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REVIEW

The kinetic behaviour of cardiac glycosides *in vivo*, measured by isotope techniques

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The usual clinical doses of cardiac glycosides are low (0·002–0·02 mg/kg) because of their potent pharmacological activity and relatively narrow therapeutic range. Accordingly, the classical analytical procedures are not sensitive enough for the quantitative determination of the low concentrations of these drugs in biological material. The introduction of radioactively labelled cardiac glycosides, however, has greatly facilitated investigations of the fate of therapeutic amounts of the drugs in the organism and also enabled kinetic studies in isolated organ systems to be made. The radiochemical analysis has proved so sensitive that the kinetic behaviour of cardiac glycosides even in *subthreshold* dosage or in concentrations, devoid of any pharmacological or toxic action, can be examined. Such investigations have led to a better understanding of the kinetic and clinical properties of these valuable drugs.

In earlier reviews (Rothlin & Bircher, 1954; Wright, 1960; Zwieten, 1967) comprehensive compilations of references on the subject have been given. The present paper aims rather to present a critical appreciation of current development in this field, partly based on our own experience. At first, the kinetic behaviour of the drugs in isolated organ systems will be discussed. Investigations in organs, incubated in Tyrode solution will be emphasized, but also studies made in isolated organs incubated in oxygenated whole blood will be considered. *In vivo* studies on the fate of radioactively labelled cardiac glycosides have been made in animals and in man. Distribution, absorption, elimination and metabolism may thus be examined under normal and also pathological circumstances. The sequence: isolated organs (Tyrode solution) $<$ isolated organs (whole blood) $<$ animals $<$ man shows an increasing complexity of the systems studied, although it should be recognized that conclusions drawn from these studies are, at the same time valuable in throwing light on the behaviour of cardiac glycosides under clinical circumstances.

Kinetic studies on isolated atria, incubated in oxygenated Tyrode solution

Electrically driven isolated atria, suspended in oxygenated Tyrode solution provide a simple system for the determination of kinetic properties of drugs under reproducible circumstances, the number of variables in the system being limited and well-defined. Moreover, the influences of, for example, changes in drug concentration, frequency of beat, and ion concentration on the kinetic behaviour may be conveniently examined in such an experimental system. The results probably allow relevant conclusions about the kinetic behaviour of the drugs in human heart muscle tissue if the experiments are made with atria of the guinea-pig, a species that shows pronounced sensitivity to cardiac glycosides. Although Sjoerdsma & Fischer (1951) studied the uptake of [^{14}C]digitoxin by isolated perfused hearts of the rabbit, systematic investigations on

uptake and release of the various cardiac glycosides by isolated heart muscle preparations have only been done recently. In isolated atria kept in Tyrode solution, the kinetic properties of 4 different glycosides were established: [^{3}H]digoxin (Kuschinsky, Lahrtz, & others, 1967; Kuschinsky, Lüllmann & others, 1967); [^{3}H]ouabain and [^{3}H]digitoxin (Kuschinsky, Lüllmann & Zwieten, 1968a); peruvoside (= α-thevetoside of cannogenin) (Kuschinsky, Lüllmann & Zwieten, 1968b). These experiments were extended to [^{3}H]digitoxigenin to establish whether the sugar moieties in the glycoside molecules are pertinent to their kinetic and pharmacological properties (Kuschinsky, Lüllmann & Zwieten, 1968c).

The uptake of the four glycosides and the aglycone showed some similarity. For all five substances the uptake process reached an equilibrium phase after a given time of incubation. The time course of the uptake could be described algebraically by means of e-functions; that is to say, the well-known equation $Y = A\,(1 - e^{-kt})$ could be applied, Y being the uptake at time t, A the uptake at equilibrium and k the rate constant. Apart from k, the tissue/medium (T/M) radioactivity ratio at equilibrium is also a parameter of particular importance for the character of the uptake process since it allows a direct comparison of the relative accumulation of the various compounds examined. The rate constants (k), the half lives ($t_{\frac{1}{2}}$) and also the T/M ratios obtained for "therapeutic" medium concentrations of the glycosides and the aglycone are listed in Table 1. [^{3}H]Ouabain is seen to be taken up much more rapidly than the other drugs. The rate of the ouabain uptake process suggests that the drug is restricted mainly to the extracellular space, since its uptake rate is similar to that of the uptake rate of molecules of similar size in this compartment (Lüllman & Zwieten, 1967). The other glycosides and the genin also penetrate into the cell and become attached to intracellular structures (see literature quoted above).

Table 1. *Rate constants* (k), *the half lives* ($t_{\frac{1}{2}}$) *and also the* T/M *ratios obtained for therapeutic medium concentrations of the glycosides and the algycone*

Drug	Medium concn (g/ml)	Positive inotropic action (%)	Rate constant k s^{-1} ($\times 10^{-4}$)	$t_{\frac{1}{2}}$ min	T/M
[^{3}H]Ouabain	5×10^{-7}	132	31·0	3·7	0·6
[^{3}H]Digoxin	$2{\cdot}5 \times 10^{-7}$	100	5·0	23·1	2·8
[^{3}H]Peruvoside	$2{\cdot}5 \times 10^{-7}$	120	5·2	22·2	3·2
[^{3}H]Digitoxin	1×10^{-7}	90	14·0	8·3	9·3
[^{3}H]Digitoxigenin	1×10^{-7}	150	5·7	20·0	8·0

Although the pharmacological effects of the various drugs in the given concentrations occur within the same range (compare the relative increases of the contractile force in Table 1), there are quite large differences between the T/M ratios achieved at equilibrium. Whereas the ouabain content of the atrial tissue is but 60% of that in the medium, a more than nine-fold accumulation relative to the concentration of the bath fluid was seen with digitoxin. The decreasing polarity in the sequence: ouabain > digoxin > digitoxin ≈ digitoxigenin (Waldi, 1962) is obviously accompanied by an increased cellular accumulation. This finding would suggest that an important part of the cardiac glycosides (and the aglycone) that accumulate is probably bound to lipid-, and protein-containing, structures of the cells. The binding of the labelled drugs to serum proteins also increases in the same sequence: no measurable protein binding could be demonstrated for ouabain, whereas about 80% of the [^{3}H]digitoxin

or its genin are firmly attached to serum proteins (Scholtan, Schlossmann & Rosenkranz, 1966; Kuschinsky, 1968). Moreover, previous removal of lipid material from atrial tissue upon extraction with aqueous glycerol reduced the T/M ratio for [^{3}H]-digitoxin to approximately 4·5 without changing that obtained for [^{3}H]ouabain (0·5) (Kuschinsky, Lüllman & Zwieten, 1968d). Obviously the removal of lipid material reduces the binding capacity of the tissues for digitoxin without affecting the size of the compartment that contains ouabain.

If we assume that a certain degree of receptor occupation is related to a given positive inotropic action and that this degree will be about the same for all the glycosides used in our experiments, the large differences in T/M ratio achieved suggest that not all of the accumulated drug is involved in the therapeutic effect. In other words, the higher the relative accumulation, the larger the amount of drug that is bound to those cellular structures or dissolved in those cellular lipids that are not involved in the development of the pharmacological effect. For [^{3}H]digitoxin and its aglycone, especially, the major part of the amount taken up is probably bound to or dissolved in cellular compartments that are not involved in drug action. For [^{3}H]ouabain the picture seems to be different: the relatively small amount of drug bound by the tissue is probably of vital importance for the pharmacological effect. Concomitantly, [^{3}H]ouabain probably combines to a major degree with specific "receptors", necessary for the development of positive inotropic action. Such "receptors" will probably be accessible from the extracellular space quite easily, since [^{3}H]ouabain is mainly contained in the extracellular space (see below). The "receptors" might for instance be located on the outward membrane or otherwise in the T-tube system, the latter being accessible from the extracellular space. If the membrane location of the "receptors" is assumed, these structures would occupy but 0·006% of the membrane surface (Kuschinsky, Lüllmann & Zwieten, 1968a).

The comparison between the uptake process of the cardiac glycosides and their pharmacological effect has provided some information about the existence of glycoside "receptors" and cellular structures to which an unspecific binding of the drugs takes place. It also seemed of interest to compare the release of initially bound [^{3}H]glycosides (or aglycones) with the disappearance of the pharmacological effect during wash-out with glycoside-free Tyrode solution (Lüllmann, Weber & Zwieten, 1968). The release processes could not be described by means of e-functions, since the rate constant gradually decreased upon prolonged incubation. It drew the attention, however, to the fact that the loss of [^{3}H]labelled glycosides from the tissue occurred at roughly the same rate for each of the five drugs studied: after a wash-out period of 2 h about 40–50% of the initially accumulated drugs had been released by the tissues. The disappearance of the positive inotropic effect, however, took place far more rapidly, no measurable effect being left after 15–20 min of wash-out for ouabain, digoxin or digitoxin. The positive inotropic effect of digitoxigenin disappeared after only 5 min, although the total tissue concentration determined by isotope studies was 50% of its original value 2 h after wash-out. There seemed to be no correlation between the disappearance of the pharmacological effect and the reduction in total tissue issue concentration of all 4 drugs studied. The disappearance of the positive inotropic action must be a reflection of the dissociation of the glycoside or aglycone molecules from the "receptors". Since these "receptors" are easily accessible from the extracellular space, it might be assumed that the diffusion of the glycoside molecules from this space would be the rate-limiting step in the wash-out process of the

pharmacological effect. Indeed, experimental evidence is available to show that the diffusion of the glycoside molecules from the extracellular space largely determines the rate by which the positive inotropic effect is washed out, although the major part of the glycosides bound to cellular structures or dissolved in cellular lipids disappears more slowly (Lüllmann & others, 1968). These findings once more confirm that, particularly with digitoxin and its genin but also with digoxin and peruvoside, by far the greater part of the accumulated drug plays no part in the pharmacologic effect. Obviously, the differences in "fixation" to heart muscle tissue, postulated by clinicians to explain the differences in loss of activity per day for the various glycosides does not really exist. The different clinical decay rates of ouabain, digoxin and digitoxin must be caused by differences in the elimination rate, a parameter that is governed by a much longer half life than the "wash-out" process from myocardial tissues.

Kinetic studies on isolated atria, incubated in oxygenated whole blood

The use of oxygenated blood for the incubation of isolated organs has given rise to insurmountable experimental difficulties like excessive foam formation and haemolysis. However, with the aid of a specially designed oxygenator, Lüllmann, Peters & Zwieten have developed an experimental procedure allowing them to incubate beating guinea-pig atria and other organs in circulating, oxygenated whole blood of the same species. No significant haemolysis occurred over several hours and the usual serum electrolyte concentrations were also maintained. Foam formation could be avoided. This newly developed technique proved convenient for kinetic studies with radioactively labelled cardiac glycosides. Of course, this method more closely approaches *in vivo* conditions than does the incubation of isolated atria in Tyrode solution. In whole blood both the binding of the glycosides to serum proteins and also the presence of erythrocytes give rise to a more complicated, although a more realistic, over-all picture. On the other hand, elimination by either the liver or kidneys cannot occur, thus metabolic degradation hardly takes place. Therefore, these processes cannot interfere with the partition of the radioactively-labelled drug over the various biological compartments within the given system.

It can be demonstrated that both [^{3}H]digitoxin and its aglycone are taken up by erythrocytes, much less, however, than by atrial tissue. Neither [^{3}H]ouabain nor [^{3}H]digoxin are taken up by the erythrocytes (Lüllmann, Peters & Zwieten, to be published).

At equilibrium, the following apparent T/M radioactivity ratios were reached for the partition between the blood and the isolated atria: [^{3}H]ouabain 0·52; [^{3}H]digoxin 1·25; [^{3}H]digitoxin 1·12; [^{3}H]digitoxigenin 0·61 (serum = 1). These values are clearly different from those obtained in the experiments with oxygenated Tyrode solution as a medium. However, the binding of the drugs to serum proteins (mainly albumin) should be taken into consideration. By means of the Sephadex gel filtration method, Kuschinsky (1968) obtained the following values for the amount of *free* drug in guinea-pig serum: ouabain $\approx$ 100, digoxin 70, digitoxin 12, digitoxigenin 8%. These determinations were made with [^{3}H]labelled drugs in "therapeutic" concentrations. If the ratio of glycoside concentration in the tissues to *free* glycoside concentration in the serum is calculated, the following "true" T/M values are obtained: ouabain 0·52, digoxin 1·79, digitoxin 9·32, digitoxigenin 7·63. These ratios are in satisfactory agreement with the T/M ratios obtained in Tyrode solution as the medium of incubation (see Table 1). It is evident, then that the cardiac glycosides are taken up by heart

muscle tissue only when they are available in the free form, that is to say, not bound to serum proteins. The same conditions may be expected to hold true for the partition of cardiac glycosides administered to man. The pharmacologically *active* concentration of the different glycosides probably lies within the same range.

Fate of [^{3}H]*labelled glycosides in animals and man*

The distribution of radioactively labelled cardiac glycosides between various organs has been investigated in a number of animal species (Repke, 1958; Gonzalez & Layne, 1960; Bretschneider, Doering & others, 1962; Dutta, Marks & Smith, 1963; Marks, Dutta & others, 1964; Fauconnet & Widmer, 1965; Lüllmann & Schaum, 1968) In most of these studies no particular affinity of the cardiac glycosides for heart muscle tissue could be demonstrated. No particular accumulation in heart muscle tissue was observed, since the highest concentration was found in the excretion organs like liver, colon and kidneys. However, myocardial tissue accumulated somewhat more of the glycosides than did striated or smooth muscle.

Virtually the same observations were made by Okita, Talso & others (1955) for the distribution of [^{14}C]digitoxin, administered to moribund patients shortly before death. A five-fold accumulation in the serum level, however, was found for [^{3}H]-ouabain in human auricle tissue obtained by biopsy (Marks & others, 1964). The usually modest accumulation of [^{3}H]labelled cardiac glycosides *in vivo*, both in animals and man is in agreement with the unpublished observations of Lüllmann, Peters & Zwieten on the accumulation of the drugs by isolated atria, incubated in circulating, oxygenated whole blood (p. 4).

From clinical experience it is well known that the enteral absorption of cardiac glycosides shows large differences for the various drugs: thus, whereas digitoxin is absorbed completely, the absorption of ouabain is particularly uncertain and in most cases negligible. These clinical observations confirmed early animal studies with the Hatcher procedure (for reviews see Rothlin & Bircher, 1954; Wright, 1960). In recent experiments (Lahrtz, Sattler & Zwieten, 1968) it was shown that in the cat, *intraduodenally* applied [^{3}H]labelled digitoxin reached a much higher serum level than did [^{3}H]digoxin by the same route. [^{3}H]Ouabain on the other hand gave widely varying though usually low serum levels when administered in this manner. No measurable radioactivity could be demonstrated in the serum of human subjects who had been given an oral, therapeutically subthreshold dose of [^{3}H]ouabain. The low urine radioactivity in these subjects indicated that about 0·5–2% of the given dose had been absorbed (Lahrtz, Sattler & Zwieten, 1968). These isotope studies in both animals and man have confirmed the empirically obtained clinical experience about the absorption of cardiac glycosides—the uselessness of oral administration of ouabain is once more emphasized.

The time course of the serum radioactivity after intravenous or oral administration of radioactively labelled cardiac glycosides has been studied in animals (Harrison, Brandenburg & others, 1964; Katzung & Meyers, 1965; Abel, Luchi & others, 1965; Lüllmann & Schaum, 1968) and also in man (Okita & others, 1955; Doherty, Perkins & Mitchell, 1961; Doherty & Perkins, 1962; Marcus, Pavlovitch & others, 1967). Usually, serum concentration of the cardiac glycosides was found to decline by at least two e-functions, the first process being much faster than the second one. The exact significance of these two phases is not yet known. Possibly the first phase may represent the rapidly occurring uptake of the [^{3}H]cardiac glycosides in the blood by

the various organs, whereas the second phase might reflect the elimination process. The rate of the elimination is *not* determined by the partition of the drug between blood and tissues, but by the kidney or the liver, or both. The presumed tissue "fixation" to the heart does *not* determine this rate. During renal failure the excretion of cardiac glycosides is retarded (Doherty, Perkins & Wilson, 1964; Doherty & Flanigan, 1967; Lahrtz & Zwieten, 1968a, 1968b). However, the exact mechanism of the retarded excretion is not fully understood (Lahrtz & Zwieten, 1968b). Although in man ouabain is almost entirely excreted in the urine (Lahrtz & Zwieten, 1968b), [^{3}H]-digitoxin and its metabolites are eliminated via kidneys and liver (Lahrtz & Zwieten, unpublished). The excretion via the kidney by glomerular filtration may be expected to become preponderant if the binding to serum proteins of the glycoside in question is low. Most of the protein-bound glycoside becomes attached to serum albumin, a molecule that normally cannot be filtered by the glomerular system.

Impairment of the glycoside excretion via the liver as a result of a pathologically reduced biliary flow does not seem to give rise to an increased tissue concentration in patients (Marcus & Kapadia, 1964; Lahrtz & Zwieten, 1968a, 1968b). Accordingly, no accumulation of cardiac glycosides can be expected during liver disease, in contrast to the observations made in patients suffering from renal failure. Again, the isotope studies have confirmed previous clinical observations.

CONCLUSIONS

The kinetic experiments with tritium-labelled cardiac glycosides in isolated atria incubated either in oxygenated Tyrode solution or in oxygenated whole blood have shown that the distribution of the drugs between the various compartments in the *in vitro* system occurs rapidly. Since this rate of distribution is much higher than that of the drug elimination *in vivo*, the latter process exclusively determines the decay rate *in vivo*. The elimination depends rather upon the activity of the excretion organs like the kidney and the liver. The kinetic behaviour of cardiac glycosides in heart muscle is not primarily responsible for the duration of the cardiac effect elicited by these drugs.

The different T/M ratios obtained for the four cardiac glycosides and for the genin in isolated atrial tissue, incubated in an aqueous, protein-free medium suggest that their distribution between the various compartments within the cell differs widely from drug to drug. With those glycosides achieving a high T/M ratio at equilibrium, most of the accumulated glycoside seems to be bound to cellular structures or is present in compartments that have nothing to do with the positive inotropic effect. A small part of the molecule (e.g. digitoxin or its aglycone), however, combines with more specific "receptors" that are obviously involved in the development of the positive inotropic glycoside effect. With ouabain, the *major* part of the tissue bound drug combines with these "receptors", that are probably located on the outward membrane surface or may also be in the T-tube system. The kinetic studies with [^{3}H]ouabain suggest that the "receptors" must be easily accessible from the extracellular space. The combination of the drugs with the "receptors" probably reaches the equilibrium phase quite rapidly.

In vivo, and also in isolated atria incubated in oxygenated whole blood, the relative accumulation of the glycosides by the tissues *apparently* shows little difference for the drugs studied. However, if the protein-binding of the drugs is considered, a different picture emerges: if the amount of *free* glycoside in the blood is taken as a base for the calculation of the T/M ratios at equilibrium, approximately the same values are

obtained as for isolated atria, incubated in Tyrode solution where protein-binding cannot occur. Concomitantly, cardiac glycosides are taken up by heart muscle in accordance with the concentration of free and not protein bound, molecules.

In vivo, the protein-binding of the cardiac glycosides may be expected to influence their elimination through the kidneys. The protein-bound drugs cannot pass through the glomerular system; on the other hand, if free glycosides have passed the glomerular system, part of the molecules may be subject to reabsorption in the tubular system, provided the compound in question is lipid soluble, as for example is digitoxin.

The main conclusion that may be drawn from the kinetic investigations is probably the fact that no particular "fixation" of the drugs to heart muscle takes places *in vivo*. Although this parameter is frequently discussed by clinicians, there seems to be no experimental evidence for its real existence. The different properties of the various cardiac glycosides *in vivo* are most probably caused by differences in *elimination* or metabolic degradation or both, and not by a different kinetic behaviour towards myocardial tissues.

SUMMARY

In this review a critical evaluation of current developments in kinetic research on the behaviour of cardiac glycosides both *in vitro* and *in vivo* is given. The results recently obtained in this field suggest that in heart muscle tissue digitoxin and to a lesser extent digoxin predominantly combine with cellular structures that are not immediately involved in the development of the pharmacological effect. Only a part of the drugs combine with specific "receptors", and thus give rise to positive inotropic action. Probably, these "receptors" are easily accessible from the extracellular space which may be the membrane surface or T-tube system. Ouabain, however, chiefly combines directly with these "receptors". Thus, the different properties of the various cardiac glycosides *in vivo* are probably caused by differences in elimination or metabolism and not by a different kinetic behaviour towards heart muscle tissues. The kinetic studies suggest that *in vivo* no particular "fixation" of these drugs to the heart really takes place.

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Examination of the hallucinogen 2,5-dimethoxy-4-methylamphetamine

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Physical characteristics are reported for a tablet form of a new hallucinogenic drug previously circulating in the USA under the name "STP". High resolution mass, nmr, ultraviolet and infrared spectrometric evidence, which identify the extracted base as 2,5-dimethoxy-4-methylamphetamine, and its chromatographic behaviour, are compared with the experimental compound "DOM". Polymorphic modifications exhibiting distinct solid phase infrared spectra have been studied by X-ray diffraction and by differential calorimetry. Animal behavioural tests indicate that the psychotomimetic activity of the base is comparable with mescaline but up to 50 times more potent.

