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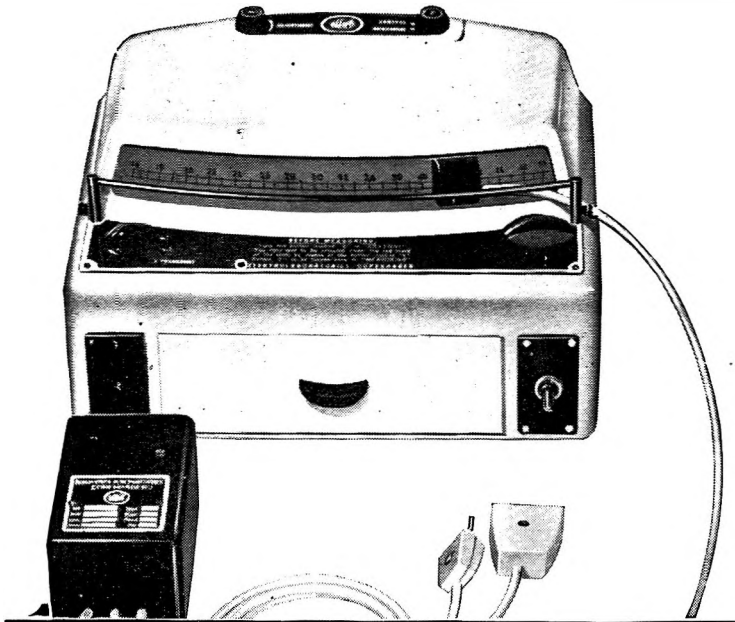

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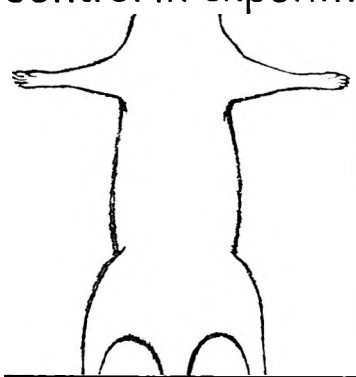

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J. Pharm. Pharmac., 1966, **18**, 697–708 Received May 2, 1966

Accelerated crystal growth of sulphathiazole by temperature cycling

J. E. CARLESS AND A. A. FOSTER

A novel method is suggested for accelerating the physical changes in suspensions. The suspension is stirred in a jacketed vessel and is alternately heated and cooled. Results indicate a rectilinear relationship between the median particle size of sulphathiazole in suspension and the number of temperature cycles. Crystal growth is measured by the Coulter counter and growth rates are compared arithmetically to indicate which additives (surface-active agents) may be used to inhibit crystal growth.

THE crystal growth of particles suspended in fluids may be due to polymorphic change, Ostwald ripening or temperature fluctuations during storage. The last two factors will become more significant when the range of the particle size is wide, since the small particles will tend to dissolve and recrystallise on the large particles. This effect is described by the Ostwald-Freundlich equation (Ostwald, 1900; Freundlich, 1930) and is usually insignificant above 5 μ, because there is little change in solubility with change in size above this level (Jones & Partington, 1915; Fischer & Ferguson, 1950; Rumford & Bain, 1960). The relationship between solubility and particle size was correlated with the charge on the particles by the equations of Lewis (1909) and Knapp (1922). Polderman (1962) reviewed the relationship of surface-active agents, temperature changes, polymorphism and particle size to crystal growth in pharmaceutical suspensions and many references are available in the literature about crystal growth in essentially saturated suspensions and from supersaturated solutions.

Nogami & Nagai (1958) and Hasegawa & Nagai (1958) studied the effect of pH, surface-active agents and time on the crystal growth of aqueous suspensions of sulphadiazine stored at one of four constant elevated temperatures. They concluded that all of the factors interacted, but the most significant was temperature.

In general, the first polymorphic form to crystallise from a supersaturated solution will be the metastable form and this may quickly revert to the more stable form. This has been demonstrated clearly for cholesterol (Higuchi & Saad, 1965; Saad & Higuchi, 1965). Furthermore, milling may introduce polymorphic change (Polderman, Bloo & Fokkens, 1958; Lees, 1963) and the formation of the hydrates and solvates of organic materials may also be important in this context (Shefter & Higuchi, 1963). In some instances the metastable form of a medicament may have a greater pharmacological activity than the stable form and, where this is so, reversion to the less energetic, stable modification must be prevented (Higuchi & Lau, 1962).

Many workers have studied crystal growth from supersaturated solutions and have concluded that impurities, surface-active agents and other

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additives, markedly retarded crystal growth rates (Fysh, 1951; Albon & Dunning, 1957, 1960; Okano, Kojima & Satake, 1957; Okano, Kojima & Akahosi, 1958; Michaels & Colville, 1960; Allen, Milosovich & Mattocks, 1965; Higuchi & Saad, 1965; Saad & Higuchi, 1965). However, our technique of producing crystal growth not only involves crystallisation rates, but also dissolution rates, because the large particles are made to grow at the expense of the small particles. The dissolution rates of several pharmaceutical materials have been studied with the Coulter counter (Edmundson & Lees, 1965; Higuchi & Saad, 1965; Saad & Higuchi, 1965).

It is desirable that an accelerated storage test should simulate, as closely as possible, the conditions of normal shelf storage. When a preparation is exposed to ambient conditions, the range of temperature experienced is variable within fairly closely defined limits, but it is always variable. The accelerated storage test imposed should supply exactly similar temperature fluctuations, but at greatly increased frequencies. However, accelerated tests have previously been designed to effect the acceleration of degradation by using elevated temperatures. The present test design demonstrates the effect of altering the frequency of temperature fluctuations at fairly low temperatures resulting in particle size changes, which are dependent upon (*a*) the slope of the solubility curve, (*b*) the rates of dissolution, crystallisation and diffusion, (*c*) the range and frequency of the temperature cycles.

Sulphathiazole was chosen since it showed a large increase in water solubility with temperature rise, i.e. over the temperature range 23–33° the solubility almost doubled in some vehicles. However, the solubility at constant temperature was insufficient to render particle size determinations difficult when using the Coulter counter or sedimentation techniques.

Materials and apparatus

The crystal growth studies were made on sulphathiazole B.P. micromilled and of mean size 6 μ. Differential thermal calorimetry (Perkin Elmer D.S.C.I.*) indicated that two polymorphic forms were present and, in this respect, the sulphathiazole corresponded to the authentic sample of sulphathiazole supplied by the Department of Pharmaceutical Sciences, Pharmaceutical Society of Great Britain.

In some experiments the sulphathiazole, at a concentration of 0·3% w/v, was suspended in an aqueous vehicle containing 0·1% v/v Nonidet P42 (Shell Chemical Co. Ltd.) as wetting agent. This was used without further purification and contained 27% of a polyethylene oxide condensate. In other experiments, 0·3% w/v concentrations of sulphathiazole suspended in aqueous vehicles containing 0·006, 0·06 or 1·0% w/v of cetomacrogol 1000 B.P.C. were examined.

All concentrations of the wetting agents were in excess of their critical micelle concentrations.

* Perkin-Elmer Ltd., Beaconsfield, Bucks.

TEMPERATURE CYCLING APPARATUS

Suspensions were alternately heated and cooled in a jacketed, stainless-steel reaction vessel. The vessel was lagged and fitted with an inlet pipe to the base of the jacket and an outlet pipe at the top, diametrically opposite the inlet pipe. The reaction vessel had an essentially airtight lid, in the form of a cupola, so designed that condensation occurring on the lid could run back into the reaction suspension.

The suspension was stirred by a stainless-steel paddle-type stirrer at 680 rpm and the temperature of the suspension was recorded by a conventional thermometer passing through the lid.

"Hot" water was allowed to flow from a thermostatic bath, through the vessel jacket and back to the same bath during 8 min, then "cold" water from a second thermostatic bath was allowed to flow for 8 min. The 16 min cycle was repeated for the desired number of temperature fluctuations. This time cycle was selected to effect an accelerated temperature fluctuation simulating daily variations in temperature. A shorter cycle was less reproducible because the bath temperatures were necessarily more divergent.

The results quoted in this paper were obtained by the manual operation of the temperature cycling apparatus during continuous periods of up to 33 hr. This was avoided in later work by automating the process using more suitable water circuitry, and a "cyclothermostat" (Carless, Foster & Jolliffe, 1966). A comparison of the temperature hysteresis of manual and automatic cycling techniques is made in that paper.

COULTER COUNTER

A Coulter counter, model "A",* with a 100 μ orifice was used for the particle sizing. The electrolyte solution used for counting was prepared by saturating with sulphathiazole a solution containing 0·9% w/v sodium chloride and the preselected concentration of surface-active agent, shaking for three days at 23° and filtering through Whatman No. 1 filter paper and then through a No. 4 sintered glass filter. Corrections for background counts and coincidence were made in the usual way.

The sample of suspension at 23° was always removed from the reaction vessel $15\frac{3}{4}$ min after the beginning of a temperature cycle (see Carless & others, 1966, for details). A suitable aliquot was added to 150 ml of saturated electrolyte solution in the Coulter beaker and was stirred for 10 min before counting was commenced. Total counting time was approximately 9 min.

Methods and results

CRYSTAL GROWTH

Fig. 1 describes the change in particle size and particle size range when a suspension of sulphathiazole was submitted to temperature cycling

* Coulter Electronics Ltd., Dunstable, Beds., England.

in a vehicle containing 0·1% v/v Nonidet P42 in a saturated solution of sulphathiazole.

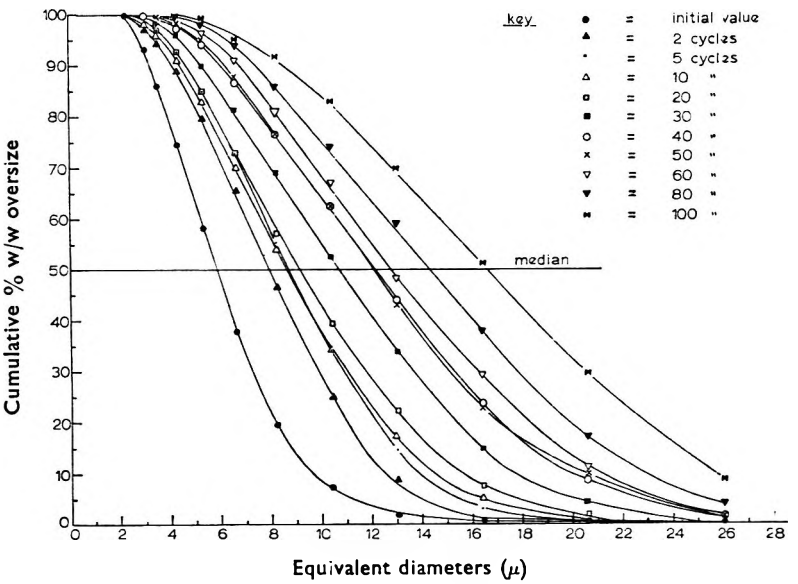
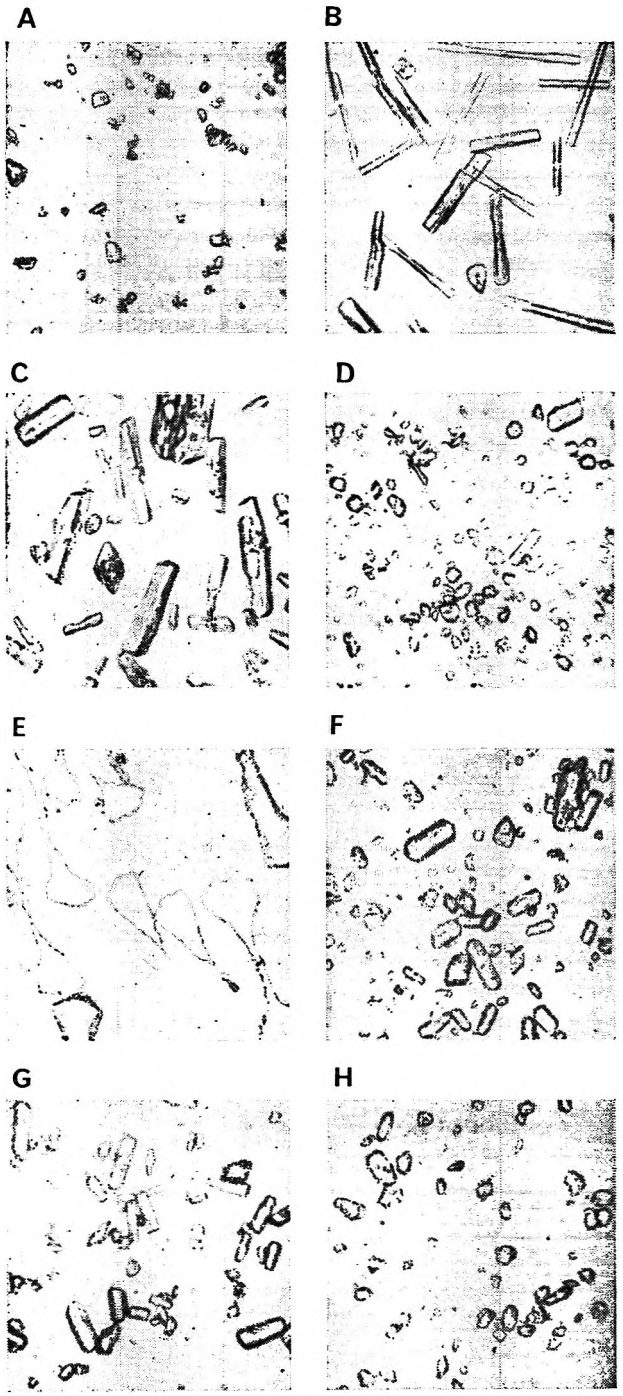


FIG. 1. Crystal growth of sulphathiazole in 0·1% Nonidet P42.

The temperature was taken through a cycle from 23° to 43°. Fig. 2C shows the crystal shape and size after growth under these conditions, while Fig. 2 A represents the original suspension. When the median values from Fig. 1 were plotted against the number of temperature cycles, a rectilinear increase in the equivalent diameter occurred, after an initial period of 2–4 cycles (Fig. 3). However, many subsequent experiments indicated that the initial phase usually corresponded to 8–10 cycles. Fig. 3 shows that the data from Fig. 1 could be plotted as the median size derived from curves of percentage number oversize (N/N), or as percentage weight oversize (w/w) and that in both cases a rectilinear relationship existed.

Straight lines were obtained by the method of least squares, the significance limits of the Student *t*-distribution were calculated and P (the probability value) was found to be much less than 0·001, confirming that the experimental results fell on the straight line. Subsequently, more than 40 similar experiments have shown P to be less than 0·001. Therefore, it is safe to assume that, under these growth conditions, the response

FIG. 2. Key to photographs: A, Original suspension, no treatment. B, 0·4% v/v Nonidet P42 vehicle, 32 cycles. C, 0·1% v/v Nonidet P42 vehicle, 32 cycles. D, 0·1% v/v Nonidet P42 vehicle, stored at 43° during 63 hr. E, 0·1% v/v Nonicet P42 vehicle, stored at 43° during 63 hr, then 36 cycles, then stored at 43° during further 15 hr. F, 0·006% w/v cetomacrogol vehicle, 28 cycles. G, 0·06% w/v cetomacrogol vehicle, 28 cycles. H, 1·00% w/v cetomacrogol vehicle, 32 cycles.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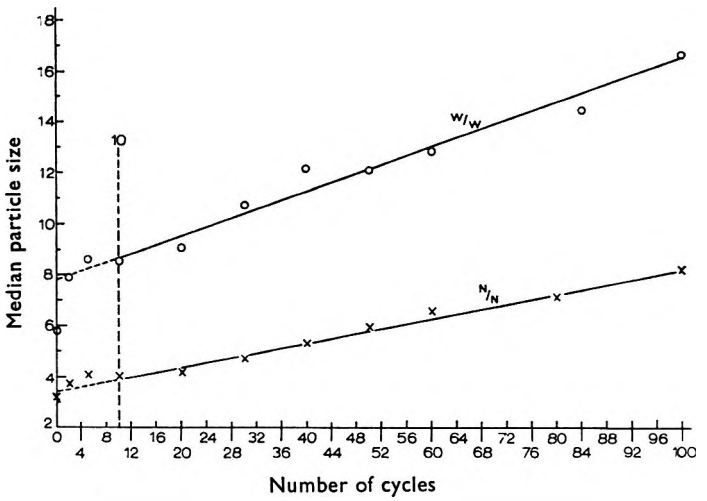

FIG. 3. Crystal growth of sulphathiazole in 0·1% v/v Nonidet P42. Lines of least squares, $P \ll 0{\cdot}001$.

of crystal growth to temperature cycling is rectilinear, after the first 8–10 cycles.

EFFECTS OF CONSTANT AND CYCLING TEMPERATURES

The results have clearly established the induction of rapid crystal growth by suitable temperature cycling. However, it became necessary to check that no significant growth occurred under steady temperature conditions and an experiment was undertaken to observe the growth produced when the suspension was stored: (i) at a constant temperature of 23°; (ii) at a constant temperature of 43°; (iii) at a constant temperature of 23° after a period of cycling; (iv) at a constant temperature of 43° after a period of cycling, and to observe whether the storage time before the cycling period influenced the final particle size.

The results (Fig. 4) indicated that there was little crystal growth at either constant temperature, with or without a previous history of temperature cycling. However, growth during the cycling period was considerable. Several replicates of this experiment were made and there was little correlation between the length of storage at constant temperature before cycling and the final particle size. Every replicate gave a qualitatively similar graph, but there was little quantitative correlation. A comparison of Fig. 2 C with D and E gives some indication that the reason for the quantitative discrepancy may be related to habit modification of the crystals.

EFFECT OF CONCENTRATION AND NATURE OF SURFACE-ACTIVE AGENT

Figs 1 and 3 show the growth resulting when the sulphathiazole was suspended in a saturated solution of sulphathiazole containing Nonidet P42 and exposed to temperature cycles between 23° and 43°.

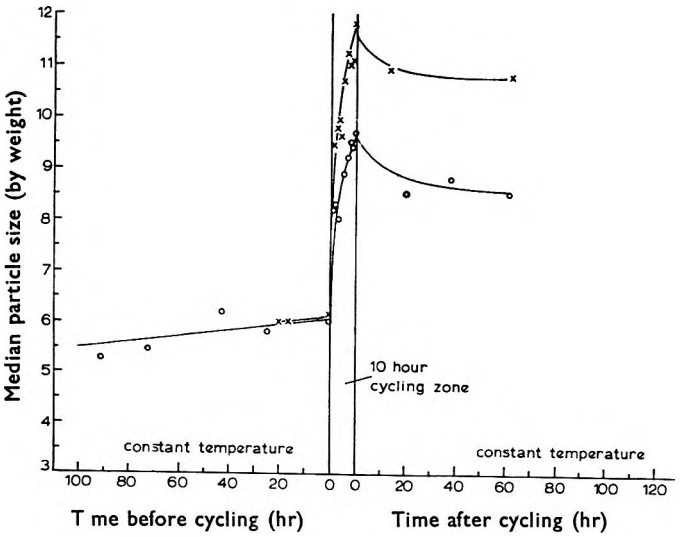

FIG. 4. Crystal growth at constant and cycling temperatures. X, under constant temperature of 23°. O, under constant temperature of 43°.

Fig. 5 represents the crystal growth of sulphathiazole, suspended in triple distilled water containing cetomacrogol, when exposed to temperature fluctuations between 23° and 33°. This Figure includes averaged data from experiments replicated up to five times.

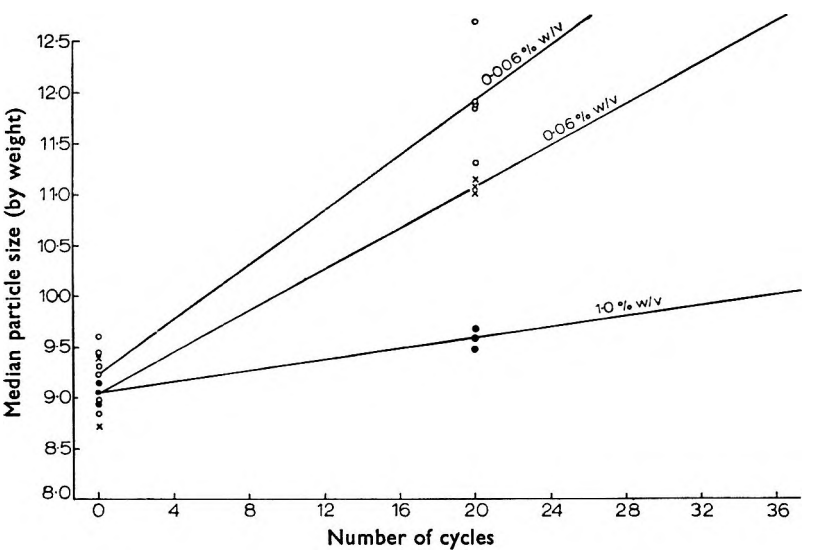

FIG. 5. Effect of concentration of cetomacrogol as % w/v on crystal growth of sulphathiazole.

Comparison of Figs 3 and 5 indicates that a change in the conditions of growth produced essentially linear growth rates, even though the shape and form of the final crystals were markedly different (Fig. 2 F, G and H). The same observation holds for other concentrations of Nonidet P42 (Fig. 2 B and C).

Fig. 5 also indicates that, under similar conditions, crystal growth rates were inhibited by increasing concentrations of cetomacrogol. It was considered that the observed change in growth rate may have been due to a change in the solubility of sulphathiazole resulting from the introduction of cetomacrogol.

Fig. 6 describes the solubility of sulphathiazole in several concentrations of cetomacrogol over the range of temperature studied.

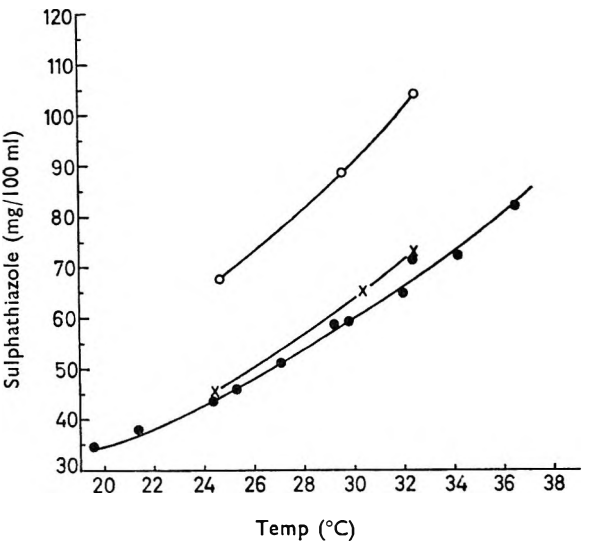

FIG. 6. Solubility of sulphathiazole in solutions of cetomacrogol. ○ = 1·0% w/v, × = 0·06% w/v, ● = 0·006% w/v.

