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Research Papers

The nature of the adrenergic receptors of the trachea of the guinea-pig

R. W. FOSTER

The adrenergic receptors of the guinea-pig isolated trachea have been characterised as β-receptors by established criteria. No evidence was obtained that any α-receptors are present. (−)-Isoprenaline was 17 times more potent than (−)-adrenaline which was 10 times more potent than (−)-noradrenaline. High concentrations of piperoxan, thymoxamine and dihydrogenated ergot alkaloids did not antagonise the catecholamines, while phentolamine and phenoxybenzamine potentiated them, isoprenaline almost as much as noradrenaline. Propranolol, pronethalol and the 3,4-dichloro-analogues of (±)-noradrenaline, adrenaline and isoprenaline each specifically antagonised the catecholamines, isoprenaline moreso than noradrenaline. The characteristics of this blockade by pronethalol and propranolol fulfilled established criteria for competitive antagonism; propranolol (pA_2 against noradrenaline 6·56 ± 0·21) was 18·6 (11·4 to 30·5) times more potent than pronethalol, (pA_2 against noradrenaline 5·29 ± 0·07).

MOST analyses of the actions of sympathomimetics, and of the interactions of this group of drugs with others, have been made on tissues with α-receptors. The neglect of the β-receptor may stem from the lack of a convenient and reliable test tissue.

Quantitative studies on the catecholamines are made difficult if the drugs have two opposite actions on the test tissue, or if they interact with two types of receptor. Such complexities have been shown to exist in intestinal muscle. Axelsson, Bueding & Bulbring (1961) found adrenaline to have both inhibitory and excitatory actions on the electrical activity of the guinea-pig taenia coli. Ahlquist & Levy (1959), Furchgott (1960) and Wilson (1964) have each presented evidence for the presence of both α- and β-adrenergic receptors in the small intestine of the dog, rabbit and guinea-pig respectively; in each animal, interaction between catecholamines and either α- or β-receptors produced inhibition of the intestine. An investigation of the adrenergic receptors of the bronchioles of the anaesthetised dog by Castro de la Mata, Penna & Aviado (1962) showed that sympathomimetic bronchodilation was subserved by β-receptors but that α-receptors subserving bronchoconstriction were present and could be revealed by β-receptor blockade with dichloroisoprenaline.

The isolated paired tracheal chain preparation of the guinea-pig, described by Foster (1960) as a development of the preparations of Castillo & de Beer (1947) and Akcasu (1952) would provide a convenient and reliable test tissue for pharmacological analyses if it were shown to be equipped with β-receptors only.

Experimental

METHODS

Pairs of tracheal chain preparations (Foster, 1960) were suspended in identical jacketed organ baths containing 5 ml of Krebs solution at 37·5°,

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bubbled with 95% oxygen and 5% carbon dioxide. Relaxations were recorded on smoked paper with isotonic balanced balsa wood levers, magnifying 20 times; the tissue supported a load of 200 to 240 mg. The load was removed from the tissue while it was washed, and replaced 5 min before the next drug addition. Washing was effected by displacement of the bath fluid with fresh pre-warmed and pre-bubbled Krebs solution. Drug-induced relaxant responses were recorded for 15 min after which 25 min of washing at 5 min intervals was necessary for recovery of full inherent tone.

(+)-Ascorbic acid, 200 μg/ml, was always included in the Krebs solution to delay catecholamine autoxidation.

All experiments were designed to yield quantitative information. They consisted of repeated recording of the log concentration: effect curves of one or more catecholamines before and after exposure of the tissue to α- or β-blocking agents. Two methods were used to record the log concentration:effect curve; the normal sequential method in which the tissue was washed to recovery between each dose of agonist, and the cumulative method of Ariëns & de Groot (1954) in which the concentration of agonist in the bath was increased fourfold every 15 min without washing. After recording the first relaxation fully only the last 7 min of each accumulated relaxation was recorded.

Near the beginning and end of each experiment a maximum relaxation was produced by addition of a suitable dose of either aminophylline or a catecholamine. These drugs produced equal maximum relaxations. Relaxations produced by drugs were expressed as a proportion (%) of this maximum possible relaxation; doses as final bath concentration of base.

DRUGS

Drugs used were: (–)-adrenaline bitartrate, aminophylline (theophylline ethylenediamine dihydrate), (+)-ascorbic acid, (±)-dichloroadrenaline hydrochloride, (±)-dichloroisoprenaline hydrochloride, (±)-dichloronoradrenaline hydrochloride, dihydroergotamine ethanesulphonate, Hydergine (a mixture of equal parts of the methanesulphonates of dihydroergocornine, dihydroergocristine and dihydroergokryptine), (–)-isoprenaline bitartrate dihydrate, (–)-noradrenaline bitartrate monohydrate, papaverine hydrochloride, phenoxybenzamine hydrochloride, phentolamine hydrochloride, piperoxan hydrochloride, pronethalol hydrochloride, propranolol hydrochloride, sodium nitrite, thymoxamine hydrochloride.

Results

JUSTIFICATION OF CUMULATIVE METHOD FOR CONSTRUCTION OF LOG CONCENTRATION: EFFECT CURVES

Log concentration: effect curves for either noradrenaline or isoprenaline were constructed by both the sequential and cumulative methods on each member of a pair of tracheal chain preparations. In this way comparison of the curves obtained could be made both within and between members. Fig. 1 illustrates and compares the methods and shows the similarity of

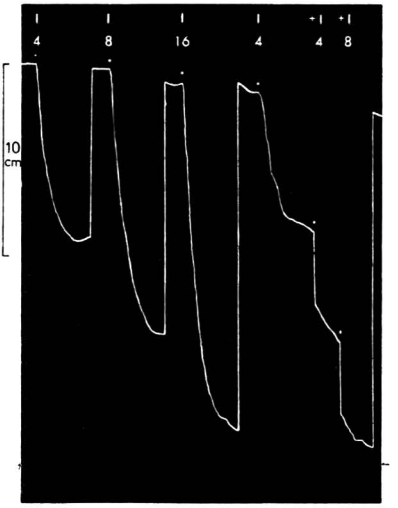

FIG. 1. The method used in recording sequential and cumulative log concentration: effect curves for isoprenaline(I). The figures refer to the final bath concentration in ng/ml. The sequential method involves three 15 min records of drug-induced relaxations, each followed by 25 min of washing. The cumulative method involves three 15 min relaxations; the first is recorded in full, the drum is switched off for the first 8 min of each accumulated relaxation to gain end-point definition. 25 min of washing restores full inherent tone. A frontal writing point was used in making this record.

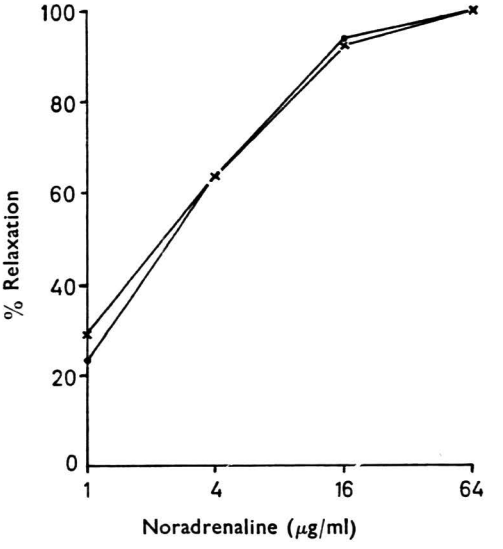

FIG. 2. Comparison of log concentration: effect curves for noradrenaline obtained with the paired tracheal chain preparation. Percentage relaxation is plotted against final bath concentration of noradrenaline (in μg/ml) on a log scale. ●——● = sequential method on one member, ×——× = cumulative method on the other member of the pair of preparations.

responses obtained by the two methods to twofold increases in concentration of isoprenaline. Fig. 2 compares the curves for noradrenaline obtained by both methods over a wider concentration range between members. No systematic difference between the methods was found in either the slope of the curve or the ED50 for either agonist. Small differences were apparent but, whether assessed cumulatively or sequentially, noradrenaline was as often more potent as it was less; the small differences were also more apparent within, than between, chains (due to slight changes in sensitivity which occur with lapse of time), and were similar in magnitude to spontaneous changes which occur when log concentration: effect curves are repeated on the same pair of preparations by the sequential method only (the maximum observed difference in ED50 was 0·22 log units).

RELATIVE POTENCY OF CATECHOLAMINES

(−)-Noradrenaline, (−)-adrenaline and (−)-isoprenaline are powerful relaxants of the guinea-pig isolated tracheal chain. If an adequate concentration is applied each can evoke the maximum relaxation of which the preparation is capable. If smaller concentrations are applied, a steep sigmoid relationship can be demonstrated between the logarithm of the concentration and the percentage relaxation produced. The mid portions of the log concentration: effect curves of these three catecholamines do not differ significantly in slope. There is a non-significant ($0{\cdot}1 < P < 0{\cdot}25$) tendency for the slope of isoprenaline to be less than that of noradrenaline. The logarithms of the concentrations of (−)-noradrenaline producing a 50% relaxation on 68 different preparations were normally distributed as shown by equality of the mean, the median and mode. The relative molar potencies of these three catecholamines are shown in Table 1, and compared with previously reported values.

TABLE 1. MOLAR POTENCIES OF CATECHOLAMINES RELATIVE TO (−)-NORADRENALINE

(−)-Isoprenaline	(−)-Adrenaline	(−)-Noradrenaline	Reference
280	8·1	1	Hawkins (1952)
32	4·6	1	McDougall & West (1953)
—	11·0	1	Lu & Allmark (1954)
—	15·0	1	Lu & Allmark (1954)
174	10·2	1	Present work with range of standard error.
151–200	9·1–11·5		

Note.—Racemic drugs are assumed to show half the activity of the (−)-form. Standard errors are not equal above and below the mean because they are converted from a logarithmic scale.

There is fair, though not excellent, agreement between these values for the relative potencies of the catecholamines. All agree that (−)-isoprenaline is more potent than (−)-adrenaline and that this is more potent than (−)-noradrenaline.

EFFECTS OF α-BLOCKING AGENTS

Piperoxan in concentrations of 10 to 80 μg/ml, produced a small contraction of the tracheal muscle but no change in its sensitivity to noradrenaline.

Thymoxamine in concentrations of 1 to 64 μg/ml caused a very small (less than $\times 2$) potentiation of noradrenaline. 64 μg/ml also caused a small contraction of the tracheal muscle; lower concentrations did not change its tone.

Dihydrogenated ergot alkaloids. Dihydroergotamine, 2 μg/ml, had no effect on either tone or sensitivity to noradrenaline. Hydergine at 6 to 14 μg/ml had no effect on tone but caused a very small potentiation of noradrenaline. 40 μg/ml caused a small ($\times$ 2) potentiation of noradrenaline without changing tone.

Phentolamine. Concentrations of 5 to 80 μg/ml caused a dose-dependent and large ($\times 20$ at 40 μg/ml) potentiation of noradrenaline. Concentrations of 10 to 80 μg/ml caused a dose-dependent and medium ($\times$ 7 at 40 μg/ml) potentiation of isoprenaline. 80 μg/ml caused a small relaxation of the tracheal muscle; lower concentrations did not change its tone.

Phenoxybenzamine in concentrations of 0·4 to 2 μg/ml caused a slowly-developing and large potentiation of all three catecholamines; noradrenaline was potentiated most. There was no change in tone.

EFFECTS OF β-BLOCKING AGENTS

The dichloro-analogues of isoprenaline, adrenaline and noradrenaline, and pronethalol and propranolol each antagonised the catecholamines and did not antagonise aminophylline, sodium nitrite or papaverine at the concentrations tested.

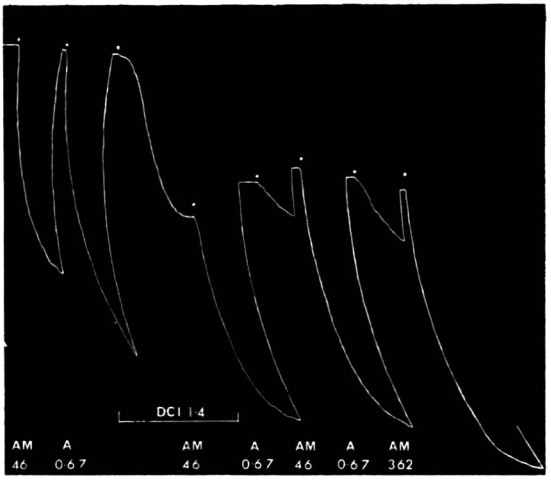

FIG. 3. Dichloroisoprenaline (DCI) produces a relaxation and antagonises adrenaline (A). Aminophylline (AM) is not antagonized. Both the relaxation and antagonism produced by dichloroisoprenaline persist after it is washed from the bath. Concentrations are in μg/ml. A side writing point was used in making this record.

Dichloroisoprenaline was most unsatisfactory because in concentrations of 0·2 to 20 μg/ml it caused a relaxation of the trachea. This relaxation was never maximal but did not seem to be dose-dependent and might

range from 10 to 80% of the existing tone; it also proved slow to wash out. Concentrations of 1 to 20 μg/ml, applied for 35 min, blocked the actions of catecholamines for several hours by an amount which increased with the concentration of dichloroisoprenaline used. This blockade was surmountable and log concentration: effect curves for each catecholamine before and after the blocking agent were parallel. Fig. 3 shows the relaxation and antagonism of adrenaline caused by 1·4 μg/ml of dichloroisoprenaline without antagonism of aminophylline.

Isoprenaline was antagonised more than noradrenaline.

Dichloroadrenaline. This was qualitatively similar to dichloroisoprenaline but less potent.

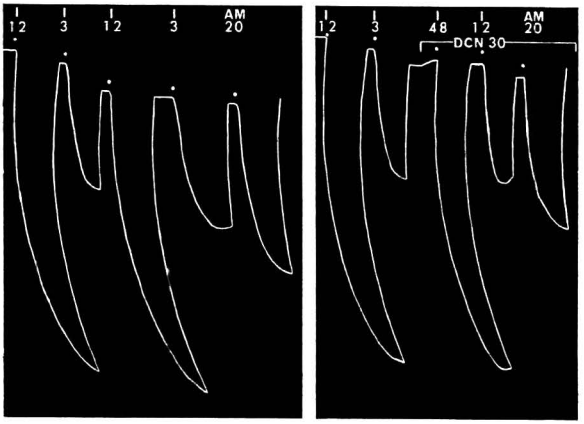

FIG. 4. Dichloronoradrenaline (DCN) antagonises isoprenaline(I) without relaxing the tracheal chain. Aminophylline (AM) is not antagonised. Note the very similar behaviour of the two members of this paired preparation—the left is used as a control to measure any spontaneous changes in drug sensitivity which may occur. A side writing point was used in making this record. Concentrations are in ng/ml for isoprenaline and in μg/ml for aminophylline and dichloronoradrenaline.

Dichloronoradrenaline was less potent still but had the advantage that a concentration could be found (about 40 μg/ml) which antagonised the catecholamines without causing a relaxation. Fig. 4 shows the antagonism of isoprenaline by dichloronoradrenaline without antagonism of aminophylline.

Propranolol in concentrations of 0·0025 to 20 μg/ml caused a dose dependent antagonism of the catecholamines without changing the tone. Catecholamine log concentration: effect curves were parallel before and after propranolol antagonism. This antagonism increased in size, if the propranolol was maintained in the bath, over several hours. It was thus impossible to perform experiments at a true equilibrium. Fig. 5 shows the effect on the adrenaline log concentration: effect curve of increasing concentrations of propranolol added 30 min before each cumulative noradrenaline challenge. Fig. 6 shows the results of six similar experiments with noradrenaline plotted after Arunlakshana & Schild (1959)—logarithm (noradrenaline dose ratio − 1) against negative

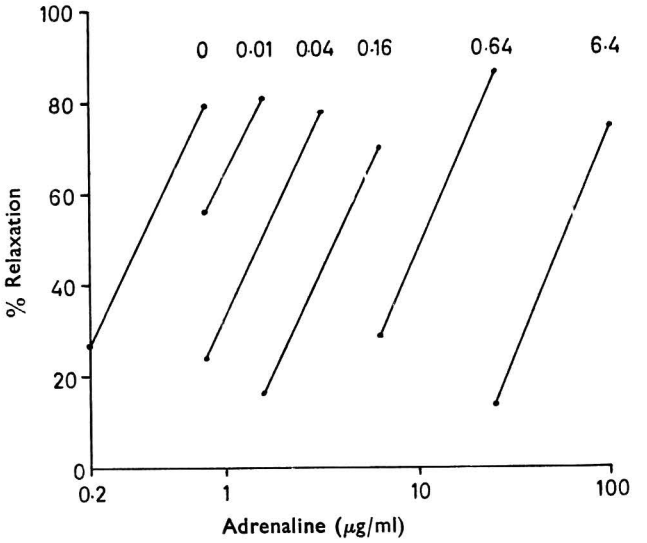

FIG. 5. Log concentration: effect lines for adrenaline obtained on the same preparation. The effect of increasing concentrations of propranolol (in μg/ml) added 30 min beforehand is shown. Propranolol causes a concentration-dependent parallel shift to the right of the adrenaline log concentration: effect line over a 640-fold range of increasing concentration.

