30 min. after the injection of an equivalent dose of morphine, but less intense than that observed in the first 4 hr. after the injection of a similar dose of chlorpromazine.

Reserpine potentiates the action of morphine. This potentiation can be observed 2 hr. after the injection of reserpine. The early potentiating period is followed by a later period of summation of effects. When morphine is injected 24 or 48 hr. after reserpine, the intensity of the analgesic effect is less than when administered only 2 hr. after reserpine.

The initial potentiating effect can be clearly seen when threshold doses of reserpine and morphine are used.

Comparing the analgesic action of tranquillisers and their effects upon morphine analgesia, it seems apparent that a striking difference can be observed between the two classes of drugs, the one represented by chlorpromazine and the other by reserpine. Chlorpromazine has a definite and early analgesic activity, which disappears after a few hours; reserpine on the other hand shows an analgesic action only 24 or 48 hr. after the injection.

When tested in combination with morphine, both drugs enhance its effects. Reserpine, however, in the first hours after the injection, definitely potentiates the analgesia induced by morphine; the later summation of effects of reserpine and morphine, appearing 24 hr. after the injection of the rauwolfia alkaloid, has no parallel with chlorpromazine since the effect of chlorpromazine is not detectable after a few hours.

Our experiments are more concerned with the relation between reserpine and morphine and with the differences seen between a short or a longer interval after the injection of reserpine. In the early period a true potentiation is observed, since reserpine by itself has no analgesic action or only a moderate one, but after 24 or 48 hr., a definite increase in RT is observed. When morphine is then injected, a simple summation of the analgesic effects of reserpine and morphine occurs.

One might speculate on the mechanism of this dual effect of reserpine when combined with morphine. As the analgesic action of reserpine is observed only a long interval after the injection of the drug and remains for some days it could be interpreted as an indirect action, since after 48 hr., only traces of it are detectable in the brain (Plummer, Sheppard, and Schulert, 1957; Sheppard, Hui, Plummer, Peets and Giletti, 1958). This action could involve a participation of catechol and indole amines, since there is some indication that the time required for the injected animal to behave like the controls is the time necessary to restore those amines in the nervous structures of the brain after depletion by reserpine. But note that these last experiments were carried out on rabbits (Pletscher, Shore and Brodie, 1956). A short interval after injection, reserpine does not exert any analgesic effect, but is able to potentiate morphine analgesia. This could be interpreted as a direct action of the drug, if we assume that at this stage the highest concentrations of reserpine would be present in the brain.

Reserpine by itself is able to potentiate the analgesic action of morphine, but its analgesic effect would be an indirect one, probably following depletion of catechol or indole amines.

Acknowledgement. We are indebted to Mr. Helgio L. Werneck for constructing the apparatus and drawing the figures.

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A NOTE ON AN EFFECT OF PYROGALLOL ON DUODENAL MOTILITY

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Pyrogallol injected intravenously exerts an initial depressor and a secondary stimulatory effect on the duodenal motility of dogs. The first effect is similar to that of adrenaline. The duodenal excitation is thought to be due to a peripheral cholinergic mechanism. Pyrogallol enhances the duration of the inhibitory action of adrenaline on duodenal motility and converts the action of histamine to an inhibition.

Pyrogallol is known to be an inhibitor of catechol-*o*-methyl transferase (Axelrod and Laroche, 1959; Axelrod, 1960) and its action as a potentiator of the effects of catecholamines has thereby been explained (Axelrod, 1960). It has been shown to enhance actions of adrenaline (Wylie, Archer and Arnold, 1960; Axelrod, 1960; Bacq, 1935; Ludueña, 1938; Lembeck and Resch, 1960; Izquierdo, Izquierdo, Kaumann and Coussio, 1961a, b), noradrenaline (Wylie and others, 1960; Izquierdo and others, 1961a, b), sympathetic nerve stimulation (Bacq, 1935; Izquierdo and others, 1961b), isoprenaline (Konzett, 1960; Izquierdo and others, 1961a, and unpublished results) and is presumed to exert its own adrenergic effect by the potentiation of circulating catecholamines (Izquierdo and others, 1961a).

An entirely different action of pyrogallol is described in this paper, which refers to studies on the duodenal motility *in situ* of dogs.

Methods

Twenty mongrel dogs of both sexes were used in this experiment. They were anaesthetised with intraperitoneal pentobarbitone (35–40 mg./kg.). Carotid blood pressure and duodenal motility, by intraduodenal balloon, were recorded on smoked paper.

All drugs were injected into a cannulated external iliac vein.

Results

Pyrogallol (5, 10, 20, 25 and 50 mg./kg.) produced an initial and immediate reduction in tonus and a decrease or disappearance of duodenal contractions in all dogs. This effect closely resembled that of 1 or 2 μg./kg. of adrenaline injected as a control, and lasted 1 to 3 min.

Also the duration of the inhibitory action of adrenaline upon the duodenal activity was always increased.

Two to 10 min. after its injection, pyrogallol provoked a marked increase in the amplitude of contractions (Fig. 1), which sometimes reached very high levels. This was seen in 19 out of 20 dogs. The tonus was usually also increased. This effect lasted up to 15–40 min. from the injection of the polyphenol. A dose-response curve could not be obtained since all doses over 5 mg./kg. gave the same response.

This enhancement of duodenal motility was not inhibited by mepyramine (5 mg./kg., 2 dogs) or hexamethonium (3–8 mg./kg., 4 dogs) but was completely abolished by atropine (0·5–2 mg./kg., 9 dogs) (Fig. 2). It appeared in animals with their vagi intact or with one or both of them sectioned. It was also observed in animals previously treated with hexamethonium but it was not possible to evoke it after the administration of atropine, unless (1 dog) large doses of neostigmine were given beforehand.

The potentiating effect of pyrogallol on the duodenum was intensified after the administration of tolazoline (5 mg./kg., 3/3 dogs), phentolamine

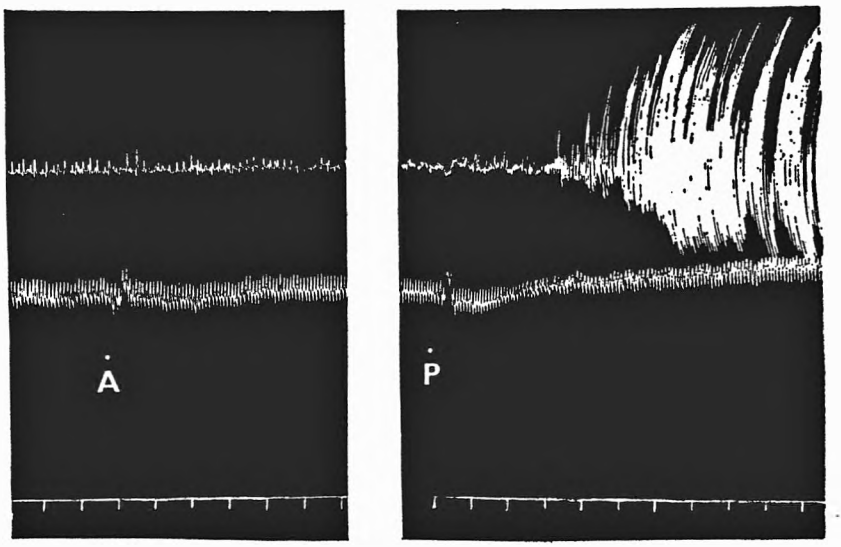

FIG. 1. From above down: duodenal motility, blood pressure, time (min.). A: 3 ml. 1 per cent ascorbic acid. P: pyrogallol, 20 mg./kg. Note the increase in duodenal motility seen after pyrogallol.

(10 mg./kg., 1/2 dogs) and yohimbine (0·5 mg./kg., 1 dog). It could be evoked in the presence of one or more of these drugs, or of dichloroisoprenaline (3 mg./kg., 1 dog).

The excitatory action of histamine (1, 2, 4 μg./kg.) on the duodenum is converted into a brief inhibition after pyrogallol. This inhibition resembles that produced by adrenaline.

Pyrogallol produces its excitatory effect on duodenal activity either when dissolved in 1 per cent ascorbic acid or when diluted, a few seconds before its injection, in saline solution. 3 ml. 1 per cent ascorbic acid did not itself exert any appreciable effect on duodenal motility (2 dogs).