In the summer of 1967, references appeared in the British press to "a new and dangerous drug" circulating under the description "STP" in the U.S.A. and Canada. Some medical reports suggested that this drug, the nature of which was not then known, was significantly more potent and longer acting than lysergide (LSD), and that certain phenothiazines—customarily used as antidotes for LSD—potentiate its action and may cause respiratory collapse. The initials STP were not thought to be chemically significant; some press reports suggested the substance to be 5-methoxy-*NN*-dimethyltryptamine (i.e. the methyl ether of the natural hallucinogen bufotenine) or confused the drug with a military incapacitating agent "BZ".

Only very limited supplies of the drug were believed to have reached this country but the Home Office Drugs Branch was able to obtain a single tablet, thought to be of Californian origin; the tablet was submitted to this Laboratory for examination. A few milligrams of a base hydrochloride were extracted, sufficient to investigate the thin-layer chromatographic behaviour and to record high resolution mass, ultraviolet and infrared spectra; in addition animal behavioural tests with a small portion of the tablet and with aqueous and chloroform extracts were undertaken on our behalf at the Chemical Defence Experimental Establishment. From accurate mass measurement and the spectral characteristics it was possible to deduce with fair certainty the structure to be 1-methyl-2-(2,5-dimethoxy-4-methylphenyl)ethylamine: it is convenient to refer to this structure as 2,5-dimethoxy-4-methylamphetamine.

Concurrently, workers at the U.S. Food and Drug Administration (FDA) had encountered several dosage forms (white, orange or light blue tablets) loosely described as STP and by a similar deductive process had reached an identical conclusion about the structure of the psychotomimetic ingredient (Martin & Alexander, 1968). The weight, dimensions, lactose excipient and well finished appearance were all comparable with the tablet we had examined [U.S. Bureau of Drug Abuse Control (US-BDAC), personal communication]. Moreover the FDA identified‡ the base with

‡ Press release 2nd August, 1967; cited, *inter alia*, in *Chem. Engng News*, 14th Aug., 1967, p. 39.

an experimental compound, DOM, developed by the Dow Chemical Co. but for which no information had hitherto been published nor had there been an "Investigational New Drug" application.

Through the courtesy of the US-BDAC a small sample of DOM was supplied to the Home Office. Access to this material permitted a fuller examination of the spectrometric, chromatographic and chemical characteristics of 2,5-dimethoxy-4-methylamphetamine, a refined estimate of its psychotomimetic activity, and comparison with the base extracted from the STP tablet. A preliminary account of the tablet examination was given restricted publication (Maunder, 1967); the more detailed results are now reported as an aid to the identification of this substance should its use become more prevalent in this country. To date, the relatively few samples detected have been either the pale blue tablet or clear gelatin capsules filled with a similarly coloured powder apparently diluted with lactose.

EXPERIMENTAL

Melting points (uncorrected) were determined in sealed evacuated capillaries or with Kofler hot-stage microscope or inferred from calorimetric transitions.

Ultraviolet absorption spectra were recorded as aqueous or acidified solutions using a Unicam SP.800 spectrophotometer.

Thin-layer chromatography systems. (A), silica gel (30 g) impregnated with NaOH (60 ml 0·1 N), developed with chloroform–methanol (9 : 1) (Genest & Farmilo, 1964); (B), adsorbent as (A) but with methanol as solvent; (C), silica gel, developed with methanol–ammonia (sp. gr.: 0·880) (100 : 1·5) (Sunshine, 1963). Visualization was by spraying with 1% iodine in methanol or by examining the fluorescence under ultraviolet (360 nm) illumination.

Colour reagents. Froehde: sodium molybdate (50 mg) in sulphuric acid (10 ml). Mandelin: ammonium vanadate (1 g) in sulphuric acid (100 ml). Marquis: 40% formalin (8–10 drops) in sulphuric acid (10 ml). Mecke: selenious acid (0·25 g) in sulphuric acid (25 ml).

Infrared absorption spectra were recorded as Nujol mulls using a Grubb Parsons GS 2 grating spectrometer.

X-ray powder diffraction patterns were recorded photographically using a Unicam 9 cm camera and vanadium-filtered chromium Kα radiation. Measurements of I/I_0 were made with a Joyce recording microdensitometer.

Mass spectra were obtained initially with an Associated Electrical Industries MS-9 spectrometer (at the National Physical Laboratory) and subsequently in this Laboratory with an MS-902. Both instruments are double focussing, electron bombardment mass spectrometers with a resolution better than 1 : 10,000. Accurate mass measurements were made with reference to heptacosafluorotributylamine and all fragment formulae assigned agree within 15 ppm (MS9), or 5 ppm (MS-902), with the theoretical values.

100 *MHz proton magnetic resonance spectra* were recorded (at the National Physical Laboratory) with a Varian HA-100 spectrometer using solutions in carbon tetrachloride. Chemical shifts were calculated as τ values using tetramethylsilane as standard.

Differential scanning calorimetry was carried out with a Perkin-Elmer DSC-1B apparatus, using dry nitrogen at 20 ml/min as carrier gas and a heating rate of 8°/min.

RESULTS

Tablet form. The tablet was circular, biconvex and half-scored; the turquoise colour approximately matched G5 in the *Chemist and Druggist* Identification Guide. It weighed 189·6 mg. The maximum thickness was 3·74 mm (almost 5/32 inch) and diameter 7·16 mm (10/32 inch). The physical characteristics were such that expertise in its production could be inferred. The only crystalline component detected by X-ray diffraction was lactose monohydrate.

The tablet was substantially soluble in water and completely soluble in dilute hydrochloric acid. With small quantities (less than 10 mg) dispersed in sodium hydroxide solution the first chloroform extraction preferentially removed small amounts of fatty material; acidification of the alkaline phase, followed by an excess of dilute ammonia or sodium bicarbonate, with chloroform extraction yielded essentially pure base. On a larger scale (upwards of 20 mg) the base was not significantly retained in the initial alkaline phase. It was discovered that the free base is somewhat volatile and isolation as the salt is safer. Altogether approximately 2·0 mg of amorphous base, m.p. 59° (Kofler block), was extracted from 42 mg of the tablet.

The ultraviolet absorption spectrum of an acidified solution of 1 mg of the tablet [λ_{max} 288 nm (E 1%, 1 cm = 7·5) λ_{min} 255, shoulder 220/225 (E 1% 1 cm *ca* 22) and λ_{max} <215] was very similar to that of methoxamine [λ_{max} 291, λ_{min} 253, λ_{max} 226, shoulder 215], which has the structure 2-amino-1-(2,5-dimethoxyphenyl)propan-1-ol. The aqueous extract also included a blue dyestuff, λ_{max} 626 nm, and chloride ion, confirmed conventionally.

Limited examination with the thin-layer system (A) using methanolic iodine visualization suggested that the STP base could be distinguished from methamphetamine but not easily from mescaline, phentermine and amphetamine. In addition a weaker, more mobile, spot, visualized only by its fluorescence in 360 nm illumination, overlapped secondary spots of methoxyphenamine and methoxamine. In general the limited mobility in system (A) is disadvantageous and a more detailed comparison, using several thin-layer systems, was undertaken after the DOM sample was obtained—see below.

Active drug form. The DOM sample was a white powder; it proved to be a hydrochloride salt, m.p. (evacuated capillary) 184–5°. With the Marquis reagent it gave a bright lemon yellow colour slowly turning pink, in contrast to the brown becoming olive green given by amphetamines not substituted in the aromatic ring. Colours were developed with other common alkaloid reagents, including Froehde (yellow turning to lime green), Mandelin (bright green tending to brown), Mecke (varying from brown through green hues back to brown) and sulphuric acid itself (faint pink becoming yellow).

On all three thin-layer systems when visualized with methanolic iodine, DOM gave a single spot comparable with that observed with the base extracted from STP, whereas in 350 nm illumination there was no sign of the latter's weaker more mobile spot. In a detailed study of 31 variously substituted amphetamine, phenethylamine and phenethanolamine salts, using systems (B) and (C) (to be published), only ephedrine, pseudoephedrine, and methamphetamine exhibited mobility overlapping DOM; the first two substances may be distinguished by their bright yellow colour with methanolic iodine spray (DOM gives a pinkish-brown) while methamphetamine may be resolved in the otherwise less favourable system (A).

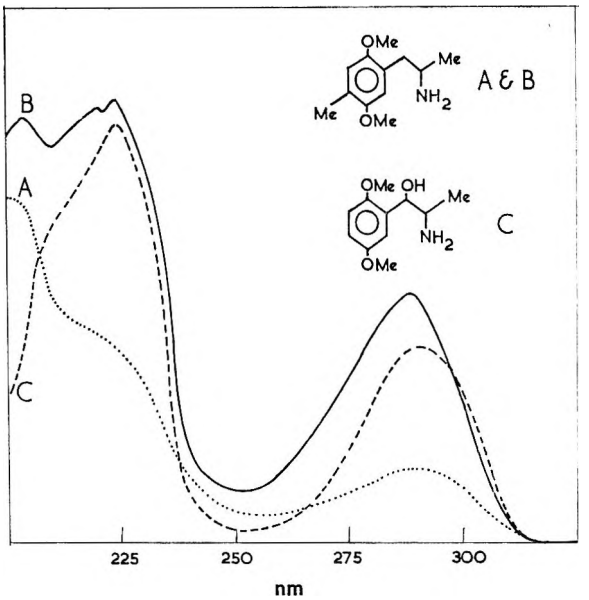

FIG. 1. A = aqueous extract of STP tablet (c = 36 mg %). B = aqueous solution of DOM hydrochloride (c = 6·6 mg %). C = aqueous solution of methoxamine hydrochloride (c = 5·5 mg %).

Note that at wavelengths greater than 220 nm, B and C are virtually superposable, and that from the ratio A/B, the STP tablet must contain about 4·5% DOM base, or 5·3% expressed as the hydrochloride.

An aqueous solution of DOM produced an ultraviolet absorption spectrum [λ_{max} 288 nm (E 1%, 1 cm = 166), λ_{min} 251 (33), λ_{max} 224 (272), λ_{max} 209 (254)] which above 215 nm is in satisfactory agreement with that obtained from the aqueous extract of the STP tablet and moreover is virtually superposable on that previously demonstrated for methoxamine (see Fig. 1); in the latter there is a slight bathochromic shift of the centre of the aromatic band to 291 nm, possibly attributable to interaction of one methoxyl with the side-chain hydroxyl. From the specific absorbance at 288 nm observed for DOM it may be calculated that the STP tablet contained about 4·5% of 2,5-dimethoxy-4-methylamphetamine (that is, 8·6 mg base or 10 mg as the hydrochloride).

Chloroform extraction of an alkaline solution of DOM yielded a base giving the same infrared spectrum as that normally obtained from the STP tablet. Two polymorphic forms were obtained: material recovered from evaporation of chloroform solution was shown to be substantially form II containing a small proportion of form I, detectable by its infrared absorption at 924 cm^{-1}. On standing for one month this material was wholly converted to form II; form I was obtained in a pure state by crystallization from a melt. Infrared spectra and X-ray diffraction patterns of the hydrochloride as received and of the two forms of the base are shown in Fig. 2 and Table 1 respectively. The melting points of the two forms differ widely: differential scanning calorimetry of form II showed a broad endothermic transition commencing at 55° (melting of form II) followed by an exothermic transition (recrystallization as form I) and a second broad endothermic transition beginning at 110° (melting of form I). The two forms are apparently enantiotropic, since form I slowly reverts to form II at room temperature.

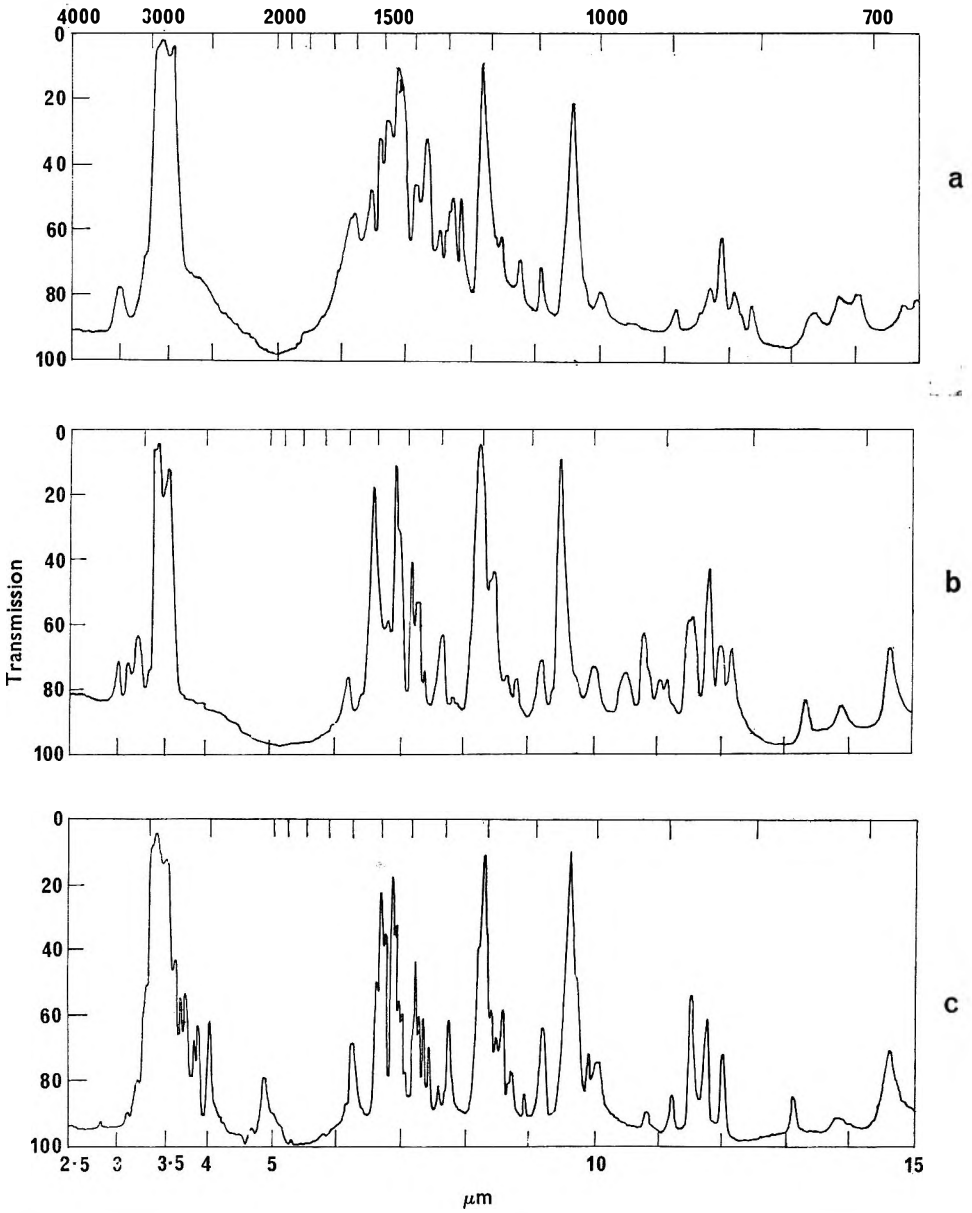

FIG. 2. Infrared spectra (Nujol mulls) of **a**, DOM base form I; **b**, DOM base form II; **c**, DOM hydrochloride.

The high resolution mass spectrum of DOM base was in general agreement with that of the base from the STP tablet and with that given by Bellman (1968). There were, however, some additional peaks in the STP spectrum, most of which were apparently derived from fatty acids used as binders in the tablet, but those at m/e 281, 192 and 116 remain unidentified; accurate mass measurement suggested the formulae $C_{15}H_{23}NO_4$, $C_{12}H_{16}O_2$ and $C_5H_{10}NO_2$ respectively. The high resolution nuclear magnetic resonance spectrum of the DOM base was also recorded.

Table 1. *X-Ray powder diffraction patterns*

Base form II		Base form I		Hydrochloride	
d (Å)	I/I_0	d (Å)	I/I_0	d (Å)	I/I_0
13·9	5	8·59	6	9·97	25
10·8	15	7·81	100	7·28	4
8·59	47	7·43	72	6·64	6
7·76	22	7·18	5	6·17	20
7·08	100	5·67	5	5·77	28
6·64	8	5·21	44	5·59	100
5·71	14	4·49	18	5·34	77
5·50	15	4·24	66	5·04	46
5·18	11	4·02	19	4·70	10
4·77	5	3·89	5	4·35	35
4·63	5	3·79	37	4·24	42
4·47	8	3·73	23	4·11	3
4·31	16	3·62	72	3·94	76
4·24	20	3·43	6	3·80	14
4·13	38	3·18	15	3·66	17
3·89	8	3·11	5	3·54	37
3·79	4	2·99	3	3·46	35
3·69	4	2·77	10	3·37	37
3·56	38	2·61	4	3·31	36
3·49	16	2·59	5	3·08	5
3·33	10	2·50	5	3·02	5
3·04	11	2·42	3	2·91	22
2·38	6	2·39	3	2·81	4
		2·27	2	2·73	3
		2·24	4	2·66	4
		2·19	2	2·57	14
				2·51	7
				2·45	6
				2·40	3
				2·33	10
				2·18	5

Elucidation of structure

The structure 2,5-dimethoxy-4-methylamphetamine was deduced from the spectrometric investigation of the base extracted from the STP tablet and from the subsequently supplied sample of DOM hydrochloride.

Accurate measurement of the parent ion peak gave a mass of 209·1415($C_{12}H_{19}NO_2$ requires 209·1416). The fragment peaks at m/e 166 ($C_{10}H_{14}O_2$) and 44 (C_2H_6N) are both consistent with the parent molecule being a primary amine with a methyl group attached to the α-carbon atom. The presence of an isopropylamine side-chain was confirmed by the 100 MHz nmr spectrum (see Fig. 3). This showed a doublet centred at τ 9·0 due to the CH_3 group adjacent to the CH group in the propyl chain, and a series of eight bands centred at τ 7·56 due to the inequivalent *gem* hydrogens adjacent to the asymmetric carbon atom (AB part of an ABX spin system), while the CH proton itself gave a complex broad signal at τ 7·02 and the NH_2 protons a broad band at τ 8·66.

The presence of two methoxy groups attached to the ring was indicated by the pair of singlets at τ 6·32 and 6·34 in the nmr spectrum, the successive mass differences between the fragments at m/e 166, 135 ($C_9H_{11}O$) and 105 (C_8H_9), the strong infrared absorptions due to aryl ether groups at 1212 and 1045 cm^{-1} (with no evidence of any other oxygen functions) and the ultraviolet correlation with methoxamine. Similarly the aryl methyl group was indicated by the nmr singlet at τ 7·90 and the mass difference between the fragments of m/e 166 and 151 ($C_9H_{11}O_2$), the latter suggesting that a methyl group can be lost without destroying the stable tropylium structure. The two

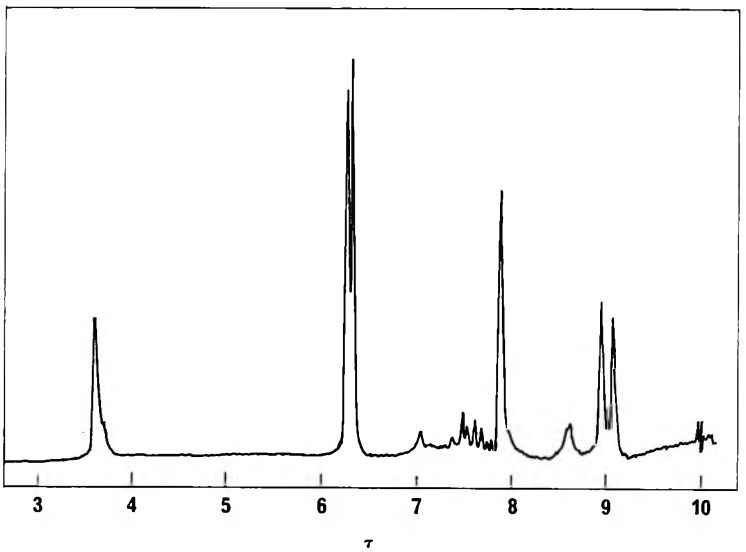

FIG. 3. The 100 MHz nuclear magnetic resonance spectrum of DOM base. For convenience of illustration, the separation of the signals at τ 6·32 and 6·34 has been exaggerated.

aromatic protons gave a single nmr absorption at τ 3·54 with no resolvable fine structure (Martin & Alexander, 1968, reported a rather complex signal); the lack of coupling between these protons, and the absence of strong infrared absorption at 800–830 cm^{-1}, confirm that the two ring protons cannot be adjacent to each other. The *para* relationship of the two methoxy groups is demonstrated by reference to comparison spectra: thus, in the ultraviolet, the position of the π–π^* transition band centred at 288 nm is consistent with, for example, hydroquinone dimethyl ether (λ_{max} 292, 288, 282, λ_{min} 250) rather than the 1,3-dimethoxy derivatives of benzene and toluene (λ_{max} 283, 279, 274, λ_{min} 244) or the 1,2-dimethoxy system in veratrole (λ_{max} 282, 277, 272, λ_{min} 245). A similar inference may be drawn from the position of the C–O stretching absorption in the infrared spectrum at 1212 cm^{-1} (cf. methoxamine 1220 cm^{-1}); *ortho* substitution would give absorption near 1250 cm^{-1} and *meta* substitution near 1150 cm^{-1}. Moreover the fact that the two methoxy groups give a single absorption in the infrared and the very close signals τ 6·32 and 6·34 in the nmr spectrum shows that their environments are very similar, leaving 2,5-dimethoxy-4-methylamphetamine as the only possible structure.

