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REVIEW

Salicylate and enzymes

M. J. H. SMITH AND P. D. DAWKINS

Department of Biochemical Pharmacology, King's College Hospital Medical School, Denmark Hill, London, S.E.5, U.K.

Salicylate inhibits the activities of a number of cellular enzymes and in some instances the mechanisms of inhibition have been established (Smith, 1968a). Reported inhibitory actions of the salicylate ion on important enzyme systems *in vitro* are now reviewed and assessed in relation to the known clinical and toxic effects of the drug.

RELATION BETWEEN *in vitro* INHIBITIONS AND *in vivo* EFFECTS

The demonstration that salicylate, or indeed any drug, inhibits the activity of an enzyme *in vitro* is not sufficient evidence that one or more of the *in vivo* actions of the drug are due to such inhibition. The composition of *in vitro* incubation mixtures are varied over much wider ranges than those existing in body cells. The relative proportions of enzyme, substrates and cofactors and the pH and ionic constituents of the incubation media may bear little resemblance to physiological requirements. There may be foreign chemicals present either to activate the system or to trap reaction products. Such extreme and artificial conditions are frequently used to obtain an enzyme activity that can be measured conveniently. Any inhibition observed under *in vitro* conditions so different from those obtaining *in vivo* must obviously be interpreted with caution. Thus, *in vivo*, the concentration of the drug may vary with both tissue and time in the body whereas *in vitro* it remains effectively constant. The following factors must be taken into account when considering the possible relation between a particular enzyme inhibition and a clinical or toxic action of the salicylates.

Tissue concentrations of drug

If an interaction between salicylate and an enzyme is to be relevant to an *in vivo* effect then it must be elicited at drug concentrations equivalent to those occurring in the body tissues of man or experimental animals. It is not possible to predict accurately the tissue concentrations of salicylate from a knowledge of the exact size of the ingested dose since, in individual subjects, factors such as absorption from the gut and subsequent distribution among the various body fluids, organs and tissues are neither simple nor standard.

The total plasma salicylate concentrations observed during therapy or found in acute salicylate poisoning have been used as the basis for comparison (Smith, 1966). However, no allowance is made for the variable degree of binding of salicylate to the circulating proteins. The drug is largely bound to serum albumin in man and only a fraction is available to enter the body cells and subsequently determine the tissue concentrations. The accurate measurement of this fraction is not easy. The analytical methods, including equilibrium dialysis, ultrafiltration, gel filtration and frontal elution techniques, yield different results on the same sample. The ratio of protein-bound to free, i.e., unbound, salicylate varies with the total salicylate concentration, a greater proportion being bound at low than at high salicylate

concentrations (Davison & Smith, 1961). At a total plasma salicylate concentration of 0·1 mM, approximately 1% of the drug is in the free form and this increases to 10% at 1 mM, 25% at 3 mM and 30% at 5 mM. Table 1 indicates the relation between the dose of salicylate and the total and free plasma salicylate concentrations expected to occur in man. It is the plasma concentrations of the free salicylate that must be used to assess whether an *in vitro* effect of salicylate is of real or potential importance *in vivo*. However, there are some normal subjects and patients who may show higher plasma concentrations of free salicylate than would be predicted from Table 1. At least two types of binding sites are concerned in the interaction of salicylate and albumin and some human albumin molecules appear to possess fewer binding sites than others (Moran & Walker, 1968). There are also conditions such as infancy, acute infections and rheumatoid arthritis which are associated with hypoalbuminaemia.

Table 1. *Plasma salicylate concentrations in man.*

Situation	Dose		Maximum plasma salicylate concentration (mM)	
	Nature	mg/kg (70 kg man)	Total	Free*
Analgesia	Single dose 1 g aspirin	15	0·5	0·005
Therapy of rheumatoid arthritis	Divided doses 6–8 g sodium salicylate or aspirin/day	100	1·5–2·5	0·15–0·60
Acute poisoning	Single dose 30 g or more of aspirin	400 or more	3·0–10·0	1·0–5·0

* In this and the subsequent Table the term free salicylate refers to that fraction of the drug which is not bound to either circulating or tissue proteins.

The human and animal data indicate that for an *in vitro* action of salicylate as an inhibitor of an enzyme system to be relevant to its clinical effects it must occur in the range 0·005 to 0·6 mM and to be implicated as the basis of one or more of the major toxic symptoms it must be elicited at concentrations between 0·6 and 5 mM.

Mechanism of inhibition

All enzyme inhibitions are the result of an interaction between the inhibitor and some component of the enzyme system. Inhibitors are usually categorized as being either reversible or irreversible. Reversibility implies that equilibrium is set up between the enzyme system and inhibitor. Thus the enzyme activity of an inhibited system returns on merely removing the inhibitor by dialysis or similar means.

An important characteristic of reversible inhibitors is that a definite degree of inhibition, dependent on the concentration of the inhibitor, is usually reached quite rapidly. Thus the extent and duration of the inhibitory action *in vivo* will depend, at least in part, on the inhibitor concentrations attained and maintained with time. The total and unbound salicylate concentrations in several mouse tissues over a period of several hours after the injection of a single dose of the drug have been determined (Sturman, Dawkins & others, 1968; McArthur, Dawkins & Smith, 1970). Corresponding data for man are not available but an approximate guide to

the expected concentrations of unbound salicylate in the plasma after varying single doses of salicylate is given in Fig. 1. The maximum plasma concentrations of unbound drug are reached quite rapidly, within 2–4 h, even after oral administration and the persistence of the drug in the circulation is dose-dependent (Levy & Leonards, 1966). The apparent half-life of salicylate elimination is about 2–4 h with a small analgesic dose but increases to 15 to 30 h with large toxic doses of the drug, and will vary with dose in the intermediate range of doses. Chronic administration of the drug will cause fluctuating plasma concentrations depending on the size and spacing of the doses. In the treatment of rheumatoid arthritis (Table 1) the dosage regime is designed to maintain the concentrations of unbound salicylate in the plasma between 0·15 and 0·60 mM for several weeks.

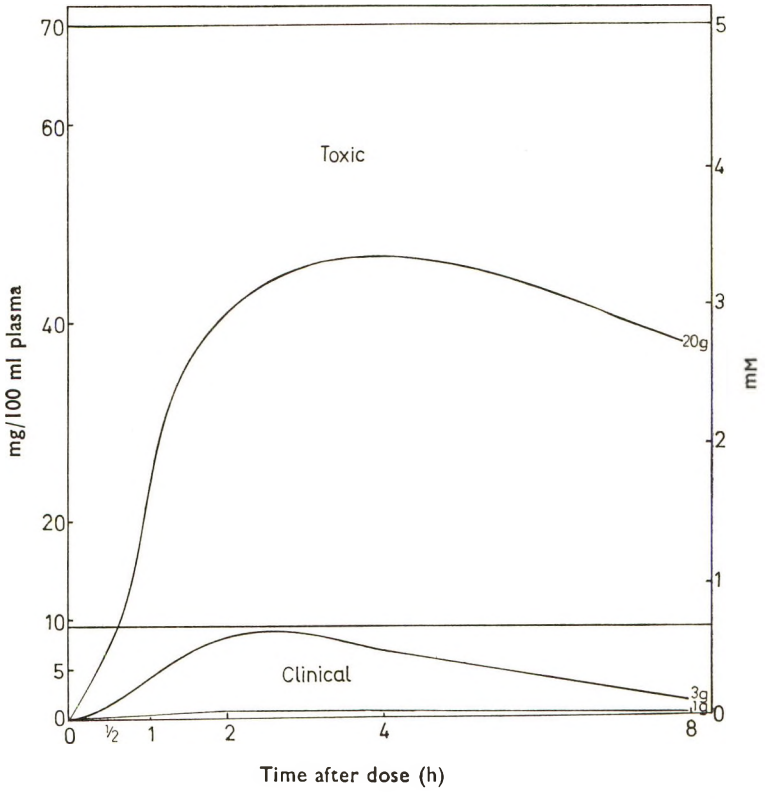

FIG. 1. Concentrations of free salicylate in human plasma after the oral administration of single doses of either 1, 3 or 20 g of sodium salicylate or aspirin.

If the inhibitor acts by a reversible competitive mechanism, involving a substrate or coenzyme, then the endogenous concentrations of the components of the enzyme system become of importance. For example, salicylate inhibits certain dehydrogenase enzymes *in vitro* by competing with the appropriate pyridine nucleotide coenzyme. The degree of inhibition achieved in any tissue *in vivo* will vary not only with the salicylate concentration but also with the coenzyme concentration in the tissue. Thus although the tissue concentration of drug may produce an initial inhibition of an enzyme activity *in vivo* this will progressively decrease with time even if the concentration of drug in the tissue is maintained. This effect will be superimposed on and enhance the diminishing degree of inhibition resulting from the elimination of the drug from the tissue.

Irreversible inhibition, in contrast to the reversible type, is characterized by a progressive increase with time ultimately reaching complete inhibition even with very dilute inhibitor, provided that the inhibitor is in excess of the amount of enzyme present (Dixon & Webb, 1964). A single dose of a drug, which is an irreversible inhibitor of an enzyme, will cause a gradual loss of the enzyme activity, the extent of which depends on the concentration of drug attained in the tissues and its rate of removal by elimination and biotransformation. The enzyme activity will return to normal at a rate depending on the rate of biosynthesis of new enzyme and the rates of synthesis of enzymes in mammalian tissues vary over a wide range (Schimke, 1969). The situation will be different if the drug is given in repeated, if smaller, doses. A balance will be set up between the rate of inactivation of the enzyme by the drug and the rate of synthesis of fresh enzyme leading to new levels of enzyme activity in the tissues. These levels may be either lower than the pretreatment values or higher if induction of the enzyme occurs.

Irreversible inhibitors form covalent bonds with enzymes. Carbamyl phosphate inactivates some enzymes by acylating the enzyme protein and it has been suggested that salicylates may act similarly (Grisolia, Santos & Mendelson, 1968). The term "chemotrophic regulation" has been used for such interactions of reagents with enzymes on sites other than the active centre and it has been assumed that such altered proteins are more susceptible to degradation by proteolytic enzymes. The formation of an initial complex between the inhibitor and the active site of an enzyme followed by the subsequent reaction of part of the inhibitor molecule and the enzyme protein to form a covalent bond compound has been proposed by Baker (1967). This type of irreversible inhibitor is described as "active-site-directed" and the chemical reactions between the inhibitor and enzyme protein include alkylation, acylation and phosphorylation. Baker, Lee & others (1960) suggested that certain substituted salicylates inhibit lactate and glutamate dehydrogenases by this mechanism although salicylate itself behaves as a strictly reversible competitive inhibitor.

The loss of activity of enzymes *in vitro* in the presence of high concentrations (>50 mM) of salicylate and other aromatic anions may be due to denaturation. This is associated with changes in the solubility and other similar properties of the enzyme protein and will be grossly evident if the proteins precipitate. Denaturation implies that a spatial change, i.e., a modification of either the secondary, tertiary or quaternary structure of the enzyme has occurred (Joly, 1965) leading to loss of activity. Inhibition is assessed and classified by kinetic measurements and in some instances, such as that of chemotrophic regulation, the distinction between the two terms is more apparent than real.

Intracellular reserve of enzyme

A major difference between *in vitro* and *in vivo* studies on enzyme inhibition is that in living cells an enzyme normally functions as one step in a chain. Its variation in activity in the cell is governed not only by the varying intracellular concentration of its substrates, which are, in turn regulated by the actions of other enzyme systems, but also by its potential catalytic activity. Some enzymes, termed equilibrium enzymes (Krebs, 1969), normally function at only a fraction of their maximum capacity and their potential activity is far in excess of the flux rate of their substrates. This excess enables them to establish near-equilibrium between starting materials and end products not only when the flux rate of the material varies widely, but also when a substantial proportion of the enzyme has been inhibited. For example, a 90% inhibition of fumarase is not likely to affect the flux rate of metabolic intermediates through the tricarboxylic acid cycle. On the other hand there are enzymes, the non-equilibrium enzymes, that normally function at near their maximum capacities

and any variation in these capacities is liable to affect the metabolic pathways in which they catalyse individual steps. Such enzymes are potentially rate-limiting and inhibition of their activities *in vivo* is of much more significance. The interpretation of the possible relation between *in vitro* inhibition and *in vivo* effects should therefore, take into account whether the enzyme being studied does or does not establish near-equilibrium in the tissues.

Intracellular compartmentation of enzymes

Enzymes are not distributed homogeneously throughout a cell but tend to be concentrated, rather than exclusively localized, in certain compartments or subcellular structures. The *in vivo* effects of drugs must therefore depend on the relative

may affect the intercompartmental barriers between enzymes and substrates and thus change metabolism without any direct effect on the enzymes involved. This is an accepted mechanism for the stabilization of lysosomal membranes by cortisol and chloroquin both *in vitro* and in living cells (Weissmann, 1967). A similar mode of action of salicylate as an anti-inflammatory agent has been suggested (Duthie, 1963) but recent work (Harford & Smith, 1970) showed that the only effect of the drug in concentrations above 1 mM is to cause an increased release of the enzymes *in vitro*.

ENZYME SYSTEMS INHIBITED BY SALICYLATE *in vitro*

In this section the reported effects of salicylate on enzyme systems are reviewed. Emphasis is placed on those inhibitions that may be related to the known metabolic, pharmacological, clinical and toxic effects of the drug. Some degree of selection has therefore been inevitable. Whenever possible an account of the discovery of each effect is followed by a description of the mechanism of inhibition and a discussion of its possible relevance to known *in vivo* actions of salicylate.

Oxidative phosphorylation reactions

The discovery of this important effect of salicylate on cellular metabolic processes has followed a time-honoured and traditional approach to the study of drug action. It was observed that administration of the drug to man (Cochran, 1952) caused a marked and progressive increase in the oxygen consumption. This effect was searched for in systems of decreasing organization and complexity until the subcellular level was reached. Salicylate was found to uncouple oxidative phosphorylation reactions in respiring mitochondrial preparations (Brody, 1956) and it appeared that the drug interfered with one or more of the sequence of enzymes involved in the phosphorylation of ADP which occurs subsequent to the electron transport chain. However, the exact site of action has not been unequivocally defined. This is not an unknown situation with uncoupling reagents principally because the mechanism of oxidative phosphorylation itself remains to be established (Boyer, 1968). It has been suggested that salicylate acts by increasing the permeability of the mitochondrial membrane to ATP causing an enhanced rate of its destruction by ATP-ase (Charnock & Opit, 1962). However, it has been shown that salicylate uncouples in mitochondrial fragments, i.e., in the absence of an intact mitochondrial membrane (Miyahara & Karler, 1965). The observation of Penniall (1958) that the terminal phosphorylation, associated with the oxidation of cytochrome c, is the most sensitive to the uncoupling action of salicylate is also consistent with a direct mechanism of action involving the phosphorylation enzymes.

There is no doubt that salicylate at concentrations in the range 0·2 to 2·0 mM, which is within that observed in the unbound form in the plasma of patients either receiving therapy or with acute poisoning (Table 1), uncouples oxidative phosphorylation reactions in mitochondrial suspensions (Brody, 1956; Packer, Austen & Knoblock, 1959; Jeffrey & Smith, 1959). Furthermore it has been established that this action is reversible (Charnock, Opit & Hetzel, 1962; Whitehouse, 1963). It has also been possible to show that the drug in concentrations of 0·1 mM and above decreases the content of ATP and creatine phosphate and increases the concentrations of inorganic phosphate in isolated rat muscle preparations (Smith & Jeffrey, 1956).

However, the direct demonstration of an uncoupling action cannot be accomplished in the whole animal. The administration of salicylate, in doses of 250 to 750 mg/kg body weight, in the rat does not reduce either the normal ATP concentrations in the liver and paw or the elevated ATP concentrations in paws rendered oedematous by pretreatment with dextran (Slater & Sawyer, 1966; Kalbhen, Domenjoz & Ehlers, 1967; Slater & Delaney, 1970). Some authors have reported morphological changes in liver mitochondria in animals given chronic doses of salicylate (Gutowaka-Grzegorczyk & Kalczak, 1968) but others have not observed changes in the liver mitochondria characteristic of uncoupling after single doses of the drug (Bullock, Delaney & others, 1970). The most probable explanation is that, unlike isolated mitochondria and rat diaphragm muscle, the whole animal can maintain ATP levels in the presence of salicylate. Under normal physiological conditions the processes of catabolism are not proceeding at a maximum rate and can show a compensatory increase if the efficiency of oxidative phosphorylation is impaired. Greater amounts of substrate are sacrificed to supply energy and to maintain ATP levels. In addition ATP may be formed by anaerobic phosphorylation reactions at the substrate level which are not affected by uncoupling reagents. This has been demonstrated in an *in vitro* system by Kalbhen & Domenjoz (1969) who showed that when neoplastic mast cells, which rely on glycolysis for most of their energy production, are incubated with salicylate, their level of ATP increases although the efficiency of ATP production by the respiratory chain is reduced.

A reduction in ATP levels in body tissues, resulting from a direct action of the drug on the phosphorylation of ADP, cannot therefore be the mechanism by which salicylate exerts either its clinical anti-inflammatory effects in rheumatoid patients (Whitehouse, 1968) or its hypoglycaemic action in patients with diabetes mellitus (Manchester, Randle & Smith, 1958). The effects of an uncoupling action due to salicylate which become evident either in experimental animals or in man are those that are caused by the compensatory increase in body catabolism. Included here are the increased oxygen consumption, the depletion of liver glycogen, the increased production of heat at the tissue level and the increased metabolic production of carbon dioxide. The increased production of heat is responsible for the dangerous hyperpyrexia which is a prominent symptom of salicylate poisoning in infants (Segar & Holliday, 1958). The enhanced rate of oxidation of both endogenous and exogenous substrates leads to augmented production of CO_2 which as a pure action can produce respiratory acidosis but more often either exaggerates or antagonizes other effects of toxic amounts of salicylate on acid-base balance (Winters, 1963).

Dehydrogenases

The oxygen uptake of isolated tissue preparations shows a biphasic response to exposure to increasing salicylate concentrations. An initial stimulation, due to the uncoupling effect of the drug, is succeeded by a marked depression of the oxygen consumption (Smith, 1958). The latter response suggested that respiratory enzymes,

less sensitive to the drug than the oxidative phosphorylation reactions, were being inhibited by the higher salicylate concentrations. Studies on the oxidation of intermediary metabolites by tissue homogenates (Kaplan, Kennedy & Davis, 1954) and the transfer of radioactivity from labelled metabolites in mitochondria (Bryant, Smith & Hines, 1963) indicated that 5–7 mM salicylate inhibits dehydrogenase enzymes in the tricarboxylic acid cycle. Later work (Baker, Lee & others, 1960; Bargoni, 1964; Hines & Smith, 1964; Smith & Sturman, 1967; Grisolia, Mendelson & Diederich, 1969) showed that the drug inhibits a wide variety of pyridine nucleotide-dependent dehydrogenases.

Euler & Ahlstrom (1943) reported that salicylate inhibited the activity of lactate dehydrogenase and that the inhibition could be reversed by the addition of coenzyme. This general mechanism also applies to the other dehydrogenase enzymes. Later work (Dawkins, Gould & others, 1967) has established kinetic constants for selected enzymes. It has been suggested (Grisolia & others, 1968) that several dehydrogenases are inhibited by salicylate by chemotrophic regulation (see p. 732), and the topic has produced an atmosphere of brisk controversy (Smith, 1968b; Grisolia, 1968). The most serious objection is that in order to demonstrate the existence of such an effect it is necessary to incubate dehydrogenase enzyme preparations for prolonged periods in the presence of salicylate concentrations which, if the kinetic constants are correct, should either largely or completely inhibit the enzyme activity. The inhibitory action of salicylate on glutamate dehydrogenase *in vitro* does not involve competition with the pyridine nucleotide coenzyme, NAD, (Gould, Huggins & Smith, 1963) but may be caused by the drug producing a dissociation of the enzyme molecule into subunits (Villela & Calcagnotto, 1970).

The pyridine nucleotide-dependent dehydrogenases are not very sensitive to inhibition by salicylate *in vitro*, the inhibitor constants ranging from 1·5 mM for liver alcohol dehydrogenase to 70 mM for the glucose-6-phosphate dehydrogenase of human red cells. Other oxidase enzymes, including succinate dehydrogenase and the L-amino acid oxidases, require high concentrations of the drug (>10 mM) to interfere significantly with their activities *in vitro* (Hines, Bryant & Smith, 1963). The only *in vivo* effects of salicylate that could be related to an inhibition of dehydrogenases would be those that occur after the administration of large toxic doses of the drug. The depressed oxygen consumption, which occurs terminally in animals given lethal amounts of salicylate, belongs to this category. A further example may be the accumulation of organic acids, including malate and citrate, in the plasma of patients with acute salicylate intoxication (Dienst & Greer, 1967). The principal disturbance in acid-base balance in the human infant (Done, 1963) and in some adults (Brown & Proudfoot, 1969) poisoned with salicylate, is a metabolic acidosis caused by the accumulation of organic acid anions in the plasma. The organic acid anions may accumulate because of other actions of the drug (see p. 736) but an inhibition of cellular dehydrogenases may be a contributory factor.

Aminotransferases

Radioactive glutamate accumulates in tissue preparations utilizing labelled pyruvate in the presence of salicylate, suggesting that the drug interferes with the further metabolism of the amino-acid (Huggins, Smith & Moses, 1961). The most important quantitative pathway for glutamate metabolism in animal tissues is that controlled by aminotransferase enzymes and it was shown subsequently (Steggle, Huggins & Smith, 1961; Yoshida, Metcoff & Kaiser, 1961; Gould & Smith, 1965a) that salicylate inhibits the activity of a number of aminotransferase activities in rat serum and in rat isolated tissues.

The mechanism of inhibition is reversible and involves competition with the amino- and 2-oxoacid substrates of the enzymes (Gould, Dawkins & others, 1966). Some of the enzymes, e.g., alanine aminotransferase are relatively sensitive to the drug, the K_I for 2-oxoglutarate being 0·6 mM, whereas others resemble the pyridine-nucleotide dehydrogenases.

The intraperitoneal injection of single doses of salicylate, in the range 150–600 mg/kg, has been observed to cause changes in the pattern and pool sizes of mouse liver amino-acids consistent with the drug differentially inhibiting aminotransferase activities (Dawkins & Smith, 1971). On the other hand, the acute or chronic administration of the drug either to man or to experimental animals increases the activities of alanine and aspartate aminotransferases in the serum and tissues and of tyrosine-2-oxoglutarate aminotransferase in the liver (Manso, Taranta & Nydick, 1956; Janota, Wincey & others, 1960; Singer & Mason, 1965; Vaughan, Steele & Korty, 1969). These findings bear little, if any, relevance to the clinical actions of salicylate but a disturbance of amino-acid interconversions caused by large amounts of salicylate may contribute to the amino-aciduria observed in acute salicylate poisoning in man (Elliot & Murdaugh, 1962; Andrews, Bruton & de Baare, 1962; Ben-Ishay, 1964) and in the rat (Berry & Guest, 1963).

Decarboxylases

The actions of salicylate on the enzymes that decarboxylate amino-acids have been studied in attempts to explain the mechanism of the convulsions that occur in acute salicylate intoxication in children and the anti-inflammatory effects of the drug. Glutamate decarboxylase was investigated because alterations in the ratio between glutamate and γ-aminobutyrate in various regions of the central nervous system may induce convulsions. However, the enzyme from bacteria and in homogenates of rat brain is only inhibited by high concentrations (>10 mM) of the drug (Gould & Smith, 1965b; McArthur & Smith, 1969).

The mammalian histidine decarboxylases are more sensitive to the drug *in vitro*. These may be subdivided into the specific enzyme, which acts on histidine only, and the non-specific L-amino-acid decarboxylase which is also capable of decarboxylating L-5-hydroxytryptophan and L-dihydroxyphenylalanine. Salicylate inhibits both types of enzyme activity *in vitro*, the specific enzyme by a mechanism involving competition with the pyridoxal phosphate coenzyme, the K_I being 2·7 mM, and the nonspecific enzyme by competing with the amino-acid substrates, the K_I being 1·5 mM (Skidmore & Whitehouse, 1966a; 1967).

One important implication of these findings is that salicylate could inhibit the biosynthesis of histamine *in vivo* (Whitehouse, 1968) and this could be the mechanism of its clinical anti-inflammatory actions. Schayer (1965) has argued that newly synthesized histamine is the major mediator of inflammatory responses and has observed raised levels of histidine decarboxylase activity during inflammation. It seems to be generally accepted that histamine is the initial pharmacological mediator of the vascular changes associated with inflammatory reactions but its action is transient and the participation of other mediators is necessary to maintain and extend inflammation (Spector & Willoughby, 1968). This does not exclude the possibility that salicylate could modify inflammatory reactions by interfering with the biogenesis of histamine in the tissues. It must also be remembered that the experimental work on tissue mediators of inflammation is virtually restricted to acute inflammatory responses induced by a bewildering variety of stimuli and may bear little, if any, relevance to the chronic inflammatory reactions characteristic of human rheumatic disorders. However, the evidence that salicylate is an anti-histamine due to its inhibitory action on the histidine decarboxylases is not

convincing. In favour is the observation that the oral administration of 4 g of aspirin per day halves the urinary excretion of histamine in patients with rheumatism and in normal subjects (Sjaastad & Sjaastad, 1965). However, the administration of 400 mg/kg of salicylate to the rat increased the levels of the enzyme in gastric tissue by several fold (Petillo, Gulbenkian & Tabachnick, 1969). Again the administration of salicylate to some patients with chronic urticaria exacerbates the condition. This effect is dependent on the size of the salicylate dose and seems to be caused by an increased rate of formation and release of histamine (Hamrin, 1957).

Aminoacyl-tRNA synthetases

The discovery of the uncoupling effect of salicylate on oxidative phosphorylation reactions stimulated several studies of the possible actions of the drug on biosynthetic reactions requiring ATP. One of these was the incorporation of amino-acids into the protein of isolated tissues. It was shown that salicylate, in high concentrations, 10–15 mM, inhibits the *in vitro* incorporation of radioactive amino-acids into the protein of rat costal cartilage (Bellamy, Huggins & Smith, 1963) and into the epithelial glycoproteins of sheep mucosal scrapings (Kent & Allen, 1968). Protein biosynthesis in the rat isolated diaphragm is more sensitive to the drug and salicylate concentrations from 0·5 to 5 mM interfere with the incorporation of a variety of amino-acids into protein (Manchester & others, 1958; Dawkins, Gould & Smith, 1966). The next steps were to show that the effect occurred in cell-free preparations, at salicylate concentrations of 0·3 mM and above, and that it was independent of the uncoupling action (Dawkins, Gould & Smith, 1966; Reunanen, Hanninen & Hartiala, 1967).

The mechanism of the inhibitory action of salicylate on protein biosynthesis *in vitro* involves a differential action of the drug on the formation of aminoacyl-tRNAs (Burleigh & Smith, 1970). The most sensitive aminoacyl-tRNA synthetases are those that incorporate glutamate, aspartate and histidine, and the drug shows a simple competitive inhibition with respect to the amino-acid substrate.

It requires high toxic doses of the drug to interfere with protein biosynthesis in the adult animal. Thus the intraperitoneal injection of 600 mg/kg significantly inhibits the incorporation of radioactive amino-acids into liver proteins of the mouse (Dawkins, McArthur & Smith, 1971). Young animals exhibiting active growth are more sensitive to the drug. The immature rat shows a decreased rate of weight grain and skeletal growth when given an injection of either sodium salicylate or aspirin between 250 and 300 mg/kg (Limbeck & others, 1966) and young chicks react similarly (Nakaue, Weber & Reid, 1967; Thomas, Nakaue & Reid, 1967).

The relative insensitivity of general protein synthesis *in vivo* does not exclude the possibility that the formation of specific proteins may be preferentially inhibited by the drug. Amongst these may be the immunoglobulins. Ambrose (1966) has shown that salicylate inhibits secondary antibody responses *in vitro* although the drug is only considered to possess weak immunosuppressive activity in the whole animal (Griswold & Uyeki, 1969). The biosynthesis of collagen in granulation tissue induced by carrageenan in the rat is inhibited by salicylate *in vivo* whereas the formation of non-collagen protein is not altered (Fukuhara & Tsurufuji, 1969). Other experimental work with collagen in the intact rat (Trnavska, Trnavsky & Kuhn, 1968) suggests that the administration of 300 mg/kg of salicylate increases the turnover of the protein, implying more rapid rates of both synthesis and breakdown. Salicylate inhibits collagen biosynthesis in tissue culture preparations and in cell-free systems (Rokosova-Cmuchalova & Bentley, 1968) but the mechanism is concerned, at least in part, with the capacity of the drug to chelate ferrous ions (Nakagawa & Bentley, 1969).

Proteases

Proteolytic enzymes may be involved in inflammatory reactions at several points. They may act on protein precursors of tissue mediators causing the release of polypeptides. The later stages of rheumatoid joint disease may involve the disruption of intra-articular lysosomes causing the release of hydrolase enzymes, including the proteolytic cathepsins, which degrade the protein-polysaccharide complexes of cartilage. The lysosomal enzymes may also degrade auto-immune bodies and the tissue debris resulting from a continuing inflammatory process.

Studies with general proteolytic enzymes, such as trypsin and chymotrypsin, have not provided evidence that salicylate is an effective inhibitor. The K_i for chymotrypsin is 9·0 mM (Skidmore & Whitehouse, 1966b) and the inhibitory effect of the drug on trypsin-induced casein breakdown is not impressive (Morsdorf, 1965). The autoproteolytic process that occurs in fresh homogenates of rat paw tissue is more sensitive to salicylate but it requires 5 mM drug to produce an approximately 50% inhibition (Morsdorf, 1965). It was claimed that kallikrein, the enzyme that releases kinins from circulating globulins, was inhibited by salicylate *in vitro* (Northover & Subramanian, 1961) but this has been disproved by Hebborn & Shaw (1963), Davies, Holman & others (1966) and Bertelli, Donati & Marek (1969). Some of the chrondrolytic enzymes are inhibited by 1 to 2 mM salicylate (Simmons & Chrisman, 1965) both *in vivo* and *in vitro*. No information appears to be available about the possible inhibitory effects of the drug on individual lysosomal proteases and such data must await the further purification and characterization of these materials.

Ribonucleic acid polymerases

The reports (Warkany & Takacs, 1959; Larsson, Ericson & Bostrom, 1963) that the administration of salicylate to pregnant rats caused several congenital malformations of the litters carried to full term stimulated interest in the effects of the drug on nucleic acids. One finding (Kazakova & Chebotar, 1969) is that sodium salicylate induces conformational changes in DNA prepared from rat liver nuclei and mitochondria. A second interaction of the drug and nucleic acids is its inhibitory effect on rat liver DNA and RNA polymerase activities *in vitro* (Janakidevi & Smith, 1969). The DNA polymerase activity was more sensitive to the drug being inhibited by salicylate concentrations of 1 mM. However, incubation of rat foetuses with 2 mM salicylate revealed that RNA synthesis, assessed by measurement of the polymerase activity and the incorporation of labelled orotic acid, was inhibited whereas DNA synthesis, followed by the incorporation of radioactive thymidine, was not (Janakidevi & Smith, 1970a). Later work showed that only the RNA polymerase stimulated by ammonium sulphate and manganese ions was sensitive to salicylate (Janakidevi & Smith, 1970b). This enzyme is thought to be responsible for the biosynthesis of certain RNA species, including messenger RNA, and the administration of 400 mg/kg of salicylate to adult mice preferentially interfered with the formation of rapidly labelled RNA (Janakidevi & Smith, 1970c). Salicylate also inhibits the incorporation of radioactive uridine into the nucleic acids of human peripheral lymphocytes (Packman, Esterly & Peterson, 1969).

The mechanism by which salicylate inhibits nucleic acid polymerase activities is unknown and it will be necessary to solubilize and purify the enzymes as a preliminary step in the investigations. The drug differs from other inhibitors such as actinomycin D and aflatoxin B_1 and resembles α-amanitin in only inhibiting the Mn^{2+}: ammonium sulphate-activated RNA polymerase (Stirpe & Fiume, 1967).

If salicylate inhibits the biosynthesis of messenger RNA *in vivo* this may be involved with its teratogenic effects in rodents. However, salicylate while frequently quoted as a teratogen in animals is not considered to be so in man (Palmer, 1969). The

main argument is the shape of the dose-response curve showing that malformation occurs over a very narrow range and embryopathic activity occurs at dosages where a maternally toxic effect is evident. Thus the only predicted chance of a human foetal malformation would be in circumstances such as an attempted suicide using a large toxic dose of salicylate by a woman 4–5 weeks pregnant who recovered without suffering a miscarriage. This may be a somewhat oversanguine conclusion since Richards (1969) considers that the results of a retrospective epidemiological study of malformations in human pregnancy showed that the taking of salicylate preparations in the first trimester is associated with significant increases in abnormalities of the central nervous system and the alimentary tract of the foetus.

Prostaglandin biosynthesis

It was shown by Piper & Vane (1969) that among the substances released during anaphylaxis in guinea-pig lung was an unidentified material (RCS) which contracted rabbit aorta. The release of this substance was blocked by salicylates. It was subsequently found that arachidonic acid, one of the precursors of prostaglandins, also released RCS from perfused lungs. Although RCS has not been isolated and its chemical nature is unknown, attention was directed to the effects of salicylates on prostaglandin production. Three different biological systems were used, cell-free homogenates of guinea-pig lung incubated with arachidonic acid (Vane, 1971), isolated perfused dog spleen stimulated by adrenaline (Ferreira, Moncada & Vane, 1971) and human blood platelets exposed to thrombin (Smith & Willis, 1971). In each instance the stimulated production of prostaglandins was inhibited by 0·005–0·2 mM aspirin but sodium salicylate was either much less potent or without action. The effect was interpreted as inhibition of the biosynthesis of prostaglandins since the tissues could be stimulated to release more prostaglandins than they contain. However, it will be necessary to perform metabolic studies on the incorporation of labelled precursors into the various prostaglandins, to demonstrate the inhibitory action of aspirin on an isolated enzyme preparation such as prostaglandin synthetase (Takeguchi, Kohno & Sih, 1971) and to exclude an action of the drug on prostaglandin breakdown before this mechanism is proven.

The relevance of this interesting and important effect of aspirin *in vitro* to the therapeutic and other actions of the drug is a matter for speculation (Collier, 1971). It will be necessary to show that the prostaglandins are mediators of pain, fever and inflammation in man. At present there is little acceptable evidence that the prostaglandins are concerned in either pathological or experimental pain. They elevate body temperature when injected in the cerebral ventricles of cats (Milton & Wendlandt, 1971) and are found in increased amounts in acute inflammatory exudates in animals (Willis, 1969). In man, it has been shown that they are present in blister fluid (Angaard, Arturson & Jansson, 1970) and accumulate in the interstitial fluid of the skin of patients with allergic contact dermatitis (Greaves, Sondergaard & McDonald-Gilson, 1971) but this is not proof that they have any role as mediators in acute inflammatory reactions. Their possible occurrence in the chronic inflammation characteristic of rheumatoid arthritis has not been studied.