DISCUSSION

The explanation of the initial sympathomimetic-type effect of pyrogallol on the duodenum may be an enhancement of the action of circulating catecholamines. In fact, it also potentiates the intestinal effect of exogenous

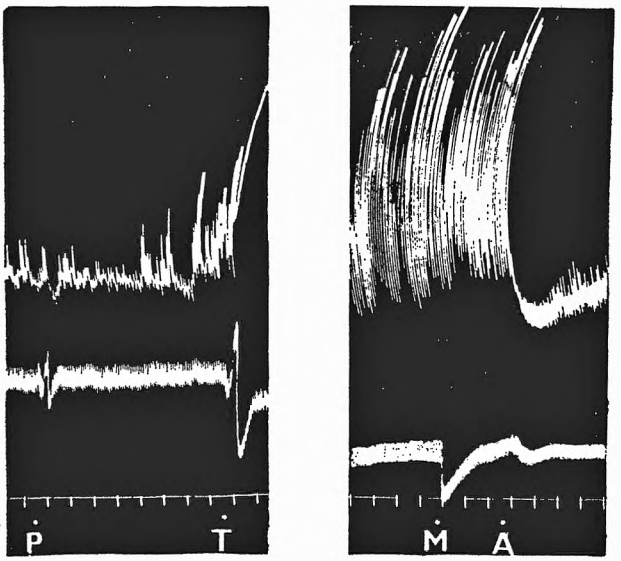

FIG. 2. P: pyrogallol, 10 mg./kg. T: tolazoline, 5 mg./kg. M: 11 min. afterwards, 4 mg./kg. hexamethonium. A: atropine, 1 mg./kg. Note that tolazoline exaggerates the effect of pyrogallol on the duodenum; hexamethonium does not inhibit it, and atropine does.

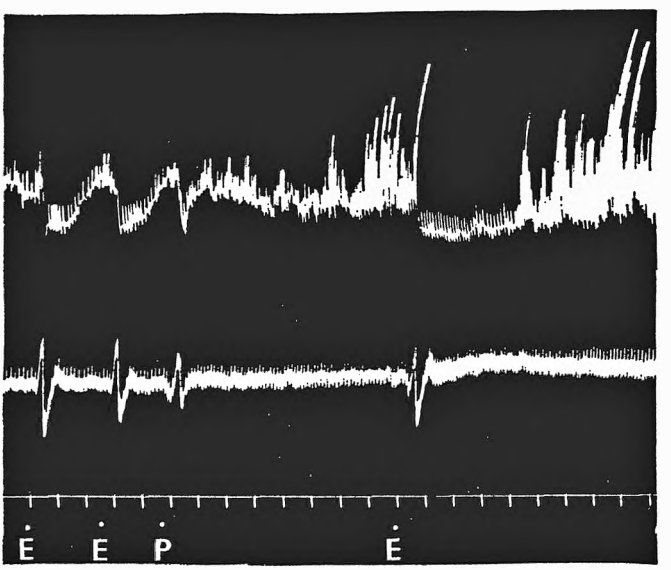

FIG. 3. E: Adrenaline, 1 μg./kg. P: pyrogallol, 20 mg./kg. Note the enhancement by pyrogallol of the effect of adrenaline on duodenal motility as well as the effect of pyrogallol itself.

adrenaline. However, an adrenaline-like effect in its own right cannot be wholly discarded.

The secondary enhancement of intestinal motility seen 2–10 min. after pyrogallol seems acetylcholine-like in nature for it is readily abolished by atropine and cannot be provoked in its presence unless neostigmine is first administered. It appears to be peripheral, because it happens in spite of vagotomy and after hexamethonium and is not inhibited by the latter.

The augmentation of this effect seen with tolazoline, phentolamine and yohimbine may be due to the complex actions on the intestine of these drugs, part of which action is acetylcholine-like and histamine-like.

The potentiator effect of pyrogallol on duodenal motility is independent of its acetylcholine-like action. In fact, it enhances the duration of the effect of adrenaline on duodenal motility while this is, at the same time, much potentiated by pyrogallol itself (Fig. 3). Moreover, the latency of this effect is much longer (2–10 min.) than that of the adrenaline-like potentiating effect, which is immediate according to the results of Wylie and others (1960).

The reversal of the effect of histamine on duodenal motility may be thought to be due to an unmasking of its known stimulatory action on the adrenal medulla by pyrogallol. An inhibition of histamine-like effects on duodenum has been observed by Juorio (unpublished results) using isolated organs.

In summary, an intense acetylcholine-like effect of pyrogallol is described on the intact duodenum of dogs, and which does not prevent the simultaneous potentiation of the action of adrenaline-like agents upon it.

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THE IDENTIFICATION AND DETERMINATION OF SOME PHENOLIC ACIDS IN URINE USING TWO-DIMENSIONAL PAPER CHROMATOGRAPHY

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The identification and determination of vanillic, homovanillic, *p*-hydroxybenzoic, *p*-hydroxyphenylacetic, *m*-hydroxybenzoic and *o*-hydroxyphenylacetic acids by the use of two-dimensional paper chromatography has been investigated.

The present investigation is concerned with the qualitative and quantitative examination of those phenolic acids that are stable to hot acid hydrolysis in the presence of 5N hydrochloric acid—vanillic, homovanillic, *p*-hydroxybenzoic, *p*-hydroxyphenylacetic, *o*-hydroxyphenylacetic and *m*-hydroxybenzoic acids. Preliminary hot acid hydrolysis does not appear to be particularly favoured by American investigators because of the lability of some phenolic acids. Hot acid hydrolysis was employed by Boscott and Cooke (1954) but the concentration of acid used was insufficient to affect the hydrolysis of glycine conjugates.

All the above phenolic acids, with the exception of *o*-hydroxyphenylacetic acid, are readily detectable on paper chromatograms prepared from normal human urine and represent different aspects of human metabolism. *m*-Hydroxybenzoic and vanillic acids are probably almost entirely of dietary origin although it has been suggested that the latter may be related to noradrenaline metabolism (Smith and Bennett, 1958) but this has been disputed (Shaw and Trevarthan, 1958). *p*-Hydroxyphenylacetic acid is an important metabolite of tyrosine and increased urinary excretion has been observed in a variety of conditions (Boscott and Cooke, 1954). It has been reported that *p*-hydroxybenzoic acid may be a metabolite of tyrosine (Booth, Masri, Robbins, Emerson, Jones and DeEds, 1960) although this substance may also be derived from *p*-cresol produced in the intestines as the result of bacterial activity (Williams, 1959). Homovanillic acid is recognised as a metabolite of 3,4-dihydroxyphenylalanine and dopamine and its determination could be of value in the study of phaeochromatocytoma. It does not appear to be derived from any dietary source. *o*-Hydroxyphenylacetic acid is derived from phenylalanine (Boscott, 1953, 1956; Armstrong, Shaw and Robinson, 1955) but appears to be only readily detectable in phenylketonuria. Booth, Masri, Robbins, Emerson, Jones and DeEds (1959) have, however, shown that in the rat and the rabbit, this substance may also be formed from coumarin.

Phenolic acids are excreted in urine mainly as conjugates with sulphate, glucuronic acid and glycine. The form of conjugation may be important in some instances but in general examinations, a mixture of conjugates may create confusion. Hydrolysis of conjugates was effected before chromatographic examination, hot acid hydrolysis in the presence of

5N hydrochloric acid being employed. Hydrolysis of sulphates, glucuronidates and glycine conjugates is effected. The methoxyl group and the parent substances—*m*-hydroxybenzoic, *p*-hydroxybenzoic, *p*-hydroxyphenylacetic, vanillic, homovanillic and *o*-hydroxyphenylacetic acids are unaffected by such treatment. Free phenolic acids are separated with ether and the extracts evaporated to dryness *in vacuo*. Volatile phenols are thus removed.

Three two-dimensional solvent systems have been used extensively for the examination of urinary phenolic acids. (1) Isopropanol : ammonia : water and benzene : propionic acid : water (Armstrong, Shaw and Robinson, 1956). (2) Benzene : acetic acid : water and 20 per cent aqueous potassium chloride (Boscott and Cooke, 1954). (3) Chloroform : acetic acid : water and 20 per cent aqueous potassium chloride (Booth, Emerson, Jones and DeEds, 1957).