The solubility values were obtained by shaking excess sulphathiazole with the solution of cetomacrogol for 3 days and filtering through a Millipore membrane filter* of nominal pore size, 0·45 μ, and assaying by the method of Bratton & Marshall (1939).

Solubility determinations were made on similar systems containing 0·9% w/v sodium chloride in addition and the results were identical to those in Fig. 6.

Thus, there was no likelihood of a change in particle size on addition of the suspension to the electrolyte vehicle in the Coulter counter. The inhibition of crystal growth by increasing concentrations of cetomacrogol occurred in spite of the enhanced solubility caused by the cetomacrogol.

* Millipore Filter Corporation, Bedford, Massachusetts, U.S.A.

Discussion

There are many combinations of large and small particles in a bulk suspension and the change in the mean diameter may be accepted as the integrated function of such combinations (Hasegawa & Nagai, 1958). However, the method employed for measuring particle size changes in suspensions must be highly sensitive, reproducible and statistically significant (Higuchi & Lau, 1962). A mean diameter may be obtained with this degree of accuracy from Coulter counter analysis and, when the particle size distribution is logarithmic normal, the statistical significance is increased further (Thornton, 1963; Lewis & Goldman, 1965).

We measured particles of 2·5 μ and over and assumed that these represented all the particles present. We noted that the particle size was log normal initially when calculated on a weight basis but that during the accelerated storage there was an increasing deviation from log normality. This agrees with the dissolution results of Higuchi & Hiestand (1963), but Hasegawa & Nagai (1958) found that the particle size distribution remained log normal even after much crystal growth in suspensions of sulphadiazine which resulted from storage at constant temperature for four months. Because of the difficulty of interpretation of distributions which were not log normal, we concluded that median values of the distributions would be a suitable criterion for crystal growth.

Under conditions of temperature cycling, up to a total of 100 cycles, the median particle size and the particle size range of the sulphathiazole suspension continued to increase. This differs from the results of Hasegawa & Nagai (1958) and Nogami & Nagai (1958), who noted that the size distribution became narrower as the median particle size increased and postulated that the effective growth rate and particle size would reach limiting values.

Allen & others (1965) recorded growth rates proportional to time, but Okano & others (1958), studying crystallisation by cooling supersaturated solutions, obtained growth curves very similar to those we report, i.e. curving steeply initially and then becoming rectilinear. Okano & others (1958) studied crystal growth in suspensions containing surface-active agents in excess of their critical micelle concentrations and this factor may account for the agreement with our results.

It is our opinion that the initial steep slope of the growth curves may be due to one or more of the following factors:

1. Milled material will have a large number of active growth sites because of particle fracture and these will gradually heal over as growth proceeds (Kolthoff & Rosenblum, 1935).

2. The inner perfection of the crystals by recrystallisation may result in the cementing together of small particles so causing a rapid loss of small particles in the initial stages (Kolthoff & Rosenblum, 1935; Kolthoff & Eggertsen, 1941).

3. The possible presence of an "easily soluble layer" (Clelland,

Cumming & Ritchie, 1952; Gibb, Ritchie & Sharpe, 1953; Bergman & Paterson, 1961; Bergman, 1962, 1963).

4. A large proportion of ultrafine material may be present initially, which would result in a very high solubility rate and hence a relatively rapid decrease in small particles.

5. Slow adsorption of the surface-active agent on to the growth sites of the crystal faces.

6. The existence of some material of a metastable modification, which rapidly reverts to the stable form.

The median particle size of the sulphathiazole studied was 6 μ and it is not to be expected that Ostwald ripening would be important. However, the process of temperature cycling involves two factors which will affect the net crystal growth, i.e. dissolution and crystallisation, and it would be impossible to separate the effects of each in the present experimental design. We have already discussed the crystal growth observed during storage at constant temperature and Edmundson & Lees (1965) have studied the dissolution rates of hydrocortisone acetate in very dilute solutions. They observed that the decrease in the diameter of the particles was proportional to time. Similar effects were noted at several agitation rates in excess of 400 rpm (Niebergall & Goyan, 1963; Wurster & Taylor, 1965). The stirring rate, used in the present work, was 680 rpm and, although it is appreciated that the rate limiting process is dependent upon the stirring rate, it has not yet been possible to carry out work at different agitation rates.

Higuchi & Saad (1965) and Saad & Higuchi (1965) noted that growth and dissolution of cholesterol were both retarded by the presence of impurity. They postulated an interfacial interaction producing a barrier to interfacial transport between solution and solid and vice versa. Similar growth retardation was observed by Michaels & Colville (1960) who stated that small particles were more sensitive to surface-active agents than were large particles. They deduced that the method of growth of small particles (i.e. two dimensional) was more sensitive to adsorbed contaminants than that for large particles (viz. growth from dislocations). Undoubtedly, this effect is significant in the crystal growth results quoted in this paper, but the nature and concentration of the surface-active agent produce two additional effects: (*a*) a net decrease in the crystal growth rate of all particles; (*b*) habit modification.

The net crystal growth retardation, observed with increasing concentrations of surface-active agents, must be a result of interfacial interaction between the cetomacrogol and sulphathiazole and occurred despite the increase in solubility of sulphathiazole in the presence of the cetomacrogol.

Reference to Fig. 2 B, C, E and H indicates the extreme habit modification and consequent crystal breakage, which Michaels & Colville (1960) and Allen & others (1965) stated was a result of selective adsorption onto specific faces of the crystals.

However, such crystal growth and modification will also be influenced by the initial polymorphic form of the crystals under study. With sulphathiazole, there are at least three polymorphs (Grove & Keenan, 1941; Miyazaki, 1947; Frederick, 1961) and Miyazaki stated that commercial samples consisted of a mixture of two polymorphs. We have shown, by differential thermal calorimetry, that the sample of sulphathiazole used was a mixture of two or more polymorphs and, although this must affect the crystal growth, we have been concerned primarily with the reproducibility of the growth without defining the actual mechanism of the physical changes.

Higuchi & Lau (1962) indicated the use of inhibiting agents in "stabilising" suspensions of the metastable modification of methyl prednisolone and the inhibition of growth of "stable" suspensions is now receiving an increasing amount of study.

To accelerate the change in particle size we subjected the suspension to temperature fluctuations producing a cycle of events resulting in saturation-undersaturation-saturation-supersaturation and then again saturation. Thus it was possible to effect accelerated storage under conditions closely simulating shelf storage, but differing in that the rate of temperature fluctuations was greatly increased and controlled.

To study the system over more protracted periods and to encourage better reproducibility and reliability, it was found desirable to automate the temperature cycling process (Carless & others, 1966).

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A calcium requirement for release of ^{3}H-guanethidine by sympathetic nerve stimulation

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Cat colons were labelled with ^{3}H-guanethidine by close intra-arterial injection. Two hr later the colons were removed and the vascular bed was perfused with Krebs solution containing 0, 2·5 or 5 mM calcium. The spontaneous efflux of ^{3}H-guanethidine was not changed by alterations in the calcium concentration, but stimulation of the post-ganglionic sympathetic nerves failed to enhance the output of ^{3}H-guanethidine unless calcium was present in the perfusion fluid. It is concluded that extracellular calcium is essential for release of ^{3}H-guanethidine by sympathetic nerve stimulation and that the neural release of the inactive transmitter substitute is related to the pharmacological action of the drug.

RECENTLY, evidence has been accumulating that the antihypertensive drug guanethidine possesses some of the properties associated with the series of β-hydroxylated phenylethylamines believed to act as "false" transmitters, in the sense postulated by Day & Rand (1963). Two of the obvious requirements of a "false" neuro-humoral transmitter are firstly, uptake into adrenergic neurones and secondly, release by sympathetic nerve stimulation. Both requirements have recently been fulfilled for guanethidine (Costa, Chang & Brodie, 1964; Chang, Costa & Brodie, 1965; Boullin, Costa & Brodie, 1966a). Another essential factor might be a dependence upon extracellular calcium.

It is well-known that this cation is required for release of acetylcholine from cholinergic nerves (Harvey & MacIntosh, 1940), and for release of catecholamines from the adrenal medulla (Douglas & Rubin, 1961); it has also been demonstrated recently that calcium is required for the release of noradrenaline from sympathetic nerve endings in the cat colon (Boullin & Brodie, 1965; Boullin, 1966). The data presented in this communication indicate that extra-cellular calcium is required for the release of ^{3}H-guanethidine from sympathetic nerve fibres by electrical stimulation.

Experimental

GENERAL

Experiments were made with a preparation of the isolated cat colon, perfused through the vascular bed from the inferior mesenteric artery to the colic vein, and described in detail elsewhere (Boullin, Costa & Brodie, 1966b). Briefly, the procedure was as follows: cats weighing 2–4 kg were anaesthetised with pentobarbitone and dissected to expose the inferior mesenteric artery to the colon. 0·5 mg (35 μc) ^{3}H-guanethidine sulphate (specific activity 17·4 mc/mM) was injected into the artery so that the drug passed into the vascular bed of the colon. After 2 hr the colon was removed for perfusion.

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PERFUSION OF THE COLON

The vascular bed was perfused at constant flow (1·2–2·4 ml/min). The perfusion medium was Krebs solution (Gillespie & MacKenna, 1961). The calcium concentration was varied from 0 to 2·5 mM or 5 mM and 1 mM disodium edetate was always included in the calcium-free solutions. It was added to the 2·5 or 5 mM calcium solutions as stated in Results.

During perfusion, the venous effluent from the vascular bed of the colon was collected at 2 min intervals before, during and after sympathetic nerve stimulation. The post-ganglionic sympathetic nerve fibres accompanying the inferior mesenteric artery were stimulated supra-maximally for 5 min with trains of 3000 rectangular impulses of 1 msec duration at a frequency

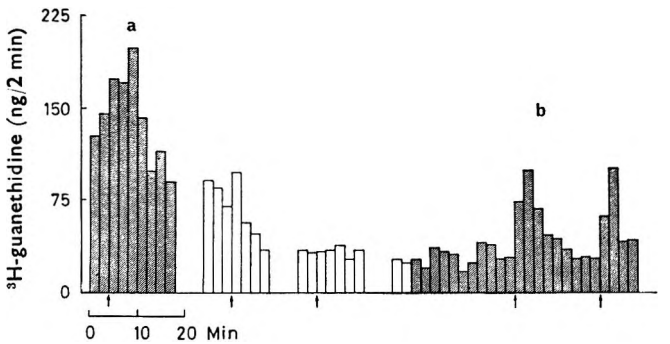

FIG. 1. Effects of changes in calcium concentration on the output of ^{3}H-guanethidine from the vascular bed of the isolated cat colon. Each histogram represents the output of drug in venous effluent from the colon collected over 2 min. Hatched histograms refer to output during perfusion with Krebs solution containing 2·5 mM calcium (without EDTA at a; with 1 mM EDTA at b) and open histograms to output during perfusion with calcium-free solution. At the arrows the nerve fibres were stimulated with 3000 impulses at 10/sec. Experimental data were obtained 40 min after commencement of perfusion.

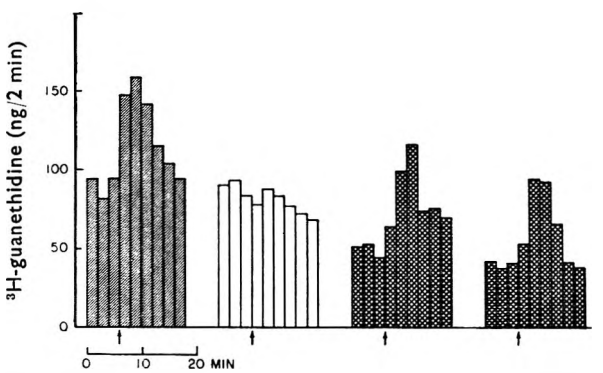

FIG. 2. Experimental details as Fig. 1 except that the cross-hatched histograms represent the output of guanethidine during perfusion with Krebs solution containing 5 mM calcium and 1mM EDTA. Hatched histograms refer to 2·5 mM calcium only, while open histograms refer to calcium-free 1mM EDTA.

of 10/sec. 1 ml of each sample of the venous effluent was assayed for total radioactivity by liquid scintillation spectrometry.

The data presented are based on experiments with 6 cats.

Results

Boullin & others (1966a) have described the pattern of efflux of ^{3}H-guanethidine from the vascular bed of the isolated cat colon during perfusion with Krebs solution containing 2·5 mM calcium. Sympathetic nerve stimulation at 10 impulses/sec caused an increase in output of radioactive guanethidine that was independent of the spontaneous efflux of drug that occurred in the absence of nerve stimulation; successive periods of nerve stimulation released similar amounts of guanethidine irrespective of whether the spontanecus output was high or low.

In the present experiments it was found that sympathetic nerve stimulation only increased the efflux of ^{3}H-guanethidine when calcium was present in the perfusion medium. During perfusion with calcium-free Krebs solution containing 1 mM disodium edetate, the spontaneous output of radioactivity was unchanged, but the response to nerve stimulation was abolished. When the cation was replaced, in the presence of disodium edetate, the response to nerve stimulation was restored. In addition there was also a small increase in the spontaneous output occurring shortly after the replacement of calcium, and lasting for several minutes (Fig. 1). Similar results were obtained when double the normal concentration of calcium was added to the perfusion fluid after perfusion with calcium-free solution (Fig. 2).

Discussion

The data indicate that extracellular calcium is required for the release of tritiated guanethidine in the same way as it is required for the release of tritiated noradrenaline from the isolated cat colon in response to sympathetic nerve stimulation (Boullin & Brodie, 1965; Boullin, 1966). In both instances replacement of the calcium ion evokes a small increase in the spontaneous release of labelled compound. Calcium also appears to exert a direct releasing action on ^{14}C-guanethidine bound in tissue slices (Boullin, unpublished). However, provided the cation is present, increase in the calcium ion concentration does not enhance the efflux of guanethidine or noradrenaline (Boullin, 1966) released in response to sympathetic nerve stimulation. Apart from these and other similarities (Boullin & others, 1966a), the essential difference between guanethidine and noradrenaline, the authentic transmitter, is that guanethidine does not possess affinity for adrenergic receptors, since noradrenaline is still effective when adrenergic blockade is maximal (Boura & Green, 1962) and phenoxybenzamine does not augment the output of radioactive guanethidine after sympathetic nerve stimulation (Boullin, unpublished). Therefore guanethidine may be termed an inactive transmitter substitute.

ห้องสมุด กรมวิทยาศาสตร์

It is well-known, particularly from the work of Cass & Spriggs (1961), that there is no close correlation between the onset of adrenergic blockade and depletion of catecholamines from tissues. In experiments with the cat spleen, Hertting, Axelrod & Patrick (1962) showed that there was a transient release of ^{3}H-noradrenaline immediately after guanethidine was administered. At this time the output of noradrenaline to sympathetic nerve stimulation was blocked (Hertting & others, 1962; Abercrombie & Davies, 1963). Thus it appears that guanethidine is bound and produces adrenergic blockade without any substantial noradrenaline loss. In the light of these observations and the present data it seems that guanethidine has two actions: it prevents release of the authentic transmitter noradrenaline and also behaves as an inactive substitute. These actions may or may not be related.

Guanethidine differs from the false transmitters such as α-methylnoradrenaline, metaraminol and octopamine in that it does not possess affinity for postsynaptic receptors. After neural release, therefore, guanethidine is either lost into the general circulation, or recaptured into the binding sites.

The duration of the pharmacological action of guanethidine may depend upon the time taken for depletion of the intra-cellular stores of the drug following release by nerve impulses and loss of the transmitter substitute into the general circulation. If this is so, then for a given dose of guanethidine, the adrenergic blockade may be related to the level of sympathetic nervous activity.

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Identification of the thiazide diuretic drugs

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A method for detecting and identifying ten thiazide diuretic drugs in tablets, gastric washings and urine by ultraviolet spectrophotometry and paper chromatography is described. The thiazides can be detected by absorption maxima in the region 264 to 294mμ at concentrations of 5 μg/ml or lower. Identification is by ascending high temperature reverse phase paper chromatography using tributyrin treated paper and developing for 20 min at 90° with a phosphate buffer (pH 7·4). Further differentiation is obtained with the solvent system amyl alcohol—0·880 ammonia (9:1) on Whatman No. 1 paper. The thiazides are located as absorbing or fluorescent spots in ultraviolet light (2534 Å) and these are confirmed by the stable red colour given by an alkaline sodium 1,2-naphthaquinone-4-sulphonate spray reagent. Many of these diuretics are co-extracted with barbiturates and may interfere with barbiturate determinations.

NON-mercurial oral diuretic drugs are unlikely suicidal agents because of their relatively low toxicity, but their widespread use ensures that they will be encountered during routine screening for poisons or in the identification of unknown pharmaceutical preparations. Most of these compounds have a thiazide structure (i.e. a substituted 1,3-benzene-disulphonamide). Chlorothiazide (6-chlorobenzo-1,2,4-thiadiazine-7-sulphonamide-1,1-dioxide) may be considered as the parent compound.

Many of the published methods for detecting and estimating these drugs rely on hydrolysis, diazotisation and coupling to form a coloured azo-dye which can be estimated colorimetrically. This is the basis of the procedure used by Sheppard, Mowles & Plummer (1960) to determine chlorothiazide and hydrochlorothiazide in urine, and by Bermejo (1961) to estimate hydroflumethiazide and bendroflumethiazide. But although the procedure is very sensitive it is not specific.

Spectrophotometric determination of chlorothiazide and hydrochlorothiazide has been reported by Marciszewski (1960), and also by Charnicki, Bacher, Freeman & DeCesare (1959) who used it for the estimation of chlorothiazide in tablets. Sunshine & Gerber (1963) have investigated the ultraviolet and infrared spectra of hydrochlorothiazide and Kaye (1961) the ultraviolet spectrum of chlorothiazide. While the British Pharmacopoeia and the British Pharmaceutical Codex 1963 use ultraviolet absorption spectra in the assay of chlorothiazide, hydrochlorothiazide, hydroflumethiazide, benzthiazide and bendrofluazide, crystal tests were used to detect chlorothiazide, hydrochlorothiazide, hydroflumethiazide and acetazolamide by Groenewegen (1960) and to detect chlorothiazide and hydrochlorothiazide by Zoetten (1960).

The spectrophotometric and chromatographic behaviour of acetazolamide and chlorothiazide has been examined by Kraemar & Vacek (1960) and by Kala (1961), who also studied chlorazanil and hydrochlorothiazide.

No systematic scheme for the identification of this group of drugs appears to have been published and many of the reported methods assume prior knowledge of the compounds sought. We now report on

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the spectrophotometric and chromatographic examination of these thiazides, and also of frusemide which has been included because it has some structural similarities and is an important diuretic.

ULTRAVIOLET SPECTROPHOTOMETRY

Standard solutions of the diuretics containing 10 μg/ml were prepared with distilled water made alkaline to pH 10 (Universal Indicator paper) with 2N ammonia solution. These solutions were stable for several days whereas standard solutions prepared in dilute sodium hydroxide deteriorate in a few hours. The ultraviolet absorption spectra of the solutions

TABLE 1. MELTING POINTS, ULTRAVIOLET ABSORTPION AND CHROMATOGRAPHIC DATA OF THE THIAZIDE DIURETICS

Approved name	M.p. °C.	pH 10		pH 2		Paper chromatography			
		λ max	E(1%, 1 cm)	λ max	E(1%, 1 cm)	Rf values system 1	Rf values system 2	Ultra-viclet light	**NQS
Polythiazide[1] ..	209	264 315	460 80	270 310	550 135	0·38	0·05	Absorbs	++
Methyclothiazide[2] ..	216	264 318	420 60	270 315	525 120	0·73	0·20	Absorbs	++
Hydroflumethiazide[3]	226 –229 decomp.	274 330	450 80	273 325	580 100	0·62	0·06	Fluores.	+
Cyclopenthiazide[4] ..	380	273 320	375 40	272 315	470 45	0·80	0·06	Absorbs	+
Hydrochlorothiazide[5]	273	274 325	620 110	272 315	760 90	0·08	0·62	Absorbs	++
Bendrofluazide[6] ..	262	275 335	480 100	273 330	620 120	0·22	0·64	Fluores.	++
Chlorothiazide[7] ..	342	294 315S	630	280 272, 294S 305S	580	0·05	0·75	Absorbs	++
Benzthiazide[8] ..	241	298 315	290 270	282 300S	190	0·36	0·56	Absorbs	+
Teclothiazide[9] ..	230 decomp.	270 315	350 80	265 310	455 80	0·72	0·21	Absorbs	++
Frusemide[10] . ..	216	272 333	810 200	275 340	860 250	0·75	0·17	Fluores. green	++

(S = Shoulder.) (++ = Strong positive. + = Positive.)

* With the exception of chlorothiazide and hydrochlorothiazide, the compounds in the Table have not been examined by both chromatographic and spectrophotometric methods so far as we are aware.

** See reagents, p. 717.

[1] 6-Chloro-3,4-dihydro-2-methyl-3-(2,2,2-trifluoroethylthiomethyl)benzo-1,2,4-thiadiazine-7-sulphonamide-1,1-dioxide[Nephril, Renese].

[2] 6-Chloro-3-chloromethyl-3,4-dihydro-2-methylbenzo-1,2,4-thiadiazine-7-sulphonamide 1,1-dioxide [Enduron].

[3] 3,4-Dihydro-6-trifluoromethylbenzo-1,2,4-thiadiazine-7-sulphonamide 1,1-dioxide [Di-Acemil, Hydrenox, Naclex, Rontyl].

[4] 6-Chloro-3-cyclopentylmethyl-3-4-dihydrobenzo-1,2,4-thiadiazine-7-sulphonamide 1,1-dioxide [Navidrex].

[5] 6-Chloro-3,4-dihydrobenzo-1,2,4-thiadiazine-7-sulphonamide 1,1-dioxide [Dichlotride, Direma, Esidrex, Esidrex, Hydril, Hydro-Diuril, Hydrodiuril, Hydrosaluric, Hydrothide, Oretic].

[6] 3-Benzyl-3,4-dihydro-6-trifluoromethylbenzo-1,2,4-thiadiazine-7-sulphonamide 1,1-dioxide [Aprinox, Centyl, Neo-Naclex].

[7] 6-Chlorobenzo-1,2,4-thiadiazine-7-sulphonamide 1,1-dioxide [Alurene, Chlorotride, Diuril, Minzil, NeoDema, Salisan, Saluric, Saluretil, Yadalan].

[8] 3-Benzylthiomethyl-6-chlorobenzo-1,2,4-thiadiazine-7-sulphonamide 1, 1-dioxide [Fovane, Dyt de].

[9] 6-Chloro-3,4-dihydro-3-trichloromethylbenzo-1,2,4-thiadiazine-7-sulphonamide 1,1-dioxide [Deplet].