There is no reason to differentiate between salicylate and acetylsalicylate with respect to either experimental (Wilhelmi, Gydnia & Ziel, 1968) or clinical anti-inflammatory (Woodbury, 1965) actions since they are equally effective. The relative lack of activity of sodium salicylate as an inhibitor of prostaglandin production cannot be reconciled with a mechanism relating an interference with the biosynthesis of prostaglandins in the tissues to clinical antirheumatic actions.

This mechanism may be of more significance in situations in which aspirin has been shown to cause different effects to salicylate. These include analgesia (Lim, 1966),

bradykinin-induced bronchoconstriction (Berry & Collier, 1964), aggregation of blood platelets (O'Brien, 1968), thurfyl nicotinate erythema (Truelove & Duthie, 1959) and hypersensitivity reactions (Samter & Beers, 1967). Clearly, the acetylsalicylate ion may act *in vivo* as an independent chemical entity and its interactions with tissue components, including the generating system for prostaglandins, may differ from those of its immediate metabolite, salicylate.

Other interactions with enzyme systems

The inhibitory effects of salicylate *in vitro* and *in vivo* on the biosynthesis of mucopolysaccharide sulphates have been described by Bostrom, Whitehouse and their colleagues (Whitehouse, 1965). The processes are affected by 2 mM drug *in vitro* and after the administration of 100 mg/kg of salicylate in whole animals. The mechanism has been explained in terms of the uncoupling action of salicylate but many enzymes are involved in mucopolysaccharide synthesis and it is likely that salicylate exerts multiple inhibitory effects (Lee & Spencer, 1969). Salicylate inhibits xanthine oxidase activity *in vitro* and *in vivo* (Bergel & Bray, 1959; Mitidieri & Affonso, 1959) and the biosynthesis of uric acid in perfused liver (Ramia, Boyal & Calvet, 1966). This action may be partially responsible for the beneficial effects of large doses (over 5 g per day) of salicylate in gout.

SUMMARY AND CONCLUSIONS

The work of the last two decades has shown that salicylate is a versatile inhibitor of many important enzyme activities *in vitro*. These include oxidative-phosphorylation reactions, dehydrogenases, decarboxylases, aminotransferases, aminoacyl-tRNA synthetases and nucleic acid polymerases. In some instances the mechanisms of inhibition have been established and resemble each other in being reversible and competitive with either a substrate or a coenzyme.

With many enzyme systems concentrations of greater than 5 mM are required to demonstrate an appreciable interference with the measured activities *in vitro*. Such interactions can bear little relevance to *in vivo* actions of the drug even in extreme cases of acute salicylate poisoning. However, there are some *in vitro* inhibitory effects of salicylates that occur within the concentration range (0·6 to 5·0 mM) of free salicylate observed either in the plasma of poisoned human subjects or in the tissues of animals given high toxic doses of the drug. The inter-relation between these *in vitro* inhibitions, the effects observed after the administration of doses of salicylate giving equivalent concentrations of salicylates in the tissues of whole animals, and known toxic symptoms in man, are indicated in Table 2.

It is therefore possible to state a reasonable case for the proposition that several of the major toxic symptoms in salicylate intoxication are caused by the drug interfering with the activities of cellular enzyme systems. This is not so with respect to the anti-inflammatory action of the drug. The only two interactions with enzyme systems *in vitro* that occur in the range 0·15 to 0·60 mM (see Table 1) are the uncoupling effect and the inhibition of protein biosynthesis. An interference with cellular energy-yielding reactions due to uncoupling could theoretically affect many aspects of the inflammatory reaction (Whitehouse, 1968) but no convincing evidence relating uncoupling potency to anti-inflammatory activity has been obtained. Salicylate inhibits general protein biosynthesis in the mouse in toxic amounts only, and the possibility that it preferentially interferes in man with the biosynthesis of specific proteins which either initiate or maintain inflammation is purely speculative. It could be concluded that the biochemical investigation of the salicylates has failed to elucidate the mechanism(s) of their anti-inflammatory and antirheumatic actions.

Table 2. *Relation between inhibitory effects of salicylate on isolated enzyme systems and toxic actions in man.*

In vitro Enzyme system	Inhibitory salicylate concentration (mM)	*In vivo* Effects in animals and man	Toxic action in man
Oxidative phosphorylation	0·2	Increased O_2 consumption Liver glycogen depletion Enhanced heat production Increased metabolic formation of CO_2	Hyperpyrexia Respiratory acidosis
Dehydrogenases	1·5–10·0	Decreased O_2 consumption Accumulation of organic acid anions in tissues	Metabolic acidosis
Aminotransferases	0·6–10·0	Changed patterns and pool sizes of tissue amino-acids	Amino-aciduria
RNA polymerases	2·0	Impaired biosynthesis of messenger RNA	Teratogenicity

Such a statement is both premature and over-simplified. The prematurity stems from the fact that only a comparatively limited number of interactions of enzymes and the drugs have been described and studied. Many enzymes, more intimately concerned with the inflammatory processes await either discovery or characterization. The enzyme systems responsible for the biosynthesis of prostaglandins and other mediators of inflammation should be considered in this connection. The anti-rheumatic properties of salicylate may be mediated by effects on such enzymes rather than on general cellular reactions catalysed by the ubiquitous dehydrogenases or aminotransferases. Secondly, it is equally possible that the drug may act at several sites rather than specifically interfere with a single enzyme system. The overall anti-inflammatory effect may be due to multiple interactions with several components; biochemical, pharmacological and immunological, of the chronic inflammatory response. Thus either uncoupling or a selective interference with protein biosynthesis need not be the only mechanism but may play a supporting role to other effects of salicylate. These may include the release of small biologically active molecules from their binding sites on circulating and tissue proteins because it has been shown that the drug will displace tryptophan (McArthur & Dawkins, 1969), long-chain fatty acid anions (Dawkins, McArthur & Smith, 1970) and pyridoxal-5-phosphate (Dempsey & Christensen, 1962) from human and bovine serum proteins.

It has been demonstrated that the clinically useful antirheumatic drugs displace protein-bound tryptophan and L-phenylalanyl-L-phenylalanine from human serum *in vitro* (McArthur, Dawkins & Smith, 1971). This action is not shared by other drugs which lack clinical anti-inflammatory actions but which bind to the serum proteins and are administered to man over similar periods of time (Smith, Dawkins & McArthur, 1971). In a comparison with normal subjects the percentage of tryptophan bound to serum proteins is significantly reduced in patients with rheumatoid arthritis receiving therapy with antirheumatic drugs (McArthur, Dawkins & others, 1971). This also occurs in pregnancy and jaundice, two conditions which are associated with an increased incidence of remissions in rheumatoid arthritis. It has been proposed by these workers that human serum contains peptide-like substances in protein-bound and free forms and that the latter protect susceptible tissues against the effects of chronic inflammatory processes. In patients with

rheumatoid arthritis the anti-inflammatory peptides are bound to an abnormal extent to the circulating proteins and the antirheumatic drugs act by redressing the bound : free ratio to that in the normal subject.

REFERENCES

AMBROSE, C. T. (1966). *Bacteriol. Rev.*, **30**, 408–417.

ANDREWS, B. F., BRUTON, D. C. & DE BAARE, L. (1962). *J. Pediat.*, **60**, 201–205.

ANGAARD, E., ARTURSON, G. & JONSSON, C. E. (1970). *Acta physiol. scand.*, **80**, 46A.

BAKER, B. R. (1967). *Design of Active-Site-Directed Irreversible Enzyme Inhibitors.* New York: John Wiley.

BAKER, B. R., LEE, W. W., SKINNER, W. A., MARTINEZ, A. P. & TONG, E. (1960). *J. mednl pharm. Chem.*, **2**, 633–657.

BARGONI, N. (1964). *Ital. J. Biochem.*, **13**, 67–74.

BELLAMY, A., HUGGINS, A. K. & SMITH, M. J. H. (1963). *J. Pharm. Pharmac.*, **15**, 559–560.

BEN-ISHAY, D. (1964). *J. Lab. clin. Med.*, **63**, 924–932.

BERGEL, F. & BRAY, R. C. (1959). *Biochem. J.*, **73**, 182–192.

BERRY, H. K. & GUEST, G. M. (1963). *Metabolism*, **12**, 760–770.

BERRY, P. A. & COLLIER, H. O. J. (1964). *Br. J. Pharmac. Chemother.*, **23**, 201–216.

BERTELLI, A., DONATI, L. & MAREK, J. (1969). In: *Inflammation, Biochemistry and Drug Interaction*, pp. 66–75. Editors: Bertelli, A. & Houck, J. C. Amsterdam: Excerpta Medica Foundation.

BOYER, P. D. (1968). In: *Biological Oxidations*, pp. 193–235. Editor: Singer, T. P. New York: Interscience Publishers.

BRODY, T. M. (1956). *J. Pharmac. exp. Ther.*, **117**, 39–51.

BROWN, S. S. & PROUDFOOT, A. T. (1969). *Biochem. J.*, **112**, 35P.

BRYANT, C., SMITH, M. J. H. & HINES, W. J. W. (1963). *Ibid.*, **86**, 391–396.

BULLOCK, G. R., DELANEY, V. B., SAWYER, B. C. & SLATER, T. F. (1970). *Biochem. Pharmac.*, **19**, 245–253.

BURLEIGH, P. M. C. & SMITH, M. J. H. (1970). *Biochem. J.*, **117**, 68P.

CHARNOCK, J. S. & OPIT, L. J. (1962). *Ibid.*, **83**, 596–602.

CHARNOCK, J. S., OPIT, L. J. & HETZEL, B. S. (1962). *Ibid.*, **85**, 190–193.

COCHRAN, J. B. (1952). *Br. med. J.*, **2**, 964–967.

COLLIER, H. O. J. (1971). *Nature, Lond.*, **232**, 17–19.

DAVIES, G. E., HOLMAN, G., JOHNSTON, T. P. & LOWE, J. S. (1966). *Br. J. Pharmac. Chemother.*, **28**, 212–217.

DAVISON, C. & SMITH, P. K. (1961). *J. Pharmac. exp. Ther.*, **133**, 161–170.

DAWKINS, P. D., GOULD, B. J. & SMITH, M. J. H. (1966). *Biochem. J.*, **99**, 703–707.

DAWKINS, P. D., GOULD, B. J., STURMAN, J. A. & SMITH, M. J. H. (1967). *J. Pharm. Pharmac.*, **19**, 355–366; see also CHESHIRE, A. M. & PARK, M. V. (1971). *Biochem. J.* In the press.

DAWKINS, P. D., MCARTHUR, J. N. & SMITH, M. J. H. (1970). *Ibid.*, **22**, 405–410.

DAWKINS, P. D., MCARTHUR, J. N. & SMITH, M. J. H. (1971). *Biochem. Pharmac*, **20**, 1303–1312.

DAWKINS, P. D. & SMITH, M. J. H. (1970). *J. Pharm. Pharmac.*, **22**, 913–922.

DEMPSEY, W. B. & CHRISTENSEN, H. N. (1962). *J. biol. Chem.*, **237**, 1113–1120.

DIENST, S. & GREER, N. (1967). *J. Maine Med. Ass.*, **58**, 11–14.

DIXON, M. & WEBB, E. C. (1964). *Enzymes.* London: Longmans, Green and Co.

DONE, A. K. (1963). In: *Salicylates. An International Symposium*, pp. 260–266. Editors: Dixon, A. St. J., Martin, B. K., Smith, M. J. H. & Wood, P. H. N. London: Churchill.

DUTHIE, J. J. R. (1963). *Ibid.*, pp. 288–291.

ELLIOT, H. C. & MURDAUGH, H. V. (1962). *Proc. Soc. exp. Biol. Med.*, **109**, 333–335.

EULER, H. von & AHLSTROM, L. (1943). *Z. Physiol. Chem.*, **279**, 175–186.

FERREIRA, S. H., MONCADA, S. & VANE, J. R. (1971). *Nature New Biology, Lond.*, **231**, 237–239.

FUKUHARA, M. & TSURUFUJI, S. (1969). *Biochem. Pharmac.*, **18**, 2409–2414.

GOULD, B. J., DAWKINS, P. D., SMITH, M. J. H. & LAWRENCE, A. J. (1966). *Molec. Pharmac.*, **2**, 526–533.

GOULD, B. J., HUGGINS, A. K. & SMITH, M. J. H. (1963). *Biochem. J.*, **88**, 346–349.

GOULD, B. J. & SMITH, M. J. H. (1965a). *J. Pharm. Pharmac.*, **17**, 83–88.

GOULD, B. J. & SMITH, M. J. H. (1965b). *Ibid.*, **17**, 15–18.

GREAVES, M. W., SONDERGAARD, J. & MCDONALD-GIBSON, W. (1971). *Br. med. J.*, **2**, 258–260.

GRISOLIA, S. (1968). *Lancet*, **2**, 1033.

GRISOLIA, S., MENDELSON, J. & DIEDERICH, D. (1969). *Nature, Lond.*, **223**, 79–80.

GRISOLIA, S., SANTOS, I. & MENDELSON, J. (1968). *Ibid.*, **219**, 1252.

GRISWOLD, D. E. & UYEKI, E. M. (1969). *Europ. J. Pharmac.*, **6**, 56–60.

GUTOWAKA-GRZEGORCZYK, G. & KALCZAK, M. (1968). *Rheumatologia*, **6**, 181–185.

HAMRIN, B. (1957). *Lancet*, **1**, 867–868.

HARFORD, D. J. & SMITH, M. J. H. (1970). *J. Pharm. Pharmac.*, **22**, 578–583.

HEBBORN, P. & SHAW, B. (1963). *Br. J. Pharmac. Chemother.*, **20**, 254–263.

HINES, W. J. W., BRYANT, C. & SMITH, M. J. H. (1963). *Biochem. Pharmac.*, **12**, 1109–1116.

HINES, W. J. W. & SMITH, M. J. H. (1964). *Nature, Lond.*, **201**, 192.

HUGGINS, A. K., SMITH, M. J. H. & MOSES, V. (1961). *Biochem. J.*, **79**, 271–275.

JANAKIDEVI, K. & SMITH, M. J. H. (1969). *J. Pharm. Pharmac.*, **21**, 401–402.

JANAKIDEVI, K. & SMITH, M. J. H. (1970a). *Ibid.*, **22**, 249–252.

JANAKIDEVI, K. & SMITH, M. J. H. (1970b). *Ibid.*, **22**, 58–59.

JANAKIDEVI, K. & SMITH, M. J. H. (1970c). *Ibid.*, **22**, 51–55.

JANOTA, I., WINCEY, C. W., SANDIFORD, M. & SMITH, M. J. H. (1960). *Nature, Lond.*, **185**, 935–936.

JEFFREY, S. W. & SMITH, M. J. H. (1959). *Biochem. J.*, **72**, 462–465.

JOLY, M. (1965). In: *A Physico-chemical Approach to the Denaturation of Proteins*, p. 3. New York: Academic Press.

KALBHEN, D. A. & DOMENJOZ, R. (1969). In: *Inflammation, Biochemistry and Drug Interaction*, pp. 334–338. Editors: Bertelli, A. & Houck, J. C. Amsterdam: Excerpta Medica Foundation.

KALBHEN, D. A., DOMENJOZ, R. & EHLERS, K. (1967). *Life Sci.*, **6**, 1883-1886.

KAPLAN, E. H., KENNEDY, J. & DAVIS, J. (1954). *Arch. Biochem. Biophys.*, **51**, 47–61.

KAZAKOVA, T. B. & CHEBOTAR, N. A. (1969). *Biochimica*, **34**, 1151–1157.

KENT, P. W. & ALLEN, A. (1968). *Biochem. J.*, **106**, 645–658.

KREBS, H. A. (1969). In: *Current Topics in Cellular Regulation*, Vol. 1, pp. 45–56. Editors: Horecker, B. L. & Stadtman, E. R. New York: Academic Press.

LARSSON, K. S., ERICSON, B. & BOSTROM, H. (1963). *Acta. paediat. scand.*, **52**, 36–40.

LEE, K. H. & SPENCER, M. R. (1969). *J. pharm. Sci.*, **58**, 464–468.

LEVY, G. & LEONARDS, J. R. (1966). In: *The Salicylates*, pp. 5–48. Editors: Smith, M. J. H. & Smith, P. K. New York: Interscience Publishers.

LIM, R. K. S. (1966). *Ibid.*, pp. 155–202.

LIMBECK, G. A., CONGER, J. D., TIPPIT, D. F. & KELLEY, V. C. (1966). *Arthritis Rheum.*, **9**, 776–782.

MANCHESTER, K. L., RANDLE, P. J. & SMITH, G. H. (1958). *Br. med. J.*, **1**, 1028–1030.

MANSO, C., TARANTA, A. & NYDICK, I. (1956). *Proc. Soc. exp. Biol. Med.*, **93**, 84–88.

MCARTHUR, J. N. & DAWKINS, P. D. (1969). *J. Pharm. Pharmac.*, **21**, 744–750.

MCARTHUR, J. N., DAWKINS, P. D. & SMITH, M. J. H. (1970). *Ibid.*, **22**, 801–805.

MCARTHUR, J. N., DAWKINS, P. D. & SMITH, M. J. H. (1971). *Ibid.*, **23**, 393–398.

MCARTHUR, J. N., DAWKINS, P. D., SMITH, M. J. H. & HAMILTON, E. B. D. (1971). *Br. med. J.*, **2**, 677–679.

MCARTHUR, J. N. & SMITH, M. J. H. (1969). *Ibid.*, **21**, 21–23.

MILTON, A. S. & WENDLANDT, S. (1971). *J. Physiol.*, **207**, 76P.

MITIDIERI, E. & AFFONSO, O. R. (1959). *Nature, Lond.*, **183**, 471.

MIYAHARA, J. T. & KARLER, R. (1965). *Biochem. J.*, **97**, 194–198.

MORAN, C. J. & WALKER, W. H. C. (1968). *Biochem. Pharmac.*, **17**, 153–156.

MORSDORF, K. (1965). In: *Non-Steroidal Anti-Inflammatory Drugs*, pp. 85–89. Editors: Garattini, S. & Dukes, M. N. G. Amsterdam: Excerpta Medica Foundation.

NAKAGAWA, H. & BENTLEY, J. P. (1969). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **28**, 883.

NAKAUE, H. S., WEBER, C. W. & REID, B. L. (1967). *Proc. Soc. exp. Biol. Med.*, **125**, 663–664.

NORTHOVER, B. J. & SUBRAMANIAN, G. (1961). *Br. J. Pharmac. Chemother.*, **17**, 107–115.

O'BRIEN, J. R. (1968). *Lancet*, **1**, 779–783.

PACKMAN, L. M., ESTERLY, N. B. & PETERSON, R. D. A. (1969). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **28**, 294.

PACKER, L., AUSTEN, F. K. & KNOBLOCK, E. C. (1959). *Proc. Soc. exp. Biol. Med.*, **100**, 239–244.

PALMER, A. K. (1969). In: *Teratology*, pp. 55–72. Editors: Bertelli, A. & Donati, L. Amsterdam: Excerpta Medica Foundation.

PENNIALL, R. (1958). *Biochim. Biophys. Acta*, **30**, 247–251.

PETILLO, J. J., GULBENKIAN, A. & TABACHNICK, I. I. A. (1969). *Biochem. Pharmac.*, **18**, 1784–1788.

PIPER, P. J. & VANE, J. R. (1969). *Nature, Lond.*, **223**, 29–35.

RAMIA, J., BOYAL, J. & CALVET, F. (1966). *Revta esp. Fisiol.*, **22**, 85–97.

REUNANEN, M., HANNINEN, O. & HARTIALA, K. (1967). *Nature, Lond.*, **213**, 918–919.

RICHARDS, I. D. G. (1969). *Br. J. prev. soc. Med.*, **23**, 218–225.

ROKOSOVA-CMUCHALOVA, B. & BENTLEY, J. P. (1968). In: *Chemical Biology of Inflammation*, pp. 315–327. Editors: Houck, J. C. & Forscher, B. K. London: Pergamon Press.

SAMTER, M. & BEERS, R. F. (1967). *J. Allergy*, **40**, 281–293.

SCHAYER, R. W. (1965). *Fedn Proc. Fedn Am. Socs. exp. Biol.*, **24**, 1295–1297.

SCHIMKE, R. T. (1969). In: *Current Topics in Cellular Regulation*, Vol. 1, pp. 77–124. Editors: Horecker, B. L. & Stadtman, E. R. New York: Academic Press.

SEGAR, W. E. & HOLLIDAY, M. A. (1958). *New Engl. J. Med.*, **259**, 1191–1198.

SIMMONS, D. P. & CHRISMAN, O. D. (1965). *Arthritis Rheum.*, 8, 960–969.

SINGER, S. & MASON, M. (1965). *Biochim. biophys. Acta*, **110**, 370–379.

SJAASTAD, O. V. & SJAASTAD, O. (1965). *Acta pharmac. tox.*, **23**, 303–311.

SKIDMORE, I. F. & WHITEHOUSE, M. W. (1966a). *Biochem. Pharmac.*, **15**, 1965–1983.

SKIDMORE, I. F. & WHITEHOUSE, M. W. (1966b). *J. Pharm. Pharmac.*, **18**, 558–560.

SKIDMORE, I. F. & WHITEHOUSE, M. W. (1967). *Biochem. Pharmac.*, **16**, 737–751.

SLATER, T. F. & DELANEY, V. B. (1970). *Biochem. J.*, **116**, 303–308.

SLATER, T F. & SAWYER, B. C. (1966). *Ibid.*, **101**, 24–28.

SPECTOR, W. G. & WILLOUGHBY, D. A. (1968). *The Pharmacology of Inflammation.* London: English Universities Press.

SMITH, J. B. & WILLIS, A. L. (1971). *Nature New Biology, Lond.*, **231**, 235–237.

SMITH, M. J. H. (1958). *Am. J. Physiol.*, **193**, 29–33.

SMITH, M. J. H. (1966). In: *The Salicylates*, pp. 49–105. Editors: Smith, M. J. H. & Smith, P. K. New York: Interscience Publishers.

SMITH, M. J. H. (1968a). *Clin. Tox.*, **1**, 387–407.

SMITH, M. J. H. (1968b). *Lancet*, **2**, 828.

SMITH, M. J. H. DAWKINS, P. D., MCARTHUR, J. N. (1971). *J. Pharm. Pharmac.*, **23**, **451**.

SMITH, M. J. H. & JEFFREY, S. W. (1956). *Biochem. J.*, **64**, 589–592.

SMITH, M. J. H. & STURMAN, J. A. (1967). *J. Pharm. Pharmac.*, **19**, 108–113.

STEGGLE, R. A., HUGGINS, A. K. & SMITH, M. J. H. (1961). *Biochem. Pharmac.*, **7**, 151–153.

STIRPE, F. & FIUME, L. (1967). *Biochem. J.*, **105**, 779–782.

STURMAN, J. A., DAWKINS, P. D., MCARTHUR, J. N. & SMITH, M. J. H. (1968). *J. Pharm. Pharmac.*, **20**, 58–63.

TAKEGUCHI, C., KOHNO, E. & SIH, C. J. (1971). *Biochemistry*, **10**, 2372–2376.

THOMAS, J. M., NAKAUE, H. S. & REID, B. L. (1967). *Poultry Sci.*, **46**, 1216–1219.

TRNAVSKA, Z., TRNAVSKY, K. & KUHN, K. (1968). *Biochem. Pharmac.*, **17**, 1493–1500.

TRUELOVE, L. H. & DUTHIE, J. J. R. (1959). *Ann. Rheum. Dis.*, **18**, 137–141.

VANE, J. R. (1971). *Nature New Biology, Lond.*, **231**, 232–235.

VAUGHAN, D. A., STEELE, J. L. & KORTY, P. R. (1969). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **28**, 1110–1113.

VILLELA, G. G. & CALCAGNOTTO, A. M. (1970). *Biochem. Pharmac.*, **19**, 2176–2178.

WARKANY, J. & TAKACS, E. (1959). *Am. J. Path.*, **35**, 315–331.

WEISSMANN, G. (1967). In: *Rheumatology.* An Annual Review, pp. 1–28. Editor: Rotstein, J. Basel: Karger.

WHITEHOUSE, M. W. (1963). In: *Salicylates. An International Symposium*, pp. 55–61. Editors: Dixon, A. St. J., Martin, B. K., Smith, M. J. H. & Wood, P. H. N. London: Churchill.

WHITEHOUSE, M. W. (1965). *Prog. Drug. Res.*, **8**, 321–429.

WHITEHOUSE, M. W. (1968). In: *Chemical Biology of Inflammation*, pp. 293–306. Editors: Houck, J. C. & Forscher, B. K. London: Pergamon Press.

WILHELMI, G., GDYNIA, R. & ZIEL, R. (1968). In: *Pain*, pp. 373–391. Editors: Soulairac, A., Cahn, J. & Charpentier, J. London: Academic Press.

WILLIS, A. L. (1969). In: *Prostaglandins, Peptides and Amines*, p. 31. Editors: Montegazza, P. & Horton, E. W. London: Academic Press.

WINTERS, R. W. (1963). In: *Salicylates. An International Symposium*, pp. 270–280. Editors: Dixon, A. St. J., Martin, B. K., Smith, M. J. H. & Wood, P. H. N. London: Churchill.

WOODBURY, D. M. (1965). In: *The Pharmacological Basis of Therapeutics*, 3rd Edition, p. 329. Editors: Goodman, L. S. & Gilman, A. New York: Macmillan Co.

YOSHIDA, T., METCOFF, J. & KAISER, E. (1961). *Am. J. dis. Child.*, **102**, 511–512.

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The significance of differences in the potency of enantiomers of anti-acetylcholine* drugs

R. W. BRIMBLECOMBE, D. M. GREEN. T. D. INCH AND PAMELA B. J. THOMPSON

Chemical Defence Establishment, Porton Down, Salisbury, Wiltshire, U.K.

The anti-acetylcholine potencies of the dimethylaminoethyl and *N*-methyl piperidin-4-yl esters of *R* and *S* 2-cyclohexyl-2-hydroxy-2-phenylacetic acid and their quaternary derivatives have been measured by *in vitro* and *in vivo* procedures. The *R*-enantiomer of dimethylaminoethyl-2-cyclohexyl-2-hydroxy-2-phenylacetate was approximately 100 times more active than the corresponding *S*-enantiomer both *in vivo* and *in vitro*. In contrast, the differences in potencies of the enantiomers of the other compounds were smaller *in vivo* than *in vitro* and moreover, the *in vivo* differences in potency decreased as the potency of the racemates increased. The relevance of these results to general studies of enantiomeric differences is discussed.

The results from pharmacological comparisons of enantiomers which differ in potency are normally used to draw conclusions concerning geometrical requirements for efficient interaction between drug and receptor (Casy, 1970). In this connection it has been suggested that the high ratio between the potencies of enantiomers for highly active drugs compared with the lower enantiomeric potency ratios observed for less potent drugs, reflects the better geometrical fit of the former for the receptor (Pfeiffer, 1956). However, although it is usually convenient and attractive, and probably correct, to invoke differences in the nature of the drug–receptor interaction as the principal reason for differences in the potency of enantiomers, the possible importance of other factors in contributing to these differences have long been recognized. For example, the following factors have been listed (Pfeiffer, 1956): (a) differences in the rates of absorption, destruction and excretion of isomers, (b) competitive inhibition between isomers, (c) differential penetration of enantiomers to the site of drug action and (d) the possible racemization *in vivo* of one or both of the enantiomers. Despite general recognition of many of these and other factors, few detailed discussions have been published concerning the type and extent of information that pharmacological comparisons of enantiomers can be expected to contribute to our understanding of drug action, or of the experimental conditions necessary for maximum information to be obtained. In this paper some results from pharmacological comparisons of the enantiomers and racemates of the hydrochlorides and methiodides of dimethylaminoethyl 2-cyclohexyl-2-hydroxy-2-phenylacetate and *N*-methyl piperidin-4-yl-2-cyclohexyl-2-hydroxy-2-phenylacetate are given.

Enantiomeric pairs of anti-acetylcholine drugs were chosen for study because it was apparent from previously reported studies (Ariëns, 1966; Casy, 1970) that drugs of this type fulfilled most of the requirements that appeared to be necessary to obtain the maximum information possible. Some of the requirements were that the enantiomers of a number of drugs within a series should be available with known

* Editorial policy has dictated the use of the term anti-acetylcholine drugs for the drugs used in the experiments described in this paper.

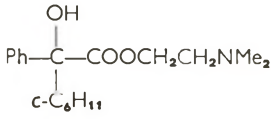
configuration and with appreciable differences in potency. Additionally there should be a variety of pharmacological test procedures sufficiently accurate to indicate whether the potency of the more active enantiomer was twice that of the racemate. Although the enantiomers of atropine and hyoscine fulfilled the above requirements (Buckett & Haining, 1965), they were not available in optically pure forms, which was another necessary prerequisite if maximum information was to be obtained. In compounds like atropine and hyoscine where the enantiomeric potency ratio approaches or exceeds 100, the presence as impurity of 1% or less of the most active isomer can account for most (or all) of the apparent activity of the less active (or inactive) isomer leading to possible misinterpretation of the pharmacological results. The enantiomers of atropine and hyoscine have only been obtained by classical resolution methods and no techniques are available that permit unequivocal determinations of the optical purity of resolved enantiomers* (Eliel, 1962). For enantiomers prepared in this way even the tedious and time-consuming procedure of purifying enantiomers to states of constant biological activity (Long, Luduena & others, 1956) although providing a good indication, is no guarantee of optical purity. Fortunately, any complications which could have arisen from the use of resolved enantiomeric pairs were avoided by the choice of dimethylaminoethyl- and *N*-methylpiperidin-4-yl 2-cyclohexyl-2-hydroxy-2-phenylacetates as the anti-acetylcholine drugs for detailed study. The methyl esters of *R*- and *S*-2-cyclohexyl-2-hydroxy-2-phenylacetic acid were prepared by a stereospecific synthetic procedure that guaranteed the optical purity of the enantiomers and permitted unequivocal configurational assignments to be made (Inch, Ley & Rich, 1968). Transesterification of the methyl esters with *N*-methylpiperidin-4-ol or dimethylaminoethanol afforded the optically pure *R* and *S* *N*-methylpiperidin-4-yl and dimethylaminoethyl-2-cyclohexyl-2-hydroxy-2-phenylacetates which were converted into their respective hydrochlorides and methiodides. Dimethylaminoethyl-2-cyclohexyl-2-hydroxy-2-phenylacetate was chosen because of the fact that the enantiomers of this highly potent anti-acetylcholine compound had already been established as having appreciably different potencies (Ellenbroek, Nivard & others, 1965). *N*-Methylpiperidin-4-yl-2-cyclohexyl-2-hydroxy-2-phenylacetate was chosen because it was known that glycollic acid esters of *N*-methylpiperidin-4-ol are amongst the most potent anti-acetylcholine drugs available (Abood, 1968).

The four tests for anti-acetylcholine potency we used were chosen because they have proved to be reliable both in our laboratory and elsewhere (Abramson, Barlow & others, 1967). Further, two of the tests, production of mydriasis in mice and antagonism of carbachol-induced contractions of the guinea-pig isolated ileum, may be used to follow the time course of drug action. It will be shown subsequently that the value of enantiomeric potency ratios in providing information about modes of action of drugs is of limited value unless due allowance, where necessary, is made for differences in the time course of action of the enantiomers.

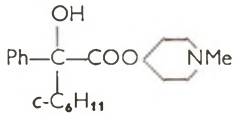
Drugs

The following drugs were examined for anti-acetylcholine activity. (Hydrochlorides are designated by a number and a configurational notation. Methiodides are designated by a number, the letter M, and a configurational notation.)

* Since this paper was submitted a detailed treatment of the biological consequences of optical impurities has appeared [Barlow, R. B. (1971), *J. Pharm. Pharmac.*, **23**, 90].



Compound I



Compound II

Compound III

Compound I. Dimethylaminoethyl(*R*)-2-cyclohexyl-2-hydroxy-2-phenylacetate hydrochloride (IR) and methiodide (IMR). Dimethylaminoethyl 2-cyclohexyl-2-hydroxy-2-phenylacetate hydrochloride (I Rac) and methiodide (IM Rac). Dimethylaminoethyl(*S*)-2-cyclohexyl-2-hydroxy-2-phenylacetate hydrochloride (IS) and methiodide (IMS).

Compound II. *N*-Methyl-piperidin-4-yl(*R*)-2-cyclohexyl-2-hydroxy-2-phenylacetate hydrochloride (IIR) and methiodide (IIMR). *N*-Methyl-piperidin-4-yl 2-cyclohexyl-2-hydroxy-2-phenylacetate hydrochloride (II Rac) and methiodide (IIM Rac). *N*-Methyl-piperidin-4-yl(*S*)-2-cyclohexyl-2-hydroxy-2-phenylacetate hydrochloride (IIS) and methiodide (IIMS).

Compound III. 2(*S*)-Dimethylaminopropan-1-yl(*R*)-2-cyclohexyl-2-hydroxy-2-phenylacetate hydrochloride (IIIRS) and methiodide (IIIMRS). 2(*R*)-dimethylaminopropan-1-yl(*R*)-2-cyclohexyl-2-hydroxy-2-phenylacetate hydrochloride (IIIRR) and methiodide (IIIMRR). The first configurational term (IIIRS) refers to the configuration of the acid moiety and the second configurational term (IIIRS) refers to the configuration of the aminoalcohol.

The enantiomeric dimethylaminoethyl- and *N*-methylpiperidin-4-yl 2-cyclohexyl-2-hydroxy-2-phenylacetates were prepared by base catalysed transesterification of the optically pure enantiomers of methyl 2-cyclohexyl-2-hydroxy-2-phenylacetate (Inch & others, 1968) with dimethylaminoethanol and *N*-methylpiperidin-4-ol. The optically pure 2(*S*) and 2(*R*)-dimethylaminopropan-1-ols which were transesterified with methyl (*R*)-2-cyclohexyl-2-hydroxy-2-phenylacetate were prepared as described by Inch & Lewis (1971). Racemic compounds were prepared by standard procedures.

METHODS AND RESULTS

Method 1

Determination of affinity constants. The terminal 2–3 cm of ileum from a guinea-pig was suspended in a bath (2·5 ml) of Ringer Tyrode solution at 37°. The ileum was attached to a Devices 2LDO1 optical wedge transducer and subjected to a load of 0·5 g and contractions were recorded on a direct writing pen recorder. The bath was connected to reservoirs and overflow so that the fluid in the bath could be changed by upward displacement either by Tyrode alone or by Tyrode containing drugs at predetermined concentrations. Events in the bath were controlled automatically by an apparatus similar to that described by Schild (1947). The method used for determining the affinity constants of the anti-acetylcholine drugs was similar to that described by Barlow, Scott & Stephenson (1963) except that carbachol instead of acetylcholine was used as agonist. The agonist was in contact with the tissue for 15 s and the interval between doses was 1·5 min. All solutions contained hexamethonium bromide ($1{\cdot}1 \times 10^{-4}$M). After regular responses were obtained for two different concentrations of carbachol, the Tyrode solution in which the ileum was suspended was replaced by Tyrode solution containing the antagonist and the concentration of the

agonist was increased to maintain comparable contractions. For drugs with low affinity constants (log k < 9) the antagonist and the ileum came into equilibrium in less than 10 min but for drugs with higher affinity constants, e.g. IMR, IIR, II Rac, IIS, IIMR, IIM Rac and IIMS much longer periods were required before a steady response to the agonist in the presence of the antagonist was obtained.