The system of Armstrong and others has been used in this investigation.

Experimental

Hydrolysis of Conjugates and Extraction of Phenolic Acids

1 volume of urine and 1 volume of 10 N hydrochloric acid were boiled under a reflux condenser for 1½ hr. The cooled mixture was extracted three times with 4 volumes of ether. The combined ether extracts were evaporated to dryness in an all glass vacuum still. The residue was dissolved in ethanol so that 1 ml. ethanolic solution was equivalent to 50 ml. urine.

Two Dimensional Paper Chromatography

20 μl. of an ethanolic solution of the urinary phenolic acids (equivalent to 1 ml. of urine) was applied to a sheet (55 × 45 cm.) of Whatman No. 1 filter paper which was then stapled in the form of a cylinder. Development was carried out by the capillary ascent method with (i) isopropanol : ammonia 0·88 : water (8 : 1 : 1) as the first solvent mixture, and (ii) benzene : propionic acid : water (2 : 1 : 1), the organic phase, as the second solvent mixture. An all glass tank capable of holding a number of paper cylinders was used.

After development, the sheets were allowed to dry and then treated by one of the following systems.

(I) Pauly's Reagent (Bolling, Block and Sober, 1949; Block, 1951).

Reagents: (*a*) 1 per cent sulphanilamide in N hydrochloric acid. (*b*) 5 per cent sodium nitrite (w/v). (*c*) 10 per cent sodium carbonate (w/v).

Procedure: 10 ml. of (*a*) and 10 ml. of (*b*) were mixed in a 100 ml. glass stoppered measuring cylinder. After standing for 1 min., 80 ml. of butanol was added and the mixture shaken. Papers were sprayed with the aqueous phase of this mixture, and when dry with 10 per cent sodium carbonate solution.

(II) Diazotised *p*-nitraniline followed by 20 per cent aqueous sodium carbonate solution (Bray, Thorpe and White, 1950).

(III) Diazotised diethylaminoethyl *p*-aminophenylsulphone (Boscott and Cooke, 1954).

Standard solutions. (1) ethanolic solutions of *p*-hydroxybenzoic, *p*-hydroxyphenylacetic, *o*-hydroxyphenylacetic, vanillic, homovanillic and *m*-hydroxybenzoic acids containing 1 mg. acid/ml. (20 μl. is equivalent to 20 μg. acid). (2) an ethanolic solution containing all the above acids at 1 mg. acid/ml. (20 μl. is equivalent to 20 μg. of each acid). 20 μl. of a standard solution was applied to a paper. Development was carried out at the same time as the unknown.

In an examination, four paper chromatograms were developed simultaneously. (1) blank, (2) urine extract applied for spraying, (3) urine extract applied, unsprayed, for the colorimetric determination of *m*-hydroxybenzoic and *p*-hydroxyphenylacetic acid, (4) standard quantities of the 6 phenolic acids applied for spraying.

TABLE I

THE REACTION (COLOUR DEVELOPMENT) OF PHENOLIC ACIDS ON PAPER CHROMATOGRAMS WHEN TREATED WITH 3 SPRAY REAGENTS

Substance	Spray Reagent		
	1	2	3
m-Hydroxybenzoic Acid	Yellow	Dark red	Yellow
p-Hydroxybenzoic Acid	Yellow	Red	Purple
o-Hydroxyphenylacetic Acid	Orange	Purple	Purple
p-Hydroxyphenylacetic Acid	Purple	Purple	Purple
Vanillic Acid	Brick red	Purple	Purple
Homovanillic Acid	Purple	Light brown	Purple

NOTE: aqueous sodium carbonate added subsequently or with the reagent.

Spray Reagent

1. Diazotised sulphanilic acid (Bolling and others, 1949; Block, 1951.
2. Diazotised *p*-nitroaniline (Bray and others, 1950).
3. Diazotised diethylaminoethyl *p*-aminophenylsulphone (Boscott and Cooke, 1954).

Quantitative Evaluation of Coloured Spots

A section of paper containing the coloured spot was separated and extracted for 2 hr. with 10 ml. of 50 per cent aqueous methanol. The extinction of the coloured solution was then read against a blank at an appropriate wavelength. A measured area of paper containing the coloured spot was always separated from the paper. A "blank" paper was prepared at the same time. The "blank" solution was prepared from this paper, using a section of identical area and R_F value.

RESULTS AND DISCUSSION

The six phenolic acids could be readily identified on paper chromatograms and although *p*-hydroxyphenylacetic acid and *m*-hydroxybenzoic acid showed some overlapping, the other phenolic acids showed good separation.

Diazotised sulphanilic acid appeared to be the most suitable spray reagent since it showed a greater differentiation in colour of spots of individual phenolic acids (see Table I). For quantitative examinations this spray reagent has the lowest blank values.

Examination of urinary extracts showed the invariable presence of homovanillic, vanillic, *m*-hydroxybenzoic, *p*-hydroxybenzoic and *p*-hydroxyphenylacetic acid. *o*-Hydroxyphenylacetic acid was just detectable (less than 1 mg./day) in conditions other than phenylketonuria. In two cases of phenylketonuria, values of 24·0 and 8·2 mg./day were found.

Values obtained from the examination of 12 normal urines are recorded in Table III. The excretion of *p*-hydroxybenzoic acid was found to be

TABLE II

WAVELENGTH AT WHICH AQUEOUS METHANOLIC EXTRACTS OF THE COLOURED COMPLEXES OF THE FOLLOWING PHENOLIC ACIDS FROM PAPER CHROMATOGRAMS ARE MEASURED COLORIMETRICALLY

Substance	Wavelength mμ
m-Hydroxybenzoic acid	460
p-Hydroxybenzoic acid	470
o-Hydroxyphenylacetic acid	470
p-Hydroxyphenylacetic acid	500
Vanillic acid	480
Homovanillic acid	500

increased by about 45 per cent after the ingestion of 10 g. of tyrosine, suggesting that this acid might be derived in part from the aromatic amino-acid. The oral ingestion of 6 g. of benzoic acid produced no appreciable effect upon the urinary excretion of *p*-hydroxybenzoic acid. It does not appear that *p*-hydroxylation occurs in the metabolism of benzoic acid.

Paper chromatograms of urinary extracts often showed a number of coloured spots close to the benzene : propionic acid base line. These were presumably due to phenolic lactic acids which were not considered in this investigation.

Substituted cinnamic acids, for example, ferulic, *o*- and *p*-hydroxycinnamic acids produce typically coloured spots but as the result of the

TABLE III

THE NORMAL URINARY EXCRETION IN MG./DAY OF SOME PHENOLIC ACIDS

Substance	Minimum	Maximum	Average
m-Hydroxybenzoic acid	8·3	38·6	23·2
p-Hydroxybenzoic acid	2·1	12·5	6·3
o-Hydroxyphenylacetic acid		less than 0·5	
p-Hydroxyphenylacetic acid	12	28	14
Vanillic acid	4·6	36·8	14·8
Homovanillic acid	0·8	1·6	1·2

hydrolytic treatment employed, these acids are converted into forms which do not react with any of the three spray reagents.

Substituted phenolic lactic acids show instability when subjected to the hydrolytic treatment. When examined by the paper chromatographic technique, 4-hydroxy-3-methoxymandelic acid showed losses of about 50 per cent. The residues obtained from this acid had a strong smell of vanillin indicating chemical changes that may have occurred

during decomposition. Vanillin itself gave little colour on chromatograms. Phenolic pyruvic acids are unstable in the presence of the first solvent mixture. Thus the methods adopted are unsuitable for the examination of substituted cinnamic acids and phenolic lactic and pyruvic acids.

Standard solutions of the phenolic acids applied to paper could be readily eluted as the coloured complexes. Extinctions were read at the wavelengths shown in Table II and were found to be linear within the range 0 to 80 μg. Whatman No. 1 filter paper contains "phenolic" material which reacts with all three spray reagents. Much of this material follows the solvent fronts in both systems. Therefore a blank sheet of Whatman No. 1 paper was developed along with the sheets to which urine extracts or standard solutions of the phenolic acids had been applied.