[10] 4-Chloro-N-furfuryl-5-sulphamoylanthranilic acid [Lasix, Furosemide].

were determined between 230 and 350 mμ with a Hilger & Watts H.999 automatic recording spectrophotometer. The sample and reference solutions were then acidified with sulphuric acid and the absorption spectra again determined.

The results indicated that small changes in the pH value do not cause significant variations in the spectra but diagnostic features can be obtained if both acid and alkaline spectra are recorded. Thus hydroflumethiazide, cyclopenthiazide, hydrochlorothiazide, bendrofluazide and frusemide exhibit little or no variation in the position of the maxima at 272 to 275 mμ but polythiazide, methyclothiazide, chlorothiazide, benzthiazide and teclothiazide show changes greater than 5 mμ. When the alkaline solutions of polythiazide and methyclothiazide are acidified and replotted the strong absorption maxima at 264 mμ shifts to a higher wavelength (270 mμ) whereas the absorption maxima of chlorothiazide, benzthiazide and teclothiazide move to lower wavelengths. Eight of the ten diuretics examined show a significant increase in the absorption maxima at 260 to 275 mμ on changing from alkaline to acid pH. Distinctive characteristics are obtained for eight of the ten diuretics studied, the unresolved pair being polythiazide and methyclothiazide.

The main purpose of this examination of the thiazide ultraviolet spectra was to detect and identify these drugs; the extinction values shown in Table 1 are therefore intended only as an indication of the sensitivity of the method and for approximate quantitative determination of the drugs. They should not be used for accurate spectrophotometric assays. Rogers (1964) has stressed that particular care is needed to avoid spuriously low results, and recommends the adoption of a procedure in which the extinction of the sample is compared with that of a reference substance under the same conditions. Table 2 gives the values published for five

TABLE 2. COMPARISON OF PUBLISHED ULTRAVIOLET SPECTROPHOTOMETRIC DATA

Compound	Solvent	Wavelength absorp-maximum mμ	$E(1\%, 1\text{ cm})$	Wavelength absorp-maximum mμ	$E(1\%, 1\text{ cm})$	Reference
Chlorothiazide	0·1N NaOH	292	430	—	—	British Pharmacopoeia 1963
	"	291	425	—	—	Marciszewski (1961)
	"	292	—	—	—	Charnicki & others (1959)
	0·5N NaOH	294	444	—	—	Kaye (1961)
	H_2SO_4	278	301	—	—	Kaye (1961)
	Ethanol	280	—	—	—	Kraemar & Vacek (1960)
Hydrochlorothiazide	Ethanol	271	660	317·5	120	British Pharmacopoeia 1963
	Methanol and trace HCl	271	654	317	130	Merck Index (1960)
	Methanol	271	—	—	—	Rehm & Smith (1960)
	0·1N NaOH	273	525	—	—	Marciszewski (1961)
	0·1N NaOH	272	520	322	90	Sunshine & Gerber (1963)
	H_2SO_4	271	650	314	110	Sunshine & Gerber (1963)
Bendrofluazide	0·01N NaOH	273	413	329	80	British Pharmacopoeia 1963
Hydroflumethiazide	0·01N NaOH	274	460	333	95	British Pharmacopoeia 1963
Benzthiazide	0·01N ethanolic HCl	283	284	—	—	British Pharmaceutical Codex 1963

of these drugs and shows the variations in both wavelength and extinction values. These variations are probably due to instrumental differences, type of solvent, and possibly to the instability of some of these compounds. A note on the stability of the benzothiazines has been published by Yamana & Koike (1961), and Arizan & Sterescu (1960), Rehm & Smith (1960) and Marciszewski (1960) have reported the presence of 4-amino-6-chlorobenzene-1,3-disulphonamide as an impurity in some samples of chlorothiazide and hydrochlorothiazide.

PAPER CHROMATOGRAPHY

The two solvent systems chosen, namely the high temperature reverse-phase system (Street, 1962) and the amyl alcohol-ammonia system (Jackson, 1958) give good distribution of this group of drugs and are already widely used. Brief details of these systems are given below. The Rf values obtained are shown in Table 1. The use of both systems permits unequivocal identification of any particular thiazide diuretic (see Table 3).

TABLE 3. Rc VALUES OF THE THIAZIDE DIURETICS, WHERE Rc = DISTANCE MOVED BY THIAZIDE/DISTANCE MOVED BY CHLOROTHIAZIDE

Name	Rc values for system 1	Rc values for system 2
Chlorothiazide	1	1·0
Bendrofluazide	4	0·85
Hydrochlorothiazide	6	0·83
Benzthiazide	7	0·75
Hydroflumethiazide	12	0·08
Teclothiazide	14	0·28
Frusemide	15	0·23
Methyclothiazide	15	0·27
Cyclopenthiazide	17	0·08
Polythiazide	18	0·07

Location. Most of the common location reagents did not react with these compounds, but experiments with sodium 1,2-naphthaquinone-4-sulphonate indicated that it might provide a selective group reagent. Feigl (1954) mentions the use of this compound to detect substances containing two removable hydrogen atoms attached to carbon or nitrogen. Coloured paraquinoid condensation products are formed.

The drugs were located with ultraviolet light (2534 Å), and an alkaline solution of sodium naphthaquinone-4-sulphonate (NQS) was sprayed on the chromatograms giving stable orange-red colours with the thiazide diuretics. This reagent did not react with other solvent-soluble acidic and neutral drugs (e.g., salicylates, barbiturates, carbamates, hydantoins, phenacetin, glutethimide), and provides a useful confirmatory test for the thiazide drugs, distinguishing them from other drugs which give absorbing or fluorescent spots on chromatograms. Mercurous nitrate solution may also be used to locate synthetic diuretics (Kala, 1961), but this reagent reacts with many other drugs including barbiturates.

Experimental

REAGENTS

Tributyrin solution. Prepare a solution containing 10% v/v glycerol tributyrate (tributyrin) in acetone. *Buffer solution pH* 7·4. Dissolve 1·5 g potassium dihydrogen phosphate and 7·9 g disodium hydrogen phosphate in 1 litre distilled water. *NQS reagent.* Prepare a saturated solution of sodium 1,2-naphthaquinone-4-sulphonate (NQS) in 50% v/v ethanol and water. *Solvent system* 1. Mix together amyl alcohol (180 ml) and concentrated ammonia solution (20 ml) (sp.gr. 0·880). Shake well. *Thiazide standards.* Prepare a solution containing 10 mg/ml of the relevant thiazide diuretic in acetone.

GENERAL PRCCEDURE

System 1. Samples of the thiazide standard and of the unknown were applied in 1 μl and 5 μl quantities on the baseline of a Whatman No. 1 paper to give spot sizes less than 5 mm. The paper was then developed by the ascending method with solvent system 1 at room temperature for 6½ hr. No equilibration is necessary with this solvent.

System 2. A sheet of Whatman No. 3 paper cut to fit a small chromatography tank was dipped in the tributyrin solution, blotted and dried at room temperature. Samples of the thiazide standards and the unknown were applied to the prepared paper as before and the chromatogram was developed by the ascending method in the phosphate buffer solution at 90° $\pm$ 5° for 20 min.

Location. The dried chromatograms were examined by ultraviolet light (2534 Å) and the fluorescent or absorbing spots marked with pencil. The chromatograms were then lightly sprayed with 0·1N sodium hydroxide solution and with the NQS reagent. The thiazides appeared as stable orange spots within 5 to 15 min.

EXTRACTION

The methods of extraction were those used in routine screening of pharmaceutical and biological samples submitted for qualitative analysis for poisons and drugs. They are not necessarily the most efficient, but they have been developed to meet the particular problem of searching for and identifying an unknown substance.

Tablets. The tablet is crushed in a mortar and triturated with distilled water (3 $\times$ 20 ml). The combined water extracts are filtered through a Whatman No. 1 paper and the filtrate is examined at pH 10 and pH 2 with a recording ultraviolet spectrophotometer. If a diuretic drug is indicated the solution is acidified, saturated with ammonium sulphate, and extracted three times with an equal volume of ether. After drying with anhydrous sodium sulphate, the solvent is filtered and evaporated and the residue taken up in a small amount of ethanol for paper chromatography.

Urine or gastric washings. The sample is acidified with dilute hydrochloric acid and saturated with solid ammonium sulphate, filtered, and the

filtrate extracted with 2 volumes of ether by shaking in a separating funnel for ½ min. The ether layer is separated and the aqueous layer returned to the separating funnel. The ether extraction is repeated and the ether extracts pooled. The aqueous phase is retained for extraction of basic drugs if required. The ether extracts are washed with 2 × 10 ml of freshly prepared saturated sodium bicarbonate solution. The bicarbonate washings are separated, and labelled "A". The ether is now extracted with 2 × 20 ml of 0·1N sodium hydroxide solution and these extracts are labelled "B".

The "A" and "B" extracts contain the strong and weak acidic drugs respectively. Most of the thiazide diuretics are extracted into the "B" group but chlorothiazide, hydrochlorothiazide and frusemide are in group "A".

The bicarbonate extracts "A" are acidified, re-saturated with ammonium sulphate, filtered and ether extracted. The ether extract is dried with anhydrous sodium sulphate, treated with a small amount of charcoal, filtered and evaporated. The residue is taken up in a known volume of distilled water and filtered. The filtrate is made alkaline with ammonia, and examined in an ultraviolet spectrophotometer. If the solution exhibits an absorption maximum between 264 and 300 mμ it is acidified and replotted.

The sodium hydroxide extract "B" may sometimes be examined directly in the ultraviolet spectrophotometer but if this extract is cloudy or coloured it should be treated as extract "A". The thiazides may be recovered from these solutions after ultraviolet examination by acidification, saturation and ether extraction. The residues obtained after evaporation of the ether are dissolved in the minimum volume of acetone for chromatographic examination.

Discussion

The qualitative analysis of pharmaceuticals is frequently required in forensic science. Many of these analyses are of the "general unknown" type, that is to say the analyst has no prior knowledge of the type of drug; it is important therefore that any scheme of analysis should be generally applicable and without too many specialised procedures. Once such a scheme has been established it is essential to know how new drugs will behave with the reagents and solvent systems employed, and whether they will interfere with the familiar chromatographic and spectral patterns of the established drugs and poisons. The use of the diuretic drugs in heart diseases and in the treatment of barbiturate poisoning by "forced diuresis" indicated that an examination of their analytical behaviour should be useful to the forensic scientist and clinical biochemist.

This investigation of the ultraviolet spectrophotometric properties of the diuretic thiazides shows that the $E(1\%, 1\text{ cm})$ values of these drugs are so good that, despite their low water solubility, they can be easily detected by simple aqueous extraction of tablets and ultraviolet spectrophotometric screening. Thus, maxima in the 270 to 280 mμ and 310 to 315 mμ regions

at pH 2 provide the analyst with a good clue to the presence of these drugs and confirmation is obtained by replotting at pH 10 followed by paper chromatographic examination.

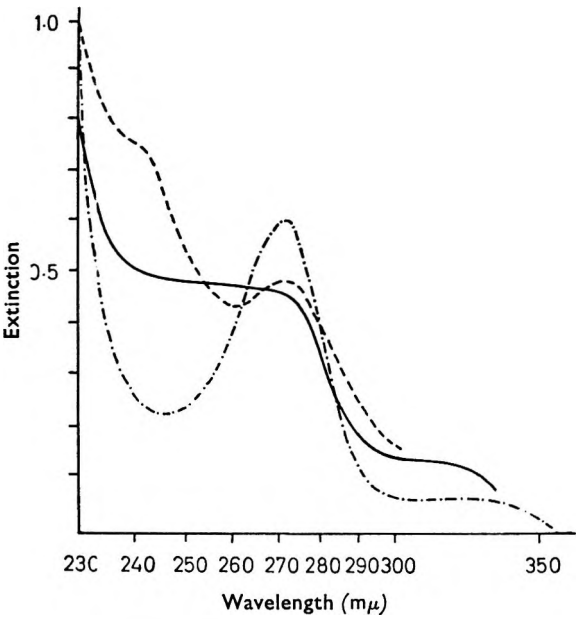

FIG. 1. Ultraviolet spectra of the weak acid group (Residue 'B') obtained from a urine containing equal quantities of pentobarbitone and polythiazide. The typical 240 mμ maximum of pentobarbitone at pH 10 is partially obscured and the 255 mμ maximum at pH 13 is obliterated by the presence of polythiazide. — · — · — pH 2; — — — pH 10; —— pH 13.

Many of these drugs can be extracted along with the barbiturates. This can cause distortion of the typical barbiturate spectra, e.g. the characteristic 240 mμ peak at pH 10 may be partially obscured and the 255 mμ peak at pH 14 of the 5,5-di-substituted barbiturates may be almost obliterated by the presence of these diuretics. The spectra obtained from the weak acid group (residue B) when equal quantities of polythiazide and pentobarbitone were added to a urine sample, are shown in Fig. 1. Quantitative barbiturate estimations based on the difference between the extinction at 260 mμ at pH 14 and pH 10 are seriously affected. Methods based on the 240 mμ differential absorption at pH 10 and pH 2 are less disturbed.

Additional maxima obtained between 270 and 315 mμ should however warn the analyst that he is dealing with a mixture; paper chromatographic examination would then confirm this fact. The presence of unusual absorbing or fluorescing spots on the chromatogram which do not react with the common acidic group location reagents (e.g. ferric chloride, cobalt nitrate) should be investigated by spraying with the NQS reagent.

The thiazide diuretics therefore do not offer an insurmountable obstacle to the detection and determination of the barbiturates if a full spectral plot and paper chromatographic examination are made.

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A comparison of aralkylamines and aralkylguanidines as antagonists of adrenergic neurone blockade

R. FIELDEN AND A. L. GREEN

Structure-activity relationships have been studied for aralkylamines and aralkylguanidines which restore the responses of the nictitating membranes to nerve stimulation in anaesthetised cats given sympathetic blocking drugs. This reversing action was largely specific for adrenergic neurone blockade; blockade of sympathetic ganglia or of α-adrenergic receptors was unaffected. (+)-Amphetamine was the most active amine and *N*-benzyl-*N*-methylguanidine was the most active guanidine. In mice, ptosis resulting from adrenergic neurone blockade was much more readily prevented or abolished by the aralkylamines and aralkylguanidines than was ptosis caused by other types of sympathetic blocking agent. The most potent antagonist of ptosis was *N*-(2-phenylcyclopropyl)guanidine which was about ten times as active as amphetamine. The relative antagonistic potencies of 2 amines and 8 guanidines were virtually identical for all types of adrenergic neurone blocking drug, regardless of whether or not they cause noradrenaline depletion. The prevention of guanethidine-induced ptosis was always accompanied by some reduction in the extent of heart-noradrenaline depletion, but the minimum dose of antagonist required to prevent ptosis completely was always lower than that required to eliminate depletion.

AMPHETAMINE and some other sympathomimetic amines are known to counteract adrenergic neurone blockade produced by xylocholine, bretylium or guanethidine (Bain & Fielden, 1956; Day, 1962). We have shown (Fielden, Green & Willey, 1965; Fielden & Green, 1965) that adrenergic neurone blockade could also be prevented, and in some circumstances reversed, by certain aralkylguanidines. The mechanism of this reversing action for one particular compound, (+)-*N*-(1-phenylethyl) guanidine, has been discussed in detail elsewhere (Fielden & Green, 1966). In the present paper we have compared a variety of aralkylamines and aralkylguanidines as antagonists to different types of adrenergic neurone blocking agent in cats and mice in an attempt to obtain further insight into the mechanism of adrenergic neurone blockade.

Experimental

METHODS

Cats were anaesthetised with chloralose (100 mg/kg, intravenously). The contractions of both nictitating membranes were recorded on smoked paper by frontal writing levers (magnification 15 times, load 3–4 g). The preganglionic cervical sympathetic nerves were periodically stimulated, through shielded platinum electrodes, with 200 rectangular pulses (0·5 msec duration, 10 to 15 V amplitude) at frequencies of 1, 3, 10 or 30/sec. Drugs were dissolved in 0·9% saline and injected through a cannula into a femoral vein.

Groups of 6 male mice (weight range 20 to 30 g) were injected subcutaneously with the drugs dissolved in sufficient 0·9% saline to give a volume of 10 ml/kg. Ptosis was estimated by direct observation on a 0 to 8 scale (Rubin, Malone, Waugh & Burke, 1957; Fielden & Green,

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1966). The hearts from the 6 mice were pooled and the noradrenaline was extracted with butanol and assayed fluorimetrically (Fielden & Green, 1965). The heart-noradrenaline content of treated mice was calculated as a percentage of that in control groups treated with saline alone.

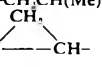
The aralkylguanidines were prepared as described by Fielden & others (1965). Doses are expressed in terms of free amine or guanidine base except where the salt is specifically mentioned.

Results

STRUCTURE-ACTIVITY RELATIONSHIPS FOR ANTAGONISM OF ADRENERGIC NEURONE BLOCKADE IN CATS

Antagonistic potency was assessed in anaesthetised cats from reversal of the blocking action of (−)-*N*-(1-phenylethyl)guanidine sulphate (2 mg/kg) on the responses of the nictitating membranes to sympathetic nerve stimulation. This drug is a powerful adrenergic neurone blocking agent in anaesthetised cats, but, unlike many related drugs, it has little contracting action of its own on the nictitating membranes (Fielden & others, 1965), hence it is well suited to quantitative reversal studies. A typical experiment is shown in Fig. 1. The response of the nictitating membranes at all rates of stimulation up to 30 pulses/sec was greatly reduced 1 hr after the (−)-*N*-(1-phenylethyl)guanidine. The antagonist was then injected intravenously and the responses of the membranes were re-examined 30 min later. If recovery was incomplete, additional doses of antagonist were given. The percentage recovery was generally much the same at all rates of stimulation, but with large doses of the most potent amines, the responses to low rates of nerve stimulation after recovery from block were sometimes greater than those observed before injection of the adrenergic neurone blocking drug. These amines did not potentiate responses to nerve stimulation when given alone. The relative potency of each antagonist was estimated from the total dose required to cause 50% recovery in the responses to stimulation at 10 pulses/sec. Each drug was tested on the contractions of both membranes in 2 or 3 cats. The doses listed in Table 1 are only approximate, since some variations occurred with different cats. *N*-Phenethylguanidine (IIIb) and

TABLE 1. REVERSAL BY ARALKYLAMINES AND ARALKYLGUANIDINES OF ADRENERGIC NEURONE BLOCKADE PRODUCED BY (−)-*N*-(1-PHENYLETHYL)GUANIDINE

X and R in Ph·X·NRZ		Dose (mg/kg) causing 50% reversal of block			
X	R	Amine (Z = H)	Cpd No.	Guanidine (Z = C(NH)NH$_2$)	Cpd No.
$-CH_2-$	H	No effect at 6	Ia	3	Ib
$-CH_2-$	Me	30% recovery at 10	IIa	0·2–0·4	IIb
$-(CH_2)_2-$	H	No effect at 6	IIIa	Blocks (see text)	IIIb
$-(CH_2)_3-$	H	No effect at 13	IVa	No effect at 6	IVb
(+)-CH(Me)−	H	4	Va	Blocks (see text)	Vb
$-CH(Me)CH_2-$	H	0·4	VIa	1·5–3	VIb
(+)-$CH_2CH(Me)-$	H	0·01–0·02	VIIa	10% recovery at 2	VIIb
	H	0·05–0·1	VIIIa	0·5–1	VIIIb

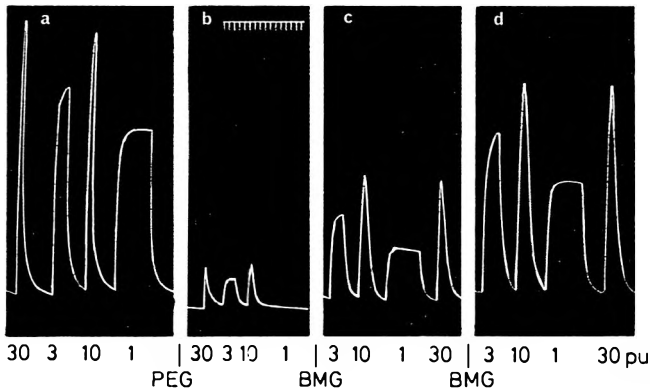
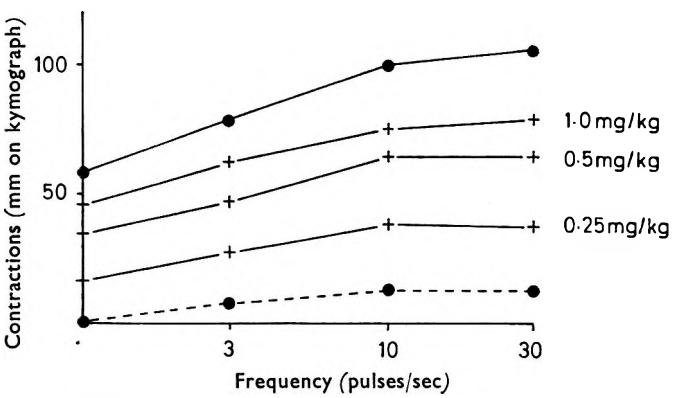


FIG. 1. A. Effect of drugs on contractions of the nictitating membranes produced by stimulation of the cervical sympathetic nerve in cats anaesthetised with chloralose. 200 Rectangular pulses (15V, 0·5 msec) were applied at frequencies of 1, 3, 10 or 30/sec. (a) Contractions of the right nictitating membrane before drugs. (b) 1 hr after 2 mg/kg of (−)-*N*-(1-phenylethyl)guanidine sulphate (PEG). Panels (c) and (d) show the responses 30 min after two consecutive doses of 0·25 mg/kg of *N*-benzyl-*N*-methylguanidine sulphate (BMG). (Time: 30 sec).



B. Mean contractions (mm on kymograph) of both membranes:— ●——● responses before drugs, ●- - -● responses 1 hr after PEG, +——+ responses 30 min after subsequent consecutive injections of 0·25, 0·25 and 0·5 mg/kg of BMG, giving the total doses specified on the graphs.

(+)-*N*-(1-phenylethyl)guanidine (Vb) exert some blocking action of their own in anaesthetised cats, hence reversal could not be conclusively demonstrated. However, we have shown previously that (+)-*N*-(1-phenylethyl)guanidine will prevent the blocking action of the (−)-isomer in anaesthetised cats if given either beforehand or simultaneously with it (Fielden & others, 1965). The parent amine of (−)-*N*-(1-phenylethyl)-guanidine, namely (−)-1-phenylethylamine, produced 50% reversal at about 1 mg/kg.

Although all the potent amines in Table 1 are primary amines, reversal of block also occurred readily with some secondary and tertiary amines;

for example, *N*-methylamphetamine produced 50% reversal at about 0·05 mg/kg, and *NN*-dimethylamphetamine at about 0·15 mg/kg.