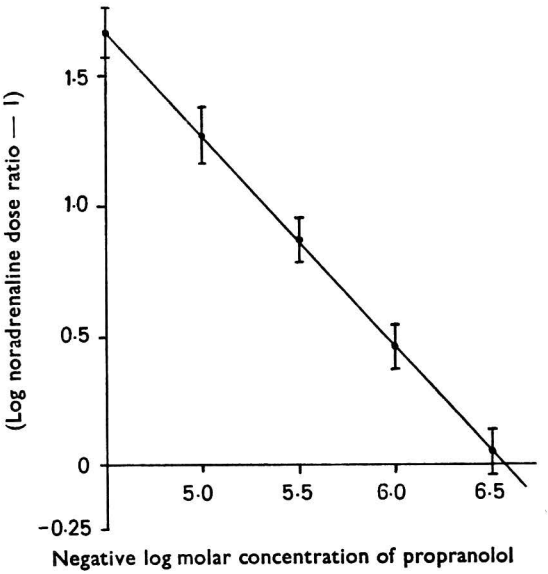

FIG. 6. Plot of log (noradrenaline dose ratio minus one) against negative log molar concentration of propranolol. Each point (with its standard error) is a mean derived from six experiments similar to that illustrated in Fig. 5 but using noradrenaline as agonist. Note that the points lie on a straight line over a 100-fold range of antagonist concentration. $pA_2 = 6{\cdot}56$; $pA_2 - pA_{10} = 1{\cdot}16$.

logarithm of molar concentration of propranolol. The mean pA_2 with standard error was 6·56 ± 0·21 and the mean $pA_2 - pA_{10}$ with standard error was 1·16 ± 0·05.

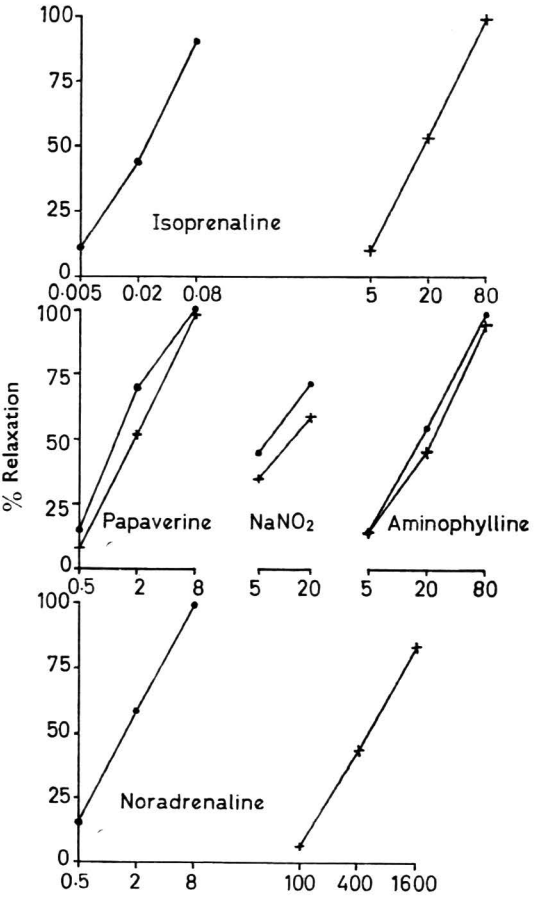

FIG. 7. Specificity of propranolol. Log concentration: effect curves obtained with five agonists on each member of the same paired preparation. One member (●———●) was bathed in normal Krebs solution, the other (+———+) was continuously exposed to propranolol, 20 μg/ml, the highest concentration used. Papaverine, sodium nitrite and aminophylline are insignificantly affected; noradrenaline is blocked by a factor of 320, isoprenaline by a factor of 780. All concentrations are expressed in μg/ml on the same logarithmic scale.

Both isoprenaline and adrenaline were blocked much more than noradrenaline by the same concentration of propranolol. This difference emerged from 3 types of experiment. (1) A concentration of propranolol could be found (about 12·5 ng/ml) which would significantly antagonise isoprenaline without antagonising noradrenaline. (2) When tested on the same preparation any one concentration of propranolol always antagonized isoprenaline more than noradrenaline (see for example

Fig. 7). (3) Single pA_2 values determined as in Figs 5 and 6 were larger for isoprenaline and adrenaline than for noradrenaline.

Fig. 7 shows the specificity of propranolol. One member of a pair of tracheal chains was continuously exposed to the β-blocking agent in very high concentration and cumulative log concentration:effect curves for sodium nitrite, aminophylline, isoprenaline, noradrenaline, and papaverine were determined on each. Only the catecholamines are antagonised and isoprenaline more than noradrenaline.

Attempts to wash the propranolol from the tissues were only partially successful even though continued for many hours.

Pronethalol was qualitatively very similar to propranolol. It was less potent by a factor (with range of standard error) of 18·6 (11·4 to 30·5). Its reported (Black & Stephenson, 1962) sympathomimetic action was only occasionally observed and is certainly much less of a problem than with dichloroisoprenaline.

Five determinations of the pA_2 of pronethalol against noradrenaline gave a mean value, with standard error, of $5{\cdot}29 \pm 0{\cdot}07$. The $pA_2 - pA_{10}$ was $1{\cdot}03 \pm 0{\cdot}07$.

Discussion

The time cycle of 40 min necessary for the tracheal chain preparation when drug potency is assessed by the sequential method is inordinately long compared with that applicable to most α-receptor containing tissues. It is effectively halved by the use of the paired preparation, since the two members forming the pair behave identically (Foster, 1960); even so, the amount of information obtainable in one day is limited. The use of the cumulative method reduces the effective time cycle still further by requiring only one 25-min wash period after every two or three drug additions. That the method is valid is indicated by the close similarity of log concentration:effect curves produced by it to those produced by the sequential method.

Analysis of the nature of the adrenergic receptors in any tissue is based on two fundamental experiments: the relative potency of catecholamines (Ahlquist, 1948) and the identity of specific blocking agents.

The decending order of potency of the catecholamines on the trachea is isoprenaline, adrenaline and noradrenaline. This is highly suggestive of interaction with β-receptors and this suggestion is strengthened by the sizes of their potency differences.

Even after very large concentrations of β-blocking agents (e.g. 20 μg/ml of propranolol) noradrenaline has never caused a contraction of the trachea such as described by Castro de la Mata & others (1962) on dog bronchioles *in vivo*.

Piperoxan, thymoxamine and the dihydrogenated ergot alkaloids have no significant actions on the trachea in concentrations many times larger than those which cause antagonism of noradrenaline on recognised α-receptor containing isolated tissues (Leitch, Liebig & Haley, 1954; Paterson, 1965). For instance, on the guinea-pig isolated vas deferens,

appreciable antagonism to noradrenaline (pA_2) was seen with 0·08 μg/ml, 0·008 μg/ml and 0·004 μg/ml of piperoxan, thymoxamine and dihydroergotamine respectively (Leach, 1956; Birmingham & Szolcsányi, 1965). Phentolamine and phenoxybenzamine, again in concentrations much greater than those necessary for α-blockade, produce a potentiation not only of noradrenaline but also of isoprenaline; this potentiating action is thus almost certainly unrelated to α-receptor blockade and has been observed on a variety of other tissues equipped with β-receptors. Holzbauer & Vogt (1955) found that phenoxybenzamine, 0·2 μg/ml, potentiated adrenaline and isoprenaline on the rat isolated uterus. Huković (1959) observed an increase in the effect of adrenergic nerve stimulation on the isolated rabbit atria with phenoxybenzamine, 25 μg/ml. Benfey & Greeff (1961) used isolated guinea-pig atria and showed that both phentolamine, 17 μg/ml, and phenoxybenzamine, 25 μg/ml, potentiated noradrenaline.

Direct evidence has recently become available which attributes this potentiating action of phenoxybenzamine to inhibition of noradrenaline uptake by the storage mechanism. Hertting, Axelrod & Whitby (1961) found that phenoxybenzamine markedly reduced the uptake of intravenous ^{3}H-noradrenaline into the heart, spleen and adrenal of the cat but phentolamine did not do this. Farrant, Harvey & Pennefather (1964) have shown that phenoxybenzamine blocks the uptake of noradrenaline into the stores of the cat kidney (but not the uterus) and rat heart, spleen and uterus. Iversen (1965b), working with the rat isolated perfused heart, found phenoxybenzamine, 10 μg/ml, to reduce the uptake of ^{3}H-noradrenaline by 92% and phentolamine, 10 μg/ml, to reduce it by 66%.

The present results were that phenoxybenzamine and phentolamine potentiated isoprenaline quite markedly, though less than noradrenaline. The uptake of noradrenaline into stores is greater than that of adrenaline (Iversen & Whitby, 1962; Iversen, 1965a). Isoprenaline has been ignored until recently. Evidence is appearing that there are two distinct uptake mechanisms. Uptake I operates at low catecholamine concentrations, has a greater affinity for noradrenaline than adrenaline and very little for isoprenaline; it is blocked by cocaine and desipramine (Iversen, 1963, 1965a). Uptake II operates at high catecholamine concentrations and is blocked by metanephrine (Iversen, 1965c); it does accumulate isoprenaline (Callingham, 1965). On this basis it would seem reasonable to suppose that phenoxybenzamine and phentolamine block uptake II in the guinea-pig trachea, since both potentiate isoprenaline. This point will be investigated further.

Arunlakshana & Schild (1959) have stated that "If two agonists act on the same receptors they can be expected to be antagonised by the same antagonist, and, if the antagonism is competitive, they can be expected to be antagonised by the same concentration of antagonist and to produce with it the same pA_x or dose ratio". While this is theoretically true if the competitive antagonist has only this action, quantitative work has demonstrated that various α-blocking agents antagonise noradrenaline

more than adrenaline on the guinea-pig isolated vas deferens (Leach, 1956).

A similar difference in the degree of antagonism to noradrenaline, adrenaline and isoprenaline is here reported for dichloroisoprenaline, pronethalol and propranolol and the question arises whether a different receptor is involved for each agonist. Dichloroisoprenaline and pronethalol are other agents known to block the uptake of noradrenaline into tissue stores. Muscholl (1961) found that dichloroisoprenaline completely blocked the uptake of noradrenaline into the rat heart and spleen. Farrant & others (1964) found it to block the uptake of noradrenaline into the cat uterus (but not the kidney); Iversen (1965b) has shown that dichloroisoprenaline, 2 μg/ml, and pronethalol, 5 μg/ml, reduced the uptake of ^{3}H-noradrenaline into the rat isolated perfused heart by 51% and 36% respectively. It is suggested that propranolol will be found to share this property and that this is the basis for the differential antagonism of β-blocking agents towards the catecholamines. It seems likely that the catecholamines are all blocked to the same extent at the receptor level but that at the same time noradrenaline is potentiated: this potentiation is "hidden" but serves to reduce the degree of antagonism experimentally observed.

The present work confirms the potency difference of about ten noted by Black, Crowther, Shanks & Dornhorst (1964) between propranolol and pronethalol and extends their basic pharmacology by demonstrating the competitive nature of their antagonism. The evidence for this may be summarised.

1. Over a 100-fold range of concentration propranolol or pronethalol causes a progressive parallel rightward shift of the log concentration: effect curve of each catecholamine. The maximum relaxation is not reduced.

2. Over the same range of concentration for propranolol or pronethalol there is a linear relation between the dose ratio of agonist minus one and the concentration of antagonist. The mean $pA_2 - pA_{10}$ values of $1{\cdot}16 \pm 0{\cdot}05$ and $1{\cdot}03 \pm 0{\cdot}07$ were in acceptable agreement with the theoretical value of 0·95 for competitive antagonism.

Acknowledgements. I am grateful to the following companies for gifts of drugs; Denver Labs Ltd. (thymoxamine); Eli Lilly & Co., Ltd. (the catecholamine dichloro-analogues); Ward, Blenkinsop & Co., Ltd. [(–)-isoprenaline) and I.C.I. (pronethalol and propranolol)].

The work on the relative potency of catecholamines, and the actions of their dichloro-analogues, was performed in the Pharmacology Department, King's College, London, and formed part of a thesis which has been accepted for the degree of Ph.D. in the University of London.

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The detection and identification of synthetic steroids in horse urine

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A scheme for the detection of synthetic steroids which could be used in the "doping" of race-horses is described. The method involves extraction from urine into ethyl acetate : ether (1 : 1) followed by initial two-dimensional thin-layer chromatography using (1) ethyl acetate (2) methylene chloride : dioxan : water (100 : 50 : 50). Elution of the ultraviolet absorbing spots for extinction measurements is followed by further one way thin-layer chromatography as free alcohols in amyl acetate : acetone (1 : 1). Further chromatography of the steroid alcohols in chloroform : ether : water (80 : 20 : 0·5) on formamide-impregnated plates and chromatography of the acetates in ether helps to identify the unknown steroid. Additional identification is made by colour reactions with (1) a tetrazolium reagent (2) vanillin : perchloric acid.

ANTI-INFLAMMATORY steroids are now widely used both in veterinary and human medicine. Their possible use to influence the performance of racehorses caused us to investigate methods for their detection. We report a method for the detection of the steroids most likely to be used in this way. The method enables a separation to be made between any of these synthetic steroids and the naturally occurring steroids in horse urine. For the purpose of identifying the presence of "dope", we developed a method which would detect unchanged drug because (i) this was easier than looking for conjugated forms of drug which would need to be hydrolysed; (ii) it made the diagnosis of "doping" more certain in cases where metabolites might be the same as those from endogenous steroids, e.g. metabolites from prednisolone and hydrocortisone; (iii) detailed metabolism studies were not necessary (although work on this aspect has already commenced). For speed, we considered a method based on thin-layer chromatography would be preferable to any based on paper chromatography. From our attempts to obtain samples, we found that the only accessible anti-inflammatory steroids were: betamethasone, dexamethasone, fludrocortisone, 6α-methylhydrocortisone, 6α-methylprednisolone, prednisolone, prednisone and triamcinolone. These were used as the alcohols since their 21-esters would be hydrolysed to the parent alcohols in the body (Voigt, 1959; Melby & St. Cyr, 1961). These steroids, and hydrocortisone and cortisone, and their separation, form the subject of this paper.

Experimental

MATERIALS

Triamcinolone, dexamethasone and betamethasone were gifts from Dr. R. W. H. Edwards. Further samples of these steroids together with fludrocortisone, 6α-methylhydrocortisone and 6α-methylprednisolone and the corresponding acetates were gifts from Mr. J. S. Wragg (Boots Pure Drug Co. Ltd.). Prednisolone, cortisone, hydrocortisone, and the

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corresponding acetates, and prednisone were obtained from Koch-Light Laboratories. Other acetates were prepared by the method of Edwards (1960).

METHODS

A Unicam SP.800 recording spectrophotometer was used for ultraviolet and visible absorption measurements.

The ethyl acetate was redistilled before use; other solvents and chemicals were of analytical grade where this was available (with the exception of the ether for chromatography which was solvent ether B.P.)

Thin-layer chromatography was on silica gel containing a fluorescent additive (Kieselgel GF.254-Merck) and of layer thickness 0·25 mm. The plates were prepared by slurrying 50 g silica gel, particle size 5-25 μ, with 100 ml distilled water, spread, dried in air at room temperature for 15 min, and dried and activated at 110° for 1 hr in an oven with internal fan.

Formamide-impregnated plates were prepared by ascending development of the plates with 20% formamide in acetone, followed by drying for 30 min in air at room temperature before use. A Hanovia 'Chromatolite' was used for ultraviolet (254 mμ) examination of the plate after chromatography. Reducing steroids were detected by spraying with a solution of 0·05% w/v 2,5-diphenyl-3-(4-styrylphenyl) tetrazolium chloride and 8% w/v sodium hydroxide in methanol. This was made immediately before use by mixing equal volumes of 0·1% tetrazolium solution and 16% sodium hydroxide solution, the strength used by Clifford, Wilkinson & Wragg (1964) to prepare their spray reagent containing tetrazolium blue.

Steroids generally were detected by spraying with vanillin-perchloric acid reagent. This reagent is made immediately before use by mixing equal volumes of 10% w/v vanillin in glacial acetic acid and 72% perchloric acid. Colours due to the steroids appear after heating at 90° for 10 min. This is essentially an application of the method of Few (1965).

URINE FRACTIONATION

All solvent extracts were taken to dryness under reduced pressure (water-pump) in a rotary film evaporator keeping the temperature below 40°. When these residues were submitted to chromatography, they were transferred to the plate (completely) using sodium sulphate-dried ether as solvent.

Urine (100 ml) was extracted with ethyl acetate:ether (1:1, 100 ml) by mechanical rolling in a bottle at one 1 rev./sec for 15 min. The solvent was *completely* separated, washed without delay with 1/10th volume ice-cold 0·1 N sodium hydroxide and then with 1/10th volume 0·1 N hydrochloric acid. The solvent layer was filtered and after thorough drying (sodium sulphate), evaporated to dryness and submitted to two dimensional thin-layer chromatography. The plates were first run in ethyl acetate and then in the system of Hall (1964) prepared by mixing methylene chloride:dioxan:water (100:50:50) and using the lower layer. Spots were located by ultraviolet examination.