Psychotropic function

By courtesy of their Director, the Chemical Defence Experimental Establishment undertook rodent behavioural tests with the STP tablet and, subsequently, with the DOM salt. Hall's "Open Field" test with rats (Brimblecombe, 1964), and a "Head Twitch" count method (Corne & Pickering, 1967) for groups of 10 mice, were selected as convenient means of broadly distinguishing between amphetamine-like (analeptic) and mescaline-like (psychotomimetic) activity. In preliminary tests, residues of chloroformic and aqueous extracts of 10 mg STP tablet, and 10 mg of tablet dissolved in dimethyl sulphoxide, were compared with mescaline hydrochloride and (±)-amphetamine sulphate; the solutions were administered by subcutaneous injection.

It appeared that the chloroform extract and the original tablet both produced behavioural changes very similar to those evoked by mescaline and quite distinct from the amphetamine effect on motor activity; the aqueous extract was devoid of activity. From this limited single dose experiment one may only cautiously infer a broadly equipotent response between 10 mg of this tablet and 10 mg of pure mescaline hydrochloride. Similar tests were subsequently undertaken with the DOM salt and it was confirmed that 2,5-dimethoxy-4-methylamphetamine has a psychotomimetic activity qualitatively similar to mescaline and that a dosage of 0·2–0·4 mg/kg has a potency in mice comparable with 10 mg/kg mescaline, i.e. 25 to 50 times more potent.

Two independent studies of the effect of DOM on human volunteers in the U.S.A. were reported by Snyder, Faillace & Hollister (1967); of 16 adults of either sex, those receiving more than 5 mg drug suffered "marked" hallucination. Assuming the minimum adult human hallucinogenic doses of mescaline and lysergide to be 300 and 0·1 mg, it would appear that in man 2,5-dimethoxy-4-methylamphetamine is about 60 times more potent than mescaline but has only one fiftieth the activity of lysergide. It is also of interest to compare this result with Shulgin's (1964) value of 17 times the hallucinogenic potency of mescaline for 2,4,5-trimethoxyamphetamine.

Thus, on the basis of the clinical report of Snyder and his colleagues, and the limited rodent studies with DOM and the STP tablet undertaken by Brimblecombe for us, it appears that early press accounts of a psychotomimetic potency in excess of that of lysergide were unjustified. The clinical study also discounts reference to longer lasting (up to 72 h) effects and moreover no adverse reaction on concomitant administration of chlorpromazine was found. However, this—as was recognized—still leaves open the possibility that several drugs may have been circulating under the description STP. Alternatively the alleged effects may have been due to variable impurities in illicit preparations, or more potent homologues, or even compound dosage forms. With the single tablet available to us it was not possible to investigate further the material of mass 281 ($C_{15}H_{23}NO_4$) but its presence did not appear to enhance the potency relative to DOM. Nevertheless the presence of this substance may be of diagnostic value in comparing different illicit preparations of 2,5-dimethoxy-4-methylamphetamine; it was certainly absent in the mass spectrum of DOM.

Legal status

Possession of both mescaline and amphetamine (α-methylphenethylamine) is controlled by regulations made under the Drugs (Prevention of Misuse) Act, 1964 but the generic description in the Schedule to that Act, and in Schedule 4B to the 1967 Poisons Rules, cannot be held to subsume aromatic ring substituted derivatives of "β-aminopropylbenzene" (see discussion in Phillips, 1967). Thus it was held that 2,5-dimethoxy-4-methylamphetamine was not regulated in Great Britain in 1967; a similar situation prevailed in the U.S.A. until April 2, 1968. No steps have since been taken explicitly to control this substance under the 1964 Act but revision of the generic entries in the Poisons List and Poisons Rules made October 23, 1968 should ensure restriction of supply of a variety of aromatic ring substituted analogues of mescaline (phenethylamines) and amphetamine (α-methylphenethylamines).

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The extraction of azovan blue from precipitated plasma proteins

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The paper describes a method for the estimation of azovan (Evans) blue in plasma proteins precipitated by trichloroacetic acid. The precipitate is dissolved in a strong solution of urea and an anionic detergent; the dye is absorbed on a cellulose column, from which it can be eluted by alkaline acetone-water. The method allows information to be obtained about drug uptake and plasma volume without the need for additional blood samples. It has been used with cat, rabbit, rat and guinea-pig plasma.

In the course of studies on the uptake of choline from the circulation of cats and rabbits, azovan (Evans) blue (T-1824) was added to the solutions of choline administered to act as unabsorbable tracer. Free choline in the plasma was estimated essentially by the method of Bligh (1952) and it was noticed during the precipitation of the proteins by trichloroacetic acid that the dye was retained by the precipitate. Since the problem of blood loss is always troublesome with small animals when repeated blood samples are taken, the possibility of extracting the dye from the precipitate was investigated. It was found that the proteins were soluble in strong urea solution and that the dye could be removed from the resulting solution by absorption onto cellulose. The method described below permits azovan blue and free choline to be estimated on the same sample of the plasma and thus halves the volume of blood required.

EXPERIMENTAL

Materials

Urea/Teepol solution: Solutions A. Equal volumes of water and Teepol CH-53 (Shell Malaysia Limited). Solution B. 16 g urea dissolved in 20 ml solution A; solution may be hastened by warming. Solution C. 20 g urea dissolved in 25 ml water. Solution D. 19 ml solution C + 1 ml solution A + 1 ml 12% w/v trichloroacetic acid. Solution E. 24 ml water + 1 ml solution A.

Eluting solution. 50 ml water + 50 ml acetone + 2 ml of 2-amino-2 methyl propan-1-ol (B.D.H. Ltd.).

Cellulose absorbent. Cellulose powder (10 g; Whatman, Standard Ashless Grade or Whatman CF11) is suspended in 100 ml of 1% w/v disodium EDTA and washed in the form of a column (approximately 2 cm diameter) with a further 200 ml 1% disodium EDTA followed by 200 ml water. The treated cellulose is washed by decantation several times with water to remove "fines"; and stored under water. It is kept in a refrigerator at 0–4° to reduce bacterial and fungal growth; any unused after 2 weeks is discarded.

Absorption columns. A column consists of a 50 mm length of 5 mm bore glass tubing; the lower end constricted down to a 1 mm hole and a wide bore reservoir section fixed to the upper end. The tube was constricted by heating the extreme tip in a fierce flame, this produced a flat topped ledge. On this ledge rests a 5 mm disc of

glass fibre filter paper (Whatman GF/A) which in turn supports the cellulose absorbent. Ordinary cellulose filter paper is not suitable since the resulting flow rate is too slow.

The columns are packed with a suspension of the treated cellulose in solution E. Care is taken to ensure a reasonable constancy of column height (27–28 mm.) The columns are allowed to drain and are then washed with 2 × 1 ml solution E. Shortly before the addition of the protein solutions they are washed with 1 ml solution B.

Method

Blood (0·5 ml) is added to 0·05 ml heparin solution (100 u/ml 0·9% NaCl) and centrifuged to separate the plasma. Plasma (0·25 ml) is transferred to a narrow 3 ml polypropylene tube (type 59400 M.S.E. Ltd.). Water (0·5 ml) is added followed by 12% trichloroacetic acid (0·5 ml) and the mixture shaken; after standing for 10 min the precipitate is separated by centrifuging at 1000 *g* for 5 min. The supernatant is poured off and its choline content assayed; the precipitate is drained for a few minutes by inverting the tube on filter paper and then 1 ml of solution B is added. The precipitate is brought into solution with the aid of a glass pestle. This consists of a piece of 4 mm glass tubing with a bulb blown at the end so that it fits the bottom and sides of the centrifuge tube closely. By gentle up and down strokes the precipitate is "homogenized" and dissolves. Frothing is prevented by lightly smearing the pestle shank with silicone antifoam A (Hopkin & Williams Ltd.). When the precipitate has dissoved, the pestle is withdrawn while being washed with a further 0·2 ml solution B. At this stage the solution may be left for some hours at room temperature.

The protein solution is passed through the prepared column to remove the azovan blue. Unaided, the flow is slow and the rate is best increased by the cautious application of compressed air at a pressure of about 20 cm water. The column must

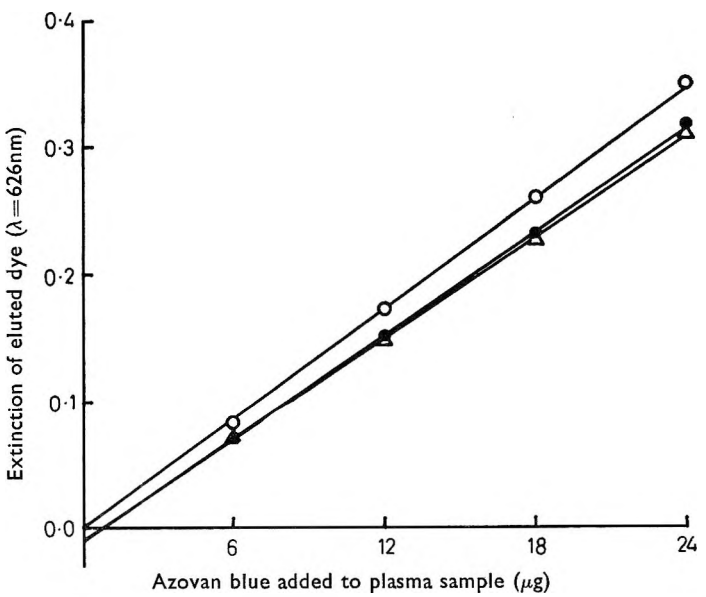

FIG. 1. Recovery of azovan blue added to rabbit plasma. ○ Original dye solution, ● Eluted from column after absorption from undenatured plasma and solution E (1 : 1), △ Eluted from column after absorption from precipitated and redissolved plasma proteins as described in method. Each point is the mean from duplicate samples. The lines shown are the calculated regression lines extended to the extinction axis.

be kept wet and too fast a flow will result in "break-through" of the dye. The centrifuge tube and column are washed free of protein with 2 × 1 ml solution D, each portion being allowed to pass through the column before the next is added. The column is washed with 2 × 1 ml solution E to remove most of the urea and the dye eluted by 3 × 1 ml of eluting solution. The dye solution is collected in 5 ml graduated stoppered test-tubes and made up to volume with eluting solution.

The extinction of the dye solution is read at 626 nm with eluting solution as blank. The relation between the extinction and the amount of dye in the plasma sample is linear from 0 to at least 30 μg of dye. The line for the recovered dye is almost parallel to that of the original dye solution but with a negative intercept on the extinction axis suggesting that there is a constant loss of dye independant of concentration (see Fig. 1). When 24 μg of dye is added to the plasma sample before the trichloroacetic acid precipitation, the recovery is 85–90%, this compares with 90–95% for the more usual procedure using undenatured plasma.

Standardization

At the start of an experiment four initial samples are taken before any dye is administered to the animal and known amounts of the injection solution added to the plasma just before the precipitation stage. Usually the amounts correspond to 6, 12, 18, 24 μg azovan blue and the regression line of extinction against amount of azovan blue is calculated by the method of Aldridge, Berry & Davies (1949). The dye concentration in the later samples is calculated from their extinction readings.

DISCUSSION

The method is a variant of many previously described for the extraction of azovan blue from plasma by the use of anionic detergents and cellulose. The use of cellulose treated with EDTA as absorbent and the addition of an organic amine to the eluting solution were adopted since they had been found to give higher recovery of dye from undenatured plasma. The nature of the amino-alcohol is probably not critical, ethanolamine would probably be satisfactory but none was available in the laboratory. Similarly the kind of Teepol used is a mixture of ionic and non-ionic detergents; Teepol 514 has been used on an earlier occasion, but it too is not obtainable in Singapore. Detergents other than Teepol CH-53 may require modification of the solutions, especially the amount of trichloroacetic acid in Solution D.

The plasma proteins can be brought into solution with strong urea solutions containing less Teepol but the absorption and recovery of the dye is not satisfactory.

The method although developed for the bioassay of choline can obviously be used with any other determination involving the removal of the plasma proteins by trichloroacetic acid. It has been used successfully with cat, rabbit, rat and guinea-pig plasma.

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Inhibition of glutamate decarboxylase by salicylate *in vitro*

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Salicylate inhibits the activity of glutamate decarboxylase from *Escherichia coli* by a reversible mechanism, but high concentrations of the drug denature the enzyme protein

Salicylate inhibits the activity of glutamate decarboxylase prepared from *Escherichia coli* and rat brain (Gould, Huggins & Smith, 1963; Gould & Smith, 1965). It was suggested that the mechanism of inhibition involved an irreversible combination of the drug with the enzyme protein. This mechanism has not been confirmed in the present work. High concentrations of salicylate denature, and lower concentrations cause a reversible inhibition of the bacterial enzyme.

EXPERIMENTAL

Materials

Type 1. Glutamate decarboxylase (L-glutamate-1-carboxylyase, EC 4.1.1.15) from *E. coli* was obtained from the Sigma Chemical Co., St. Louis, and the [^{14}C]salicylic acid from the Radiochemical Centre, Amersham, Bucks. Other chemicals were of analytical grade and deionized water was used throughout.

Measurement of glutamate decarboxylase activity

The decarboxylase activity was determined at 37° by the Warburg technique, using a Gilson Respirometer. The final concentrations were 1·25 mg/ml of glutamic acid and 0·5 mg/ml of glutamic decarboxylase in all experiments. All solutions were prepared in 0·1 M acetate buffer, pH 5·0. The reaction was started by the addition of glutamic acid from the side arm, except that in some experiments the addition of enzyme from the side arm was used to initiate the reaction. The activity was measured by the rate of evolution of carbon dioxide over a 5 or 10 min period and calculated as Qco_2 (μl of CO_2/mg of dry wt of protein/5 or 10 min).

Dialysis experiments

The enzyme preparation (5 mg) in 5 ml of either M or 0·1 M acetate buffer, pH 5·0, was dialysed against 50 ml of the same buffer at 0°; the dialysing medium was replaced ten times over a period of 48 h. Further samples of the enzyme suspensions were exposed for 15 h at 0° to either 10 μCi (10 μ mole) of [^{14}C]salicylate or to 0·25 M salicylate plus 10 μCi of radioactive salicylate and dialysed as described above. The radioactivity in triplicate samples (0·5 ml) of the mixture, before and after dialysis, were counted in a Beckman LS 200B scintillation counter, using as phosphor 5 ml of 0·4% 2,5-diphenyloxazole, 0·02% 1,4-bis-2(4-methyl-5-phenyl-oxazole)benzene and 60% naphthalene in 1,4-dioxan.

RESULTS AND DISCUSSION

The results in Table 1 show that salicylate inhibits the activity of glutamate decarboxylase from *E. coli* and the degree of inhibition becomes greater as the salicylate concentration is increased. Table 2 shows the effects of exposing the enzyme preparation to salicylate concentrations, ranging from 20 to 250 mM, for 1 h and then diluting with buffer before the reaction is started by the addition of the substrate. If the inhibitory effect of salicylate is irreversible, then the observed degree of inhibition should have been determined by the concentration of salicylate present at the preincubation stage, whereas if it is reversible, then the observed inhibition should depend on the salicylate concentration present in the diluted reaction mixtures. The results show that the inhibition produced by preincubation with 20 mM salicylate is completely reversible but that an increasing degree of irreversible inhibition occurs as the salicylate concentration is raised from 50 to 250 mM. Salicylate, therefore, inhibits glutamate decarboxylase *in vitro* by two separate mechanisms, one reversible and one irreversible. The concentration of the drug present in the reaction mixtures determines which type of inhibition predominates when the enzyme preparation is exposed to the drug for 1 h.

Some precipitation of the enzyme preparation was observed in the presence of salicylate concentrations greater than 150 mM, suggesting that chemical denaturation of the enzyme protein may have occurred. Further evidence supporting this mechanism is provided by the results in Table 3, which show that the degree of inhibition

Table 1. *Effect of salicylate on* E. coli *glutamate decarboxylase activity.* Each value represents the mean of six determinations. In the control experiment 93 μl of CO_2 were evolved during 10 min.

Final concn of salicylate (mM) in reaction mixture	0	5	10	15	20	50	100	150	250
Inhibition (%) ± s.e.	0	9 ±2·6	13 ±3·0	20 ±3·4	56 ±2·1	85 ±1·8	99 ±0·5	99 ±0·4	

Table 2. *Effect of preincubating enzyme with salicylate for* 1 *h.* The enzyme preparation was preincubated at room temperature (20°) with salicylate for 1 h, diluted 1 to 20 with 0·1M acetate buffer, pH 5·0 and there action started by tipping the glutamate from the side arm into an aliquot of the diluted mixture contained in the centre well of the Warburg flask. The evolution of CO_2 was followed manometrically for 10 min and inhibitions were calculated from the Q_{CO_2}/10 min (91 μl), observed with an enzyme preparation treated as above, except that it was preincubated in the absence of salicylate.

Salicylate concentration (mM)		Theoretical inhibition (%)		
Preincubated with enzyme	In diluted mixture	Irreversible	Reversible	Observed Inhibition (%)
20	1	20	0	0
50	2·5	56	0	16
100	5·0	85	0	70
150	7·5	99	5	85
250	12·5	99	12	99

Table 3. *Effects of preincubating enzyme with salicylate with time.* Experimental details were as described in Table 2. The results are given as percentage inhibitions calculated from corresponding control experiments in which the enzyme preparation was incubated in the absence of the drug.

Salicylate concn (mM) used for preincubation	Time of preincubation (h)			
	1	6	24	48
20	0	0	7	13
50	16	24	42	50
100	70	76	82	86

increased as the period of preincubation of the enzyme preparation with the drug was extended. Denaturation could explain the complete loss of activity which occurred when glutamate decarboxylase was exposed for 15 h to 250 mM salicylate but not the reported binding of radioactive salicylate to the enzyme protein (Gould, Huggins & Smith, 1963). The glutamate decarboxylase preparation was therefore exposed to either 250 mM salicylate plus 10 μmole of [^{14}C]salicylate or to 10 μ mole of the radioactive salicylate for 15 h at 0° and dialysed as described in the Experimental section. The average counts/min were 63,000 $\pm$ 1500 per ml in the dialysis sac at the beginning of the experiments. With and without carrier salicylate the corresponding figure at the end of dialysis was 100 $\pm$ 15 counts/min while outside the dialysis sac there were 90 $\pm$ 10 counts/min ml^{-1}. These results show that negligible binding occurred either with the denatured enzyme protein preincubated with 250 mM salicylate or when the enzyme protein was exposed to only a tracer amount of radioactive salicylate (10 μ mole). We are unable to explain the discrepancy between the experimental results of the earlier work and of the present investigation. Neither substitution of 3 M acetate buffer, pH 5·0, which was used in the previous experiments, nor continuous stirring of the contents of the dialysis sac during the dialysis, affected the removal of the radioactivity from the enzyme preparation.

It must be concluded that salicylate does not irreversibly combine with glutamate decarboxylase but that the drug chemically denatures the enzyme protein, the extent of denaturation depending on the salicylate concentration and on the time of exposure. Very high concentrations of the drug (250 mM) caused practically complete denaturation within an hour (Table 2) whereas the effect did not become evident with 20 mM salicylate until the preincubation period was extended to 24 h. The present results also show (Table 2) that part of the inhibitory effect of salicylate is due to a reversible mechanism and it is known from previous work (Gould & others, 1963) that this does not involve competition with the substrate.

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Biological half-lives of [4-^{14}C]testosterone and some of its esters after injection into the rat

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Biological half-lives for ^{14}C from labelled testosterone in muscle and whole body of rats have been measured after intramuscular injection of [4-^{14}C]testosterone and its lower esters (formate to valerate). A relation has been observed between ethyl oleate–water distribution coefficients, biological half-lives in the rat and "times of maximum effect" in the rat and fowl.

After intramuscular injection, testosterone has a shorter duration of biological action, than its esters. A possible reason is that the rate determining process is the transfer of the hormone from the globules of solution, at its site of injection in the muscle, to the surrounding tissues. Thus, testosterone, which would be expected to have an oil to water distribution coefficient more heavily in favour of water than the coefficients of the esters, would be transferred the more rapidly. Similarly, the differences in duration of biological action between esters would be related to their distribution coefficients. This theory is tested below by comparing the distribution coefficients between water and ethyl oleate, of testosterone and its esters, with retention times in the rat and in the muscle into which injected.

EXPERIMENTAL

Solubility determinations

Saturated solutions of the esters in ethyl oleate were prepared by percolation (James & Roberts, 1968) and assayed by measuring the absorbance of the carbonyl stretching peak at 1680 cm^{-1}, which had been found to be linearly related to steroid concentration. Gravimetric analysis and ultraviolet spectrophotometry were precluded by the low volatility and irrelevant absorption of the solvent.

Preparation of radioactive doses

[4-^{14}C]*Testosterone and testosterone*[4-^{14}C]*propionate* were obtained from the Radiochemical Centre, Amersham. For each preparation, 50 mg of inactive steroid was added to 50 μCi of the radioactive solution, which was carefully evaporated to dryness.