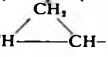
Reversal experiments with guanethidine as the blocking drug gave essentially similar results, although additional effects sometimes arose which rendered interpretation more difficult. Blockade by guanethidine sulphate (4 mg/kg) was half-reversed by 0·05 to 0·1 mg/kg of (+)-amphetamine (VIIa) or by 0·5 mg/kg of *N*-benzyl-*N*-methylguanidine (IIb). However, although *N*-(2-phenylpropyl)guanidine (VIb) (0·5–2 mg/kg) or *N*-(2-phenylcyclopropyl)guanidine (VIIIb) (0·5–2mg/kg) caused some restoration of responses after guanethidine, there was, at the same time, a pronounced and sustained contraction of the unstimulated nictitating membranes which made it impossible to assess quantititatively the response to nerve stimulation. These sustained contractions did not occur when either of these two drugs was injected into cats which had not had guanethidine.

(+)-Amphetamine and *N*-benzyl-*N*-methylguanidine failed to restore contractions of the nictitating membranes blocked by pempidine (5 mg/kg) or by phenoxybenzamine (2·5 mg/kg).

STRUCTURE-ACTIVITY RELATIONSHIPS FOR ANTAGONISM OF PTOSIS IN MICE

Antagonism experiments in mice were done to compare the potency of aralkylguanidines as antagonists of those adrenergic neurone blocking agents which also deplete the peripheral tissues of noradrenaline, such as guanethidine, with their potency against blocking agents which do not markedly lower noradrenaline levels, such as (−)-*N*-(1-phenylethyl)-guanidine. As the latter has only a short-lasting action in mice, the related, more persistent drug, *N*-[1-(2,4-xylyl)ethyl]guanidine (Fielden & Green, 1965), was used instead. Table 2 shows the relative potencies of eight aralkylguanidines and two aralkylamines in preventing ptosis when given subcutaneously to mice together with 10 mg/kg of guanethidine sulphate or *N*-[1-(2,4-xylyl)ethyl]guanidine sulphate. We have shown previously (Fielden & Green, 1965) that adrenergic neurone blockade in mice can be assessed from the extent of ptosis. None of the aralkylguanidines used as antagonists caused significant ptosis in mice when

TABLE 2. PREVENTION BY ARALKYLGUANIDINES AND ARALKYLAMINES OF PTOSIS PRODUCED BY ADRENERGIC NEURONE BLOCKADE IN MICE

Antagonist $Ph{\cdot}X{\cdot}N(R){\cdot}C(:NH)NH_2$			Approximate dose of drug causing 50% reduction in ptosis score when injected together with 10 mg/kg of	
X	R	Cpd No.	Guanethidine sulphate	*N*-[1-(2,4-Xylyl)ethyl]-guanidine sulphate
$-CH_2-$	H	Ib	3	2
$-CH_2-$	Me	IIb	0·4	0·2
$-(CH_2)_2-$	H	IIIb	2	0·6
$-(CH_2)_3-$	H	IVb	>20	20
(+)-CH(Me)–	H	Vb	2·5	1
$-CH(Me)CH_2-$	H	VIb	0·3	0·1
(+)-$CH_2CH(Me)-$	H	VIIb	15	not tested
	H	VIIIb	0·12	0·08
(+)-Amphetamine		VIIa	1·0	0·7
2-Phenylcyclopropylamine		VIIIa	3·0	4·0

given alone at doses up to 20 mg/kg, although some of them produced slight ptosis at doses higher than this.

Similar results to those in Table 2 were obtained when the antagonists were given after the blocking drug, as in the experiments with cats. *N*-(2-Phenylcyclopropyl)guanidine sulphate (VIIIb) (0·5 mg/kg) or *N*-benzyl-*N*-methylguanidine sulphate (IIb) (1·5 mg/kg) injected 1 hr after 10 mg/kg of guanethidine sulphate or *N*-[1-(2,4-xylyl)ethyl]guanidine sulphate reduced the ptosis score within a further 2 hr from between 5 and 6 to less than 2.

The antagonism displayed by these compounds is generally selective for ptosis resulting from adrenergic neurone blockade. This is illustrated for *N*-benzyl-*N*-methylguanidine in Table 3. However, ptosis

TABLE 3. PREVENTION BY *N*-BENZYL-*N*-METHYLGUANIDINE OF PTOSIS PRODUCED BY VARIOUS SYMPATHETIC BLOCKING DRUGS

Drug	Dose (mg/kg)	Time after injection (hr)	Ptosis score	
			Drug alone	Drug + *N*-benzyl-*N*-methylguanidine sulphate (5 mg/kg)
Xylocholine bromice	50	1	5·8	0
Guanethidine sulphate	20	2	6·2	1·0
Pempidine tartrate	20	1	4·0	3·3
Pentolinium tartrate	10	1	4·8	3·5
Phentolamine methanesulphonate	10	1	4·7	4·8
Phenoxybenzamine hydrochloride	10	1	5·3	5·2
Reserpine	0·25	4	5·7	3·0
Chlorpromazine hydrochloride ..	2·5	1	6·3	5·7

caused by other types of drug may be affected to some extent. As shown in Table 3, *N*-benzyl-*N*-methylguanidine caused some reduction in the ptosis produced by reserpine or by ganglion blocking agents. A rather more marked effect on ganglion blockade was found with (+)-amphetamine or *N*-(2-phenylpropyl)guanidine (VIb), both of which produce a distinct exophthalmos in mice. However, doses of these drugs sufficient to reduce the extent of ptosis do not significantly decrease the mydriasis caused by ganglion blocking agents.

COMPARISON OF EFFECTS ON GUANETHIDINE-INDUCED PTOSIS AND HEART-NORADRENALINE DEPLETION

As shown in Table 4, the reduction in the extent of ptosis, which occurs 4 hr after the subcutaneous injection of some aralkylguanidines into mice together with guanethidine sulphate (10 mg/kg), was accompanied by a decrease in the extent of heart-noradrenaline depletion. Larger doses (5–20 mg/kg) of *N*-(2-phenylcyclopropyl)guanidine (VIIIb) or *N*-benzyl-*N*-methylguanidine (IIb) almost completely eliminated the noradrenaline depletion as well as preventing ptosis.

When given after guanethidine, both (+)-amphetamine and *N*-benzyl-*N*-methylguanidine abolished ptosis without any dramatic recovery occurring in the heart-noradrenaline levels. Thus 6 hr after 10 mg/kg of guanethidine sulphate, mice had a ptosis score of 4–5 and noradrenaline levels about 20% of normal. In mice given (+)-amphetamine sulphate

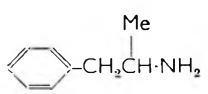
TABLE 4. EFFECT OF ARALKYLGUANIDINES ON GUANETHIDINE-INDUCED PTOSIS AND NORADRENALINE-DEPLETION IN MICE
Where more than one group of mice was used, the range of results is given in parentheses

Drug	Cpd No.	Dose (mg/kg)	Heart-noradrenaline content (% of control)		Ptosis score
			Drug alone	Drug + guanethidine	Drug + guanethidine
None		—	100(82–112)	20(18–21)	5·3(4·5–6·3)
N-(2-Phenylcyclopropyl)guanidine sulphate	VIIIb	0·1	77	35(34–36)	3·1(3·0–3·2)
		0·5	70	59(53–64)	0·2(0·0–0·3)
N-(2-Phenylpropyl)guanidine sulphate	VIb	0·1	98	25(25–25)	3·9(3·5–4·3)
		0·5	86	37(35–39)	0·8(0·5–1·0)
N-Benzyl-*N*-methylguanidine sulphate	IIb	0·3	75	30(29–30)	3·9(3·8–4·0)
		1·5	75	43(43–43)	0·4(0·3–0·5)
N-Phenethylguanidine sulphate	IIIb	2·5	49	32(32–32)	2·5(2·5–2·5)
		20	38	34(32–36)	0·5(0·3–0·7)

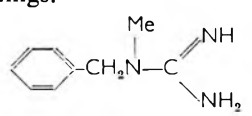
(5 mg/kg) or *N*-benzyl-*N*-methylguanidine sulphate (5 or 20 mg/kg) 4 hr after the guanethidine, ptosis had disappeared completely within a further 2 hr, but the noradrenaline level was still less than 30% of normal.

Discussion

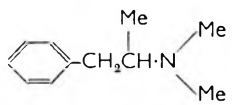
It has been shown that numerous aralkylamines with sympathomimetic properties can reverse adrenergic neurone blockade, but that the restoration of responses to nerve stimulation is not directly related to the sympathomimetic action (Day, 1962). Table 1 shows the effect of variation in the structure of the aralkyl group on the abolition by aralkylamines and aralkylguanidines of adrenergic neurone blockade in anaesthetised cats. Although there is no direct correlation between the aralkyl groups required for optimal activity in the two series—the guanidine derived from the most active amine, (+)-amphetamine (VIIa), had only marginal antagonistic activity, whereas the most potent guanidine, *N*-benzyl-*N*-methylguanidine (IIb), is derived from an almost inactive amine—there is some stereochemical similarity between the most active compounds. This stereochemical similarity is more striking between *N*-benzyl-*N*-methylguanidine (IIb) and *NN*-dimethylamphetamine (IX), which is less active than amphetamine and only slightly more active than *N*-benzyl-*N*-methylguanidine. These results are consistent with both types of compound acting at the same sites on the nerve endings.



(VIIa)



(IIb)



(IX)

The structural requirements for optimal antagonistic activity differ somewhat in mice, but even so, high activity is retained by both classes of

compound. However, (+)-amphetamine is no longer more potent than any of the guanidines, while in the guanidine series itself, the size of the aralkyl group required for maximum activity is increased. *N*-(2-Phenylcyclopropyl)guanidine (VIIIb) and *N*-(2-phenylpropyl)guanidine (VIb) are particularly potent in mice.

The selectivity displayed by these compounds in both cats and mice for counteracting adrenergic neurone blockade in preference to sympathetic blockade produced by other types of drug, suggests that reversal of adrenergic neurone blockade most likely results from displacement of the blocking drug from its binding sites at the nerve endings. Pre-treatment of rats with amphetamine has been shown to prevent the specific uptake of guanethidine into sympathetically-innervated tissues, and, when given subsequent to guanethidine, to displace the latter from its binding sites in these tissues (Chang, Costa & Brodie, 1965). Amphetamine also blocks the specific uptake of bretylium (Brodie, Chang & Costa, 1965).

However, additional actions must also be present. Besides having a weak anti-reserpine activity, which we have attributed elsewhere to monoamine oxidase inhibition (Fielden & Green, 1966), some of the guanidines can also partly relieve the ptosis caused by ganglion blocking agents. (+)-*N*-(1-Phenylethyl)guanidine (Vb) has been shown to produce a short-lasting antagonism of the relaxation of the nictitating membranes produced by pempidine in conscious cats. In contrast, the mydriasis caused by ganglion blockade is not significantly decreased by low doses of these drugs, hence the alleviation of ptosis probably results from an action peripheral to the ganglia. This action may be the local release of noradrenaline at the nerve endings, particularly since ptosis produced by α-receptor blocking drugs is resistant to either amphetamine or the guanidines.

It has been disputed whether guanethidine, which causes a marked loss of noradrenaline from sympathetically-innervated tissues, blocks sympathetic transmission by the same mechanism as drugs, such as xylocholine and bretylium, which do not cause noradrenaline depletion. Brodie and his co-workers (Brodie & others, 1965; Kuntzman, Costa, Gessa & Brodie, 1962) argued that it does not, and that whereas bretylium-like drugs simply block the outflow of noradrenaline from the nerve endings, guanethidine causes adrenergic neurone blockade by persistent activation of the normal noradrenaline-releasing mechanism at the nerve endings, so that no further response can occur on nerve stimulation. The parallelism found between the relative potencies of the amine and aralkylguanidine antagonists in preventing adrenergic nerve block by guanethidine and by *N*-[1-(2,4-xylyl)ethyl]guanidine, which does not cause noradrenaline depletion, suggests that these two types of drug block sympathetic transmission by an action at a common site.

Amphetamine not only prevents the adrenergic neurone blocking action of guanethidine but also reduces the noradrenaline depletion produced by this drug (Chang & others, 1965). Doses of the aralkylguanidines sufficient to decrease the extent of guanethidine-induced ptosis also always lessen the extent of noradrenaline depletion. However, the minimum dose

required to prevent ptosis completely is always lower than that required to eliminate depletion. It has been demonstrated that more than one uptake mechanism for drugs is present on nerve cell membranes (Iversen, 1965), consequently, guanethidine may reach the noradrenaline storage vesicles, from which it ultimately displaces the noradrenaline, by more than one route. One of these may be transference from the binding sites on the nerve cell membrane at which adrenergic neurone blockade is produced, and another an uptake mechanism not directly associated with liberation of the transmitter. If amphetamine and the aralkylguanidines can block the former process without themselves blocking nerve transmission, then the adrenergic neurone blocking action of guanethidine may be completely prevented with only partial loss of the noradrenaline-depleting action.

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Metal binding tendencies of various antibiotics

P. J. NIEBERGALL, D. A. HUSSAR*, W. A. CRESSMAN*, E. T. SUGITA AND J. T. DOLUISIO

A method to determine the presence of metal-drug complexes in dilute solutions is described. Using this method cycloserine was found to complex with cupric, nickelous, zinc and cobalt ions; streptomycin and novobiocin complexed with cupric ions; erythromycin complexed with cobalt ions, and chloramphenicol exhibited no metal binding tendencies. Various penicillins were found to interact with zinc and cupric ions. Preliminary investigations suggest that cupric ions, rather than simply complexing with penicillin as suggested by previous workers, promote the degradation of penicillin to penicilloic acid. Evidence is presented to confirm the presence of penicilloic acid in reaction mixtures initially containing penicillin G or V and cupric ions, and to establish that the reaction follows second order kinetics and ceases when all available cupric ion has been consumed. Good correlation was noted for these results and previous work which showed the effects of metal ions on the antibacterial properties of penicillin.

SINCE metal complexation may markedly change the physico-chemical properties of drugs, it is not surprising that, in certain instances, the *in vitro* stability (Guenther, 1950), as well as the *in vivo* distribution, storage, biotransformation and elimination of a drug may be influenced by the presence of metals (Beckett, 1958; Albert, 1960; Brock, 1962). It would appear, therefore, that a quantitative knowledge of the metal ion complexing tendencies of antibiotics would be of great value. Although investigations have been made previously, preliminary data from our laboratories showed discrepancies, in a few instances, with literature data. This investigation was made to expand earlier work, and to compare data for the possible interactions of chloramphenicol, erythromycin, cycloserine, streptomycin, novobiocin and various penicillins with certain divalent cations. The results both agree and disagree with those in some of the previous reports. The principal difference was with the penicillin G-cupric ion interaction reported by Weiss, Falab & Erlenmeyer (1957). We therefore discuss this in detail.

Experimental

REAGENTS AND EQUIPMENT

Methicillin sodium and oxacillin sodium were donated by Bristol Laboratories. Penicillin V potassium and penicillin G potassium were donated by Chas. Pfizer. Eli Lilly donated erythromycin and cycloserine. Novobiocin sodium, streptomycin sulphate, and chloramphenicol were donated by Upjohn, E. R. Squibb and Sons, and Parke, Davis respectively. The reagent grades of copper chloride ($CuCl_2 \cdot 2H_2O$), zinc chloride ($ZnCl_2$), nickel chloride ($NiCl_2 \cdot 6H_2O$), magnesium chloride ($MgCl_2 \cdot 6H_2O$), cobalt chloride ($CoCl_2 \cdot 6H_2O$), and calcium chloride ($CaCl_2 \cdot 2H_2O$) were obtained

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from the Mallinckrodt Chemical Works or J. T. Baker Chemical Company. Distilled, deionised water which was boiled for 1 hr to remove carbon dioxide was used. It had a specific conductance of $2{\cdot}50 \times 10^{-6}$ ohm^{-1} cm^{-1} or less, as determined by a Serfass conductivity bridge.

PROCEDURE

Thirty ml of a $1{\cdot}00 \times 10^{-3}$M solution of the antibiotic was diluted to 50·0 ml with water. The solution was then titrated potentiometrically, at $30 \pm 0{\cdot}2°$ with a standard solution of $2{\cdot}00 \times 10^{-2}$N sodium hydroxide at not greater than 0·4 ml increments. Absorption of carbon dioxide was shown not to be a factor, by duplicate titration on solutions both in the presence and absence of nitrogen. A Leeds and Northrup model 7401 pH meter or a Radiometer TTT-1 titrator was used to determine pH values.

To test for metal complexation, 15·0 ml of a $1{\cdot}00 \times 10^{-3}$M solution of divalent cation was mixed with 30·0 ml of the antibiotic solution. The resulting solution was diluted to 50·0 ml and titrated with standard sodium hydroxide solution in the manner described above.

With the penicillin salt derivatives and erythromycin, an equivalent amount of hydrochloric acid (1·50 ml of a $2{\cdot}00 \times 10^{-2}$N solution) was added, before dilution with water, to convert ionising groups to their acid form. In addition, with the penicillin salt derivatives, titrations were also made on solutions to which no acid had been added.

The metal solution was titrated alone with the standard sodium hydroxide solution after diluting 15·0 ml of the divalent cation solution to 50·0 ml.

Results and discussion

The method of Doluisio & Martin (1963) was used to evaluate complexes between drugs and metal ions. In this, at least two titration curves are required. The first is the potentiometric titration of the drug (Curve I) and the second is the potentiometric titration of the same concentration of drug reacted with divalent cation (Curve II). A downward displacement of Curve II, due to metal displacement of a proton, serves as a qualitative test for metal complexation *providing* Curve II is not simply a summation of a metal hydroxo curve and Curve I. The greater the tendency of the metal to combine with a given co-ordinating agent, the greater the drop in pH. When the apparent metal complexation takes place in pH regions where metal hydroxo formation may also occur, a third potentiometric titration curve (Curve III), that of metal ions alone, is required. The sum of curves I and III must then be compared with Curve II. When Curve II deviates from the summation curve (Curve IV), it may be assumed that metal-drug complexation is occurring. This analysis is based on the assumption that when two simultaneously occurring reactions, metal hydroxo formation and proton dissociation of drug, are independent and non-interacting the total base consumed at a particular pH should equal the sum of the base consumed when each reaction is run separately. However, if interaction between metal and

drug occurs, deviations should be apparent. Fig. 1 illustrates the type of curves resulting when there is no interaction, or only weak interaction, between metal and drug. In these cases Curve II coincides with Curve IV

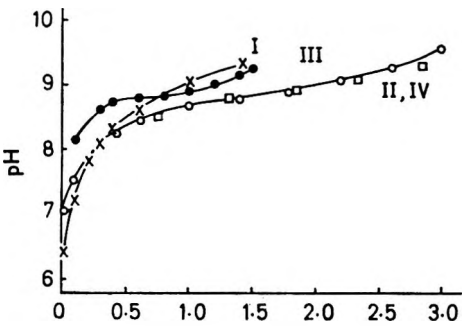

FIG. 1. Potentiometric titration of –●–● Co^{++}, –X–X– novobiocin sodium, –○–○– novobiocin sodium and Co^{++}; –□–□– summation of curves I and III. Titrant used was $2{\cdot}00 \times 10^{-2}$ N sodium hydroxide. Data indicate no complexation.

over the entire pH range investigated. Fig. 2 illustrates the type of curves resulting when simple mononuclear metal-drug complexes are formed. In these cases, Curve II is depressed from Curve I and bears no resemblance to either curves III or IV. The stoichiometric ratio of the drug to metal and the stability constants can be calculated from these data using the method described previously (Doluisio & Martin, 1963).

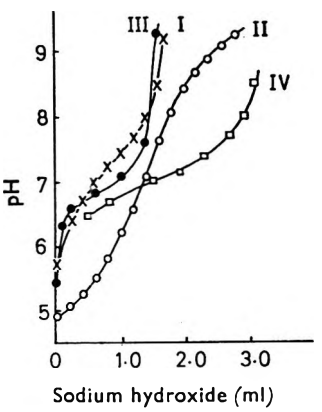

FIG. 2. Potentiometric titration of –X–X– cycloserine, –○–○– cycloserine and Cu^{++}, –●–● Cu^{++}; –□–□– summation of curves I and III. Titrant used was $2{\cdot}00 \times 10^{-2}$ N sodium hydroxide. Data indicate formation of a 1:1 and a 2:1 complex.

Table 1 summarises the metal complexing tendencies of erythromycin, cycloserine, novobiocin,chloramphenicol and streptomycin.

TABLE 1. METAL BINDING TENDENCIES FOR VARIOUS ANTIBIOTICS

Antibiotic	Complex formation	Probable weak complex formation	No Apparent complex formation*
Chloramphenicol ..	—	—	Ca^{++}, Co^{++}, Cu^{++} Mg^{++}, Ni^{++}, Zn^{++}
Cycloserine	Cu^{++}, Ni^{++}	Co^{++}, Zn^{++}	Ca^{++}, Mg^{++}
Erythromycin	—	Co^{++}	Ca^{++}, Cu^{++}, Mg^{++} Ni^{++}, Zn^{++}
Novobiocin sodium ..	—	Cu^{++}	Ca^{++}, Co^{++}, Mg^{++} Ni^{++}, Zn^{++}
Streptomycin sulphate ..	Cu^{++}	—	Ca^{++}, Co^{++}, Mg^{++} Ni^{++}, Zn^{++}

* The potentiometric method is not suitable for the study of weak complexation and, thus, it is possible for weak complexes to form and not be detected.

Cycloserine. Cycloserine was found to form 1:1 (log $K_1 = 5{\cdot}5$) and 2:1 (log $K_2 = 4{\cdot}7$) complexes with cupric ion. The over-all logarithmic stability constant of 10·2 compares favourably with the value of 9·7 reported by Neilands (1956). Weak interactions were also noted with nickel, zinc and cobalt ions. These results agree with those reported by Neuzil & Breton (1958). There was no evidence of complexation occurring between cycloserine and either magnesium or calcium ions.

Chloramphenicol. There was no evidence of complexation between chloramphenicol and the divalent cations investigated. This is in agreement with the findings of Ujiie (1957), who demonstrated that the antibiotic activity of this antibiotic is not affected by zinc, copper, magnesium and calcium ions.