Substances in the areas of absorption corresponding to any abnormally present steroid were eluted with 1 × 2 ml ethanol by shaking mechanically for 10 min. The ultraviolet absorption curve was then determined on the ethanolic solution after centrifuging. The Δ^4-3 ketones give a maximum at approximately 240 mμ (see Table 1). The remaining area of the plate was sprayed with the tetrazolium reagent followed by the vanillin: perchloric acid reagent. This detects any other non-ultraviolet absorbing steroids which may be present and also indicates whether they are reducing. This technique is useful in revealing the presence of certain metabolites of these drugs. After ultraviolet examination the ethanolic extracts were taken to dryness at room temperature, and chromatographed on silica gel using amyl acetate: acetone (1 : 1) as solvent. The steroids were located by ultraviolet light and their presence confirmed by spraying with the tetrazolium reagent, and then overspraying with the vanillin reagent (see Table 1).

Further confirmation of the presence of any of these steroids was obtained by submitting a urine extract (from 500 ml urine) to the two dimensional chromatography described and eluting the spots as before with ethanol. Approximately half of each eluate was taken to dryness, dissolved in 1 ml pyridine and 1 drop acetic anhydride added (Edwards, 1960). After leaving overnight at room temperature, the solution was taken to dryness under vacuum (water-pump), dissolved in ether and the acetates submitted to thin-layer chromatography using ether as solvent. The spots were located by ultraviolet light and then sprayed with the tetrazolium reagent to indicate the presence of the reducing side-chain.

The remaining ethanolic eluates were spotted on formamide impregnated plates and run in chloroform: ether: water (80 : 20 : 0·5) (Clifford & others, 1964) and the steroids were located as above.

Results and discussion

Rf values, determined on standard solutions and on steroids in urine extracts, are summarised in Table 1. At least five assessments were made of each Rf value, and these were usually obtained from different plates and in most instances on different days. In no case did the standard deviation of the results exceed 0·06. When identifying an unknown it is, of course, essential to run standards on the same thin-layer plate. For the best results, all solvent systems should be freshly prepared every 2 days and allowed to equilibrate for at least 1 hr before use.

The Rf values obtained from urine extracts do not necessarily correspond with those from pure standards. In the ethyl acetate system, separation of standards of cortisone or hydrocortisone and prednisolone or prednisone is not great, but in urine extracts the separation is marked. Triamcinolone in urine extracts gives a much increased Rf value compared with standard solutions. The Rf values of pure steroids and steroids in urine extracts are given in the Table for the systems used for the original separation, i.e. ethyl acetate and methylene chloride: dioxan: water. In the event of an abnormal steroid being detected, the Rf value for identification is listed in Table 1 under urine extract.

TABLE 1. Rf VALUES OF STEROIDS IN DIFFERENT SOLVENT SYSTEMS (on Silica gel plates)

Steroid	Ethyl acetate		Methylene chloride: dioxan: water		Chloroform: ether: water on formamide plates	Amyl acetate: acetone	λ_{max}*	Colour with vanillin-perchloric acid sprayed over the tetrazolium reagent	Rf of steroid acetate in ether
	Standard solutions	Steroids in urine extracts	Standard soln.	Steroids in urine extracts					
Betamethasone	0·47	0·40	0·48	0·45	0·33	0·69	240	Grey	0·44
Hydrocortisone	0·35	0·46	0·45	0·47	0·38	0·62	241	Purple/Brown	0·42
Cortisone	0·36	0·45	0·62	0·60	0·77	0·66	238	Red/Brown	0·34
Dexamethasone	0·47	0·41	0·45	0·45	0·34	0·69	242	Grey/Purple	0·45
Fludrocortisone	0·49	0·45	0·46	0·46	0·29	0·71	244	Purple/Brown	0·55
6α-Methylhydrocortisone	0·40	0·37	0·48	0·47	0·57	0·64	241	Red/Brown	0·42
6α-Methylprednisolone	0·38	0·35	0·41	0·41	0·45	0·66	243	Red/Brown	0·37
Prednisolone	0·30	0·27	0·38	0·38	0·25	0·61	243	Green/Grey	0·40
Prednisone	0·30	0·32	0·55	0·53	0·60	0·64	238	Grey-Purple	0·29
Triamcinolone	0·23	0·29	0·24	0·36	0·04	0·56	243	Light Brown	0·22

* Determined for ethanol solutions containing 20 μg/ml of steroid

The ethyl acetate conditions are critical and considerable variations in Rf values can occur if either the extract added to the plate is wet, or impurities, including water, are present in the ethyl acetate. Also all traces of ethyl acetate must be removed before the second run is commenced. The position of the original spot and the solvent used to transfer the steroid to the plate can also alter the Rf values. This is particularly true of triamcinolone. The papers by Quesenberry & Ungar (1964), Dallas (1965) and Clifford & others (1964) are of interest with reference to these points.

In the separation of the acetates using ether as solvent, the tank should be prepared 24 hr before use. The grade of ether can be critical and we have found that solvent ether B.P. (Boots Pure Drug Co. Ltd.), 'aged' for 24 hr as above, gave the greatest separations. The values quoted in Table 1 have been determined in this way.

The scheme has been applied to urines containing added steroids and it has been possible to detect and identify any of the steroids concerned at levels of 2 μg/ml in original urine. Although detection of 1 μg/ml is possible on the 2-dimensional chromatogram at this level there is usually insufficient material to allow the ultraviolet detection after chromatography in the amyl acetate : acetone system. The losses involved in manipulations and elutions at this level appear to be relatively large. The volume of urine available for extraction then becomes the limiting factor to the certain diagnosis of "doping".

We have found no materials present normally in horse urine which run within the same area and give an ultraviolet maximum within the same region as the steroids under discussion. There is a frequently ocurring "normal" spot in horse urine which does show up on the initial two-dimensional chromatogram in the area of these synthetic steroids, but usually only faintly, and the ultraviolet extinction of its eluate is virtually zero between 235 and 260 mμ. (Weak maxima are observed at approximately 230 and approximately 285 mμ; the identity of this spot is unknown). The initial two-dimensional chromatography and ultraviolet absorption measurement together serve as a quick and useful screen for the detection of foreign steroids in horse urine.

Where difficulty is experienced in obtaining a satisfactory ultraviolet absorption curve the acetate may be eluted, after chromatography in ether, and used for ultraviolet measurement.

For complete differentiation of dexamethasone from betamethasone, the acetate is submitted to chromatography on alumina as suggested by Hall (1964). The Rf of betamethasone acetate is 0·45 and that of dexamethasone acetate 0·59.

We feel that the scheme gives a quick and sound identification of any of these foreign steroids. The evidence of identification can be summarised as follows:

1. A neutral material which, on thin-layer chromatography, shows as an ultraviolet-absorbing spot on the original two-dimensional chromatogram in a region normally free from ultraviolet absorbing material.
2. Rf values in agreement with those found in four systems: (i) ethyl

acetate; (ii) methylene chloride: dioxan: water; (iii) amyl acetate: acetone and (iv) in a partition system formamide-chloroform: ether: water for the parent steroid.

3. An Rf value for the steroid acetate in ether.

4. An ultraviolet maximum (in ethanol) between 235–245 mμ (see Table 1), the reaction with the tetrazolium reagent and the colour produced with the vanillin-perchloric acid reagent.

Any substance conforming is an 'abnormal' in horse urine belonging to the *neutral* group of drugs. This 'abnormal' would contain in the same molecule a group giving absorption in the ultraviolet (of which a Δ^4-3-one structure is one possibility), a fairly easily esterifiable group (presumably a primary or unhindered secondary alcohol), a reducing structure capable of reducing the tetrazolium reagent in the *cold* (of which an α-ketol structure is the most likely), a grouping reacting with vanillin-perchloric acid (see Few, 1965) and agreeing in Rf value, in 5 systems, with a standard steroid.

In experiments on the metabolism of prednisolone and prednisone which we hope to publish shortly, we found that the administration of either gave a mixture of both in the urine. Therefore as a further confirmation of prednisolone (or prednisone) administration, we expect to identify by our procedures both the 11-hydroxy- and the 11-oxo-compound in the urine.

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Biotransformations *in vitro* undergone by phenothiazine derivatives in a liver preparation

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Characterisation of the products of biotransformations resulting when a wide range of medicinally used phenothiazine derivatives were incubated with a "microsomal and soluble" fraction of livers from albino Wistar rats, is described. The compounds were chlorpromazine, demethylchlorpromazine, dedimethylchlorpromazine, triflupromazine, promazine, acepromazine, propiomazine, diethazine, promethazine, isopromethazine, ethopropazine, trimeprazine, methotrimeprazine, ethylmemazine, proquamezine, cyamemazine, trifluoperazine, prochlorperazine, thioproperazine, fluphenazine, pecazine, and also the sulphoxides of the first three compounds and of promazine. The predominant reactions were those of dealkylation and of hydroxylation of the aromatic ring. Sulphoxidation did not occur to a significant extent with any of the compounds examined.

APART from the distribution in the tissues, the rate of biotransformation of a foreign compound and the chemical change or changes effected by the organism will inevitably affect the observed pharmacological action of the compound. A comparative study of the changes undergone by pharmacologically diverse phenothiazine derivatives on incubation with a rat liver preparation was undertaken to determine whether the predominant pharmacological action of a given compound might be related to the observed biotransformations.

Experimental

LIVER PREPARATION

The livers of male albino Wistar rats, 200–250 g, were homogenised in the cold with two volumes of potassium chloride solution (1·15% w/v). The homogenate was centrifuged at 9000 rcf. for 15–20 min at 0° to sediment unbroken cells and mitochondria. The supernatant fraction was withdrawn and used (2 ml per reaction mixture) as "microsomal and soluble" fraction. The reaction medium, total volume 8 ml, was as described by Mueller & Miller (1955) but using glucose 6-phosphate in place of the diphosphate and adding the phenothiazine derivative (2·5 μ moles). Five replicate flasks were used for each compound examined. After incubating the reaction vessels with shaking at 37° for 1½ hr, the contents of each set of five flasks were pooled, solid sodium hydroxide (to 3% w/v) was added and the mixture heated on a boiling water-bath for 1 hr. This procedure ensured the subsequent extraction of the phenothiazine derivatives. After the hydrolysis, the extract was transferred to a liquid/liquid extractor and extracted continuously with ether (approximately 125 ml) for at least 2 hr. The ether extract was washed free from alkali and distilled to a low volume from a warm water-bath under reduced pressure. Where storage before further examination was unavoidable, the flask containing the extract was flushed with nitrogen before being sealed and stored in the dark.

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THIN-LAYER CHROMATOGRAPHY

Thin-layer plates, approximately 250 μ thick, were prepared from Aluminium Oxid G (Merck) and from silica gel (Kieselgel, Merck). The alumina plates were oven-dried immediately before use. Solvent development was allowed to proceed to 10–12 cm from the point of sample application, which was 1·5 cm from the lower edge of the plate. After drying, the plates were examined for fluorescence under ultraviolet light.

Solvents. (1) Chloroform containing ethanol (10% by volume) as described previously (Robinson & Beaven, 1964) was used for alumina plates. For silica gel plates, the most useful were (2) chloroform : ethanol : ammonia solution (0·89) 80 : 20 : 1, by volume, and (3) n-propanol : water : acetic acid 85 : 15 : 2, by volume.

The chloroform used for these solvents contained approximately 1% ethanol by volume as a stabiliser. Solvent was replaced after the development of six plates.

Spray reagents. (1) Sulphuric acid 50% v/v; (2) Analar hydrobromic acid (B.D.H., 46–48%); (3) ferric chloride solution (1% w/v in dilute nitric acid); (4) sodium metaperiodate 1% w/v in water (Posner, Culpan & Levine, 1964); (5) ammonium persulphate 5% w/v in water; (6) Gibb's reagent (2,6-dichloro-1,4-benzoquinone-4-chloroimine 1% w/v in ethanol, followed by exposure of the chromatogram to ammonia vapour); (7) sodium nitroprusside-acetaldehyde reagent for secondary amines (Walkenstein & Seifter, 1959).

Rf values. The Rf values for a parent compound and its metabolites quoted in any of the tables were obtained from a single chromatogram. Mean Rf values for chlorpromazine, promazine and triflupromazine were 0·80, 0·70 and 0·80 with standard deviations of 0·06, 0·11 and 0·11 respectively for 30 results obtained on different silica gel plates using solvent (2). The relative positions of the metabolites with respect to the parent compound were constant despite small variations in the absolute Rf values due to minor changes in the experimental conditions.

ABSORPTION SPECTROSCOPY

Spectra were recorded using a Unicam SP 800 spectrophotometer.

The dry residue obtained from evaporation of an ether extract was shaken with 4 ml of sulphuric acid (0·01 N) and the solution filtered through a sintered-glass filter (porosity 3). An aliquot portion was diluted suitably with the acid and the ultraviolet absorption curve was recorded. To the remaining filtrate was added an equal volume of concentrated sulphuric acid and, after cooling, the visible absorption curve was recorded; the ultraviolet absorption curve of this solution was measured after diluting one in ten with sulphuric acid (50% v/v).

Differential spectrophotometry was carried out by replacing the solvent in the reference cuvette with an aqueous solution of the drug or its sulphoxide or a solution of the drug to which an equal volume of concentrated sulphuric acid had been added.

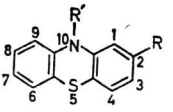
The presence of the sulphoxide of the original drug may be detected by inspection of the ultraviolet absorption curve of an extract in dilute acid solution, in particular by examining the shape of the curve between 270–350 mμ. Differential spectra obtained using both the parent compound and the sulphoxide (when available) confirmed the validity of results obtained by this method. Solutions of the extracts prepared as described above are not contaminated significantly by coenzymes derived from the incubation medium.

Differential measurements on the coloured solutions obtained on adding sulphuric acid to the extracts permitted evaluation of the spectra of some derivatives produced during the biological reaction (see Figs 1 and 3); the sulphides and the corresponding sulphoxides give rise to similar spectra and dealkylation of the 10-dialkylaminoalkyl substituent does not alter the spectrum of the parent compound significantly.

COMPOUNDS

The phenothiazine derivatives were kindly donated by various pharmaceutical companies. Samples which had melting-points corresponding

TABLE 1. FORMULAE OF PHENOTHIAZINE DERIVATIVES EXAMINED



Compound	R	R'
Chlorpromazine	Cl	$CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}NMe_2$
Demethylchlorpromazine	Cl	$CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}NHMe$
Dedimethylchlorpromazine	Cl	$CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}NH_2$
Triflupromazine	CF_3	$CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}NMe_2$
Promazine	H	$CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}NMe_2$
Acepromazine	CO·Me	$CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}NMe_2$
Propiomazine	CO·Et	$CH_2{\cdot}CH(Me){\cdot}NMe_2$
Diethazine	H	$CH_2{\cdot}CH_2{\cdot}NEt_2$
Promethazine	H	$CH_2{\cdot}CH(Me){\cdot}NMe_2$
Isopromethazine	H	$CH(Me)CH_2{\cdot}NMe_2$
Ethopropazine	H	$CH_2{\cdot}CH(Me){\cdot}NEt_2$
Trimeprazine	H	$CH_2{\cdot}CH(Me){\cdot}CH_2{\cdot}NMe_2$
Methotrimeprazine	O·Me	$CH_2{\cdot}CH(Me){\cdot}CH_2{\cdot}NMe_2$
Ethylmemazine	Et	$CH_2{\cdot}CH(Me){\cdot}CH_2{\cdot}NMe_2$
Proquamezine	H	$CH_2{\cdot}CH(NMe_2){\cdot}CH_2{\cdot}NMe_2$
Cyamemazine	CN	$CH_2{\cdot}CH(Me){\cdot}CH_2{\cdot}NMe_2$
Trifluoperazine	CF_3	$CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}N$ ⟨piperazine⟩ $N{\cdot}Me$
Prochlorperazine	Cl	$CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}N$ ⟨piperazine⟩ $N{\cdot}Me$
Thioproperazine	$SO_2{\cdot}NMe_2$	$CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}N$ ⟨piperazine⟩ $N{\cdot}Me$
Fluphenazine	CF_3	$CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}N$ ⟨piperazine⟩ $N{\cdot}CH_2{\cdot}CH_2OH$
Pecazine	H	CH_2–⟨piperidine⟩$N{\cdot}Me$

with those given in the literature and which migrated as single substances on thin-layer chromatoplates were used without further purification. The compounds examined, with their formulae, are listed in Table 1.

Results and discussion

CHLORPROMAZINE AND ITS DEMETHYLATED AND SULPHOXIDISED ANALOGUES

Chloropromazine. The results using chlorpromazine were similar to those described previously using the whole liver homogenate (Robinson & Beaven, 1964) except that hydroxylated demethylchlorpromazine and traces of chlorpromazine sulphoxide were detected as well. There was no evidence of oxidation at the amino-nitrogen atom.

TABLE 2. CHARACTERISTICS OF CHLORPROMAZINE METABOLITES, RECOVERED FROM THE INCUBATION MEDIUM, ON SILICA GEL CHROMATOPLATES

Compounds	Rf on silica gel plates	
	Solvent (2)	Solvent (3)
Chlorpromazine	0·80	0·30
Chlorpromazine sulphoxide	0·62	0·08
Demethylchlorpromazine	0·53	0·45
Hydroxychlorpromazine	0·47	0·23
Demethylhydroxychlorpromazine	0·15	0·03
Dedimethylchlorpromazine	*	0·60

* Runs with demethylchlorpromazine in this solvent.