Testosterone[4-^{14}C]*formate* was prepared by heating labelled testosterone of activity 50 μCi (50 mg), with 85% formic acid (0·6 ml) at 60° for 2 h. The crystals, which separated when the hot solution was poured into cold water, were recrystallized from n-hexane.

Testosterone[4-^{14}C]*acetate, -butyrate and -valerate* were prepared by heating labelled testosterone of activity 50 μCi (50 mg) with pyridine (1·5 ml) and the appropriate acid anhydride (0·3 ml). The esters separated on pouring the hot mixture into cold water,

and were recrystallized from 95% ethanol. All melting points agreed with those quoted in the literature.

Solutions for injection were prepared by dissolving about 50 mg of the ester in 5 ml of ethyl oleate. Exact concentrations were determined by weighing.

Determination of elimination rates

Albino male rats, 250 g, were used. 0·1 ml of injection, containing 1 μCi of activity and 1 mg of steroid, was introduced into the left gluteus muscle of each rat. The syringe was weighed before and after injection to determine the precise quantity of materials used. For whole body elimination, urine and faeces were collected at 24 h intervals, and the activity remaining in the body calculated by difference. For muscle elimination, animals were killed after the required period of time and the whole leg removed. Expired air was not examined because this has been shown by Ashmore, Elliot & others (1953) to be inactive after administration of [4-^{14}C]testosterone.

Preparation of extracts for counting

Faeces were extracted as described by Martin (1966). The solution for counting was prepared by dissolving the residue in benzene and adjusting to 10 ml.

Urine was added directly to the scintillator.

Muscle. The whole upper leg was digested in 50 ml of 2N methanolic potassium hydroxide at 60° for 4 h. The solution was centrifuged, after removing the bone, and samples taken from the methanolic layer. The fat layer gave negligible counts.

Determination of Carbon-14

Hall and Cocking's scintillator (1965) (PPO–POPOP–toluene–2-ethoxyethanol) was used for faeces extracts, and PPO–POPOP–naphthalene–dioxan (Graham & Nicholls, 1959) for muscle digests and urine. 0·1 ml of sample was added to 7·5 ml of scintillator, and counted in an I.D.L. Tritium Scintillation Counter No. 6012. Internal standards were prepared by dissolving the original injections in benzene and dioxan respectively.

RESULTS AND DISCUSSION

The plots of the logarithms of radioactivity remaining against time, were linear for elimination from both whole body and muscle. First order constants were obtained by least squares analysis. Biological half-lives, the times at which only half the initial activity is retained, were calculated from the rate constants, and are given in Table 1. The variation in biological half-life, as the homologous series is ascended, is shown in Fig. 1A and B.

Miescher, Wettstein & Tschopp (1936) measured the biological responses in rat and fowl after one injection of testosterone or its esters, and their results, which were shown graphically, have been interpreted by Dorfman & Shipley (1956) as "times of maximum effect", the times at which Miescher's graphs reached a maximum. The change in Dorfman's figures, as the homologous series is ascended, is shown in Fig. 2A.

Solubilities in ethyl oleate (% w/v) are: testosterone, 0·69; formate, 5·27; acetate 3·14; propionate, 5·16; butyrate, 5·10; valerate 3·98. The logarithms of the distribution coefficients, calculated as the ratio of these solubilities to those in water (James & Roberts, 1968), are plotted against the position of the ester in the homologous series in Fig. 2B.

Figs 1A, 2A and B suggest that retention of the carbon-14 in rat is related to time of maximum effect in rat and fowl, as determined by Miescher & others (1936), and all

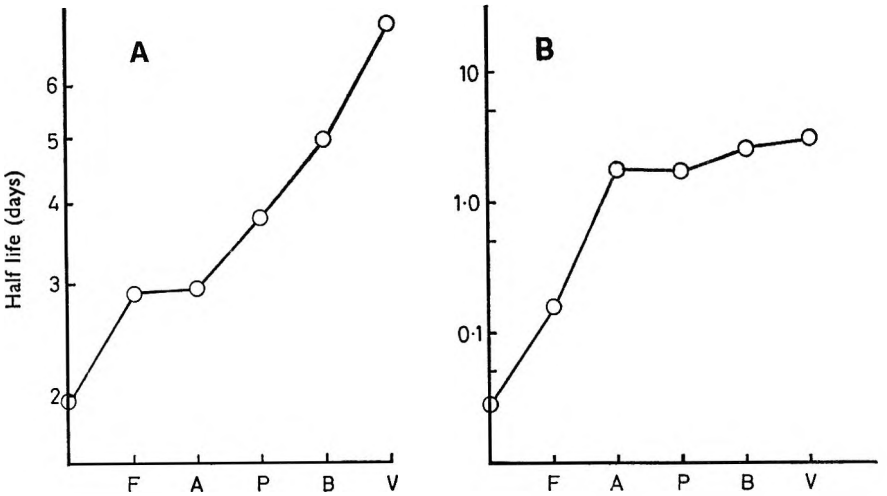

FIG. 1. Biological half-lives in A, rat and B, muscle; F, formate; A, acetate; P, propionate; B, butyrate; V, valerate.

are in turn related to the distribution coefficient. This was established more critically by plotting the logarithms of the above biological results against log distribution coefficient. Three approximately parallel straight lines were obtained, all having correlation coefficients greater than 0·95. However, the points for log biological half life in muscle were scattered (correlation coefficient = 0·71), and gave a regression line steeper than the other three lines. This is confirmed by Fig. 1B, in which the profile for half-life in muscle is different from the profiles in Figs 1A, 2A and B. It appears therefore that the rate of elimination from muscle has little connection with distribution coefficient, and therefore is not dependent on the rate of transfer from the globules of injection to the surrounding tissues. It is further evident that the rate determining step in the duration of activity of testosterone and its esters is not centred in the muscle tissue where the injection was given. This conclusion also follows from the half-lives in muscle, which are less than those in the whole rat (Table 1).

Samuels (1966) has pointed out that a distribution coefficient favouring lipids leads to concentration in fatty tissue, because the distribution coefficient is proportional to the rate of entry into fat and inversely proportional to the rate of release. Plotz & Davies (1957) have detected significant levels of progesterone in body fat, after

Table 1. *Biological half lives of carbon-14 in rat after intramuscular injection of [4-^{14}C]testosterone and its esters*

	Half life (days)	
	Muscle	Whole body
Testosterone	0·029 (10)	1·99 (3)*
Formate	0·155 (10)	2·82 (2)
Acetate	1·74 (10)	2·94 (2)
Propionate	1·63 (10)	3·75 (4)
Butyrate	2·54 (10)	4·94 (3)
Valerate	2·97 (15)	7·43 (4)

* No of animals

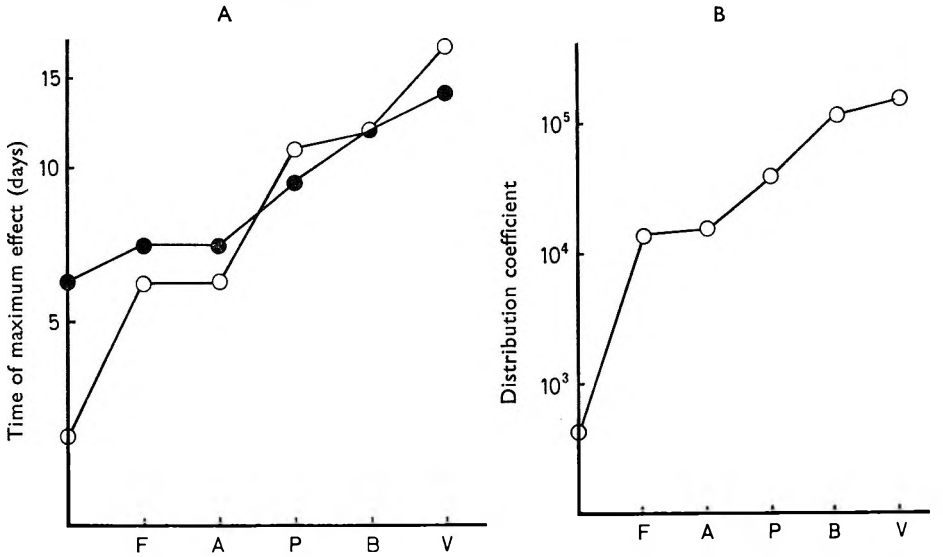

FIG. 2. A. Times of maximum effect. B. Distribution coefficients. F, formate; A, acetate; P, propionate; B, butyrate; V, valerate. ○, rat; ●, fowl.

intramuscular injection, and infer that absorption from an oily muscular depot must be fast, and that progesterone and/or its metabolites diffuse promptly from blood to fat. The results obtained in this investigation favour a similar mechanism for testosterone and its esters, and suggest that differences in times of maximum effect are a consequence of the differences between their distribution coefficients, which affect the relative rates of release from body fat.

It is probable that the rates of elimination from muscle do not correlate with distribution coefficients because ethyl oleate is absorbed at a rate similar to that for the steroids. Support for this suggestion is provided by Deanesly & Parkes (1933) who showed that appreciable quantities of olive and sesame oils are absorbed from subcutaneous tissue within 2 days of injection in rat.

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The dissolution of commercial aspirin

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Two samples of commercial aspirin showing a difference in intrinsic dissolution rate have been studied. Crystallographic examination and solubility determinations failed to reveal any difference between them. The effects of agitation and temperature on intrinsic dissolution rates showed that the samples had different thermodynamic activities and, depending on the conditions, the metastable form was capable of rapid reversion to a more stable form.

Tawashi (1968), has reported the existence of two polymorphic forms of aspirin, one of which dissolves 50% faster than the other. Differences in dissolution rate have been observed between samples of commercial aspirin (Mitchell & Saville, 1967). The two samples showing the greatest difference in dissolution rate have been examined further. These are designated form A† and form B‡.

Factors affecting the dissolution of solids have been reviewed by Bircumshaw & Riddiford (1952) and more recently by Wurster & Taylor (1965a) who paid particular attention to the pharmaceutical literature. Higuchi (1967) has discussed the use of physical models to describe dissolution rate mechanisms.

EXPERIMENTAL

Measurement of dissolution rate. Dissolution rates in 0·1 N hydrochloric acid were measured using the beaker method of Levy & Procknal (1964) and the rotating disc method of Wood, Syarto & Letterman (1965). Samples were removed at suitable time intervals and assayed for aspirin and salicylic acid as described by Mitchell & Saville (1967). At least two replicate determinations were made and the results averaged. Reproducibility varied with the method, the form of aspirin and the experimental conditions of agitation and temperature but was normally within $\pm$ 4%. Using the conditions of Wood & others, the intrinsic dissolution rates of form A and form B were 0·995 and 1·75 mg/cm^{2}min^{-1} respectively.

Determination of solubility. Excess aspirin was equilibrated with 0·1 N hydrochloric acid by rapid stirring in a water-jacketed beaker maintained at the appropriate temperature. Samples were removed using a pipette fitted with a filter-stick, diluted immediately with 0·1 N hydrochloric acid and assayed for aspirin and salicylic acid.

RESULTS AND DISCUSSION

Representative dissolution curves are shown in Fig. 1A and B. Form B behaves as expected but with form A the linear dissolution curve is preceded by a steeper non-linear portion which occurs at all rotation rates and temperatures using both the beaker and rotating disc methods. Intrinsic dissolution rates per unit area were calculated from the slopes of the straight lines for form B and the linear part of the curve for form A.

† Asagran 4D ‡ Aspirin No. 3 special; both from Monsanto Chemicals, Australia

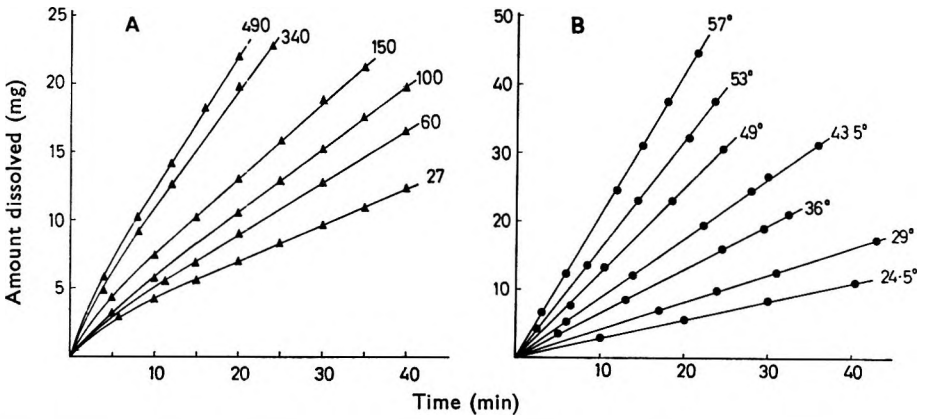

FIG. 1A. Dissolution curves of form A aspirin. Beaker method using compressed discs (diameter 1·3 cm) at various stirring rates (rev/min) in 0·1N HCl at 37°.

B. Dissolution curves of form B aspirin. Rotating disc method at various temperatures in 0·1N HCl at 150 rev/min.

Examination of the discs of aspirin after a dissolution experiment showed that much pitting had occurred in discs of form A but not in discs of form B.

Crystallographic examination. Polarized light microscopy showed that both forms A and B belonged to the monoclinic system. In view of the differences in dissolution rate the samples were examined for polymorphism by X-ray diffraction powder patterns, infrared spectra (Nujol mull) and attenuated total reflectance of infrared. These methods failed to reveal any difference between forms A and B. It is possible however, that one or both forms may be a mixture of polymorphs and that the initial rapid dissolution and pitting of form A is due to the presence of a small amount of a more rapidly dissolving form such as B.

Effect of agitation on dissolution rate. An increase in agitation intensity (rate of stirring or velocity of the dissolution medium across the surface of the dissolving substance) will increase the dissolution rate of a system in which the transport of solute molecules from the solid-solution interface is wholly or partially the rate-determining step. If the rate-determining step is the rate of the interfacial reaction, the overall dissolution rate will be independent of agitation.

The influence of agitation on the transport process is a result of its effect on the thickness of the diffusion layer. Levich (1942) calculated that for a disc rotating in a volume where wall effects are minimal, the thickness of the diffusion layer, "h", should be related to the angular velocity of the disc, "w", by the expression

$$h = 1{\cdot}612\ D^{\frac{1}{3}}v^{\frac{1}{6}}w^{-\frac{1}{2}} \quad .. \quad .. \quad .. \quad .. \quad (1)$$

where D is the solute molecule diffusion coefficient and v the viscosity. Therefore, for laminar flow

$$k_t \propto w^{\frac{1}{2}} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (2)$$

where k_t, the rate constant for the transport process, $= D/h$. For other types of stirring

$$k_t \propto w^a \quad .. \quad .. \quad .. \quad .. \quad .. \quad (3)$$

where the value of a, which lies between 0 and 1, depends on the type of agitation (laminar or turbulent), the geometrics of the stirrer and vessel and the position of the stirrer with respect to the dissolving substance (Bircumshaw & Riddiford 1952).

Levy & Procknal (1964) have expressed the relation between the dissolution rate, DR, and the rotation rate RR, according to the general equation

$$DR = K(RR)^{a'} \quad .. \quad .. \quad .. \quad .. \quad (4)$$

where K is a constant and a' depends on the nature of the dissolution control as well as the experimental conditions. For transport controlled dissolution using the beaker or rotating disc methods under conditions of laminar flow, $a = a'$. When plotted as log DR against log RR a straight line of slope approximately 0·5 has been taken as evidence that dissolution is transport controlled. Dissolution under total interfacial control is independent of agitation and the expected slope is zero. If both transport and interfacial processes are of the same order of magnitude however, the overall rate will be a function of both processes and the observed rate constant, k_{obs}, is related to k_t and the rate constant for the interfacial reaction, k_i, by

$$k_{obs} = \frac{k_i\, k_t}{k_i + k_t} \text{ (Wurster \& Taylor, 1965b)} \quad .. \quad .. \quad (5)$$

Using equation 5 it can be shown that an increase in interfacial control with rotation rate will produce a non-linear plot of log k_{obs} (or log DR) against log RR with a maximum slope of 0·5 ($k_i \gg k_t$) decreasing to zero when dissolution is under total interfacial control ($k_i < k_t$). Dissolution can be under 15% interfacial control (85% transport control) however, before the slope of the log DR against log RR curve changes significantly from 0·5, i.e. to $< 0{\cdot}45$. Moreover the change in slope is very gradual and in studies which cover a limited range of rotation rates the slope will appear to be linear.

The effect of agitation on the dissolution rate of aspirin has been reported previously (Mitchell & Saville, 1967). For convenience, results using the beaker method plotted as log DR against log RR are given in Fig. 2A. The results for form A fall on a straight line of slope 0·45. Form A also shows a slope of approximately 0 45 at two different temperatures using the rotating disc method, Fig. 2B. As discussed above such a plot may indicate that the dissolution process is under partial interfacial control.

The log DR against log RR profile for form B, Fig. 2A, is unusual. A change in the relative dissolution rates of polymorphs with variation in agitation has been reported

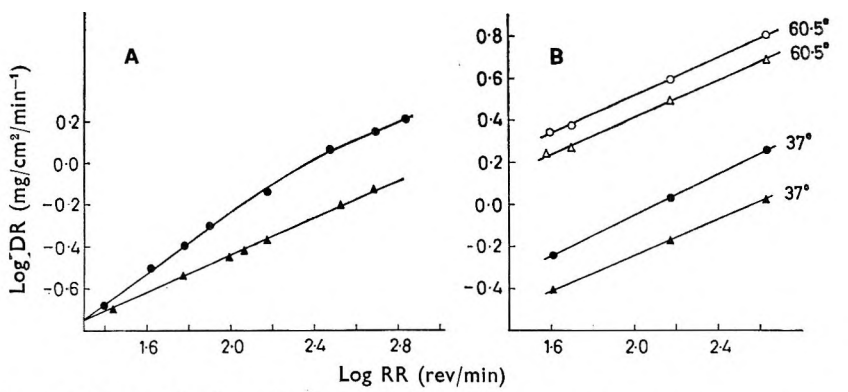

FIG. 2A. Variation of intrinsic dissolution rate with stirring rate. Beaker method using compressed discs of aspirin in 0·1N HCl at 37°. Form A, ▲; form B, ●.

B. Variation of intrinsic dissolution rate with rotation rate. Rotating disc method in 0·1N HCl. Form A, ▲; form B, ●.

previously for methylprednisolone (Hamlin, Nelson & others, 1962; Levy & Procknal, 1964) and in this case the dissolution rate of the metastable form approaches that of the stable form with increase in agitation intensity. From Fig. 2A, however, it can be seen that at an estimated rotation rate of about 20 rev/min the dissolution rates of forms A and B are the same but with increase in rotation rate up to about 300 rev/min the dissolution rate of form B increases relatively faster than A. The slope of the curve is 0·65 whereas under the experimental conditions used, the maximum slope for transport or mixed transport-interfacially controlled dissolution should be about 0·5. Above 300 rev/min the slope of the curve changes to 0·45 and the relative dissolution rate of form B is about 75% faster than form A. At rotation rates less than about 300 rev/min it is evident that some process additional to the normal interfacial and transport processes is involved.

Solubility of aspirin. On the basis of the dissolution behaviour it seems reasonable to suggest that the two forms have different thermodynamic activities. The solubilities of form A and form B are the same at each temperature studied, however, and at no time during solubility determinations on either form did the concentration of aspirin in solution rise above the equilibrium solubility as reported, for example, with metastable crystalline forms of prednisone (Wurster & Taylor, 1965b). Hence if the differences in dissolution rate are due to a difference in thermodynamic activity, it follows that one form must be reverting rapidly to the other during the solubility determination. Solubility data reported by Edwards (1951) for aspirin are shown in the van't Hoff type plot, Fig. 3, together with additional data obtained in this work. As expected from thermodynamic considerations the curve is linear only at lower temperatures (Moelwyn-Hughes, 1961).

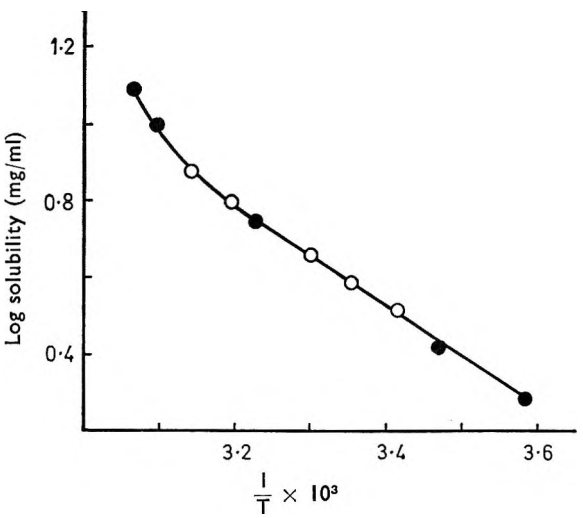

FIG. 3. Solubility of aspirin as a function of temperature. Edwards (1951) in 0·1N H_2SO_4, ○; this work in 0·1N HCl, ●.