Streptomycin. Of the cations tested, streptomycin was found to complex only with cupric ion (log $K_1 = 4{\cdot}3$). Foye, Lange, Swintosky & others (1955) reported the formation of chelates with cupric, cobalt, magnesium and nickel ions in solutions that were approximately 0·1 molar with respect to metal ions and streptomycin. Evidence for chelate formation was a drop in pH during the formation, production of characteristic colours, decreased water solubility (except for magnesium), and absence of metal ions in solutions having a pH greater than 5·0. Metal hydroxo formation, however, could also partially explain these observations. Indeed, nickel and cobalt hydroxo compounds were present in the precipitates obtained. It was also stated that a 3:1 cupric ion-streptomycin chelate formed. However, using a potentiometric titration method, Zahn & Eisenbrandt (1964) found only a 1:1 cupric ion-streptomycin complex to be present. When solutions containing cupric ion-streptomycin in the ratio of 3:1 were studied, the 1:1 chelate formed, and was followed by a precipitate of copper hydroxo compounds. The conclusion formed was that cupric ion and streptomycin formed only a 1:1 chelate. Zahn & Eisenbrandt (1964) also found no evidence for a magnesium chelate.

Novobiocin. Of the metals tested only cupric ions were found to complex with novobiocin and even this interaction appeared slight. Brock (1962) reported a magnesium complex with novobiocin and has suggested that this interaction may affect the antibacterial activity of the drug.

Erythromycin. Of the metals tested only cobalt ions were found to complex with erythromycin and this interaction appeared slight.

PENICILLIN ANALOGUES

The penicillin derivatives exhibited similar results when titrated in the presence of divalent cations. No interactions were evident with calcium, cobalt, magnesium or nickel ions. Deviations in the titration curves were noted in the presence of cupric and zinc ions. However, the deviations varied depending on the time lapse between the preparation and titration of the test solutions. The nature of the zinc-penicillin interaction has not yet been investigated, but preliminary investigations suggest that cupric ions, instead of simply complexing, promote the degradation of penicillins.

Weiss & others (1957) investigated the interaction of cupric ions and penicillin G potassium and found log K_1 to be 4·8. These authors stated that the stability constant is not related to the acidity constant. They suggested that the β-lactam ring is involved in the complexation and that this ring, after 3 hr, is unaltered by the presence of cupric ions. Before this report, Guenther (1950) noted that cupric ions split penicillin G into penicillamine and corresponding cleavage products. His qualitative results indicated that the reaction proceeds rapidly via the penicilloic acid intermediate in mildly acid solutions.

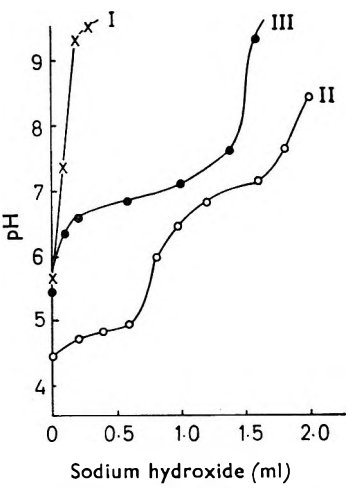

FIG. 3. Potentiometric titration of –X–X– penicillin V potassium, –○–○– penicillin V potassium and cupric chloride 30 min after mixing, and –●–●– cupric chloride. Titrant used was 2·00 × 10^{-2} N sodium hydroxide.

It was apparent, from our data, that when copper-penicillin interaction occurred, protons were displaced (Fig. 3). This proton displacement occurred after proton dissociation from the carboxyl group (pK_a = 2·8), and, as mentioned previously, was found to increase with time. This variation with time suggested that degradation was occurring. The

addition of disodium ethylenediaminetetra-acetic acid (EDTA) prevented this proton displacement.

The penicilloic acids, which form upon rupture of the β-lactam ring, give an intense blue colour with an arsenomolybdic acid-mercuric chloride reagent described by Pan (1954). A small amount of this reagent was added to aliquots of a pH 5·50 reaction mixture that initially contained equivalent amounts of penicillin G or V and cupric chloride. A blue colour, which intensified markedly with time appeared. When a similar reaction mixture was prepared without cupric ion, a very light blue colour appeared immediately. This colour did not intensify with time, and was attributed to a slight degradation of the penicillin caused by the reagent itself.

The ultraviolet absorption spectra of $3{\cdot}00 \times 10^{-4}$M solutions of penicillin G and V potassium in 0·12M acetate buffers at pH 4·00, 4·50, 5·00 and 5·50 were determined. Identical spectra were obtained for the four solutions. Solutions were made in the same buffers, and at the same penicillin concentrations, but containing an equimolar concentration of cupric chloride. The presence of the cupric ion did not immediately alter the spectra, which were determined by means of a Photovolt model 43 linear-log recorder attached to a Beckman model DB spectrophotometer.

Solutions containing $3{\cdot}00 \times 10^{-4}$M penicilloic V or G acid were made in the same acetate buffers. The spectra obtained were identical with the respective penicillin spectra down to 250 mμ. Solutions containing equimolar $3{\cdot}00 \times 10^{-4}$M amounts of penicilloic acid and cupric chloride were observed. The absorption spectra for penicilloic V acid-cupric ion

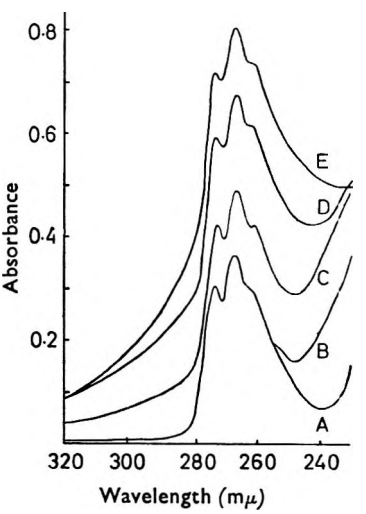

FIG. 4. Absorption spectra of A, penicilloic V acid $3{\cdot}00 \times 10^{-4}$ M; B, penicillin V $3{\cdot}00 \times 10^{-4}$ M both with and without equimolar concentrations of cupric ion; C, penicillin V and cupric chloride after 20 min; D, penicillin V and cupric chloride after 40 min; E, penicilloic V acid and cupric chloride immediately after mixing at pH 5·50, using 1 cm cells.

solutions show that the peaks at 273 mμ and 266 mμ, unlike those for the penicillin V-cupric ion solutions, rose markedly and were similar to spectral curves produced by penicillin V-cupric ion solutions after about 18·5 hr (Fig. 4). These data indicate that the conversion of penicillin V into penicilloic V acid in the presence of cupric ion could be followed at either 273 or 266 mμ. Parallel results were obtained with penicillin G at 245 mμ. The penicilloic acids were obtained by hydrolysis of penicillin at pH 12 for 20 min at room temperature (Rapson & Bird, 1963). These solutions were then adjusted to pH 6·50 and either used or discarded within 4 hr.

To establish the order of the reaction with respect to each of the reactants, a series of solutions was prepared containing the same amount ($3{\cdot}00 \times 10^{-4}$M) of penicillin G or V potassium, but varying the initial amounts of cupric chloride from 0·50 to $3{\cdot}00 \times 10^{-4}$M. The change in absorbance for these solutions was measured as a function of time, using a Beckman model DB spectrophotometer with the cell compartment thermostated at 32°. The initial rates, obtained graphically from plots of absorbance against time were plotted against cupric chloride concentration. The resulting linear relationship indicated a first order dependence upon cupric ion. The experiment was repeated, keeping the cupric chloride concentration constant at $3{\cdot}00 \times 10^{-4}$M while varying the penicillin concentration from 0·13 to $3{\cdot}00 \times 10^{-4}$M. The plots of initial rate against penicillin concentration were also linear, indicating a first order dependence upon penicillin concentration.

In view of the spectral differences between penicillin V and penicilloic V acid in the presence of cupric ion, we decided to conduct kinetic studies by following the change in absorbance at 273 mμ. The percent penicillin V remaining at any time can be obtained using the general method of Frost & Pearson (1961), and the following:

$$A_0 = c\,a \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

$$A_0 = c\,(a-x) + c'\,x \quad .. \quad .. \quad .. \quad (2)$$

$$A_\infty = c'\,x = c'\,a \quad .. \quad .. \quad .. \quad .. \quad (3)$$

in which A_0 is the absorbance of a solution containing equimolar amounts of penicillin V potassium and cupric chloride at initial concentration a, assuming that any absorbance contribution due to the cupric chloride would be negligible, c is the molar absorptivity of penicillin V, x is the amount of penicilloic V acid formed, A_∞ is the absorbance of the solution after complete conversion to penicilloic V acid and c′ is the molar absorptivity of penicilloic acid in the presence of an equimolar amount of cupric ion. Thus we find that:

$$\%\ \text{Penicillin V remaining} = \frac{a-x}{a} = \frac{A_\infty - A_t}{A_\infty - A_0} \times 100 \quad .. \quad .. \quad (4)$$

The metal caused degradation of the penicillins is generally spoken of as the "metal catalysed" degradation. If this were true, by the classical definition for catalysis, the reaction should follow pseudo first order

kinetics. In this event, a plot of the logarithm of the absorbance terms in equation 4 against time would yield a straight line.

Solutions containing equimolar 3×10^{-4}M penicillin V and cupric chloride at pH 4·00, 4·50, 5·00 and 5·50 were made in 0·12M acetate buffers. The value for A_∞ was obtained using a solution containing equimolar 3×10^{-4}M amounts of penicilloic V acid and cupric chloride. The change in absorbance of the solutions was followed at 273 mμ, at $30 \pm 0{\cdot}1°$.

In all instances, first order plots of the data showed marked curvature, indicating that cupric ion was being consumed in the reaction. The reaction would therefore be expected to follow second order kinetics, and the following equation would apply for this system:

$$\frac{A_\infty - A_0}{A_\infty - A_t} = a\,k\,t + 1 \qquad .. \qquad .. \qquad .. \quad (5)$$

in which t represents time, k is the second order rate constant and the other terms have the meaning previously given. The data plotted according to equation 5, as shown in Fig. 5, indicate a second order

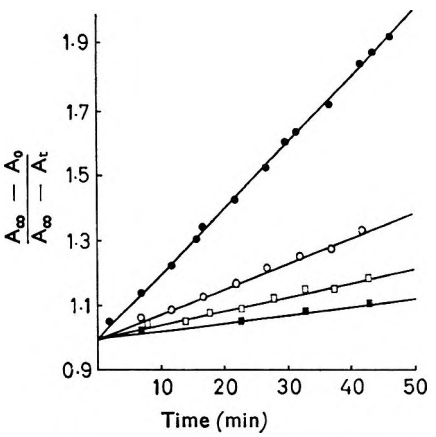

FIG. 5. Second order plots for reaction mixtures consisting of equimolar $3{\cdot}00 \times 10^{-4}$ M penicillin V potassium and cupric chloride at –■–■– pH 4·00, –□–□– pH 4·50, –○–○– pH 5·00, –●–●– pH 5·50 at 32°.

overall reaction. It should be noted that a further test of the suitability of the plots would be the observation that the intercept of this equation should be equal to 1·00. A *t*-test was made on all of the intercepts, which had been calculated through the method of least squares. In all instances, the intercepts did not differ significantly from the expected value at the 95% confidence level.

The rate constants were evaluated by the method of least squares, and found to increase markedly with pH within the range studied. The half lives for the reaction mixtures at the concentration used were found to

vary between 50–120 min depending upon pH. It should be emphasised that the kinetic data are introduced at this point only to demonstrate the order of the reaction, and to help estimate the speed of the reaction. Rate constants are not shown, since the runs were made at only one concentration of reactants, and only the appearance of penicilloic V acid was followed.

Since the reaction was shown to be second order, it was felt that it would be reasonable to assume that cupric ion is not catalysing the reaction, in the classic sense, but is promoting it, appearing as a penicilloic acid-cupric ion complex. The distinction between the terms "catalysed" and "promoted" may shed some light on the mechanism by which additives such as citrates, phosphates and EDTA stabilise penicillin solutions. Since it is well known that heavy metal ions inactivate the penicillins, these metal ion (cupric) chelators have been added to penicillin solutions to prevent deterioration by removing free metal ion which may have been introduced as a contaminant. It has also been suggested (Schwartz & Buckwalter, 1962) that their principal effect is to buffer the solutions to a pH range where the degradation rate is minimal. We prepared solutions containing penicillin V and cupric chloride in the ratio of 10:1, in a pH 5·50 0·12M acetate buffer. The reaction should have ceased when 10% of the penicillin V had been degraded, since the pH used gives excellent stability in the absence of cupric ions. The reaction, as followed at 273 mμ, apparently ceased with 90·6% penicillin V remaining (calculated using equation 4). Thus it would appear that in solutions containing trace amounts of metal ion, the effect of citrates would be primarily one of a buffering action.

Another interesting development during these investigations was the effect of cupric ion upon the re-arrangement of both penicillins G and V into their corresponding penicillenic acids. At pH 5·50 in 0·12M acetate buffer at 32°, penicillin G (3×10^{-4}M) in the absence of cupric ion develops a marked 322 mμ peak after 25 hr and increasing up to 72 hr which is characteristic of the penicillenic acids; in the presence of an equimolar amount of cupric chloride, the 322 mμ peak does not appear. The absorbance at 322 mμ in the presence of cupric ion is due to the spectral shift accompanied by the hydrolysis of the penicillin into the penicilloic acid. The same spectral shift, with no 322 mμ peak can be seen in Fig. 4 for penicillin V (3×10^{-4}M) in 0·12M acetate buffer at pH 5·50, although in the absence of cupric ion, a peak does appear. Thus it appears that the mechanism of degradation, as well as the rate appear to be influenced by the presence of cupric ions.

Our results compare well with those obtained by Ujiie (1957), who found that magnesium and calcium ions did not affect zones of inhibition produced by penicillin. Our results showed that these ions do not interact with penicillin. Ujiie (1957) also found that cupric and zinc ions decreased the zones to almost zero; our findings were that cupric ions promoted the degradation of penicillins to penicilloic acids, and that zinc interacted with penicillin. In the present investigation EDTA prevented the cupric ion promoted degradation of penicillins G and V while

Ujiie (1957) found that EDTA removes the zone decreasing effect of cupric ion.

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The antifungal activity of some thiocyanatoaniline derivatives

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A series of derivatives of *p*-thiocyanatoaniline has been synthesised and tested *in vitro* for activity against dermatophytes; some structure-activity relationships are discussed. One of the more highly active compounds, 2,6-dichloro-4-thiocyanatoaniline, showed some activity against experimental dermatophyte infections when applied topically, was of low toxicity to animals, and did not sensitise them. In a clinical trial the compound also showed some therapeutic activity but caused skin sensitisation.

THE fungicidal activity of a variety of organic sulphur compounds is well known but only a few such investigations of aromatic thiocyanates have been reported (Wilcoxon & McCallan, 1935; Walker, 1937; Walker, Morell & Foster, 1937; Morel, 1946; Davies & Sexton, 1946; Muncie & Morofski, 1949). More recently Landis, Kley & Ercoli (1951) examined a number of oxyalkyl thiocyanate derivatives against several dermatophytes *in vitro* and Pohloudek-Fabini & Weuffen (1960; 1964) have recorded the marked fungistatic activity of a variety of aryl thiocyanates. We have therefore prepared a series of simple derivatives of *p*-thiocyanatoaniline and have tested them against a range of dermatophytes *in vitro*. Because of its higher activity 2,6-dichloro-4-thiocyanatoaniline was further examined *in vitro* and *in vivo*.

Experimental

4-Thiocyanatoanilines were readily prepared by the method of Kaufmann & Oehring (1926). Some were brominated in position 2 and/or position 6 as described by Dienske (1927). Acetyl derivatives were obtained by known methods (Dienske, 1927; Smith & Orton, 1908). Benzoylation was accomplished with benzoyl chloride in pyridine, and chloroacetyl and dichloroacetyl derivatives were prepared by using the acid chloride in benzene. Melting points are given in Table 1. Satisfactory analyses were obtained for all novel compounds.

BIOLOGICAL METHODS

The fungistatic activity of acetone solutions of the compounds was determined *in vitro* by agar-dilution tests using Sabouraud agar. Plates were surface-inoculated using a multi-point inoculator (Hale & Inkley, 1965) with 0·01 ml of suspensions of fungal spores (prepared from 7–21 days old agar slopes with 2 ml of Sabouraud broth). Some compounds were also tested in Sabouraud liquid medium using twofold serial dilutions in 2 ml amounts. The inoculum was 0·02 ml of a spore suspension prepared as above. Fungistatic tests on compounds formulated in oil-in-water creams (Polawax 15%, liquid paraffin 25% and distilled water to 100%) were made by the diffusion method of Burlingame & Reddish (1939).

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TABLE 1. THE ANTIFUNGAL ACTIVITY OF *p*-THIOCYANATOANILINE AND SOME HALOGENATED DERIVATIVES

Compound No.	R	R′	X	m.p.	Minimum inhibitory concentration (μg/ml) after 7 days at 25°		
					Trichophyton sp.*	*Microsporon* sp.†	*Epidermophyton floccosum* WB2
1	H	H	–	57°	50	12·5–50	50
2	H	H	2-Cl	67°	3·0–12·5	3·0	3·0
3	H	H	2-Br	82°	3·0–12·5	1·0	3·0
4	H	H	3-Cl	75°	12·5	3·0–12·5	3·0
5	H	H	3-I	85–86°	12·5–50	12·5	12·5
6	Me	Me	2-SCN	94·5–95·5°	3·0	1·0	1·0
7	Me	Me	3-Cl	72–73°	3·0	3·0	3·0
8	Pr	H	2-Cl	40–41°	3·0	3·0	0·3
9	Ac	H	2-Cl	141–142°	12·5	3·0	3·0
10	Ac	H	2-Br	144–145°	3·0–12·5	3·0	3·0
11	Ac	H	3-I	194°	12·5–50	12·5	12·5
12	$ClCH_2·CO$	H	2-Cl	105–107°	0·3–1·0	0·3	0·3
13	$Cl_2CH·CO$	H	2-Cl	93–94·5°	1·0–3·0	3·0	1·0
14	H	H	3-Cl,2-Me	112–113°	12·5	3·0	3·0
15	H	H	2,6-Cl_2	96·5–97°‡	0·1–1·0	0·3	0·3
16	H	H	2-Br,6-Cl	93–94°	0·1–3·0	0·3	0·3
17	H	H	2,6-Br_2	123°	0·3–1·0	0·3	1·0
18	H	H	2-Br,3-Me,6-Br	104–105°	1·0	0·3	1·0
19	H	H	2,3-Cl_2	141–142°	3·0	1·0	1·0
20	H	H	2,5-Cl_2	118–119°	3·0	1·0	1·0
21	H	H	3,5-Cl_2	166–167°	3·0	1·0	1·0
22	Me	Me	2,3-Cl_2	62–62·5°	3·0	1·0	1·0
23	Me	H	2,5-Cl_2	94°	3·0	1·0	1·0
24	Me	Me	3,5-Cl_2	165–166°	12·5–50	12·5	50
25	Ac	Ac	2,6-Cl_2	95°	12·5	12·5	12·5
26	Ac	H	2,6-Cl_2	161–162°	50	12·5	12·5
27	$ClCH_2·CO$	H	2,6-Cl_2	158–159°	12·5	12·5	12·5
28	$Cl_2CH·CO$	H	2,6-Cl_2	191°	3·0	3·0	3·0
29	Ph·CO	Ph·CO	2,6-Cl_2	176–177°	50	12·5	50
30	Ph·CO	H	2,6-Cl_2	142°	50	12·5	50
31	$Cl_2CH·CO$	Me	2,5-Cl_2	103°	3·0–12·5	12·5	12·5
32	Ac	H	3,5-Cl_2	156–157°	12·5	1·0–3·0	1·0
33	H	H	2,6-Me_2	87–88°	50	50	200
34	Me	Me	–	72–73°	50	50	50

* *Trichophyton* sp. = *T. mentagrophytes* A280, *T. interdigitale* NCTC 175, *T. verrucosum* NCTC 168 *T. tonsurans* WB1, *T. rubrum* WB2.

† *Microsporon* sp. = *M. audouini* NCTC D617, *M. canis* Arkland 834E.

‡ On treatment with aqueous alcoholic sodium hydroxide solution, di(4-amino-3,5-dichlorophenyl) disulphide, m.pt 174°, was obtained. Lempert, Beke & Borovansky (1956) give m.pt 171·5–172·5°.

Fungicidal activity was determined using the Coates, Drain, Macrae & Tattersall (1959) modification of the method of Golden & Oster (1947). In tests with formulated compounds the mycelium-covered membranes were embedded in the creams for 1 or 2 hr, washed and plated.

In vivo tests were made in guinea-pigs infected with *T. mentagrophytes* (A280) using the method of Martin (1959). Commencing on the day after infection, the formulated compounds were applied once daily for 7 days, one animal being used for each preparation. Other guinea-pigs were infected with *M. canis* and after 5 days, when this infection was established, topical treatment with formulated compound was given once daily for 6 days.

Results and discussion

The fungistatic activity of the compounds is shown in Table 1. Our results confirm reports of the activity of *p*-thiocyanatoaniline, Compound 1 (Ortel & Weuffen, 1959; Zsolnai, 1962). The introduction of a halogen atom into the ring (Compounds 2–5) enhanced activity and this was further

increased by the introduction of a second halogen atom (Compounds 15–17 and 19–21). The insertion of a second thiocyanato-group, a "pseudo-halogen" (Compound 6), was also highly effective in increasing fungistatic activity. There are indications that halogen substitution in position 2 or 6 (Compounds 15–17) is more effective than substitution in other positions. Halogenation may enhance the lipid solubility of *p*-thiocyanatoaniline and so facilitate penetration into the dermatophytes. However, Pohloudek-Fabini & Weuffen (1964) found that although halogenation increased the antifungal activity of Schiff's bases of thiocyanatoaniline, there was no definite relationship between the activity of the compounds and their solubility in organic solvents or water. Acylation of, or substitution in, the amino-group generally diminished activity, and alkyl substitution in the ring did not enhance it (e.g. compare Compounds 1 and 33).

It has been suggested that the thiocyanato-group reacts with sulphydryl groups of fungal enzymes, because the antifungal activity of several organic thiocyanates is antagonised by cysteine (Zsolnai, 1962). However, bacteria also possess essential sulphydryl-containing enzymes, yet concentrations of less than 50 μg/ml of the thiocyanates that we have examined did not inhibit the growth of a range of pathogenic bacteria. Further work is needed to elucidate the mode of action of these relatively specific antifungal compounds.