Thin-layer chromatographic characteristics of the metabolites are given in Table 2. Demethylchlorpromazine gave a blue colour with the sodium nitroprusside-acetaldehyde reagent, as did the metabolite referred to as demethylhydroxychlorpromazine. The two hydroxylated metabolites consistently gave a purple colour with sulphuric acid, hydrobromic acid and ferric chloride, and an immediate blue colour with Gibb's reagent. Most of the phenothiazine compounds examined gave a blue-grey colour with Gibb's reagent and with sodium nitroprusside-acetaldehyde on standing, presumably due to an oxidising reaction. The colour produced by the hydroxylated compounds with sodium periodate was usually a brick red, as is given by 7-hydroxychlorpromazine, but in some of the extracts examined a definite pale emerald-green colour was obtained. The ammonium persulphate reagent gave similar colours and was preferred because the colours were more intense and more persistent. The green colour with these reagents was seen only on plates developed in a non-acidic solvent; the presence of traces of acid resulted in a lavender-purple colour.

The ultraviolet absorption characteristics of the extract in dilute acid solution were not remarkable and easily resolved by differential measurements into sulphide (λ_{max} 255 and 305 mμ), which predominated, and a trace of sulphoxide (λ_{max} 239, 275, 290 and 342 mμ).

Differential visible absorption spectra in strong acid solution indicated the presence of a hydroxy-compound (λ_{max} 570 mμ) as well as chlorpromazine (λ_{max} 530 mμ). The ultraviolet absorption peaks of dilutions

(with 50% v/v) sulphuric acid of the coloured solutions of the extracts were at 218, 270 and 277 mμ; chlorpromazine has peaks at 213, 268 and 277·5 mμ under the same conditions.

In the current work a greater proportion of the initial chlorpromazine underwent biotransformation reactions than was found previously (Robinson & Beaven, 1964). This was partly due to an increase in the incubation time but mainly due to the use of a fraction of the liver rather than the whole homogenate, thus minimising the non-specific interactions with proteins (Gabay & Harris, 1965) other than those associated with enzymes involved in the biotransformation reactions. The contamination, assessed spectrophotometrically, of the solutions with coenzymes was negligible.

TABLE 3. SUMMARY OF CONCLUSIONS OF THE BIOTRANSFORMATIONS FOUND FOR CHLORPROMAZINE ANALOGUES

Compound incubated	Transformation products found*
Chlorpromazine	Chlorpromazine sulphoxide (trace) Demethylchlorpromazine Dedimethylchlorpromazine Hydroxychlorpromazine Demethylhydroxychlorpromazine
Chlorpromazine sulphoxide	Chlorpromazine (trace)
Demethylchlorpromazine	Demethylchlorpromazine sulphoxide Demethylhydroxychlorpromazine
Demethylchlorpromazine sulphoxide Dedimethylchlorpromazine Dedimethylchlorpromazine sulphoxide	None

* In each case the original compound incubated was also detected in the preparation at the end of the incubation.

Since no chlorpromazine sulphoxide could be detected when a control chlorpromazine solution was carried through the entire experimental procedure, omitting only the addition of the tissue preparation to the incubation mixture, it seems unlikely that the chlorpromazine sulphoxide found after incubation was an artifact. Similar stability was found with the other compounds included in this series of experiments.

The hydroxylated chlorpromazine derivative which gave the unusual green colour with periodate and persulphate reagents was obtained on several occasions. In other experiments, a substance having similar chromatographic characteristics was obtained; this gave a reddish-brown colour with these reagents. The spectral absorption properties of the extracts containing these different metabolites were similar although minor differences could have been masked by the predominance of non-hydroxylated compounds. It is considered that these two metabolites may differ only in the position of the hydroxyl group in the phenothiazine ring system.

Chlorpromazine analogues. Thin-layer chromatographic data for the extracts obtained after incubation of the demethylated and sulphoxidised analogues of chlorpromazine with the liver preparation, together with the absorption spectral data for the dilute and strong sulphuric acid solutions of the extracts, yielded the conclusions summarised in Table 3.

These results are relevant to the current polemics concerning the mechanism of biological hydroxylation of phenothiazine derivatives. Under the appropriate chemical conditions some phenothiazine-5-oxides rearrange to the corresponding 3-hydroxyphenothiazine (see Craig & Tate, 1961), which has caused speculation that biological sulphoxidation could precede rearrangement to a ring hydroxy-derivative; the latter reaction could be effected either biologically or non-biologically. Alternatively, sulphoxidation and hydroxylation could be separate biological reactions.

The biotransformation reactions seen in the present work for chlorpromazine and demethylchlorpromazine include hydroxylation and, to a lesser extent, sulphoxidation; comparatively more of the initial chlorpromazine was metabolised (A. E. Robinson and S. E. Selim to be published). Since the three sulphoxides (except for a trace of a reduction product of chlorpromazine sulphoxide) were recovered unchanged from the incubation mixtures, an enzyme-catalysed rearrangement to a hydroxy-compound appears to be unlikely. It is possible that if the enzyme is located within the hepatic microsomes the physical characteristics, including the more nearly planar shape and the partition coefficient may prevent the sulphoxide gaining access to the enzyme site(s).

TABLE 4. THIN-LAYER CHROMATOGRAPHIC CHARACTERISTICS OF EXTRACTS FROM INCUBATION MEDIA CONTAINING TRIFLUPROMAZINE

Solvent 1*	Solvent 2**	Solvent 3**	Fluorescence	Colours given with reagents: 1 & 2	3	4	5	6	7	Identity concluded
0·84	0·84	0·28†	Green	O	O	pO	O	—	—	Triflupromazine
0·80	0·70	0·10	Blue	O	—	O	O	—	—	Triflupromazine sulphoxide
0·52	0·60	0·50	Green	O	O	O	O	—	B	Demethyltriflupromazine
0·30	0·54	0·35	Green	Pu	Pu	Pi/Pu	Pi/Pu	G	—	Hydroxytriflupromazine
0·02	0·23	0·62	Green	Pu	Pu	Pi/Pu	Pi/Pu	G	B	Demethylhydroxy-triflupromazine

* Alumina chromatoplate. ** Silica gel chromatoplate. † Ran immediately behind hydroxy triflupromazine. Colour code: O = Orange; B = Blue; Pu = Purple; Pi = Pink; G = Grey-green

Triflupromazine. The chromatographic and spectral absorption properties of the extracts obtained after incubation of triflupromazine with the liver fraction are summarised in Tables 4 and 5. Fig. 1 shows the visible absorption spectra obtained using (*a*) normal reagent blank (curve 1), (*b*) triflupromazine (curve 2) and (*c*) 7-methoxytriflupromazine (curve 3) in the reference beam. Clearly, the extract contains two absorbing components: unchanged triflupromazine and a hydroxy-derivative (cf. Table 5), and possibly a third. In most instances, the absorption peak of the hydroxylated triflupromazine was at about 570 mμ; this metabolite appears to be 7-hydroxytriflupromazine. However, a metabolite having the same Rf value in systems 1 and 2 and giving the same colour reactions with all reagents except periodate and persulphate was recovered from a developed thin-layer chromatoplate and gave a well-defined spectrum in strong sulphuric acid with peaks at 217–8, 270,

TABLE 5. LIGHT ABSORBING PROPERTIES OF SOLUTIONS OF TRIFLUPROMAZINE, EXTRACTS FROM INCUBATION MEDIA TO WHICH TRIFLUPROMAZINE WAS ADDED, AND REFERENCE COMPOUNDS

Compound	Ultraviolet absorption peaks* in 0·01 N sulphuric acid solution	Comments	Ultraviolet and visible absorption peaks* in 50% v/v sulphuric acid solution	Comments
Triflupromazine	(235) 256 306		207 (250) 275 505	Reference compound
Triflupromazine sulphoxide.. ..	237 274, 298 340		207 (250) 275 505	Reference compound
Extract from incubation with triflupromazine	(235) 256 306	Slight distortion at 235–40 and 270–85	210 276 520 and 568 505, 568	Unresolved Resolved†
Extract from incubation with triflupromazine and proadifen	256 304		206 276 504–5	
7-Methoxytriflupromazine	(240) 258 308		225 282 (370) (450) 573	Reference compound
7-Methoxy-2-trifluoromethyl-phenothiazine	217 (240) 262 314	In ethanol	222, 249 279 348, 376 436 558	Reference compound
8-Methoxy-2-trifluoromethyl-phenothiazine	235, 262 303 (335)	In ethanol	226 280 350 (460) 559	Reference compound

* Wavelengths in mμ. Those in brackets refer to shoulders on the curves. † see text.

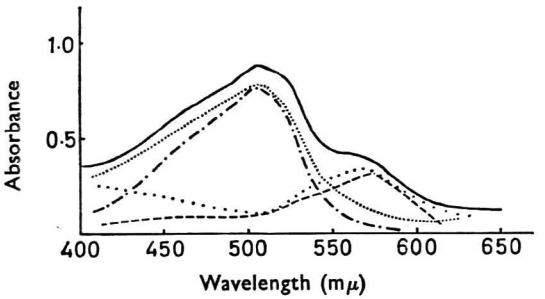


FIG. 1. Visible absorption curves in 50% v/v sulphuric acid for an extract derived from incubation of the liver fraction with triflupromazine. 1. ——— Extract against 50% v/v sulphuric acid. 2. . . . . Extract against triflupromazine in 50% v/v sulphuric acid. 3. - - - - - Extract against 7-methoxytriflupromazine in 50% v/v sulphuric acid. 4. — · — · — Solution of triflupromazine in 50% v/v sulphuric acid used as reference for 2 against 50% v/v sulphuric acid. 5. — — — Solution of 7-methoxytriflupromazine in 50% v/v sulphuric acid used as reference for 3 against 50% v/v sulphuric acid.

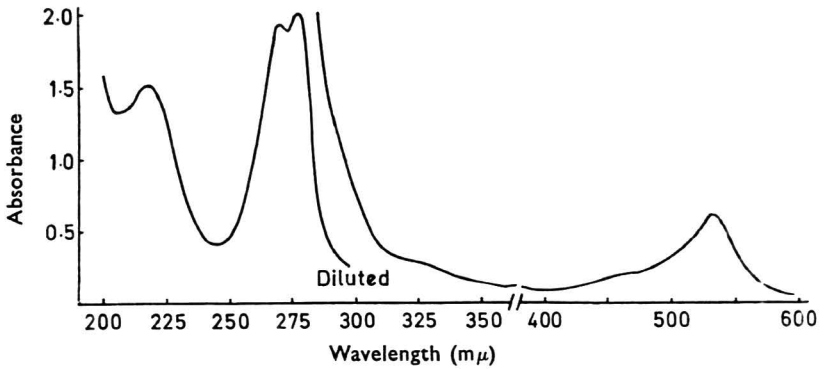


FIG. 2. Absorption spectrum of a hydroxylated triflupromazine derivative in 50% v/v sulphuric acid recovered from a thin-layer chromatoplate.

277·5, 350 (shoulder), 376 and 533 mμ (see Fig. 2). This metabolite is thought to be a position isomer, probably 3-hydroxytriflupromazine or, possibly, 8-hydroxytriflupromazine (see Table 5 and cf. chlorpromazine).

Qualitatively similar results were obtained when the initial concentration of triflupromazine in the incubation mixtures was varied between 1 and 5 μmoles per flask.

Addition of proadifen hydrochloride (SKF 525-A; 2-diethylaminoethyl-αα-diphenylvalerate hydrochloride) to the incubation mixture containing triflupromazine at a molar ratio of between 1 to 6 and 1 to 2 prevented hydroxylation of the compound; demethylation was unaffected and no greater proportion of sulphoxide was noted. This inhibition of a detoxication reaction would explain the enhanced or prolonged pharmacological action in animals treated with the inhibitor. The hydroxylated phenothiazine derivatives as a whole seem to be less active pharmacologically than the parent compounds.

OTHER PROMAZINE DERIVATIVES

The remaining compounds of this group which were examined included promazine and the corresponding sulphoxide, acepromazine, and propiomazine.

TABLE 6. THIN-LAYER (SILICA GEL) CHROMATOGRAPHIC CHARACTERISTICS OF EXTRACTS FROM INCUBATION MEDIA CONTAINING PROMAZINE AND FOR REFERENCE COMPOUNDS

	Rf		Reagents			
	Solvent 2	Solvent 3	1 & 2	4	5	Compound
Extract	0·75	0·19	O/Pi	pPi	O/Pi	Promazine
	0·46	0·24	Pu	Pi/Br	Pu/Br	3-Hydroxypromazine
	0·43	0·37	O/Pi	Pi/Br	Pi/Br	Demethylpromazine
	0·16	0·4	Pu	Pi/Br	Pu/Br	Demethyl-3-hydroxy-promazine
Reference compounds	0·74	0·22	O/Pi	pPi	O/Pi	Promazine
				L	L	1-Hydroxypromazine
	0·4	0·24	Pu	T	T	2-Hydroxypromazine
	0·45	0·25	Pu	R/Pu	G/Br	3-Hydroxypromazine
	0·62	0·24	pPi/Br	Pi/R	Cr	4-Hydroxypromazine
	0·65		O/Pi	pPi	O/Pi	Promazine sulphoxide

Colour code: O = Orange; B = Blue; Pu = Purple; Pi = Pink; G = Grey-green; Br = Brown; Cr = Crimson; L = Lavender; R = Red; T = Turquoise.

Promazine. The thin-layer chromatographic results, which are summarised in Table 6, confirm and extend the previous results with the whole liver homogenate system (Robinson & Beaven, 1964). The ultraviolet absorption spectrum of the dilute acid solution of the extract corresponded exactly with that of promazine: there was no indication of sulphoxidation. The visible absorption spectrum in strong acid solution showed an absorption peak at 519 mμ together with a shoulder at 560–580 mμ; difference measurements with promazine in strong acid in the reference beam gave the absorption peak of the hydroxylated derivative as 566–8 mμ. 3-Hydroxypromazine has an absorption peak at 566–8 mμ under these conditions.

Promazine sulphoxide was recovered unchanged after incubation with the liver preparation.

Acepromazine and propiomazine. The data obtained for these compounds are included in Tables 7 and 8. Both derivatives underwent hydroxylation but demethylation was detected only with acepromazine.

MISCELLANEOUS ALKYLAMINOALKYLPHENOTHIAZINE DERIVATIVES

Various compounds were investigated to determine whether slight differences in chemical structure influenced the pathways of biotransformation. The data obtained are summarised in Tables 7 and 8.

The length of the alkyl chain attached to position 10 of the phenothiazine nucleus is reduced to two carbon atoms in diethazine. A branched methyl group is present on the two-carbon chain in promethazine and isopromethazine while ethopropazine is the diethylamino-analogue of promethazine.

TABLE 7. CHARACTERISTICS OF METABOLITES EXTRACTED FROM INCUBATION MIXTURES (SILICA GEL PLATES AND SOLVENT SYSTEM 2)

Compound added to incubation medium	Metabolites recovered from incubation mixture		
	Unchanged compound	Dealkylated analogues*	Hydroxylated analogues**
Acepromazine[1]	0·78 Orange†	0·42 Orange†	0·48 Orange L (1, 2, 3)‡ O (4) 0·22
Propiomazine[2]	0·92 Orange†		0·45 Orange L (1, 2, 3) R (4)
Diethazine	0·89 Blue†	0·75 Blue†	0·62 Dark blue† Pu (4) O (5) 0·47 blue Y/Br (4 & 5)
Promethazine	0·88 G (4) I (5)	0·76 G (4 & 5)	0·67 L (1, 2, 3) R/Pu (4 & 5) 0·60 L (1, 2, 3) G/Y (4) Y (5)
Isopromethazine[3]	0·94 G (4) B/G (5)	0·88	0·80 B (1, 2, 3) R/Br (4 & 5) 0·7 B (1, 2, 3) Y (4 & 5)
Ethopropazine[4]	0·80 Blue†	0·66 Blue†	0·38 Blue† L (1, 2, 3) Pi with 4
Trimeprazine	0·89 Pi (1, 2, 3)	0·57	0·63 O (4) Pi (5) 0·23 O (4) Pi (5)
Methotrimeprazine ..	0·89 B/Pu (1, 2, 3) Pi (4)	0·63, 0·23 } Pu (5)	0·57, 0·18 } Y (5)
Ethylmemazine	0·82 Pi (1, 2, 3)	0·53	0·58 L (1, 2, 3) 0·36 Pu (5)
Proquamezine	0·63 Pi (1, 2, 3) G (5)	0·43	0·35 Pi (4) G/Pi (5)
Cyamemazine	0·78 Yellow/green† O (1, 2, 3)	0·42	0·49 Pi (4) Y/Br (5)
Trifluoperazine	0·80 Green† O (1, 2, 3, 5)	0·39 Green†	0·51 L (1, 2, 3) 0·14 Pi/O (4) L (5)
Prochlorperazine	0·72	0·24	0·38 Li (1, 2, 3) Pi/Br (4)
Thioproperazine	0·59 Green†	0·32	0·25 L (1, 2, 3, 4)
Fluphenazine	0·68 Green† O (1, 2, 3) B/G (5)	(0·27—see text)	Pu (1, 2, 3) O/Br (4, 5) 0·32
Pecazine	0·86 O/Pi (1, 2, 3) Pi/Br (5)	0·28	0·52 Pu (1, 2, 3) O/Br (4) Pi (5)

* Positive reaction with reagent 7. ** Positive reaction with Gibb's reagent, etc. † Fluorescence under ultraviolet light. ‡Spray reagents—see text. [1–4]Sulphoxides: positive reaction with sulphuric acid but negative with ferric chloride reagents; [1]0·56 blue (trace)†; [2]0·52 blue (trace)†; [3]0·76 (trace)†; [4]0·13 (trace)†. For colour code see Table 6.