Effect of temperature on dissolution rate. An increase in temperature will increase the rates of both the interfacial reaction and transport processes (Bircumshaw & Riddiford, 1952). For a transport controlled dissolution process the dependence of k_t on temperature can be expressed by the Arrhenius equation

$$k_t = Z_t e^{-Et/RT} \quad \ldots\ldots\ldots\ldots \quad (6)$$

where k_t is given by the ratio of the dissolution rate, DR, and solubility, C_s, provided the experimental conditions are such that the concentration of dissolved solute is negligible compared with C_s. Hence for a transport controlled process a plot of log DR/C_s against 1/T should be linear where the slope of the line is related to the activation energy of the transport process, E_t. E_t changes only slightly with agitation intensity so that plots of log DR/C_s against 1/T at different rotation rates should have approximately the same slope.

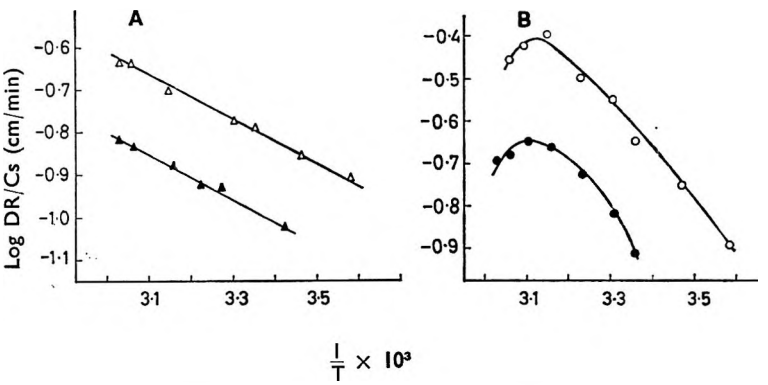

FIG. 4A. Dependence of log DR/C_s, form A, on temperature. ▲ 150 rev/min; △ 430 rev/min in 0·1N HCl, rotating disc method.

B. Dependence of log DR/C_s, form B, on temperature. ● 150 rev/min; ○ 430 rev/min in 0·1N HCl, rotating disc method.

Log DR/C_s against 1/T curves for aspirin forms A and B are plotted in Fig. 4A, B respectively. The activation energy of form A calculated from the slope of the line is 2·4 kcal/mole which is slightly below the range of 2·8–6·5 kcal/mole expected for a transport controlled process (Bircumshaw & Riddiford, 1952). The curves for form B are unusual and show a maxima at 50°. A change in slope indicates a shift in dissolution control but a change in sign is thereoretically impossible. It is concluded therefore that the true solubility of form B is greater than the equilibrium solubility, C_s, used in plotting Fig. 4B, and that forms A and B are thermodynamically different species of aspirin.

Dissolution rate and crystal reversion. Higuchi, Bernardo & Mehta (1967) have developed a model for the dissolution of a mixture of two polymorphs. The theory also provides a qualitative explanation of the "anomalous" effect of agitation intensity on the dissolution of the metastable polymorph of methylprednisolone first reported by Hamlin & others (1962). During the dissolution of the metastable form it is suggested that a layer of the stable form crystallizes at the solid-solution interface. After a time-lag the effect on the dissolution curve is the same as dissolution from a mixture of the two forms. With increase in agitation intensity the thickness of the diffusion layer, *h*, decreases and the dissolution rate quickly approaches that of the pure stable form. The theory requires the assumption that the reversion rate of the metastable form is neither very rapid nor very slow.

The dissolution of aspirin differs from the model proposed for methylprednisolone in several respects. The effects of agitation and temperature on the dissolution of

form B indicate that it is a metastable form, but no time-lag is apparent in the dissolution curves (Fig. 2 and Fig. 1 of Mitchell & Saville, 1967). The results for form A suggest that it is a stable form which contains a small amount of a less stable form. The intercepts of the steady-state dissolution curves on the ordinate (Fig. 1A) are not related to the agitation intensities (and therefore to the diffusion layer thickness, h), however, as required by the model of Higuchi. Finally the dissolution rates of forms A and B appear to be the same at low agitation intensities using the beaker method, but diverge with increase in stirring rate (Fig. 2A) whereas with methylprednisolone polymorphs the dissolution rates differ at low agitation intensities and approach each other as the agitation rate is increased (Levy & Procknal, 1964).

At low stirring rates and in the presence of excess solid, as in the solubility determinations, it is apparent that form B undergoes rapid reversion to a more stable form. The effect of the increase in rotation rate on the dissolution rate of form B suggests an inverse relation between rate of reversion and agitation intensity. Thus the dissolution rate approaches that expected of a more soluble form and gives rise to the unusually high value of 0·55 for the slope of the log DR against log RR curve (Fig. 2A). The change in slope from 0·65 to approximately 0·45 at stirring rates greater than about 300 rev/min indicates that the rate of reversion has reached a minimum limiting value and that dissolution is taking place as if from the more soluble form. The slope of log DR against log RR for form B using the rotating disc method shows no change with rotation rate and is approximately the same as form A (Fig. 2B). From the values of dissolution rate it is apparent, for a given rotation rate, that agitation intensities using the rotating disc method are much greater than for the beaker method, e.g. 150 rev/min rotating disc method = 400 rev/min beaker method. It is likely therefore that minimal reversion occurs with the rotating disc method under the given experimental conditions.

According to the Noyes-Whitney law the ratio of dissolution rates should correspond to the solubility ratio (Hamlin, Northam & Wagner, 1965). Hence, using the difference in dissolution rates found at high rotation rates, it can be postulated that the true solubility of form E at 37° is approximately 75% greater than form A and therefore the interfacial concentration, C_i, is greater than the equilibrium solubility, C_s. During the dissolution of form B the diffusion layer will be supersaturated with respect to a more stable form and polymorphic reversion may occur simultaneously with dissolution. From a consideration of the factors controlling crystal growth it is possible to offer a qualitative explanation for the dependence of reversion rate on agitation intensity. Crystal growth rates are highly dependent on the degree of supersaturation (Taylor & Wurster, 1965). The degree of supersaturation at the solid-solution interface of a metastable crystal undergoing dissolution is given by C_i/C_s where $C_i > C_s$. For a mixed transport-interfacial dissolution process the results of Wurster & Taylor (1965b) show that C_i decreases with increase in agitation intensity. It is suggested therefore that the corresponding reduction in the degree of supersaturation is responsible for the decrease in reversion rate.

A comparison of our results with those of Tawashi (1968) reveals another problem. The more stable of the two forms of aspirin recrystallized by Tawashi was very similar to commercial aspirin U.S.P. We have found that most commercial samples are similar to form B which by comparison with form A must be metastable. It is possible that both forms of aspirin recrystallized by Tawashi are metastable polymorphs and that another more stable form exists.

An important conclusion from a practical viewpoint is that a study of intrinsic rates may be more useful in selecting a suitable drug sample than techniques such as infrared and X-ray diffraction since these will probably fail to distinguish between samples containing different mixtures of polymorphs.

Acknowledgements

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Effects of nicotine on motor co-ordination and spontaneous activity in mice

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Doses of nicotine (0·2 and 0·4 mg/kg subcutaneously) which depress spontaneous activity, improve the ability of mice to remain on a rotating rod, indicating that the reduction in activity is not due to non-specific disruption of motor ability.

The performance of rats trained to press a bar for water rewards is stimulated by small subcutaneous doses (0·05, 0·1 mg/kg) of nicotine; larger doses (0·2, 0·4 mg/kg) briefly reduce the rate of bar-pressing before increasing it (Morrison, 1967). The larger doses also depress spontaneous motor activity in mice (Morrison & Armitage, 1967). In the present experiments a rotating rod (Dunham & Miya, 1957) was used to test whether the depresssion of spontaneous activity caused by nicotine is a result of motor incapacity.

EXPERIMENTAL

Methods

Rotarod. Batches of 100 young male T.O. mice were trained to remain on a wooden rod 2 inches in diameter, rotating at a speed of 11 rev/min. Approximately 40% of the mice did not learn to stay on the rod at this speed and were rejected. The effects of nicotine were tested on the remaining mice with the rod rotating at 14 or 20 rev/min. At the slower speed most control mice could stay on the rod for 5 min or more but at 20 rev/min most fell off within 5 min. An improvement or impairment of performance could therefore be detected. The mice were tested 3 at a time; two of the animals were injected subcutaneously with nicotine while the third control mouse received physiological saline. After a delay of either 2 or 5 min the mice were placed on the rotarod and the time for which each mouse remained on the rod was recorded. After 5 min any mice remaining on the rod were removed.

Motor activity. This was recorded in boxes measuring 24 × 36 cm fitted with 3 photoelectric cells (Rossum, 1962). The mice were placed singly in the boxes for 1 h and their activity allowed to subside. They were then removed and injected subcutaneously with saline or nicotine, returned to the boxes and their activity recorded for a further 30 min.

RESULTS

Table 1 shows the effect of nicotine on rotarod performance in five experiments. The control mice varied greatly in their ability to stay on the rod, some falling off almost immediately and others remaining for the full 5 min of the test period. The majority fell off between 10 and 40 s. The variability in performance was not related to body weight or to time of day. Because of scores within each group of mice were not normally distributed, means and standard errors have not been presented. Instead, the time at which half the mice in a group had fallen off was calculated and the

results have been analysed statistically using the non-parametric Mann-Whitney U test. In four groups of mice (Experiments 1, 2 and 4) nicotine significantly increased the time the mice remained on the rod. In only one group did the mice injected with nicotine show a slightly poorer performance than the corresponding control group (Experiment 3). In the earlier experiments the mice were tested 2 min after injection, but when it became apparent that the depression of spontaneous activity caused by nicotine was at its greatest between 5 and 10 min after injection (see below) the delay between injection and testing was increased to 5 min. In one experiment (no. 4) mice were tested either 2 or 5 min after receiving 0·4 mg/kg of nicotine. Both experimental groups showed a significant improvement over the controls but this was greater after the shorter delay.

Table 1. *Effects of nicotine (Nic) on the ability of mice to remain on a rod rotating at 20 rev/min*

Expt	Treatment (dose in mg/kg)	Time between injection and test	No. of mice	Time for half the mice to fall off (s)
1	Saline	2 min	20	16
	Nic 0·2	,,	20	55·5**
2	Saline	2 min	19	25
	Nic 0·1	,,	19	75
	Nic 0·2	,,	18	186*
3	Saline	2 min	18	40
	Nic 0·2	,,	18	61·5
	Nic 0·4	,,	18	30
4	Saline	2 min	20	21
	Nic 0·4	2 min	20	66***
	Nic 0·4	5 min	20	28·5*
5	Saline	5 min	20	23·5
	Nic 0·2	,,	20	28
	Nic 0·4	,,	20	47·5

* P <0·05, ** P <0·01, *** P <0·001 Statistical significance of difference between nicotine injected and corresponding control group. Mann-Whitney U test.

In order to test the effects of very high doses of nicotine, a group of mice were trained and tested at 14 rev/min. At this speed most of the control mice remained on the rod for the whole of the 5 min period. Doses of nicotine up to 3·2 mg/kg did not affect their ability to stay on the rod. At a dose of 6·4 mg/kg of nicotine the average

Table 2. *Mean activity scores of groups of mice tested singly in activity boxes after nicotine administration*

Treatment	No. of mice	Activity counts (frequency of breaking light beam) 0–5 min	5–10 min	10–15 min	15–30 min
Saline	22	88	64	48	156
Nicotine 0·2 mg/kg	22	62	35	35	131
Nicotine 0·4 mg/kg	22	64*	22***	24***	73***

Mann-Whitney U test * P <0·05, *** P <0·001 Statistical significance of difference between nicotine and saline injected groups.

time on the rod of 16 mice was reduced to 3 min. Eight of these mice had convulsions and 4 of them died; the remainder showed severe tremors.

Table 2 shows the effects on motor activity of 0·2 and 0·4 mg nicotine/kg. Nicotine reduced activity and this effect was most pronounced between 5 and 10 min after the injection. For the 0·4 mg/kg dose the reduction was highly significant ($P < 0.001$).

DISCUSSION

Motor activity, when measured by the boxes used here, is depressed by nicotine in rats as well as mice (unpublished observations) and no consistent evidence for a stimulant action of nicotine has been found in these studies. Nicotine, however, has a biphasic action on bar-pressing behaviour, a period of increased response following the initial phase of depression (Wanner & Battig, 1966; Morrison & Armitage, 1967; Morrison, 1967). In contrast to its effect on motor activity as measured by the activity boxes, nicotine did not depress activity in the rotarod test, its only detectable effect being improvement of performance.

The reduction in activity caused by nicotine in the activity boxes is unlike the effect of amphetamine in the same test, but there are similarities in the actions of these two drugs on bar-pressing behaviour. Nicotine also resembles amphetamine in its effect on rotarod performance since it has been shown that amphetamine also improves performance in these tests (Plotnikoff, Reinke & Fitzloff, 1962). Nicotine, therefore, can either decrease or increase performance, and the nature of the effect depends on the test used. The reduction in spontaneous motor activity caused by nicotine does not appear to be the result of a non-specific depression since amounts of nicotine which depress activity actually improve performance on the rotarod.

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The mechanism of action of 2-halogenoethylamines at the adrenergic α-receptor and a further investigation of the "spare receptor" hypothesis*

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The evidence for "spare-receptors" has been examined in the adrenergic α-receptor system of the rat vas deferens using *N*-(2-bromoethyl)-*N*-ethyl-1-naphthylmethylamine (SY.28) and *NN*-dimethyl-2-phenylethylamine hydrobromides as the antagonist species. It is clearly demonstrated that "spare-receptors" are not present in this tissue and that the observed parallel shift of the dose-response curve of noradrenaline produced by SY.28 is due to a competitive reversible phase of action of the corresponding ethyleneimine species rather than to the presence of "spare-receptors". Additionally, these experiments have revealed the existence of a significant non-competitive reversible phase of action of SY.28. From radiochemical studies and the observed first order recovery of tissue response following blockade by *NN*-dimethyl-2-bromo-2-phenylethylamine, it is concluded that the α-receptors in the rat vas deferens are indistinguishable from the α-receptors in the rabbit aorta in their behaviour towards this agent. From these results and those of others, it may be concluded that there is no existing evidence for "spare receptors" in adrenergic α-receptor systems.

In his original treatment of drug-receptor interactions Clark (1933, 1937) assumed that tissue response is proportional to the concentration of drug-receptor complex and that maximum response occurs with complete occupation of the receptors. During the last decade these assumptions have been subjected to extensive challenge and it now appears to be widely accepted that maximum tissue response does not necessitate 100% occupation of the receptors and that a substantial receptor "reserve" ("spare-receptors") may exist (Furchgott, 1954; Nickerson, 1956; Stephenson, 1956; Ariëns, Rossum & Koopman, 1960; Paton, 1961; Rossum & Ariëns, 1962; Burgen, 1966; Mackay, 1966a, b; Rossum, 1966). Estimates of the percentage of receptors occupied at maximum response have ranged from 1% to 0·0001% (Nickerson, 1956; Paton & Rang, 1966; Schild, 1962).

The concept of "spare-receptors" appears to be supported principally by experiments involving the use of 2-halogenoethylamines which interact irreversibly at adrenergic, cholinergic and histaminergic receptor sites. In many instances it is observed that treatment of the tissue with such agents produces a shift of the dose-response curve of the agonist to the right and that this shift precedes any decrease in the

* Previous paper May, Moran & others (1967).

maximum response obtainable. Such shifts would normally be regarded as representative of competitive reversible antagonism but for the one fact that 2-halogenoethylamines are known to be alkylating agents which can bind covalently to various tissue sites. It has, therefore, been assumed quite generally (Nickerson, 1956; Ariëns, and others, 1960; Rossum, 1966; Furchgott, 1967) that such shifts of the agonist dose-response curve without depression in the maximum response indicate the presence of a receptor "reserve" which must be irreversibly inactivated before a reduction in the tissue response can be obtained. However, from theoretical considerations of the mechanism of action of 2-halogenoethylamines we have proposed (Triggle, 1965a) that the portion of the dose-response curves considered to depict receptor "reserve" is in fact best attributed to a *competitive reversible* binding of the ethyleniminium ion at the receptor.

Recent work from our laboratory (Moran, May & others, 1967; May, Moran & others, 1967) concerned with the adrenergic α-receptor system of the rabbit aorta and utilizing a different experimental approach to that outlined above, has provided strong evidence that "spare-receptors" do not exist in this system. In view of these results and because of the importance of the "spare-receptor" concept both to quantitative pharmacology and to problems of receptor isolation, we have initiated a program to re-examine the experimental evidence upon which the "spare-receptor" hypothesis is founded and to provide a mechanistic explanation for the observed shifts in dose-response curves.

METHODS

Pharmacology. Pairs of rat vas deferens were suspended in water-jacketed baths containing 20 ml of modified Krebs-bicarbonate solution (Huković, 1961) maintained at 37° and bubbled with 5% carbon dioxide in oxygen. Cumulative dose-response curves were constructed from recording of isotonic contractions obtained by means of an ink-writing lever on a kymograph. Two control dose-response curves for noradrenaline were always determined on each tissue before treatment of one tissue with blocking agent: the paired tissue was not treated with blocking agent and served as a control.

Solutions of the 2-bromoethylamines were made up in neutral saline and allowed to stand at room temperature for 30 min before placing on ice. This insured that formation of the derived ethyleniminium ions was essentially complete.

Tissues were blocked with *NN*-dimethyl-2-bromo-2-phenylethylamine and cumulative dose-response curves of noradrenaline were obtained during the recovery of the response at the stated time intervals. Repetition of dose-response curves at intervals of less than 30 min occasionally resulted in erratic responses so that in general only two curves, determined at 30 min intervals, were obtained from the same tissue; the paired tissue being used as a control. The first-order rates of recovery of tissue response were determined as previously described (May & others, 1967).

Other tissues were blocked to varying degrees with SY.28, [*N*-(2-bromoethyl)-*N*-ethyl-1-naphthylmethylamine hydrobromide], followed by determination of the dose-response curves in a cumulative manner. In some cases, this was followed by treatment with 10^{-3} M sodium thiosulphate containing an ED50 (determined from control curve) of noradrenaline for 4 min followed by a 15 min washout period after which the dose-response curve was redetermined.

Radioactivity measurements. The rate of tritium loss from tissue blocked with [^{3}H]*NN*-dimethyl-2-bromo-2-phenylethylamine was determined by taking aliquots of the bath fluid at 5 min intervals. Disintegration rates were determined by liquid scintillation counting using internal standards for quench correction (Rogers & Moran, 1966). In all cases a minimum of 10,000 counts was collected. The first order rate of tritium loss was obtained by plotting this data according to Rose (1964) as previously described (May & others, 1967).

Partition experiments. [^{3}H]*NN*-Dimethyl-2-hydroxy-2-phenylethylamine (May & others, 1967) was added to a mixture of 5 ml $CHCl_3$ and 5 ml H_2O and allowed to equilibrate with shaking for 24 h. Aliquots from the two phases were counted and the ratio of the disintegration rates determined.

RESULTS

Fig. 1 shows the dose-response curves of noradrenaline determined on the rat vas deferens before and after exposure to SY.28 at a concentration of $2{\cdot}5 \times 10^{-7}$ M for 1 min followed by washout of excess SY.28. It is apparent that, after this treatment with SY.28, the dose-response curve of noradrenaline is shifted to the right with no decrease in maximum response, a finding which accords with previously published data of other workers (Ariëns & others, 1960; Ariëns, Simonis & Rossum, 1964) and which is normally interpreted as evidence for a receptor "reserve" despite the fact that this phenomenon is typical of competitive antagonism. After the blockade by SY. 28, sodium thiosulphate (10^{-3} M) and noradrenaline (3×10^{-6} M, ED50) were added for 15 min, washed out, and the dose-response curve to noradrenaline redetermined. It can be seen (Fig. 1) that the dose-response curve is shifted back to the left and is essentially super-imposable upon the control curve at response levels of greater than 60%. The inset in Fig. 1 shows that sodium thiosulphate alone has no significant effect.

Some tissues were blocked with SY. 28 to a degree sufficient to cause a decrease in maximum response and were then treated with sodium thiosulphate in the presence of

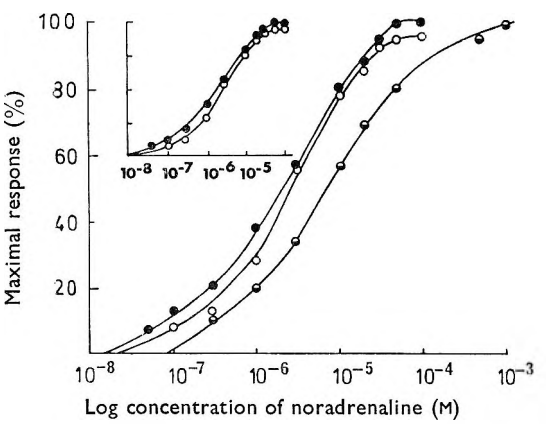

FIG. 1. Effect of sodium thiosulphate on blockade mediated by SY. 28. Rat vas deferens was blocked with SY. 28 ($2{\cdot}5 \times 10^{-7}$ M) for 1 min and the dose-response curve for noradrenaline determined after washout (◒). Tissues were then treated with 10^{-3} thiosulphate for 15 min and the dose-response curve redetermined (○). Controls were treated with saline (●). Inset shows the effect of 10^{-3} M sodium thiosulphate alone (○) as compared to controls (●). Dose-response curves are the average of 9 experiments.