The fungistatic activity of four of the most active compounds was much greater than that of a number of agents used in the treatment of dermatophyte infections (Table 2). When the activity of the formulated thiocyanates was compared with that of aqueous suspensions in diffusion tests, 2,6-dichloro-4-thiocyanatoaniline (Compound 15) was satisfactorily released from the cream and was studied further. In fungistatic tests in Sabouraud broth, it showed little decrease in activity in the presence of

TABLE 2. THE ACTIVITY IN LIQUID MEDIA OF SOME AROMATIC THIOCYANATES COMPARED WITH OTHER ANTIFUNGAL AGENTS

Compound		Minimum inhibitory concentration μg/ml after 7 days at 26°							
		T. mentagrophytes A280	*T. tonsurans* WB1	*T. interdigitale* NCTC 175	*T. rubrum* WB2	*T. verrucosum* NCTC 168	*M. audouini* NCTC D617	*M. canis* Arkland, 834E	*Epid. floccosum* WB2
15	2,6-Dichloro-4-thiocyanatoaniline	1·56	1·56	0·39	0·39	0·19	0·78	0·78	0·78
17	2,6-Dibromo-4-thiocyanatoaniline	1·56	3·1	0·39	0·39	0·39	0·78	0·78	0·39
16	2-Bromo-6-chloro-4-thiocyanatoaniline	0·78	1·56	0·39	0·39	0·39	0·39	1·56	0·39
12	α,2-Dichloro-4-thiocyanatoacetanilide	1·56	1·56	0·78	0·39	0·39	0·19	0·78	0·39
	3-(*p*-Bromophenoxy) propyl thiocyanate*	6·25	6·25	3·1	3·1	3·1	6·25	3·1	6·25
	Undecenoic acid B.P.	12·5	12·5	6·25	12·5	12·5	12·5	12·5	6·25
	Griseofulvin B.P.	12·5	3·1	12·5	1·56	1·56	0·39	1·56	1·56
	Dichlorophen B.Vet.C.	6·25	3·1	1·56	6·25	3·1	3·1	6·25	3·1
	N-Butyl-4-chloro-2-hydroxybenzamide†	6·25	6·25	6·25	1·56	3·1	3·1	3·1	6·25

* Landis, Kley & Ercoli (1951).
† This was tested as a 10% tincture also containing 1% salicyclic acid (Korger & Nesemann, 1960).

10% ox serum but there was an eightfold loss in activity when the concentration of serum was increased to 50%. A 0·5% aqueous suspension or a 1% formulation of the compound was not fungicidal to *T. mentagrophytes* or *M. canis* after 2 hr contact.

Although the lower alkyl thiocyanates are highly poisonous to mammals due, perhaps, to the liberation of cyanide (see Negherbon, 1959) aromatic thiocyanates are much less toxic. Compound 15 was not found to be acutely toxic or irritant to laboratory animals. It is a white solid without unpleasant odour or lachrimatory properties. Dr. B. Lessel (private communication) determined that the acute LD50 of this compound for albino mice was about 250 mg/kg by the oral route and 1,300 mg/kg when injected subcutaneously. When applied as a 2% oil-in-water cream it was not irritant to the skin of rabbits and did not cause sensitisation reactions in guinea-pigs. Further experiments were therefore made *in vivo*.

In guinea-pigs infected with *T. mentagrophytes* the topical application of Compound 15 as a 2·0% cream, the highest concentration tested, reduced the lesions but this was less effective than similar application of griseofulvin 1·0%. Orally or subcutaneoulsy administered Compound 15, at 200 mg/kg daily for 6 days, failed to reduce the extent of lesions; griseofulvin at 60 mg/kg had a significant effect. Topical application of Compound 15 did not completely eliminate the *M. canis* infection but the extent of fluorescence under Wood's light was reduced, particularly with a 2·0% cream. The reduction in the extent of fluorescence was greater with the topical application of a 0·1% cream than with one containing 2% 3-(*p*-bromophenoxy)propyl thiocyanate and was similar to that of a glycol solution containing 1% griseofulvin. Although highly inhibitory to dermatophytes *in vitro* the topical administration of Compound 15 is thus only moderately effective in reducing experimental infections. It is well known that many compounds which are highly active *in vitro* show disappointing activity when used for topical medication. Probably, as Campbell (1964) has recently re-emphasised, this is partly due to the difficult problem of the penetration of keratin.

Unfortunately this compound can cause sensitisation in man. In a limited clinical trial of a stable cream formulation containing 0·1% of Compound 15 the preparation showed some therapeutic effect but skin reactions appeared in 6% of patients. Positive patch tests to this thiocyanate were obtained in several of them (Dr. P. T. Main, private communication). This result exemplifies the inadequacy of animal tests to detect compounds which can cause skin sensitisation in man (see Carter & Griffith, 1965). We are unaware of previous reports of organic thiocyanates acting as skin sensitisers.

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Evidence for a persistent central depressant action seen after recovery from anaesthesia

K. THURÁNSZKY, G. RABLOCZKY AND A. KÉKES-SZABÓ

The fate of the classical adrenaline reversal in anaesthetised and in conscious cats was investigated. It seems that anaesthesia has some late effects on pharmacological responses that can be demonstrated for up to 72 hr after recovery of consciousness. The adrenaline reversal typical of the anaesthetised cat persisted for up to 72 hr after recovery of consciousness, thereafter the response to intravenous adrenaline was entirely pressor.

IT has been shown previously (Thuránszky, 1966a) that adrenergic blocking agents like dihydroergotoxine reverse the pressor effect of adrenaline in anaesthetised animals but that this is not so in non-anaesthetised animals.

We often observed in the animals after they had awakened from anaesthesia and when their behaviour was apparently quite normal, that the pressor effect of adrenaline remained reversible by dihydroergotoxine. We decided to follow the response to adrenaline after dihydroergotoxine-pretreatment to see for how long after recovery from anaesthesia the response remained reversed.

Experimental

METHOD

The blood pressure of cats in anaesthesia and during and after the awakening period was recorded by Thuránszky's method (1966b).

After awakening, the animals were regarded as normal if the behavioural responses, feeding habits and blood pressures responses evoked by exteroceptive stimuli appeared to be the same as those of animals which had not been anaesthetised (Thuránszky, 1966c).

The variations of the pressor effect of adrenaline in a dose of 10 μg/kg were investigated under ether or hexobarbitone anaesthesia and at various intervals after awakening. The cats were all pretreated with dihydroergotoxine.

One of our experiments made during and after hexobarbitone anaesthesia is shown in Fig. 1. In panel (1) the effect of adrenaline (10 μg/kg i.v.) on the mean blood pressure of a cat anaesthetised with 0·1 g/kg hexobarbitone is shown. In (2) is recorded the effect of the same dose of adrenaline after intravenous pretreatment with 0·5 mg/kg dihydroergotoxine. In panels (3)–(6) are shown the responses to 10 μg/kg adrenaline given 15 min after 0·5 mg/kg dihydroergotoxine, recorded at 24, 36, 48 and 72 hr respectively after recovery from anaesthesia. The response was still reversed at 24 hr, biphasic at 36 and 48 hr and entirely pressor at 72 hr and subsequently.

In Fig. 2 the degree of elevation or depression of the blood pressure is demonstrated in mm Hg related to the starting blood pressure level. These animals were pretreated with 0·5 mg/kg dihydroergotoxine 15 min

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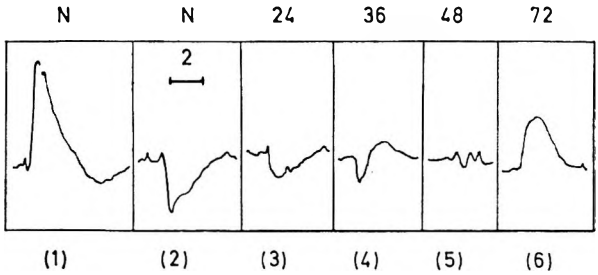

FIG. 1. Panel 1 shows the rise in blood pressure induced by 10 μg/kg adrenaline given intravenously in a cat anaesthetised with hexobarbitone (0·1 g/kg). After 0·5 mg/kg dihydroergotoxine the response to the same dose of adrenaline was reversed (panel 2). Panels 3, 4, 5 and 6 show the gradual change in the response to adrenaline from adrenaline-reversal to an entirely pressor response in the cat at 24, 36, 48 and 72 hr after recovery of consciousness. The injections of adrenaline were given 15 min after pre-treatment with 0·5 mg/kg dihydroergotoxine. N represents the anaesthetised state. Time: 2 min.

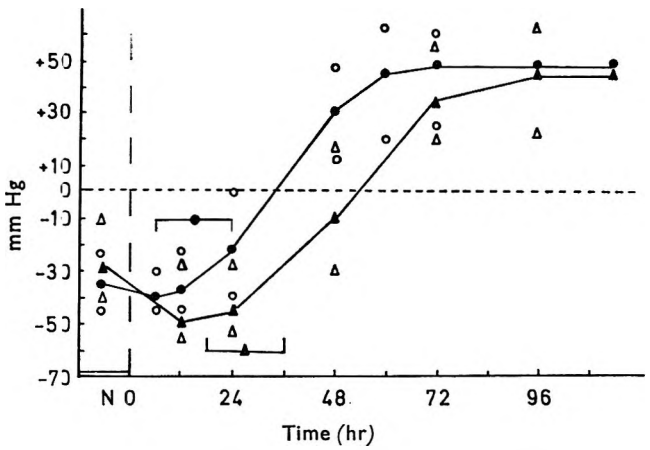

FIG. 2. The change in the response of the blood pressure of the cat to intravenous adrenaline after pretreatment with dihydroergotoxine, related to the anaesthetised state and the time after recovery of consciousness. Ordinate: change in b.p. in mm Hg; 0 is the baseline b.p.; + indicates a rise and — a fall in b.p. Abscissa: N is the anaesthetised state; 0 is the time of recovery of consciousness; 24, 48, 72, 96 hr after recovery of consciousness. Mean results obtained with ether as the anaesthetic are shown thus ●, and with hexobarbitone thus ▲. The bracketed circle and triangle show the period after awakening when the behavioural responses and blood pressure reflexes evoked by exteroceptive stimuli are normal. Open symbols show the range.

before each injection of adrenaline. The effect of adrenaline was investigated under ether or hexobarbitone anaesthesia and at various time intervals after awakening. It is obvious from Fig. 2 that there is a reversed effect of adrenaline both under anaesthesia with ether or hexobarbitone and in the early period after awakening.

After hexobarbitone anaesthesia the effect of adrenaline is still reversed after 48 hr, and becomes a pressor response only after the 72 hr. With

ether anaesthesia the reversed effect is abolished somewhere between 48 and 72 hr. In these experiments the anaesthesia persisted from 5 to 45 min. The variations of duration of the anaesthesia between such limits did not affect significantly the time for post-anaesthetic recovery of the pharmacological response mentioned above. Following a short-term anaesthesia with chlorethyl (shorter than 5 min) the effect of adrenaline became normal in 48 hr.

Discussion

We conclude that there is a difference between the time necessary for post-anaesthetic recovery of behavioural responses and recovery of some pharmacological responses. The observation of the reversed effect of adrenaline produced by dihydroergotoxine is a suitable test to demonstrate the late effect of anaesthesia. It was reported by Thuránszky (1966a) that in conscious animals adrenergic blocking drugs could not reverse the effect of adrenaline whereas they reversed it in the anaesthetised state. In conscious animals adrenaline also produces a pressor response after administration of dihydroergotoxine. The present study makes it evident that after recovery from the anaesthetic it is possible to produce typical adrenaline-reversal with dihydroergotoxine, although at this time the animal is visually quite normal and awake and its behaviour and vegetative responses evoked by exteroceptive stimuli are normal, too. There is also a late effect of anaesthesia after 24 hr. The animal can only be regarded as normal pharmacologically when the typical adrenaline-reversal, after administration of dihydroergotoxine, has already disappeared and the adrenaline effect has returned to a pressor response. This total "awakening" takes places in the 72 hr after anaesthesia.

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A note on the effects of some psychotropic drugs on the aggressive behaviour in the ant, *Formica rufa*

WOJCIECH KOSTOWSKI

AGGRESSIVE behaviour in lower animals has not been analysed pharmacologically. Inhibition of the motor activity with simultaneous outbursts of aggressiveness seems to be characteristic in ants treated with reserpine (Kostowski, Beck & Mészáros, 1965; 1966). Among the invertebrates, ants are a suitable subject for studies on aggressiveness because of their tendency to attack other species.

We have assessed the influence of drugs on aggressive behaviour in ants and mice.

Methods

ANTS

A plastic container 35 × 35 × 120 mm was divided by a removable wall into two compartments. Into one of these compartments, 10 ants (*Formica rufa*) were placed and into the other, one beetle (*Geotrupes* sp.). This species, because of its massive structure, was resistant to ant bite and poison. After 15 min for calming and adaptation the dividing wall was removed, when immediately the ants attacked the beetle. The number of attacking ants was counted after one, two and three min. Accumulation of formic acid secreted by ants during the attack was prevented by ventilating the container by means of small holes. The beetles could be used repeatedly after one-day intervals.

An index of aggressiveness was assessed. This was the number of attacking ants × 10.

Drugs. Reserpine, lysergic acid diethylamide, phenobarbitone and (±)-amphetamine were given orally with honey; chlordiazepoxide and chlorpromazine because they were rejected in food, were injected in 0·6% saline into the abdominal cavity in 0·3–0·5 μl amounts with a microsyringe.

MICE

The modified fighting-mice test of Tedeschi, Tedeschi, Muche, Cook, Mattis and Fellows (1959) was used. The amplitude of impulses (50 msec duration, 3 cycles/sec) was calculated as 10 V less pain threshold voltage. The aggressiveness was calculated as a percentage of fighting pairs during 3 min stimulation. The same drugs as for the ants were used. They were injected in 0·9% saline, intraperitoneally.

Results

ANTS

In control experiments the index of aggressiveness was 39, 41 and 44, after one, two and three min respectively. Because the values obtained

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for each minute were in general not statistically distinguishable, the results are presented as a mean index of aggressiveness obtained from all three counts (Fig. 1).

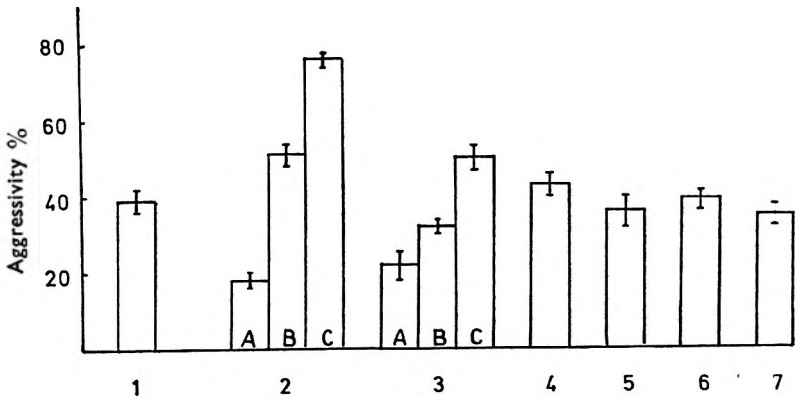

FIG. 1. The average index of aggressivity in ants obtained from 3 counts during 3 min observation. Statistically significant difference calculated accordingly to Student *t*-test at the 5% level. $P < 0{\cdot}05$ for 2A, 2C and 3A. The vertical bars show the standard error. A, 1–3 hr after drug administration; B, after 6–8 hr; C, 18–24 hr. 1, control. 2, reserpine. 3, lysergic acid diethylamide. 4, amphetamine. 5, phenobarbitone. 6, chlordiazepoxide. 7, chlorpromazine.

Reserpine (0·5 μg/mg body weight) decreased aggressiveness after 2–3 hr but in 18–24 hr aggressiveness was markedly increased. Lysergic acid diethylamide (0·025–0·1 μg/mg weight) acts similarly, causing first decrease and subsequently an increase in aggressiveness. Amphetamine (1·0 μg/mg weight), phenobarbitone (5 μg/mg weight), chlordiazepoxide

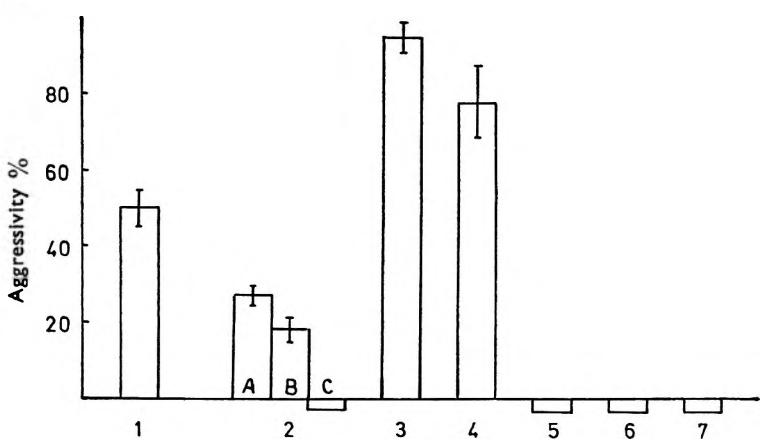

FIG. 2. Aggressivity of mice as a percentage of fighting pairs. 1, control. 2, reserpine (A, after 1–1·5 hr; B, after 6–8 hr; C, after 12–24 hr). 3, lysergic acid diethylamide (after 1–2·5 hr). 4, amphetamine (after 2·5 hr). 5, phenobarbitone (after 3–6 hr). 6, chlordiazepoxide (after 2·5 hr). 7, chlorpromazine (after 3–6 hr). The vertical bars show the standard error. $P < 0{\cdot}05$ for 2A, 2B, 3 and 4.

(0·5 μg/mg weight) and chlorpromazine (0·5 μg/mg weight) did not influence the aggressive behaviour of the ants (Fig. 1). Some calming and ataxia were observed after reserpine and chloropromazine; the other drugs did not produce this effect.

MICE

The percentage of fighting pairs of mice during the 3 min stimulation was 51 in control experiments (Fig. 2). Reserpine (2 mg/kg weight) markedly decreased aggressiveness 1–6 hr after injection and completely suppressed it after 18–24 hr. Lysergic acid diethylamide (0·1 mg/kg) and amphetamine markedly increased aggressivity, with 95% and 78% fighting pairs respectively. Phenobarbitone (10 mg/kg), chlordiazepoxide (1 mg/kg) and chlorpromazine (5 mg/kg) abolished aggressiveness, and calming and ataxia were observed.

Discussion

The results show that the influence of psychotropic drugs on aggressive behaviour differs in ants and mice. Drugs which were potent in mice, such as chlorpromazine, phenobarbitone and amphetamine, had no effect in ants. Reserpine has a biphasic effect in ants—depression of aggressiveness initially, followed by prominent hyperaggressiveness. Lysergic acid diethylamide acted similarly. It seems possible that this biphasic effect may be related to disturbance of brain 5-hydroxytryptamine since this neurohormone was detected in the central nervous system of certain insects (Gersch, Fischer, Unger & Kabitzer, 1961). 5-Hydroxytryptophane decarboxylase has also been detected in small amounts in insect brains (Colhoun, 1964). This 5-hydroxytryptamine or related substances may play a role of inhibitory transmitter in the central nervous system of the ant.

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Recovery of noradrenaline levels after reserpine compared with the life-span of amine storage granules in rat and rabbit

SIR,—After reserpine treatment the noradrenaline in peripheral tissues and in the central nervous system (CNS) rapidly decreases to very low levels, and the recovery takes place slowly over several weeks (Carlsson, Rosengren, Bertler & Nilsson, 1957). The mechanism appears to be a longlasting blockage of the storage mechanism in the amine granules (Bertler, Hillarp & Rosengren, 1961, Carlsson, Hillarp & Waldeck, 1963; Lundborg, 1963; Dahlström, Fuxe & Hillarp, 1965). However, the recovery time for the cell bodies is much shorter (24–48 hr) both centrally (Dahlström & Fuxe, 1964a) and peripherally (Norberg & Hamberger, 1964). Thus, a great difference exists between the recovery time after reserpine treatment in the adrenergic nerve cell bodies and in their terminals. This difference may be explained by the fact that the storage granules are formed in the pericarya of the neurones and transported down the axons to the terminals (see, *inter alia*, Dahlström, 1965).

The recovery time for noradrenaline after reserpine has in this study been examined in different tissues of the rat and rabbit. Brain, heart and skeletal muscle (gastrocnemius) were examined.

Male albino rats (Sprague-Dawley, 200 g) and male albino rabbits (1·4–2·0 kg) were injected with reserpine (Serpasil ampoules, 2·5 mg/ml, diluted with isotonic glucose solution) intraperitoneally (rats, 1 and 10 mg/kg) and intravenously (rabbits, 0·2 and 2 mg/kg). The animals were killed 48 hr, 1, 2, 3, 4, 5, and 6 weeks (rat) or 48 hr, 1, 2, 4 and 6 weeks (rabbit) after the injection. Noradrenaline was measured spectrophotofluorimetrically (Bertler, Carlsson & Rosengren, 1958; Häggendal, 1963).

The recovery of noradrenaline after reserpine administration could be represented graphically as an approximately straight line for all the tissues examined. The time required for a total recovery was between 4 and 5 weeks for the rat after both 1 and 10 mg/kg doses. For the rabbit the corresponding time was about 7 weeks after the high dose (2 mg/kg); at this dose the amines decreased initially to very low levels. After the lower dose of 0·2 mg/kg, the initial decrease was less marked and the recovery time was about 6 weeks. However, if the recovery curve for this latter group of rabbits was extrapolated to zero level of noradrenaline the total time for recovery was about 7 weeks.

These findings are supported by results obtained by Häggendal & Lindqvist (1964) on the effect of a single dose of reserpine (0·2 and 1 mg/kg) on the catecholamine levels in brain and heart of the albino rabbit. If the recovery curves of both brain and heart noradrenaline were extrapolated to zero and to normal levels the time required for a total recovery would be about 6 weeks.

The storage granules being synthesised in the cell body and transported via the axons to the adrenergic terminals have a course of transport which has been found to be linear (Dahlström & Häggendal, 1966a, b). The adrenergic nerve terminals are thus supplied with newly formed granules at a rate which is in all probability fairly steady. The time required for a total exchange of granules in the terminals (the life-span of the granules) has been calculated for hind-leg skeletal muscle of rat to be about 5 weeks (Dahlström & Häggendal, 1966a), and for the rabbit about 7 weeks (Dahlström & Häggendal, 1966b).

The straightness of the noradrenaline recovery curve of rat and rabbit after reserpine treatment and the fact that the time required for a total recovery for both species is close to the calculated life-span of the amine granules, indicate that the course of the noradrenaline recovery after reserpine reflects the downward-transport of the newly formed storage granules, unaffected by reserpine.

The straight course of the noradrenaline recovery is contrary to the view that *in vivo* the reserpine-blocked granules in the terminals would be able to regain their normal ability to take up and store noradrenaline again during their lifetime (at least after a high dose of reserpine). In the latter instance the recovery curve ought to resemble a logarithmic curve rather than a straight line.

It has been found that after axotomy of monoamine-containing nerve fibres in the CNS accumulations of the respective amine rapidly occur proximal to the lesion (Dahlström & Fuxe, 1964a, b). This indicates that the transport of amine granules is high also in the CNS. Since the recovery time for noradrenaline in both rat and rabbit brains was about the same as in the peripheral tissues of the respective species, and likewise followed an approximately straight line, it seems likely that the life-span of amine granules in the CNS is about the same as in the peripheral terminals.