The affinity constant of the antagonist using the four point assay procedure was calculated by the method of the Edinburgh group (Pharmacological Experiments on Isolated Preparations, Staff of the Department of Pharmacology, F. & S. Livingstone, 1968).

In Table 1 the logarithms of the affinity constants are expressed as a mean of four to ten determinations using two to five different concentrations of antagonist. One preparation was used for only one concentration of antagonist. For compounds of affinity constant Log k < 10·0 dose ratios in the range 10–400 were used whereas dose ratios in the range 50–1000 were used for compounds of affinity constant log k > 10·0. It will be seen from Table 1 that with the exception of compounds IIM Rac the standard error associated with the measurements of affinity constants was small. Plots of dose ratio against concentration for IMR and IMS and for IIMR and IIMS are shown in Fig. 1. The times taken for compounds II Rac, IIM Rac

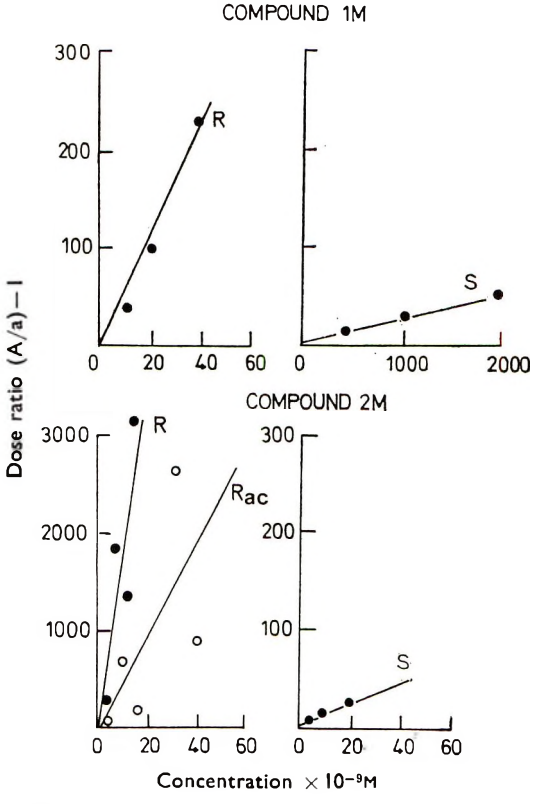


FIG. 1. Effect of isomers of compound 1M and isomers and racemate of compound 2M in antagonizing carbachol induced contractions on the guinea-pig isolated ileum. Each point on the graph represents the mean dose ratio obtained from two determinations. A linear relation between dose ratio $\left(\frac{A}{a}\right)-1$ and antagonist concentration demonstrates that the compounds are competitive antagonists.

Table 1. *Summary of pharmacological results.*

Compound	Method 1	Method 2. Production of mydriasis in mice			Method 3 (Salivation)	Method 4 (Tremors)
	log k ± s.e. (n results)	Potency relative to atropine with 95% confidence limits in brackets	Time to peak response (min)	Duration (min) ± s.e.	ED50 μmol/kg with 95% confidence limits in brackets	ED50 μmol/kg with 95% confidence limits in brackets
IR	*9·06 ± 0·01 (5)	0·76 (0·56–0·96)	10	73 ± 3	0·76 (0·44–1·29)	5·07 (3·87–6·9)
I Rac	*8·73 ± 0·02 (4)	0·34 (0·28–0·41)	10	58 ± 5	1·40 (1·11–3·33)	22·7 (7·39–41·4)
IS	*7·07 ± 0·01 (5)	0·006 (0·004–0·008)	<5	65 ± 6	>100	>100
IMR	9·66 ± 0·08 (6)	2·81 (2·34–3·44)	10	70 ± 10	0·06 (0·02–0·07)	> 50
IM Rac	*9·36 ± 0·03 (7)	1·67 (1·29–2·1)	20	90 ± 12	0·20 (0·08–0·52)	> 50
IMS	7·38 ± 0·02 (6)	0·074 (0·06–0·09)	5	34 ± 3	8·84 (5·15–15·2)	> 25
IIR	10·92 ± 0·10 (6)	2·36 (2·04–2·72)	80	901 ± 85	0·18 (0·04–0·31)	0·56 (0·30–1·3)
II Rac	10·53 ± 0·07 (4)	1·17 (1·02–1·36)	90	552 ± 45	0·31 (0·18–0·54)	3·29 (1·35–8·44)
IIS	8·48 ± 0·04 (4)	0·12 (0·10–0·14)	5	32 ± 4	7·75 (3·8–16·2)	9·42 (5·54–16·6)
IIMR	11·08 ± 0·10 (8)	2·76 (2·33–3·32)	80	1024 ± 48	0·06 (0·03–0·13)	24·32 (13·8–46·9)
IIM Rac	10·39 ± 0·17 (10)	2·1 (1·68–2·60)	10	780 ± 68	0·05 (0·03–0·11)	> 50
IIMS	9·08 ± 0·05 (6)	1·17 (0·99–1·39)	5	41 ± 2	1·05 (0·52–1·88)	73·8 (39·9–91·4)
IIRS	10·00 ± 0·04 (6)	1·63 (1·35–1·99)	20	143 ± 10	0·55 (0·31–0·98)	0·72 (0·14–3·62)
IIRR	9·88 ± 0·05 (6)	2·78 (2·29–3·40)	20	102 ± 7	0·55 (0·31–0·96)	2·52 (1·46–4·39)
IIIMRS	10·04 ± 0·03 (6)	3·16 (2·47–4·19)	40	645 ± 110	0·27 (0·15–0·49)	> 50
IIIMRR	10·08 ± 0·05 (6)	4·25 (3·4–5·24)	40	267 ± 28	0·20 (0·10–0·40)	> 25

* Determinations made by Dr. R. B. Barlow, Department of Pharmacology, University of Edinburgh.

and their enantiomers and compound IMR to reach equilibrium at different concentrations are listed in Table 4. The time to reach equilibrium was determined when contractions of the ileum elicited by the agonist in the presence of the antagonist had reached a constant height.

Method 2

Production of mydriasis in mice. Male mice (18–25 g) were used. Drugs were injected into a tail vein. Preliminary experiments were made on single animals to obtain an indication of suitable dose levels. Then, using groups of 10 mice at each of three dose levels, the pupil diameter was measured at different times after injection, to cover as far as possible the total period of action of the drug. The eyes were held 20 cm from a Watson microscope lamp and the measurement was made using an eyepiece graticule in a ×20 microscope. The mean pupil diameter from the two eyes were used and the mice were kept in the dark before and between readings. Graphs were plotted of pupil diameter against time. The time to peak effect varied with dose so in calculations of potency relative to atropine the maximum mean pupil diameter reached at each dose was used, irrespective of time and the results calculated on the basis of a six-point assay. Estimates of the duration of action were obtained from the graphs and were the times from injection, through peak pupil diameter, to 50% maximum pupil diameter. Estimates of the time to reach peak pupil diameter were also made. The results are listed in Table 1 and results for compounds IR and IS and IIR and IIS are shown in Fig. 2.

Methods 3 *and* 4

Antagonism of oxotremorine effects in mice. Male mice (18–25 g) were used. The drugs were injected intraperitoneally 15 min before the injection into a tail vein of 100 μg/kg oxotremorine. Animals were examined at 5, 10 and 15 min after the oxotremorine injection for the presence of salivation or tremors. No attempt was made to grade the severity of either response, it was noted as being present or absent. Four groups each containing five mice were used and ED50s for block of salivation (Method 3) and of tremors (Method 4) were calculated by probit analysis.

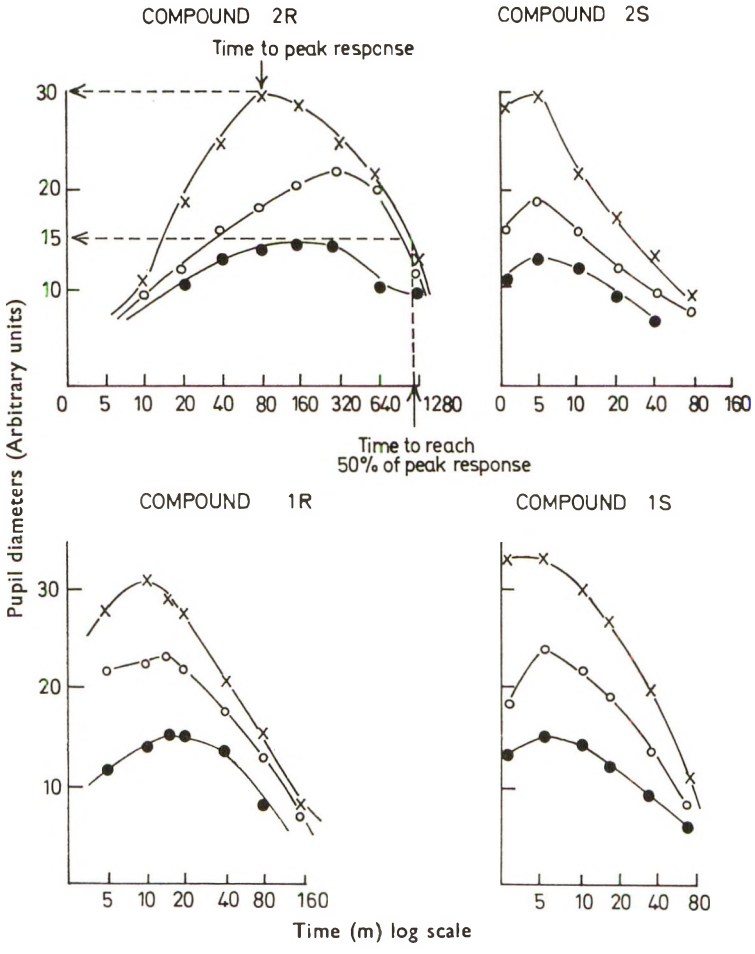


FIG. 2. Graphs showing production and duration of action of mydriasis induced by isomers of compounds 1 and 2. Compounds 1R 2R: × = 0·1, ○ = 0·05, ● = 0·025 μmol/kg. Compound 2S: × = 2, ○ = 1, ● = 0·5 μmol/kg. Compound 1S: × = 4, ○ = 2, ● = 1 μmol/kg.

DISCUSSION

The results for I Rac and its enantiomers and the corresponding methiodides (Table 1) showed that the three tests used for assessing peripheral anti-acetylcholine potency (measurement of affinity constants on the guinea-pig isolated ileum, blockade of oxotremorine-induced salivation in mice and production of mydriasis in mice) were consistently accurate. For example, the *R*-enantiomers IR and IMR were approximately twice as active as their respective racemates I Rac and IM Rac in all three tests (Table 2) as required for compounds where the inactive isomer is appreciably less potent than the active isomer. Also, the IR : IS potency ratio (Table 3) was very similar in the three tests, thereby providing evidence for the dependability of the three tests. Reasons for the greater variation in the enantiomeric potency ratios of IMR and IMS will be discussed later. The consistent results obtained from the three peripheral anti-acetylcholine assay procedures for compounds I Rac and IM Rac and their enantiomers made an interesting contrast with the much more variable

results obtained for compounds II Rac, IIM Rac and their enantiomers. Thus although IIR was approximately twice as active as its racemate in these three tests, IIMR was five times more active than its racemate in antagonizing the effects of carbachol-induced contractions of guinea-pig ileum but only of similar potency to its racemate in producing mydriasis in mice or in antagonizing the production of oxotremorine-induced salivation (Table 2). Even more striking variations were exhibited by the IIR : IIS and IIMR : IIMS potency ratios (Table 3). The *in vitro* ratio was 272 for IIR : IIS, 20 in the mydriasis test and 43 in the test for antagonism of oxotremorine-induced salivation. Similarly the *in vitro* ratio was 100 for IIMR : IIMS and 2·3 and 17·6 in the *in vivo* tests. The following conclusions may be drawn from the above results.

It was probable that the differences in potency of IR and IS, and IMR and IMS, as determined by the guinea-pig ileum assay reflected accurately the differences in affinity of the drugs for the receptor because the straight line dose ratio–concentration plots obtained for the enantiomers and the racemates (Abramson & others, 1969) indicated that they elicited their effects by a similar mechanism. Under the experimental conditions which were used, differences in rates of destruction (if any) of enantiomers were unlikely to make any significant contribution to differences in enantiomeric potency and the fact that the R-enantiomers were twice as active as their racemates showed that no racemization of the enantiomers occurred. If the conclusion is correct that the observed *in vitro* differences in enantiomeric potency for IR and IS and IMR and IMS resulted from factors associated only with the drug–receptor interaction, a similar conclusion about the *in vivo* differences in the potencies of IR and IS can be drawn from the facts that the enantiomeric potency ratio for IR : IS was similar *in vivo* and *in vitro* and that *in vivo*, as *in vitro*, compound IR was approximately twice as active as the racemate I Rac. Further, the consistency of the IR : IS enantiomeric potency ratios provide an indication that the anti-acetylcholine receptors in the guinea-pig ileum, mouse eye and mouse salivary gland are essentially identical.

Table 2. *Relative anti-acetylcholine activities of* R *and* S *enantiomers compared with racemate.*

Compound	Method 1	Method 2	Method 3	Method 4
IR	2·14	2·23	1·84	4·47
IS	0·022	0·018	<0·01	<0·2
IMR	2·00	1·68	3·33	—
IMS	0·01	0·044	0·022	—
IIR	2·45	2·01	1·72	5·86
IIS	0·009	0·10	0·04	0·607
IIMR	5·0	1·31	0·83	—
IIMS	0·05	0·55	—	—

Table 3. *Enantiomeric potency ratios.*

Enantiomers	Method 1	Method 2	Method 3	Method 4
IR : IS	100	123	>100	>22
IMR : IMS	200	38	147	—
IIR : IIS	272	20	43	38
IIMR : IIMS	100	2·3	17·6	—

It must be pointed out that conclusions, such as those reached above, will usually only be valid if the time profiles of pharmacological action of the enantiomers and their racemate are taken into account. This is important because the law of mass action requires that the rate of drug action be proportional to the concentration of the drug and thus where the differences in potency of enantiomers are large, concentration effects can be exceedingly important where comparisons are made at the ED50 level. The time–activity profiles for IR, I Rac and IS were found to be similar both in Method 1 (affinity constants) and Method 2 (mydriasis) and consequently the fact that possible differences in time–activity profiles were not taken into account in Method 3 was unlikely to affect seriously the IR : IS enantiomeric potency ratio obtained using this method. However, as will be demonstrated subsequently, differences in the time profile of compounds such as IIMR and IIMS are large and have a pronounced effect on the pharmacological results.

The results that the enantiomeric pairs IMR and IMS, IIR and IIS, and IIMR and IIMS exhibited considerable variation in enantiomeric potency ratios, and that compound IIMR was not twice as active as its racemate (IIM Rac) in any of the three tests for peripheral anti-acetylcholine activity, seem at first sight to contradict the conclusions reached above. We feel that an explanation can be offered which provides interesting and important new information about the behaviour of anti-acetylcholine drugs. Examination of the changes in potency and changes in enantiomeric potency ratios caused by quaternization of IR and IS, and IIR and IIS reveals an explanation for the facts that *only* the IR : IS potency ratio was similar *in vivo* and *in vitro* and the enantiomeric potency ratios of the other pairs were much smaller *in vivo* than *in vitro*. Quaternization of IIS increased mydriatic potency (Method 2) from 0·12 to 1·17 and the potency of antagonism of salivation from an ED50 of 7·75 to an ED50 of 1·05 μmol/kg (Method 3), whereas quaternization of IIR caused corresponding increases from 2·36 to 2·76 and 0·18 to 0·06. Thus the changes produced by quaternization of IIR were much smaller than the changes which resulted from the quaternization of IIS. In contrast, the increases in potency caused by quaternization of IR [0·76 to 2·81 (Method 2) and 0·76 to 0·06 μmol/kg (Method 3)] and IS [0·006 to 0·074 (Method 2) and $>$100 to 8·84 μmol/kg (Method 3)] were similar. These results, and the observations that IR was much less potent than IIR whereas the quaternary derivatives of IR and IIR have equivalent *in vivo* potencies even though the affinity constant of IIMR (log k = 11·08) was greater than IMR (log k = 9·66), suggest that there is a minimum dosage below which maximum anti-acetylcholine effects cannot be obtained *in vivo*, and that the potencies of IMR, IIR and IIMR all approached this minimum value. The fact that IIMR was only slightly more potent than IIMS in causing mydriasis also favoured the concept that a minimum dose is required to produce a maximum biological effect for anti-acetylcholine drugs.

As mentioned previously, the rates of onset of anti-acetylcholine effects *in vivo* and the rates at which the drugs equilibrate with the guinea-pig ileum are considerably influenced by dose or concentration of the drug. Thus Fig. 2 illustrates that, with the possible exception of compound IIR, the times to onset of mydriatic effects decrease as drug dosage is increased and similarly the results in Table 4 show that drugs equilibrate more rapidly with the guinea-pig ileum at high concentration than at low concentration. However the results also show that concentration effects alone do not control the rate at which anti-acetylcholine drugs act. Thus, although

Table 4. *Equilibration times with the guinea-pig ileum.*

Compound	Bath concentration (mol)	Time to equilibrium* (Plateau time) min	Mean log k
IMR	1×10^{-8}	24	9·66
	2×10^{-8}	15	
	4×10^{-8}	12	
IIR	2×10^{-9}	60	10·92
	4×10^{-9}	44	
	8×10^{-9}	40	
II Rac	4×10^{-9}	58	10·53
	8×10^{-9}	44	
IIS	2×10^{-7}	18	8·41
	5×10^{-7}	15	
IIMR	4×10^{-9}	107	11·08
	8×10^{-9}	77	
	$1{\cdot}2 \times 10^{-8}$	40	
	$1{\cdot}6 \times 10^{-8}$	59	
IIM Rac	4×10^{-9}	67	10·32
	$1{\cdot}2 \times 10^{-8}$	65	
	$1{\cdot}6 \times 10^{-8}$	75	
	$3{\cdot}2 \times 10^{-8}$	70	
	4×10^{-8}	56	
IIMS	4×10^{-9}	28	9·10
	$8{\cdot}0 \times 10^{-9}$	28	
	2×10^{-8}	24	

* Each time represents the mean obtained from two determinations.

compounds IR and IIR both produce maximum mydriatic effects at a dose of 0·1 μmol/kg, the effects appear much more rapidly for IR than for IIR (Fig. 2). Also, at a concentration of 4×10^{-9}M, compound IIMR required 107 min to equilibrate with guinea-pig ileum, whereas IIMS equilibrated in 28 min. From the results in Tables 1 and 4 there is some indication that drugs with high affinity constants take longer to produce mydriatic effects and longer to reach equilibrium with the ileum than compounds with lower affinity constants, and also that drugs with high affinity constants have a more prolonged effect. The result that IIMS, which has a similar affinity constant and *in vivo* potencies to compound IMR, also has a similar time–activity profile, indicated that the factors which control the time course of drug action are not critically dependent on drug stereochemistry. Also some evidence is available which suggests that the time–activity profiles are not significantly influenced by metabolic factors. For example, the affinity constants for IIMR are the same (Fig. 1) whether measured at low concentration with a long period of time for equilibrium to be reached or at a high concentration with a short period of time to equilibrium (Table 4). The reason why anti-acetylcholine drugs with high affinity constants require longer to produce mydriatic effects than anti-acetylcholine drugs with lower affinity constants even where equimolar doses are administered is not understood.

The obvious importance of the differences in the time–activity profiles for the pairs of enantiomers IMR and IMS, IIR and IIS, and IIMR and IIMS is possibly reflected by the results that the enantiomeric potency ratios for these pairs were smaller in the mydriasis tests (Method 2), where time effects were considered, than in the salivation tests (Method 3), where a set time schedule was adhered to for the injection of drugs and the making of observations. The higher enantiomeric potency ratios in the salivation test (Method 3) compared with the mydriasis test can be explained if the

less active isomers produced their maximum anti-acetylcholine effects *before* the oxotremorine was administered, and if there was little difference between the observed potency of the *R*-enantiomers and their maximum potency at the optimum time. In preliminary experiments, the time–activity profile of IIR and IIS in antagonizing oxotremorine-induced salivation has been measured by modifying Method 3 so that in separate experiments oxotremorine was administered 5, 10, 15, 20, 80, and 160 min after the administration of IIR or IIS. It was found that IIR was most potent (ED50, 0·09 μmol/kg) 80 min after its administration and that IIS showed a peak effect (ED50, 2·5 μmol/kg) after 10 min. Thus the potency ratio IIR : IIS fell from 43 to 28, the latter value being close to the IIR : IIS ratio observed in Method 2. However, it must be pointed out that in Method 3 some error may result because of the mode of administration (intraperitoneal injection) of the drugs in the salivation experiments. The preliminary experiments showed that whereas IIR had similar time–activity profiles in the mydriasis and salivation experiments, IIS had a sharp peak of activity in the mydriasis test but showed no such obvious peak of activity in the salivation test. These results may indicate that whereas for IIR the rate of diffusion within the body to the receptor is the controlling feature, for IIS the rate of diffusion from the peritoneum into the body is the rate controlling step. As a general screening procedure and for comparisons of compounds with similar time–activity profiles, such as IR and IS, Method 3 is perfectly adequate. However for compounds with different time–activity profiles it is clear that intravenous administration of the anti-acetylcholine drugs would be more appropriate than intraperitoneal injection and that measurements of ED50s should be made at the optimum times. Method 4 (antagonism of oxotremorine induced tremors) suffers from the same deficiencies as Method 3.

Another consequence of large differences in the time–activity profiles of enantiomers is that measurements of the affinity constant of their racemate may be made more difficult. For example, there was a far greater random error in the experimental measurements of the affinity constant of IIM Rac (Fig. 1) than for any other compound and it was for the enantiomers for IIM Rac that the largest difference in equilibration times was observed (Table 4). It is not unreasonable to suppose that, under the conditions used for measuring the affinity constant of IIM Rac, the rate of equilibration between IIMR, IIMS (both highly potent drugs) and the ileum was so slow that the equilibrium situation was difficult to assess experimentally, with the consequence that reproducible results were difficult to obtain. Under these circum-

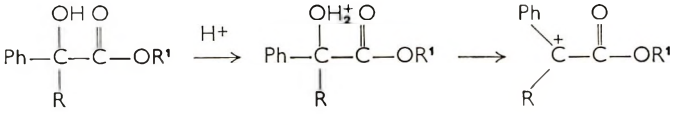
The general implications of the above discussion are that comparisons of enantiomeric potency ratios can provide evidence concerning the similarity or otherwise of receptors at different sites in the body or in different species providing that due allowance is made for differences in the time course of the enantiomers and that the potency of the more active isomer does not approach the minimum dose that is necessary to cause maximum biological effects. Nevertheless, if the concentration requirements cause a uniform decrease in *in vivo* enantiomeric potency ratios compared with *in vitro* ratios, it is possible that comparisons of these *in vivo* ratios can still provide evidence in favour of receptor similarity, if not of receptor differences. For example, the similarity of the IR : IS peripheral and central enantiomeric potency ratios provides evidence to suggest that the central receptors with which anti-acetylcholine

drugs interact are similar to those in the peripheral nervous system. This conclusion is supported by the similarity of the IIR : IIS *in vivo* peripheral and central enantiomeric potency ratios and also by the *in vivo* central and peripheral ratios obtained for *dextro* and *laevo* hyoscyamine (Buckett & Haining, 1968).

Conclusions that were reached from previous studies of enantiomers obviously require reappraisal in terms of the results obtained above. For example, it was suggested by Pfeiffer in 1956 that the enantiomeric potency ratios for highly active drugs should be greater than the enantiomeric potency ratios for less active drugs. This suggestion has subsequently been alluded to as Pfeiffer's Rule (Ariëns, 1965). It has also been claimed that where the ratio of the potency of the more active enantiomer to the racemate falls appreciably below 2 there is a lack of receptor selectivity and that in the limiting case where this ratio is 1 and the enantiomers are equiactive, there is a complete lack of stereochemical sensitivity (Casy, 1970). The results which have been described in this paper show that these generalizations are not valid, at least for anti-acetylcholine drugs, in *in vivo* experiments although they may possibly be justified for some isolated tissue experiments.

Ariëns and his coworkers (Ariëns, 1966) modified Pfeiffer's rule to the effect that the enantiomers of highly active drugs only have large differences in potency if the asymmetric centre is in a region of the molecule which is essential to the drug–receptor interaction. Ariëns' proposition was based on the values of the affinity constants (pA_2 values) for the rat jejunum of the choline and β-methylcholine esters of benzilic acid and 2-cyclohexyl-2-hydroxy-2-phenylacetic acid. It was found that the activity of these esters depended little on the configuration of the β-methylcholine moiety and that the β-methylcholine esters were less potent than the corresponding choline esters. These results were considered to imply that the β-methyl region of the β-methylcholine anti-acetylcholine esters contributed little to the potency of the drugs. The results in Table 1, which showed that the α-methylcholine esters IIIMRR and IIIMRS [and the tertiary analogues IIIRR and IIIRS] of *R*2-cyclohexyl-2-hydroxy-2-phenylacetate had similar affinity constants and were equally potent in antagonizing the effects of oxotremorine induced salivation (Method 3), and moreover were more potent than the corresponding choline derivative (IMR) [and the tertiary analogue IR] do not support Ariëns, modification of Pfeiffer's rule. There can be little doubt that the α-methyl group in IIIRR, IIIRS, IIIMRR and IIIMS makes a considerable contribution to the observed anti-acetylcholine potency of these compounds which is independent of stereochemistry. The conclusion follows from these results that Pfeiffer's rule (or Ariëns' modification) does not apply generally to *in vitro* or *in vivo* experiments.

In a recent review (Abood, 1970) and elsewhere (Abood, 1968; Gabel & Abood, 1965), Abood and his coworkers have made a number of suggestions concerning the mode of action of anti-acetylcholine drugs. Some of these suggestions are invalidated by the results that are summarized in Table 1. For example, it was suggested that these drugs may act by a carbonium ion mechanism, e.g.



Since carbonium ion formation necessitates destruction of asymmetry it cannot be consistent with the results that enantiomers have different potencies and different

durations of action. Also the suggestion that the duration of action of the drugs is related to their ease of acidic hydrolysis cannot be valid since pairs of enantiomers which are hydrolysed at the same rate have quite different durations of action.

It has been suggested by Abood, in detailed discussions of the relation between anti-acetylcholine activity and molecular conformation that the activity of glycollic acid esters of tertiary aminoalcohols was very dependent on the availability of the lone pair of electrons on nitrogen, particularly in cyclic aminoalcohols. It was also stated that in reaching this conclusion, little distinction was made between central and peripheral anti-acetylcholine effects. Since in this paper and elsewhere it has been shown that quaternary compounds are usually more potent peripherally than tertiary compounds, the contribution of the lone pair of electrons of nitrogen to the peripheral anti-acetylcholine activity of the glycollate esters is clearly of little consequence. This evidence must also detract from the possibility of there being any connection between the lone pair availability and the effects of glycollate esters in the central nervous system.

In summary it appears that, even without knowledge of the configuration of enantiomers, the results from studies of enantiomers and their racemates can provide critical tests of certain aspects of drug action and indicate whether or not receptors in different species and in different parts of the body are similar. Other workers have demonstrated the utility of this approach previously with experiments with (+)- and (−)-amphetamine (Taylor & Snyder, 1970) and with (+)- and (−)-noradrenaline (Patel, 1969) and provided that due consideration is given to time–activity profiles and minimum dose requirements, this approach should be of potential relevance to many active types of drug. Where the absolute configuration of the enantiomers are known, studies of enantiomers are able to provide additional information. For example, since *S*(−)hyoscyamine, dimethylaminoethyl-*R*-2-cyclohexyl-2-hydroxy-2-phenylacetate, 2-cyclohexyl-2-phenyl-4-dimethylaminomethyl-1,3-dioxolan (Brimblecombe & Inch, 1970) and 4-dimethylaminomethyl-2-(1-cyclohexyl-1-hydroxy-1-phenyl)methyl-1,3-dioxolan (Brimblecombe, Inch & others, 1971) are configurationally related, it can be assumed that they act in the same way. In contrast, since the stereochemical requirements of acetylcholine-like and anti-acetylcholine drugs differ in some cases (Brimblecombe, Green & Inch, 1970) it is possible that both kinds of drugs do not share points of attachment with a common receptor. Knowledge of the precise stereochemistry of drugs is also important from the viewpoint of design of new drugs. Hopefully in the future it is possible that knowledge of the absolute stereochemical requirements for high potency will help in the elucidation of receptor structure.

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REFERENCES

ABOOD, L. G. (1968). In *Drugs Affecting the Central Nervous System.* Editor: Burger, A. New York: Marcel Dekker.

ABOOD, L. G. (1970). In *Psychotomimetic Drugs*, pp. 67–74. Editor: Efron, D. H. New York: Raven Press.

ABRAMSON, F. B., BARLOW, R. B., MUSTAFA, M. G. & STEPHENSON, R. P. (1969). *Br. J. Pharmac.*, **37**, 207–233.

ARIËNS, E. J. (1966). *Adv. Drug. Res.*, **3**, 325–285.

BARLOW, R. B., SCOTT, K. A. & STEPHENSON, R. P. (1963). *Br. J. Pharmac. Chemother.*, **21**, 509–522.

BRIMBLECOMBE, R. W., GREEN, D. M. & INCH, T. D. (1970). *J. Pharm. Pharmac.*, **22**, 951–953.

BRIMBLECOMBE, R. W. & INCH, T. D. (1970). *Ibid.*, **22**, 881–888.

BRIMBLECOMBE, R. W., INCH, T. D., WETHERELL, J. & WILLIAMS, N. (1971). *Ibid.*, **23**, 649–661.

BUCKETT, W. R. & HAINING, C. G. (1965). *Br. J. Pharmac. Chemother.*, **24**, 138–146.

CASY, A. F. (1970). In *Medicinal Chemistry*, p. 81. Editor: Burger, A. New York: Wiley-Interscience, 1970.

ELLENBROEK, B. W. J., NIVARD, R. J. F., VAN ROSSUM, J. M. & ARIËNS, E. J. (1965). *J. Pharm. Pharmac.*, **17**, 393–404.

ELIEL, E. L. (1962). *Stereochemistry of Carbon Compounds*, p. 83. McGraw Hill.

GABEL, N. & ABOOD, L. G. (1965). *J. mednl Chem.*, **8**, 616–619.

INCH, T. D. & LEWIS, G. J. (1971). *Carbohydrate Res.*, **16**, 455–458.

INCH, T. D., LEY, R. V. & RICH, P. (1968). *J. chem. Soc.* (C), 1693–1699.

LONG, J. P., LUDUENA, F. P., TULLAR, B. F. & LANDS, A. M. (1956). *J. Pharmac. exp. Ther.*, **117**, 29–38.

PATEL, P. N. (1969). *J. Pharm. Pharmac.*, **21**, 628–629.

PFEIFFER, C. C. (1956). *Science, N.Y.*, **124**, 29–31.

SCHILD, H. O. (1947). *Br. J. Pharmac. Chemother.*, **2**, 189–206.

TAYLOR, K. M. & SNYDER, S, H, (1970), *Science, N.Y.*, **168**, 1487–1489.

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Decreased distribution of oxotremorine to brain after pharmacological blockade of its peripheral acetylcholine-like effects

BO KARLÉN,* LIL TRÄSKMAN† AND FOLKE SJÖQVIST†

**Department of Organic Chemistry, Faculty of Pharmacy, Box* 6804, 113 86 *Stockholm, and †Department of Pharmacology (Division of Clinical Pharmacology), Karolinska Institutet, Stockholm* 60, *Sweden.*

The distribution of [^{3}H]oxotremorine, after intravenous injection, to brain and tissues of rats and mice has been studied. In rats, oxotremorine (0·3 mg/kg) rapidly reached the brain with a peak concentration of 1200 ng/g at 1 min. Blockade of the peripheral muscarinic effects of oxotremorine with amitriptyline methyl iodide or atropine methyl nitrate (25 mg/kg, s.c.) markedly decreased the brain content of oxotremorine (peak concentration 400 ng/g), probably as a result of its more rapid distribution to peripheral tissues and an increased volume of distribution. The ratio of drug distribution between plasma and brain is unaffected by pretreatment with anticholinoceptive drugs. Also, the total body concentrations of oxotremorine are similar in pretreated rats and controls. A small dose of oxotremorine (10 μg/kg) is distributed similarly in pretreated rats (amitriptyline methyl iodide, 25 mg/kg, s.c.) and in controls. In mice there was a linear relation between dose, up to 180 μg/kg, and brain concentration of oxotremorine. At higher doses, relatively more drug reached the brain. Oxotremorine induced a marked fall in blood pressure in the unanaesthetized rat that was reduced by amitriptyline methyl iodide and reversed by atropine methyl nitrate. It seems likely that differences in blood flow between pretreated rats and controls are responsible for the differences in the distribution of the drug.

Tremorine (1,4-dipyrrolidino-2-butyne) and oxotremorine [1(2-oxopyrrolidino)-4-pyrrolidino-2-butyne] induce in laboratory animals a syndrome resembling the symptoms of Parkinson's disease, i.e. ataxia, tremor and rigidity (Everett, Blockus & Shepperd, 1956), that can be blocked by antiparkinsonian drugs. Tremorine and oxotremorine, therefore, have been used widely in the screening of compounds with potential activity in this disease (Jenden, 1967). Since tremorine owes its effect to its biotransformation to oxotremorine (Cho, Haslett & Jenden, 1961), and various blockers of tremorine metabolism interfere in the screening procedure (Sjöqvist, Hammer & others, 1968), oxotremorine has replaced tremorine in this test. The assumption is made that the intensity of induced tremor depends on the concentration of oxotremorine in the brain and that the antagonists interfere with the drug's action solely at central receptor sites.

Oxotremorine also produces hypothermia, and drugs that counteract this effect may enhance its disappearance from the brain (Hammer, Karlén & Sjöqvist, 1968a). Such drugs decrease the intensity and duration of tremor partly by lowering the concentration of oxotremorine at receptor sites in brain (loc. cit.).

We report a marked impairment of the distribution of the drug to the brain after its peripheral acetylcholine-like effects had been blocked. This represents a new type

of drug interaction. The investigation was precipitated by the finding that the hypothermic effect of oxotremorine in mice was reduced by amitriptyline methyl iodide although this compound should not pass the blood brain barrier readily.