The zones occupied by *m*-hydroxybenzoic and *p*-hydroxyphenylacetic acids exhibit much overlapping. Although the two acids may be distinguished visually, quantitative determination by the elution of the coloured complexes is not practical. These acids were determined in residues of alcohol eluates obtained from an unsprayed paper chromatogram which was developed at the same time. *m*-Hydroxybenzoic acid was determined by the chloroimide reaction (Tompsett, 1958) and *p*-hydroxyphenylacetic acid with 1-nitroso-2-napthol (Tompsett, 1958). Neither acid interferes with the colorimetric determination of the other.

It is very difficult to distinguish between *m*-hydroxybenzoic and *m*-hydroxyphenylacetic acids since both acids possess similar R_F values in the solvent systems employed and have similar reactions towards spray reagents.

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THE NEUROMUSCULAR BLOCKING ACTION OF TETRAETHYLAMMONIUM

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The abnormally low activity of tetraethylammonium at the neuromuscular junction of the rat-diaphragm has been investigated. It seems possible to explain this lack of action in terms of the charge delocalisation and the stereochemistry of tetraethylammonium. The neuromuscular blocking activities of tetraethylammonium, isomers of it and related quaternary ammonium compounds have been determined and the structure activity relationships are discussed.

Jacobj and Hagenberg (1902) demonstrated that the pharmacological activity of tetraethylammonium differed from that of other simple monoquaternary ammonium compounds. While toxic doses of tetramethylammonium given to frogs gave rise to both muscarinic and curariform responses, tetraethylammonium in similar doses produced neither.

The actions of a series of quaternary ammonium compounds on the anaesthetised mammal were investigated by Marshall (1913, 1914). The successive replacement of the methyl groups in tetramethylammonium by ethyl groups was shown to lead to a decline in neuromuscular blocking activity, tetraethylammonium being eighty times less active than tetramethylammonium as a neuromuscular blocking agent. These results were confirmed by Ing and Wright (1933), who used the frog rectus abdominis preparation.

The autonomic ganglionic blockade produced by tetraethylammonium was described by Acheson and Periera (1946), who stated that tetraethylammonium over a wide range of doses had no action other than a specific ganglionic effect. Atkinson (1952), and Jepson, Simeone and Lynn (1953), reported that tetraethylammonium injected intra-arterially in cats and dogs had no significant effect in doses below 10 mg./kg. With higher doses a transient neuromuscular depression occurred preceded by a small increase of muscle responses to both direct and indirect stimulation. Stovner (1957), investigated the action of tetraethylammonium bromide on the isolated phrenic nerve-diaphragm preparation of the rat. He showed that concentrations of tetraethylammonium up to 2 millimolar caused no neuromuscular block to either single or tetanic nerve stimulation. A neuromuscular block was produced with higher concentrations which could be antagonised with potassium but not with anticholinesterases.

In a review of the curariform action of onium salts, Ing (1936) stated that curariform activity appeared to depend primarily on the ionic character of onium ions and not on their detailed chemical structure but that it was difficult to account for the abnormally low activity of tetraethylammonium.

Holmes, Jenden and Taylor (1947), considered the charge delocalisation in onium ions in relation to their curariform activity and suggested that the charge density on the central atom was critical for curariform activity. The amount of charge delocalisation of onium ions is dependent on the electronegativity of the organic radicals linked to the central atom. Hence, it was suggested that the charge density of the nitrogen atom in the tetraethylammonium ion was unusually low because ethyl groups are more electronegative than other aliphatic groups, and this explained the lack of neuromuscular blocking action of tetraethylammonium. The evidence quoted as a basis for this postulate depended on the dissociation constants of aliphatic amines and carboxylic acids.

It would be expected, according to this postulate, that the replacement of a methyl group by an ethyl group in any quaternary ammonium compound should lead to a reduction in neuromuscular blocking activity. Ing and Wright (1933), however, demonstrated for a number of compounds that replacement of a methyl group by an ethyl group in quaternary ammonium compounds and quaternary arsonium compounds led to an increase in curariform activity.

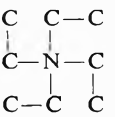
The work of Thomas (1961b) on the anti-acetylcholinesterase activity of aliphatic quaternary ammonium compounds provides a possible explanation for the lack of curariform activity of tetraethylammonium. While the block of neuromuscular transmission is a more complex process than the anti-acetylcholinesterase action, involving a sequence of events and a number of different mechanisms (Van Rossum, Ariens, Linssen, 1958), the primary event in both actions is the adsorption of a compound onto a receptor and both receptors have the same natural substrate, acetylcholine. It appeared possible that the factors governing the adsorption of onium ions onto acetylcholinesterase would also have an influence on the adsorption of the ions onto the neuromuscular receptor and consequently on neuromuscular blocking action. The action of tetraethylammonium on the cholinesterases has been studied by a number of workers (Barlow and Ing, 1948; Bergman and Shimoni, 1951; Kensler and Elsner, 1951; Takagi, 1953; Thomas, 1961a) who all came to broadly the same conclusions, even though different sources of both acetylcholinesterase and cholinesterase were used. Tetraethylammonium is a very weak inhibitor of cholinesterase, particularly at low substrate concentrations, and under some conditions can even potentiate the enzyme.

Thomas (1961b) postulated that the anti-acetylcholinesterase activity of simple quaternary ammonium compounds is related to the forces of adsorption between these ions and the active site of the enzyme and that the factors involved in the adsorption process are coulombic attraction and van der Waal's forces, with the distribution of the onium ion between the surface and the bulk of the solution also playing a part. It was further considered that the δ^+ charge on the α-carbon atoms of the quaternary ammonium group provided the major contribution to the coulombic attraction. The charge on the nitrogen atom was considered to make a relatively minor contribution to the total electrostatic binding

force. Since the configuration of a quaternary ammonium nitrogen atom is tetrahedral then the maximum number of α-carbon atoms which can be directed towards a surface is three, for example in tetramethylammonium (I). The abnormally low adsorption forces between tetraethylammonium and the enzyme, reflected by the lack of anti-acetylcholinesterase activity of the ion, may be explained only after a consideration of its stereochemistry.

Tetraethylammonium is an open chain molecule and consequently its configuration is not fixed because of possible rotation about carbon to nitrogen and carbon to carbon bonds. The configuration of tetraethylammonium in the crystalline state has been determined, however, by Waite and Powell (1958). It was shown that in projection the structure was that of a Nordic Cross (II).

(I)



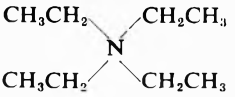
(II)

The inner carbon atoms were reported to be at the vertices of a regular tetrahedron with the nitrogen atom at the centroid. The N—C bond length was 1·49 Å and the N—C—C angle, 121° 44′, 12° greater than the normal regular tetrahedron angle. From studies in conformational analysis it is suggested that statistically the same arrangement of groups will predominate in aqueous solution where the restricting environment of a crystal lattice is absent. An examination of molecular models of such a molecule indicated that only two unhindered α-carbon atoms could be directed towards a surface or receptor whichever way the model was orientated. Consequently the coulombic forces of attraction between the tetraethylammonium ion and the anionic site of acetylcholinesterase are low. Since tetraethylammonium is the lowest homologue in which such a situation occurs then its anomalous behaviour as an anti-acetylcholinesterase agent may be explicable on this basis.

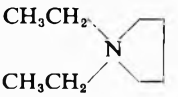
As methylene groups are added to tetramethylammonium in a symmetrical manner to produce tetraethylammonium, two factors are at work to modify the anti-acetylcholinesterase activity of the molecule; (*a*) the increase in methylene groups will tend to increase the activity as the van der Waal's forces and distribution factor are increased, and (*b*) the progressive decrease in the availability of the α-carbon atoms will decrease activity. Further addition of methylene groups to tetraethylammonium to produce n-alkyltriethylammonium compounds will increase activity by increasing van der Waal's forces and the distribution factor while coulombic forces remain constant.

The theory is supported by the fact that 1,1′-spirobipyrrolidinium and *NN*-diethylpyrrolidinium have been found to be more powerful inhibitors of acetylcholinesterase than tetraethylammonium (Thomas, 1961a). The only difference between these compounds is that pairs of ethyl groups of tetraethylammonium are linked together to form the pyrrolidine ring.

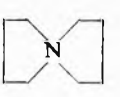
The effect of this is to make the α-carbon atoms more available and this gave an increased activity. There is virtually no change in the van der Waal's forces or the distribution factor from one compound to the next.