After a single dose of reserpine (or after cessation of chronic reserpine treatment) the functional recovery occurs largely within 1 to 3 days, both in central and peripheral neurones. The first granules, formed in the cell bodies and unaffected by reserpine, have reached the mid-part of the sciatic nerve of the rat as early as 18 hr after a single dose of the drug (Dahlström, 1966). Hence it is reasonable to assume that fresh granules have reached the terminals at the time when the first signs of functional recovery are observed. The possibility of a causal relationship between the two phenomena should be considered. Such a relationship may not be contradicted by the findings of Andén, Magnusson & Waldeck (1964) that during reserpine recovery (during the second to third day after a single dose of reserpine to a rat) the capacity of the tissues to take up and retain labelled noradrenaline is high compared with the noradrenaline levels. It might be that such a high capacity is characteristic of the newly formed downward-transported granules.

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Diethyldithiocarbamate and amphetamine stereotype behaviour

SIR,—Pfeifer, Galambos & György (1966) described the sedative effects of diethyldithiocarbamate (DDC) including antagonism of amphetamine-induced hypermotility. The authors assumed this antagonism to be due to the effect of DDC in decreasing brain noradrenaline by inhibiting the synthesis of noradrenaline from dopamine.

We found the effect of DDC on the stereotype behaviour (constant sniffing, licking or biting) exhibited by rats (16) and mice (20) made hyperactive by amphetamine (3 and 6 mg/kg base s.c. respectively) was not antagonised by DDC 500 mg/kg s.c. or even after repeated doses (2 or 3 × 500 mg/kg) given from 7 hr to $\frac{1}{4}$ hr previous to the amphetamine. Also, two groups of six mice given amphetamine (6 mg/kg) and either DDC or a placebo 7, 4 and 1 hr previously, showed a similar onset of stereotype activity (68 min ± 29 s.d.) but the activity terminated at 289 min ± 52 (s.d.) for DDC and at 151 min ± 9 (s.d.) for the placebo. All the animals treated with DDC were strongly sedated and the reduction in amphetamine hypermotility (locomotion) reported by Pfeifer & others (1966) was obvious. Thus the effect of DDC is in sharp contrast to compounds which decrease the synthesis of both dopamine and noradrenaline by inhibition of the tyrosinehydroxylase (α-methyltyrosine and some of its derivatives). These compounds in relatively low doses change the amphetamine-induced stereotype hyperactivity into a more varied behaviour, which besides sniffing includes locomotion and grooming. This effect of α-methyltyrosine is reversed by dopa (Randrup & Munkvad, 1966; Weissmann, Koe & Tenen, 1966). The reversing effect of dopa is not inhibited by DDC (4 rats, two with 500, two with 2 × 500 mg/kg s.c. DDC given before amphetamine + dopa). The evidence thus indicates that dopamine rather than noradrenaline is associated with the stereotype behaviour, while the motility may be more related to noradrenaline.

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Bowel 5-hydroxytryptamine levels in the immunosympathectomised mouse

SIR,—Mammals treated at birth with the antiserum to Levi-Montalcini's nerve growth factor (NGF) show an almost complete absence of peripheral sympathetic nerves in organs supplied by the thoracic ganglia (Cohen, 1960; Levi-Montalcini & Booker, 1960; Levi-Montalcini & Angeletti, 1962; Levi-Montalcini, 1964; Vogt, 1964; Zaimis, Berk & Callingham, 1965). Levi-Montalcini & Angeletti (1962) demonstrated that in such immunosympathectomised animals, the noradrenaline content was markedly decreased in various tissues. Subsequently, Hamberger, Levi-Montalcini, Norberg & Sjöqvist (1965), using fluorescence microscopy, showed that in the peripheral effector organs of the superior cervical and stellate ganglia of the rat, a complete loss of fluorescent axons and terminals occurred in immunosympathectomised animals. Levi-Montalcini & Angeletti (1962) have reported lower levels of monoamine-oxidase activity in the gastrointestinal tract of immunosympathectomised mice, whilst cells exhibiting a yellow fluorescence typical of 5-hydroxytryptamine (5-HT) were found in increased numbers in the intestines of immunosympathectomised rats compared to controls (Hamberger & others, 1965). Iversen, Glowinski & Axelrod (1966) have recently reported increased 5-HT levels in mouse bowel associated with no depression of monoamine oxidase activity.

Presented here are mucosal 5-HT levels for 14 bowel areas in the gastrointestinal tract of the immunosympathectomised mouse.

Five litters of mixed sexes from the same randomly bred, closed colony of Swiss-Webster white mice maintained at UCLA were obtained within a three week period. The first was injected subcutaneously on the first six days of life with 0·02 ml anti NGF serum per gram of body weight;* the second received 0·02 ml/g 0·9% saline in place of the anti NGF serum and the third was weighed and handled but not injected. The fourth and fifth litters were left unhandled apart from cage cleaning until the time of assay. All assays were made on mice 160–180 days old. The diet was Purina rat chow with a tryptophan content of 0·22%. Mice were killed between 8 a.m. and 9 a.m. on the day of assay by exsanguination after ether induction, and the following tissues were rapidly excised: oesophagus, stomach fundus and body, pyloric antrum, upper and lower duodenum (first and fourth centimetre from pyloric sphincter), mid jejunum, mid and terminal (last centimetre) ileum, appendix (caecal tip), ascending, transverse, and descending colon, and proximal rectum. After removal the segments were opened longitudinally, cleared, blotted dry, and the mucosa separated from the muscle, and assayed spectrophotofluorometrically for 5-HT content (Thompson, 1966). Where tissues were available duplicate samples were run. P values between the immunosympathectomised group, and the three control groups (Table 2) were compared with the pooled figures from the normal, the saline injected, and the handled mice. This procedure was justified since the analysis of variance with unequal groups (Emmens, 1948) demonstrated no significant difference between the normal, the saline injected, and the handled mice. At the time of assay, specimens of the posterior thoracic wall were removed for sympathetic ganglia cell counts in the immunosympathectomised, and the saline injected mice. The tissues were fixed in bichromateformaldehyde and serial histological sections cut at 10 μ and stained with cresyl violet.

Table 1 gives the weight and sex of the immunosympathectomised and saline injected mice, as well as the mean ganglion cell counts on serial sections of four

* Abbott Laboratories, Chicago. This antiserum has a potency of 22,000 antiunits/ml based on an assay preparation of sensory ganglia from 8 day old chick embryos.

ganglia of the thoracic sympathetic chain. The reduction in nerve cell density is 85%. The results presented in Table 2 demonstrate an increased level of mucosal 5-HT in the proximal gastrointestinal tract of the immunosympathectomised mice, compared to the normal, the saline injected, and the handled animals. Such differences range from a $P < 0.02$ (pyloric antrum, and lower duodenum) to $P < 0.001$ (stomach body, mid jejunum, and ileum), with the upper duodenal samples exhibiting no significant difference. The data presented for the large bowel uniformly shows no significant difference in all tissues sampled. Undetectable values for the oesophageal mucosa are not unexpected due to the paucity of argentaffin cells present (Ham, 1965).

Hamberger & others (1965) also demonstrated increased fluorescence typical

TABLE 1. MEAN GANGLION CELL COUNTS OF FOUR THORACIC SYMPATHETIC GANGLIA. THE MEAN IMMUNOSYMPATHECTOMISED (IMS) CELL COUNT AS A PERCENTAGE OF THE MEAN SALINE INJECTED GROUP CELL COUNT IS INDICATED

IMS group			Saline group			
Sex	Body weight	Ganglion cell count	Sex	Body weight	Ganglion cell count	IMS cel count as % of control
M	25·5	56	F	26·0	367	
M	20·5	69	F	26·0	549	
F	26·0	90	M	32·5	455	
M	25·0	74	F	26·5	463	
F	24·0	82	M	31·5	462	
M	30·0	82	F	24·0	538	
F	22·0	78	M	31·0	754	
M	31·0	85			—	
Mean ganglion cell count		77			513	15%

TABLE 2. GASTROINTESTINAL 5-H LEVELS IN μG/G MUCOSA FOR THE FOUR GROUPS OF MICE. [Values are expressed as means ± standard error. The number of samples is shown in brackets. In calculating the P values the IMS group was compared to the pooled data from the normal, the saline injected, and the handled mice (see text for explanation).]

Tissue	Mouse groups: IMS	Mouse groups: Controls	P
Oesophagus	0 (8)	0 (29)	—
Stomach fundus	22·72 ± 0·97 (5)	15·96 ± 0·95 (22)	<0·01
Stomach body	20·12 ± 2·62 (6)	11·79 ± 0·79 (22)	<0·001
Pyloric antrum	84·93 ± 8·45 (8)	66·60 ± 2·92 (28)	<0·02
Upper duodenum	27·11 ± 2·17 (7)	23·66 ± 1·37 (29)	NS*
Lower duodenum	20·11 ± 2·93 (7)	15·69 ± 0·63 (27)	<0·02
Mid jejunum	18·89 ± 1·96 (8)	14·94 ± 0·92 (29)	<0·001
Mid ileum	13·95 ± 1·40 (6)	9·35 ± 0·52 (23)	<0·001
Terminal ileum	16·87 ± 3·36 (6)	10·16 ± 0·73 (22)	<0·001
Appendix	12·57 ± 2·30 (6)	15·81 ± 1·11 (26)	NS*
Ascending colon	54·74 ± 1·55 (7)	47·50 ± 3·01 (24)	NS*
Transverse colon	51·93 ± 3·07 (6)	45·09 ± 1·56 (28)	NS*
Descending colon	47·95 ± 3·41 (6)	42·92 ± 2·39 (23)	NS*
Proximal rectum	44·78 ± 1·81 (6)	41·88 ± 3·47 (17)	NS*

* Not significant.

of 5-HT in the small intestine (exact location unspecified) of immunosympathectomised rats compared to controls. Iversen & others (1966) who reported increased bowel 5-HT levels in immunosympathectomised mice compared with controls did not indicate which portion of the small bowel was involved.

There appear to be two possible mechanisms for this increase in small bowel mucosal 5-HT in the immunosympathectomised animals. Firstly, incomplete degradation of 5-HT by monoamine oxidase could be postulated, in view of the lower levels of this enzyme reported by Levi-Montalcini & Angeletti (1962) in the small bowel after immunosympathectomy. However, Iversen & others (1966) assert that after this treatment no alteration of monoamine oxidase occurred in the bowel. Secondly, although it is generally considered that argentaffin cells do not migrate up the villus, little is known about their ageing processes. The demonstration by Dupont, Biggers & Sprinz (1965) of a decreased transit time of epithelial cells in the jejunum of immunosympathectomised rats suggests that some similar effects may be operating for the argentaffin cell.

The differential changes in 5-HT levels seen between the large and small bowel samples may be related to a selectivity in the effects of the anti NGF serum on the sympathetic nervous system. This has already been demonstrated both by Vogt (1964) and Zaimis & others (1965) who have shown in rodents that the thoracic, coeliac and mesenteric ganglia are not equally affected by the anti NGF serum.

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Uptake of tritiated tyramine and (+)-amphetamine by mouse heart slices

SIR,—The sympathomimetic action of tyramine and amphetamine is of the "indirect" type, by releasing noradrenaline from a minor, labile store (Trendelenburg, 1963). Cocaine antagonises the action of these amines (Tainter & Chang 1927; Burn & Rand, 1958). It has been proposed that cocaine counteracts the action of these amines by inhibiting their uptake into the noradrenergic neurones in the same way as it inhibits the uptake of noradrenaline at the neurone membrane transfer sites (cf. Muscholl, 1966). In a previous investigation we could demonstrate that tritiated tyramine and (+)-amphetamine were accumulated into mouse brain cortex slices (Ross & Renyi, 1966). Only the uptake of the former amine was, however, decreased by cocaine, desipramine and ouabain. Amphetamine seems therefore to be accumulated into the tissue mainly owing to its lipid solubility while tyramine in part is actively taken up by the tissue. Since it is of interest to know whether cocaine inhibits the uptake of amphetamine into tissues in which this amine affects the effector organs by the "indirect" way we have now examined the uptake of tritiated tyramine and (+)-amphetamine by mouse heart slices and the influence of cocaine upon this uptake.

Heart ventricle slices (100 mg) were incubated at 37° with 0·1 nmole/ml of the tritiated amine (tyramine hydrochloride, generally labelled, 1·73 c/mmole; (+)-amphetamine sulphate, generally labelled, 1·82 c/mmole, New England Nuclear Corp.) in 2 ml of Krebs-Henseleits solution, pH 7·4. The amines were extracted from the tissues in the same way as described for noradrenaline (Ross & Renyi, 1964). The radioactivity in the slices was regarded as the amount of the amine being taken up and was expressed as nmole/g. Each value is the mean of four determinations.

The results obtained are presented in Table 1. While both amines were accumulated into the tissue only that of tyramine was decreased by cocaine. Thus the heart tissue resembles the brain cortex tissue regarding the uptake mechanisms for tyramine and amphetamine.

TABLE 1. EFFECT OF COCAINE ON THE ACCUMULATION OF TRITIATED TYRAMINE AND (+)-AMPHETAMINE INTO MOUSE HEART SLICES

Amine	Incubation time, min	Amine uptake nmole/g ± s.e.	
		Control	Cocaine HCl 10 µg/ml
Tyramine-^{3}H	5	0·125 ± 0·003	0·083 ± 0·006‡
	30	0·160 ± 0·005	0·130 ± 0·003†
(+)-Amphetamine-^{3}H	5	0·162 ± 0·005	0·163 ± 0·007
	30	0·195 ± 0·010	0·199 ± 0·009

† $0{\cdot}01 > P > 0{\cdot}001$ ‡ $P < 0{\cdot}001$

The lack of effect of cocaine on the amphetamine uptake seems to contradict the possibility that cocaine antagonises the "indirect" action of amphetamine by inhibiting its uptake. The possibility remains that cocaine counteracts the amphetamine induced release of noradrenaline from an intra- or extra-neuronal site. If from an intra-neuronal site it has to be assumed that cocaine acts at different sites when inhibiting the noradrenaline uptake or when antagonising the action of the "indirectly" acting amphetamine. If an extra-neuronal site of release of noradrenaline is postulated the possibility exists that cocaine acts directed at the same site, especially if it is assumed that the "indirectly" acting

amine releases noradrenaline bound to the transfer (uptake) sites of the neurone membrane (Ross & Renyi, 1966).

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Reserpine and the neuromuscular junction

Sir,—The ineffectiveness of reserpine on the neuromuscular transmission (Bein, 1956) was questioned by Liebmann & Matthies (1964) who claimed that the drug displayed a powerful anticurare activity by increasing the acetylcholine release from the motor nerve endings. Their results, however, were not confirmed by Ledda & Baldi (1965).

We now summarise investigations made to establish whether reserpine affects both the amount of acetylcholine stores in the motor nerve, and the pattern of the end-plate potentials.

The experiments were made on the isolated phrenic nerve-diaphragms of the guinea-pig and rat; the methods for detecting acetylcholine and for intracellular recordings were those previously described (Beani, Bianchi & Ledda, 1966).

The right and left hemidiaphragms of five guinea-pigs were separately incubated for 2 hr in oxygenated Tyrode solution with dyflos 500 μg/ml, at 38°. After washing out the dyflos, the preparations were indirectly stimulated at 50/sec for 10 min. They were then kept at rest for 1 hr and one hemidiaphragm of each pair was maintained in the presence of reserpine 1×10^{-5}M. Immediately after a second period of stimulation at 50/sec (10 min) the tissue acetylcholine was extracted both in the control and treated preparations.

TABLE 1. TOTAL TISSUE ACETYLCHOLINE (NG/HEMIDIAPHRAGM ± S.D.) AT THE END OF THE SECOND PERIOD OF STIMULATION AT 50/SEC IN HEMIDIAPHRAGMS KEPT IN TYRODE SOLUTION, AT 38°, WITH OR WITHOUT RESERPINE 1×10^{-5}M. PREINCUBATION WITH DYFLOS 500 μG/ML FOR 2 HR (each value is the mean of five experiments).

Treatment	Guinea-pig weight g ± s.d.	Hemidiaphragm weight mg ± s.d.	Acetylcholine ng/hemidiaphragm ± s.d.
Controls	330 ± 23	223 ± 33	81·5 ± 11·3
Reserpine	330 ± 23	250 ± 49	76·0 ± 8·0

As shown in Table 1, reserpine does not change the transmitter stores; in similar experimental conditions, the drug does not modify the acetylcholine release (Ledda & Baldi, 1965). The end-plate potentials were recorded from curarised rat diaphragms, kept in oxygenated Tyrode solution at 33°. The phrenic nerve was stimulated at 1, 10 and 100/sec for 10 sec.

TABLE 2. EFFECT OF RESERPINE ON END-PLATE POTENTIAL HEIGHT (MV ± S.D.) OF RAT CURARISED DIAPHRAGMS (TUBOCURARINE 1×10^{-6} G/ML). (Stimulation of phrenic nerve at 1, 10 and 100/sec for 10 sec at 33°.)

Treatment	Stimulation rate		
	1/sec	10/sec	100/sec
Controls (161*)	2·53 ± 0·69	1·39 ± 0·45	0·69 ± 0·34
Reserpine (22) 1×10^{-5}M	2·50 ± 0·58	1·44 ± 0·39	0·75 ± 0·22

* No. of end-plates.

Different end-plates were usually impaled both before and 40–50 min after adding reserpine 1×10^{-5}M. In three cases, the potentials of the same end-plate were recorded throughout the experiment. As shown in Table 2, the drug does not affect the end-plate potential amplitude at different stimulation rates. The inactivity of the drug was again confirmed in six non-curarised diaphragms in which the pattern of the frequency of the miniature end-plate potentials and amplitude was followed. The average frequency of the miniature end-plate potential of 14 normal end-plates was 152/min (range 78–240); after reserpine the value was 161/min (range 80–264). Also the average height of the miniature end-plate potentials remained the same (0·80 mV) before and during treatment, without any change in the amplitude distribution. The obvious conclusion is that reserpine has no detectable effect on the transmitter stores nor on the spontaneous and stimulus-triggered end-plate bioelectric events. Moreover, the post-synaptic chemosensitivity seems to be unaltered, as suggested by maintained end-plate potential height. These results strengthen the hypothesis that the change of acetylcholine stores and release observed after reserpine in other tissues, as in the brain (Malhotra & Mehta, 1966), are mediated through the effect of the drug on the physiological dispostion of other biogenic amines (Beani, Ledda, Bianchi & Baldi, 1966).

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Pargyline on blood pressure in spinal and decerebrate cats

SIR,—Acutely administered pargyline produces a slowly developing long-lasting hypertensive effect in spinal cats, but in decerebrate animals, under similar conditions, it has no action on blood pressure.

Five cats were anaesthetised with ether, and cannulae were placed in the right carotid artery, the left external jugular vein and the trachea. Both vagi were cut and the cord sectioned at C-2, the cats being maintained subsequently under artificial respiration. One hr after the ether anaesthesia was discontinued, pargyline hydrochloride was given intravenously in doses of 10 mg/ kg at 30 min intervals for a cumulative dosage of 60 mg/kg. The systolic and diastolic blood pressures were read 15 min before and after each injection. Immediately after each dose, a small hypotensive response of short duration was obtained, but during the course of each experiment pargyline gradually produced a long-lasting hypertensive effect. After 20 mg/kg (Table 1), the mean systolic blood pressure was increased by 26·8 mm Hg, whereas after 60 mg/kg, the increase was 54·8 mm Hg. In this series of experiments, the mean heart rate was not significantly altered although a positive chronotropic trend was apparent according to rank-sum analysis (Rümke & de Jonge, 1964).

Five more cats were decerebrated (Burn, 1952), and maintained subsequently under artificial respiration. In contrast to the results in spinal animals, pargyline did not raise the blood pressure, and a slight, but not statistically significant, depression was obtained (Table 1). Mean heart rates were again not significantly altered, although analysis by the method of de Jonge (Rümke & de Jonge, 1964) indicated a decreasing trend.

TABLE 1. THE EFFECT OF PARGYLINE HYDROCHLORIDE ON BLOOD PRESSURE AND HEART RATE IN SPINAL[1] AND DECEREBRATE[2] CATS. (Readings taken 15 min after injection. Paired mean differences from control values[3] ± s.e.)

Cumulative intravenous dose mg/kg	Spinal cats (n = 5)			Decerebrate cats (n = 5)		
	Blood pressure (mm Hg)		Heart rate beats/min	Blood pressure (mm Hg)		Heart rate beats/min
	Systolic	Diastolic		Systolic	Diastolic	
10	1·8 ± 2·6	2·6 ± 3·7	−6·0 ± 9·4	0·8 ± 2·0	−5·8 ± 1·7	4·8 ± 6·9
20	26·8 ± 8·6*	26·2 ± 15·5	−5·6 ± 13·3	−1·6 ± 2·2	−6·8 ± 4·0	0·4 ± 5·9
30	42·0 ± 11·5*	33·0 ± 9·8*	18·8 ± 17·9	−1·8 ± 3·5	−6·0 ± 4·6	−2·4 ± 6·3
40	47·2 ± 13·0*	38·6 ± 8·8*	21·6 ± 21·1	−4·6 ± 4·4	−9·2 ± 4·5	−9·6 ± 5·5
50	48·8 ± 9·7*	38·6 ± 7·2*	18·0 ± 17·2	−6·8 ± 6·3	−12·2 ± 6·8	−12·0 ± 9·5
60	54·8 ± 10·3*	49·2 ± 9·0*	13·0 ± 18·0	−9·6 ± 9·3	−13·8 ± 6·0	−20·4 ± 12·7

[1] Control values: Blood pressure 97·6/46·6; heart rate 154·8.
[2] Control values: Blood pressure 128·2/86·0; heart rate 199·2.
[3] Analysis by the method of Walker & Lev (1953).
* $P < 0.05$.

Ten cats were then decerebrated according to the method of Smith, Budris & Paul (1963). In five of these animals an insulated, bipolar, twisted, nichrome wire electrode was placed in the medulla at Horsley-Clarke coordinates P12, L2, H-8. Stimulation at this site with square pulses (100/sec; 1 msec duration) resulted in striking hypertensive responses. Three of these preparations were given pargyline and two received iproniazid phosphate intravenously. Pargyline at 40 mg/kg caused a reduction in the pressor response; higher doses, with a maximum of 100 mg/kg, produced further reduction but in no case was the response abolished. Iproniazid had similar effects over the same dosage range. In the other five decerebrate animals, a bipolar shielded electrode was placed on the central end of the cut sciatic nerve. Stimulation (100/sec; 1 msec duration)

produced typical pressor responses. In three of the animals given pargyline, this response was decreased in magnitude by doses of 40 mg/kg. In the two other cats, iproniazid (50 mg/kg) caused similar effects.