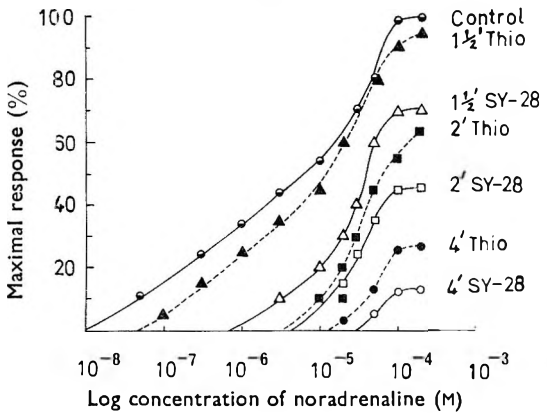

FIG. 2. Effect of sodium thiosulphate on the response of vas deferens blocked to varying degrees with SY. 28. Dose-response curves for noradrenaline were determined on control tissues (◒) and compared to the curves obtained from tissues blocked with SY. 28 ($2{\cdot}5 \times 10^{-7}$) for the designated times before (——) and after (- - - - -) treatment with 10^{-3} M sodium thiosulphate.

an ED50 of noradrenaline (3×10^{-6} M). From Fig. 2 it can be observed that thiosulphate relieves a portion of the inhibition as measured by the maximum response, e.g. 70% response after SY.28 block increases to 95% response after treatment with sodium thiosulphate. Furthermore, there is an apparent shift of the dose-response curves from tissues treated with thiosulphate back towards the control curve similar to that observed in Fig. 1. This shift is more pronounced in those tissues treated for shorter periods of time with SY.28. Tissues which were blocked to the extent of 100% with SY.28 showed no reversal of inhibition when treated with noradrenaline and thiosulphate under identical conditions; this result would be expected if all receptors were alkylated.

It was not possible to make similar experiments with sodium thiosulphate using *NN*-dimethyl-2-bromo-2-phenylethylamine as the α-blocking agent since it was impossible to obtain a shift to the right of the dose-response curve of noradrenaline

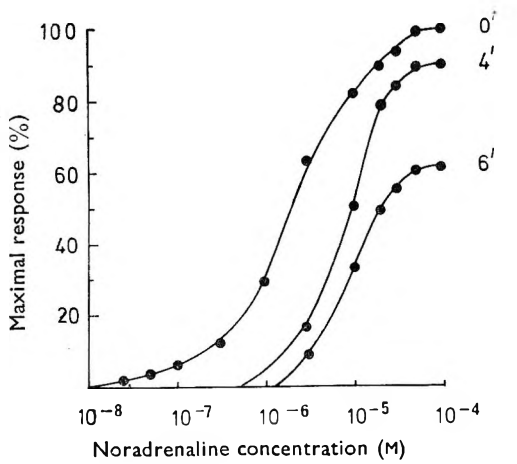

FIG. 3. Dose-response curves of noradrenaline following exposure of vas deferens to *NN*-dimethyl-2-bromo-2-phenylethylamine. Tissues were treated with *NN*-dimethyl-2-bromo-2-phenylethylamine ($1{\cdot}3 \times 10^{-6}$ M) for the times indicated followed by washout and determination of the dose-response relation. Dose-response curves represent the average of 3 experiments.

without a simultaneous decrease in the maximum response (Fig. 3). This is probably due to the greater reactivity of the *NN*-dimethyl-2-phenylethyleniminium ion (Triggle, 1964, 1965a). The situation is further complicated by the fact that *NN*-dimethyl-2-bromo-2-phenylethylamine produces an irreversible blockade of rather short duration (Triggle, 1964, 1965a; Kimelberg & Triggle, 1965; May & others, 1967) and significant recovery occurs via intramolecular hydrolysis during washout and determination of one dose-response relation.

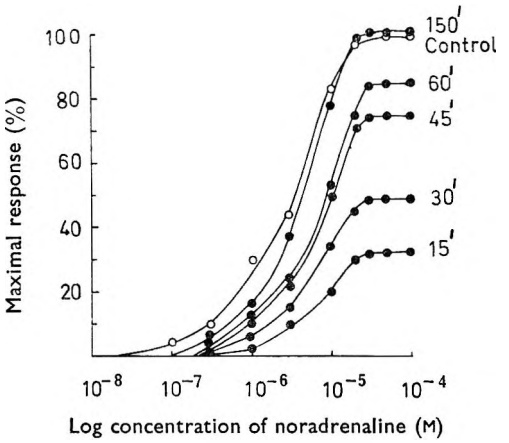

FIG. 4. Recovery of response of vas deferens blocked with *NN*-dimethyl-2-bromo-2-phenylethylamine. After complete blockade of the noradrenaline response of rat vas deferens by *NN*-dimethyl-2-bromo-2-pheny ethylamine ($1{\cdot}3 \times 10^{-5}$ M for 5 min) tissue response was determined at the times indicated.

However, it was possible to determine dose-response curves for noradrenaline during the period when the response of the vas deferens was recovering from exposure to *NN*-dimethyl-2-bromo-2-phenylethylamine. Dose-response curves were determined every 15 or 30 min following complete blockade by *NN*-dimethyl-2-bromo-2-phenylethylamine ($1{\cdot}3 \times 10^{-5}$ M for 5 min). Normally, only 2 curves were determined on each tissue at 30 min intervals; the paired tissue served as a control. The results of these experiments are shown in Fig. 4 from which it is apparent that the maximum response is always obtained at essentially the same concentration of noradrenaline. Furthermore, it is important to note that there is no dose-response curve showing full recovery of maximum response with a simultaneous shift to the right relative to the control curve.

The results from Fig. 4 have been plotted as previously described in order to determine the $t_{\frac{1}{2}}$ for recovery of tissue response to noradrenaline after blockade by *NN*-dimethyl-2-bromo-2-phenylethylamine. From Fig. 5, the $t_{\frac{1}{2}}$ was found to be 22 min. In duplicate experiments, using [^{3}H]*NN*-dimethyl-2-bromo-2-phenylethylamine, the rate of radioactivity appearing in the bath fluid during the recovery process was determined and found to have a $t_{\frac{1}{2}}$ of 24 min.

In the partition experiments it was found that *NN*-dimethyl-2-hydroxy-2-phenylethylamine partitions between $CHCl_3$–H_2O in a ratio of 51 : 49 as compared to a ratio of 96 : 4 for *N*-ethyl-*N*-(2-hydroxyethyl)-1-naphthylmethylamine (Moran & others, 1967).

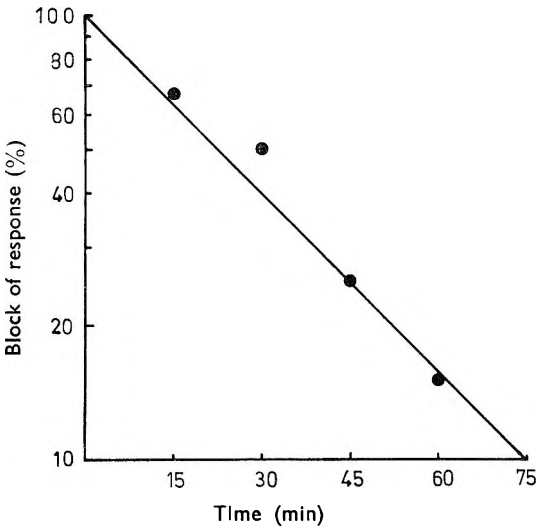

FIG. 5. Plot of first order rate of recovery of response of vas deferens to noradrenaline following blockade by *NN*-dimethyl-2-bromo-2-phenylethylamine. Data from Fig. 4 were plotted as previously described (Moran & others, 1967) to determine first order rate constants.

DISCUSSION

Sodium thiosulphate is known to react rapidly with ethyleniminium ions to form Bunte salts (Bunte, 1874; Fruton, Stein & Bergmann, 1946) and to prevent, *but not reverse*, the irreversible blockade of adrenergic α-receptors produced by 2-halogenoethylamines (Graham, 1962). However, sodium thiosulphate should reverse any blockade of α-receptors caused by reversible binding of ethyleniminium ions. This effect will be most readily demonstrable in the presence of an agonist which, through competition with the ethyleniminium ions, should make the latter more accessible for reaction with sodium thiosulphate. This effect is shown clearly in Fig. 1. Brief treatment of the vas deferens with SY.28 produces a shift to the right of the noradrenaline dose-response curve, unaccompanied by any decrease in the maximum height of response. This shift is completely reversed by subsequent treatment with sodium thiosulphate and can, therefore, be attributed to a reversible antagonism produced by SY.28, rather than to the presence of "spare-receptors". However, the shift in the dose-response curve of noradrenaline shown in Fig. 1 is not very large, a finding in accord with the work of Ariëns & others (1960, 1964) who have reported only a small "reserve" or absence of "spare-receptors" in rat vas deferens.

It appears to be generally accepted that the progressive decline in the response to an agonist of a tissue which has been treated with SY.28 or other related 2-halogenoethylamines is due to an irreversible inactivation of an increasing proportion of the receptor sites. The data presented in Fig. 2 show that the decline in the response to noradrenaline of rat vas deferens pretreated with SY.28 can be *partially, but not completely, reversed* by treatment of the tissue with sodium thiosulphate. It is highly improbable that the partial reversal of blockade can be attributed to any reaction of sodium thiosulphate with the alkylated receptor, a conclusion which is strengthened by the finding that complete blockade of the α-receptors by SY.28 is unaffected by sodium thiosulphate. It is, therefore, probable that the progressive depression by SY.28 of the maximum height of the response to noradrenaline shown in Fig. 2 may be attributed

to a dual mode of action of SY.28—an irreversible phase unaffected by sodium thiosulphate and a reversible non-competitive phase which is abolished by incubation of the tissue with sodium thiosulphate*. The presence of the latter component of action of 2-halogenoethylamines active at the adrenergic α-receptor does not appear to have been explicitly recognized hitherto, but its existence is in good agreement with our previous studies involving the use of [^{3}H]SY.28 which indicated clearly the lack of specificity of this agent (Moran & others, 1967).

The previous discussion indicates that the existence of a significant reversible phase of action of an (ultimately) irreversibly acting agent is sufficient to produce dose-response curves which have been interpreted as evidence for the existence of "spare-receptors". This interpretation is rendered invalid, at least for the α-receptors of the rat vas deferens, by the finding that sodium thiosulphate can reverse the parallel shift of the dose-response curves produced by SY.28. A valuable further test of the "spare-receptor" hypothesis would be provided by the use of an irreversibly acting agent that has no significant phase of reversible interaction at the receptor. The dose-response curves for noradrenaline from vas deferens treated with *NN*-dimethyl-2-bromo-2-phenylethylamine (Fig. 4) do not show parallel shifts unless accompanied by a decline in maximum response (compare Fig. 1), suggesting the absence of a reversible phase of action. However, the use of this agent in progressively inactivating the α-receptor system is complicated by its short duration of action ($t_{\frac{1}{2}}$ for recovery, 22 min), so that during washout procedures a significant recovery of response occurs via intramolecular hydrolysis (Triggle, 1965a, b; Kimelberg & Triggle, 1965). This difficulty was avoided by a procedure in which the tissue was completely blocked to noradrenaline by *NN*-dimethyl-2-bromo-2-phenylethylamine and washed thoroughly under conditions demonstrated to remove completely [^{3}H]*NN*-dimethyl-2-hydroxy-2-phenylethylamine from the tissue (May & others, 1967). The dose-response curves of noradrenaline obtained during the recovery of tissue response we believe to be uncomplicated by the presence of reversibly bound antagonist. The curves shown in Fig. 4 demonstrate that the maximum response to noradrenaline at any level of recovery of tissue response occurs at the same concentration of noradrenaline and, furthermore, when full recovery of response is obtained there is no dose-response curve showing a maximum response with a simultaneous shift to the right relative to the control curve.

We have previously demonstrated the absence of "spare-receptors" in the rabbit aorta α-receptor system (May & others, 1967). Since this conclusion was based on a different experimental approach to those described here and because Rossum (1965; Rossum & Mujić, 1965) has suggested that the α-receptors in different tissues may have different properties, it was desirable to determine whether the characteristics of irreversible α-receptor blockade were the same in both the rabbit aorta and the rat vas deferens. From Fig. 4 it is possible to obtain a first-order plot (Fig. 5) for recovery of tissue response with a $t_{\frac{1}{2}}$ of 22 min essentially identical with our previously determined

* The terms, competitive and non-competitive, are used with regard to the site of action of the 2-halogenoethylamines whereas the terms, reversible and irreversible, refer to the chemical mechanism of action (Kimelberg, Moran & Triggle, 1965). Experimentally, it is difficult to distinguish competitive irreversible inhibition from non-competitive reversible or irreversible inhibition. In the present situation, thiosulphate will distinguish between reversible and irreversible inhibition produced by SY.28 but *a priori* it cannot be said that the decrease in maximum response observed after treatment with SY. 28 and sodium thiosulphate is due to competitive or non-competitive irreversible blockade. In agreement with Waud (1962), we have shown that agonists can protect non-specific sites as well as the receptor sites (Moran & others, 1967; May & others, 1967).

value of 23 $\pm$ 6 min for the rabbit aortic strip system. Furthermore, in experiments using [^{3}H]*NN*-dimethyl-2-bromo-2-phenylethylamine it was found that the $t_{\frac{1}{2}}$ for the process of tritium loss from the recovering tissue was 24 min, in good agreement with the above figures and with our previously determined value (20·7 + 4·1 min) for the rabbit aortic strip system (May & others, 1967). The conclusion is thus permissible that the α-receptors in the rat vas deferens and rabbit aorta exhibit an identical behaviour towards *NN*-dimethyl-2-bromo-2-phenylethylamine.

The ability of an ethyleniminium ion to produce reversible, rather than irreversible, blockade probably depends upon a combination of factors determined by the reactivity and lipophilic character of the ion. Clearly a low reactivity towards nucleophiles will result in a slow rate of reaction with the receptor grouping providing a long-lasting reversible phase of action. This represents the behaviour exhibited by the *N*-ethyl-*N*-1-naphthylmethylethyleniminium ion (from SY.28) which is of lower reactivity and greater lipophilic character* than the *NN*-dimethylphenylethyleniminium ion with which it has not proved possible to obtain dose-response curves indicating any significant reversible phase of action (Fig. 3). Furthermore, it might be expected that the binding of antagonists having a high lipophilic character, such as SY.28, to non-receptor lipophilic sites could produce the non-competitive blockade observed in the present study (Fig. 2).

Previous studies (Moran & others, 1967) have demonstrated that it is not possible to effectively wash tissues free of the alcohol corresponding to SY.28. Thus, despite washout times of 10–20 min, sufficient ethyleniminium ion corresponding to SY.28 is probably left in the tissues to cause both reversible competitive and non-competitive blockade.

The data reported in this paper provide clear evidence that "spare-receptor" do not exist in the rat vas deferens α-receptor system thus substantiating the conclusion drawn earlier from unrelated studies on the rabbit aortic strip α-receptor system (May & others, 1967). This is in agreement with the data of Lewis & Miller (1966) on the rat seminal vesicle preparation. It is noteworthy that early experiments of other workers (Chen & Russell, 1950; Graham & Lewis, 1953), demonstrated that, with low doses of 2-halogenoethylamines, the inhibition was competitive (reversible), whereas with larger doses, the inhibition became non-competitive (irreversible or non-competitive reversible).† It seems probable that, at least for the adrenergic α-receptor system, "spare-receptors" are of little or no significance. The work reported in the present paper also demonstrates that the use of irreversibly acting antagonists in quantitative pharmacology may yield results which are subject to misinterpretation unless the mechanisms of action of the antagonist are carefully examined. It is appropriate to note that SY.28 and several related 2-halogenoethylamines are also irreversible antagonists at the cholinergic receptor and similar shifts are noted which have been interpreted as indicative of a receptor reserve. However, relatively high concentrations and prolonged incubation times are necessary to produce irreversible blockade. Apparently, the reactivities of the derived ethyleniminium ions towards the

* See footnote to p. 44.

† Since it is not possible to obtain partition data for the ethyleniminium ions we have taken the partition ratios between $CHCl_3$ and H_2O of *N*-ethyl-*N*-(2-hydroxyethyl)-1-naphthylmethylamine and *NN*-dimethyl-2-hydroxy-2-phenylethylamine as giving a measure of the relative lipophilicities of the corresponding ethyleniminium ions. Evidence was presented in a previous paper in this series for the pronounced ability of *N*-ethyl-*N*-(2-hydroxyethyl)-1-naphthylmethylamine to bind to tissue fractions.

alkylatable site at the cholinergic receptor are relatively low and consequently a pronounced phase of reversible antagonism might be expected. A similar phenomenon at the active site of acetylcholinesterase is discussed by Belleau & Tani (1966). We have, therefore, re-examined the mechanisms of action of such compounds in cholinergic systems. The complex results of this investigation will be published in a separate paper.

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LETTERS TO THE EDITOR

An improved cannula suitable for chronic implantation into a lateral cerebral ventricle of the rat

A cannula suitable for chronic implantation into a lateral cerebral ventricle of the rat should be simple and inexpensive. It should also be easy to accurately manipulate into the ventricle, rigidly attached to the skull and subsequently facilitate the passage of an injection needle. Several cannulae have been described for this purpose (Wagner & de Groot, 1963; Decima & George, 1964; Feldberg & Lotti, 1967; Khavari, Feider & others, 1967; Myers, Casaday & Holman, 1967), but only that of Hayden, Johnson & Maickel (1966) appeared to meet all these criteria. Experience with this cannula, however, revealed disadvantages in its design and several modifications have now been evolved which greatly improve its implantation, patency and efficiency during the injection of drugs. The modified cannula is illustrated in Fig. 1.

The trochar hole in the original cannula was superfluous, as implantation into a lateral cerebral ventricle is readily carried out visually using fixed co-ordinates (1·4 mm caudal to the bregma and 1·3 mm lateral to the mid-line). Moreover, as the Perspex base was bulky and of an awkward shape for the implantation procedure, a ledge was cut into its two sides, and the central turret so formed was rounded off. A notch was cut into the posterior wing of the cannula to accommodate a bone screw in the region of thick bone just rostral to lamda. This screw together with two other bone screws, sited just lateral and rostral to the cannula respectively, were used as anchoring points for Surgical Simplex C which firmly secured the cannula to the skull. The modified cannula has the advantage that the wings are easily covered with Surgical Simplex C and the skin can be neatly sutured around the turret. A dummy needle was also incorporated into the assembly as the guide needle of the original cannula tended to become blocked by tissue particles and debris.

The modified cannula may be adapted for injecting small quantities of drugs into other discrete areas of the brain by varying the length of the guide needle.

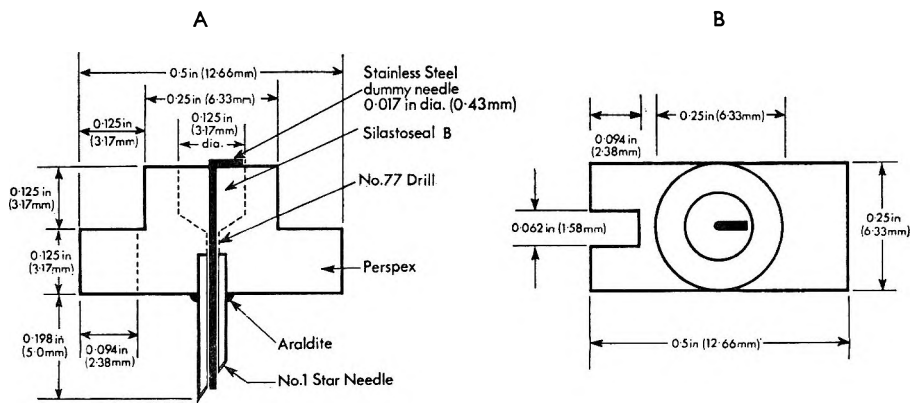

FIG. 1. Side (A) and top (B) view of a modified cannula suitable for implantation into a lateral cerebral ventricle of the rat.

Thanks are due to S. W. Smith for technical assistance and to R. B. Murdoch who acted as a personal assistant while on a sandwich course from Bath University of Technology.

Department of Pharmacology,
Allen & Hanburys Ltd.,
Ware,
Herts, England.

S. I. ANKIER

October 24, 1968

Silastoseal B is obtainable from Midland Silicones Ltd., Barry, Glamorgan.

Bone screws—nickel silver cheese head 16 BA × 1/8 inch obtainable from Laubscher Brothers, London, E.C.1.

Surgical Simplex C is an autopolymerizing acrylic resin obtainable from North Hill Plastics Ltd., London, N.16.

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Effect of chelating agents on copper content and tyramine response of the rat heart

Of the three enzymes involved in the conversion of tyrosine to noradrenaline, two have been shown to be metalloproteins. Tyrosine hydroxylase, which converts tyrosine to dopa in what is generally considered to be the rate-limiting step, contains iron (Udenfriend, 1966) and dopamine-β-hydroxylase, which converts dopamine to noradrenaline, contains copper (Friedman & Kaufman, 1965). Pharmacological inhibition of either of these two enzymes or of dopa decarboxylase, which converts dopa to dopamine, has been shown to lower catecholamine levels in guinea-pig heart to varying degrees (Spector, 1966). Among the substances which have been shown to inhibit dopamine-β-hydroxylase *in vitro* are various chelating agents (Goldstein, Lauber & McKereghan, 1964). *In vivo*, the acute administration of chelating agents has led to decreased levels of noradrenaline in the rat heart (Collins, 1965; Carlsson, Linqvist & others, 1966).