(III)
Tetraethylammonium



(IV)
NN-Diethylpyrrolidinium



(V)
1,1′-Spirobipyrrolidinium

The three compounds, III, IV, V, together with their isomers n-butyldimethylethylammonium bromide and n-pentyltrimethylammonium bromide, have now been examined for neuromuscular blocking activity.

Experimental

Chemical

1,1′-Spirobipyrrolidinium bromide and NN-*diethylpyrrolidinium bromide* were prepared as described by Thomas (1961a) and *tetraethylammonium bromide* was obtained commercially. *n-Pentyltrimethylammonium bromide* was prepared by condensing n-pentyl bromide with trimethylamine, m.p. 194–195°. Found: C, 45·9; H, 9·5. Calc. for $C_8H_{20}BrN$. C, 45·7; H, 9·5.

n-Butyldimethylethylammonium bromide was prepared by standard reactions from dimethylamine, ethyl bromide and n-butyl bromide, m.p. 208–209·5°. Found: C, 45·8; H, 9·3. Calc. for $C_8H_{20}BrN$. C, 45·7; H, 9·5.

Pharmacological

The isolated phrenic nerve-diaphragm preparation of the rat was used.

Female albinos weighing 200 g. ± 20 g., were dissected as described by Bülbring (1946). The preparation was immersed in a modified Tyrode solution (Taugner and Fleckenstein, 1950) in a perspex organ bath of rectangular cross section, similar to that described by Raventos (1959). The solution, pH 7·2, at 29° ± 1°, was aerated with a mixture of oxygen 95 per cent and carbon dioxide 5 per cent.

The phrenic nerve was stimulated by square wave pulses of 10–20 volts and 0·1–1·0 msec. duration, delivered at a rate of 3/min., were used (Attree, 1950).

The molar concentration in the suspending medium which would produce a 50 per cent reduction of the response to indirect stimulation in 3 min. was determined for each compound. The assumption was made that, over the range of concentrations used, the response increased linearly with the dose. A statistical analysis of the results showed that this was valid.

An increase in the sensitivity of the preparation to the compounds under test was developed whatever the time interval between doses. Consequently, to obtain a reliable estimate of neuromuscular blocking activity, the results were calculated from observations of the effects of four different doses of each compound applied to four different rat phrenic nerve-diaphragm preparations.

Preliminary experiments were made with each compound and suitable doses chosen. Four doses, in constant ratio, which would be expected to produce blocks of between 20 per cent and 80 per cent in 3 min., were selected. The four doses were applied each to four rat phrenic nerve-diaphragm preparations in a randomised order by a Latin square design. Only four doses were applied to any one preparation. The experimental data from the four tissues was analysed statistically (Starmer, 1961). The results are given in Table I.

TABLE I

THE NEUROMUSCULAR BLOCKING ACTIVITIES OF TETRAETHYLAMMONIUM AND ITS ISOMERS ON THE RAT PHRENIC NERVE-DIAPHRAGM PREPARATION

Compound	Molecular weight	Concentration in m-moles/ml. to produce neuromuscular block*	Confidence limits (P = 0·95)	Relative activity T.E.A = 1
Tetraethylammonium ..	210	$3{\cdot}68 \times 10^{-3}$	$(3{\cdot}48–3{\cdot}88) \times 10^{-2}$	1
NN-Diethylpyrrolidinium ..	208	$2{\cdot}27 \times 10^{-2}$	$(2{\cdot}16–2{\cdot}38) \times 10^{-2}$	1·6
1,1 -Spirobipyrrolidinium ..	206	$1{\cdot}19 \times 10^{-2}$	$(1{\cdot}15–1{\cdot}23) \times 10^{-2}$	3·1
Dimethylethylbutylammonium	210	$1{\cdot}83 \times 10^{-3}$	$(1{\cdot}75–1{\cdot}91) \times 10^{-3}$	20
Trimethylpentylammonium	210	$4{\cdot}81 \times 10^{-4}$	$(4{\cdot}47–5{\cdot}17) \times 10^{-4}$	77

* Concentration per ml. of bath fluid required to produce a 50 per cent block in 3 min.

RESULTS AND DISCUSSION

The factors which constitute the total binding force appear to be those suggested by Thomas (1961b) to be important for the adsorption of quaternary ammonium compounds onto acetylcholinesterase.

The explanation for the anomalously low activity of tetraethylammonium at the neuromuscular junction can therefore be explained in terms of stereochemistry and charge delocalisation.

Trimethylpentylammonium bromide. The structure of this compound is such that the conditions for its adsorption onto an anionic receptor are the best possible, three α-carbon atoms being completely available for electrostatic binding whichever way the molecule approaches the surface. Consequently, the coulombic forces would be at a maximum for an aliphatic onium ion. Since the pentyl chain is normal once the onium ion had become associated with the anionic area, it could orientate itself to come into close contact with the surface of the receptor. Thus potentially a van der Waal's bond could be formed between all the carbon atoms of the chain and the atoms of the enzyme surface. Finally, because of the polar–nonpolar asymmetry of the molecule it would be expected that it would concentrate at the surface of a solution. These three factors lead to the conclusion that *a priori* trimethylpentylammonium would be adsorbed onto an anionic receptor more avidly than any other of the compounds examined and, therefore, should be the most active neuromuscular blocking compound of the series.

Dimethylethylbutylammonium bromide. Statistically, the availability of the α-carbon atoms is less with this structure because of the substitution of one of the methyl groups, but the van der Waal's forces and the

concentration of the ion at the surface of the solution would be similar to trimethylpentylammonium. Consequently, dimethylethylbutylammonium should be a weaker neuromuscular blocking agent than trimethylpentylammonium.

Tetraethylammonium bromide. We have already shown that the binding forces between tetraethylammonium and the anionic site on acetylcholinesterase are unusually low, with the result that tetraethylammonium is a very weak inhibitor of acetylcholinesterase. It can be seen from Table I that it is also the weakest neuromuscular blocking agent of the series.

Diethylpyrrolidinium bromide. The structure of this compound is similar to that of tetraethylammonium but two of the ethyl groups are linked through the β-carbon atoms to form a pyrrolidine ring. From a stereochemical point of view the α-carbon atoms are made more available for binding to the anionic site of acetylcholinesterase than with tetraethylammonium. The other factors, such as potential van der Waal's forces and distribution between bulk and interface in solution, should be similar in both tetraethylammonium and *NN*-diethylpyrrolidinium. The effect of the change in structure from tetraethylammonium would be to increase the binding forces between an anionic receptor and *NN*-diethylpyrrolidinium. *NN*-Diethylpyrrolidinium is a more powerful inhibitor of acetylcholinesterase than tetraethylammonium (Thomas, 1961a) and from Table I it may be seen that it has also a more powerful neuromuscular blocking action.

1,1′-*Spirobipyrrolidinium bromide.* In this compound the rotation of all the ethyl groups is restricted with the result that, theoretically, the α-carbon atoms are more available for binding than in the previous compound. Since the other factors are virtually constant it would be expected that 1,1′-spirobipyrrolidinium would be more active than *NN*-diethylpyrrolidinium. However, to offset the advantage conferred by making the molecule more rigid, it may be that the potential van der Waal's forces would be less because the ethyl groups would not be able to orientate towards a surface under the influence of the free energy of the surface atoms of the receptors. On balance, it is difficult to assess which factors would have the greater influence, but the spiran compound should be more active than tetraethylammonium in all tests. It may be seen from Table I that 1,1′-spirobipyrrolidinium is a more powerful neuromuscular blocking agent than tetraethylammonium and it has also been shown (Thomas, 1961a) that the spiran compound is more active as an inhibitor of acetylcholinesterase than tetraethylammonium. However, when *NN*-diethylpyrrolidinium and 1,1′-spirobipyrrolidinium are compared it is seen that the spiran is a more powerful neuromuscular blocking agent (Table I) but less active as an inhibitor of acetylcholinesterase (Thomas, 1961a). This may be taken to reflect the opposition of the two factors.

If the compounds listed in the table are analysed from the point of view of charge delocalisation and stereochemistry the order of activities becomes explicable.