Dealkylation and hydroxylation, separately, were undergone by each of these compounds. The hydroxylated analogues all gave characteristic colours with the sodium periodate and ammonium persulphate reagents (Table 7) and possessed absorption peaks in strong sulphuric acid solution in the visible region at wavelengths longer than those of the parent

TABLE 8. LIGHT ABSORBING PROPERTIES OF THE DRUGS AND SOLUTIONS OF EXTRACTS OBTAINED FROM INCUBATION MIXTURES

Compound added to incubation medium	λ_{max} in mμ of ultraviolet absorption peaks in 0·01 N sulphuric acid solution	λ_{max} in mμ absorption peaks in 50% v/v sulphuric acid solution
Acepromazine	205 243 278	212 235 (252, 290) 515
Extract	207 243 278	(230) 254, 290 522 and 570 unresolved 515 and 572 resolved*
Propiomazine	204 241	204 241 289 522
Extract	204 241 (275) 342, 358 (ill-defined)	239 277 528 and 580 unresolved 522 and 583 resolved*
Diethazine	250 300	211 266 274 517
Extract	(235) 247 295 Trace of sulphoxide	214 266 275 518 and shoulder 560–80 518 and 572 resolved*
Promethazine	202 250 298	211 267 275 (440) 518–20
Extract	204 249 296–8	212 267 276 (438) 523 and 572 unresolved 518–20 and 573 resolved*
Isopromethazine	202 252 303	213 257 (270–5) 520–5
Extract	(235) 251 295 Trace of sulphoxide	212 262 273 Wide band 520–580 520, 565–70, 600 resolved*
Ethopropazine	249 302	211 266–271 (broad) 519
Extract	248 295 Trace of sulphoxide	213 268 276 (372, 440) 520 and 560 519 and 570 resolved*
Trimeprazine	252 300	213 268 275 517
Extract	252 298	212 268 275 (450) 518 and shoulder 570 516 and 576 resolved*
Methotrimeprazine	250 300	223 281 572
Extract	249 298	223 (253) 282 572
Ethylmemazine	251 300	211 266 276 531
Extract	250 298	218 (270) 278 534 distorted 531 and 579 resolved*
Proquamezine	253 301	250 275 520
Extract	250 300	250 274, 440 519 and shoulder 570 516 and 570–575 resolved*
Cyamemazine	232 268	(235) (250) 286 513
Extract	235 265	516 distorted 513 and 570 resolved*
Prochlorperazine	254 303	212 269 277 530
Extract	254 303	216 269 277 530 and 575 (weak)
Thioproperazine	265 315	210 (251) 282 518 distorted
Extract	263 315	210 251 283 503 and 570 resolved*
Fluphenazine	256 306	210 (250) 275 503 distorted
Extract	256 306	210 (250) 275 503 and 565 resolved*
Pecazine	253 303	213 267 274 516
Extract	253 300	213 267 274 516 and 563 resolved*

* Wavelengths are those obtained from differential measurements.

compounds (Table 8, and cf. Fig. 3). The value of using both of the aforementioned reagents for the location and characterisation of hydroxy-derivatives is seen particularly in the case of promethazine; both of the 'hydroxy' spots on the chromatoplates gave the same colours with the acidic reagents (1, 2 and 3, p. 20) but differed in their reactions with periodate (4) and persulphate (5). One of the two compounds reacted differently with each of the reagents. Similarly with isopromethazine, differentiation of the hydroxy-derivatives was possible only with the periodate and persulphate reagents on neutral or alkaline chromatoplates; if acid was present in the developing solvent or in the spray reagent, as in the persulphate reagent used by Goldenberg & co-workers (1964), the colours seen with the acidic reagents were obtained. Although two hydroxy-derivatives were apparent on thin-layer plates of extracts from promethazine incubation mixtures, only one was detected spectrophotometrically (λ_{max} 573 mμ in 50% v/v sulphuric acid), perhaps implying that the compounds differ in the nature of the alkyl chain attached to the ring nitrogen atom. However, the two hydroxy-derivatives present in the isopromethazine extracts did give two different absorption peaks (λ_{max} 565–570 and 600 mμ) in strong acid solution suggesting that, in this case, the difference involves the chromophore directly.

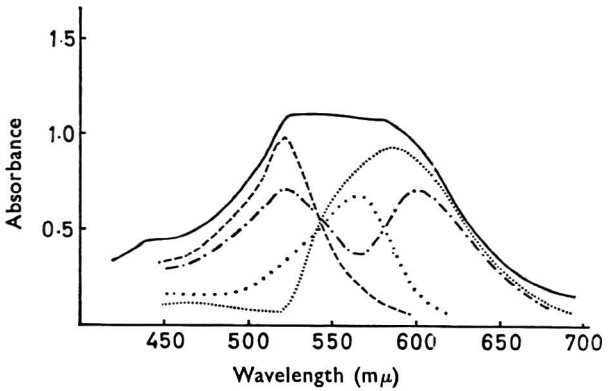


FIG. 3. Visible absorption curves in 50% v/v sulphuric acid for an extract derived from incubation of the liver fraction with isopromethazine. 1. ——— Extract against the acid. 2. - - - - - Extract against isopromethazine in 50% v/v sulphuric acid. 3. — — — Solution of isopromethazine in 50% v/v sulphuric acid used as reference for 2 against 50% v/v sulphuric acid. 4. — · — · — Extract against 3-hydroxypromazine in 50% v/v sulphuric acid. 5. . . . . Solution of 3-hydroxypromazine in 50% v/v sulphuric acid used as reference for 4 against 50% v/v sulphuric acid.

The wavelengths of the absorption peaks of the hydroxylated analogues of diethazine, promethazine, ethopropazine and one of the isopromethazine derivatives in strong acid solution are as expected in the absence of other ring substituents. In all cases where hydroxylation had occurred, observation of the sequence of colour changes seen on addition of concentrated sulphuric acid to the dilute acid solution of the extract

was informative. Usually, when the acid was added dropwise and with mixing, the purple colour associated with the hydroxy-compound developed first and became pinker or more orange, depending upon the parent compound, as the concentration of the acid was increased. It was sometimes possible to obtain a spectrum for the hydroxy-compound before that of the parent compound was fully developed, but the stability of the spectra for the former was poor compared with those for the latter compounds.

Despite the relative ease of oxidation of these compounds, sulphoxides were either totally absent or were present in trace amounts only.

The effect of introducing an additional group to the 3-dimethylaminopropyl substituent present in promazine was also studied. These compounds included trimeprazine, methotrimeprazine, ethylmemazine, proquamezine and cyamemazine.

Demethylation was undergone by each of these compounds although, with proquamezine, it was impossible to determine at which dimethylamino-group the reaction occurred. The hydroxylated analogues again showed absorption peaks in strong acid solution at wavelengths longer than those of the parent compounds. None of the compounds in this group yielded a sulphoxide.

SOME 10-HETEROCYCLIC-SUBSTITUTED PHENOTHIAZINE DERIVATIVES

The phenothiazine derivatives containing a heterocyclic ring as part of the substituent in position 10 examined were trifluoperazine, prochlorperazine, thioproperazine, fluphenazine and pecazine. With each of the compounds containing the 4-methylpiperazinyl group, a spot on the developed chromatoplate was obtained which gave a positive reaction with the nitroprusside-acetaldehyde reaction for secondary amines. A secondary nitrogen group could arise either from demethylation of the methyl piperazinyl group or by opening of the piperazine ring. Similarly, pecazine also formed a substance which reacted as a secondary amine. Fluphenazine, which has a hydroxyethyl group on the piperazine ring gave a similarly placed spot on the developed chromatoplate in solvent 2 but it did not react with the reagent.

Each compound in this group was hydroxylated on the phenothiazine ring. However, the reduced contribution to the observed spectrum in strong acid solution in the visible region, compared with the corresponding promazine derivatives, suggested that hydroxylation occurs less readily with these compounds which, consequently, are effective at lower dose levels. The sulphoxides, which all show fluorescence at a shorter wavelength than the parent compounds when viewed under ultraviolet light, were not detected in the extracts and it seems unlikely that biological sulphoxidation precedes hydroxylation in these systems. Thus, all of the phenothiazine derivatives studied were susceptible to the same pathways of hepatic biotransformation and there is no obvious correlation between chemical structure and pharmacological activity on this basis. The pharmacological potency of each compound would, therefore,

appear to depend on the quantitative relationships between the tissue levels of the drugs and their rate of biotransformation and subsequent elimination.

The type of action effected by any particular compound may be related to the actual distribution of the compound and the possible metabolic products formed in, or transported to, the various tissues. This aspect is being studied further.

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Release of tissue histamine by the babesicidal agents quinuronium and amicarbalide

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Quinuronium sulphate liberated appreciable quantities of histamine from tissues of mice, rats and sheep. The signs of quinuronium poisoning in mice and rats were more severe than for compound 48/80. The animals acquired tolerance to the latter drug. Thus the toxicity of quinuronium probably depended on factors other than histamine release. Amicarbalide released amounts of histamine comparable with those liberated by quinuronium only in rat tissues.

THE two babesicidal agents investigated are both in common use in Britain for the treatment of bovine piroplasmosis.

Quinuronium sulphate [*NN'*-diquinol-6-ylurea 1,1'-dimetho(methyl sulphate)] has been in use since the early 1930's (Sergent, Donatein, Parrot & Lestoquard, 1933). Its marked toxicity was first recorded by Cernaianu, Schuldner & Magureanu (1935), who described profuse salivation, defaecation and micturition as invariable effects, usually accompanied by dyspnoea, muscular spasm and collapse. These were followed occasionally by death and were held to be cholinergic in nature. In 1935, however, Kikuth observed that the toxic symptoms bore a strong resemblance to "shock". Adrenaline was recommended as a suitable antidote to quinuronium poisoning, as it could partly antagonise certain cholinergic effects and also "shock" from histamine release. In 1959, Kronfeld published evidence of a "respiratory" type of death produced by quinuronium. He showed that quinuronium depressed cellular oxygen uptake in the brain and that sympathomimetic amines were useless in preventing death in these circumstances. In 1960, Rummler & Laue showed that quinuronium inhibited circulating cholinesterase, and atropine and pyridine 2-aldoxime methiodide together provided good clinical improvement in sheep "poisoned" by quinuroniun.

Recently a new babesicidal drug with a higher therapeutic index than quinuronium has been introduced, namely, amicarbalide [*NN'*-di(*m*-amidinophenyl) urea di-isethionate] (Ashley, Berg & Lucas, 1960; Beveridge, Thwaite & Shepherd, 1960). So far, the only untoward effect has been local swelling at the injection site and some inco-ordination.

The evidence for the cholinergic and central inhibitory aspects of quinuronium toxicity was clear, but in view of the shock-like nature of quinuronium poisoning, there was a possibility of histamine release. It therefore seemed important to investigate the role of histamine in the toxicity of quinuronium and to include amicarbalide in the investigations in view of its own toxic reactions.

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Experimental

METHODS

Male albino mice weighing 20–30 g, and male and female albino rats weighing 150–200 g obtained from the University animal breeding unit were used. Sheep diaphragm sections were obtained at slaughter.

In vitro *release of histamine from perfused rat hind-quarters.* The technique was described by Feldberg & Mongar (1954). The posterior part of the bisected body of the rat was perfused through the dorsal aorta with oxygenated Krebs solution and perfusate collected from the vena cava. Perfusion was with solutions containing quinuronium, 500 μg/ml; amicarbalide, 2 mg/ml and compound 48/80, 100 μg/ml. 100 ml each of control and drug perfusates were collected, treated with hydrochloric acid according to Feldberg & Talesnik (1953), reduced in volume by boiling, and assayed for histamine after neutralisation.

In vitro *release of histamine from sheep diaphragm strips.* Small areas of diaphragm were taken from freshly killed sheep, the area chosen being at the junction of the muscular and tendinous portions. From the peritoneal side of the muscle a thin sheet of tissue was prepared by splitting the musculature with a clean razor blade to leave peritoneum and a thin layer of attached muscle, about 3 mm thick. This sheet was then divided into a number of strips approximately 1 cm wide and 3 cm long, which were washed in warm (37°) saline for 1 min, carefully dried on cellulose tissue and weighed.

Sixteen such strips were prepared and each was placed in a tube containing 8 ml of Krebs solution. Four tubes remained as controls and the second, third and fourth groups of four contained, respectively: quinuronium, 500 μg/ml; amicarbalide, 2 mg/ml; and compound 48/80, 100 μg/ml (i.e. the same concentrations as those perfused through rat tissue). All tubes were incubated for 30 min at 37°, after which the tissues were removed and the solution assayed for histamine.

In vivo *histamine release in mice.* Experiments were designed according to the recommendations of Riley & West (1955), with a slight modification of the sub-acute experiment. Drugs were dissolved daily in fresh normal saline and injected on three successive days intraperitoneally into groups of six or eight mice. The doses measured in μg/g in the case of the babesicidal compounds approximated to between one and two times the therapeutic dose, and for 48/80 were within the doses recommended by Riley & West (1955), which were as follows: quinuronium (1) 1·0, (2) 1·5, (3) 2·0; amicarbalide (1) 20·0, (2) 25·0, (3) 30·0; Compound 48/80 (1) 2·0, (2) 2·5, (3) 3·0. A fourth group of mice received an equivalent volume of normal saline.

On the third day of injections, 3 hr after the final injection, four mice from each group were killed and skinned. A representative sample of skin was extracted and assayed for histamine.

In vivo *histamine release in rats.* A similar experiment was devised using white rats, which were injected intraperitoneally on three successive days, the daily dosage (μg/g) being as follows: quinuronium (1) 2·0

(2) 2:5, (3) 3·0; amicarbalide (1) 50·0, (2) 65·0, (3) 70·0; Compound 48/80 (1) 2·0, (2) 2·5, (3) 3·0. A fourth group received an equivalent volume of normal saline.

HISTAMINE EXTRACTION AND ASSAY

Perfusate and supernatant. The method has been described by Feldberg & Talesnik (1953).

To each sample, concentrated hydrochloric acid was added to produce an acid concentration of 20% v/v HCl. The samples were heated for 30 min in a boiling water-bath, then neutralised with concentrated sodium hydroxide solution using universal indicator paper. The volume was measured.

Histamine assays were performed on atropinised guinea-pig ileum (1×10^{-8} g atropine) in Tyrode solution. To exclude any non-histamine component, the solutions were re-assayed in Tyrode containing mepyramine 1×10^{-9} g.

Tissues. A portion of tissue was weighed, ground with silver sand and added to a small quantity of 20% v/v hydrochloric acid, which was boiled for 10 min, filtered through Whatman No. 1 filter paper and washed with three volumes of distilled water. The clear filtrate was neutralised with sodium hydroxide solution diluted with Tyrode as required and assayed as described above.

Results

In vitro *release of histamine from perfused rat hind-quarters.* Assays indicated that there was no detectable histamine released from the saline perfused tissues. Compound 48/80 released the largest amount of histamine, whereas quinuronium and amicarbalide released less than half this amount (Table 1).

In vitro *release of histamine from sheep diaphragm strips.* Table 1 shows that there was an appreciable quantity of histamine released from the control tissue. Compound 48/80 liberated a large quantity of histamine and quinuronium about half this amount, whereas the amount of histamine released by amicarbalide was not significantly different from that of the controls.

In vivo *histamine release in mice.* Within a few minutes of an intraperitoneal injection of quinuronium or compound 48/80, the mice became restless and showed signs of cyanosis. Mice which received quinuronium were very severely affected, showing also marked dyspnoea and collapse before finally recovering. Compound 48/80 was less severe in its effects than quinuronium and over a period of 3 days the mice acquired some tolerance to compound 48/80, but did not seem to show any tolerance to the action of quinuronium. Amicarbalide produced no marked toxic signs but the mice became slightly restless.

Table 1 shows that compound 48/80 and quinuronium reduced the skin histamine content by 50%, whereas amicarbalide did not show any measurable histamine release at the given dosage.