MATERIAL AND METHODS

Drugs and animals

[^{3}H]Oxotremorine was prepared and checked for radiochemical purity (Karlén & Telč, 1966; Hammer, Karlén & Sjöqvist, 1968b) and diluted with unlabelled oxotremorine oxalate to yield a specific activity (10 mCi; mmol) which was convenient for analysis.

Male Sprague Dawley rats, 180–200 g, or male Swiss albino mice (N.M.R.I., Bethesda), 18–22 g, were used. Oxotremorine (0·3 mg/kg as base or as otherwise stated) was administered into the tail vein in a volume of 1 or 5 ml/kg (rat or mouse respectively). Thirty min before the administration of drug, rats or mice were injected subcutaneously with either saline, or atropine methyl nitrate (25 mg/kg), or amitriptyline methyl iodide (25, 12·5 or 6·25 mg/kg). Rats were also pretreated with *N*(5-pyrrolidino-3-pentynyl) succinimide citrate (BL 14) (10 mg/kg as base, i.p.) in volumes of 1 ml/kg, 15 min before oxotremorine.

Determination of [^{3}H]oxotremorine

Rats were killed with ether, decapitated and blood collected. Plasma and various tissues were removed and analysed.

Plasma. Plasma (1 ml) was made alkaline with NaOH (1 ml; 0·5N) and extracted with toluene (6 ml) containing 1·5% isoamylalcohol. After centrifugation, 4 ml of the organic phase was mixed with toluene (10 ml) mix (PPO/POPOP) and counted by liquid scintillation. The extraction of oxotremorine was not affected by the presence of amitriptyline methyl iodide or atropine methyl nitrate.

Brain, liver, kidney and lung. The tissues were dissected out, weighed and homogenized in an Ultra-turrax homogenizer in seven parts of ice-cold water and a sample of the homogenate (2 ml) was made alkaline, extracted, and counted as described above.

Carcass. This was homogenized in a Waring blendor with four parts of ice-cold water. A sample of the homogenate (2 ml), filtered through gauze, was made alkaline, extracted and counted as described above.

Red cells. To red cells (1 ml) was added NaOH (1 ml; 0·5N). The mixture was frozen and the resulting thick emulsion was diluted with water (2 ml), extracted and and counted.

Bladder plus urine. The ureters were tied off and the bladder containing the urine prepared free and weighed. The bladder was homogenized with an Ultra-turrax homogenizer with ten parts of water and a 2 ml aliquot was analysed as above.

Internal standards. To an aliquot of plasma or tissue homogenate from untreated animals was added a known amount of [^{3}H]oxotremorine in a volume of 10 μl. The standards and samples of unknown concentration were analysed together.

Specificity of the extraction procedure. The extraction procedure is specific for oxotremorine (Hammer & others, 1968a).

Recording of body temperature. The rectal temperature of the mice (rats show no hypothermia after the drug) was recorded with an electrothermometer at intervals of 30 min throughout the experiments, which were made at 20–22°.

Recording of blood pressure. Rats were anaesthetized with ether. A polyethylene tube was inserted in a carotid artery and connected to a Statham pressure transducer model P 23 DC. The rats were allowed to recover from anaesthesia for about 1 h and were then restrained in a suitably sized plastic tube. Oxotremorine was then injected in the tail vein and the blood pressure recorded for about a minute, whereupon the rat was removed from the restrained position and placed free on the laboratory bench.

Surgical procedures

The distribution of oxotremorine was studied after bilateral nephrectomy performed transabdominally in a few rats anaesthetized either with sodium pentobarbitone (20–40 mg/kg, i.p.) or ether. After ligating the blood vessels and suturing the abdomen the rats were allowed to recover the righting reflex before oxotremorine was administered.

RESULTS

Effect of amitriptyline methyl iodide on the distribution of tremorine in rats

Rats were pretreated with saline, then given the drug (0·3 mg base/kg, i.v.) and killed at various times thereafter. The drug disappeared from plasma polyphasically. A peak brain concentration of 926 ng/g was reached within 1 min, after which the concentration declined monophasically (Fig. 1). When rats were pretreated with amitriptyline methyl iodide (25 mg/kg, s.c.) 30 min before the injection of the drug, 1 min after the injection the plasma concentration in the pretreated rats was 300 ng/ml (compared with 1200 ng/ml in the controls), and the peak brain concentration of the pretreated animals was only 380 ng/g which was reached 2 min after the injection.

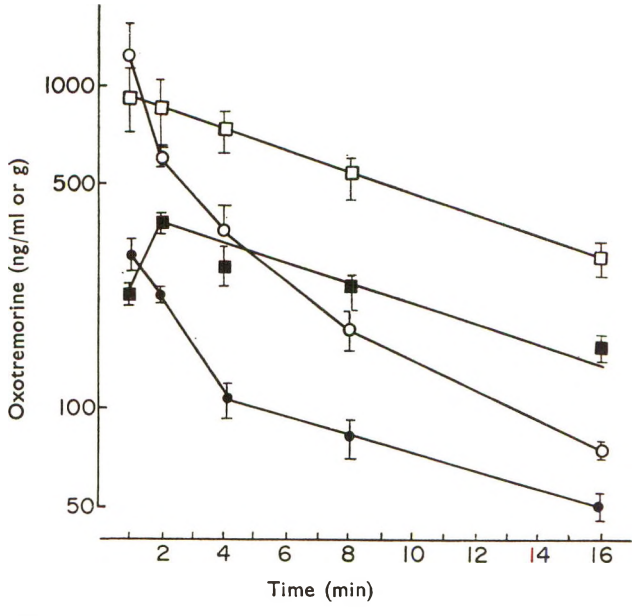

FIG. 1. Distribution of oxotremorine (0·3 mg/kg, i.v.) in rats after pretreatment with amitriptyline methyl iodide (25 mg/kg, s.c). Data are given as means ± s.d. (n = 3). ○—○ Plasma and □—□ brain controls. ●—● Plasma and ■—■ brain amitriptyline methyl iodide.

The ratio between the concentrations of drug in plasma and brain were similar in the absence and presence of amitriptyline.

The control rats developed tremor and the usual peripheral symptoms after the drug while pretreated rats showed tremor but no peripheral symptoms.

Distribution of oxotremorine after different pretreatment doses of amitriptyline methyl iodide

Rats were pretreated with amitriptyline methyl iodide (6·25, 12·5 and 25 mg/kg) and then given oxotremorine (0·3 mg base/kg, i.v.). At all doses of the amitriptyline salt, plasma and brain concentrations of oxotremorine (after 8 min) were significantly lower (about 85 ng/ml and 250 ng/g, respectively regardless of the dose of amitriptyline) than in the controls (181 ± 4 ng/ml and 458 ± 47 ng/g respectively); data are means ± s.d. of three animals. All pretreated rats were protected from the peripheral effects of the drug.

Distribution of a small dose of oxotremorine

To test whether the distribution of oxotremorine could be related to its pharmacological effects, the distribution and elimination of a small dose (10 μg/kg, i.v.), insufficient to produce any visible symptoms, was studied. Brain concentrations at 4 and 16 min were almost identical in control rats (9·8 ± 0·9 and 6·6 ± 0·4 ng/g, respectively) and in rats pretreated with amitriptyline methyl iodide (25 mg/kg, s.c., 9·8 ± 1·9 and 6·5 ± 0·9 ng/g, respectively). Plasma levels in the control rats (3·8 ± 0·6 and 1·5 ± 0·2 ng/g, respectively) were somewhat lower than in pretreated rats (4·3 ± 0·7 and 2·0 ± 0·5 ng/g, respectively). Data are expressed as means ± s.d. of three or four animals.

Distribution of oxotremorine in tissues

Rats were pretreated with amitriptyline methyl iodide (25 mg/kg, s.c.) or saline followed by oxotremorine (0·3 mg/kg, i.v.) 30 min later and killed after 8 min. The concentration of drug was lower in plasma, brain and lung of pretreated animals compared to controls (Tables 1 and 2), whereas the concentrations in kidney, liver and carcass did not differ significantly between the groups (Table 1). The ratio between the concentration of oxotremorine in carcass and plasma was doubled in the pretreated animals. The urine from bladders of pretreated rats was greater in volume and had about 30 times higher concentration of the drug compared to controls.

Table 1. *Concentrations of oxotremorine (OTMN) in different tissues after pretreatment with amitriptyline methyl iodide in rats.*

		Concn of OTMN (ng/ml or g ± s.d.)						
Pretreatment	Dose (mg/kg, s.c.)	Plasma	Brain	Lung	Kidney	Liver	Urine + bladder (pooled)	Carcass
Controls ..	Saline	177 ± 10	583 ± 74	312 ± 69	1018 ± 361*	56 ± 17*	220 (0·76 g)	206 ± 14*
Amitriptyline methyl iodide	25	94 ± 23	319 ± 66	210 ± 23	990 ± 347*	40 ± 3*	6315 (2·07 g)	182 ± 23*

Rats were pretreated 30 min before the administration of OTMN (0·3 mg/kg, i.v.) and killed 8 min thereafter. The data are expressed as means ± s.d. from three animals.
* N.S.

Table 2. *Plasma, red cell and brain concentrations of oxotremorine (OTMN).*

Pretreatment	Dose (mg/kg, s.c.)	Time (min)	Concn of OTMN		
			Plasma (ng/ml)	Red cells (ng/g)	Brain (ng/g)
Control	Saline	1	887 (2)	765 (2)	1012 (2)
,,	,,	8	155 ± 27	129 ± 21	637 ± 292
Amitriptyline methyl iodide ..	25	1	389 ± 15	340 ± 7	392 ± 43
,,	,,	8	105 ± 7	92 ± 9	297 ± 91

Rats were pretreated 30 min before the administration of OTMN (0·3 mg/kg, i.v.).
The data are expressed as means ±s.d. of three animals.

Red cells as well as plasma and brain were analysed for oxotremorine in pretreated (amitriptyline methyl iodide, 25 mg/kg, s.c.) and control rats at 1 and 8 min after the drug (Table 2). Its concentration in the red cells was lower than in plasma both in control and pretreated rats at each time. There was no difference between the two groups in the ratio between the concentration of drug in plasma and red cells.

Distribution of oxotremorine in nephrectomized rats

To exclude the possibility that differences in renal clearance of oxotremorine between pretreated and control rats could explain the phenomena observed, the kidneys of one group of rats were removed under anaesthesia, while two other groups of rats were sham-operated. The nephrectomized and one of the sham-operated groups were pretreated (30 min) with amitriptyline methyl iodide (6·25 mg/kg, s.c.), while the third group was given saline. Oxotremorine (0·3 mg/kg, i.v.) was then given and the animals killed 2 min later. The results in Table 3 show no marked difference between the pretreated nephrectomized and sham-operated rats, but both groups were significantly different from the controls. Thus the brain and plasma concentrations of drug were significantly higher in the control rats. The concentrations of drug in the carcass were the same in all three groups.

Table 3. *Plasma and brain concentrations of oxotremorine (OTMN) in nephrectomized rats.*

	Pretreatment	Dose (mg/kg, s.c.)	Experiment No.*	Concn of OTMN		
				Plasma (ng/ml)	Brain (ng/g)	Carcass (ng/g)
Nephrectomized	Amitriptyline methyl iodide	6·25	I	281 (2)	633 (2)	208 (2)
	,, ,,	,,	II	460 (2)	763 (2)	—
Sham-operated	Amitriptyline methyl iodide	6·25	I	275 (2)	676 (2)	234 (2)
	,, ,,	,,	II	394 ± 12	671 ± 10	—
Sham-operated	Controls	Saline	I	491 (2)	991 (2)	210 (2)
	,,	,,	II	670 ± 39	1187 ± 210	—

Rats were pretreated 30 min before the administration of OTMN (0·3 mg/kg, i.v.) and killed 2 min later. The data are expressed as means of two animals or as means ± s.d. of three animals.
* In exp. I rats were anaesthetized with sodium pentobarbitone, 20–40 mg/kg, i.p.
In exp. II rats were anaesthetized with ether.

Brain concentrations of oxotremorine in mice

Parts of the above experiments were repeated in mice using the brain only. The data indicate a similar difference in the distribution of oxotremorine as in rats between controls and pretreated (amitriptyline methyl iodide, 25 mg/kg, s.c.) mice (controls 407 ± 23, 378 ± 20; pretreated animals 230 ± 3, 267 ± 13 μg drug/g brain) at 2 and 8 min respectively.

The brain concentration of oxotremorine was also analysed after oxotremorine at doses of 60, 180, 300 and 360 μg/kg; the concentrations of drug in the brain at 2 min were 60 ± 9, 182 ± 20, 407 ± 23 and 501 ± 49 ng/g, respectively (means ± s.d. of five animals).

Up to doses of 180 μg/kg of oxotremorine there was a linear relation between the dose and concentration in the brain. However, at higher doses relatively more drug was distributed to the brain.

Hypothermic effect of oxotremorine in mice

The hypothermic effect of oxotremorine in mice was partly blocked by amitriptyline methyl iodide.

Distribution of oxotremorine after pretreatment with atropine methyl nitrate and N(5-*pyrrolidino*-3-*pentynyl*)*succinimide* (*BL* 14)

Rats were pretreated with either saline or atropine methyl nitrate (25 mg/kg, s.c.) 30 min before receiving oxotremorine (0·3 mg/kg, i.v.), and were killed at various times thereafter. Plasma and brain concentrations of drug were essentially the same as those obtained with amitriptyline methyl iodide. The animals showed fully developed tremor but no peripheral effects of oxotremorine.

The tertiary amine, BL 14, which antagonizes both central and peripheral symptoms of oxotremorine (Karlén, Lindeke & others, 1970), was also used for pretreatment (10 mg/kg, i.p., 15 min before oxotremorine, 0·3 mg/kg, i.v.). Results were similar to those obtained with the two other anti-acetylcholine drugs. The pretreated rats showed no salivation or lacrimation and no (or very slight) tremor after oxotremorine.

Blockade of blood pressure lowering effect of oxotremorine in the unanaesthetized rat with amitriptyline methyl iodide and atropine methyl nitrate

Oxotremorine (0·3 mg/kg, i.v.) evoked a dramatic fall (−105 ± 13·7 mm Hg) in the arterial blood pressure of the unanaesthetized rat. Pretreatment with atropine methyl nitrate in the dose used in the pharmacokinetic experiments (25 mg/kg, s.c.) reversed this effect and in contrast a rise (+82 ± 7·2 mm Hg) in blood pressure was induced by oxotremorine (Walker & Weetman, 1970). Pretreatment with amitriptyline methyl iodide (25 mg/kg, s.c.) partially reduced (−68 ± 6·5 mm Hg) the hypotensive effect of oxotremorine (means ± s.d. of 3–7 animals).

DISCUSSION

Plasma and brain concentrations of oxotremorine after intravenous injection were much lower in rats and mice in which the peripheral anti-acetylcholine effects of the drug were blocked, than in control animals. In contrast, the distribution of a small, pharmacologically ineffective dose of oxotremorine is similar in control rats and in rats pretreated with amitriptyline methyl iodide. These findings indicate that the pharmacological effects of oxotremorine greatly influence its own distribution, a new

example of dose-dependent pharmacokinetics (cf. Levy, 1968). Data in mice show that relatively more of the drug is distributed to the brain when the dose is increased above a certain level. Oxotremorine has a profound hypotensive effect in the unanaesthetized rat. This will result in marked changes in the blood-flow to different tissues.

After pretreatment with anti-acetylcholine drugs it therefore seems likely that the distribution of oxotremorine will be different compared to that in animals with the fully developed oxotremorine-syndrome.

Our results indicate that oxotremorine is much more rapidly distributed from plasma to tissues when its peripheral acetylcholine-like and hypotensive effects have been blocked fully or partly. The kinetic data suggest that the volume of distribution of the drug is increased in rats pretreated with anti-acetylcholine drugs. Already after 1 min the plasma level of oxotremorine is one third of that in the control rats (Fig. 1).

The low plasma concentrations of oxotremorine in pretreated animals are responsible for the lower brain concentrations compared to controls. There is no evidence for an effect of the anti-acetylcholine drugs on the diffusion of oxotremorine from plasma to brain. The small increase in the renal excretion of the drug after pretreatment with amitriptyline methyl iodide cannot account for the much lower plasma and brain concentrations of the drug in pretreated animals compared to those in controls. This is further shown in the experiments with nephrectomized rats. Also, the oxotremorine-level in the carcass was similar in all experimental groups.

The pharmacological significance of our findings is obvious since lowered brain concentrations of the drug in the pretreated animals will result in less marked central symptoms. This may therefore be unrelated to an antagonism between oxotremorine and the pretreatment drug at receptor sites in brain. In fact, amitriptyline methyl iodide although a quaternary ammonium base thought not to pass the blood-brain barrier, reduced the hypothermia caused by oxotremorine. Our experiments illustrate the fallacies with drug screening procedures utilizing drug-induced "models" of human diseases. It is important in such procedures to measure the concentrations of the interacting drugs in body fluids or tissues, a fact which is usually disregarded.

Acknowledgements

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REFERENCES

Cho, A. K., Haslett, W. L. & Jenden, D. J. (1961). *Biochem. biophys. Res. Commun.*, **5**, 276–279.

Everett, G. M., Blockus, L. E. & Shepperd, J. M. (1956). *Science, N.Y.*, **124**, 79.

Hammer, W., Karlén, B. & Sjöqvist, F. (1968a). *Life Sci.*, **7**, 205–211.

Hammer, W., Karlén, B. & Sjöqvist, F. (1968b). *Biochem. Pharmac.*, **17**, 935–942.

Jenden, D. J. (1967). In: *Methods of Pharmacological Testing*, pp. 337–361. Editor: Burger, A. New York: Marcel Dekker Inc.

Karlén, B. & Telč, A. (1966). *Acta pharm. suecica*, **3**, 197–200.

Karlén, B., Lindeke, B., Lindgren, S., Svensson, K.-G., Dahlbom, R., Jenden, D. J. & Giering, J. E. (1970). *J. mednl Chem.*, **13**, 651–657.

Levy, G. (1968). In: *Importance of Fundamental Principles in Drug Evaluation*, pp. 141–172. Editors: Tedeschi, D. H. & Tedeschi, R. E. New York: Raven Press.

Sjöqvist, F., Hammer, W., Schumacher, H. & Gillette, J. R. (1968). *Biochem. Pharmac.*, **17**, 915–934.

Walker, L. J. & Weetman, D. F. (1970). *Br. J. Pharmac.*, **39**, 490–500.

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A method for the continuous recording of peripheral vascular conductance

D. G. GARDINER, T. C. HAMILTON AND M. W. PARKES

Pharmacology Department, Roche Products Limited, Welwyn Garden City, Herts, U.K.

A method is described for the continuous recording of the conductance of a perfused vascular bed, derived from simultaneous measurement of blood flow and perfusion pressure. For the auto-perfused hindquarters of the anaesthetized cat, it is shown that for vasoconstrictor or vasodilator drugs, injected either intravenously or into the arterial perfusion circuit, a record of changes in conductance may be of considerable aid in clarifying the responses of the vessels.

The prospect of following variations in the properties of a vascular bed by changes in its resistance to flow rather than by flow itself, offers the advantage of detecting those changes independently of changes in perfusion pressure, thus permitting the study of vascular effects of drugs in auto-perfused areas. Changes in vascular capacitance are, however, less readily reflected by changes in resistance than by its reciprocal, namely, conductance (Stark, 1968). This value is represented by flow divided by pressure and provides a more ready interpretation of the effects of drugs upon the vessels.

Methods for the continuous recording of peripheral vascular resistance have been described by Hughes (1970) and Jancsó & Galgócry (1969). The method described here records both blood flow and perfusion pressure and continuously relates these parameters by an electronic divider circuit, to give a record of vascular conductance.

METHODS

Blood flow is measured by the insertion of an electromagnetic flowmeter (Statham E-3002) into the arterial perfusion circuit using an extra-corporeal flow probe; the principle of the method would, however, apply if cuffed flow sensors were to be used. Perfusion pressure is measured with a pressure transducer attached to the distal side of the extra corporeal circuit. Signals from both the blood flow and perfusion pressure transducers are amplified and their outputs fed into a dividing module (Figs 1 and 2).

Using blood flow as the numerator and perfusion pressure as the denominator, the divider output represents conductance of the vascular bed.

In the demonstrations of the applications of the method presented in this paper, cats intraperitoneally anaesthetized with chloralose (100 mg/kg) were used. After cannulation of the trachea, a blood pressure transducer was inserted into the common carotid artery and the external jugular vein cannulated for the intravenous administration of drugs.

For the study of vascular changes in the hindquarters, the extra-corporeal circuit was inserted into the abdominal aorta just proximal to the iliac bifurcation; for the study of the splanchnic vascular bed the circuit was connected to the superior mesenteric artery. Blood was thus allowed to be propelled naturally through the extra-corporeal circuit and perfused bed without the intervention of a perfusion pump.

Records were obtained of the heart rate (HR), systemic arterial blood pressure (BP), perfusion pressure (PP), conductance (C) and regional blood flow (F). Drug solutions were injected either intravenously or intra-arterially through a side arm on the extra-corporeal circuit. Maximum dose volume administered intra-arterially was 0·3 ml.

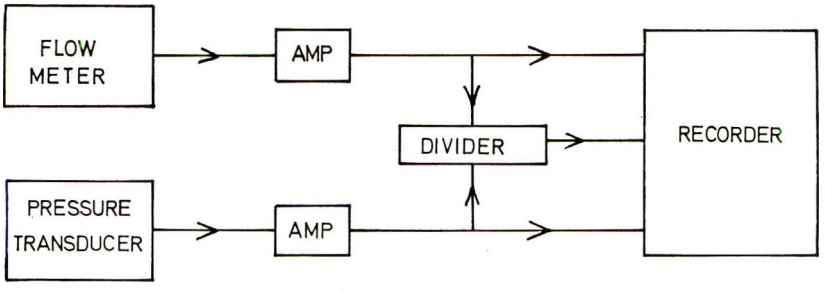

FIG. 1. Block diagram of arrangement for recording blood flow, perfusion pressure and vascular conductance. AMP = D.C. amplifier.

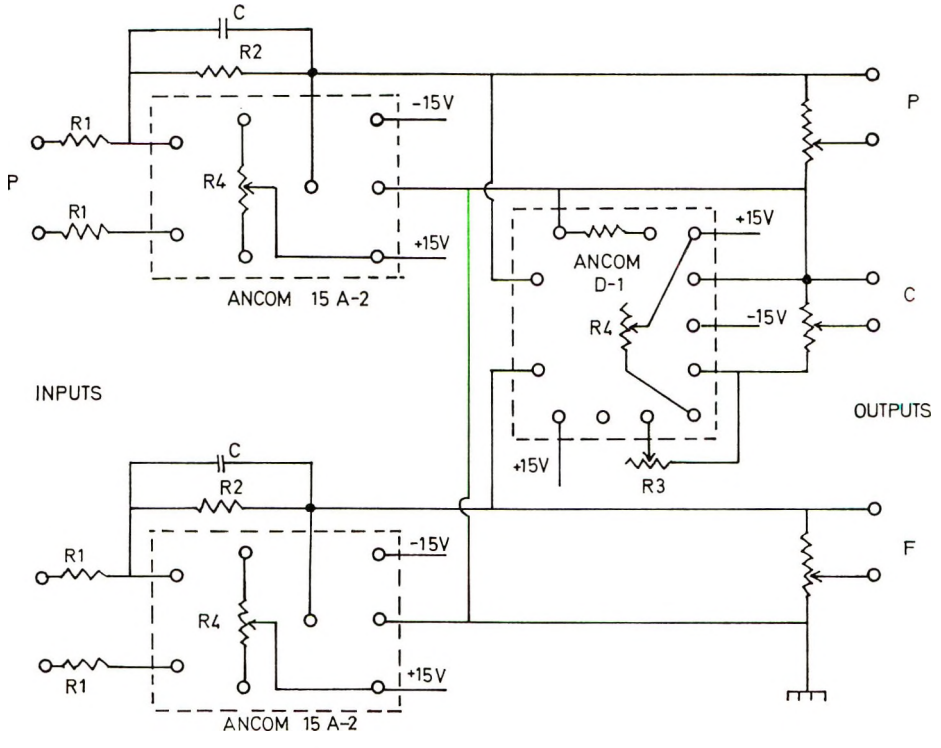

FIG. 2. Circuit diagram. C = 0·1 μF. R1 = 2·2 KΩ; R2 = 2·2 MΩ; R3 = 2KΩ. R4 = 50KΩ. P = Perfusion pressure. F = Blood flow. C = Vascular conductance = F/P.

RESULTS

In both the hindquarters and the splanchnic region, the effects of intra-arterial or intravenous injection of vasoconstrictor or vasodilator agents upon the vessels could be detected by changes in vascular conductance and distinguished from the

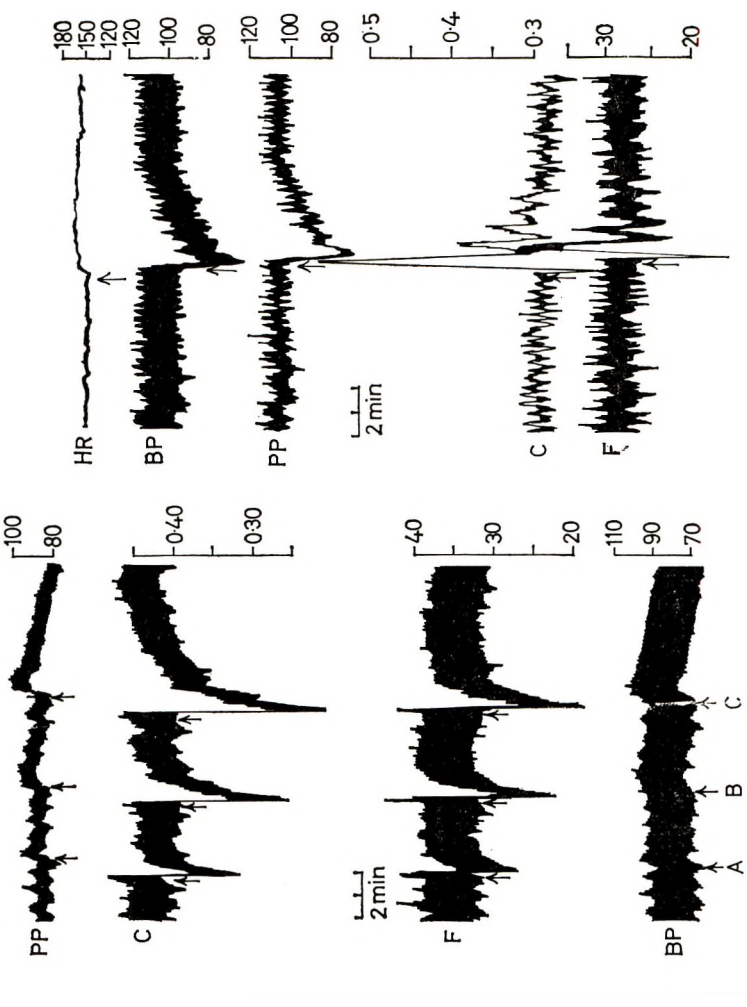

FIG. 3. Part of recording of hindquarters perfusion in a chloralosed cat. Traces: Heart rate (HR). Vascular conductance (C). Perfusion pressure (PP). Blood flow (F). Carotid blood pressure (BP) mm Hg. At the arrows, an injection of noradrenaline, 0·2 μg/kg, intravenously: these show corresponding points on the traces and indicate the offset of the recording pens.

FIG. 4. Recording as Fig. 3. At each set of arrows, an injection of noradrenaline into the perfusion circuit: these show corresponding points on the traces and indicate the offset of the recording pens. The doses of noradrenaline were as follows: A—0·01 μg; B—0·02 μg; C—0·05 μg.

FIG. 5. Recording as Fig. 3. At the arrows, an injection of papaverine, 1 mg/kg, intravenously: these show corresponding points on the traces and indicate the offset of the recording pens.

consequences of their effects on blood pressure. Thus, Fig. 3 shows that noradrenaline (0·2 μg/kg) injected intravenously was followed by an increase in both pressure and blood flow in the hindquarters. These increases arose from changes outside the perfused bed, within which conductance rose briefly as the vessels were distended by the increased perfusion pressure. The conductance then showed a secondary fall, similar to the effect after injection of noradrenaline into the perfusion circuit (Fig. 4); this may therefore be attributed to the direct action of the amine on the vessels of the perfused bed. The flow record in Fig. 3 shows a sharp fall at a time when the pressure is still elevated, consequent upon the reduced conductance.

These secondary effects were maximal some 24 s after intravenous injection (Fig. 3) while the corresponding direct effects reached their peak 12 s after injection into the perfusion circuit (Fig. 4). These figures indicate a circulation time of some 12 s which is similar to that reported from precise studies (Groom, Morris & Rowlands, 1957).

Similarly, as shown in Fig. 5, the intravenous injection of papaverine (1 mg/kg) caused a fall in perfusion pressure, owing to widespread vasodilatation; as a consequence, the flow in the perfused hindquarters decreased initially and the conductance record also shows an initial decrease, i.e., passive reduction in tone of the vessels. Secondarily, however, the conductance is seen to increase as the drug reached the perfused bed, with a similar further delay whereupon flow increased, despite the low perfusion pressure.

The injection of vasoconstrictor or vasodilator drugs into the arterial perfusion circuit caused the expected rise in conductance and flow, with a fall in perfusion pressure (Fig. 4), although large doses resulted in secondary changes in systemic pressure when sufficient of the drug reached the general circulation. The relations between dose of drug and effect on conductance, flow and perfusion pressure of the perfused hindquarters are illustrated in Fig. 6 for intra-arterially injected isoprenaline.

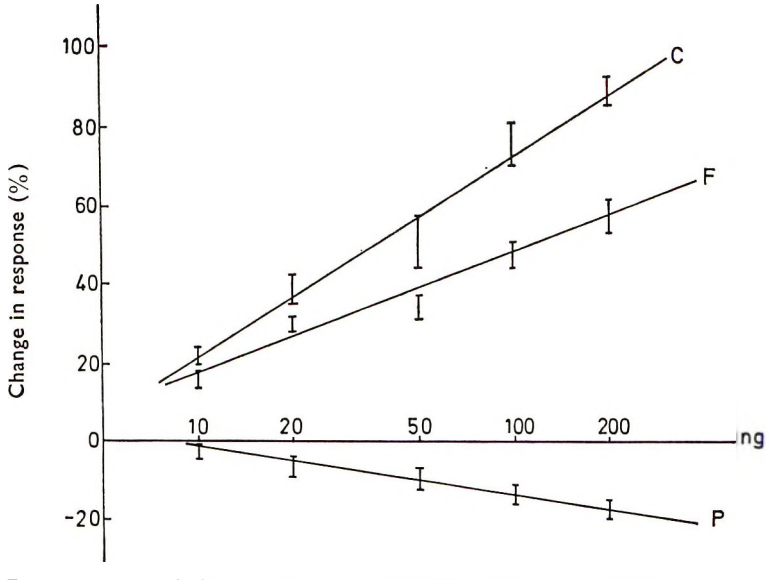

FIG. 6. Dose-response relations for the effect of isoprenaline, injected into the perfusion circuit, upon blood flow (F), perfusion pressure (P) and vascular conductance (C). The vertical bars represent the mean values ± s.e. obtained with each dose level. The lines were calculated from 60 values.

The effects are plotted as percentage change from the value recorded before injecting the dose; multiple regression analysis showed that the highest correlations were obtained by expressing results in this way.

DISCUSSION

By deriving a value for the conductance of the perfused vascular bed it is claimed that a more informative representation of the responses of the vessels to drugs is achieved than may be obtained by observing only changes in flow or perfusion pressure. The consequences of the intravenous injection of vasoconstrictors or vasodilators, illustrated by Figs 3 and 5, may be analysed into components of direct effects on the perfused vessels and indirect systemic effects.

For drugs injected into the perfusion circuit, the evidence again suggests that changes in conductance most nearly represent the effects of the drugs on the vessels. From multiple regression analysis applied to the results plotted in Fig. 6, the changes in flow with various doses of isoprenaline were more closely correlated with changes in conductance ($r_{FC} = 0{\cdot}86$) than with changes in perfusion pressure ($r_{FP} = -0{\cdot}6$); at the highest dose used, systemic changes may have affected pressure. The partial correlation of flow changes on conductance changes was somewhat less than the overall correlation ($r_{FC.DP} = 0{\cdot}72$) while, when dose and conductance were held constant, the negative correlation of flow changes with changes in perfusion pressure became positive ($r_{FP.DC} = 0{\cdot}51$).

This suggests that the increase in conductance affected flow to an extent that more than compensated for the fall in perfusion pressure, as may be seen by comparing the dose-response relations in Fig. 6.

REFERENCES

GROOM, A. C., MORRIS, W. B. & ROWLANDS, S. (1957). *J. Physiol., Lond.*, **136**, 218–225.
HUGHES, R. (1970). *Br. J. Pharmac.*, **39**, 232P–233P.
JANCSÓ, T. & GALGÓCRY, G. (1969). *Acta physiol. Hung.*, **35**, 173–182.
STARK, R. D. (1968). *Nature, Lond.*, **217**, 779.

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The self-association in aqueous solutions of morphine sulphate and some related salts

J. H. PERRIN AND A. ISHAG*

School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53706, U.S.A.

Conductivity and optical rotatory dispersion investigations suggest that morphine sulphate and some related salts aggregate in aqueous solution. This association is probably due to the stacking of the aromatic rings.

Recently optical rotation and ellipticity have been shown to depend upon the state of aggregation of optically active surfactants (Bonkoski & Perrin, 1968, 1969; Mukerjee, Perrin & Witzke, 1970; Perrin & Witzke, 1971). The change in optical properties was attributed to changes in degree of ionization (Bonkoski & Perrin, 1968, 1969; Perrin & Witzke, 1971) when the differently charged species have dissimilar optical rotatory dispersion (ORD) or circular dichroism (CD) curves and/or a "medium" effect arising from changes in refractive index at the micellar surface (Mukerjee, & others, 1970).

Concentrated solutions of morphine salts appeared to be surface-active (Perrin, unpublished observations), and it was decided to investigate the possible aggregation of some morphine related salts by conductivity and optical rotation. Anomalous ORD curves have been reported for morphine and related compounds (Bobbitt, Weiss & Hanessian, 1959), Cotton effects occurring at wavelengths near 300 nm; these are probably associated with the aromatic rings. Observations at lower wavelengths are complicated by the high absorbances of the compounds.

MATERIALS AND METHODS

Materials

The following salts were used as supplied by the manufacturer. Codeine phosphate U.S.P., codeine sulphate N.F., morphine sulphate U.S.P. (Mallinckrodt, St. Louis, Mo.); hydromorphone hydrochloride (Dilaudid, Knoll Pharmaceutical Co., Orange, N.J.). All solutions were prepared in deionized water.