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THE ISOLATION AND IDENTIFICATION OF 1-ETHINYLCYCLOHEXYL CARBAMATE AND ITS METABOLITE FROM TOXICOLOGICAL SPECIMENS

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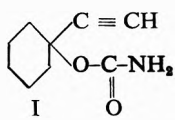
Sensitive tests involving the formation of the mercuric and silver salts of ethinamate (1-ethinylcyclohexyl carbamate) are reported for the detection and identification of μg. quantities of the drug. Conditions for the isolation of this compound and its metabolite from toxicological specimens are described. The infra-red spectra of both the carbamate and its metabolite isolated from a human brain are also given.

Ethinamate (Valmid,1-ethinylcyclohexyl carbamate; I) is a central nervous depressant, hypnotic and sedative which has found popular use in Singapore and is a problem in forensic chemistry. Although several papers have been published on its pharmacological actions (Langecker, Schumann and Junkmann, 1953; Gruber, Kohlstaedt, Moore and Peck, 1954; Franke, 1954; Foltz, Dracos and Gruber, 1955; and Swanson, Anderson and Gibson, 1956) there is a scarcity of chemical methods for its isolation and identification from toxicological specimens.



I

Langecker, Schumann and Junkmann (1953) and Nakamura (1958) have recommended the extraction of ethinamate at pH 8. The disadvantages of extraction at this pH are that many other substances which react with the mercuric chloride test of Langecker and which also interfere with the colour reactions of Nakamura are extracted as well. The precipitation method with ammoniacal silver nitrate of Langecker and others lacks specifiity in that halide, aldehydes and methylpentynol, another commonly used sedative, interfere with the test. Recently Moss and Jackson (1961) have reported a paper chromatographic method for the detection of this compound and other carbamates using furfural and concentrated hydrochloric acid as the colour developing reagents.

This paper describes some sensitive and specific tests for the detection and identification of ethinamate. A study was also made on the metabolite isolated from a human brain.

Experimental

Reagents

Aqueous ethinamate solution: 50 μg./ml. *Ammoniacal potassium mercuric iodide solution:* dissolve 2·5 g. potassium iodide and 5 g. mercuric iodide in 100 ml. of water, add 50 ml. 5 per cent ammonia, boil for 10 min., allow the solution to stand overnight and filter through a filter paper.

Alkaline potassium mercuric iodide solution: dissolve 2·5 g. potassium iodide and 5 g. mercuric iodide in 100 ml. of water; add 50 ml. of 5 per cent potassium hydroxide and filter. 10 per cent *aqueous solution potassium cyanide. 2,4-Dinitrophenylhydrazine solution:* dissolve 3 g. of 2,4-dinitrophenylhydrazine in 20 ml. concentrated sulphuric acid, 60 ml. water and 20 ml. 95 per cent ethanol; allow the solution to stand overnight and filter. 5 per cent *aqueous mercuric chloride solution. Tollen's reagent:* prepare by addition of 125 ml. 0·1 N silver nitrate, 15 ml. 6 N sodium hydroxide, 20 ml. 25 per cent ammonium hydroxide and 90 ml. of water. *Isobutanol:* A.R. grade.

Preparation of Mercuric Salt of Ethinamate

Procedure 1: To a small test tube containing an aqueous solution of ethinamate is added 5 drops of ammoniacal potassium mercuric iodide reagent; this is warmed over a micro burner for 2 min. The mercuric salt of ethinamate is obtained as a white precipitate or gives a turbidity to the solution depending on the amounts of carbamate present. The sensitivity is 10 μg. of carbamate (in 1 ml.). Addition of a drop of 10 per cent potassium cyanide solution dissolves the precipitate. If obtained in mg. quantities, the precipitate can be recrystallised from ethanol: benzene (1 : 1) solution yielding a crystalline product, m.p. 209°. Found N, 5·19. Calc. for $C_{18}H_{24}HgN_2O_4$, N, 5·27.

Procedure 2: Alkaline potassium mercuric iodide solution is used instead of ammoniacal potassium mercuric iodide solution. If ethinamate is present an immediate precipitate is obtained. On warming a brown precipitate is obtained because of the hydrolysis of the carbamate to an ammonium salt which then reacts with the alkaline potassium mercuric iodide to give the brown precipitate.

Bromural, carbromal, formaldehyde, acetaldehyde, formic acid and methylpentynol do not interfere in procedure 1 but do so in procedure 2.

Preparation of 2,4-*Dinitrophenylhydrazone of Cyclohexene Methyl Ketone from the Mercuric Salt of Ethinamate*

The mercuric salt obtained in procedure 1 is centrifuged and the supernatant liquid is pipetted off. To the residue is added 0·5 ml. concentrated hydrochloric acid. The solution is heated over a small flame until the residue dissolves. 0·5 ml. of 2,4-dinitrophenylhydrazine solution is added and the resulting solution is heated to boiling. The 2,4-dinitrophenylhydrazone of cyclohexene methyl ketone is obtained as a red fluffy precipitate. When recrystallised from 95 per cent ethanol it has a m.p. 206°. The limit of detection is 200 μg. of carbamate (in 1 ml.). Found: C, 55·17; H, 5·54; N, 45·81. Calc. for $C_{14}H_{16}N_4O_2$, C, 55·24; H, 5·26; N, 45·70.

Addition Product of Ethinamate and Mercuric Chloride

To an aqueous solution containing the carbamate is added 5 drops of mercuric chloride solution. The addition product is formed as a white precipitate. The sensitivity is 20 μg. of the carbamate (in 1 ml.). The compound can be recrystallised from 95 per cent ethanol, m.p. 195°.

Modified Test for Ethinamate with Tollen's Reagent

One ml. of Tollen's reagent is added to a solution containing the carbamate and allowed to stand for 10 min. A few drops of isobutanol is added and shaken. If ethinamate is present the organic layer attains a reddish brown colour. The sensitivity of this test is 15 μg. of the carbamate (in 1 ml.).

Isolation of Ethinamate from Gastric Lavage

In most cases of poisoning by sedatives or hypnotics stomach washouts are available for toxicological analysis. The carbamate can be isolated by the following procedure.

A suitable portion of the stomach washout is acidified with 10 ml. of 10 per cent hydrochloric acid and the acidic solution is extracted with ether. The ethereal solution is evaporated and the residue tested for the presence of the carbamate. If the residue contains oil the latter can be removed by boiling with water, cooled and extracted with light petroleum (b.p. 40–60°). The light petroleum fractions are rejected and the aqueous layer is extracted with ether. The residue obtained after the evaporation of the ethereal solution is dissolved in the minimum amount of water and tested for ethinamate by the procedures already described.

Isolation of Ethinamate and its Metabolite from Viscera

A death from this drug took place early in 1959 in Singapore. At the time of the investigations there were no indications of the type of poisons taken and hence all the organs were submitted for toxicological analysis. Because only 15 ml. of urine and the same volume of blood were available for examination, no significant conclusions could be obtained from the analyses. The extraction of the drug from the other organs are given below.

Extraction of the stomach. Half of the stomach was macerated, treated with 400 ml. 95 per cent ethanol and 20 ml. 10 per cent tartaric acid and boiled for 1 hr. The alcoholic solution was filtered, evaporated and the residue treated with absolute ethanol. The ethanolic solution was again filtered and evaporated. The residue, an oily substance, was treated with light petroleum which was decanted off. The residue on trituration with 50 per cent ethanol and chilling deposited crystals which were filtered and sublimed under reduced pressure. The melting point of the white sublimate (3·1 g.) was 96–98° alone or mixed with an authenticated sample of ethinamate. The infra-red absorption spectra of the compound in chloroform and potassium bromide were taken and found to be similar to those of the carbamate itself. The substance also gave positive results for all the tests for the carbamate already described.

Extraction of the brain. The whole brain (475 g.) was macerated and digested with 300ml. of 95 per cent ethanol and 20 ml. 10 per cent tartaric acid. The alcoholic solution was filtered, evaporated and the residue was treated with absolute ethanol. The solution was again filtered and evaporated. The residue was taken up in ether and filtered.

The ethereal solution was shaken with 30 ml. 0·1 N hydrochloric acid to remove any basic material that might be present. The ether layer was washed with water, 30 ml. of 0·5 per cent sodium hydroxide solution and finally with water again. The ethereal solution was evaporated and the residue on tituration with 50 per cent ethanol yielded crystals (84 mg.) m.p. 112–116°. On repeated recrystallisation from the same solvent the m.p. was raised to 142°. The crystals can be sublimed under reduced pressure (0·1 mm. Hg) without decomposition. Found C, 83·52; H, 11·74; O, 5·26.