In vivo *histamine release in rats.* Within a few minutes of the injection of quinuronium, the rats began to show hyperactivity and face washing, followed by frenzied jumping movement and respiratory distress. A

TABLE 1. THE RELEASE OF HISTAMINE FROM THE TISSUES OF SHEEP, RAT AND MOUSE

	Tissue						
	In vitro			*In vivo*			
	Rat hind-quarters	Sheep diaphragm		Mouse		Rat	
Drug treatment	Mean histamine released in 100 ml perfusate (μg ± s.e.)	Mean histamine released μg/g ± s.e.	Hist-amine released %	Mean histamine content of skin μg/g ± s.e.	Hista-amine released %	Mean Histamine content of skin μg/g ± s.e.	Hist-amine released %
Physiological saline	0·00	1·63 ± 0·60	13	26·1 ± 2·27	0	33·21 ± 2·80	0
Quinuronium Sulphate ..	101·0 ± 40·0	5·62 ± 0·66	45	14·1 ± 1·85	46	16·80 ± 3·44	49
Amicarbalide Isethionate ..	125·0 ± 26·0	3·11 ± 0·44	25	27·35 ± 1·25	0	23·09 ± 1·91	30
Compound 48/80	298·0 ± 32·0	9·60 ± 1·45	77	12·31 ± 1·90	53	6·00 ± 1·82	82

Mean histamine content of sheep diaphragm/peritoneum = 12·50 μg/g ± 1·50.
Number of estimations for all mean histamine values = 4.

period of quiescence preceded recovery. Amicarbalide produced some slight excitement in the rats, some of which showed face washing movements. Compound 48/80 produced similar signs to quinuronium with the addition of facial oedema. After three days of injections the rats became tolerant to the action of compound 48/80 but not to quinuronium. At the stated dosage levels, compound 48/80 released the most histamine; quinuronium released about half this quantity and amicarbalide about a third.

Discussion

Preliminary *in vitro* evidence from perfused rat hind-quarters indicated that quinuronium and amicarbalide were both capable of releasing significant quantities of histamine from rat tissues.

In sheep diaphragm, amicarbalide in the given concentration did not release histamine, whereas quinuronium and compound 48/80 did. A difficulty in the sheep experiments was a variable spontaneous release of histamine. Rocha e Silva & Schild (1949) reported this to occur in the rat diaphragm. These authors incubated rat diaphragms in oxygenated Tyrode at 37°, whereas in the sheep experiments described here, Krebs solution was not oxygenated. Factors which may have contributed to the variable release were (1) uneven thickness of tissue, (2) absolute thickness making oxygen and drug diffusion variable, and (3) trauma of tissue.

In vivo experiments in rats and mice were more informative. In mice, quinuronium and compound 48/80 were approximately equally active, but amicarbalide failed to liberate histamine at the doses used. In the rat,

quinuronium and amicarbalide released comparable quantities of histamine which were less than those produced by compound 48/80. The signs of toxicity in mice showed that although quinuronium and compound 48/80 released similar amounts of histamine, quinuronium was much more toxic. In addition mice acquired some tolerance to this dosage of compound 48/80 over a period of 3 days, but not to quinuronium.

In rats where quinuronium and amicarbalide released similar amounts of histamine, quinuronium was distinctly more toxic,. This suggested that although quinuronium was a potent histamine liberator, the drug possessed toxic properties other than histamine release, which agreed with the observations of central respiratory inhibition by Kronfeld (1959) and the anticholinesterase activity observed by Rummler & Laue (1960).

In the experiments described, it was clear that histamine release was a significant part of the overall toxicity of quinuronium in mice, rats and sheep. Amicarbalide at these doses only appeared to release comparable quantities of histamine in rats.

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Liver function and pyrexia caused by a pyrogen from *Escherichia coli*, lysergic acid diethylamide and dinitrophenol

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Rabbits with liver damaged by carbon tetrachloride do not respond with hyperthermia to a lipopolysaccharide pyrogen from *Escherichia coli* or dinitrophenol, but lysergic acid diethylamide develops its usual effect. Rabbits with obstructive liver damage react with hyperthermia to all three pyrogenic factors. The results support the concept of a peripheral action of pyrogen. It is suggested that the liver plays a rôle in the process of transforming the bacterial pyrogen into the endogenous pyrogen.

IN our previous studies it was possible to demonstrate that a lipopolysaccharide from *Escherichia coli*, when administered *in vitro* or *in vivo* increases oxygen consumption in sections of liver (Venulet & Desperak, 1957; Desperak-Naciążek & Venulet, 1960). This effect is connected with the activation of succinic acid dehydrogenase (Venulet & Desperak-Naciążek, 1960). The rôle of liver function seems to be so important that in rabbits with carbon tetrachloride liver damage the pyrogenic response disappears during the time of positive liver function tests and reappears with their return to initial values.

Because this phenomenon might help to evaluate the significance of peripheral factors in different kinds of hyperthermia we decided to study the effects of lysergic acid diethylamide (Sandoz) and 2,4-dinitrophenol in rabbits with damaged livers.

Material and methods

In all experiments only animals with reactivity to pyrogen were used. Liver damage was developed by two different methods. In the first group, freshly distilled carbon tetrachloride was injected subcutaneously in a dose of 1·5 ml/kg. In the second group the common hepatic duct was ligated to produce obstructive liver lesion (McLuen & Fouts, 1961). As we have found previously (Venulet & Desperak-Naciążek, 1963) the peak liver damage in both types of injury is reached 72 hr after injection or ligation. The tests were still positive during the first two weeks but during the third and fourth week they gradually returned to normal.

Animals, in 3 groups, received bacterial pyrogen, lysergic acid diethylamide or dinitrophenol intravenously at weekly intervals for four weeks. The rectal temperature was measured every 30 min by thermocouples during 1 hr before and 3 hr after the injection. The results were evaluated statistically by means of the analysis of variance and the *t*-test.

Results and discussion

From Table 1 it can be seen that with both pyrogen and dinitrophenol the pyrogenic reaction disappears during the onset of liver damage but

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reappears with return of its function. The results with lysergic acid diethylamide are independent of the degree of the liver lesion, the hyperthermia persisting. These differences are statistically significant and throw new light on the rôle played by the liver in pyrogen and dinitrophenol hyperthermia and its non-participation in lysergic acid diethylamide hyperthermia.

TABLE 1. THE INFLUENCE OF CARBON TETRACHLORIDE LIVER DAMAGE ON THE PYROGEN LYSERGIC ACID DIETHYLAMIDE OR DINITROPHENOL HYPERTHERMIA

Drug and dose	No. of animals	Δ t°C						Smallest difference when P=0·95
		Before damage	After damage on day					
			4	11	18	25	32	
Pyrogen, 0·02 μg/kg ..	38	+0·85	+0·85	+0·55	+0·41	+0·59	+1·0	0·2
Lysergic acid diethylamide, 5 μg/kg	20	+0·71	+0·82	+0·80	+0·86	+0·85	+0·80	0·15
Dinitrophenol, 10 mg/kg ..	20	+0·97	+1·1	+0·52	+0·51	+0·6	+0·8	0·23

These results furnish some new evidence for the generally accepted opinion about the mechanism of action of lysergic acid diethylamide and dinitrophenol. Moreover they supply further proof for the underestimated rôle of peripheral factors in the postpyrogenic fever. It seems likely that endotoxins other than of *E. coli* origin also develop fever partially through the peripheral mechanism. The fact that on the fourth day after carbon tetrachloride injection, when the lesion is most pronounced, the fever reaction still persists to disappear only a few days later is difficult to explain. In agreement with the theory that bacterial pyrogens are either transformed into endogenous pyrogen or stimulate its production, we assume the existence of an endogenous activator which is produced by the liver and which circulates in the blood for some days, even after its production has been stopped by the carbon tetrachloride. Therefore on the fourth day its level is still high enough to start the whole reaction.

TABLE 2. THE INFLUENCE OF OBSTRUCTIVE JAUNDICE ON THE PYROGEN, LYSERGIC ACID DIETHYLAMIDE OR DINITROPHENOL HYPERTHERMIA

Drug and dose	No. of animals	Δ t°C						Smallest difference when P=0·95
		Before damage	After damage on day					
			3	7	10	14	17	21
Pyrogen 0·02 μg/kg ..	20	1·02	1·03	1·32	1·13	1·32	0·93	0·86	0·37
Lysergic acid diethylamide 5 μg/kg ..	20	0·97	0·66	0·81	0·80	1·25	0·62	1·03	0·47
Dinitrophenol, 10 mg/kg ..	20	0·95	0·67	0·53	0·60	0·88	0·48	0·93	0·55

Table 2 shows that not all kinds of liver damage lead to the same result. In spite of highly positive liver tests in rabbits with ligated hepatic ducts, the pyrogenic response to our pyrogen, lysergic acid diethylamide or

dinitrophenol remains unchanged throughout the experiment. This indicates the complex character of the observed phenomena and the specificity of involved mechanisms. The two kinds of damage are so different in their mechanism and morphology that their consequences also differ.

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Protein binding and the excretion of some azo dyes in rat bile

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The kinetics of biliary excretion of four dyes, amaranth, geranine, lissamine and dechlorolissamine have been examined in the rat. The protein binding of each dye to blood and liver *in vitro* was examined, and a correlation found between excretion rate and the relative degree of binding of dye to the liver compared with the blood.

THE ability of a compound to be excreted in the bile appears to be related to molecular size and metabolic pathway (Williams, Millburn & Smith, 1965). The transport of material from the blood to the bile often involves active transport processes, for example, with anions (Sperber, 1959) and some cations (Schanker & Solomon, 1963).

Ryan & Wright (1961, 1962) reported the almost quantitative biliary excretion of some water-soluble azo dyes. In contrast to the compounds studied by Williams & others (1965), these dyes were excreted unchanged. There appeared to be no correlation between the amount of dye excreted within 6 hr of intravenous injection and chemical structure or molecular weight.

Few studies on the biliary excretion of drugs have involved the determination of the kinetics of the process. These azo dyes, rapidly excreted and not metabolized, would appear to be ideal compounds for use in the study of biliary excretion kinetics. Four dyes were chosen and an attempt made to determine their biliary excretion kinetics and to relate this to their tendency to bind to protein both in the liver and in the blood.

Experimental

MATERIALS

Three of the dyes, amaranth (Colour Index No. 16185), geranine 2GS (Colour Index No. 18050) and lissamine fast yellow 2 G (Colour Index No. 18965) were commercial samples, recrystallised from ethanol:water. Dechlorolissamine [the di-sodium salt of 1-(*p*-sulphophenyl-)3-methyl-4-(*p*-sulphophenylazo-)5-pyrazolone] was kindly supplied by Dr. A. J. Ryan. All dyes were chromatographically and analytically pure.

METHOD

Collection of bile. Albino male rats (250-350 g) were anaesthetised with urethane (125 mg/100 g). A longitudinal incision was made just below the diaphragm, and the bile duct isolated. After ligation of the duct at the duodenal end, a cannula consisting of a 23 gauge hypodermic needle shaft attached to a length of polyethylene tube was introduced into the bile duct, and secured with cotton thread. The dose of dye was injected, in aqueous solution, into a femoral vein and bile was collected

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at 5–15 min intervals for 2–2½ hr. The body temperature of the rats was maintained by exposure to a radiator.

Analysis of samples. The dye content of the bile samples was determined by diluting the samples with 0·01M hydrochloric acid to suitable volume, and reading the optical density in a Bausch and Lomb Spectronic 20 spectrophotometer at the wavelength of maximum absorption. Where bile pigments interfered with the optical density readings, these were precipitated by adding equal volumes of 40% zinc sulphate solution and 11·2% potassium hydroxide solution, eluting the dye from the precipitate by washing with hot water before adjusting to a suitable volume with dilute acid.

PROTEIN BINDING

The degree of protein binding in both whole rat blood and rat liver homogenates was determined *in vitro* for the four azo dyes. 4·0 ml of dye (2×10^{-4}M) in Krebs-Ringer-phosphate buffer, pH 7·4 (Umbreit, Burris & Stauffer, 1957), and 4·0 ml of the blood or liver homogenate, diluted to 14 mg/ml protein (equivalent to bovine serum albumin), as a standard with Krebs-Ringer-phosphate buffer pH 7·4, were placed in a centrifuge tube and shaken at 37° for 10 min. 1·0 ml of 40% zinc sulphate solution and 1·0 ml 11·2% potassium hydroxide solution were added to precipitate the protein and protein-bound dye. The mixtures were centrifuged and the supernatant diluted and read in a spectrophotometer, to determine free dye. Rat liver homogenates were prepared in an all-glass Dounce homogeniser with a loose-fitting, unground plunger, and both blood samples and liver homogenates were standardised for protein concentrations by the biuret method of Cleland & Slater (1953).

The method of protein precipitation outlined above was used because it involves only brief exposure of the preparation to extremes of pH. Thus it gives a more physiological measure of protein binding than precipitation with trichloroacetic acid, where acidic conditions may abstract some bound dye from the protein. The purpose of the binding experiments used here was to determine the relative binding capacities of liver and blood and not the absolute capacity in each case. For this reason a fixed concentration of dye was reacted with blood and liver preparations standardised to the same amount of protein.

Results and discussion

The dyes appear in the biliary cannula within 3–5 min of injection. Fig. 1 shows the kinetic analysis of results obtained with the dyes after a dose of 20μmoles had been administered in each case. Two types of semilogarithmic plots can be used with cumulative excretion results of this type (Wagner, 1963). The more common plot of log amount not excreted [log (a-x)] against time, requires that the total amount excreted (a) be known. During the time of biliary collection it was difficult to make an accurate estimate of (a). The method used was a plot of log excretion

rate against the mid-point of the collection interval. This plot is independent of the total amount excreted and the first order rate constant can be calculated from the slope of the curve.

Fig. 1 indicates that the excretion process may be described by two first order processes, an initial rapid excretion of most of the dye followed by a slower residual excretion. This biphasic excretion may be related to changes in bile flow rate or to storage of some of the dye in a depot from which slow release occurs after the unbound dye has been excreted. Wiseman, Schreiber & Pinson (1964) have reported a similar biphasic curve in their study of blood levels of benzquinamide and explain the slow secondary release of drug as due to release of unchanged drug or metabolites from tissue depots. Richard, Tyndall & Young (1959) also obtained a similar biphasic curve in studies on rate of plasma disappearance of sulphobromophthalein sodium but their measurements of biliary excretion cover an insufficient time to allow any indication of biphasic excretion to become apparent. Hence biphasic liver uptake may or may not be related to biphasic excretion and more work is required to clarify this point.

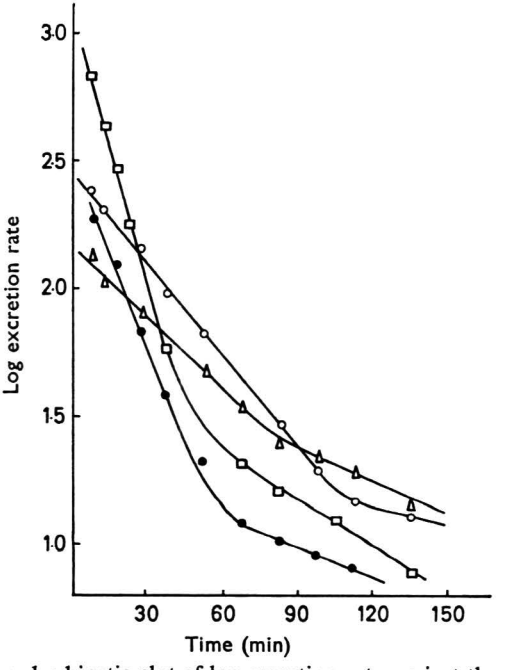

Fig. 1. First-order kinetic plot of log excretion rate against the mid-point of the collection intervals for the four azo dyes: □ Amaranth, ● Geranine, ○ Lissamine, △ Dechlorolissamine

Inspection of Table 1 shows that there is no simple relationship between the excretion rate and molecular weight of the dye anion nor do the rate constants show any correlation with the final total excretion.

Brauer & Pessotti (1949) showed that sulphobromophthalein sodium was bound to liver slices *in vitro* and also addition of bovine serum albumin reduced the degree of binding. This result indicates that the dye can be bound by liver and that plasma protein can reduce the degree of liver binding. Andrews & del Rio Lozano (1961) confirmed these results in perfused preparations. These studies suggest that protein binding in the liver could be important in the hepatic uptake, if not excretion, of compounds. To test this possibility, the ability of liver and blood preparations to bind dyes was examined. The technique used measures the amount of unbound dye left in a supernatant after precipitation of the protein contained in the system. With both liver and blood the dyes used were bound to protein to a large extent (Table 1). A relationship is apparent between excretion rate and liver : blood binding ratio (Table 1). Amaranth, the dye most strongly bound by the liver in preference to the blood, is the one most rapidly excreted. If the relative liver : blood binding ratios and excretion rates of the four dyes are compared by setting the values for dechlorolissamine at unity, then the correlation of preferential protein binding and excretion rate is obvious. For amaranth the respective figures are 2·19, 2·50; for geranine, 1·79, 1·99; for lissamine, 1·25, 1·1.

TABLE 1. KINETIC DATA FOR THE INITIAL, RAPID EXCRETION PHASE FOR ALL DYES AT A DOSE LEVEL OF 20 μMOLES AND RESULTS FOR PROTEIN BINDING IN RAT LIVER HOMOGENATES AND WHOLE RAT BLOOD

Dye	Amaranth	Geranine	Lissamine	Dechloro-lissamine
Mol. wt. of dye anion	535	463	505	436
Mean rate constant k_1 (min^{-1}) ..	0·0634	0·0507	0·028*	0·0254
Excretion half-life, $t_{\frac{1}{2}}$ (min)	10·9	13·7	24·7*	30·0
Mean % biliary excretion in 6 hr† ..	53	46	96	80
Protein bound in liver (%)‡	91·4 ± 1·1	57·2 ± 3·3	50·3 ± 3·7	43·6 ± 3·0
Protein bound in blood (%)‡	81·4 ± 4·1	62·4 ± 4·7	78·0 ± 7·3	85·0 ± 1·4
Liver : blood binding ratio	1·123	0·917	0·645	0·513

* The excretion rate constant of lissamine varies with the dose given while for the other dyes the rate constant maintains the same value until high saturation doses are reached (100 μmoles) (Priestly, 1965).
† These values are quoted from Ryan & Wright (1961, 1962).
‡ Average of six determinations each.