These experiments indicate that the presence of the medullary vasomotor centre is necessary to prevent the peripheral hypertensive action of pargyline. Further, reflexly-induced hypertensive responses are reduced by both pargyline and iproniazid.

Pargyline (as well as some other monoamine oxidase inhibitors) may have two antagonistic actions, a peripheral hypertensive effect resulting from an increase in the concentration of circulating catecholamines and a stronger depressant action on vasomotor centres in the central nervous system. The result is a reduction in blood pressure and the postural hypotension which is observed clinically.

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On the neurotoxic effects induced by alkylating agents

SIR,—During some experiments made to evaluate the chemotherapeutic effect of DL-sarcolysine applied topically to a cerebral tumour of the rat, signs of neurotoxicity were observed. We therefore investigated the central effect of several alkylating agents injected intracerebrally in normal rats. The symptoms observed, the relative potency, and some attempts to protect the animals are reported.

Male Sprague-Dawley rats, 160 ±10 g, were kept in Makrolon cages (47 × 26 × 15 cm) 6 per cage at a constant temperature of 22° and relative humidity of 60%. They had free access to food (Diet Alal 56 Alal, Milan) and water until the beginning of the experiment.

The intracerebral injection was made under light ether anaesthesia through the squamopetrosal fissure of the temporal area of the skull (Valzelli, 1964).

The drugs were dissolved in distilled water and injected in 0·02 ml volumes. The intracerebral injection of the solvent never induced any appreciable symptoms and the animals completely recovered from anaesthesia in about 10 min. The mortality was calculated after a period of 24 hr.

The drugs used were: DL-sarcolysine, L-sarcolysine, D-sarcolysine, glycine mustard, alanine mustard, cyclophosphamide, chlorambucil, 6-diazo-5-oxo-L-norleucine (DON), azaserine (all from CCNSC, N.I.H. Bethesda), tryptophan mustard (Dr. L. Otis, Stanford Research Institute, Palo Alto, California), degranol (Medimpex, Budapest), mustine, and tretamine (Simes, Milan), phenobarbitone sodium (Bayer), thiourea (Erba, Milan), phenytoin sodium (Recordati, Milan).

Several alkylating agents, when injected intracerebrally in rats, produce signs of neurotoxicity. The symptomatology is not entirely comparable for all drugs. However, a typical pattern, as with DL-sarcolysine, includes a latency time of about 30–60 min, signs of central stimulation, inco-ordinate movements, sterotyped behaviour ending with clonic convulsions and occasional tonic extensions. By increasing the dose, the latency time decreased and eventually the animals died. With DL-sarcolysine, aggregation of the animals did not change the latency time. Thus the time before the appearance of the first convulsion was 52 ±6 min when the rats were isolated and 70 ±9 min when the rats were kept 5 in a cage.

Table 1 summarises the neurotoxic dose (ED50) and the lethal dose (LD50) for each drug injected intracerebrally. Mustine, with a latency time of about 10 hr, induced only signs of spasm which never reached the stage of convulsions.

TABLE 1. NEUROTOXICITY OF VARIOUS ANTITUMOUR DRUGS GIVEN INTRACEREBRALLY

No. of rats	Drug	Neurotoxic activity ED50 (μg/rat i.c.)	LD50 (μg/rat i.c.)
150	DL-Sarcolysine	23 (39 – 13)••	40 (72 – 22)••
36	L-Sarcolysine	20 (27 – 14)	32 (48 – 21)
60	D-Sarcolysine	13 (22 – 7)	18 (30 – 11)
36	*m*-DL-Sarcolysine	47 (77 – 28)	~100
53	Glycine mustard	18 (23 – 14)	18 (21 – 15)
78	Alanine mustard	25 (29 – 21)	36 (46 – 28)
48	DL-Tryptophan mustard	1·1 (1·4 – 0·8)	4·3 (7·3 – 2·5)
18	Mannitol mustard	>100	>100
48	Mustine	•	20 (48 – 8)
18	Tretamine	>100	>100
18	Cyclophosphamide	>100	>100
18	Chlorambucil	>100	>100
18	DON	> 50	> 50
18	Azaserine	>100	>100

• = A non typical symptomatology was observed with doses from 10 to 100 μg/rat.
•• 95% fiducial limits.

All the mustard derivatives of amino-acids showed a typical pattern of neurotoxicity. The most active drug was the tryptophan mustard followed by D-sarcolysine and then DL-sarcolysine, L-sarcolysine and the nitrogen mustards of alanine and glycine. *m*-DL-Sarcolysine was less convulsant than the corresponding isomer *p*-DL-sarcolysine. The mannitol mustard and other alkylating agents including tretamine, cyclophosphamide and chlorambucil were not neurotoxic up to the dose of 100 μg/rat. DON and azaserine were tested for comparative purposes because of their chemical relation with amino-acids, but they did not show any marked effect. When DL-sarcolysine was inactivated by boiling, it lost its neurotoxic effect.

Attempts were made to protect against this neurotoxic action with anticonvulsant drugs. Phenytoin sodium (100 mg/kg i.p.) was ineffective, while sodium phenobarbitone showed a clear protective effect. In one experiment the ED50 of DL-sarcolysine changed from 15 to 50 μg/rat when phenobarbitone was given at the dose of 100 mg/kg i.p. 30 min before the intracerebral injection. The LD50 of DL-sarcolysine injected intracerebrally changed from 29 to 100 μg/rat. Also, radioprotectors were given in an attempt to decrease the neurotoxicity of DL-sarcolysine. but cysteine (1 g/kg i.p.) or thiourea (1 g/kg i.p.) were found to be inactive. It is interesting to note that previous experiments demonstrated an antagonism between these two radioprotectors and DL-sarcolysine when the latter was given intraperitoneally (Garattini, Palma & Reyers, 1965; Garattini, Palma, Reyers-Degli Innocenti & Guaitani, 1966).

The high activity of tryptophan mustard suggested the possibility that this alkylating agent was competing with the transport of tryptophan through the blood brain barrier. However, the administration of L-tryptophan (800 mg/kg i.p. or 100 μg/rat s.c.) did not protect against the neurotoxicity of 1 or 5 μg/rat of tryptophan mustard given intracerebrally. These observations may influence the choice of drugs to be injected locally in the chemotherapeutic treatment of brain tumours.

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Two different mechanisms for incorporation of ^{3}H-metaraminol into the amine-storing granules

SIR,—In a previous paper it was reported that metaraminol is incorporated into the adrenal medullary granules *in vitro* by a mechanism which does not utilise Mg^{++} and ATP (Lundborg, 1966). This mechanism is not influenced by reserpine. But it has also been shown that the ability of the heart to retain metaraminol is considerably blocked by reserpine (Shore, Busfield & Alpers, 1964; Carlsson & Waldeck, 1965). To elucidate this apparent discrepancy between *in vitro* and *in vivo* evidence the following experiments were made.

Mice, in groups of six, were given ^{3}H-metaraminol 0·04 mg/kg i.v. alone or preceeded 6 hr before by reserpine 10 mg/kg i.p. At various intervals after ^{3}H-metaraminol had been given the animals were killed. The hearts were removed and homogenised with a plastic pestle in 0·25 M sucrose containing

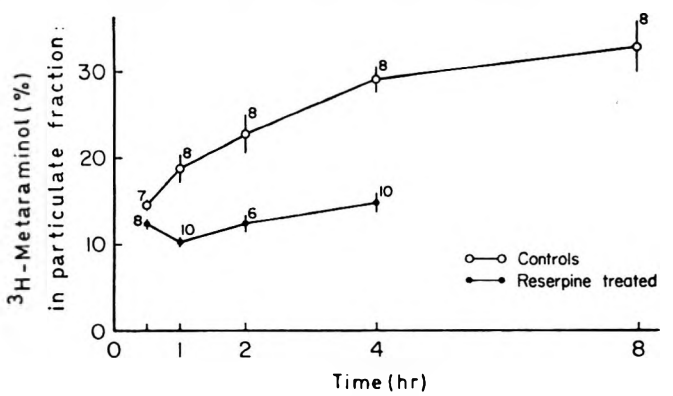

FIG. 1. Subcellular distribution of ^{3}H-metaraminol in the mouse heart. The results are expressed as ^{3}H-metaraminol in the particulate fraction as percentage of ^{3}H-metaraminol in the particulate + supernatant fractions. The bars indicate s.e.m. and the figures the number of experiments. For experimental details see text.

0·005 M phosphate buffer at pH 7·4 and 0·001 M $MgCl_2$. The homogenate was centrifuged at 2,000 × *g* for 10 min at 4° and the supernatant fluid was re-centrifuged at 100,000 × *g* in a Spinco 40 rotor for 60 min. The amount of ^{3}H-metaraminol in the "coarse" fraction (sediment 1), "particulate" fraction (sediment 2) and supernatant fractions was determined by liquid scintillation counting.

In the coarse fraction the amount of ^{3}H-metaraminol was almost constantly 30% of the total. Our results, therefore, will be expressed as the amount of ^{3}H-metaraminol in the particulate fraction as percentage of ^{3}H-metaraminol in the particulate fraction + supernatant fraction.

If ^{3}H-metaraminol is given alone there is a gradual increase of ^{3}H-metaraminol in the particulate fraction, from 14·5% after ½ hr to 32·7% after 8 hr (Fig. 1). During the same time the total amount of ^{3}H-metaraminol in the pooled 6 hearts of every group changed from 33·9 ng ± 3·5 (s.e.m.) to 27·6 ng ± 1·3. When reserpine was administered before metaraminol the percentage of metaraminol in the particulate fraction was constantly at a level of 10 to 15% while the total amount of metaraminol in the hearts changed from 26·0 ng ±1·3 after ½ hr to 7·6 ng ±0·7 after 4 hr.

Apparently there is an uptake of metaraminol in the particulate fraction even after reserpine pretreatment. This is in agreement with the *in vitro* data mentioned above, that there is a reserpine-resistant uptake of metaraminol in the amine granules. If not reserpine-treated, however, the granules seem to gradually incorporate more metaraminol. This reserpine-sensitive process is apparently so slow that it is hardly observed in an *in vitro* experiment.

These observations suggest the possible existence of at least two different mechanisms, both of importance for the incorporation of amines into the storage granules. The first, a reserpine-resistant uptake, the equilibrium being achieved rather soon. The second, a reserpine-sensitive mechanism, probably identical with the ATP-Mg^{++}-dependent uptake mechanism observed *in vitro* by Carlsson, Hillarp & Waldeck (1962, 1963) and by Kirshner (1962a, b). In the instance of ^{3}H-metaraminol (Lundborg, 1966) the incorporation is too slow to be observed *in vitro*. *In vivo*, however, ^{3}H-metaraminol may be gradually incorporated by a reserpine-sensitive mechanism resulting in an efficient retention of the amine.

A slow incorporation of metaraminol in the particulate fraction has also been observed by Giachetti & Shore (1965).

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The relation between vanilyl mandelic acid and 5-hydroxyindoleacetic acid excretion in a patient with a carcinoid tumour

SIR,—There is convincing evidence from experiments with several species (Werle & Aures, 1959; Rosengren, 1960; Lovenberg, Weissbach & Udenfriend, 1962; Reid, Riley & Shepherd, 1963; Reid & Shepherd, 1964) that the non-specific histidine decarboxylase, dopa- and 5-hydroxytryptophan decarboxylases are, in fact, one and the same enzyme. An opportunity to study this relationship in man was afforded recently by a carcinoid tumour showing an exceptionally high output of 5-hydroxyindoleacetic acid (5-HIAA).

5-HIAA was estimated by the method of Macfarlane and others (1956) and vanilylmandelic acid (VMA) by Connelian & Godfrey's (1964) modification of the method of Pisano, Crout & Abraham (1962), in 24-hr urine specimens collected from the patient (F.N., male, age approx. 50 years) over a period of six months During most of this time the patient was under treatment with methysergide to reduce the symptomatic effects of released 5-HT. The results are recorded, in chronological order, in the Table 1.

TABLE 1. VMA AND 5-HIAA CONTENT OF URINE SPECIMENS FROM A MALE WITH CARCINOID SYNDROME DURING SIX MONTHS

Date	Urinary excretion, mg/24 hr	
	VMA	5-HIAA
25/3/65	7·12	336
26/3/65	8·00	417
27/3/65	9·92	510
28/3/65	9·18	538
9/4/65	10·20	630
21/4/65	9·18	500
4/5/65	8·88	410
7/5/65	10·08	590
31/5/65	9·02	548
12/7/65	11·00	575
5/9/65	13·90	692
1/10/65	7·16	275
Upper limit of normal	7·00	10·0
	Correlation between VMA and 5-HIAA: $r = 0{\cdot}859$; $P = <0{\cdot}01$	

The highly significant correlation of the excretion levels of the metabolites of 5-HT and catechol amines supports the theory of a common decarboxylase responsible for the formation of these amines. On the other hand, in the carcinoid tumour, 5-HIAA formation is very much more rapid than VMA formation, since, in this particular patient, the output of 5-HIAA is increased to as much as 70 times the normal level, whereas the highest value for VMA excretion is only twice that of the upper limit of normal. In fact, there is no noticeable increase in VMA excretion above the normal range until the 5-HIAA excretion rises to 300–400 mg/24 hr, so that it would be unlikely that this relation would be observed in the average case of carcinoid, in which the 5-HIAA output rarely exceeds 100 mg/24 hr. This would suggest that there is a big difference in the

availability of substrates to the enzyme in the carcinoid tumour, 5-hydroxytryptophan penetrating much more readily than dopa.

I wish to thank Miss Maureen Raffan for carrying out the VMA estimations.

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Liberation of noradrenaline from the dog spleen

SIR,—Recently, Haefely, Hürlimann & Thoenen (1965) published evidence on the relation of the rate of stimulation and the quantity of noradrenaline liberated from sympathetic nerve endings in the isolated spleen of the cat. This is an account of similar experiments on the isolated spleen of the dog.

Mongrel bitches were anaesthetised with pentobarbitone, 30 mg/kg, intravenously. A midline incision was made in the abdomen and the animal eviscerated from mid-duodenum to the terminal colon. The spleen was isolated and removed to a chamber containing liquid paraffin and maintained at 37°. It was perfused with McEwen's solution maintained at 37° and at a rate of 20 ml/min. The perfusion fluid contained 1 μg/ml each of cocaine hydrochloride and phenoxybenzamine. The splenic nerves were laid over bipolar electrodes and stimulated with supramaximal square wave pulses of 1 msec duration. Stimulation was for 2 min at 0·5, 1·0, 2·0 and 5·0 c/s, applied successively without interruption. The perfusate was collected in the last 30 sec of each period of stimulation in tubes containing hydrochloric acid, ascorbic and ethylenediaminetetra-acetic acids and the noradrenaline assayed spectrofluorometrically. The method of assay also served to identify the substance as noradrenaline, and fully accounted for the vasopressor activity of the samples assayed in the pithed rat.

The combined results of three experiments are shown in Fig. 1. A resting output of approximately 20 ng/ml noradrenaline was observed; whether arising from the spontaneous release of noradrenaline from the nerve terminals, or from an indirect sympathomimetic action of cocaine is not known. The output of noradrenaline increased by 20 ng/ml at 0·5 c/s which is approximately twice the resting output; the noradrenaline output per stimulus was 1·2 ng. At a frequency of 1·0 c/s the output of noradrenaline rose to 110 ng ml, i.e. about 4 ng/stimulus. When the rate of stimulation was increased to 2·0 c/s the

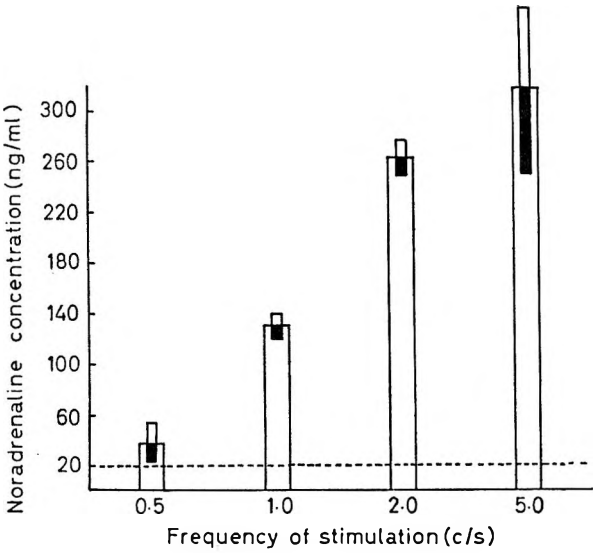

FIG. 1. Noradrenaline concentration in the perfusate of isolated dog spleen during stimulation of splenic nerves. Perfusate collected in the last 30 sec of a 2 min period of stimulation (see text). Dotted line indicates the output of noradrenaline from the spleen at rest.

concentration of noradrenaline rose to 240 ng/ml, the output per stimulus was however the same as was observed at 1 c/s. At 5 c/s the noradrenaline concentration was further increased but the increase did not parallel the increase in frequency of stimulation, thus the output per stimulus fell at 5 c/s to approximately 2 ng/stimulus. An attempt to obtain reproducible frequency/output curves for noradrenaline was unsuccessful. The results of such an experiment

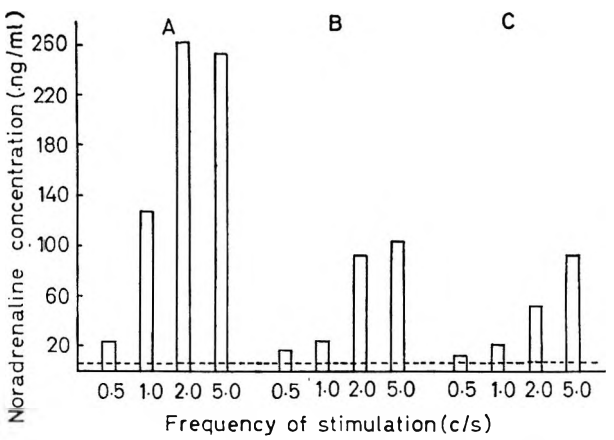

FIG. 2. Frequency/output curves for noradrenaline on isolated dog spleen. 30 min elapsed between A and B, and B and C respectively (details see text). Dotted line indicates the output of noradrenaline from spleen at rest.

are shown in Fig. 2. A period of thirty minutes was allowed between the determination of each output curve. Stimulation of the splenic nerves during the second (Fig. 2B) and third (Fig. 2 C)determinations produced much less noradrenaline in the perfusates, an effect which was most marked at the highest frequency of stimulation and may arise from exhaustion of the transmitter, as suggested by Haefely & others (1965).

Between 0·5 and 2·0 c/s the concentration of noradrenaline in the splenic perfusate during stimulation of the sympathetic nerves to the dog spleen is proportional to the frequency of stimulation employed. When the frequency of stimulation is raised from 2 to 5 c/s the concentration of noradrenaline is increased but not markedly so. In the cat this maximum lies between 4 and 8 c/s but the calculated output of noradrenaline per stimulus for the range of 0·5 to 2·0 c/s is quantitatively the same as in the dog. Thus the dog and cat spleens behave similarly in the amount of noradrenaline released by stimulation of the sympathetic nerves in the presence of phenoxybenzamine and cocaine. But, when the splenic nerves are stimulated in the absence of phenoxybenzamine and cocaine and the response of the spleen is assessed by measuring the increase in inflow pressure, maximum responses occur between 5 and 10 c/s. This response is more related to the concentration of noradrenaline in the perfusate than to the calculated output of noradrenaline per stimulus.

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Book Review

Drill's *PHARMACOLOGY IN MEDICINE.* 3rd edition. Edited by Joseph R. DiPalma. Pp. xiii + 1488 (including index). McGraw-Hill Publishing Company Ltd., Maidenhead, Berks, 1965. 180s.

Increasing specialisation within specialties is reflected in the tendency towards multi-authorship of most textbooks which purport to be comprehensive and authoritative. The obvious advantage of this arrangement is that each section can be contributed by a chosen authority on the topic; the obvious disadvantage could be discontinuity in the quality, quantity and style of the presentations. Much of the onus for the final quality of such a book falls on the editor and Professor Di Palma is to be congratulated on his successful supervision of the 3rd edition of Drill's Pharmacology in Medicine.

Drill first appeared in 1954 and was from the beginning a multi-author tome edited by Victor A. Drill. Even the first edition had 81 contributors (one of whom was Joseph R. Di Palma) and it was stated that "It is the aim of this book to present, with proper emphasis in each area, the mechanism of action, the effect on organ systems and the therapeutic uses of drugs presently used in medical practice." The success of the book and the rate of advance in the subject soon made a 2nd edition necessary and this appeared in 1958, still edited by Drill, with the number of contributors increased to 86.

For the 3rd and latest edition, published in 1965, Victor Drill has relinquished the editorship to Joseph Di Palma although he is still associated with the book

as a contributor. "Pharmacology in Medicine" now comprises 90 chapters occupying nearly 1500 pages (27 of which are taken up by the index) and is written by no fewer than 93 contributors only 35 of whom also contributed to the 2nd edition. "It is written so as to contribute as much knowledge as possible in a one-volume text which is still considered portable." Although a large book it is easily handled and a pleasure to use because of the high standard of production.

Every major topic of pharmacology is covered somewhere in the book, the only surprising omission seems to be a section on Anti-Viral agents. The text begins with an introduction to and brief history of pharmacology (in which the late Sir John Gaddum is referred to as Robert Gaddum) and then goes on to a short account of some quantitative aspects of drug administration and of the analysis of drug effects. There are some useful discussions of an introductory nature on the general principles of pharmacology such as absorption, distribution and fate of drugs. The section on psychotropic drugs includes an attempt to improve on the classification difficulties which exist in this field and it meets with limited success. There is a clear account of the out-moded analeptic drugs. In the section on autonomic pharmacology each contributor is a well-known expert in his field so that the chapters are authoritative and up-to-date. There is a section on water and salt balance which gives a particularly clear account of the diuretic drugs starting, as it should, with a consideration of modern views on renal physiology. The treatment of histamine and the antihistamine drugs is conventional while the chapter on cathartics and laxatives still refers to many old-fashioned drugs. The modern accounts of drugs used in blood disorders are followed by useful chapters on radioactive elements and gases, vapours and dusts including warfare drugs, insecticides, rodenticides and other economic [*sic*] poisons. After a comprehensive treatment of the hormones, the book ends with chapters on drugs used in the treatment of infections and infestations.

As might be expected the chapters are not all uniformly good, some appear to be more soundly-based scientifically than others and the clinical emphasis varies, but this is largely in the nature of the current disparity in the extent of knowledge and application between different fields of pharmacology and therapeutics. All the chapters supply sound basic information which can be supplemented if needed from the bibliographies which are appended. This is a book to which anyone who uses or needs to know about drugs may turn for a concise presentation of modern information.

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