TABLE I

COLOUR REACTIONS OF ETHINAMATE, ITS DERIVATIVES AND METABOLITE

Reagents	Substances			
	Ethinamate (I)	Mercuric salt of (I)	Mercuric chloride addition product of (I)	Metabolite
Sulphuric acid	Bright red	Red to orange	Light yellow	Red
Frodhe	Red	Red to orange	No colour	Orange
Marquis	Orange	Orange	No colour	Red
Erdman	Red to orange	Brown to yellow	No colour	Brown
Mecke	Brown	Brown to yellow	No colour	Violet
Mandelin	Brown to green	Red to yellow	No colour	Orange
Sulphuric acid-vanillin	Red	Red to orange	No colour	Orange
Sulphuric acid-dimethylamino-benzaldehyde	Brown	Red to orange	No colour	Red

This metabolite forms a mercuric salt, mercuric chloride addition product, and a silver salt under the conditions already described. However, its silver salt when treated with isobutanol does not give any coloration to the organic solvent. Like the parent substance this metabolite also gave colour reactions when treated with concentrated sulphuric acid or sulphuric acid-containing reagents (Table I).

The hydrochloric acid and sodium hydroxide washings obtained earlier were basified and acidified respectively and extracted with ether. The residues obtained on evaporation of the ether solutions were treated with the Tollens reagent and no precipitate was obtained from both fractions.

Extraction of the liver and kidney. A portion of the liver (285 g.) and 1 kidney were extracted separately by the same procedure as described earlier for the brain. Only traces of oil were obtained in both cases and attempts to crystallise the oils were unsuccessful. However, the oils gave precipitates with mercuric chloride, Tollens reagent and ammoniacal potassium mercuric iodide, indicating the presence of a terminal acetylenic group in the molecule. The silver salts from kidney and liver extracts gave no colour in isobutanol.

DISCUSSION

The reaction between compounds which possess the grouping $-C{\equiv}CH$ and potassium mercuric iodide in potassium hydroxide solution proceeds according to the following equation (Shriner and Fuson, 1948)—

$$2\,RC{\equiv}CH + K_2HgI_4 + 2\,KOH \rightarrow (RC{\equiv}C-)_2Hg + 4\,KI + 2\,H_2O$$

The disadvantages of using potassium hydroxide are mainly due to the interference of compounds which are easily hydrolysed to ammonia. Ethinamate itself on prolonged standing or on heating in the presence of potassium hydroxide and potassium mercuric iodide gives a brown precipitate. Traces of formaldehyde, formic acid, acetone, acetaldehyde and paraldehyde, which are subtsances commonly encountered in toxicological analysis, also interfere with the test if potassium hydroxide is used together with potassium mercuric iodide. However, when ammonia is

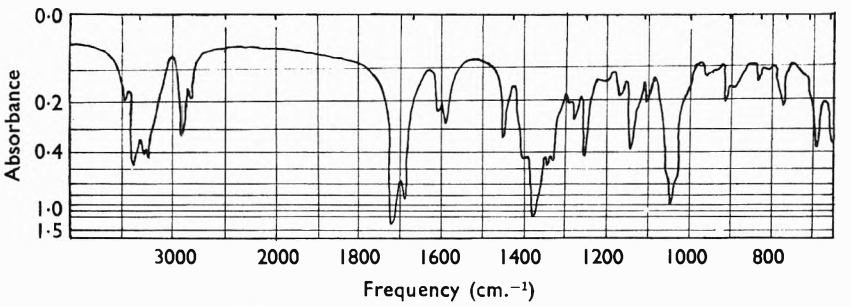


FIG. 1. Infra-red spectrum of ethinamate in potassium bromide.

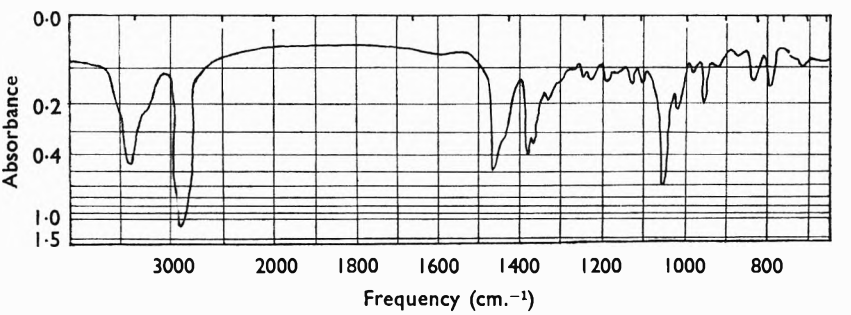


FIG. 2. Infra-red spectrum of the brain metabolite of ethinamate in potassium bromide.

used in place of potassium hydroxide the reagent is specific for the detection of ethinamate under the conditions described. The substances mentioned above do not interfere.

When the mercuric salt is treated with an aqueous solution of potassium cyanide the free carbamate is regenerated and can be extracted with ether and subjected to other confirmatory tests.

Reaction between ethinamate and Tollen's reagent. The disadvantages of using Tollen's reagent for the detection of ethinamate in toxicological specimens are mainly due to interference of halides and aldehydes in the samples. However, when the modified procedure is used it was found that only ethinamate gives a brown precipitate which is soluble in isobutanol. The precipitates formed when Tollen's reagent reacts with halides and aldehydes are not soluble in isobutanol. The modified procedure is therefore specific.

Infra-red Absorption Spectra

The infra-red spectra of ethinamate and its metabolite isolated from the human brain are shown in Figs. 1 and 2. The spectrum of the carbamate shows two intense bands at 1712 and 1685^{-1}. They reflect the vibrational characteristics of the C=O and NH_2 groups of the molecule respectively. In the –CO–NH– region two absorption bands are observed at 1610 and 1590 cm.$^{-1}$. Characteristics C–H and N–H stretching vibrations are observed in the 3500 and 2900 cm.$^{-1}$ regions.

In contrast to the marked absorption of the C=O and NH_2 groups seen in the spectrum of ethinamate these bands are absent in the spectra of the metabolite. This observation clearly indicates the absence of the carbamate group in the molecule of the metabolite. Two strong bands

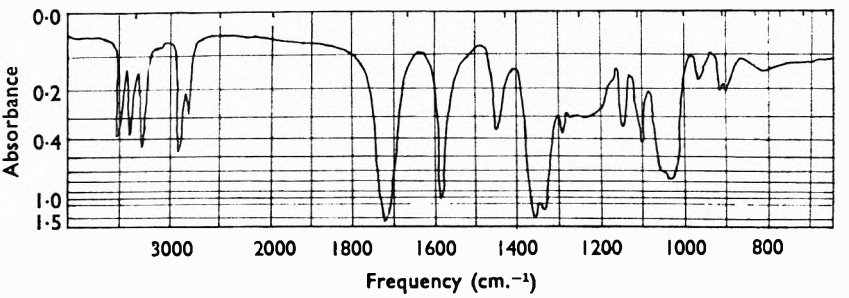


FIG. 3. Infra-red spectrum of ethinamate in chloroform.

are observed in the 3400 and 2900 cm.$^{-1}$ regions which indicate the presence of OH and $-CH_2-$ groups in the molecule. The absence of the carbamate group from the infra-red spectra is in agreement with the elemental analysis which shows the metabolite has an empirical formula of $C_{21}H_{16}O$.

The chemical reactions of the metabolite indicate the presence of a terminal acetylenic group similar to that of ethinamate in the molecule. However both the spectra of ethinamate and the metabolite do not exhibit any characteristic absorption in the 2100–2140 cm.$^{-1}$ region which is caused by the stretching of the carbon-carbon triple bond linkage (Wotiz and Miller, 1949; Wotiz, Miller and Palchak, 1950). In the ethinamate spectra the small band at 3280 cm.$^{-1}$ may be considered as indicative of the stretching of the C–H bond in the –C≡C–H grouping (Bellamy, 1958). The spectrum of a chloroform solution of the carbamate (Fig. 3) gives a better defined band at 3300 cm.$^{-1}$ which can be attributed to the terminal acetylenic group. However with the spectrum of the metabolite the detection of a similar band is made complicated by the fact that this band may be hidden in the broad OH band at 3400 cm.$^{-1}$ region. Attempts were made to acetylate the hydroxyl group of the metabolite to bring out the hidden band at 3300 cm.$^{-1}$ However the acetylated compound could not be obtained in a pure state and hence no conclusion could be drawn from its infra-red spectrum.