The method of determining protein binding is somewhat artificial and measures an equilibrium static situation compared with the kinetic situation of dye excretion. Nevertheless, it can be concluded that with the dyes studied, their rate of biliary excretion appears to be a function of their relative degree of binding to the liver proteins as against blood proteins. It would seem that dye protein binding in the liver is an integral part of the excretion process and not a storage phase delaying excretion, as appears to be the case with sulphobromophthalein sodium (Andrews & del Rio Lozano, 1961).

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A note on the absorption and excretion of ^{14}C-labelled thalidomide in pregnant mice

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After oral administration of ^{14}C-labelled thalidomide to pregnant mice, similar concentrations of the drug are found in several maternal tissues and in the placenta and foetus. Thalidomide and one of its hydrolysis products, α-(*o*-carboxybenzamido)-glutarimide, have been identified in the foetus.

THE teratogenic action of thalidomide in man and several animal species is well known (Giroud, Tuchman-Duplessis & Mercier-Parot, 1962; Somers, 1962; Bignami, Bovet-Nitti & Rosnati, 1963; Felisati, 1964). Recently, Di Paolo, Gatzek & Pickren (1964) have demonstrated that the drug produces foetal malformations in the mouse and it was of interest to determine whether thalidomide or its hydrolysis products pass from the mother to the foetus in this animal.

Methods

Thalidomide labelled with ^{14}C in both carbonyl groups of the phthalimido moiety of the molecule (specific activity 4·7 μc/g) was dissolved in dioxane (15·2 mg/ml). Pregnant albino mice (A. Tuck & Son, Essex), 8 days before expected parturition, were given water (0·2 ml) by stomach tube immediately followed by the ^{14}C-thalidomide (38 mg/kg). The animals (6) were placed in small glass metabolism cages from which expired CO_2 could be collected into sodium hydroxide (40%). Two mice each were killed after 1·5, 4 and 8 hr and the organs and tissues were removed and weighed. All of the specimens were freeze-dried. Each tissue was then ground to a powder and extracted with dioxane (3 × 10 ml). The separate tissue extracts were combined and evaporated to dryness under reduced pressure at 40°. The residues were dissolved in known volumes of dioxane and the radioactivity of the solutions was determined by scintillation counting (Graham & Nicholls, 1959) All samples were counted before and after addition of a standard ^{14}C solution to correct for quenching. ^{14}C-Thalidomide added to tissues and extracted by this procedure gave a 92% recovery.

Chromatograms of the extracts of foetal tissue were run on Whatman No. 1 paper in isopropanol:water (4:1) and in the solvent systems suggested by Schumacher, Smith, Stagg & Williams (1964). The hydrolysis products of thalidomide containing a phthalic acid moiety (Faigle, Keberle, Riess & Schmid, 1962) were also run in these solvents, alone and in mixture with the extracts. The radioactive compounds were located by autoradiography and the non-radioactive marker substances by ultraviolet light and treatment with hydrazine (Schumacher & others, 1964).

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Results and discussion

Although this was a small scale experiment, it can be seen that in the mouse the ^{14}C is so distributed that similar concentrations occur in several tissues including the foetus after administration of ^{14}C-thalidomide (Table 1). This is in agreement with similar experiments with rats (Beckmann, 1962; Faigle & others, 1962; MacKenzie & McGrath, 1962). However, in the present work, maximum amounts of ^{14}C in the tissues appear to have been achieved earlier and the levels are 5 to 10 times less than would be expected from the results of these workers. A probable explanation is that a portion of the compound administered would remain in solution and the remainder would be precipitated as fine particles in the stomach. Both these factors would promote a more rapid absorption.

TABLE 1. DISTRIBUTION OF ^{14}C IN THE TISSUES OF PREGNANT MICE GIVEN ^{14}C THALIDOMIDE[1] (38 MG/KG, ORALLY)

Time after administration of drug (hr)	Concentration of ^{14}C in tissue[2] as μg ^{14}C thalidomide g of tissue								
	Brain	Liver	Kidney	Spleen	Lung	Muscle	Placenta	Foetus	Amniotic fluid[3]
1·5	1·4	4·2	1·1	0·3	0·4	0·37	0·74	0·42	0·005
4	0·5	0·5	0·5	—	—	0·08	0·18	0·8	0·003
8	0·3	0·8	0·85	0·2	0·3	0·10	0·03	0·08	0·003

[1] Specific activity, 4·7 μc/mg. [2] Determined by scintillation counting of extract of the freeze-dried tissues in dioxane. Each value is the mean result of 2 mice. [3] Expressed as % of dose administered.

The radioactive content of the smooth muscle of the gastrointestinal tract was considerably higher than that of the other tissues studied (Table 2). Using an autoradiographic technique, Koransky & Ullberg (1964) have demonstrated a high concentration of radioactivity in the stomach wall of pregnant mice receiving ^{14}C-thalidomide. This may point to excretion at these sites. There is also a relatively large amount of ^{14}C

TABLE 2. DISTRIBUTION OF ^{14}C IN THE MUSCLE OF THE GASTROINTESTINAL TRACT OF PREGNANT MICE GIVEN ^{14}C THALIDOMIDE[1] (38 MG/KG, ORALLY)

Time after administration of drug (hr)	Concentration of ^{14}C in tissue[2] as μg ^{14}C thalidomide/g of tissue			
	Stomach	Duodenum	Small intestine	Large intestine
1·5	16·0	4·0	2·2	1·6
4	12·1	0·6	0·6	0·36
8	6·4	1·4	0·4	0·74

[1] Specific activity, 4·7 μc/mg. [2] Determined by scintillation counting of extract of the freeze-dried tissues in dioxane. Each value is the mean result of 2 mice.

in the bile. After 8 hr about 70% of the dose administered is accounted for in the urine, faeces and contents of the gastrointestinal tract (Table 3). A small amount of radioactivity was detected in the expired CO_2. A similar finding has been made with rats (Faigle & others, 1962).

Chromatography of the extracts of the foetal tissue showed that 1·5 hr after administration only thalidomide could be detected. Four and 8 hr

after the drug, thalidomide and one of its hydrolysis products α-(*o*-carboxybenzamido)glutarimide were present. The radioactive spots from the chromatogram of the 4 hr extract were eluted with dioxane and counted. This showed that the concentration of thalidomide relative to that of its hydrolysis product was 1:8.

TABLE 3. EXCRETION OF ^{14}C BY PREGNANT MICE GIVEN ^{14}C-THALIDOMIDE[1] (38 MG/KG, ORALLY)

Time after administration of drug (hr)	Concentration of ^{14}C[2] as % of dose administered				
	Blood	Bile	Urine	Faeces and gastrointestinal contents	Expired[3] CO_2
1·5	2·1	0·02	6·3	*	*
4	1·5	0·3	14·4	*	*
8	0·7	0·01	31·0	42·1	0·09

[1] Specific activity, 4·7 μc/mg. [2] Determined by liquid scintillation counting of dioxan extracts of the freeze-dried materials. Each value is the mean result of 2 mice. [3] Expired CO_2 was collected in sodium hydroxide (40%) before counting. * Not determined.

Fabro, Schumacher, Smith & Williams (1964a) have demonstrated the presence of thalidomide and this hydrolysis product in rabbit blastocysts. Their investigations of the teratogenic activity in the rabbit of several glutarimide and phthalimide compounds suggest that thalidomide itself may be the active teratogenic agent (Fabro & others, 1964b). This could also be true in the mouse, although it should be noted that the animals used in the present experiments were not in the sensitive teratogenic period for this species.

Acknowledgement. This work was made possible by a grant for apparatus from the Distillers Company Ltd. ^{14}C-thalidomide was a gift of the Distillers Company and the hydrolysis products of thalidomide were obtained from Dr. K. Schmid, Ciba Ltd., Basle. The author thanks Professor R. T. Williams for his helpful comments.

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A transistorised impulse generator for recording the heart rate from intact and isolated preparations

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A method for presenting the heart rate from intact and isolated preparations as a kymographic record is described. A transistorised impulse generator with either an integral or independent power supply is used to activate a Thorp impulse counter. The ability of the instrument to follow a wide range of heart rate changes and to measure rates in the usually encountered laboratory species is demonstrated.

THE interpretation of routine experimental blood pressure measurements is assisted by simultaneously recording the heart rate to provide a fuller analysis of the observed responses. Heart rate may be determined by simple observation, which is often tedious, or else mechanically, and it is often convenient to present the record in the form of a kymograph tracing (Beakley & Findlay, 1949; de Burgh Daly & Schweitzer, 1950; Dawes, 1951; Griffith, Innes & Kosterlitz, 1953; Perry & Wilson, 1956; and Glaser, Griffin & Knight, 1960). The methods of Beakley & Findlay, of Dawes and of Glaser & others possess the advantage of utilising the components of the electrocardiogram to determine heart rate and do not interfere with adjacent blood pressure recording apparatus.

Techniques to record the heart rate from isolated preparations have also been described (Thorp, 1948; Azarnoff & Burn, 1961).

A transistorised method for heart rate counting which is of low cost, compact and suitable for both teaching and research purposes, is now described.

Experimental

METHODS

The electrocardiogram from anaesthetised, spinal and pithed animals, was obtained from needle electrodes placed beneath the skin on either side of the thorax and fed into the input side of the impulse generator.

The electrical changes from Langendorff isolated perfused heart preparations were obtained by means of two electrodes, one attached to the metal perfusion cannula inserted into the aorta and the other from a heart clip attached to the myocardium and connected to the input stage of the counter through flexible screened leads.

In order to minimise hum pick up, connections from supporting metal structures, including the venous cannula stand, were made to the main earth source.

Kymographic records of heart rate were obtained using a Thorp impulse counter (Thorp, 1948).

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APPARATUS

A schematic diagram of the impulse generator is shown in Fig. 1 and details of the circuit in Fig. 2.

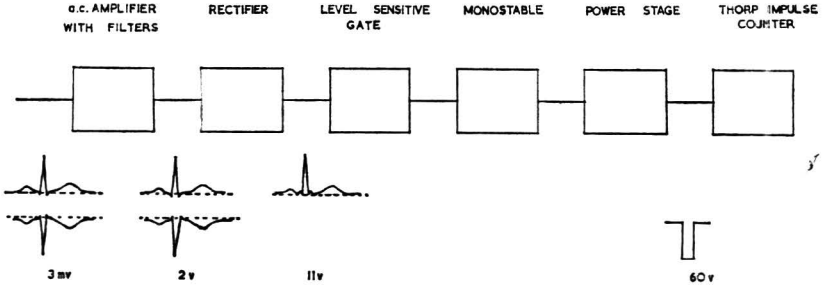

FIG. 1. Schematic diagram of impulse generator.

The AC amplifier comprises three shunt stabilised stages using close gain tolerance transistors with the consequent elimination of the need for feedback loops to stabilise the amplifier gain against production spreads. Low frequency rejection filtering (at approximately 12 db/octave) is provided for by switching interstage coupling capacitors. This is particularly advantageous when using small animals with fast heart rates, so that hum level can be reduced without affecting the QRS complex. At the lowest settings of the low frequency filter the response of the amplifier is flat down to input frequencies of 8 c/sec and the instrument will satisfactorily respond to all ranges of heart rate encountered in laboratory conditions. The upper frequency response of the amplifier is filtered by simple resistance capacitance 6 db/octave filters with options at 1, 5 and 15 Kc/sec as the corner frequencies, and mitigates against high frequency interference due to electric motors or brushes, for example. A jack plug socket at the output stage of the amplifier enables the use of low gain pre-amplifier oscilloscopes to display and check that undistorted

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FIG. 2. Circuit diagram of transistorised impulse generator. R1, 10 K Log. R2, 100 K. R3, 100 K. R4, 15 K. R5, 10 K. R6, 100Ω 1 W. R7, 100 K. R8, 100 K. R9, 10 K. R10, 39 K. R11, 39 K. R12, 1 K. R13, 3·3 K. R14, 5 K Lin. R15, 330 K. R16, 8·2 K. R17, 33 K. R18, 4·7 K. R19, 5 K Lin. R20, 50 K Lin. R21, 3·3 K. R22, 1·5 K. R23, 3·3 K 1 W. R24, 39 K. R25, 1 K. R26, 100 K. R27, 100Ω 3 W. R28, 10 K 1 W. R29, 22 K. All resistors ½ W 10% unless otherwise stated. C1, 5 μF 60 V Polyester. C2, 0·5 μF. C3, 50 μF 25 V Electrolytic. C4, 1 μF. C5, 0·05 μF Paper. C6, 0·1 μF Paper. C7, 8 μF. C8, 0·001 μF Paper. C9, 1 μF. C10, 0·05 μF Paper. C11, 0·1 μF Paper. C12, 8 μF. C13, 8 μF. C14, 500 μF 50 V Electrolytic. C15, 0·005 μF Paper. C16, 0·03 μF Paper. C17, 0·002 μF Paper. C18, 8 μF. C19, 25 μF 50 V Electrolytic. C20, 5 μF. C21, 25 μF 50 V Electrolytic. C22, 500 μF 50 V Electrolytic. C23, 500 μF 50 V Electrolytic. Condensers may be Polyester or Electrolytic unless otherwise stated. VT1, ZT 44 Ferranti. VT2, ZT 44 Ferranti. VT3, ZT 44 Ferranti. VT4, ZT 44 Ferranti. VT5, ZT 44 Ferranti. VT6, ZT 44 Ferranti. VT7, ZT 1484 Ferranti. D1, OA 81 Mullard. D2, OA 81 Mullard. D3, OA 81 Mullard. D4, OA 81 Mullard. D5, OA 81 Mullard. D6, ZS 70 Ferranti. D7, ZS 70 Ferranti. D8, ZS 70 Ferranti. D9, ZS 70 Ferranti. D10, ZS 70 Ferranti. T1, TMT 12 1:6 Richard Allen Radio. T2, Mains Transformer TMT 12 6:1 Richard Allen Radio. F1, 500 mA.

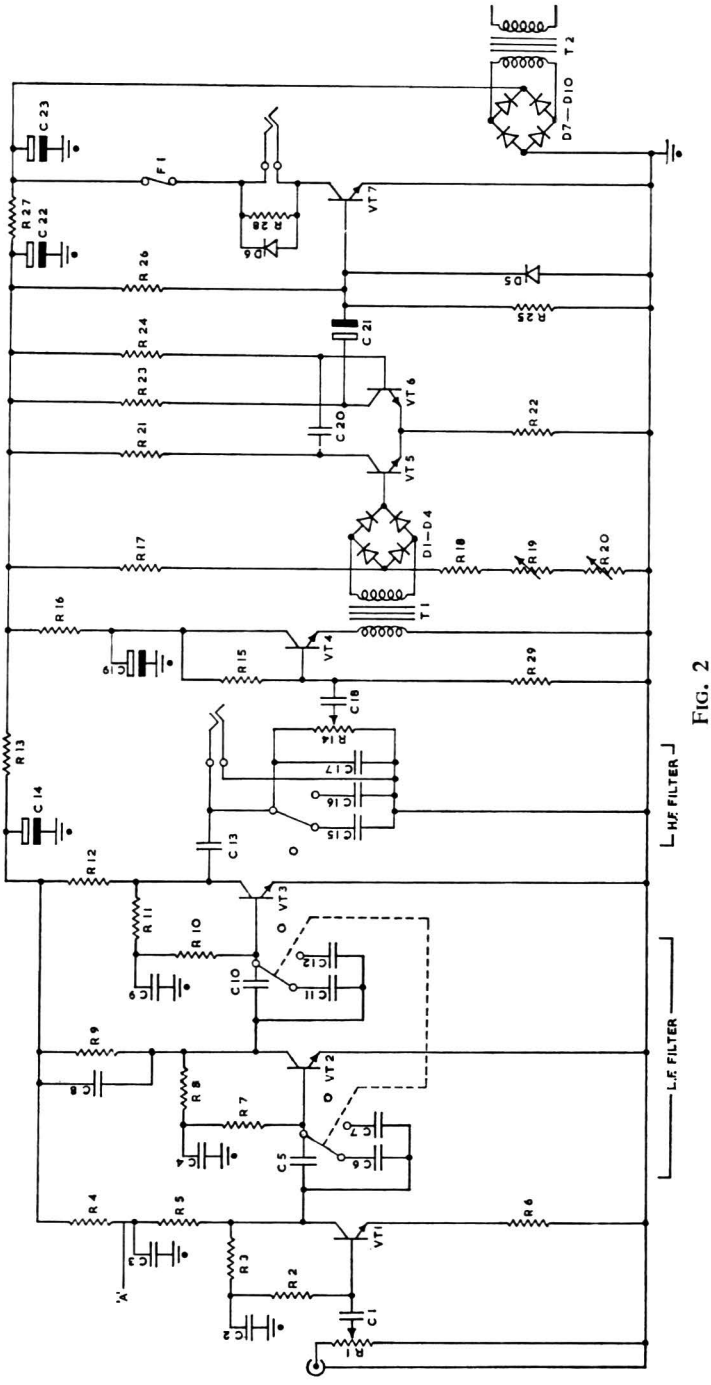
T2
D7—D10
C23
F1
VT7
R27
C22
R26
D6
D5
R25
C21
R24
R23
VT6
C20
R22
R21
VT5
D1—D4
R17
R18
R19
R20
T1
R16
VT4
C19
R15
R29
C18
R14
C17
C16
C15
R13
C14
C13
H.F. FILTER
R12
VT3
R11
R10
C12
C10
C11
L.F. FILTER
R9
C8
VT2
R8
R7
C7
C6
C5
C4
R4
R5
R6
VT1
C3
R3
R2
C2
C1
R1

FIG. 2

amplification of the QRS complex is being applied to the rectifier stage. Assuming the amplifier is functioning correctly, distortion will only be present when overloading occurs on the first stages of the amplifier, and it is recommended that the waveform be monitored at the amplifier output jack plug. The input sensitivity control can then be operated to prevent overloading which, in extreme cases, can result in random firing of the trigger.