While studying the effect of chronic administration of three chelating agents on the copper levels of various tissues in rats, the response to tyramine of atria isolated from these rats was determined. An apparent relation between the copper levels of the heart tissue and the chronotropic response of the atria to tyramine was observed. The three chelating agents were: γ-thujaplicin (5-isopropyltropolone) (Bryant & Fernelius 1954), plicatic acid (Bock, L. H., personal communication; Gardner, Swan & others, 1966) and penicillamine (Walshe, 1960). The dosages of drugs in Table 1 are shown as equimolar quantities calculated as the sodium salt of γ-thujaplicin (GT), the potassium salt of plicatic acid (P) and free penicillamine (PA).

Table 1. *Copper content of whole heart and chronotropic response to tyramine of isolated atria after prolonged administration of three chelating agents to rats on normal and copper-supplemented drinking water*

Treatment	N	Heart copper μg/g dry weight ± s.e.	P*	Isolated atria N	Basal rate beats/min ± s.e.	P*	Response to tyramine beats/min ± s.e.	P*	Increase in rate as % of control response
				Normal diet					
Untreated control	4	24·89±0·98		12	212±5		379±10		100
GT 2 mg/day	4	21·00±0·61	=0·02	5	208±5	<0·6	304±13	<0·001	58
P 5 mg/day	4	21·79±1·24	<0·2	3	216±11	<0·8	337±15	<0·05	73
PA 1·6 mg/day	4	20·18±1·84	<0·1	7	217±6	<0·6	334±14	<0·02	70
				Excess dietary copper					
GT 2 mg/day	4	25·82±0·93	<0·6	5	197±5	=0·05	338±7	<0·005	84
P 5 mg/day	4	23·09±1·26	<0·4	3	204±8	<0·8	353±20	<0·5	89
PA 1·6 mg/day	4	22·01±0·78	<0·1	4	251±7	<0·001	387±10	<0·6	81

* Statistical significance of the difference between treated and control groups.

GT = γ-thujaplicin
P = potassium salt of plicatic acid
PA = free penicillamine

Rats (Wistar strain; male, 130 g average weight) were divided into a control group and one group for each chelating agent. A normal diet of Purina Lab Chow and tap water was supplied *ad libitum* to the control group and to half of each drug-treated group. The remaining half of each drug-treated group received the same food but had, in place of tap water, distilled water containing sufficient $CuCl_2$, $2H_2O$ to give a final copper concentration of 100 μg/ml. A preliminary estimate had indicated this would result in an approximate seven-fold increase in daily copper intake. Drugs were given daily by the intraperitoneal route on six days each week for a total of 12–13 weeks. The dose of each drug was stoichiometrically in excess of the amount of copper estimated to be absorbed.

At the end of the treatment period, hearts were removed from some rats of each group for analysis of copper. The hearts were rinsed in distilled water to remove excess blood, blotted dry and trimmed of all vessels and fat, leaving only atria and ventricles. Two hearts were pooled into one sample and after dry ashing in a muffle furnace and acid extraction of the ash, the copper content was determined colorimetrically by the method of Eden & Green (1940).

Isolated atrial preparations were obtained from the remaining rats. The atria were suspended in Krebs solution containing double glucose and gassed with a mixture of 5% CO_2 in oxygen. Activity was recorded on a Grass Model 5D Polygraph using an FT 0·03 force displacement transducer. After allowing 2 h for equilibration, a control contraction rate was determined followed by measurement of the chronotropic response to a single exposure to 10^{-6} g/ml of tyramine, an amount in the middle range of the dose-response curve. It is widely accepted that tyramine acts through liberation of endogenous catecholamines (Burn & Rand, 1958; Davey & Farmer, 1963; Muscholl, 1966). The response of isolated rat atria is reduced or abolished upon depletion of noradrenaline (Kuschinsky, Lindmar & others, 1960). Lee, Yoo & Kang (1964) found that there was a significant correlation between the catecholamine content and rate of contraction of isolated rabbit atria. In the present work therefore, a reduction in the chronotropic response to tyramine was assumed to indicate a reduction in noradrenaline content of the myocardium.

The results (Table 1) show that in rats on a normal diet all three chelating agents lowered the copper content of heart tissue, though only significantly so in γ-thujaplicin-treated rats. At the same time responses to tyramine of atria taken from rats treated similarly with the chelating agents were reduced. Table 1 shows further that addition of copper to the diet tended to offset both of these effects of the chelating agents. Extra dietary copper was most effective in offsetting the effects of γ-thujaplicin and least effective against the effects of penicillamine. This probably reflects the much greater effectiveness of penicillamine in promoting copper excretion. In our laboratory we have found that penicillamine is much more effective than plicatate which in turn is only slightly more effective than γ-thujaplicin in promoting urinary excretion of copper. The reason for the high basal rates of atria from rats which had received both copper and penicillamine is not known.

In general, the changes in copper content of the heart paralleled the changes in responses to tyramine. This relation was clearest in rats treated with γ-thujaplicin, a powerful copper chelator belonging to a class of compounds, the tropolones, shown to be potent inhibitors of dopamine hydroxylase *in vitro* (Goldstein, Lauber & McKereghan, 1964). These data are consistent with the hypothesis that chelating agents lower tissue catecholamines due to chelation of heavy metals necessary for the activity of enzymes involved in catecholamine biosynthesis.

This work was supported by the grant from Medical Research Council of Canada and a Warner-Lambert Research Fellowship. We thank Dr. L. H. Bock of Rayonier Canada (B.C.) Ltd. for samples of potassium plicatate and γ-thujaplicin and Merck Sharp and Dohme of Canada Ltd. for penicillamine.

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Bradykinin antagonism by dimethothiazine

Dimethothiazine [10-(2-dimethylaminopropyl)-2-dimethylsulphamoylphenothiazine] is now in clinical use as an antihistamine-anti-5-hydroxytryptamine agent. Joulou, Ducrot & others (1966) reported that it had fifteen times the activity of aspirin against pain induced by intraperitoneal injection of bradykinin in mice but there appear to be no other studies of this effect. We have compared the bradykinin antagonism of dimethothiazine with that of cyproheptadine (Garcia Lemme & Rocha e Silva, 1965) in several preparations.

The central vein of the rabbit ear perfused by the method of de la Lande & Rand (1965) is exceptionally sensitive to kinins but responds to few other substances (Mashford & Horowitz, 1968). On this preparation both dimethothiazine and cyproheptadine showed moderate antagonism to the effects of bradykinin (Fig. 1). The concentration of dimethothiazine necessary to produce a definite effect was usually 10 μg/ml (Fig. 1a). The non-competitive nature of this antagonism became evident at higher concentrations where there was a distinct reduction of the slope of the dose-response plot. A similar situation pertained with cyproheptadine (Fig. 1b). Since the rabbit ear vein responds to few agonists other than kinins, the specificity of the antagonism could not be tested, but bradykinin antagonism was also demonstrated

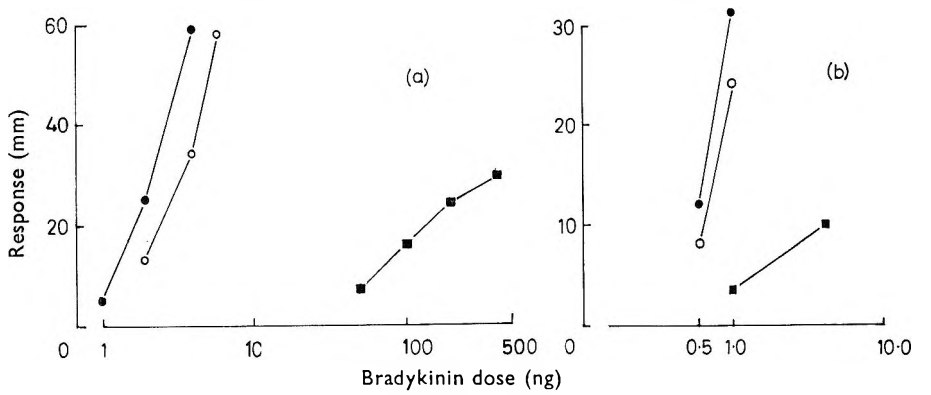

FIG. 1. Dose-response curves of rabbit isolated ear vein to bradykinin. a. Effect of dimethothiazine. b. Effect of cyproheptadine. ●——● control. ○——○ 10 μg/ml of drug. ■——■ 50 μg/ml of drug in a and 100 μg/ml of drug in b.

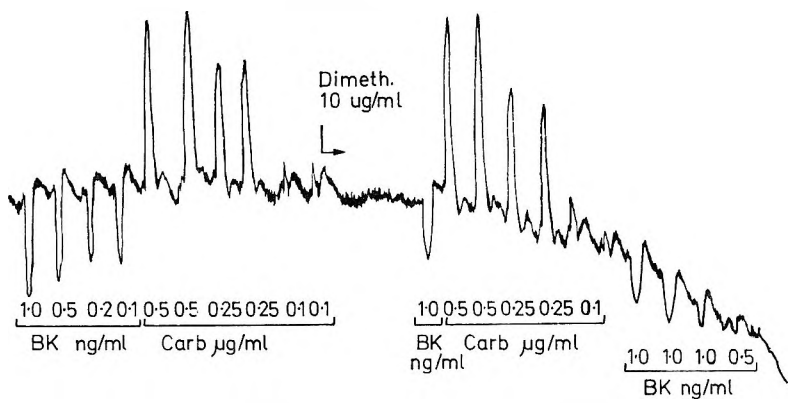

FIG. 2. Responses of superfused duodenum to bradykinin (BK) and carbachol (Carb). Addition of 1·0 μg/ml dimethothiazine to the superfusion fluid caused a relaxation and impaired subsequent response to bradykinin. Carbachol responses were unaltered.

TABLE 1. *Antagonism of various agonists by dimethothiazine and cyproheptadine in some* in vitro *preparations*

Preparation	Agonist	Degree of antagonism: Dimethothiazine μg/ml			Cyproheptadine μg/ml		
		1	10	100	1	10	100
Rabbit ear vein	Bradykinin	0	+	++++	±	+	++
Rat uterus	Bradykinin	0	++	+++	0	++	+++
	Angiotensin	0	0	0	0	0	++
Rat colon	Bradykinin	+++	++++	—	+	+	—
	Angiotensin	0	0	±	0	0	—
Rat vas deferens	Bradykinin	+		++ (50 μg/ml)	— —	—	—
	Acetylcholine	0		0	—	—	—
Rat duodenum	Bradykinin	+++	++++	—	0	+	+
	Carbachol	0	0	—	—	—	—
	Noradrenaline	0	—	—	0	0	0

— = effect not tested.
0 = no antagonism.
+ = definite slight antagonism.
++ = approximate halving of response.
+++ = marked antagonism.
++++ = response abolished.

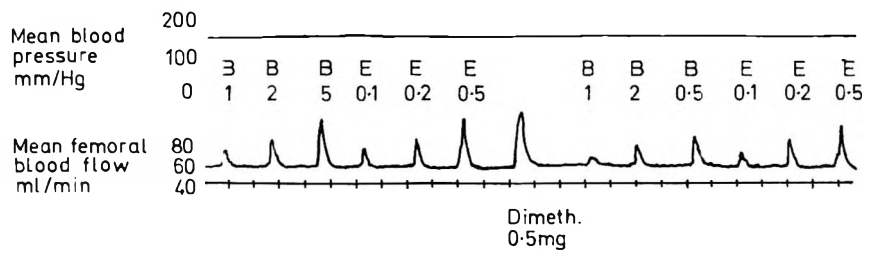

FIG. 3. Mean aortic blood pressure and mean femoral blood flow measured with an electromagnetic flowmeter in an anaesthetized dog. Injections of bradykinin (B) and eledoisin (E) into the femoral artery caused marked increase in femoral flow. Dimethothiazine 0·5 mg also increased flow but subsequent responses to bradykinin were much reduced; eledoisin responses were less effected.

with several other preparations. These were the rat uterus, duodenum, vas deferens and colon. Both the contractile effects as seen in the uterus, colon and vas deferens and the relaxation caused by bradykinin in the duodenum were antagonized by both agents. Although they are also potent antagonists to histamine and 5-HT there is some specificity since the responses of the various preparations to other agonists, such as angiotensin, carbachol, and acetylcholine, were not diminished by the presence of either compound in concentrations causing marked blockade of bradykinin responses (Table 1). Dimethothiazine appears to be a partial kinin-like agonist in the rat colon and duodenum as it caused contraction and relaxation (Fig. 2) respectively on its first application. Dimethothiazine also had a similar pattern relative to bradykinin in the dog femoral vascular bed, its injection causing a transient dilatation followed by a depression of responses to bradykinin (Fig. 3).

Dimethothiazine thus appears to act as a partial kinin-like agonist in a number of preparations and to produce a degree of non-competitive blockade in all tissues examined. At doses up to 10 μg/ml, the bradykinin antagonism was not due to non-specific depression of the preparations since response to angiotensin of uterus and colon, to carbachol of the duodenum, and to acetylcholine in the vas deferens were

unimpaired. Increasing the dose to 100 μg/ml often depressed responses to all agonists. The bradykinin-induced vasodilation in the dog femoral vascular bed and the relaxation of the rat duodenum were affected by dimethothiazine to the same extent as the contraction responses of the other varieties of smooth muscle studied. If the bradykinin blockade can be interpreted as some sort of interaction with kinin receptors, these observations provide no evidence of the sort of heterogeneity revealed by antagonists in the case of catecholamines.

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5-Hydroxyindole compounds in the perfusates from frog head

A series of investigations on the significance and role of 5-hydroxytryptamine (5-HT) have been made *in vitro*, in which the content of 5-HT and its metabolites in the brain of dead animals was measured. We have now made experiments with the perfused frog head, with the object of detecting whether there are in the perfusate any substances with properties of 5-hydroxyindoles (5-OH indoles) and also whether their content changes after administration of substances which interfere with biosynthesis, liberation or metabolism of 5-HT.

Male frogs (*Rana esculenta*, L.) were perfused as described by Trendelenburg (1938) with slight modifications: the perfusion fluid was administered through the truncus arteriosus and the collection of the perfusion fluid was via a polyethylene tube inserted in the sinus venosus. After a 1 h washing period, the specimens of perfusate were collected during the next 2 h. In these samples 5-OH indoles were estimated fluorimetrically (Ashcroft & Sharman, 1962). The following substances were used: 5-hydroxytryptophan (DL-5-hydroxytryptophan, Aldrich Chemical Co.), reserpine (Serpasil, Ciba), α-methyldopa (Aldomet, Merck), chlorpromazine (Largactil, Specia) and iproniazid (Marsilid, Roche). The doses of chlorpromazine and iproniazid are expressed as the free base, and the substances were administered subcutaneously.

In the perfusates of untreated animals we found substances with fluorescent properties of 5-OH indoles, the content of which was altered by administration of the above mentioned drugs. The results are summarized in Table 1. 5-Hydroxytryptophan induced a remarkable increase of the concentration of the total 5-OH indoles. After reserpine there was also a significant rise of 5-OH indole compounds in the perfusion fluid. α-Methyldopa lowered the content of total 5-OH indoles. Perfusion fluid from frogs pretreated with chlorpromazine showed an increased amount of 5-OH indoles. Samples from frogs treated with iproniazid showed no measurable fluorescence.

Our results with experiments *in vivo* obtained after administration of 5-hydroxytryptophan, reserpine and α-methyldopa are in good agreement with the results of related experiments *in vitro* (Udenfriend, Weissbach & Bogdanski, 1957; Pletscher,

Table 1. *Concentration of total 5-OH indoles in the perfusates of male frog head* (concentration expressed as μg 5-hydroxyindoleacetic acid/ml perfusate)

Drug	Dose (mg/kg)	Administered before perfusion	5-OH indoles	P (*t*-test)
Control	Isotonic saline	2 h	0·110 ± 0·007* (8)†	
5-Hydroxytryptophan	100	2 h	1·970 ± 0·290 (8)	<0·001
Control	Isotonic saline	2 h	0·071 ± 0·008 (11)	
Reserpine	20	2 h	0·180 ± 0·010 (10)	<0·001
Control	Isotonic saline	1 h	0·071 ± 0·004 (6)	
α-Methyldopa	75	1 h	0·026 ± 0·001 (6)	<0·001
Control	Isotonic saline	1 h 15 min	0·084 ± 0·004 (7)	
Chlorpromazine	25	1 h 15 min	0·106 ± 0·003 (8)	<0·001
Control	Isotonic saline	12 h	0·079 ± 0·005 (6)	
Iproniazid	100	12 h	no measurable fluorescence	—

* Values represent the means ± standard error of the mean.

† The figures in parentheses are numbers of experiments.

Shore & Brodie, 1955; Giarman & Schanberg, 1961). Opinions differ about the action of chlorpromazine on brain 5-HT (Bartlet, 1960; Pletscher & Gey, 1960). Our findings may be related to the action of chlorpromazine in decreasing the permeability of the storage site for 5-HT and this may be the reason why we obtained an increase of 5-OH indoles in the perfusates. Our results with iproniazid are unusual in that we failed to find any detectable fluorescence in the perfusates. On the other hand Gertner, Paasonen & Giarman (1957) observed an increased amount of 5-HT in the perfusion fluid of the isolated cervical ganglion.

Two questions arise. Firstly, which of the 5-OH indoles are present in perfusates, and secondly, is the brain the only source of these compounds.

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The transport and life-span of amine storage granules in bulbospinal noradrenaline neurons of the rat

In previous studies on peripheral sympathetic adrenergic nerves, accumulations of noradrenaline in distorted axons were seen proximal to a ligation with the use of fluorescence histochemistry (Dahlström & Fuxe, 1964a; Dahlström, 1965; Kapeller & Mayor, 1967). Convincing evidence appears to exist supporting the view that these accumulations are signs of a rapid and steady proximo-distal transport of amine storage granules down the axons (Dahlström & Häggendal, 1966 a; 1967). The rate of this transport was found to vary in different mammals, ranging between 2–10 mm/h. The storage granules are manufactured in the nerve cell bodies and transported via the axons to the nerve terminals, where they function as factories and stores for the transmitter. Their life-span in the varicosities of the nerve terminals has been calculated as about 5 weeks (rat) to 10 weeks (cat) (Dahlström & Häggendal, 1966a).

In the central nervous system (CNS) widespread systems of noradrenaline-, dopamine- and 5-hydroxytryptamine (5-HT)-containing neurons have been described (Dahlström & Fuxe, 1964b). The spinal cord has been shown to contain noradrenaline and 5-HT-containing nerve terminals and axons, the cell bodies of the neurons being situated in the medulla oblongata (Dahlström & Fuxe, 1965). After a transection of the spinal cord, the monoamines below the section disappeared within a few days (Andén, Häggendal & others, 1964). Above the transection, increases in noradrenaline content were observed biochemically (Häggendal & Magnusson, unpublished observation) and, in the same year, fluorescence histochemical studies revealed accumulations of noradrenaline and 5-HT within bulgy, enlarged axons proximal to the cut (Dahlström & Fuxe, 1964c) The same phenomenon also occurred in other monoamine neurons in the CNS after lesions (see *e.g.* Andén, Dahlström & others, 1966).

Thus, in the CNS also, a proximo-distal transport of granules in all probability occurs. We have now attempted to estimate the rate of this transport of noradrenaline storage granules in the spinal cord, and to calculate their life-span.

In normal rats most noradrenaline in the spinal cord is located in the nerve terminals and only a small amount is found in the axons (Dahlström & Fuxe, 1965). Accumulations of noradrenaline above a section would therefore probably be difficult to demonstrate, since the net increase would probably be comparatively small. From several studies it is known that two days after the administration of one large dose of reserpine (giving a long-lasting or even irreversible block of granular storage mechanisms, Dahlström & Häggendal, 1966b) the nerve terminals are still depleted of noradrenaline, while the cell bodies and axons have already recovered (Dahlström, Fuxe & Hillarp, 1965, Dahlström & Fuxe, 1965). In the peripheral nervous system it was observed that the amounts of noradrenaline accumulated above a 6 hr ligation of the sciatic nerve had returned to about normal levels on the second to third day after the reserpine treatment. Therefore, all rats used in the present experiments were given reserpine (10 mg/kg i.p.) 4 days before death.

The spinal cord of one group of rats was sectioned at the level of Th 6–7 under ether anaesthesia 12 or 24 h before death. After death one cm of the spinal cord just above the transection was dissected and assayed (2 or 4 together) for noradrenaline content (Häggendal, 1963). Another group of animals in which the spinal cord was not sectioned, had one cm parts of the thoracic cord dissected, pooled (5–10 together), and analysed for noradrenaline.

The noradrenaline content in the one cm part rose steadily from $3{\cdot}5 \pm 0{\cdot}55$ ng in unoperated rats, to $4{\cdot}4 \pm 0{\cdot}15$ ng 12 h after section and to $6{\cdot}0 \pm 0{\cdot}30$ ng in rats

transected 24 h earlier (mean values ± standard errors, n = 4). The gradual increase of noradrenaline (Fig. 1) followed a curve similar to the one observed for sciatic nerve (Dahlström & Häggendal, 1966a). However, the inclination of the curve was less for the spinal cord than for the sciatic nerve, indicating a slower rate of transport of the amine granules. For the spinal cord this rate of transport was calculated to be 0·7 mm/h. Thus, it seems that the transport of noradrenaline granules in the central noradrenaline neurons is much slower than in the peripheral sympathetic neuron (5 mm/h in the rat).