Methods

The conductivities were measured using a Beckman RC 16B2 conductivity bridge (Beckman Instrument, Cedar Grove, N.J.) at $25 \pm 0{\cdot}01°$. ORD curves were obtained using a Cary Model 60 spectropolarimeter (Applied Physics Corporation, Monrovia, Calif.). The measurements were made at $25 \pm 0{\cdot}2°$ in 5 cm cells, taped to the cell carriage to aid reproducibility. The solutions were scanned at wavelengths between 450–410nm, a region far from any Cotton effect. This minimized absorption by the concentrated solutions necessary for these investigations and enabled a long path-length cell to be used, so increasing the precision of the experiment.

* Present address: Chelsea School of Pharmacy, Chelsea College of Science and Technology, Manresa Road, London, SW3, U.K.

RESULTS

Fig. 1 shows the plot of specific conductance versus concentration for the four salts investigated. All the curves show breaks which are usually associated with micelle or aggregate formation in ionic surfactants (Mukerjee, 1967). The critical micelle concentrations (cmc) obtained are shown in Table 1. A plot of observed rotation against concentration at a given wavelength also showed a break in all cases. To emphasize the break, a deviation plot was made as shown in Fig. 2. The theoretical rotation at a given concentration was found by multiplying the concentration by the slope of the straight line plot of observed rotation against concentration for concentrations well below the cmc. The deviation at a given concentration is this theoretical rotation minus the observed rotation. The cmc's estimated from these plots are shown in Table 1 together with the wavelength of measurement. The specific rotations [α] in Table 1 are calculated from the formula

$$[\alpha] = \frac{100}{l} \cdot \frac{\Delta \text{ rot.}}{\Delta c}$$

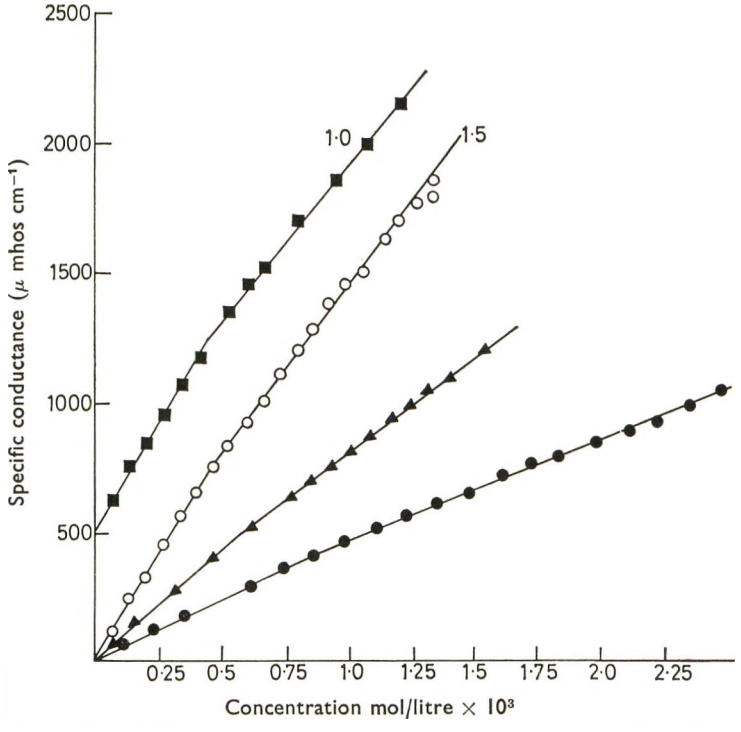

FIG. 1. Specific conductances for the salts of morphine related compounds as a function of concentration. ◆–◆–◆, codeine sulphate; ○–○–○, morphine sulphate; ◀–◀–◀, hydromorphine hydrochloride; ●–●–●, codeine phosphate.

where l = pathlength in decimeters and Δ rot. is the change in rotation in degrees for a change in concentration, Δc, in g/100 g of solution. The wavelengths reported in Table 1 are the lowest, and hence the rotations the highest, at which measurements could be accurately made with the system employed.

Table 1. *Summary of conductivity and ORD data.*

Drug	cmc mol/litre × 10_3		Specific rotations		
	Conductivity	ORD	Below cmc	Above cmc	Wave-length nm
Morphine sulphate U.S.P. $(C_{17}H_{19}NO_3)_2H_2SO_45H_2O$	4·35	4·35	224·0	218·0	420
Codeine phosphate U.S.P. $C_{18}H_{21}NO_3H_3PO_4\frac{1}{2}H_2O$	8·61	8·61	223·2	213·4	427·5
Codeine sulphate N.F. $(C_{13}H_{21}NO_3)_2H_2SO_43H_2O$	4·39	4·00	225·6	236·4	425·0
Hydromorphone hydrochloride $C_{17}H_{11}NO_3HCl$	5·59	4·97	438·6	444·6	415·0

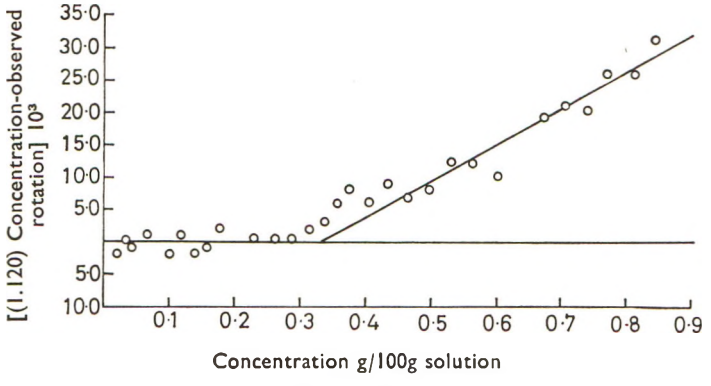

FIG. 2. Interpretation of ORD for morphine sulphate using a deviation plot. The theoretical rotation is calculated from concentration below the cmc (see text).

DISCUSSION

The break in the specific conductance versus concentration curves suggests that these molecules aggregate in large numbers at a well-defined concentration. This association is probably due to the stacking of the aromatic rings. The plots of observed optical rotatory dispersion at a given wavelength also show breaks at similar concentrations to the cmc's found by conductivity. The cmc's of the sulphates of morphine and codeine are very similar, suggesting the substitution of the methoxy for the hydroxy has little effect on the stacking of the molecules in the aggregate. From Table 1 it can be seen that a mole of codeine sulphate contains twice the number of equivalents of codeine as does the phosphate, and in terms of equivalents the cmc's of the two salts are similar and the cmc's appear to be independent of these counterions. The molecules are not planar and the packing could involve some sort of helical arrangement. The loss of the double bond and the substitution of the keto for the hydroxy group in hydromorphone does not seem from models to greatly alter the shape of the molecule as far as the proposed stacking is concerned. The cmc of hydromorphone (Table 1) is approximately 20% higher than the other drugs. The hydrochloride of hydromorphone is very much more soluble in water than are the hydrochlorides of codeine and morphine (Merck Index, 1968). This suggests that the base is more hydrophilic than the codeine and morphine, probably due to the keto group hydrogen bonding with the water molecules. It is also possible that

the hydromorphone molecules could associate by the interaction of the phenolic group of a second molecule. This type of interaction as well as the increased hydrophilicity of the hydromorphone are competitive reactions to the predominantly hydrophobic reaction envisioned for the aggregate formation, and so would result in the higher observed cmc. The reasons for the changes in the specific rotations on micelle formation are difficult to evaluate at these wavelengths so far from the absorption maximums, and as can be seen from the data in Table 1 no general trend is noticed. The rotation of codeine sulphate is enhanced on micelle formation. whereas codeine phosphate is not. This is possibly due to the different degrees of ionizations of the two salts in the micellar and non-micellar forms. The morphine sulphate, unlike codeine sulphate, shows a decrease in rotation, on aggregation yet both have similar basic pK_a's (Merck Index, 1968) and so differing degrees of ionization are unlikely to explain the observations. Interpretation of these changes involves investigation of the medium effect (Bonkoski & Perrin, 1969) and the changes in ionization on micelle formation (Bonkoski & Perrin, 1968, 1969; Perrin & Witzke, 1971). This is currently being attempted using circular dichroism to investigate the optical changes.

Acknowledgement

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REFERENCES

Bobbitt, J. M., Weiss, U. & Hanessian, D. D. (1959). *J. org. Chem.*, **24**, 1582–1585.
Bonkoski, S. & Perrin, J. H. (1968). *J. Pharm. Pharmac.*, **20**, 934–940.
Bonkoski, S. & perrin, J. H. (1969). *J. pharm. Sci.*, **58**, 1428–1429.
Merck Index (1968). 8th edn. Rahway, N. J.: Merck and Co.
Mukerjee, P. (1967). *Adv. Colloid Inter. Sci.*, **1**, 241–175.
Mukerjee, P., Perrin, J. & Witzke, E. (1970). *J. pharm. Sci.*, **59**, 1513–1515.
Perrin, J. H. & Witzke, E. (1971). *J. Pharm. Pharmac.*, **23**, 76–77.

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Flow properties of aluminium soap-hydrocarbon systems

J. S. STEPHENS*

School of Pharmacy, Portsmouth College of Technology, Park Road, Portsmouth, Hampshire, U.K.

Organogels prepared from aluminium stearate and liquid paraffin in concentrations of 0·5 to 17·0% by weight were examined using a rotational viscometer fitted with cone and plate. Parameters used to characterize the flow curves of these gels were plastic viscosity, apparent viscosity at fixed rates of shear and the estimated area of the hysteresis loop. The influence of temperature and aluminium stearate concentration on the shapes of the curves was examined. Concentration of aluminium stearate and temperature were found to influence both thixotropic behaviour and the rate of recovery after shear. A minimum concentration of 1·5 to 2·0% by weight of aluminium stearate appeared to be necessary for the formation of the gel structure.

The use of a beeswax-peanut oil base as a vehicle for a repository form of penicillin was described by Romansky & Rittman (1944). Later, Buckwalter & Dickison (1948) described a depot injection containing a suspension of pencillin salts in vegetable oil gelled with aluminium stearate. The viscosity of these gels was found to depend on the aluminium stearate used, its concentration, the type of oil, and the conditions under which the gel was made.

The rheological properties of the aluminium organogels reported by various workers have shown some discrepancies. The effect of concentration of aluminium stearate, and of temperature, on the rheological properties of a range of gels, together with subsequent changes in gel properties on storage and recovery after shear have now been examined.

MATERIALS AND METHODS

Gel preparation

Gels were formed with liquid paraffin B.P. and aluminium stearate (Manox†) in concentrations ranging from 0·5 to 17·0% by weight of the metal soap. Quantities of 300g were prepared with raw materials drawn from the same batch throughout. The method of manufacture followed the principles described by Morrison & Stephens (1967).

The finely powdered aluminium stearate was slowly added to about half the quantity of oil with the aid of a small electric stirrer. After 20 min mixing, the remainder of the oil was added and agitation continued for a further 10 min. With the mixer running, the mixture was heated through 2 to 3°/min to 160°, allowed to cool to between 130 and 140° and maintained within this range for a further hour, any

* Present address: Pharmaceutical Department, Imperial Chemical Industries Limited Pharmaceuticals Division, Macclesfield, Cheshire, U.K.

† Hardman and Holden Ltd., Manchester.

entrained air or steam being removed by manipulation of the stirrer. The preparation was then allowed to cool to 120° before removal of the stirrer and insulated to ensure a cooling rate of between 5 and 10°/h from 120° to room temperature.

Rheological examination of the gels

The instrument used was the Haake Rotovisco Rotational Viscometer fitted with cone and plate. Test sample, cone, and plate were allowed to attain test temperature (5 min) and any excess sample extruded was removed before readings were made. Rates of shear were varied manually; temperature was maintained within 0·2° throughout.

Viscosity was measured at 25° on a series of gels ranging in concentration from 0·5 to 9·0% of aluminium stearate by weight using a 15 s time cycle. Readings were then repeated at 10, 37 and 45°. Samples of 3·0 and 9·0% by weight gels were stored at 25° and allowed to remain undisturbed for 2, 7 and 21 days to test for reproducibility of the hysteresis loop.

To determine the effects of gel ageing, material containing 1·0, 3·0 and 9·0% by weight of aluminium stearate was transferred to sealed containers with the minimum of disturbance and stored at 25°. Viscosity at 25° was measured daily for three days and thereafter at weekly intervals for up to two years.

RESULTS

The 0·5 and 1·0% gels gave plastic curves at 25, 37 and 45°. Curves have been characterized by calculated values for plastic viscosity and yield stress; both increased with concentration of aluminium stearate. At 10° curves showed a yield value but were curvilinear and thus a plastic viscosity was not calculated.

The evaluation of yield stress by extrapolation to 0 s^{-1} rate of shear has been shown to provide an additional parameter for comparing curves obtained under standardized conditions of measurement. It is appreciated that if measurements had been possible at very low rates of shear, the curve would incline towards the origin of the graph and, in theory, pass through it at zero rate of shear.

At concentrations of 1·5% by weight aluminium stearate and above, hysteresis loops were formed when shear stress was plotted against rate of shear. Above 9·0% measurements were no longer possible as material was extruded.

The following relations have been established:

1. A non-linear relation was shown between the estimated areas of the hysteresis loop and both temperature and gel concentration. The loop area increased with increase in aluminium stearate concentration, and for a set concentration decreased with temperature (Table 1).

2. Yield stress values increased with concentration of aluminium stearate and decrease with temperature (Fig. 1).

3. At fixed rates of shear a relation between aluminium stearate concentration and apparent viscosity has been demonstrated. At the highest rate of shear used (9923 s^{-1}) the curve is almost linear (Fig. 2); at a low rate of shear on the up-curve (1103 s^{-1}) the curve is sigmoidal. The tangent to the curve is decreased with increase in temperature (Fig. 3).

4. Some degree of recovery after shearing was seen and depended upon concentration of aluminium stearate (Table 2).

On storage, the 1·0 and 9·0% gels increased in consistency for the first 188 h after manufacture and then changed negligibly over a 2 year period. The 3·0% gel increased in consistency during the initial 3 weeks storage and then remained constant (Table 3).

DISCUSSION

Yield stress values were determined from the curves by extrapolation of the "up-curve" to the shear stress axis. At a given temperature, yield stress increased progressively with concentration throughout the range of gels and for a fixed concentration the yield stress decreased with temperature rise (Fig. 1). Shiba (1960) described the

Table 1. *The relation between estimated area of the hysteresis loop and gel strength at varying temperatures.*

Gel strength (% w/w aluminium stearate)	Measurement temperature (°C)	Estimated area of hysteresis loop (cm^2)	Gel strength (% w/w aluminium stearate)	Measurement temperature (°C)	Estimated area of hysteresis loop (cm^2)
1·5	10·0	14·3	5·0	10·0	42·0
	25·0	5·80		25·0	16·8
	37·0	8·30		37·0	15·0
	45·0	3·40		45·0	7·00
2·0	10·0	33·0	7·0	10·0	—
	25·0	20·8		25·0	28·2
	37·0	7·50		37·0	22·0
	45·0	6·60		45·0	15·6
3·0	10·0	39·5	9·0	10·0	—
	25·0	21·8		25·0	46·3
	37·0	18·0		37·0	24·5
	45·0	9·60		45·0	11·0

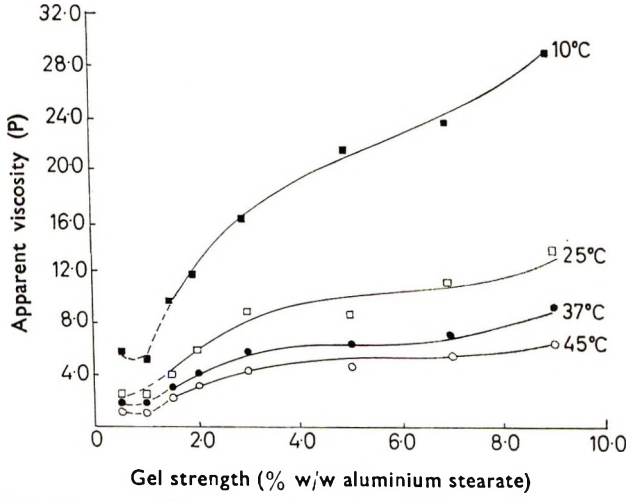

FIG. 1. The relation between yield stress and concentration of aluminium stearate in liquid paraffin derived by extrapolation of the up-curve to the shear stress axis at 0 s^{-1} rate of shear.

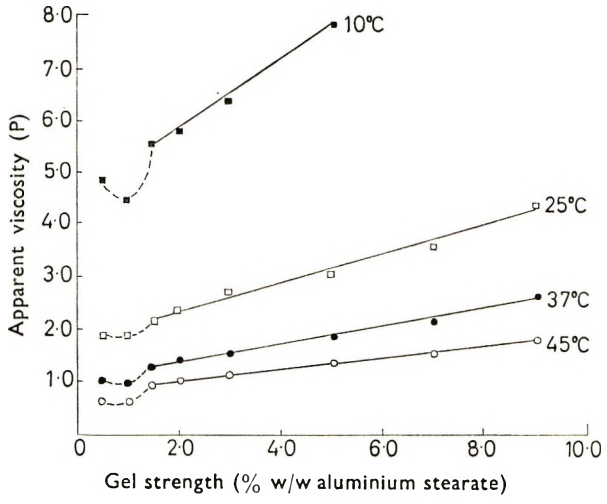

FIG. 2. The relation between apparent viscosity and concentration of aluminium stearate in liquid paraffin at high rates of shear (9923 s^{-1}). Shear rate employed maximum.

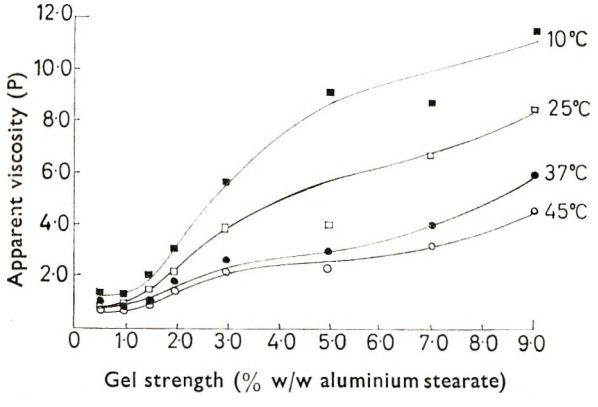

FIG. 3. The relation between apparent viscosity and concentration of aluminium stearate in liquid paraffin at low rates of shear (1103 s^{-1}) on the up-curve.

relation between yield stress and concentration as approximately linear. Results obtained in this study showed a sharp increase in yield stress at concentrations of aluminium stearate of 1·5% and higher.

Determinations of apparent viscosity at both high and low rates of shear showed that in the first case the apparent viscosity increased linearly with increasing concentration of aluminium stearate; at low rates of shear the curve was sigmoidal (Figs 2 and 3). A family of curves was obtained in both cases, the slope increasing in temperature. These observations were made for concentration of aluminium stearate greater than 1·5%. Shiba (1960) described the relation between apparent viscosity and concentration as approximately linear with aluminium stearate concentrations of 0·5 to 4·0% and maximum rates of shear of 1×10^3 s^{-1}. Differences between results obtained by Shiba and those obtained in this study may be attributed to several factors. These include: variations in instruments used; the maximum soap

Table 2. *Recovery behaviour of 3·0 and 9·0% gel after shearing.* Up-curve +, down-curve o.

Rate of Shear (s^{-1})	Shear stress (10^{-1} N m^2)											
	t = 0		t = 18 h		t = 0		t = 7 days		t = 0		t = 21 days	
	+	o	+	o	+	o	+	o	+	o	+	o
	3·0% gel											
61	5440	2720	3570	2890	4590	2720	2720	2380	4930	2890	2890	2720
123	5950	3230	3400	3230	5270	3230	3230	2720	5270	3060	3570	2890
184	6630	3570	3740	3400	5780	3400	3740	3060	5780	3230	3740	3060
368	7990	4250	4590	4250	6970	4080	4080	3740	6970	4080	4420	3740
551	8670	4760	5100	4760	7820	4590	4420	4080	8160	5270	5100	4420
	9·0% gel											
61	9690	3740	3570	3400	8840	3400	3560	3400	8610	2665	2635	2550
123	11 220	4080	4080	3910	10 030	3740	3470	3520	10 650	3115	3145	3060
184	10 030	4590	4420	4420	10 030	4420	4360	4240	9800	3740	3740	3655
368	11 790	5270	5270	5100	11 300	5150	5150	4980	11 270	4535	4505	4505
551	12 240	6290	6290	6120	11 810	6180	6130	6120	12 070	5725	5695	5525

Table 3. *Consistency changes in gels on storage at 25°.*

Gel concentration (% w/w aluminium stearate)		Time after preparation								
		18 h	24 h	48 h	72 h	7 days	14 days	21 days	7 weeks	2 years
1·0	Plastic viscosity (Poise)	1·80	1·76	1·69	1·65	1·85		1·82		2·05
	Apparent viscosity (Poise) (1103 s^{-1} rate of shear)	2·67	2·65	2·71	2·65	2·77		2·77		2·86
	Apparent viscosity (Poise) (9923 s^{-1}) rate of shear)	1·89	1·85	1·78	1·75	1·82		1·91		2·16
	Estimated area of hysteresis loop (ergs cm^{-3} s^{-1} × 10^7)	3·84	2·96	2·46	2·18	2·30	2·42	2·18	2·94	4·25
3·0	Apparent viscosity (Poise) (1103 s^{-1} rate of shear)	15·2	8·32	9·02	9·63	10·2	10·1	8·79	10·3	16·1
	Apparent viscosity (Poise) (9923 s^{-1} rate of shear)	1·95	2·36	2·42	2·73	2·77	2·70	2·78	2·81	3·7
	Estimated area of hysteresis loop (ergs cm^{-3} s^{-1} × 10^7)	4·42	4·56	4·70		4·42		4·63		5·40
9·0	Apparent viscosity (Poise) (1103 s^{-1} rate of shear)	13·8	13·4	13·6		13·9		13·5		15·4
	Apparent viscosity (Poise) (9923 s^{-1} rate of shear)	4·53	4·46	4·41		4·51		4·41		4·93

All determinations made at 25·0°

concentration used; the purity of the soap; the rate of cooling of the gel during preparation.

The flow properties of the gel changed with time and they depended upon concentration of aluminium stearate and storage temperature. The consistency of non-Newtonian samples may only be accurately compared using the complete flow curves. However, to provide a numerical comparison between samples, values for the plastic viscosity, apparent viscosity at high and low rates of shear on the "up-curve" and the estimated area of the hysteresis loop have been calculated (Table 3). Shiba (1960) considered that consistency reached a maximum some 4 h after gel preparation, but his study was restricted to a 2% gel only. Matsumura, Iguchi & others (1958) also found that storage temperature influenced structural formation. A refrigerated sample of 2% aluminium stearate-peanut oil gel required some 38 days to reach a constant viscosity whereas similar material stored at 37° reached equilibrium in 4 days. These results are not strictly comparable with those in the present study since the aluminium content and oil used differed, but it may be concluded that gels stored at 25° should be allowed to age for a minimum period of 3 weeks to reach equilibrium.

The rate of gel formation appeared to be related to the aluminium stearate content. At 1·0% aluminium stearate concentration, sufficient soap was present for the structure to form within 18 h. Above a concentration of 1·5% a more complex structure existed and its rate of formation appeared to depend upon the amount of metal soap present. With 9·0% of aluminium stearate, structural formation was rapid and complete within 18 h, but at 3·0%, structural formation occurred at a slower rate, insufficient material being present for a rapid build-up of the gel and some three weeks were necessary for consistency to reach equilibrium.

There is a disagreement in the literature about the time effects of flow behaviours on aluminium-hydrocarbon systems. Complete recovery was reported by Goldberg & Sandvik (1947), and Carver & van Wazer (1947). Evans & Matthews (1954) considered that permanent change in the structure of the gel was produced after shearing. Shiba (1960) thought that the paraffin gels were thixotropic at high temperatures or under very low rates of shear. Results in this paper show that a gradual recovery did occur with the 3·0% gel and that the system was thixotropic. No evidence of recovery was observed with the 9·0% gel; it is suggested that recovery may in fact occur, but at an extremely slow rate due to the viscosity of the system (Table 2).

Yield stress is an important factor. From results obtained, systems containing 0·5 and 1·0% of the aluminium stearate behaved as dilute suspensions of the metal soap dispersed in oil and they possessed a yield value. This yield value provided evidence of a weak structure. At soap concentrations above 1·5% the sharp rise in yield stress value coupled with the formation of hysteresis loops has provided evidence of a strong structural build-up (Fig. 1).

One possible explanation for the shape of the flow curves is as follows. At 10° both 0·5 and 1·0% gels yielded a curvilinear flow curve with yield values of 102 and 97 N m^{-2}. These values were within the limits of experimental error. It is suggested that at this temperature the thermal energy of the system was so low that some degree of molecular structure was present even under shearing conditions. On raising the temperature to 25° this weak structure was broken down and plastic flow resulted.

In an attempt to explain the shape of the curvilinear flow curve it is suggested that under low rates of shear the tendency of the long chain molecules to align in the direction of flow was negligible. They remained in a random state in which case the

rate of shear remained proportional to the shearing stress. As the rate of shear was increased, the molecules aligned. The frictional resistance between adjacent layers was reduced and a greater rate of shear was obtained for a given stress than would have occurred otherwise. The curve became concave to the stress axis.

Shearing material with concentrations of aluminium stearate of 1·5% and above resulted in the formation of a hysteresis loop. It is suggested again that under low rates of shear the molecules remained in a random state. At high rates of shear the molecules were aligned linearly and the shearing stress fell markedly. As the shearing rate was then decreased, the molecules required an appreciable time, in excess of the measuring cycle (total time 5 min) to regain their former state of entanglement. The results showed that loop area decreased with increase in temperature showing that thermal energy will break down structure; also the loop area increased with soap concentration thus showing the formation of a more rigid structure.

On the basis of the rheological studies it is possible to suggest evidence of gel structure. The aluminium soap was composed mainly of aluminium hydroxy di-soaps with small amounts of aluminium hydroxide, free fatty acid and a trace of water. The polymeric nature of the soap has been illustrated by the ability to make molecular chains when aluminium stearate in concentrations greater than 1·5% was dispersed by heat in liquid paraffin. These polymer chains imparted a rigid structure to the gel and the consistency increased with concentration. The rigidity of the structure increased lowering the temperature. Results also illustrated that at concentrations of soap less than approximately 1·5%, insufficient material was present to form and maintain such a coherent structure.

Acknowledgement

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REFERENCES

Buckwalter, F. H. & Dickison, H. L. (1948). *J. Am. pharm. Ass.*, **37**, 472–4.

Carver, E. K. & van Wazer, J. R. (1947). *J. phys. Colloid Chem.*, **51**, 751–63.

Evans, D. & Matthews, J. B. (1954). *J. Colloid Sci.*, **9**, 60–9.

Goldberg, H. & Sandvik, O. (1947). *Analyt. Chem.*, **19**, 123–31.

Matsumura, H., Iguchi, S., Yamamoto, M. & Goto, S. (1958). *Chem. pharm. Bull., Tokyo*, **6**, 55–7.

Morrison, J. C. & Stephens, J. S. (1967). *Am. Perfum. Cosmet.*, **83**, 53–6.

Romansky, M. J. & Rittman, G. E. (1944). *Science, N.Y.*, **100**, 196.

Shiba, S. (1960). *Bull. Chem. Soc. Japan*, **33**, 1563–8.

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The identification of tablets and capsules containing barbiturates by MATR infrared spectroscopy

J. E. ATKINSON

The School of Pharmacy, Robert Gordon's Institute of Technology, Aberdeen AB9 1FR, U.K.

The technique of MATR infrared spectroscopy has been used to record the infrared spectra of a variety of barbiturate-containing tablets and capsules. The production of different spectra for preparations containing different active ingredients, and the absence of spectral variations arising from the source of manufacture and the presence of tablet and capsule additives, indicate the technique to have possible application in the identification of tablets and capsules, especially where only small samples (1–2 mg) are available.

The introduction of the technique of attenuated total reflectance (ATR) to infrared spectroscopy and its modification to multiple attenuated total reflectance (MATR) have mainly found application in the examination of solid surfaces, like paper coatings and paints (Szymanski, 1967).

The technique involves the placing of the sample, which may be in the solid phase, against the surface of a plate that has suitable optical properties. For MATR the infrared light passes into the plate and, owing to the angle of incidence, dimensions of the plate, and refractive index of the material, undergoes a number of internal reflections. At each of these the light is able to penetrate the sample and undergo absorption thus making it possible to obtain an infrared spectrum for the sample.

In view of the sampling procedure, the technique was thought to have useful application in the identification of tablets and capsules. Preparations containing the barbiturate group of drugs were chosen because: (a) The barbiturates are closely related chemically. An examination of the spectra of tablets and capsules containing barbiturates would test the possibility of distinguishing between such preparations even though their formulation requires the addition of other substances (Remington, 1965). (b) Differences in the crystalline form of the barbiturates produced by grinding are known to produce variations in the halide disc infrared spectra of these compounds (Clarke, 1969). By using barbiturates the possible production of such differences during the process of tablet manufacture could be examined. (c) This technique had a possible use in tablet and capsule identification.

RESULTS AND DISCUSSION

The inclusion in tablets and capsules of diluents, disintegrants, binders and lubricants gives rise to the possibility that these compounds might contribute to the spectra of tablets and capsules. To examine this a batch of tablet granules was prepared containing (g) phenobarbitone sodium 60, lactose 296, starch 40, magnesium stearate 4 and 2% gelatin solution 50 ml. A corresponding batch of blank granules was also prepared. From these two batches of granules a number of phenobarbitone sodium

tablets (155 mg*; 23 mg†) and blank tablets (130 mg*; 0 mg†) were compounded. The MATR infrared spectrum of a sample of one of the phenobarbitone sodium tablets (1·5 mg‡) had major peaks at 1660, 1555, 1422, 1342, 1292, 1260 cm^{-1}. This spectrum was found to be identical with the MATR infrared spectrum of the batch of phenobarbitone sodium used to prepare the tablet granules. The spectrum of the blank tablet (5·6 mg‡) had no major peaks although a base line drift did occur in the ranges 4000–2000 and 950–650 cm^{-1}.

The possibility that proprietary phenobarbitone sodium tablets could differ from the formulation above and that this could alter the MATR infrared spectrum of the tablet was examined by running the spectrum of phenobarbitone sodium tablets (55–104 mg*; 30 mg†) produced by seven different manufacturers. The tablet spectra obtained were found to be identical with the spectrum obtained for the phenobarbitone sodium tablets prepared as described above. To examine further any possible differences in the MATR infrared spectrum of tablets due to variations in manufacture, the spectra of phenobarbitone tablets (57–87 mg*; 30 mg†) from eight manufacturers were run and found to be identical. The spectrum of phenobarbitone tablets had major peaks at 1702, 1419, 1350, 1312 and 1224 cm^{-1}. A comparison of the MATR infrared spectrum of the phenobarbitone sodium tablets and the phenobarbitone tablets indicated that this method of analysis could be used to distinguish between them.

Table 1. *The main peaks in the MATR infrared spectra of tablets and capsules containing one pharmacologically active compound and the weight of sample required to obtain an MATR infrared spectrum in relation to the weight of the tablet and the weight of the active ingredient.*

Barbiturate tablet	Wavelength (cm^{-1})	Wt of tablet or capsule content (mg)	Wt of active ingredient (mg)	Wt of sample (mg)
Amylobarbitone	1695, 1425, 1375, 1352, 1310, 1265, 1237, 1207, 1162, 1042, 815	80	30	0·6
Amylobarbitone sodium (Amytal sodium)	1699, 1609, 1559, 1425, 1369, 1335, 1315, 1285, 1260, 1155, 1050, 995, 788, 758	715	200	0·9
Barbitone	1757, 1667, 1408, 1372, 1315, 1229, 1039, 937, 860	425	324	1·5
Barbitone sodium (Medinal)	1695, 1662, 1540, 1456, 1445, 1430, 1410, 1370, 1335, 1310, 1015, 840, 787, 747	399	320	3·0
Butobarbitone	1697, 1422, 1357, 1304, 1237, 1209, 1032, 794	142	100	0·4
Cyclobarbitone	1689, 1409, 1339, 1289, 1209, 1026,	332	194	0·3
Hexabarbitone	1715, 1441, 1375, 1355, 1270, 1200, 1044, 775	342	260	0·5
Pentobarbitone sodium (Nembutal)	1693, 1658, 1650, 1553, 1425, 1350, 1308, 1257	128	30	0·6
Phenobarbitone	1702, 1419, 1350, 1312, 1224, 1336, 764, 692	93	30	0·7
Phenobarbitone sodium	1695, 1660, 1555, 1422, 1342, 1292, 1260, 1032, 777, 686	69	32	0·6
Quinalbarbitone sodium (Seconal sodium)	1693, 1655, 1551, 1426, 1336, 991, 921, 786	109	50	0·9
Nealbarbitone (Censedal)	1695, 1435, 1365, 1320, 1270, 1215, 1030, 992, 932, 842	84	60	0·4

* Total weight of tablet.
† Weight of the active ingredient in the tablet.
‡ Weight of sample used to obtain spectrum.

All of the tablets that had so far been examined were plain white and if the technique is to have wide application in tablet and capsule identification the addition of colouring agents and coatings to these preparations should ideally not affect the MATR infrared spectrum. To examine coloured tablets the spectrum of a pink butobarbitone tablet (142 mg*; 100 mg†), a dark pink Soneryl tablet (249 mg*, 100 mg†) and butobarbitone were examined and found to be identical. The spectrum of a Spansule (286 mg*; 97 mg†), a capsule containing phenobarbitone as white and blue granules, was identical with the spectrum previously obtained for phenobarbitone tablets. Secondly the spectrum of a Berbenzyl-30 tablet (93 mg*; 30 mg†), a dark red coated tablet, was identical with one previously recorded for phenobarbitone tablets. The possible effect of tablet coatings was further investigated using a blue-coated amylobarbitone sodium tablet (718 mg*; 200 mg†). The spectrum was run for a crushed tablet and a sample of the inner white core. Both spectra along with that obtained for the white powder present in an amylobarbitone capsule (219 mg*; 200 mg†) were identical.