Although no less than 6 g. of ethinamate was isolated from the stomach only 84 mg. of a metabolite containing an ethinyl group was obtained from 475 g. of the brain and insignificant amounts from the liver and the kidney. It seems that the ethinyl group is easily metabolised. Experiments with rat tissue slices and on dogs blood bear out the metabolic liability of this structural group (Perlman and Johnson, 1952).

McMahon (1958) and Murata (1960) have recently made extensive studies on the metabolism of ethinamate in man and they have both established that one of the metabolites isolated from the urine is a monohydroxy derivative. The metabolite obtained from the human brain during this study is different from the one obtained by these workers although the structure of the former has not yet been established.

Acknowledgements. I wish to thank Mr. Pwee Sye Chow for technical assistance rendered; Mrs. H. K. Tong, Department of Chemistry, University of Malaya, for carrying out the C and H analysis reported in this paper. I thank Dr. Loke Kwong Hung, Biochemistry Department, University of Malaya for his helpful criticism and the Chief Chemist, Mr. Chia Chwee Leong for his interest in this work. I also wish to record my appreciation to Dr. Leo Levi, Food and Drugs Directorate, Ottawa, Canada for running the infra-red spectra.

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BOOK REVIEW

RADIOACTIVE ISOTOPES IN BIOCHEMISTRY. By E. Broda. Pp. x + 376 (including index). Elsevier Publishing Company, Amsterdam, 1960. Distributed by D. Van Nostrand Company, Ltd., London. 60s.

While application of radioactive isotopes is now commonplace in biochemical research, their intelligent use still demands a smattering of knowledge of the subject of nuclear physics. This book adequately meets the requirement. It is an excellent translation into English of the first German edition published in 1958. It is essentially a concise and scholarly review by a master of his subject rather than a practical manual and touches briefly on all aspects of isotopes of interest to the biochemist. Chapters 1–7 (68 pages) are concerned with general principles including the biology of radiations. Chapters 8 and 9 (57 pages) describe methods of measurement, and the remaining chapters 10–16 (196 pages) quote examples of the application of isotopes to biochemical problems, many of which are classical studies. Stable isotopes, while not treated in detail, are mentioned where appropriate. To assist the reader to devise approaches to specific problems there is an impressive collection of 3190 (not ca. 3700 as claimed) references and a good author and subject index totalling 45 pages.

Any short account of a wide subject can easily be criticised on the grounds of omission or brevity depending on the bias of the reader. Thus the 4 pages dealing with drugs are all too short for the reviewer who was left with the impression that too much attention was paid to the biology of heavy metals. There are few easily detectable typographical errors but some curiosities of terminology appear, for example, the "metabolism of radon" (page 1), "abiosynthesis" (pages 36 and 162) and "super heavy water" (page 139). The book is well produced, not too expensive and can be recommended as an introduction to isotopic methods on a broad basis.

A. McCoubrey.

LETTER TO THE EDITOR

Paper and Gas Chromatographic Analysis of Cannabis*

SIR,—Recent interest in the chemical and physical methods of analysis of cannabis has improved the position of the forensic chemist. Reviews of the work in this field in connection with the classical botanical-colour test procedures, paper chromatography and electrophoresis, ultra-violet and infra-red spectroscopy had been made by Farmilo (1956, 1961) and Farmilo and Genest (1959). Recently we reported the analysis of the essential oil fraction of cannabis by gas liquid partition chromatography, and the use of this procedure in the identification of cannabis (Farmilo, 1960; Martin, Smith and Farmilo, 1961). Further work has just been completed on the gas and paper chromatographic methods applied to the steam volatile oil of fresh cannabis to determine the presence of cannabidiol. The methods are sensitive and sparing of material. Gas chromatography also provides information of relative concentrations of the main cannabinols that have been identified and are now characterised. These are cannabidiol, cannabinol and tetrahydrocannabinol, which often occur in the cannabis materials. Farmilo and others (1960) have shown that the cannabidiol acid, cannabidiol, cannabinol and tetrahydrocannabinol content changes with climatic conditions from northern, through mediterranean to tropical regions. The methods now described will assist in determining the origin of cannabis.

Method of preparation of samples for paper and gas chromatography. The sample of cannabis is dried at room temperature, and, after removal of seeds and stems in the case of flowering tops, it is powdered in a mortar and transferred to a 0·1 ml. centrifuge tube. 10 mg. of hashish is sufficient: larger samples of leafy material may be required. The dry powder is wetted with a few drops of methanol from a micropipette (1 μl.). The mixture is stirred and centrifuged and the supernatant fluid is transferred to a clean dry centrifuge tube (0·1 ml.). The residue remaining in the first tube is dried in a stream of nitrogen, cooled and weighed. From the empty tube weights and the weight of the methanol used the concentrations of the sample can be obtained. The solution of cannabis in methanol is relatively free of nonacosane, and if desired can be further purified by freezing at dry-ice acetone temperatures which removes further plant waxes.

Recent work in this laboratory with the Research Specialties Co., Gas Chromatographic apparatus equipped with a beta ray ionisation detector has been carried out under the following conditions.

A methyl-silicone gum rubber (3 per cent SE-30) on Chromosorb W column packing was activated by heating at 225° for 12 hr. in the argon gas stream in the chromatographic column oven, and then at 325° for 24 hr. without the gas flow. A twenty or thirty inch column was used, at 174–190°, for the assay of the samples. The argon carrier gas flow rate was 100 ml./min. Typical sample sizes that have been used are: steam distilled oils, 0·5 μl.; light petroleum (30–60°) extracts of fresh and dry green leaf and flowering tops, 40 μg.; hashish extracts with methanol, 3 μg.; with light petroleum, 10 μg. Police seizures from northern countries involving green plant parts found in reefers, 10 to 40 μg. depending on the quality of the product. Standards of cannabidiol, cannabinol and tetrahydrocannabinol gave good chromatograms at 0·5 to 1 μg.

The method of de Ropp (1960) with minor modifications was used for paper chromatography of the cannabis extracts. The solvent was cyclohexane

* Cannabis means and includes the flowering tops (bhang, Kif, etc.), the resin (hashish, Charis, etc.) of the plant *Cannabis sativa* L. and its varieties.

saturated with dimethylformamide, and the Whatman No. 1 paper on which the sample was spotted was saturated with the lower dimethylformamide layer. The paper should not be allowed to dry before development. It is sufficient to blot the excess dimethylformamide before chromatographing. Detection and identification with diazotised *p*-nitraniline and the Gibbs reagent recommended by Korte and Seiper (1960) have been found to be satisfactory for police work. The system is affected by draughts and ambient temperature changes.

The gas chromatography retention values (RT-values) for cannabidiol, tetrahydrocannabinol and cannabinol are 9·3, 13·5 and 18·3 min. at 174°/30 in. and 109 ml./min. On standing the cannabidiol standard developed a new material which gave a peak at 8·5 min. The material was also found in an extract from Canadian hemp. Pyrahexyl has an RT-value of 23·5 min. for the main band at 180°/30 in. and 100 ml./min. The relative retention time values (RRT-values) for tetrahydrocannabinol in terms of cannabidiol are 1·83, 1·58 and 1·42 for Pyrahexyl, natural tetrahydrocannabinol and synthetic tetrahydrocannabinol respectively. When using light petroleum or methanol extracts of cannabis it is recommended that RRT-values relative to cannabinol be used for identification purposes, i.e. 0·55 and 0·76 for cannabidiol and tetrahydrocannabinol respectively.

The R_F values of cannabidiol, cannabinol and tetrahydrocannabinol are 0·12, 0·36 and 0·56. These are obtained from the orange to yellow coloured spots given by the diazo reagent. Cannabidiol acid did not stain with this reagent but gave a light blue colour under ultra-violet light at 3660 Å, the spot having an R_F value of 0·06. At least ten phenolic compounds are present in the light petroleum extract, which provides more points of comparison and identification. A complete analysis of seven samples takes 4 hr. About 20 to 40 μg. of the standards and extracts are required for identification.

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