As seen from the data, about 2·5 ng of noradrenaline, probably stored within granules, was transported down the spinal cord each 24 h. To get information on the approximate number of nerve terminals these transected axons would have to supply with granules, the spinal cord below the level of Th 6 was assayed in normal, untreated rats. The noradrenaline content in this part of the spinal cord was found to be 78 ± 6·8 ng (mean ± s.e. n = 7). According to the calculations made in experiments with the sympathetic neuron system of the sciatic nerve (see Dahlström & Häggendal, 1966a) the life-span of amine storage granules in the spinal cord was found to be about 31 days (compare 35 days in the peripheral nervous system).

Reserpine, used as a tool in this study, may, however, to some extent influence the transport of the granules. During the accumulation period, that is, the fourth day after resperpine administration, there may be a slightly increased rate of accumulation, as seems to be the case above a constriction of peripheral adrenergic nerves (Dahlström & Häggendal, to be published). The increase appears, however, to be about 140% of normal.

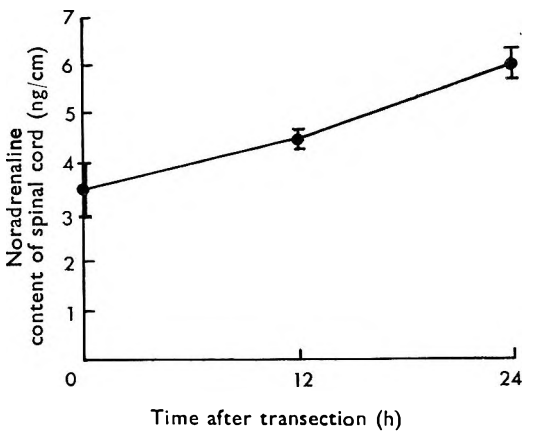

FIG. 1. The accumulation of noradrenaline in the 1 cm part of thoracic spinal cord just above a transection at the level of Th 6-7. All the animals were killed on the fourth day after a single dose of reserpine (10 mg/kg, i.p.). The values are given in means ±, indicated by vertical bars. Four estimations were made in all cases.

It was earlier found that after one large dose of reserpine, the recovery of noradrenaline to 100% of normal levels was completed after about 5 weeks in the rat and after about 7 weeks in the rabbit, both in peripheral tissues and in the CNS (Dahlström & Häggendal, 1966b). Since the recovery followed an approximately straight line with time, and since the time needed for full recovery coincided with the calculated life-span of granules in the peripheral adrenergic neurons in both species, it was suggested that a large dose of reserpine blocked the storage mechanism of the granules irreversibly and that the down transport of newly synthesized granules (from the cell body to the terminals) was essential for recovery of noradrenaline levels in the

terminals. Since the recovery time after reserpine was the same in the CNS as in the peripheral adrenergic neurons, it was suggested that in the CNS also the granular life-span was about 5 weeks. Thus, the results obtained in the present experiments are in agreement with earlier findings.

It may be mentioned that preliminary experiments with the accumulation of 5-HT in the spinal cord have revealed a higher rate of transport of presumably 5-HT-storing granules than of noradrenaline granules in reserpinized rats.

The present experiments, together with previous ones indicate that the life-span of the amine storage granules together with the size of the nerve terminal net of the neurons constitute important factors in regulating the rate of formation and the rate of proximo-distal transport of amine storage granules in both central and peripheral adrenergic neurons.

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Effect of gastrin I and caerulein on gastric acid secretion in rats

Anastasi, Erspamer & Endean (1967) isolated a polypeptide from the skin of an Australian amphibian *Hyla caerulea*, which they called "caerulein". Caerulein [Pyr.Gln.Asp.Tyr(SO_3H).Thr.Gly.Trp.Met.Asp.Phe–NH_2] is a dekapeptide amide (*M* 1352), whose C-terminal tetrapeptide amide is the same as that of gastrin.

Caerulein has a powerful stimulating effect on several exocrine organs of the gastrointestinal tract and has been reported to be more active on gastric acid secretion in rats and dogs than gastrin (Erspamer, Bertaccini & others, 1967). According to Mantegazza, Naimzada & Riva (1968), with caerulein "a clear dose-response relationship was not obtained due to the individual variation in sensitivity to the peptide".

In the perfused rat stomach preparation (Lai, 1964) we established a dose-response relation for synthetic human gastrin I and caerulein. The infusion time was 15 min for each dose (Fig. 1). The threshold doses were $<2 \times 10^{-12}$ mole/kg min^{-1} for caerulein and $<1 \times 10^{-10}$ mole/kg min^{-1} for gastrin I. From the dose-response curves it can be calculated that caerulein is on a molar basis about 45 times, and on a weight basis about 70 times, more active than gastrin I.

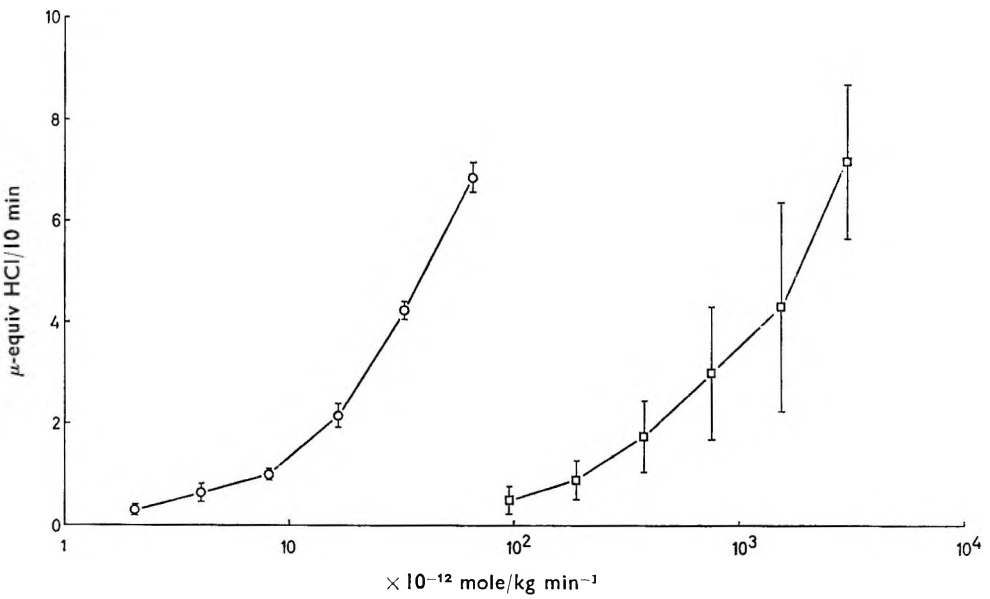

FIG. 1. Dose-response curves for synthetic human gastrin I □——□ and caerulein ○——○ in rats. Each point on the curves represents the mean value of 6 experiments in different animals. Vertical bars: s.e.

Morley, Tracy & Gregory (1965) assayed many synthetic peptides resembling gastrin I. They were unable to detect one which was more active than gastrin I. Stening & Grossman (1968) could not estimate relative potencies for gastrin I and caerulein in gastric fistula dogs since the dose-response curves were not parallel over a dose range of 1–4 μg/kg for gastrin and of 0·03–0·75 μg/kg for caerulein. 0·75 μg/kg caerulein was less active than 1 μg/kg gastrin I.

Our results demonstrate a clear dose-response relation for both gastrin I and caerulein for gastric acid secretion in rats. From the data given by Stening &

Grossman (1968) it can be calculated that in dogs caerulein is less active than gastrin I or II on a molar basis.

Gastrin I was purchased from the American Gastroenterological Association, caerulein was kindly donated by Professor Bertaccini (Parma, Italy) and supplied as a methanolic extract from the skin of *Hyla caerulea* containing 55 μg/ml caerulein.

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On the mechanism of lipomobilizing effect of chlordiazepoxide

Previously, Arrigoni Martelli & Tóth (1968) have shown that chlordiazepoxide provokes hyperglycaemia possibly through an activation of adrenergic mechanisms. The potentiation of hyperglycaemia observed in rats treated with chlordiazepoxide and theophylline or with chlordiazepoxide and cyclic 3′,5′-AMP led us to suppose that chlordiazepoxide, like theophylline (Butcher & Sutherland, 1962), interferes in some of the biochemical steps connecting the release of noradrenaline with phosphorylase activation. Since theophylline, through a blockade of phosphodiesterase, enhances lipolysis (Hynie, Krishna & Brodie, 1966), we decided to examine the effects of chlordiazepoxide on free fatty acid mobilization.

Female albino rats, Wistar strain, weighing about 250 g were used. Plasma free fatty acids (FFA) were determined according to Dole (1966). The experimental design and the results obtained are reported in Table 1. Chlordiazepoxide (40 mg/kg, i.p.) produced a sustained elevation of the plasma FFA levels; a similar effect was elicited by theophylline (20 mg/kg, i.p.).

The lipomobilizing effect of noradrenaline was potentiated by pretreatment of rats with chlordiazepoxide or with theophylline. Cyclic 3′,5′-AMP (10 mg/kg, i.p.) had no effect on plasma FFA levels. When the same dose was given to rats pretreated with chlordiazepoxide the FFA levels rose about 3-fold. Likewise, in rats pretreated with theophylline, cyclic 3′, 5′-AMP produced a significant elevation of plasma FFA. The

Table 1. *Effect of chlordiazepoxide and theophylline on plasma* FFA

1st treatment mg/kg	Time interval min	2nd treatment mg/kg	Time interval min	FFA μ-equiv/ml $\pm$ s.e.* Plasma level	FFA μ-equiv/ml $\pm$ s.e.* $\triangle$ due to treatment
None	None	None	None	0·29 ± 0·05	
Chlordiazepoxide, 40, i.p.	,,	,,	60	0·68 ± 0·07	0·39 ± 0·06
,, ,, ,,	,,	,,	90	0·53 ± 0·06	0·24 ± 0·04
,, ,, ,,	,,	,,	120	0·47 ± 0·05	0·18 ± 0·06
Theophylline, 20, i.p.	,,	,,	60	0·69 ± 0·06	0·40 ± 0·05
,, ,, ,,	,,	,,	90	0·57 ± 0·04	0·28 ± 0·09
,, ,, ,,	,,	,,	120	0·50 ± 0·05	0·21 ± 0·06
Noradrenaline, 0·05, s.c.	,,	,,	60	0·76 ± 0·08	0·47 ± 0·06
Chlordiazepoxide, 40, i.p.	30	Noradrenaline, 0·05, s.c.	60	1·23 ± 0·12	0·94 ± 0·11
Theophylline, 20, i.p.	30	Noradrenaline, 0·05, s.c.	60	1·31 ± 0·06	1·02 ± 0·05
Cyclic 3′,5′-AMP 10, i.p.	—	—	60	0·30 ± 0·04	0·01 ± 0·003
Chlordiazepoxide, 40, i.p.	60	Cyclic 3′,5′-AMP, 10, i.p.	60	0·85 ± 0·09	0·56 ± 0·07
Theophylline, 20, i.p.	60	Cyclic 3′,5′-AMP 10, i.p.	60	0·86 ± 0·09	0·57 ± 0·08
Theophylline, 20, i.p.	30	Chlordiazepoxide, 40, i.p.	60	1·03 ± 0·10	0·74 ± 0·09
Chlordiazepoxide, 40, i.p.	30	Theophylline, 20, i.p.	60	0·97 ± 0·08	0·68 ± 0·07

* Mean from 12 rats.

lipomobilizing effect of chlordiazepoxide was almost double in rats pretreated with theophylline, and vice versa.

Noradrenaline raises the cyclic 3′,5′-AMP and hence lipase activity: theophylline, by blocking phosphodiesterase, prevents the inactivation of cyclic 3′,5′-AMP and hence of adipose tissue lipase (Hynie & others, 1966). The surprising similarity of the effects of chlordiazepoxide and theophylline on plasma FFA levels, like that observed on blood glucose levels (Arrigoni Martelli & Tóth, 1968), suggests that these two drugs act through a similar mechanism.

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Noradrenaline turnover in renal hypertensive rats

Various types of experimental hypertension are thought to be associated with alterations in the metabolism of catecholamines. Thus, the levels of endogenous noradrenaline were reduced and the fall in specific activity after injection of [^{3}H]noradrenaline was greater and more prolonged in the hearts of rats made hypertensive with desoxycorticosterone and a high salt diet than in normal rats (Champlain, Krakoff & Axelrod 1966; Krakoff, Champlain & Axelrod 1967a, b). Similar results were obtained when hypertension was induced by kidney capsulation followed by contralateral nephrectomy in rats (Volicer, Scheer & others 1968) or by sinoaortic denervation in rabbits (De-Quattro, Maronde & others 1968).

In the course of studies on the antihypertensive effect of α-methyldopa, I observed normal levels of endogenous monoamines in renal hypertensive rats (Henning 1967). Obviously, this finding does not exclude an alteration in the turnover of these amines. Studies of this kind may conveniently be made by means of inhibitors of monoamine synthesis (Andén, Corrodi & Fuxe 1968). It has been found that the reduction of noradrenaline in various tissues after inhibition of tyrosine hydroxylase by α-methyl-*p*-tyrosine methyl ester (H44/68) is largely dependent on the nerve impulse flow (Corrodi & Malmfors 1966; Andén, Corrodi & others 1966). This method has been used in the present investigation to compare the turnover of noradrenaline in various organs of normal and renal hypertensive rats.

The hypertensive rats used in this study were randomly selected from a population of male Sprague-Dawley animals which had been made hypertensive by partial infarction of one kidney in combination with contralateral nephrectomy (Sokabe & Grollman 1963; Henning 1967). Blood pressure was checked in some of the rats by direct recording in the conscious state from a catheter previously implanted in the thoracic aorta (Henning 1967). Nine out of the 22 hypertensive rats used had a mean arterial

Table 1. *Tissue noradrenaline levels in normotensive and renal hypertensive rats normally and after treatment with α-methyl-*p*-tyrosine methyl ester* (H44/68) *as indicated.* The values are means in μg/g tissue and the small figures indicate number of experiments. P values were calculated with analysis of variance; n.s. = not significant

		Brain	Spinal cord	Heart	Femoral muscle	Salivary gland
A.	Normal rats, no treatment	0·502 (5)	0·315 (5)	1·158 (5)	0·090 (5)	1·651 (5)
B.	Normal rats, 4 h after H44/68, 250 mg/kg, i.p.	0·245 (6)	0·188 (6)	1·041 (6)	0·095 (5)	1·075 (6)
C.	Hypertensive rats, no treatment	0·549 (5)	0·309 (5)	1·123 (5)	0·107 (5)	1·728 (5)
D.	Hypertensive rats, 4 h after H44/68, 250 mg/kg, i.p.	0·316 (6)	0·207 (6)	0·730 (6)	0·074 (6)	1·598 (6)
Variance within groups	A–D:	0·0012	0·0013	0·0212	0·0002	0·0441
P value, %;	A–B:	<0·1	<0·1	n.s.	n.s.	<0·1
	A–C:	n.s.	n.s.	n.s.	n.s.	n.s.
	A–D:	<0·1	<0·1	<0·1	n.s.	n.s.
	B–C:	<0·1	<0·1	n.s.	n.s.	<0·1
	B–D:	<0·5	n.s.	<0·5	<2·5	<0·1
	C–D:	<0·1	<0·1	<0·1	<0·5	n.s.

blood pressure of 160 mm Hg (s.e. = 5·5). Normal and hypertensive rats 180–250 g were injected intraperitoneally with H44/68, 250 mg/kg, dissolved in saline. After 4 h, the animals were killed by exsanguination under light chloroform anaesthesia. Brain, spinal cord, heart, left submaxillary gland and femoral muscle were dissected and analysed for noradrenaline (Bertler, Carlsson & Rosengren 1958). The organs from two animals were pooled. Analysis of variance (Davies 1949), with P values equal to or less than 0·025 were regarded as significant.

The noradrenaline content of the brain and the spinal cord was the same in normal and hypertensive rats and was lowered to about the same extent by treatment with H44/68 (Table 1). Heart and femoral muscle noradrenaline levels were also the same in untreated animals from the two groups. However, in both heart and muscle, H44/68 lowered noradrenaline significantly more in hypertensive rats than in normal animals. Salivary glands were depleted significantly in normal rats but not in hypertensive animals; the difference between the two groups was statistically significant.

If the lowering of noradrenaline after tyrosine hydroxylase inhibition mainly depends on nerve impulse flow (Corrodi & Malmfors 1966; Andén & others 1966; Andén, Corrodi & Fuxe 1968), the present results indicate that renal hypertension in rats is associated with an increased impulse flow in the sympathetic nerves to the heart and femoral muscle, when compared to normal animals. By the same reasoning, a decreased sympathetic activity may exist in the case of the salivary glands. In our experiments, the basal levels of endogenous noradrenaline were the same in normal and hypertensive animals. This is in contrast to the observations by Champlain & others (1966) and Krakoff & others (1967a, b) but in agreement with those of Volicer & others (1968) who used rats with a type of hypertension similar to that induced in the present experiments. The changes in the storage and release of noradrenaline found by the above-mentioned investigators may be interpreted in terms of an increased impulse flow in the sympathetic nerves. On the other hand, the results of the present study do not exclude an alteration in the transmission mechanisms in these nerves, as suggested by these authors. Further, there may be differences between various types of experimental hypertension.

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Enhancement of the convulsant action of thiosemicarbazide in mice

Reserpine-like agents enhance seizure suceptability to leptazol (Chen, Ensor & Bohner, 1954) and reduce the effectiveness of most, if not all, anticonvulsants in mice (Chen & others, 1954; Gray, Rauh & others, 1958, 1963; Mennear & Rudzik, 1966). I have now made experiments on the effects of two catecholamine-depleting agents on the convulsant activity of thiosemicarbazide.

Male albino mice (Harlan Industries), 18–22 g, were housed in groups of 25 before experimentation and then individually after intraperitoneal injection of 100 mg/kg of thiosemicarbazide. Ten mice were used in each experimental group.

α-Methyltyrosine, suspended in corn oil, was administered intraperitoneally in three daily doses of 150 mg/kg. Ninety min after the administration of the third dose the mice received an intraperitoneal injection of thiosemicarbazide. The second catecholamine depletor, Ro4-1284 (2-ethyl-1,2,3,4,6,7-hexahydro-2-hydroxy-3-isobutyl-9,10-dimethoxy-11b*H*-benzoquinolizine), was administered in an intraperitoneal dose of 20 mg/kg simultaneously with the dose of thiosemicarbazide. Three end points were measured; the onset time for clonic seizures; the onset time for tonic seizures and the time of death.

The results summarized in Table 1 show the potentiating effect of Ro4-1284 on the convulsant action of thiosemicarbazide. In control mice the mean latency time for the onset of the initial clonic seizure was 41 ± 4 min and tonic seizures developed

Table 1. *Effect of* Ro4-1284* *and α-methyltyrosine‡ (α-MT) on the convulsant action of thiosemicarbazide†*

	Ro4-1284*			α-Methyltyrosine		
End point	Treatment	Min to end point ± s.e.	P	Treatment	Min to end point ± s.e.	P
Clonic seizure	Control	41 ± 4	<0·05	Control	31 ± 2	n.s.
	Ro4-1284	30 ± 2		α-MT	36 ± 4	
Tonic seizure	Control	46 ± 4	<0·01	Control	36 ± 2	n.s.
	Ro4-1284	30 ± 2		α-MT	36 ± 4	
Death	Control	54 ± 2	<0·001	Control	49 ± 3	<0·05
	Ro4-1284	30 ± 2		α-MT	37 ± 4	

* 20 mg/kg i.p. † 100 mg/kg i.p. ‡ 150 mg/kg/day for 3 days.

after a further latency of approximately 5 min. In these animals the initial tonic seizure was seldom terminal and the mean survival time after the onset of seizure activity was 13 min. In mice pretreated with Ro4-1284 the effects of thiosemicarbazide were produced significantly sooner than after the administration of the convulsant alone. The initial clonic seizure appeared after only 30 $\pm$ 2 min and proceeded immediately into the tonic phase. Similarly, all animals which had received both Ro4-1284 and thiosemicarbazide died during this initial convulsive episode.

Unlike Ro4-1284, α-methyltyrosine did not significantly alter the onset time of the initial clonic seizure induced by thiosemicarbazide (Table 1). A striking similarity between the effects of α-methyltyrosine and Ro4-1284 was however noted. The initial clonic seizure in the α-methyltyrosine-treated animals uniformly proceeded directly into the tonic phase of the seizure pattern. Also, all but one of the α-methyltyrosine-treated animals died during the first seizure. The animal which did not die during the initial seizure survived only 3 min after the onset of convulsive activity. The mean survival time after onset of convulsions in control mice was 18 min.

The results of these experiments clearly demonstrate that both Ro4-1284 and α-methyltyrosine markedly enhance the convulsant activity of thiosemicarbazide. The mechanism of potentiation of thiosemicarbazide and other seizure-inducing treatments by these agents remains obscure. Because both Ro4-1284 and α-methyltyrosine are known to influence the disposition of brain catecholamines, it is tempting to attribute these results to a lowering of brain amines.

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