Table 2. *The main peaks in the MATR infrared spectra of tablets and capsules containing more than one pharmacologically active compound and the weight of sample required to obtain an MATR infrared spectrum in relation to the weight of the tablet and the weight of the active ingredient.*

Tablet	Wavelength (cm^{-1})	Wt of tablet or capsule content (mg)	Wt of active ingredient (mg)	Wt of sample (mg)
Carbrital	1692, 1547, 1427, 1369, 1092	437		0·6
Pentobarbitone			100	
Carbromal			250	
Epanutin and phenobarbitone	1708, 1552, 1392, 1347, 1292, 1012	152		0·3
Epanutin			100	
Phenobarbitone			50	
Nembudeine	1694, 1650, 1600, 1550, 1538, 1505, 1470, 1451, 1410, 1365, 1240, 1112, 920, 822, 790	619		1·3
Codeine sulphate			15	
Aspirin			210	
Phenacetin			150	
Caffeine			30	
Nembutal			15	
Phenobarbitone and theobromine	1691, 1537, 1411, 1346, 1299, 1218, 1026	437		1·3
Phenobarbitone			30	
Theobromine			300	
Sonalgin	1701, 1658, 1602, 1551, 1506, 1480, 1445, 1411, 1370, 1321, 1241, 1173, 1114, 1045, 922, 835, 733	604		1·7
Butobarbitone			60	
Phenacetin			225	
Codeine Phosphate			10	
Sonergan	1693, 1408, 1351, 1300, 1233, 1030	303		0·8
Promethazine HCl			15	
Butobarbitone			75	
Tuinal	1691, 1651, 1544, 1429, 1309, 1151, 987, 915, 730	165		0·8
Quinalbarbitone sodium			50	
Amylobarbitone sodium			50	

In view of these findings, the MATR infrared spectra of a number of capsules and tablets containing a barbiturate as the only pharmacologically active ingredient were

run. The main peaks in the spectra of these tablets and those for the previously examined barbiturate tablets are recorded in Table 1. The spectra of a variety of capsules and tablets containing a barbiturate with other pharmacologically active ingredients have been examined and the main peaks in these spectra are presented in Table 2. An examination of the results in Tables 1 and 2 shows that it is possible to obtain infrared spectra of tablets and capsules using the MATR technique for sample presentation. These spectra are characteristic of a particular pharmacologically active ingredient or combination of ingredients and are not affected by the presence of tablet and capsule additives. The demonstration of a correlation between the spectra of a barbiturate and a tablet containing that drug, as with phenobarbitone sodium and

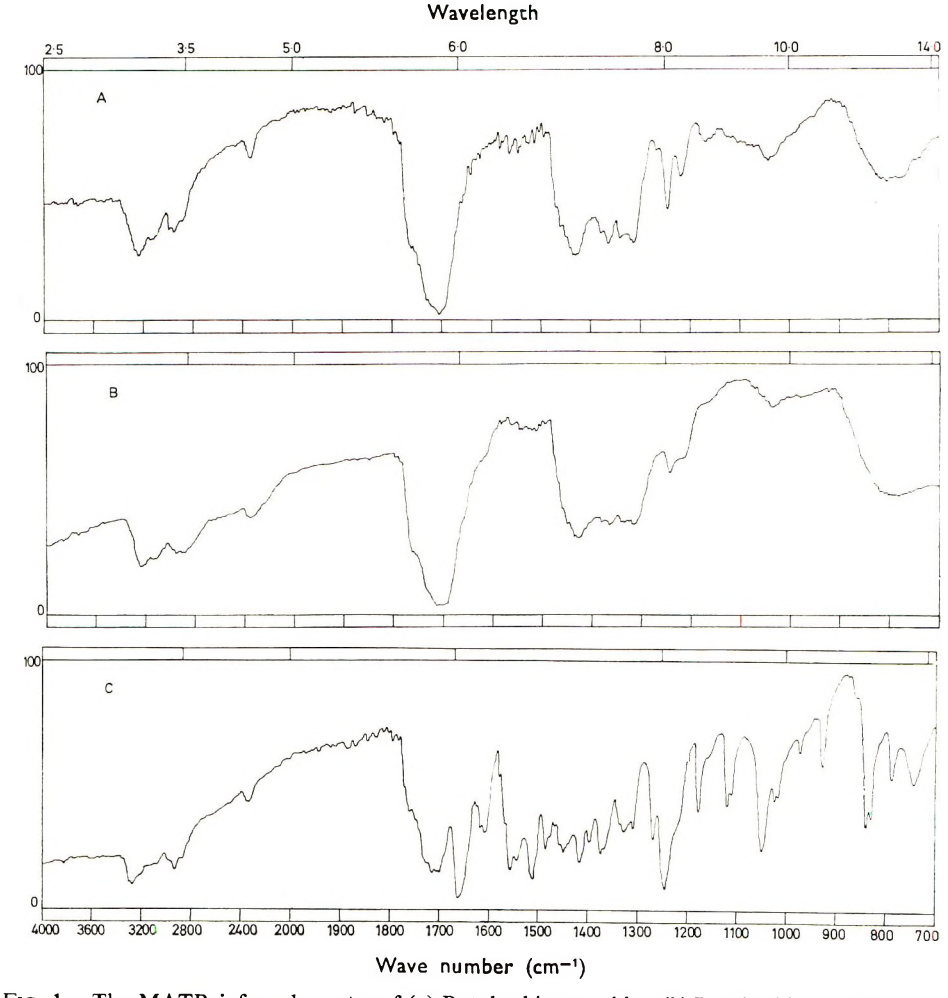

FIG. 1. The MATR infrared spectra of (a) Butobarbitone tablet, (b) Butobarbitone B.P. and (c) Sonalgin tablet.

butobarbitone could have useful application in tablet identification (see Fig. 1). This application would be limited to some extent by the number of types of tablet on the market containing only one pharmacologically active ingredient. The wider application of the technique to the identification of an unknown tablet would

necessitate the running of many reference tablet and capsule spectra. The recording of this spectral information using the method suggested by Curry, Read & Brown (1969), supplemented with other information, such as the shape, colour and size of the capsule or tablet, could be used as a rapid method of capsule and tablet identification.

METHODS

Instrument. Spectra were run on a Hilger and Watts Infrascan Spectrophotometer, fitted with a MIR-1 Teflon (Wiltec Scientific) internal reflector holder and a KRS-5, 2 mm, 45° reflector plate. An AT-30 (RIIC) attenuator was fitted in the reference beam. The energy level of the instrument was set at X2 and a scan time of 16 min was used. A wavelength correction was applied to each spectrum using a polystyrene film as a reference standard.

Sampling technique. The tablet or capsule content was crushed and a sample (Tables 1 and 2) was placed on one side of the KRS-5 plate. One to two drops of acetone were added to the sample and allowed to evaporate at room temperature (21°).

The placing of a sample of the crushed material against both sides of the KRS-5 plate which was then held in a MIR-2 (Wiltec Scientific) solid sample holder was found to give very poor spectra.

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REFERENCES

CLARKE, E. C. C. (1969). *Isolation and Identification of Drugs*, p. 117. London: The Pharmaceutical Press.

CURRY, A. S., READ, J. F. & BROWN, C. (1969). *J. Pharm. Pharmac.*, **21**, 224–231.

Remington's Pharmaceutical Sciences (1965). 13th Edn, p. 562, Easton Pennsylvania: Mack Publishing Co.

SZYMANSKI, H. A. (1967). *Progress in Infrared Spectroscopy*, Vol. 3, p. 39. New York: Plenum Press.

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Nuclear magnetic resonance studies of interactions between cetomacrogol and phenol

J. J. JACOBS, R. A. ANDERSON AND T. R. WATSON

Pharmacy Department, University of Sydney, Sydney, 2006, N.S.W., Australia

Phenol solubilized in cetomacrogol micelles appears to accumulate principally in the polyoxyethylene region and appears not to be randomly distributed along the polyoxyethylene chains. When concentrations of phenol are sufficiently high, a phenol–cetomacrogol-rich phase separates. This phase contains relatively little water and the phenol molecules may be randomly distributed along the polyoxyethylene chains. Transfer of molecules between the phases of these cloudy systems is relatively slow.

Nuclear magnetic resonance (nmr) spectroscopy may conveniently be used to follow the change of environment of a molecule upon solubilization by a surfactant, because the proton chemical shift depends (among other factors) on its physical environment. The total shielding which a proton experiences can be expressed as

$$\sigma = \sigma_d^L + \sigma_p^L + \sigma_p^N + \sigma_d^N + \sigma_s \qquad (1)$$

where the terms on the right hand side represent, in order, local diamagnetic, local paramagnetic, neighbouring paramagnetic, interatomic diamagnetic and solvent shielding contributions. The solvent shielding can be further expressed as a function of five separate effects:

$$\sigma_s = \sigma_B + \sigma_w + \sigma_a + \sigma_E + \sigma_c \qquad (2)$$

where the terms on the right hand side of the equation are associated respectively with bulk susceptibility, van der Waals interactions, diamagnetic anisotropy of the solvent, electric polarization and polarizability of the solvent, and interactions such as hydrogen bonding and charge transfer (Jackman & Sternhell, 1969a). Only the last two terms of equation (2) may differ significantly for different non-polar solutes in a particular solvent. It is generally found that if a molecule is transferred from a more polar to a less polar environment it experiences a high-field shift if the change in σ_c and σ_a is small (Buckingham, Schaeffer & Schneider, 1960; Eriksonn & Gillberg, 1966).

Thus the signals of the ring protons of phenol shift to higher field on addition of sodium dodecyl sulphate as increasing proportions of the phenol molecules are solubilized in the less-polar micellar environment (Jacobs, Anderson & Watson, 1971). Water protons also show a high-field shift on addition of sodium dodecyl sulphate due to disruption of water structure (Clifford & Pethica, 1964.)

Donbrow & Rhodes (1966) noted that the signals of the alkyl protons and the polyethylene oxide protons of cetomacrogol shift upfield when benzoic acid is solubilized in the surfactant solution. The change of chemical shift for the alkyl protons was the larger and this was interpreted as supporting other evidence that solubilized benzoic acid is located at the junction of the hydrocarbon nucleus and the polyoxyethylene region.

In the present study the chemical shifts observed in series of phenol–cetomacrogol–water systems are interpreted in terms of interactions between the components.

MATERIALS AND METHODS

Materials

Cetomacrogol 1000, B.P.C.; phenol, reagent grade; deuterium oxide (99·9% D_2O) was obtained from the Australian Atomic Energy Commission, Lucas Heights, Sydney; water was freshly distilled from an all-glass still.

Instrumental techniques

Spectra were obtained in water (unless otherwise specified) using a Perkin-Elmer R-12 high resolution spectrometer equipped with double resonance accessory, and locking to the signal of tetramethylsilane (TMS) used as an external standard. The operating temperature of the probe was 35° and all samples were allowed to equilibrate for at least 10 min before spectra were recorded.

Small chemical shifts were measured by expanding the field to 50 or 100 Hz per chart width. All shifts are quoted as changes with respect to the position of the peak concerned under some standard condition. The precision varies from 0·2 Hz for narrow peaks to 0·5 Hz for broad peaks such as that for polyethylene oxide protons in the presence of relatively high concentrations of phenol.

Each spectrum was run at least five times and the average change of signal position is reported. Corrections for bulk susceptibility have been applied where necessary.

Susceptibility corrections

When comparing chemical shifts of solutes at different concentrations or in different solvents, corrections for changes in volume diamagnetic susceptibility of the systems should be made, especially where the change in chemical shift is small.

The volume diamagnetic susceptibility of a mixture is given by

$$\chi_v^{1,2} = x_1 \chi_v^1 + x_2 \chi_v^2 \qquad (3)$$

where x_1 and x_2 are the volume fractions of the components and $\chi_v^{1,2}$, χ_v^1 and χ_v^2 are the volume susceptibilities of the mixtures and the pure components respectively (Pople, Schneider & Bernstein, 1959a).

The corrections were made using the relation

$$\sigma = \sigma_{obs} + \tfrac{2\pi}{3}(\chi_{v,ref} - \chi_v) \qquad (4)$$

where σ and σ_{obs} are the true and observed chemical shifts and $\chi_{v,ref}$ and χ_v are the volume susceptibilities of the reference system and the system under consideration (Pople, Schneider & Bernstein, 1959b).

Using volume susceptibility values of $0{\cdot}675 \times 10^{-6}$ and $0{\cdot}721 \times 10^{-6}$ for phenol and water respectively (*Handbook of Chemistry & Physics*, 1966) in equations (3) and (4) it may be calculated that the chemical shift in a 1% phenol–water solution requires a correction of −0·063 Hz when compared with that in pure water. Using Pascal constants, the volume susceptibility of cetomacrogol was estimated to be $-0{\cdot}730 \times 10^{-6}$; a 10% solution in water would therefore require a correction of +0·125 Hz. For the concentrations used in this study the necessary corrections are very small and of about the same magnitude as the experimental error.

RESULTS AND DISCUSSION

Solubilization of phenol by cetomacrogol leads to a change of environment for both phenol and cetomacrogol molecules, with subsequent changes in the chemical shifts shown in nmr spectra. (The peak positions observed are the weighted means of the positions of the free and solubilized species, as transfer is relatively rapid).

The signals of the phenolic ring protons shift upfield with increasing cetomacrogol concentration (Fig. 1), indicating a change to a less-polar environment as a greater proportion of the phenol molecules is solubilized in the cetomacrogol micelles.

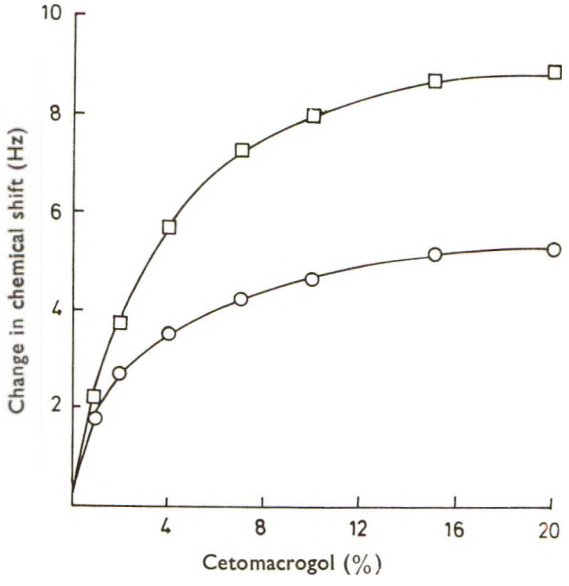

FIG. 1. Changes in chemical shift of ring protons of phenol (1%) in the presence of varying concentrations of cetomacrogol (measured with respect to the peak positions of 1% phenol in water): □ *meta* and *para* protons, ○ *ortho* protons.

An upfield shift could also be due to increased diamagnetic shielding of the aromatic rings, if the rings are randomly arranged. However, the changes of shift of the phenol ring protons in 10% cetomacrogol, with respect to the peaks in the corresponding phenol–water solutions, become smaller as the phenol concentrations increase from 0·5 to 3% (Fig. 2). Since the distribution coefficient favours solution in the micellar environment, increasing the overall phenol concentration will result in a greater increase in concentration in the micelles than in the true aqueous phase. Consequently the observed reduction in shift with increasing phenol concentration suggests a small degree of order in the arrangement of the aromatic rings which would lead to decreased diamagnetic shielding. Hence the high-field shift of the phenol ring protons with increasing cetomacrogol concentration (Fig. 1) must be due to the less polarizable environment of the micelles rather than increased diamagnetic shielding.

The phenol molecules may be solubilized in one or more of three regions of the micelles; (a) in the lipophilic centres of the micelles, (b) in the outer polyoxyethylene region and (c) at the junction of these two regions. The solubilized phenol molecules would be expected to cause a change in the chemical shift of cetomacrogol protons

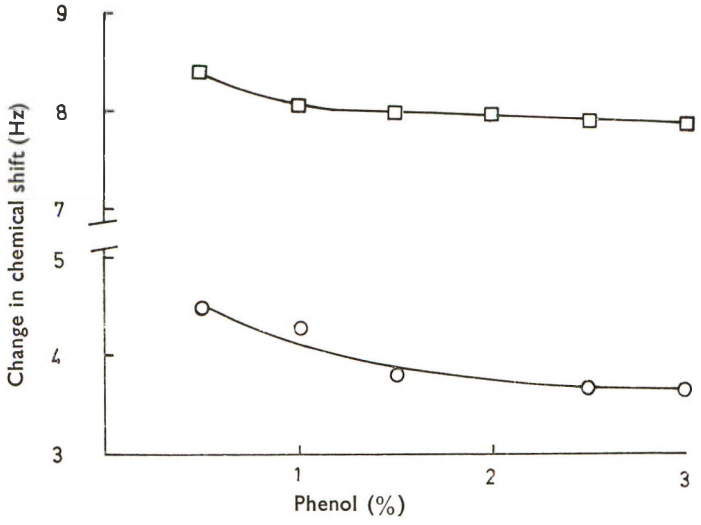

FIG. 2. Changes in chemical shift of ring protons of phenol with varying concentrations of phenol in the presence of 10% cetomacrogol (measured with respect to the peak positions of corresponding concentrations of phenol in water): □ *meta* and *para* protons, ○ *ortho* protons.

in the region or regions of solubilization, and nmr spectra may provide data to help determine where the solubilizate is concentrated in cetomacrogol micelles (Donbrow & Rhodes, 1966).

Fig. 3 shows the change in chemical shifts for both the polyethylene oxide protons and the alkyl methylene protons in the presence of increasing phenol concentrations. The greater total shift for the polyethylene oxide protons suggests that the phenol accumulates mainly in this region. This conclusion, however, is only valid if, when phenol is introduced, the changes in σ_E and σ_c of equation (2) are relatively small or about the same for both alkyl and polyethylene oxide protons. In this case, it is assumed that the diamagnetic anisotropic shielding contribution (σ_a) would outweigh

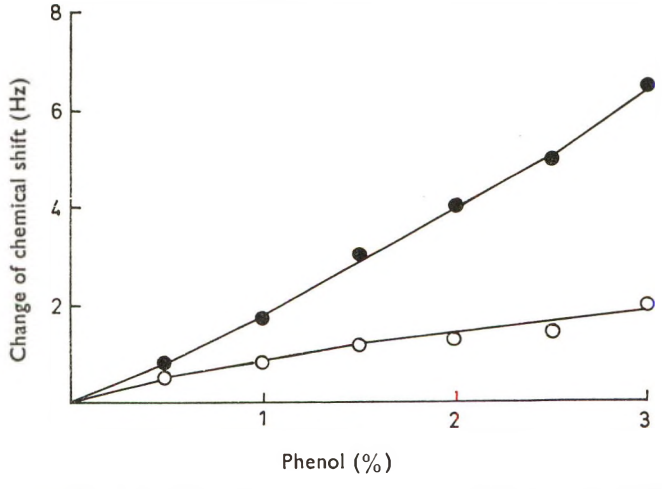

FIG. 3. Changes in chemical shift of protons of cetomacrogol (10%) in the presence of varying concentrations of phenol (measured with respect to the peak positions of 10% cetomacrogol in water): ● polyethylene oxide protons (major peak), ○ alkyl protons.

any other contributions. Since it is likely that σ_a is about the same for both alkyl and polyethylene oxide protons, a greater shielding would represent either a greater concentration of phenol or a specific orientation of the aromatic rings (σ_c). However, much orientation of the aromatic rings seems unlikely and the greater shielding of the polyethylene oxide protons may be attributed to a greater concentration of phenol in this region.

This conclusion is supported by the greater relative broadening of the polyethylene oxide peak compared with the alkyl peak. The presence of phenol would be expected to hinder the rotational freedom of the polyoxyethylene chains, leading to shorter transverse relaxation times and consequently to broader peaks. This evidence does not preclude solubilization at the junction between the lipophilic micelle centre and the polyoxyethylene region (where the solute molecules will have some association with each region) as well as solubilization of some phenol molecules in the lipophilic centre with most of the phenol being associated with the polyoxyethylene shell.

It is noteworthy that the protons in the polyoxyethylene chains have identical chemical shifts in aqueous solution. However, on the addition of phenol, this equivalence no longer exists and as the phenol concentration is increased, the apparent singlet at about δ 4·2 separates into three peaks as shown in Fig. 4. This may be

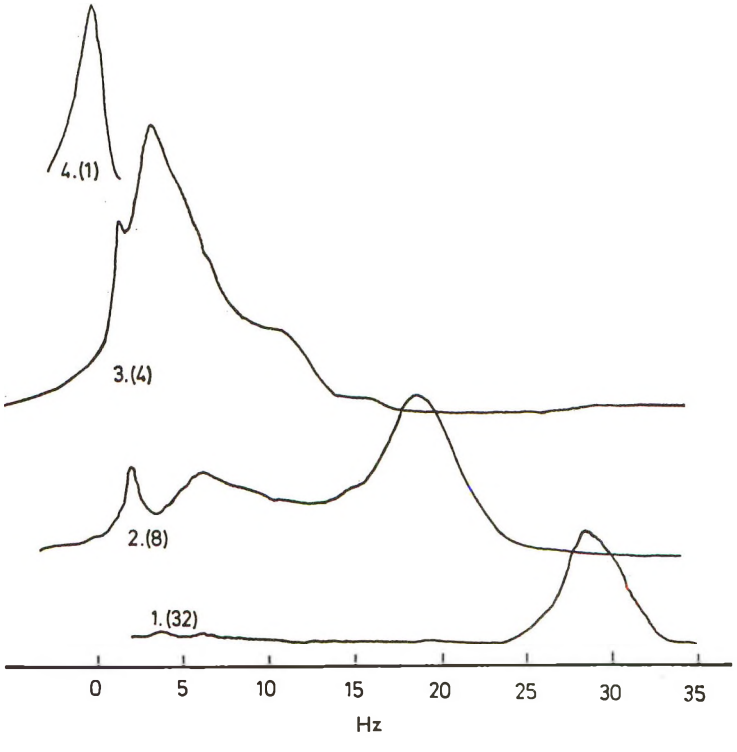

FIG. 4. Polyethylene oxide proton peaks of phenol–cetomacrogol–water systems.

Curve	Composition
1	3% phenol, 1% cetomacrogol
2	3% " , 6% "
3	3% " , 15% "
4	— " , 10% "

Numbers in brackets refer to the relative instrumental sensitivity.

caused by a non-homogeneous distribution of phenol in the polyoxyethylene region of the micelles.

As the phenol concentration is increased further, the system becomes cloudy owing to the formation of a phenol–cetomacrogol-rich phase, and a fourth peak appears. This peak has been assigned to the polyethylene oxide protons of the disperse phase. This is confirmed by the spectrum of the separated phenol–cetomacrogol-rich phase (Fig. 5). In contrast with the corresponding absorption in the aqueous system, this peak appears as a broad apparent singlet, indicating that in this system all polyethylene oxide protons are approximately equivalent.

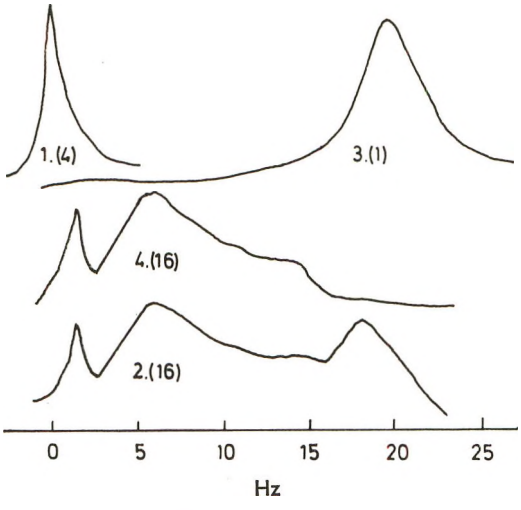

FIG. 5. Polyethylene oxide proton peaks of phenol–cetomacrogol–water systems.
Curve: 1. Cetomacrogol 7% in water. 2. Cloudy system before separation of phases (cetomacrogol 7%, phenol 3%). 3. Lower (cetomacrogol-rich phase) of system 2 after separation. 4. Upper (aqueous) phase of system 2 after separation. Numbers in brackets refer to the relative instrumental sensitivity.

The two separate absorptions which occur for the polyethylene oxide protons in the aqueous and phenol–cetomacrogol-rich phases of the cloudy systems indicate that transfer of molecules between the two phases is relatively slow, with residence times of at least 30 ms (Jackman & Sternhell, 1969b).

The changes in chemical shift of the polyethylene oxide protons with varying concentration of surfactant in the presence of 3% phenol are shown in Fig. 6. At low concentrations of cetomacrogol, and hence relatively high ratios of phenol to surfactant, the small amount of disperse phase contains most of the cetomacrogol along with high concentrations of phenol and the upfield changes of chemical shift of the polyethylene oxide protons are large. This phase contains relatively little water (see later) and the large upfield shift reflects the great change in environment of the polyethylene oxide protons. Little or no cetomacrogol is present in the aqueous phase and no signal from polyethylene oxide protons in the aqueous phase is apparent until the surfactant concentration reaches about 6%. As the overall surfactant concentration is increased to about 5%, the amount of the disperse phase increases, the phenol to cetomacrogol ratio in it falls and the upfield change of the signal from the polyethylene oxide protons becomes smaller.

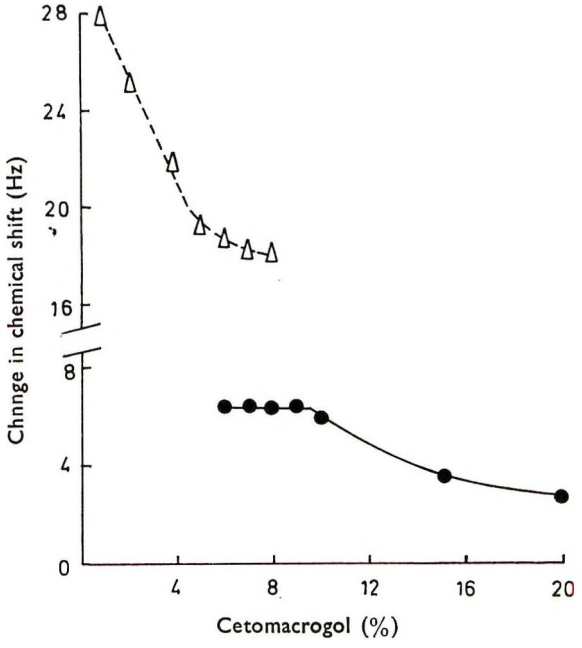

FIG. 6. Changes in chemical shift of polyethylene oxide protons of cetomacrogol in the presence of 3% phenol (measured with respect to the corresponding concentration of cetomacrogol in water): △ signal from protons in phenol–cetomacrogol-rich phase (in turbid systems), ○ signal from protons in aqueous phase (systems turbid up to 10% cetomacrogol, clear above 10% cetomacrogol).

When the cetomacrogol concentration is between 6 and 8%, sufficient surfactant is present in each phase to give two measurable signals. Both are upfield from the reference peak of the polyethylene oxide protons in water, but the signals from the protons in the disperse phase are shifted much more than that from the aqueous phase. The relative amount of the disperse phase changes over this concentration range; at 10% cetomacrogol the system has become transparent and further addition of cetomacrogol causes shifts to lower fields as the phenol in the micelles undergoes more and more dilution.

Over the range of 6 to 9% cetomacrogol, the intensities of the signals from the polyethylene oxide protons alter, reflecting changes in the relative amounts of surfactant present in each phase but there is no change in the chemical shifts observed, and the compositions of the phases would seem to be unchanged. This is supported by an estimation, from integrated spectra, of the composition of the phenol–cetomacrogol-rich phases separated from cloudy systems containing 6% and 7% cetomacrogol and 3% phenol. The molar ratio of phenol : ethylene oxide units : water was found to be the same in each sample (about 3 : 10 : 12·5). The number of water molecules per ethylene oxide unit is thus much less than for cetomacrogol in the form of micelles, where about six water molecules are incorporated per ethylene oxide unit (Elworthy, 1960).

It is generally believed that about two water molecules are associated with each ether oxygen of the polyoxyethylene chains, the remainder being trapped inside cone-shaped cavities formed by the ethylene oxide chains. It is likely then, that all the water molecules in the phenol–cetomacrogol-rich phase are associated with

the ether oxygens, and that little or no water is trapped in cavities in polyoxyethylene cones. A possible arrangement would be a lamellar structure, with the cetomacrogol molecules arranged side by side; the phenol would be concentrated in the polyoxyethylene region of this phase.

The water peak in water–cetomacrogol systems shifts to higher field with increasing concentration of cetomacrogol (Fig. 7). The addition of a solute to water generally has two opposing effects. Solutes may break up the water structure and give rise to high-field shifts; on the other hand possible hydrogen bonding between water and the solute would lead to downfield shifts. It may be assumed that the hydrogen bonding between water and the ether oxygens is less effective overall than inter-water hydrogen bonds.

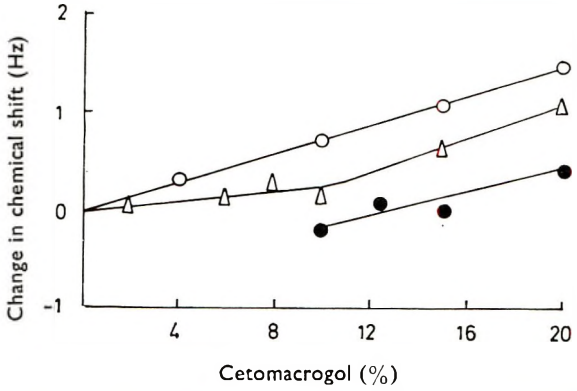

FIG. 7. Changes in the chemical shifts of water protons in the presence of: ○ varying concentrations of cetomacrogol (measured with respect to water), △ 1% phenol and varying concentrations of cetomacrogol (measured with respect to water containing 1% phenol), ● 3% phenol and varying concentrations of cetomacrogol (measured with respect to water containing 3% phenol).

When phenol is present along with cetomacrogol and water, the water shifts, with respect to the corresponding phenol–water solutions, are reduced. Phenol also disrupts water structure causing shifts to higher field (Jacobs & others, 1971), so the downward displacements shown in Fig. 7 for phenol-containing systems are due to removal of phenol from the bulk water by the cetomacrogol.

Acknowledgement

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REFERENCES

BUCKINGHAM, A. D., SCHAEFFER, T. & SCHNEIDER, W. G. (1960). *J. chem. Phys.*, **32**, 1227–1233.

CLIFFORD, J. & PETHICA, B. A. (1964). *Trans. Faraday Soc.*, **60**, 1483–1490.

DONBROW, M. & RHODES, C. T. (1966). *J. Pharm. Pharmac.*, **18**, 424–428.

ELWORTHY, P. H. (1960). *Ibid.*, **12**, 260T–266T.

ERIKSONN, J. C. & GILLBERG, G. (1966). *Acta chem. scand.*, **20**, 2019–2027.

Handbook of Chemistry and Physics (1966). 47th Edn, pp. E-112, E-113. Ohio: Chemical Rubber Co.

JACKMAN, L. M. & STERNHELL, S. (1969a). *Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry*, 2nd Edn, p. 104. London: Pergamon.

JACKMAN, L. M. & STERNHELL, S. (1969b). *Ibid.*, p. 56.

JACOBS, J. J., ANDERSON, R. A. & WATSON, T. R. (1971). *J. Pharm. Pharmac.*, **23**, 148–149.

POPLE, J. A., SCHNEIDER, W. G. & BERNSTEIN, H. J. (1959a). *High-Resolution Nuclear Magnetic Resonance*, p. 8. New York: McGraw Hill.

POPLE, J. A., SCHNEIDER, W. G. & BERNSTEIN, H. J. (1959b). *Ibid.*, p. 81.

J. Pharm. Pharmac., 1971, **23**, 794

LETTERS TO THE EDITOR

The inhibitory transmitter: GABA or its choline ester?

Recently, Curtis, Duggan & others (1970) reported studies on the action of the alkaloid bicuculline on the central nervous system; they claimed that this alkaloid was a relatively selective antagonist of γ-aminobutyric acid (GABA). Since their pharmacological evidence indicated that GABA was likely to be a transmitter at certain inhibitory synapses, the possible interaction of bicuculline with synaptic GABA receptors was discussed in the light of a comparison of Drieding stereomodels of GABA and the alkaloid. The comparison showed that GABA was indeed isosteric with part of the bicuculline molecule (Fig. 1), and this, of course, supported the view that bicuculline antagonizes the action of GABA by competing for the GABA receptors.

Examination of Drieding stereomodels of esters of choline, however, shows that many of these compounds also bear a close resemblance to that part of the bicuculline molecule which Curtis & others (1970) suggest bears a resemblance to GABA; in particular Drieding models of γ-aminobutyrylcholine (GABACh) and bicuculline demonstrate that the GABACh molecule corresponds particularly well also with that part of the alkaloid molecule under consideration (Fig. 2). The GABACh molecule matches exactly with the bicuculline molecule as far as the C-2 of the acid moiety of the choline ester, and there is only a slight discrepancy in the position of the C-3 of this part of the ester. It is obvious also that simple choline esters such as acetylcholine and propionylcholine will be isosteric with the same portion of bicuculline, but these simple esters possess no chemical group that would interact in the same way as the methylene di-oxy ring of the alkaloid. The nitrogen of bicuculline would be expected to be positively charged at physiological pH, as would the quaternary nitrogen of GABACh, and it is likely too that the methylene carbon atom of the methylene di-oxy ring would carry a partial positive charge as a result of the electron withdrawing effect of the oxygen atom. It is also likely that as the methylene di-oxy rings of the alkaloid will repel one another, forcing the bicuculline molecule into a linear form, the spatial arrangement of its nitrogen and its ester groups will then correspond well with the preferred conformation of acetylcholine esters (see for example Chothia, 1970; Inch, Chittenden & Dean, 1970). Thus, it might be suggested that bicuculline would react with a receptor for GABACh and in some ways (cf. Figs 1 and 2) could be expected to have more affinity for such a receptor than for a GABA receptor.

The possible role of GABACh in the mammalian central nervous system has been neglected by comparison with that of GABA; even though it is more than ten years since the presence of GABACh was first demonstrated in brain tissue and it was

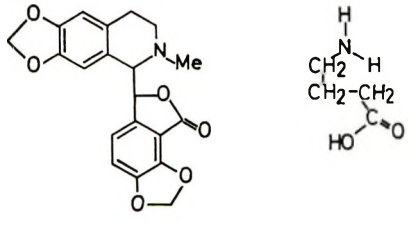

FIG. 1

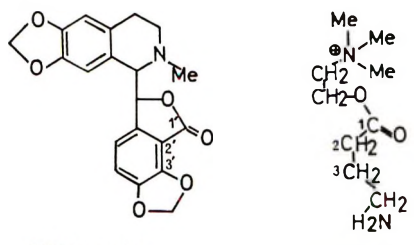

FIG. 2

proposed as an inhibitory transmitter (Kuriaki, Yakushiji & others, 1958; Kewitz, 1959a, b), there have been only a limited number of publications concerned directly with this compound. This paucity of information about GABACh could possibly be due to the lack of a specific assay for the ester and the lack of readily-available radioactively-labelled GABACh. However, it should be noted that the true role of GABA has still not been established definitively despite intensive study and much evidence for its relation with the activity of inhibitory neurons (Krnjević, 1970).

Since GABACh is known to exhibit inhibitory activity in certain parts of the cns (Takahashi, Nagashima & Koshino, 1958; Ashida, Takeuchi & others, 1965), the possibility that it might be an inhibitory transmitter should not be overlooked; this choline ester might even be the active form of GABA in inhibitory transmission. Although many of the requirements for a transmitter are apparently satisfied in the case of GABA (Krnjević, 1970), the extensive involvement of this compound in brain energy and ammonia metabolism found by Krnjević and its high concentration in brain tissue compared with that of acetylcholine (Kewitz, 1959a) could suggest that it is not the true transmitter. If this is so, some of the observed effects of GABA on inhibitory pathways might be explained by factors such as the release of stored GABACh, the enhanced synthesis of GABACh or the decreased breakdown of ester in the presence of added GABA (or both). Thus, in view of the known activity of GABACh (Takahashi & others, 1958; Ashida & others, 1965; Asano, Noro & Kuriaki, 1960) and of the still ill-defined function of GABA in the central nervous system, (Krnjević, 1970), these simple observations with molecular models prompt the question "Is the inhibitory transmitter GABA or its choline ester"?

Chemical Defence Establishment, D. J. HOWELLS
Porton Down,
Salisbury,
Wiltshire, U.K.

July 12, 1971

REFERENCES

ASANO, M., NORO, T. & KURIAKI, K. (1960). *Nature, Lond.*, **185**, 848–849.
ASHIDA, H., TAKEUCHI, N., MORI, A. & JINNAI, D. (1965). *Ibid.*, **206**, 514–515.
CHOTHIA, C. (1970). *Ibid.*, **225**, 36–38.
CURTIS, D. R., DUGGAN, A. W., FELIX, D. & JOHNSTON, G. A. R. (1970). *Ibid.*, **226**, 1222–1224.
INCH, T. D., CHITTENDEN, R. A. & DEAN, C. (1970). *J. Pharm. Pharmac.*, **22**, 954–956.
KEWITZ, H. (1959a). *Naturwissenschaften*, **46**, 495.
KEWITZ, H. (1959b). *Arch. exp. Path. Pharmak.*, **237**, 308–318.
KRNJEVIĆ, K. (1970). *Nature, Lond.*, **228**, 119.
KURIAKI, K., YAKUSHIJI, T., NORO, T., SHIMIZU, T. & SAJI, SH. (1958). *Ibid.*, **181**, 1336–1337.
TAKAHASHI, H., NAGASHIMA, A. & KOSHINO, C. (1958). *Ibid.*, **182**, 1443–1444.

Maximal responses in guinea-pig isolated ileum preparations: influence of longitudinal and circular muscle

Segments of the guinea-pig ileum prepared as described by Magnus (1904) are frequently used for the analysis of drug-receptor mechanisms involving agonists and antagonists. The maximal contraction (E_{max}) attainable with an agonist is an essential parameter for characterizing the effects of a drug in terms of affinity, intrinsic activity (Ariëns, Simonis & van Rossum, 1964) and efficacy (Stephenson, 1956). Preliminary investigations of the activities of choline and related compounds were made by using terminal segments of the guinea-pig ileum bathed in Tyrode solution at 37°. Cumulative concentration-effect curves (van Rossum & Ariëns, 1959) were obtained using an isotonic frontal writing lever (magnification × 10, and weighting 1 g) on a smoked drum.

Studies with choline and tetramethylammonium (TMA) which possess both nicotinic and muscarinic receptor stimulant properties (Triggle, 1965), showed that the maximal responses obtained with these compounds could be increased in the presence of the ganglion blocking drug hexamethonium. This unexpected effect was further evaluated using other drugs and procedures known to affect acetylcholine release. The mean maximal responses (± s.e.) of choline and TMA expressed as intrinsic activity (α), where α for acetylcholine is taken as unity, were 0·84 ± 0·05 for choline and 0·97 ± 0·10 for TMA. The mean concentrations (± s.e.) required to produce 50% of these maximal responses (EC50) were 0·09 ± 0·01, 56·0 ± 8·9 and 4·02 ± 1·55 μg/ml for acetylcholine, choline and TMA respectively. Table 1 shows the effects of various drugs and procedures on maximal responses and on EC50 concentrations for acetylcholine, choline and TMA. These values are expressed as percentage changes from control values obtained in Magnus preparations at 37°. Changes in EC50 values were calculated with respect to that response obtained under control conditions in order to avoid the influence of altered maximal responses on this parameter.

Concentrations of hexamethonium (12·5 μg/ml) which were sufficient to abolish responses to nicotine (2 μg/ml) produced only a small shift to the right of the concentration-effect curves to choline and TMA as shown by an increase in EC50 values. It would therefore appear that the predominant stimulant activity of choline, like TMA (Trendelenburg, 1961), does not involve nicotinic receptor stimulation in this tissue. In addition this treatment with hexamethonium produced increases of approximately 40% in the maximal responses attained with choline and TMA. Similar increases in maximal response were produced in the presence of morphine (0·5 μg/ml) and in tissues cooled to 25° for 15 min. These treatments are known to inhibit responses mediated indirectly through acetylcholine release in the guinea-pig ileum (Paton, 1957; Innes, Kosterlitz & Robinson, 1957). Atropine (12·5 ng/ml) shifted curves to three agonists to the right but did not increase their maximal responses.

Nicotinic receptor stimulation has been shown to produce a hexamethonium-sensitive release of catecholamines in various tissues (see Burn & Rand, 1965; Rand & Stafford, 1967, for references). However, catecholamine release was not responsible for the "inhibited" maximal responses to choline and TMA in the Magnus preparation at 37° as E_{max} was unaltered in the presence of adrenoreceptor blockade produced by a combination of phentolamine (0·1 μg/ml) and propranolol (0·1 μg/ml).

Careful observation of the ileal segments showed that at the higher concentrations used in the concentration-effect curves, choline and TMA produced a spasm within the tissue which was seen on the trace as irregular oscillations. TMA was more potent than choline in this respect. The spasm appeared to involve the circular

Table 1. *Effects of various treatments on % changes in maximal responses and EC50 values to acetylcholine, choline and tetramethylammonium.* Mean values (± s.e.) from 4 to 6 experiments of % change in maximal responses (E_{max}) and concentrations required to produce 50% of the control maximal response (EC50, cont) for acetylcholine, choline and tetramethylammonium (TMA) in Magnus preparations of the isolated guinea-pig ileum at 37°. Control values (Cont) and values obtained in the presence of hexamethonium (Hex, 12·5 μg/ml), morphine (Mor, 0·5 μg/ml) and adrenoreceptor blockade (α/βA) with phentolamine (0·1 μg/ml) and propranolol (0·1 μg/ml), and in tissues cooled to 25° (25°) are indicated. Values obtained in longitudinally cut preparations at 37° (acetylcholine = 100%) are also shown (L. cut, 37°).

		Magnus preparation (% change)					
		Cont	Hex	Mor	25°	α/βA	L. cut 37°
Acetylcholine	E_{max}	100	97 ± 5	88 ± 5	70 ± 9	95 ± 5	100
	EC50 (cont)	100	135 ± 14	190 ± 28	185 ± 37	145 ± 18	136 ± 14
Choline	E_{max}	100	147 ± 17	133 ± 5	124 ± 16	120 ± 2	168 ± 13
	EC50 (cont)	100	251 ± 48	315 ± 84	491 ± 62	109 ± 4	130 ± 28
TMA	E_{max}	100	137 ± 14	136 ± 19	124 ± 14	102 ± 2	152 ± 20
	EC50 (cont)	100	330 ± 10	450 ± 45	438 ± 38	135 ± 8	214 ± 36

muscle as evidenced by tight constrictions within the segment used. The possibility therefore existed that the "inhibited" maximal response might be due to a mechanical factor involving circular muscular contraction. Under experimental conditions where the maximal response was increased, no such constrictions within the ileal segments were noted.

To assess the influence of circular muscle activity on longitudinal muscle contractions, concentration-effect curves were produced in intact preparations, after which the ileal segments were cut longitudinally and curves to the agonists were re-established. In these cut preparations the maximal responses and EC50 values for acetylcholine were similar to those obtained in the intact preparation. In the cut preparations the maximal responses attained with choline and TMA with respect to acetylcholine (= 100%) were similar to, or greater than, those obtained in intact preparations at 37° which had been pretreated with morphine or hexamethonium or which had been cooled to 25°. Hexamethonium or morphine pretreatment had little further effect in enhancing maximal responses to choline and TMA in the cut preparations.

These results suggest that choline and TMA are producing differential concentration-dependent effects in the longitudinal and circular muscle coats of the guinea-pig ileum. The longitudinal muscle appears to be the most sensitive to muscarinic receptor stimulation. Higher concentrations are required for stimulation of circular muscle by choline and TMA, either directly, or indirectly through acetylcholine release. Brownlee & Harry (1963) have previously shown that there are differences in the sensitivities of strips of longitudinal and of circular muscle of the guinea-pig ileum to a wide variety of agonists.

The results of the present experiments show a limitation in the use of Magnus preparations of the guinea-pig ileum. Estimates of intrinsic activity or values of E_{max} which are essential for calculating other parameters, may be undervalued

because of mechanical inhibition of isotonic longitudinal muscle shortening through circular muscle activity.

Department of Pharmacology,
University of Melbourne,
Parkville, Victoria 3052,
Australia.

A. R. POMEROY
C. RAPER

June 9, 1971

REFERENCES

ARIËNS, E. J., SIMONIS, M. & VAN ROSSUM, J. M. (1964). *J. mednl Chem.*, **3**, 119.
BROWNLEE, G. & HARRY, J. (1963). *Br. J. Pharmac. Chemother.*, **21**, 544–554.
BURN, J. H. & RAND, M. J. (1965). *Ann. Rev. Pharmac.*, **5**, 163–182.
INNES, I. R., KOSTERLITZ, H. W. & ROBINSON, J. A. (1957). *J. Physiol., Lond.*, **137**, 396–409.
MAGNUS, R. (1904). *Pflügers Arch. ges. Physiol.*, **102**, 123–151.
PATON, W. D. M. (1957). *Br. J. Pharmac. Chemother.*, **12**, 119–127.
RAND, M. J. & STAFFORD, A. (1967). *Physiol. Pharmac.*, **3**, 1–95.
STEPHENSON, R. P. (1956). *Br. J. Pharmac. Chemother.*, **11**, 379–393.
TRENDELENBURG, U. (1961). *Arch. exp. Path. Pharmak.*, **241**, 452–466.
TRIGGLE, D. J. (1965). *Chemical Aspects of the Autonomic Nervous System*, p. 83, London: Academic Press.
VAN ROSSUM, J. M. & ARIËNS, E. M. (1969). *Archs int. Pharmacodyn. Thér.*, **118**, 418–446.

Analysis of the supersensitivity to noradrenaline induced by amphetamine in the isolated vas deferens of the rat

The development of supersensitivity in sympathetically innervated tissues may be of two different types. One type is specific to noradrenaline or other closely related sympathomimetic amines and dependent on a presynaptic mechanism, probably an impairment of the first step of the uptake process (Trendelenburg, 1963, 1966).

The second type of supersensitivity is non-specific and seems to be linked to a postsynaptic mechanism: modifications in the physiological state of the responding cells (Hudgins & Fleming, 1966; Westfall & Fleming, 1968a, b), or a change in the configuration of the adrenergic receptors (Carrier & Holland, 1965; Varma, 1966; Barnett, Greenhouse & Taber, 1968; Reiffenstein, 1968). Amphetamine is known to inhibit the uptake of noradrenaline (Axelrod, Hertting & Potter, 1962; Burgen & Iversen, 1965; Iversen, 1965, 1967; Häggendal & Hamberger, 1967). Recently it was suggested that amphetamine induces presynaptic supersensitivity to noradrenaline (de Moraes, Carvalho & Wherle, 1970). This report describes an investigation of the specificity of the amphetamine-induced change in sensitivity to noradrenaline.

Vasa deferentia of rats, 200–250 g, were mounted in Krebs-Ringer bicarbonate solution (de Moraes, Carvalho & Wherle, 1970). Dose-response curves to noradrenaline were made on each preparation before and after treatment with amphetamine for 20 min. Horizontal shifts of the log dose-response curves were measured at the level of the EC50. The same general procedure was employed to obtain the dose-response curves to methoxamine. Since it could not be obtained on the same vas deferens, because high concentrations of methoxamine induced spontaneous motility, vasa deferentia from the same rat were used to obtain EC50s. The error introduced is very small as vasa deferentia from the same animal had similar EC50s for methoxamine.

To measure adrenergic blocking activity, the pD'_2 value for noradrenaline-

Table 1. *Comparison of EC50 and maximum contractile response to noradrenaline and methoxamine in isolated rat vas deferens before and after exposure to amphetamine.*

Agonist	Treatment	n	EC50 (Mean ± s.e.)	Maximum contractile response (mm ± s.e.)
Noradrenaline	Control	6	4·733 ± 0·053	31·50 ± 1·52
	Amphetamine ($2 \times 10.^{5}$M)[a]	5	5·571 ± 0·084[b]	29·75 ± 2·17[c]
	Amphetamine ($2 \times 10.^{4}$M)[a]	5	6·507 ± 0·050[b]	31·54 ± 1·89[c]
Methoxamine	Control	4	4·767 ± 0·405	24·50 ± 2·66
	Amphetamine ($2 \times 10.^{4}$M)[a]	4	4·634 ± 0·326[c]	25·75 ± 1·43[c]

n Number of experiments.
EC50 Negative logarithm of the molar concentration producing 50% of the maximum response
[a] Exposure time of 20 min followed by three washes.
[b] Significantly different from control ($P < 0{\cdot}01$).
[c] Not significantly different from control ($P > 0{\cdot}05$).

phenoxybenzamine was calculated from the following equation (Bickerton, 1963): $pD'_2 = pD'_x + \log\left[\frac{Eam}{Eabm} - 1\right]$ where pD'_x is the negative logarithm of the molar concentration of phenoxybenzamine which reduced the maximum response to noradrenaline (Eam) to another value (Eabm). Amphetamine was used in "receptor-protection" experiments following the general procedure of Furchgott (1954). After a dose-response curve to noradrenaline was obtained, amphetamine was added to the vas and 20 min later phenxoybenzamine. After 30 min exposure to phenoxybenzamine both drugs were washed out. Post-exposure tests for sensitivity to noradrenaline were made between 60 and 100 min of the beginning of the experiment. The control vas was treated similarly except that amphetamine was absent. The reserpine powder used in these experiments was dissolved in a 20% solution of ascorbic acid and injected 5 mg/kg, i.p. 24 h before the experiment. (−)-Noradrenaline bitartrate, (+)-amphetamine sulphate and (±)-methoxamine hydrochloride were dissolved in distilled demineralized water which contained 0·02 mM of ascorbic acid. Phenoxybenzamine hydrochloride was dissolved in acidified ethanol (Benfey & Grillo, 1963) and diluted in normal saline.

Dose-response curves for noradrenaline and methoxamine obtained before and after exposure of the vas to amphetamine for 20 min followed by three washes were compared. The horizontal shifts of the log dose-response curves measured at the level of EC50 are statistically different only for noradrenaline (*t*-test). The exposure to amphetamine (2×10^{-4}M) for 20 min did not alter the sensitivity of the vas to methoxamine (Table 1).

pD'_2 values for noradrenaline-phenoxybenzamine were determined in another set of experiments. The pD'_2 values, after amphetamine exposure, in animals not pretreated with reserpine are statistically different ($P < 0{\cdot}05$) from the control value (7·498 ± 0·058; amphetamine: 2×10^{-5}M 6·986 ± 0·032, 2×10^{-4}M 6·942 ± 0·054). However, pre-treatment with reserpine did not affect the pD'_2 values in the absence or in the presence of amphetamine. Also of interest is that pD'_2 value was not altered by the reserpine treatment, in agreement with Green & Fleming (1967).

The evidence presented strongly favours the conclusion that amphetamine induces presynaptic supersensitivity to noradrenaline in the rat isolated vas deferens. Since methoxamine has a direct effect on α-receptors and is not taken up by adrenergic nerve endings (Hertting, 1964) it can be used as an experimental tool to test the role

of the uptake process in the development of the phenomenon of supersensitivity. Amphetamine does not increase sensitivity to methoxamine. This observation is consistent with the view that amphetamine-induced supersensitivity is probably due to an impairment of the uptake mechanism. Baum & Gluckman (1967) reported that amphetamine antagonized the adrenergic blocking activity of phenoxybenzamine in rabbit aortic strips. The pD'_2 values reported here show that the conclusion reached by Baum & Gluckman (1967) who used the rabbit aortic strips, can be applied to the rat vas deferens only if endogenous catecholamines are not first depleted. Amphetamine is an indirectly-acting sympathomimetic amine which releases noradrenaline and simultaneously inhibits the uptake mechanism (Lindmar & Muscholl, 1961, 1965; Iversen, 1967). This fact could explain why, in the vas deferens that has not been pretreated with reserpine, amphetamine seems to antagonize the adrenergic blocking activity of phenoxybenzamine. Perhaps the small amount of noradrenaline released by amphetamine in the non-pretreated preparation, before exposure to phenoxybenzamine, is responsible for the decreased value of pD'_2. On the other hand, the present results seem to exclude a configurational change of the adrenergic receptors to explain the amphetamine-induced supersensitivity.

Department of Pharmacology and Therapeutics,
Faculty of Veterinary Medicine,
University of San Paulo, Brasil.

S. DE MORAES*
F. V. CARVALHO

May 18, 1971

* Present address: Department of Pharmacology, West Virginia University Medical Center Morgantown, West Virginia, 26506.

REFERENCES

AXELROD, J., HERTTING, G. & POTTER, L. (1962). *Nature, Lond.*, **194**, 297.
BARNETT, A., GREENHOUSE, D. D. & TABER, R. I. (1968). *Br. J. Pharmac.*, **33**, 171–176.
BAUM, J. & GLUCKMAN, M. E. (1967). *J. Pharmac. exp. Ther.*, **158**, 510–518.
BENFEY, B. G. & GRILLO, S. H. (1963). *Br. J. Pharmac. Chemother.*, **20**, 528–533.
BICKERTON, R. K. (1963). *J. Pharmac. exp. Ther.*, **142**, 99–110.
BURGEN, A. S. V. & IVERSEN, L. L. (1965). *Br. J. Pharmac. Chemother.*, **25**, 34–49.
CARRIER, O. JR., & HOLLAND, W. C. (1965). *J. Pharmac. exp. Ther.*, **149**, 212–218.
DE MORAES, S., CARVALHO, F. V. & WERHLE, R. D. (1970). *J. Pharm. Pharmac.*, **22**, 717–719.
FURCHGOTT, R. F. (1954). *J. Pharmac. exp. Ther.*, **111**, 265–284.
GREEN, R. D. & FLEMING, W. W. (1967). *Ibid.*, **156**, 207–214.
HÄGGENDAL, J. & HAMBERGER, B. (1967). *Acta physiol. scand.*, **70**, 277–280.
HERTTING, G. (1964). *Biochem. Pharmac.*, **13**, 1119–1128.
HUDGINS, P. M. & FLEMING, W. W. (1966). *J. Pharmac. exp. Ther.*, **153**, 70–80.
IVERSEN, L. L. (1965). *Adv. Drug Res.*, **2**, 5–23.
IVERSEN, L. L. (1967). *The Uptake and Storage of Noradrenaline in Sympathetic Nerves*, pp. 147–198, London: Cambridge University Press.
LINDMAR, R. & MUSCOHLL, E. (1961). *Arch. exp. Path. Pharmak.*, **242**, 214–277.
LINDMAR, R. & MUSCHOLL, E. (1965). *Ibid.*, **252**, 122–133.
REIFFENSTEIN, R. J. (1968). *Br. J. Pharmac. Chemother.*, **32**, 591–597.
SNEDCOR, G. W. (1965). *Statistical Methods*, Iowa State College Press, Ames.
TRENDELENBURG, U. (1963). *Pharmac. Rev.*, **15**, 225–276.
TRENDELENBURG, U. (1966). *Ibid.*, **18**, 629–640.
VARMA, D. R. (1966). *J. Pharmac. exp. Ther.*, **153**, 48–61.
WESTFALL, D. P. & FLEMING, W. W. (1968a). *Ibid.*, **159**, 98–106.
WESTFALL, D. P. & FLEMING, W. W. (1968b). *Ibid.*, **164**, 259–269.

Action of methysergide on fenfluramine-induced contractions of the saphenous vein

A number of recent papers have discussed the relation between the actions of fenfluramine and 5-hydroxytryptamine (5-HT). Jespersen & Krüger-Schiel (1970) showed that the toxic hypothermic action of fenfluramine in dogs could be blocked by the 5-HT inhibitor methysergide. Funderburk, Hazelwood & others (1971) showed that the anorectic action of fenfluramine in rats could not be prevented by pretreatment with *p*-chlorophenylalanine but was inhibited by AHR-3009, a potent 5-HT antagonist. They suggested that fenfluramine could act directly by stimulating the tryptaminergic neuron.

It has been previously shown that fenfluramine causes a contraction of human isolated saphenous vein spirals (Coupar, Hedges & others, 1969). This effect has been further studied by investigating the action of methysergide on these responses. Four separate specimens of human saphenous vein spirals were set up in an isolated organ bath in Krebs-bicarbonate solution oxygenated with 5% carbon dioxide in oxygen. One end of the spiral was attached to a frontal writing lever and the responses were recorded on a kymograph. Two constant cumulative dose response curves to fenfluramine were obtained and then a further curve was obtained in the presence of the antagonist. After washing out, a further two constant fenfluramine curves were obtained before the procedure was repeated with a second dose of methysergide.

The results from one such estimation are shown in Fig. 1. Similar results were obtained in all four experiments, although the sensitivity of the tissues to fenfluramine and methysergide varied. Minimum effective doses of fenfluramine ranged from 0·5 to 10 μg/ml and of methysergide from 0·25 to 10 μg/ml.

The shift to the right and the lowered maxima of the fenfluramine dose response curves allow the conclusion that the antagonism by methysergide is non-competitive. The pA_2 value of methysergide against 5-HT on human vein is about 8·2 (Metcalfe & Turner, 1969) concentrations of 0·5 and 1 ng/ml being sufficient to produce marked inhibition of 5-HT responses. The higher concentrations necessary to inhibit fenfluramine responses may indicate a non-specific action of methysergide, and suggest that while the central nervous effects of fenfluramine may be related to 5-HT, this is not so clearly demonstrated in its peripheral actions on smooth muscle.

We thank the surgeons of this hospital for providing samples of human vein, the Peel Medical Research Trust and Chest and Heart Association for financial help, and Sandoz Products and Servier Laboratories for supplying methysergide and fenfluramine respectively.

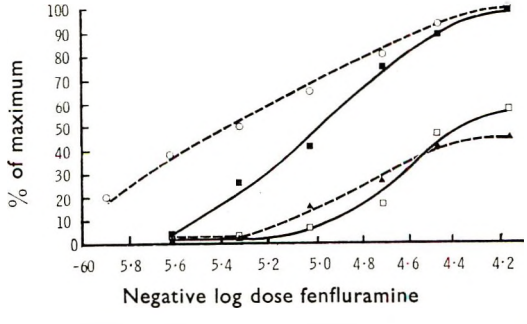

FIG. 1. The height of contraction produced by fenfluramine is expressed as a percentage of the maximum of the control curves (A ○– –○ and B ■–■) obtained before the adminstration of either 250 (□–□) or 500 (▲– –▲) ng/ml of methysergide to the bath fluid, and is plotted against the $\log_{10}$ of molar concentration of fenfluramine.

Department of Clinical Pharmacology,
St. Bartholomew's Hospital, London E.C.1, U.K.

MARILYN KIRBY
PAUL TURNER

June 2, 1971

REFERENCES

COUPAR, I. M., HEDGES, A., METCALFE, H. L. & TURNER, P. (1969). *Lancet*, **1**, 925–926.
FUNDERBURK, W. H., HAZELWOOD, J. C., RUCKART, R. T. WARD, J. W. (1971). *J. Pharm. Pharmac.*, **23**, 468–470.
JESPERSEN, S., KRUGER-SCHIEL, J. (1970). *Ibid.*, **22**, 637–638.
METCALFE, H. L. & TURNER, P. (1969). *Br. J. Pharmac.*, **37**, 519–521P.

Potentiation of the responses of the vas deferens of the guinea-pig to transmural stimulation and to noradrenaline, by triethylcholine and tetraethylammonium

The actions of triethylcholine (TEC) and tetraethylammonium (TEA) at cholinergic sites is well documented and an additional action at an adrenergic site was reported for TEA by Thoenen, Haefely & Staehelin (1967), who observed a potentiation by TEA of the contractile response of the cat spleen to stimulation of the splenic nerve. I now report a potentiation by TEC and TEA of the response of the vas deferens of the guinea-pig to adrenergic stimulation.

Adult male guinea-pigs, 500 g, were killed by stunning and bleeding and the vasa deferentia removed, stripped of their mesentery (Bentley & Sabine, 1963), and suspended in Krebs solution at 37°, bubbled with 5% carbon dioxide in oxygen. In some experiments the vasa were stimulated transmurally (Birmingham & Wilson, 1963) for 15 s every 4 min at a frequency of 20 impulses/s. Each impulse was of 500 μs duration and 100 V. In other experiments noradrenaline was added to the bath to stimulate the adrenergic receptors. Contractions were recorded by frontal writing levers on smoked paper. Concentrations of drugs are given in the weight of the base.

Each vas deferens was stimulated regularly until consecutive contractions were uniform. The tissue was then exposed to Krebs solution containing TEC (5×10^{-4} g/ml) or TEA (10^{-4} g/ml). In each experiment these concentrations of the drugs produced a potentiation of the response of the vas to transmural stimulation, although the extent of the potentiation varied from vas to vas it averaged $1\frac{1}{2}$ times. The potentiation was maintained while the drug remained in contact with the tissue, but was readily reversed. Control vasa, not exposed to the drug, showed no change in the size of the contractile response. The onset of the potentiation was rapid, maximum potentiation being reached within 30 s of administration of the drug.

Prior administration of cocaine (5×10^{-6} g/ml) or desipramine (10^{-6} g/ml) did not alter the potentiation.

A similar potentiating effect was described for choline by Bell (1967), but which differed from the present experiments in being blocked by hyoscine; the action of TEC and TEA did not appear to be at muscarinic or nicotinic ganglionic sites since neither atropine (5×10^{-6} g/ml) nor hexamethonium (5×10^{-6} g/ml) altered the potentiation.

In other experiments the effects of TEC and TEA on the response of vasa to a submaximal concentration of noradrenaline were tested. The response to $1{\cdot}6 \times 10^{-6}$ g/ml of noradrenaline (corresponding to about 30% of a maximal contraction) was, in the presence of TEC (5×10^{-4} g/ml) or TEA (10^{-4} g/ml), reproduced by a concen-

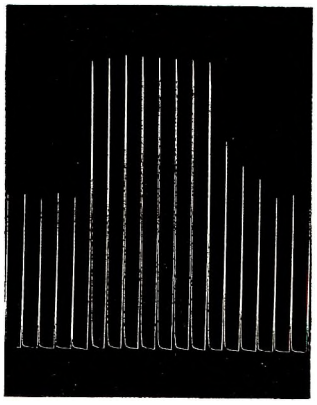

FIG. 1. The effect of tetraethylammonium (TEA) (μg/ml) on the responses of the guinea-pig vas deferens to transmural stimulation. Stimulation was for 15 s, every 4 min at a frequency of 20 impulses/s. Each impulse was of 500 μs duration and 100 V.

tration of noradrenaline of $2{\cdot}5 \times 10^{-8}$ g/ml. The action of the TEC or TEA in potentiating the response of the guinea-pig vas deferens to noradrenaline was not affected by prior administration of cocaine (5×10^{-6} g/ml) or desipramine (10^{-6} g/ml).

These experiments show TEC and TEA to have a potentiating effect at adrenergic neuroeffector junctions in the vas deferens. The action does not appear to be emdiated by blockade of the Uptake_1 mechanism since cocaine (Iversen, 1965) or desipramine, in concentrations which would be expected to inhibit the Uptake_1 process, have no effect on the potentiating action. Thoenen, Haefely & Staehelin (1967) found a measurable increase in the amount of noradrenaline released by nervous stimulation in the cat spleen after treatment with TEA; this effect may be contributing to the action of the drugs here. However, it does not explain the action of the drugs on the response to exogenous noradrenaline and for this reason it is suggested that the drugs may have a site of action at the post-synaptic membrane of the sympathetically innervated tissue.

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Department of Pharmacology,
University of London King's College,
Strand, London, W.C.2, U.K.

P. A. DUBBINS

July 30, 1971

REFERENCES

BELL, C. (1967). *Br. J. Pharmac. Chemother.*, **30**, 436–44.
BENTLEY, G. A. & SABINE, J. R. (1963). *Ibid.*, **21**, 190–201.
BIRMINGHAM, A. T. & WILSON, A. B. (1963). *Ibid.*, **21**, 569–580.
IVERSEN, L. L. (1965). *Adv. Drug Res.*, **2**, 1–46.
THOENEN, H., HAEFELY, W. & STAEHELIN, H. (1967). *J. Pharmac. exp. Ther.*, **157**, 532–540.

Storage life of prostaglandin E_2 in ethanol and saline

Few reports on the stability of prostaglandins in solution have been published. Karim, Devlin & Hillier (1968) measured the biological activity of saline solutions containing 100 ng/ml E_1, E_2, $F_{1\alpha}$ and $F_{2\alpha}$ kept at various pH values at room temperature. The PGE compounds showed 25–40% loss of biological activity after 60 days at pH 5–7, whilst the PGF compounds were still fully active after 182 days. Andersen (1969) reported 5–20% conversion to PGA_1, measured spectrophotometrically, in methanolic solutions of PGE_1 after one month at 5–10°. Now that the compounds are undergoing clinical trials, a more detailed investigation of their stability in different vehicles is necessary.

Ampoules containing 390 μg PGE_2 in 1 ml of 95% ethanol have been assayed spectrophotometrically at intervals over six months by measuring the chromophore developing at 278 nm after alkali treatment (Nugteren, Beerthuis & van Dorp, 1966). Both PGE_2, which does not itself absorb in the ultraviolet region, and PGA_2 (λ_{max} MeOH 217 nm) are converted to PGB_2 (λ_{max} MeOH 278 nm) on treatment with alkali. This assay therefore gives a measure of PGE_2 and of any PGA_2 formed by spontaneous dehydration.

After 4 weeks at 4° there was no detectable loss of total $E_2 + A_2$ and after six months at −20° <10% loss had occurred. At no time did the loss in potency of any individual ampoule exceed 11%. The average loss measured during this period was 5–6%. One ampoule which had been stored at −20° for 24 weeks was analysed by thin-layer chromatography on a silica gel G plate developed in chloroform–methanol–acetic acid–water (90:8:1:0·8). Pure PGE_2 and PGA_2 were run as markers; they were visualized by spraying with phosphomolybdic acid in ethanol and warming. The bands corresponding to PGE_2 and PGA_2 in the test sample were eluted and assayed by spectrophotometry. 378 μg (97%) was present in the PGE band and approximately 13 μg (3%) was present in the PGA band. One further ampoule stored as above was dried, taken up in saline and assayed for biological activity on the rat fundus strip against a fresh solution of PGE_2. This showed 75% potency.

For intravenous infusion, the above ampoules are usually diluted with sterile isotonic saline (pH 5–6) to give solutions containing 7·5 μg PGE_2/ml or 5 μg PGE_2/ml which are kept at 4°. Attempts were made to assess the storage life of these saline solutions. Aqueous solutions of PGE_2 show a chromophore with λ_{max} 283 nm after alkali treatment, compared with 278 nm in ethanolic solution. ϵ_{max} is also lower and takes longer to develop under aqueous conditions; conversion to PGB is incomplete (Nugteren & van Dorp, personal communication). Direct measurement of PGE content by alkali treatment and spectrophotometric measurement of the developing chromophore gives anomolous results in saline solutions, so that solutions of PGE_2 in saline were acidified to pH 3 with 2M citric acid, extracted with 2 volumes of diethyl ether and the dried extract dissolved in methanol. The methanolic solution was assayed by spectrophotometry and an aliquot analysed by thin-layer chromatography as described earlier. After ten days at 4°, 10% of the prostaglandin recovered from a 100 μg/ml solution was present as PGA. The biological activity of this solution was 70–80% (Table 1). After 6 months' storage under these conditions, one third of the prostaglandin recovered from a 7·5 μg/ml solution following extraction and thin-layer chromatography was present as PGA. Bioassay of this solution showed 50% potency. An unidentified chromophore (λ_{max} NaCl 256 nm, λ_{max} 0·5N KOH 296 nm) was separated from PGE and PGA by thin-layer chromatography and isolated.

The discrepancy between the results obtained by spectrophotometry and those from bioassay arises possibly because the spectrophotometric method measures μg quanti-

Table 1. *Composition and biological potency of solutions of PGE_2 after storage* (All values are quoted with reference to original concentrations.) The biological activity was determined on the rat fundus strip. Saline solutions of PGE_2 were extracted before spectrophotometric assay.

Concn	Storage conditions			Biological potency (%)	Spectrophotometric assay % PGE + PGA	After t.l.c.	
μg/ml	Vehicle	°C	Days	(approximate)	% Total (PGE + PGA)	PGE	PGA
390	95% ethanol	−20	182	75	94	97	3
7·5	0·9% NaCl	4	182	50	68	54	19
						34	10
100	0·9% NaCl	4	10	75	--	43	5

ties of pure compounds, whereas bioassay measures biological activity (in ng) rather than chemical composition. Bioassay is of value, however, since it indicates that after storage in ethanol, most of the biological activity remains.

It therefore appears that the potency of PGE solutions for clinical use deteriorates on storage in saline solution. This can be prevented by preparing these compounds as concentrated alcoholic solutions in ampoules. These can be kept for long periods at −20° and diluted with sterile isotonic saline as required within 24 h of use. Similar conclusions about the stability and storage of PGE compounds have been reached by Dr. D. H. Nugteren and Professor D. A. van Dorp (personal communication), who recommend that PGE_2 can be kept in ethanolic solutions for 1 year at −15° and in aqueous solution for a maximum of 1 week at 0° or 1 month at −15°.

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Institute of Obstetrics and Gynaecology,
Queen Charlotte's Maternity Hospital,
Goldhawk Road, London, W6, U.K.

HILARY C. BRUMMER

June 10, 1971

REFERENCES

ANDERSEN, N. H. (1969). *J. Lipid Res.*, **10**, 320–325.

KARIM, S. M. M., DEVLIN, J. & HILLIER, K. (1968). *Europ. J. Pharmac.*, **4**, 416–420.

NUGTEREN, D. H., BEERTHUIS, R. K. & VAN DORP, D. A. (1966). *Recl. Trav. chim. Pays-Bas Belg.*, **85**, 405–419.

The identification of blue triphenylmethane food dyes by thin-layer chromatography

Thin-layer chromatographic methods for the identification of the water-soluble synthetic dyes used as colorants in foods and drugs have been reported by authors using alumina (Mottier & Potterat, 1955), cellulose (Wollenweber, 1962), polyamide (Davidek & Davidkova, 1967), Sephadex (Parrish, 1968) and silica gel (Barrett & Ryan, 1963). Regulations governing usage of dyes vary widely from one country to another (Gill, 1962; Nieman, 1964) and reliable means of establishing unequivocal identification is essential.

Chromatography is used but the behaviour of chemically related dyes on chromatograms can be a source of error in identification among a group like the blue triphenylmethane dyes. The members of this group have a widely differing geographical acceptability particularly between North America and Europe (Gill, 1962; Nieman, 1964). Several authors have commented on the problem of their separation in particular of separating Brilliant Blue F.C.F., Blue V.R.S., Fast Green F.C.F., Green S, and Patent Blue V (Saenz Lascano Ruis, 1964; Ney, Bergner & others, 1965; Chapman & Oakland, 1968).

This paper describes a chromatographic method for differentiating between these dyes on ion exchange cellulose layers.

Thin layer chromatographic techniques were used. The plates were 10 × 5 cm diethylaminoethylcellulose pre-coated on plastic sheets by Machery-Nagel & Co. The plates were pre-treated by running in the solvent to half the length of the plate, dried and then running twice in deionized water and again dried. The spot loading was 1·0 μl of 0·01 % w/v aqueous dye solution applied using microcapillaries 2·5 cm from base of plate and developed over a distance of 5 cm.

Two solvent series were used: (*a*) Molar solutions of ammonium halides; fluoride, chloride, bromide and iodide. (*b*) 0·2M solution of ammonium salts of organic acids; acetate, citrate, carbonate and benzoate. The $R_F \times 100$ values were expressed with reference to Patent Blue V and are the mean of five determinations.

The results (Table 1) indicate that an adequate separation of these blue triphenylmethane food dyes can be obtained in the listed solvents, the best result being obtained with M ammonium iodide and 0·2M ammonium benzoate solutions. Azo dyes which are the most commonly used food dyes have very low R_F values in this system

Table 1. R_F *values relative to Patent Blue V of the dyes in the solvent series a* (M) *and b* (0·05M) *on diethylaminoethylcellulose layers.*

Dye	C.I. (1956)	Ammonium fluoride	Ammonium chloride	Ammonium bromide	Ammonium iodide
Fast Green FCF	42053	7·0	8·0	5·0	10·0
Brilliant Blue FCF.. ..	42090	33·0	46·0	66·0	65·0
Green S	44090	48·0	72·0	86·0	87·0
Blue VRS	42045	61·0	78·0	75·0	76·0
Patent Blue V*	42051	54·0	72·0	75·0	90·0
		Ammonium acetate	Ammonium citrate	Ammonium carbonate	Ammonium benzoate
Fast Green FCF	42053	10·0	10·0	6·0	19·0
Brilliant Blue FCF.. ..	42090	10·0	41·0	44·0	65·0
Green S	44090	40·0	65·0	51·0	51·0
Blue VRS	42045	160·0	85·0	59·0	78·0
Patent Blue V*	42051	14·0	24·0	64·0	74·0

* R_F value.

and as a result it is easy to separate the blue dyes from them except for Fast Green FCF.

Pharmacognosy Department, Welsh School of Pharmacy, University of Wales Institute of Science and Technology, Cathays Park, Cardiff, Glamorgan, U.K. T. D. TURNER

Elanco Division, Eli Lilly and Company Limited, Basingstoke, Hampshire, U.K. B. E. JONES

July 9, 1971

REFERENCES

BARRETT, J. F. & RYAN, A. J. (1963). *Nature, Lond.*, **199**, 372–373.
CHAPMAN, W. B. & OAKLAND, D. (1968). *Ass. publ. Analysts*, **6**, 124–128.
DAVIDEK, J. & DAVIDKOVA, E. (1967). *J. Chromat.*, **26**, 529–531.
GILL, J. P. (1962). *Chem. Prod.*, **25**, 56–62.
MOTTIER, M. & POTTERAT, M. (1955). *Analytica Chim. Acta*, **13**, 46–56.
NIEMAN, C. (1964). *Food colours recently authorised in 43 countries*, Supplement. Amsterdam: Consudel.
NEY, M., BERGER, K. G., SPERLICH, H. & MIETHKE, H. (1965). *Dt. LebensmittRsch.*, **61**, 148–150.
PARRISH, J. R. (1968). *J. Chromat.*, **33**, 542–543.
SAENZ LASCANO RUIZ, I. (1964). *Annls. Falsif. Expert. chim.*, **57**, 115–118.
WOLLENWEBER, P. (1962). *J. Chromat.*, **7**, 557–560.

Gas-liquid chromatographic estimation of paracetamol

Methods for the gas-liquid chromatographic estimation of paracetamol in plasma and urine have recently been described (Prescott, 1971). The drug could not be chromatographed directly in small amounts without significant absorption losses, and prior conversion to trimethylsilyl (TMS) derivatives was necessary. There are disadvantages however, in the use of silylating agents. The di-TMS derivatives of paracetamol formed with *N,O*-bis(trimethylsilyl)acetamide (BSA) is susceptible to hydrolysis, and silylation of other compounds in plasma extracts may give rise to unwanted peaks on the chromatograms. Although better results were obtained with *N*-trimethylsilylimidazole (TMSI), the sensitivity of the assay is limited by the broad solvent front of the slowly eluting TMSI. Furthermore, the flame ionization detector electrodes become contaminated by deposits of silica.

An improved method is now described in which both paracetamol and *N*-butyryl-*p*-aminophenol (the internal standard) are acetylated. The derivatives are stable, sensitivity is increased and the analysis can be completed in a much shorter time. Phenacetin does not interfere, and could be estimated in a sample at the same time as paracetamol.

Phosphate buffer (1·0 ml, M, pH 7·4) is added to plasma or urine (2·0 ml) containing up to 50 μg of paracetamol in a 15 ml glass-stoppered tube. Redistilled ethyl acetate (5·0 ml) containing *N*-butyryl-*p*-aminophenol (5 μg/ml) is then added and extraction effected by gentle mechanical shaking for 10 min. After centrifugation, the upper organic phase is transferred with Pasteur pipettes to 10 ml tapered stoppered centrifuge tubes and taken to dryness using a rotary vacuum evaporator. Pyridine (5 μl) and acetic anhydride (15 μl) are then added to the residue, the tubes stoppered and the contents mixed with a vortex mixer. The tubes are incubated on a water bath at 45° for 20 min and 1–3 μl aliquots are injected directly into the gas chromatograph. Samples containing paracetamol (50–500 μg/ml) are extracted with ethyl acetate containing *N*-butyryl-*p*-aminophenol (50 μg/ml), the residue is dissolved in pyridine (15 μl) and acetic anhydride (30 μl), and 1 μl aliquots are injected into the chromatograph. Appropriate dilutions are made of more concentrated samples, and total

Table 1. *Estimation of paracetamol added to plasma and urine—results of repeated analyses.*

Concentration (μg/ml)	No. of estimations	s.d. (%)
5–25	20	3·4
50–500	18	3·7
20	47*	3·4

* Each estimation was made on a different sample of plasma.

unchanged and conjugated paracetamol in plasma or urine can be determined by prior hydrolysis with glusulase as described by Prescott (1971).

A Hewlett-Packard Model 402 gas chromatograph with flame ionization detectors and a 2 ft long $\frac{1}{4}$ inch i.d. U-shaped glass tube column packed with 3% HI-EFF 8BP on 100/120 mesh Gaschrom Q (Applied Science) was used with the column temperature 220° and the nitrogen carrier gas flow rate 80 ml/min. The retention times of phenacetin, paracetamol and *N*-butyryl-*p*-aminophenol were 1·6, 3·4 and 4·5 min respectively. Satisfactory results were also obtained with 4 ft columns of 3% OV17 or 1% Carbowax 20 M on Gaschrom Q. *p*-Aminophenol will yield the same acetylated derivative as paracetamol, but this is of little consequence since, in man, *p*-aminophenol is not detectable in biological fluids following ingestion of paracetamol.

An appropriate aqueous standard of paracetamol is run with the samples and drug concentrations determined using the peak height ratios of drug to internal standard. The recovery of paracetamol added to plasma, urine or aqueous solutions is identical, and the precision of the assay is shown in Table 1.

Although interfering peaks were not normally observed with plasma extracts, a single large peak with a retention time approximately 4 times that of the internal standard was regularly encountered with both the above mentioned columns. This peak was not observed when the liquid phase was 3% XE60, but injection of acetic anhydride resulted in rapid deterioration of the column.

I am indebted to Dr. A. Robertson, Sterling Winthrop Research & Development, Fawdon, Newcastle upon Tyne, for a gift of *N*-butyryl-*p*-aminophenol, and I gratefully acknowledge technical assistance given by Mrs. M. E. Roberts and Miss N. Taylor. This work was supported by a grant from the Scottish Hospital Endowments Research Trust.

University Department of Therapeutics,
The Royal Infirmary,
Edinburgh, EH3 9YW, U.K.

L. F. PRESCOTT

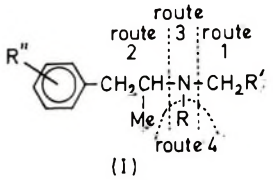
July 2, 1971

REFERENCE

PRESCOTT, L. F. (1971). *J. Pharm. Pharmac.*, **23**, 111–115.

Metabolic oxidation on aliphatic basic nitrogen atoms and their α-carbon atoms—some unifying principles

Among the pathways of metabolism of amines of type (I), metabolic oxidative cleavage from nitrogen at 1 and 4 is usually considered as dealkylation (McMahon 1966), and at 2 as deamination (Axelrod, 1955), while oxidation of the nitrogen atom at 3, when tertiary, is considered as *N*-oxidation (Beckett, Gorrod & Jenner, 1971; Beckett, Mitchard & Shihab, 1971). Some workers consider that C–N cleavage occurs after migration of O from N to α-C atoms (Ziegler & Petit, 1964). Difficulties arise in interpretation because of inability to isolate unstable metabolites.



(I)

R = H or lower alkyl
R' = H, lower alkyl or aryl
R'' = H, CF_3, halogens, etc

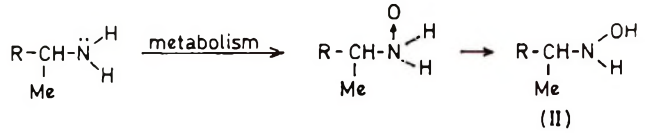
Many 'metabolites' have been identified from compounds of type (I), e.g. for benzylamphetamine (I; R=R''=H; R'=Ph), amphetamine, benzylamine, benzylmethylketone, benzaldehyde, α-methyl-β-phenylethanol, benzylalcohol, *syn*- and *anti*-benzylmethylketoximes, benzaldoxime, benzoic acid, but information is lacking concerning whether these arise by enzymatic action or by chemical changes subsequent to enzyme action.

We have observed, for instance, that storage of aqueous solutions following incubation of I, R=R''=H, R'=Ph, with liver microsomal preparations led to large changes in the concentration of some of the 'metabolites' and these changes depended on the conditions of storage, e.g. at pH 12 at 4° for 72 h the concentrations of free oximes and benzaldehyde *increased* by several hundred per cent, the rate of increase being most rapid when the solutions were shaken. The concentrations of amphetamines and benzylamines sometimes increased by as much as 50 per cent on storage under these conditions but these increases were variable, while the concentrations of benzylmethylketone *decreased* and those of benzylalcohol remained constant. At pH 1 the concentrations of benzylmethylketone and benzaldehyde increased by at least 50 per cent, whereas the concentrations of the alcohols remained constant and oximes could not be detected even after storage; increases also occurred in the concentrations of some 'metabolites' when solutions were stored at pH 7·4. Increases were obtained in benzylmethylketoxime content on storage of solutions after incubation of *N*-alkyl amphetamines with liver microsomes; oximes were generated more quickly the smaller the group R' in (I). In acidic solutions, the concentrations of oximes decreased rapidly but the concentrations of benzylmethylketone increased (Beckett, Van Dyk & others, 1971). Organic solvent extracts of the solutions immediately after incubation yielded increasing amounts of oximes on shaking with alkali and increasing amounts of aldehydes on shaking with acid.

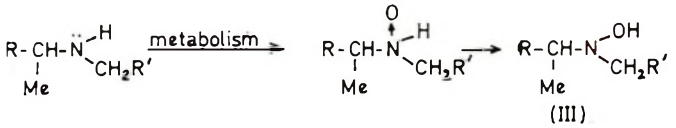
Thus, the measurement of the above substances after incubation of the parent compounds with liver microsomes may give misleading information about metabolic dealkylation or deamination of compounds of type (I) because the products of

chemical changes of metabolites* and the amount of chemical conversion is largely dependent upon the time after incubation that the analysis is made and the conditions of storage.

N-Oxidation of tertiary amines has been shown to be a metabolic route substantially inhibited by cysteamine and dithiothreitol in concentrations that have little effect on *N*-demethylation or *C*-oxidation (Beckett & others, 1971c; Gorrod & others, 1971); these inhibitors also substantially reduced the production of those metabolites which, when shaken in alkaline solution, gave oximes as metabonates, but they did not greatly reduce the formation of primary amines from metabolites of the secondary amines (I; R=H). The production of the metabolites which yield oximes from primary amines was also reduced by these inhibitors. Carbon monoxide inhibited the demethylation of methylamphetamine (I; R=R′=R″=H) without affecting the formation of oxime; SKF 525A (2-diethylaminoethyl-2, 2-diphenylvalerate hydrochloride) inhibited demethylation to a much greater extent than oxime formation. We therefore infer that the metabolites yielding oximes as metabonates from primary and secondary amines arise as follows:



(II)



(III)

Hydroxylamines of type II and III as primary metabolites are converted to ketoximes (R–C(Me)=NOH); III is also converted into traces of aldoximes (R′CH=NOH) as well as aldehydes (R′CHO) as metabonates. The rate of conversion depends on the temperature and alkalinity of the solution, upon the amount of shaking in air and on the presence of catalysts as well as upon the characteristics of group R′. Solutions containing the hydroxylamine metabolites and those containing synthetic hydroxylamines behaved similarly on polarography and by yielding oximes upon shaking in alkaline solution; on g.l.c. some breakdown of hydroxylamines to their corresponding oximes occurred. Amines were not produced when the hydroxylamines were stored in acidic, neutral or alkaline solution, and incubation of oximes and hydroxylamine derived from amphetamine did not yield amphetamine: amine metabonates must therefore have been derived from non-hydroxylamine metabolites.

We therefore conclude that these primary metabolites which yield amines from compounds of type I are produced by oxidation of the carbon α to the nitrogen atom

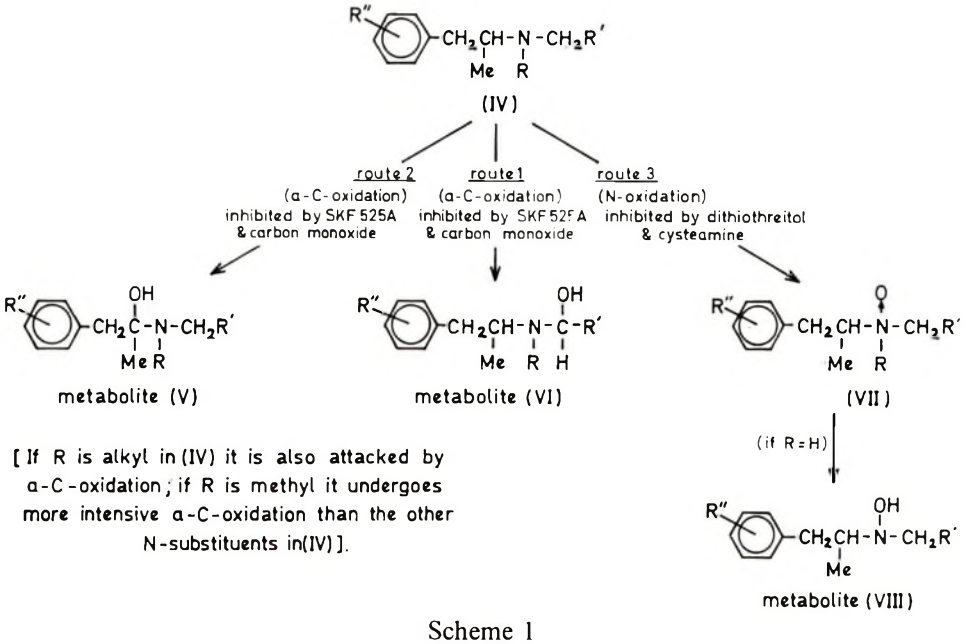
* It is necessary to distinguish between those substances formed directly by enzymatic action and those which result from chemical changes of these substances, especially if the chemical change is the rate controlling step in the formation of the compound which is finally determined.

The term *primary metabolite* is used to define a substance resulting directly from enzyme attack on an *added* substrate or a substrate formed chemically from a substance added to the biological system.

The term *secondary metabolite* will be used to define a substance derived from an enzymatic process utilising a metabolite as substrate.

Metabonate is used to define a substance which is a product of metabolism but is not a metabolite as defined above, i.e. it is formed as a result of non-enzymatic changes other than proton transfer in a metabolite, these changes occurring in the biological system or during isolation or during analytical procedures.

to yield $-N-\underset{\underset{OH}{|}}{C}-$ groups which are not very stable in neutral solution and are converted almost immediately in acidic solution to amines and ketones or aldehydes, depending upon the type of α–C group. If there are no H-atoms on the α–C-atom, this route of metabolism is not possible. Also hydroxy groups on β–C-atoms substantially inhibit the route.



Scheme 1

The metabolic oxidation on the basic nitrogen and its α-carbon atoms in aralkylamines is illustrated in scheme 1; the metabolism of benzphetamine (IV; R=Me, R′=Ph, R″=H) and benzylamphetamine (IV; R=R″=H; R′=Ph) to metabolites which are converted to metabolic products is representative of the general principles.

Incubation of benzphetamine with liver microsomes results in extensive production of benzylamphetamine and formaldehyde but much less production of those primary metabolites that yield amphetamine, benzylamine, benzylmethylketone or benzaldehyde as metabonates, i.e. the N–Me group undergoes α-C-oxidation before the other alkyl substituents. Incubation of benzylamphetamine (IV; R=R″=H; R′=Ph) yields the metabolite (V) which is converted at least 50% to the metabonates, ketone and benzylamine; after incubation, storage of the solution at acidic, neutral or alkaline pH values converts any remaining amounts of (V) to these compounds. In alkaline, but not acidic or neutral solutions, the concentrations of benzylmethylketone are slowly reduced by chemical attack to yield benzaldehyde. Only traces of benzylmethylketone produced from V are reduced to the corresponding secondary alcohol during incubation; this alcohol is regarded as a metabonate since it is not derived metabolically from an *added* substrate.

The metabolite VI, also produced during incubation of IV, suffers substantial breakdown during incubation to amphetamine and benzaldehyde but the latter is immediately reduced almost completely to benzyl alcohol. After incubation, storage of solutions of any remaining VI at acidic, neutral or alkaline pH values, yields amphetamine and benzaldehyde which remain as such, but at alkaline pH values

further amounts of benzaldehyde are produced from chemical oxidation of benzylmethylketone.

The hydroxylamine metabolite VIII is produced during incubation but is not changed significantly to metabonates during incubation. After incubation, storage of the neutral solution produces little conversion of VIII to oximes unless the solution is shaken; if the solution is made alkaline after incubation, and especially if the solution is shaken, there is rapid conversion of VIII to *anti* and *syn* benzylmethylketoximes, with the former predominating, and to small amounts of benzaldoxime. If the solution after incubation is made acidic, VIII yields no oximes.

The chemical conversions of metabolites to metabonates rather than the enzymatic conversion of substrates to metabolites are therefore the rate-controlling steps in the production of metabolic products from benzylamphetamine and substances of type I, and these rate-controlling steps are influenced greatly by changes in the conditions and environment of the metabolites in solution. Consequently, the relative importance of direct nitrogen oxidation and the various α-carbon oxidative metabolic routes can be established only by measurement of the metabolites directly, and, if these are unstable, by measurement of their metabonates only when complete conversion of metabolites to their metabonates has occurred.

Quantitative analysis of amphetamine, benzylamine, benzylmethylketone and ketoximes, benzaldehyde, benzaldoxime, benzyl alcohol and α-methyl-β-phenyl ethanol in solutions immediately after incubation of (+)-benzylamphetamine (IV; R″=R=H, R′=Ph) with rat liver microsomes plus soluble fraction, and at intervals after incubation during storage under different conditions until maxima for the concentrations of these compounds were obtained, indicated that the primary metabolites V, VI and VIII were produced in the ratio 0·5 to 1·1 to 1·0 respectively; about 90% of benzylamphetamine metabolized is accounted for by routes 1, 2 and 3 (scheme 1).

Substitution of the phenyl rings of IV alters the relative importance of oxidative routes 1, 2 and 3, as do changes in group R′ in IV from aryl to alkyl.

Department of Pharmacy,
Chelsea College,
University of London,
Chelsea, London SW3 6LX, U.K.

A. H. BECKETT
J. M. VAN DYK
H. H. CHISSICK
J. W. GORROD

August 17, 1971

REFERENCES

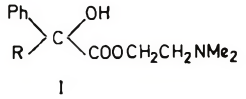
AXELROD, J. (1955). *J. biol. Chem.*, **214**, 753–763.
BECKETT, A. H., GORROD, J. W. & JENNER, P. (1971a). *J. Pharm. Pharmac.*, in the press.
BECKETT, A. H., MITCHARD, M. & SHIHAB, A. A. (1971b). *Ibid.*, **23**, 347–352.
BECKETT, A. H., MITCHARD, M. & SHIHAB, A. A. (1971c). *Ibid.*, in the press.
BECKETT, A. H., VAN DYK, J. M., CHISSICK, H. H. & GORROD, J. W. (1971d). *Ibid.*, **23**, 560.
GORROD, J. W., JENNER, P., KEYSELL, G. & BECKETT, A. H. (1971). *Chem. biol. Interactions*, **3**, 269–270.
MCMAHON, R. E. (1966). *J. pharm. Sci.*, **55**, 457–466, and references cited therein.
ZIEGLER, D. M. & PETIT, F. H. (1964). *Biochem. Biophys. Res. Commun.*, **15**, 188–193.

Since preparing this work, two communications have been published that suggest the imine or the α–C–OH hydroxylamine are precursors of oximes in metabolic deamination (see: Hucker, H. B., Michniewicz, B. M. & Rhodes, R. E. 1971. *Biochem. Pharmac.*, **20**, 2123–2128; Parli, C. J., Wang, N. & McMahon, R. E. *Biochem. biophys. Res. Commun.*, **43**, 1204–1208.

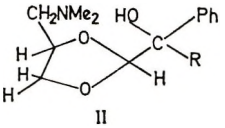
The stereoselectivity of some anti-acetylcholine* acetylenic compounds

It has been demonstrated recently that the potency of anti-acetylcholine drugs such as the glycollic ester I (Brimblecombe, Green & others, 1971) and the dioxolan derivative, II (Brimblecombe, Inch & others, 1971) depends on the absolute configuration of the benzylic carbon atom and that for I and II the isomers with the *R* configuration at the benzylic carbon are appreciably more active than where the benzylic carbon has the *S* configuration. In dioxolans such as III, the variation in potency with configuration was not so pronounced but the most active isomers were related configurationally to the most active isomers of I and II (Brimblecombe & Inch, 1970). In addition to these results Barlow (1971) has demonstrated convincingly that factors other than the configuration of the asymmetric centre can have a profound effect on the stereoselectivity of anti-acetylcholine drugs. Barlow re-examined some enantiomeric pairs of compounds of general formula IV (Duffin & Green, 1955) and showed that although the affinity constants of compounds of this type depended to a large extent on the absolute configuration at the benzylic carbon atom, the stereospecific index (the ratio of the activities of the *dextro* and *laevo* rotatory isomers) varied with the nature of the substituents on the nitrogen atom. This result indicated that the ratio of the activities of the enantiomers of anti-acetylcholine drugs depends on the *entire molecule* and that the importance of the affinity of certain groups (particularly those attached to asymmetric carbons) for the receptor can be over-emphasized. In support of this contention we now report results (Table 1) of studies of the hydrochlorides (designated by H) and methiodides (designated by M) of the acetylenic compounds V and VI, which contain a similar asymmetric centre as that found in compounds I–IV.

The acetylenic esters V, showed a similar configurational dependence as compounds such as I and II, the *R* enantiomers being appreciably more active than the *S* enantiomers. (Because only small quantities of VH (S+) and VM (S+) were available, the affinity constants which were approximately two orders lower than the corresponding *R* enantiomers were not determined accurately). In contrast, the hydrochlorides and methiodides of the enantiomers of VI showed no configurational dependence. (Although the enantiomers of VI were not completely resolved, the resolution was sufficient for any stereoselectivity to have been clearly apparent).



I



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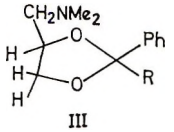
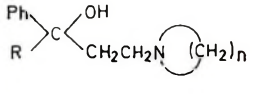
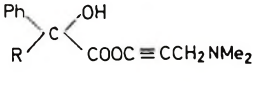


III

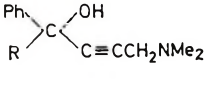


IV

R = cyclohexyl.



V



VI

* Editorial policy has dictated the use of the term anti-acetylcholine drugs for drugs used in the experiments described in this text.

Table 1. *Anti-acetylcholine activity*[1] *of derivatives of 1-dimethylaminoprop-2-yn-3-yl (2-cyclohexyl-2-hydroxy-2-phenyl)acetate* (V) *and 1-cyclohexyl-4-dimethylamino-1-phenyl-but-2-yn-1-ol.*

Compound[2]	log K ± s.e. (n of results)	Potency in antagonizing oxotremorine-induced Mydriasis Salivation (μmol/kg)	Tremors (μmol/kg)	(relative to atropine)
VH (*R*−)	9·18 ± 0·02 (3)	2·8 (1·4–4·9)	8·8 (4·5–17·2)	—
VH (±)	8·96 ± 0·03 (3)	2·5 (0·4–4·5)	15·3 (8·3–29·8)	—
VH (*S*+)	—	>25	>25	—
VM (*R*−)	8·34 ± 0·01 (4)	1·29 (0·5–2·8)	>25	—
VM (±)	8·10 ± 0·01 (3)	4·4 (2·5–7·4)	>50	—
VM (*S*+)	—	>50	>50	—
VIH (*S*+)	7·30 ± 0·02 (2)	41·8 (29·7–59·2)	>50	0·027 (0·021–0·036)
VIH (±)	7·32 ± 0·01 (3)	35·3 (20–65)	53·58	0·017 (0·013–0·02)
VIH (R−)	7·26 ± 0·02 (5)	40·5 (30·7–53·3)	>50	0·022 (0·018–0·029)
VIM (*S*+)	8·11 ± 0·04 (5)	13·7 (8–23·5)	>50	0·53 (0·4–0·7)
VIM (±)	8·11 ± 0·02	22·87 (13·23–41·34)	>50	0·4 (0·3–0·5)
VIM (*R*−)	7·98 ± 0·04 (5)	13·29 (7·5–23·8)	>50	0·17 (0·13–0·25)

[1] The four tests for measurement of anti-acetylcholine activity (measurement of affinity constants, production of mydriasis in mice, antagonism of oxotremorine-induced tremors and salivation) were as described by Brimblecombe & others (1971).

[2] All compounds had infrared and nmr spectra and analytical data consistent with the proposed structures. Methods for the preparation of the racemates of V and VI will be described elsewhere (Bebbington, Brimblecombe & others, in preparation). The enantiomers V, which were prepared by transesterification of optically pure *R*(−) and *S*(+) methyl 2-cyclohexyl-2-hydroxy-2-phenylacetate (Inch, Ley & Rich, 1968) were essentially optically pure. The enantiomers of VI were obtained by classical resolution of the tartarate salts of the racemate. The free bases which were liberated from the tartarates and converted into the hydrochlorides and methiodides by standard procedures had $[\alpha]_D^{20}$ + 11·8° (*c* 7, $CHCl_3$) and −12·5 (*c* 8, $CHCl_3$). That the *dextro* rotatory isomer had the *S* configuration and was at least 65% optically pure (i.e. a ratio of *R* : S enantiomers of 17·5 : 82·5) was established when *S*(+)1-cyclohexyl-1-phenylprop-3-yn-1-ol ($[\alpha]_D^{20}$ + 0·67°, 3% optically pure) (Cooper, Inch & Sellers, 1971) was converted into VI ($[\alpha]_D^{20}$ + 0·55°). It must be pointed out that *R*(−) V and *S*(+) VI are configurationally related in that the phenyl, cyclohexyl, and hydroxyl substituents bear the same geometrical relation to the fourth substituent on the asymmetric carbon atom in each case.

Additionally, it is of interest that whereas in the glycollates I, (Brimblecombe, Green & others, 1971) dioxolans II and III (Brimblecombe, Inch & others, 1971; Brimblecombe & Inch, 1970) and acetylenic compounds VI, quaternization with methyl iodide increased affinity constants the reverse trend was shown by the acetylenic esters V, i.e. VM (*R*−) and VM (±) had lower affinity constants than VH (*R*−) and VH (±) respectively.

Although the above results can perhaps be used in arguments which attempt to establish optimum steric requirements for drugs (and hence the most important geometrical features of the receptor) from a consideration of the nature and distance between certain key groups in anti-acetylcholine drugs (Bebbington & Brimblecombe, 1965) the value of the approach appears at this time to be of limited value. The results presented in this paper seem to provide a warning that great care must be exercised in any investigation which considers that highly specific drugs may be template models of a receptor site, and we believe that studies of enantiomeric pairs which have a high stereospecific index may be used more advantageously for comparison of receptors in different species and at different sites and for general investigations of mechanistic features of drug action (Inch, 1971; Brimblecombe, Green & others, 1971).

The technical assistance of D. B. Cooper and Mrs. J. Wetherell is gratefully acknowledged.

Chemical Defence Establishment,
Porton Down,
Salisbury, Wiltshire, U.K.

T. D. INCH
R. W. BRIMBLECOMBE

July 28, 1971

REFERENCES

BARLOW, R. B. (1971). *J. Pharm. Pharmac.*, **23**, 90–97.
BEBINGTON, A. & BRIMBLECOMBE, R. W. (1965). *Advances in Drug Research*, **2**, 143–172.
BRIMBLECOMBE, R. W., GREEN, D. M., INCH, T. D. & THOMPSON, P. M. J. (1971). *Ibid.*, **23**, 745–757.
BRIMBLECOMBE, R. W. & INCH, T. D. (1970). *Ibid.*, **22**, 881–888.
BRIMBLECOMBE, R. W., INCH, T. D., WETHERELL, J. & WILLIAMS, N. (1971). *Ibid.*, **23**, 649–661.
COOPER, D. B., INCH, T. D. & SELLERS, D. J. (1971). *Tetrahedron Lett.*, 2329–2332.
DUFFIN, W. M. & GREEN, A. F. (1955). *Br. J. Pharmac. Chemother.*, **10**, 383–386.
INCH, T. D. (1971). *Progr. Brain Res.* In the press.
INCH, T. D., LEY, R. V., RICH, P. (1968). *J. chem. Soc. C*, 1693–1699.

Acetylcholine-like action of atropine on the ciliary epithelium of the frog oesophagus during the warmer months

Atropine is known to show muscarinic actions in some experimental conditions (Burn, 1956 b & c; Hazard, Savini & Renier-Cornec, 1959; Teitel, 1961; Ashford, Penn & Ross, 1962; Goodman & Gilman, 1970). We now report another example seen in the ciliary epithelium of the oesophagus of *Rana tigrina*, the common Indian frog (60–300 g), after pithing (Burn, 1952). Experiments were made at room temperature between May 1970 and April 1971, with black seeds of *Amaranthus gangeticus* (average weight 0·95 mg/seed) instead of white poppy seeds (average weight 0·29 mg/seed). Drugs dissolved in 0·2 ml amphibian Ringer were gently measured from a syringe onto the tissue surface and readings of the time for the seed to travel 2 cm were taken over a 3–10 min period. Drugs were washed off by gently irrigating the surface with amphibian Ringer. Atropine sulphate from three sources (T. & H. Smith, U.K.; Boehringer, Germany; Indian Health Institute & Laboratory, India) was used. For control experiments carbachol (1 μg) and acetylcholine bromide (1–300 μg) were used. Hyoscine hydrobromide (0·5–3·0 μg) and eserine sulphate (1 μg) were used in some experiments.

The normal time required by the seed to travel 2 cm was about 47 s (range 11–81 s) in the summer and about 117 s (range 80–165 s) in the winter. Throughout the year, in the doses exceeding 0·1 mg, atropine usually inhibited the ciliary movement thus increasing the seed travelling time. However, smaller doses of atropine generally stimulated the cilia during the summer and inhibited during the winter (Table 1). These effects could be repeatedly elicited on the individual tissues. Hyoscine in May and June stimulated the cilia (20–68% reduction in the control seed travelling time). Throughout the year, acetylcholine, carbachol and eserine consistently stimulated the cilia (54–83% reduction in the control seed travelling time). When their individual stimulant doses were mixed together and placed onto the tissues, atropine and carbachol did not manifest clear additive or antagonistic effect.

Briefly keeping the tissues warm (90°F) in winter or cool (55°F) in summer did not alter their respective responses (Table 1) to the smaller doses of atropine.

Table 1. *Effect of atropine on ciliary movement of the frog oesophagus epithelium.*

Months, season (range of room temperature °F)	Total no. of frogs	Doses of atropine applied on the tissue surface: 0·1–30·0 μg — No. of tissues showing stimulation (range of % decrease in seed travelling time)	0·1–30·0 μg — No. of tissues showing inhibition (range of % increase in seed travelling time)	0·1–30·0 mg — No. of tissues showing inhibition (range of % increase in seed travelling time)
May-June, summer (86–102)	10*	10 (12–62%)	—	4 (7–55%)
July-October, monsoon rains (86–96)	16	15 (5–53%)	—	16 (11–68%)
December-February, winter (65–75)	8*	—	7 (6–58%)	5 (3–71%)
April, spring (92–97)	4	2 (2–21%)	2 (11–55%)	4 (5–55%)

* Higher dose of atropine was not tried in some frogs.

The *Rana tigrina* cilia, in the warmer months, respond to atropine like those of *Mytilus edulis* gill plates (Burn, 1956b). Burn, who suggested a cholinergic mechanism for the ciliary movement (1956a), has also commented upon acetylcholine-like action of atropine in some experimental conditions (1956c).

Department of Pharmacology,
All-India Institute of Medical Sciences,
New Delhi-16,
India.

M. H. AKHTER
N. K. BHIDE

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REFERENCES

ASHFORD, A., PENN, G. B. & ROSS, W. J. (1962). *Nature, Lond.*, **193**, 1082–1083.

BURN, J. H. (1952). *Practical Pharmacology*, p. 62–65. Oxford: Blackwell.

BURN, J. H. (1956). *Functions of Autonomic Transmitters*, (a) p. 78, (b) p. 84 & (c) p. 96. Baltimore: The Williams & Wilkins Company.

GOODMAN, L. S. & GILMAN, A. (1970). *The Pharmacological Basis of Therapeutics*, 4th edn, p. 527. London: Collier-Macmillan.

HAZARD, R., SAVINI, E. & RENIER-CORNEC. A. (1959). *Archs int. Pharmacodyn. Thér.*, **120**, 369–373.

TEITEL, A. (1961). *Nature, Lond.*, **190**, 814–815.

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