The rectifier stage consisting of an emitter follower driving a transformer gives voltage step up and isolates the bridge rectifier on the output from the emitter follower drive. The latter is used to obtain a good low frequency response. Inclusion of a bridge rectifier in the circuit ensures that a positive pulse is always presented to the level sensitive gate in order to fire the trigger circuit. Without this arrangement it would be necessary in some cases to carry out electrode adjustment together with oscilloscope monitoring to ensure the correct sense of the pulse. An alternative method of avoiding this disadvantage has been to use a manually operated reversing switch, but the system still requires monitoring, a feature which is avoided by the adoption of a bridge circuit.

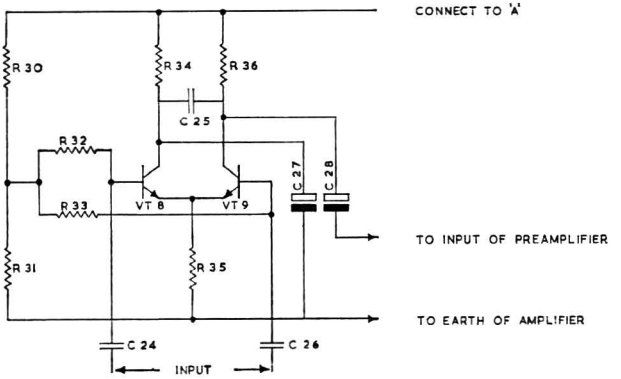

FIG. 3. Circuit diagram of differential pre-amplifier for use in conditions of limited screening. R30, 22 K. R31, 18 K. R32, 39 K. R33, 39 K. R34, 4·7 K. R35, 2·7 K. R36, 4·7 K. All resistors ½ W 10%. C24, 5 μF Polyester. C25, 0·005 μF Paper. C26, 5 μF Polyester. C27, 25 μF 50 V Electrolytic. C28, 25 μF 50 V Electrolytic. VT8, ZT 2270 Ferranti. VT9, ZT 2270 Ferranti.

The output from the rectifier actuates a monostable trigger circuit delivering a pulse into the output stage. The pulse from the output of the trigger circuit is used to drive a common emitter power stage, having a Thorp impulse counter as its load, and which in its quiescent state is held off, but is biased hard on when the base receives the trigger pulse. In order to prevent ringing due to the inductance of the Thorp impulse counter, a damping resistor and diode are used in parallel with the counter.

As the total number of transistors is small, commercial silicon transistors have been chosen on account of their greater thermal stability and reliability. Care has been taken to reduce the amount of stray noise

and hum pick up induced at the input by entirely enclosing the equipment in a metal case and having a single branched earth which is connected to the case from the earthed cable via the input jack plug. The apparatus is compact, portable and robust and the current drawn by the generator from the power supply is very low (<100 mA); thus a small power supply and simple filter is all that is required. Where AC mains are not available the generator may be driven by a battery.

In conditions where only a limited amount of screening is possible due to size of experimental subject, i.e. human heart rate, a differential pre-amplifier has been designed (Fig. 3) for use at the amplifier input, but for the laboratory conditions usually encountered, this is unnecessary.

OPERATING INSTRUCTIONS

1. The generator is more conveniently set up with the aid of a low gain oscilloscope, which may also be used to display changes occurring in the electrocardiogram as a result of experimental procedure, but it can be set up without one. The following procedure should be routinely adhered to.

(*a*) Use the oscilloscope to observe the AC amplifier output by tapping the appropriate jack plug socket.

(*b*) Adjust the input sensitivity until a suitable output is obtained from the amplifier, 5 V peak to peak. If this value is exceeded the amplifier will be overdriven and will in excessive conditions result in the possibility of unreliable counting.

(*c*) If the background noise is considerable, say more than 25% of the ECG signal, try to reduce this component of the amplifier output with the aid of the filters. (Inevitably the first stage of the amplifier generates some noise but a level of 5% of the total output should cover this adequately.)

(*d*) If hum level obscures the signal (when very large it may look like a square wave due to overdriving the AC amplifier) the object under investigation is either insufficiently screened, or else the earthing is incomplete or duplicated, causing induced hum in the resulting loops.

Note: The impulse generator itself is earth free along its mains input wire and should be earthed via the input jack plug to the table.

2. Reduce the gain control to zero.

3. Set the bistable stability control such that it just fails to actuate the Thorp impulse counter. With the setting above a certain level the the bistable runs freely as a square wave generator. The threshold between the two conditions corresponds to maximum sensitivity of the gate.

4. Gradually increase the gain control until the Thorp impulse counter starts to fire regularly. If the oscilloscope is a double beam instrument, the regular firing of the counter can be verified by observing the ECG waveform on one tract simultaneously with the output pulses to the counter on the other.

Note: Increasing the gain too much will cause the bistable to fire from the background noise of the environment and or the amplifier. If the apparatus is correctly adjusted removal of the input jack plug should cause the Thorp impulse counter to stop counting. If this is the case the amplifier noise is failing to fire the counter, and the impulse generator is functioning correctly.

GENERAL

It should be appreciated that the pulse forming and delivery circuits actuating the counter are level sensitive and fire when the amplified ECG

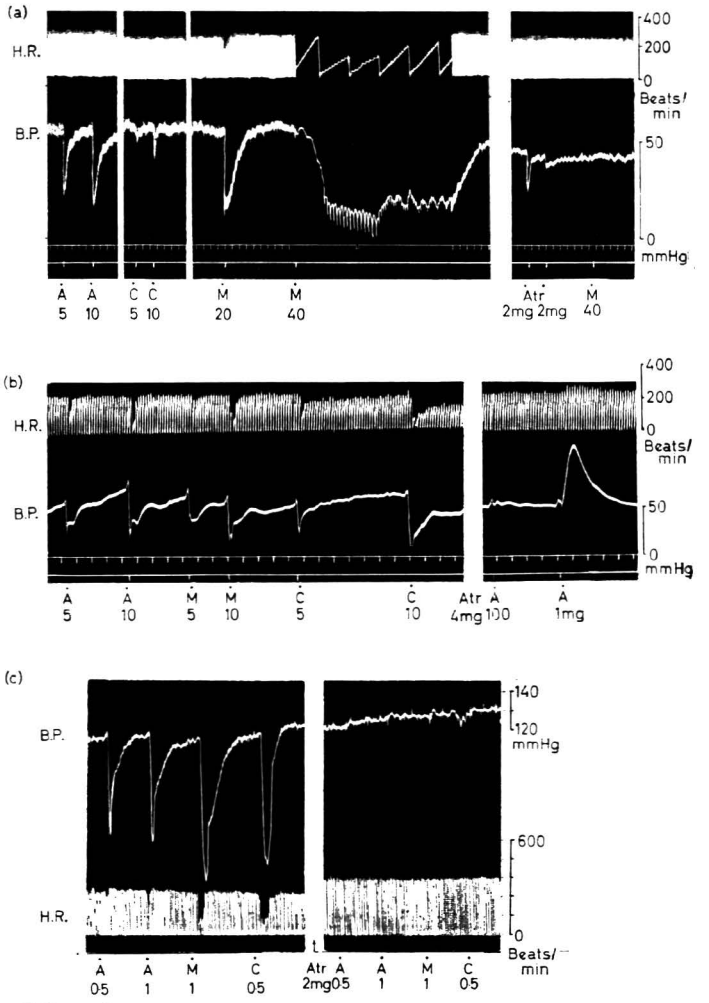

FIG. 4. Effect of parasympathomimetics on blood pressure (B.P.) and heart rate (H.R.). (a) Rabbit, 3·1 kg, anaesthetised sodium pentobarbitone 45 mg/kg intravenously; (b) Cat, 2·6 kg, spinal; (c) Rat, 220 g, anaesthetised sodium pentobarbitone 60 mg/kg intraperitoneally. Time = 30 sec. Acetylcholine (A), Carbachol (C), Methacholine (M), Atropine (Atr). Doses in μg unless otherwise stated.

reaches a suitable level of approximately 2 V. As the AC amplifier has a high gain over a selected frequency range and is typically $\times 10^4$, the background hum and noise level (often as high as 25% of the peak ECG voltage) can easily be made to fire the pulse forming circuits and thus the counter will be incorrectly actuated. This occurs when the gain control is too high.

The object of the above instructions is to adjust the amplified input signal level relative to the firing level such that only the ECG pulses are consistently used to actuate the counter.

Results

INTACT PREPARATIONS

Satisfactory heart rate counts using this apparatus have been made from anaesthetised rat, guinea-pig, rabbit, cat, spinal cat and pithed rat preparations.

Typical responses to parasympathomimetic drugs are shown in Fig. 4 and for sympathomimetic drugs in Fig. 5.

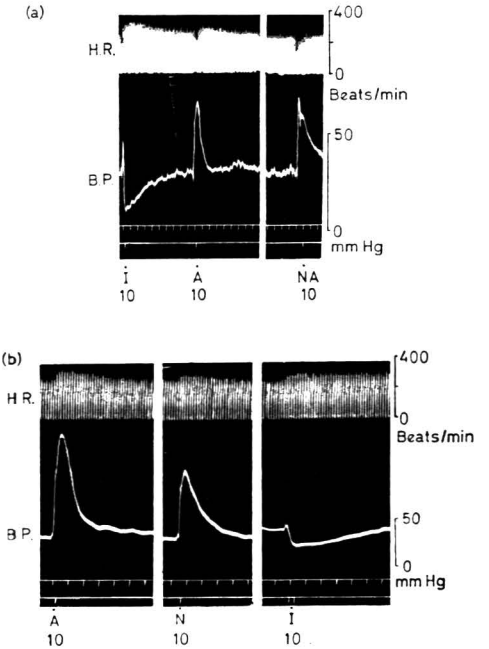

FIG. 5. Effect of sympathomimetics on blood pressure (B.P.) and heart rate (H.R.). (a) Rabbit, 3·1 kg, anaesthetised sodium pentobarbitone 45 mg/kg intravenously; (b) Cat, 2·6 kg, spinal. Time = 30 sec. Adrenaline (A), Noradrenaline (NA), Isoprenaline (I). Doses in μg.

It can be seen that in the spinal cat small doses of parasympathomimetics (5 μg) sufficient to produce a moderate lowering of the blood pressure are also able to bring about a slowing of the heart, Fig. 4 (b), whereas in

the anaesthetised rabbit and rat, Fig. 4 (a) and (c) the doses required to appreciably slow the heart produce a dramatic lowering of the blood pressure. The greater persistence of action of parasympathomimetics whose actions are terminated by cholinesterase hydrolysis is well shown in Fig. 4 (b) in which the evanescent action on the heart rate of 5 μg of acetylcholine is compared with the more prolonged action seen after 5 μg of carbachol, despite the fact that little difference can be observed in their effects on the blood pressure. Fig. 4 (a) also shows on a fast drum the ability of the counter to accurately follow heart rate changes associated with the injection of methacholine 40 μg. The blood pressure lowering effects of parasympathomimetics are completely blocked after atropine, Fig. 4 (a), (b) and (c).

The responses of the spinal cat to sympathomimetic amines, Fig. 5 (b), show that isoprenaline 10 μg and adrenaline 10 μg produce an increase in heart rate although noradrenaline 10 μg has less effect. On the anaesthetised rabbit, Fig. 5 (a), isoprenaline 10 μg produces a marked increase in heart rate; the responses to noradrenaline 10 μg and adrenaline 5 μg are complicated by the initial slowing which presumably arises from reflex vagal discharge associated with the raised blood pressure.

ISOLATED PREPARATIONS

The apparatus has also been used to record the rate of isolated Langendorff perfused heart preparations.

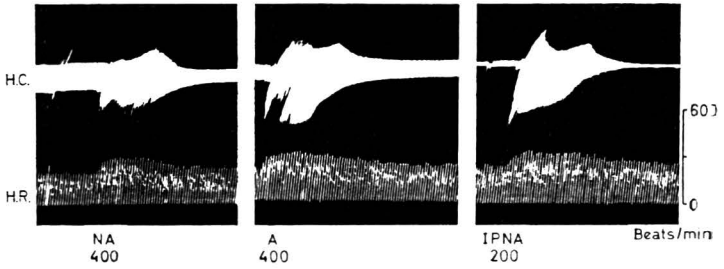

FIG. 6. Effect of sympathomimetics on responses of the isolated Langendorff perfused heart. Guinea-pig. Upper tracing heart contractions (H.C.), lower tracing heart rate (H.R.). Noradrenaline (NA), adrenaline (A), isoprenaline (IPNA). Doses in ng.

A typical record from an isolated guinea-pig heart and its responses to sympathomimetic amines is seen in Fig. 6. Isoprenaline 200 ng is seen to have a more marked stimulating action on the heart than 400 ng of noradrenaline or adrenaline. The chronotropic responses in all cases appear to last appreciably longer than the inotropic ones, and are seen to be greatest with isoprenaline.

Discussion

The need for a reliable, reasonably cheap and compact device to monitor the heart rate from either intact animal or isolated preparations

has been recognised by all experimental physiologists and pharmacologists. In this paper an apparatus is described which reasonably fulfils the above criteria, and will satisfactorily follow changes in heart rate.

In selecting the electrical changes associated with the heart contraction to actuate the recording system, the advantages of flexibility, and independence from other recording systems that may be used simultaneously, have to be weighed against the disadvantages reported by Dawes (1951) of artifacts arising from electrode movement and changes occurring in the QRS complex used to trigger the electrical circuit. In our experience the electrode movement does not constitute a problem but alterations in the character of the QRS waves as a result of drug action have proved to be a problem of real consequence. In choosing the action of parasympathomimetic and sympathomimetic drugs on the heart rate to demonstrate the ability of the counter to follow changes, these are the ones most likely to produce the maximal contrast between induced alterations of the QRS complex that are likely to be experimentally encountered (Goodman & Gilman, 1955), and provide a measure of its usefulness.

Another feature incorporated in the design of this counter is its versatility with respect to the range of species with which it will maintain a satisfactory performance. The difference in the time constants of the QRS complex obtained from a rat and a cat, which presumably is a measure of the total conduction time and size of the heart, is considerable, and the inclusion of adequate low frequency filters enables the apparatus to faithfully follow the electrical changes derived from the heart beat free from distortion and hum.

The apparatus is convenient to use and is suitable for use in both research investigations and for purposes of student demonstrations and teaching.

Difficulties encountered in operation arise from three main sources.

1. It is necessary to ensure good electrode contact by inserting the needles subdermally into the area on either side of the thorax.

2. Provision of adequate earthing on the supporting metal structures must be made to eliminate hum pick up and artifacts.

3. Random firing of the apparatus can occur if incorrect setting up procedures are adopted.

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Letters to the Editor

Gastric and duodenal ulceration: differences

SIR,—The collective term peptic ulcer which has been used in experimental and clinical work to include ulceration of the stomach and duodenum becomes less descriptive as the different roles of the factors of causation in the two types of ulceration become clearer.

Hakkinen (1960), assuming injected histamine to be the sole cause of the ensuing experimental ulceration, suggested that the anatomical site of the lesion is related to the concentration of histamine in the blood. Thus, ulceration would be expected to occur predominantly in the stomach after high blood histamine levels are achieved, and in the duodenum after lower levels. However, this hypothesis appears to be based on experiments where a total of 12 guinea-pigs (groups of 8, 2 and 2) received different doses of aqueous histamine.

We have evidence which supports Hakkinen's hypothesis and which suggests that the causes of experimental ulceration at the two sites in the gut of the guinea-pig are different.

Healthy adult male guinea-pigs of one strain (strain T as used previously; Anderson & Soman, 1965a) were kept singly in cages which prevented access to sawdust and faeces; they were denied food during the 24 hr before and after injection. Three groups received respectively 1 ml/kg depot injections containing in 10% beeswax in arachis oil: histamine acid phosphate 10 mg/ml; atropine sulphate, 1 mg/ml; histamine acid phosphate 10 mg/ml plus atropine sulphate, 1 mg/ml. The drugs were sieved (200 mesh) before incorporation.

The animals were killed 24 hr after injection. Stomach and duodenum were removed, distended with water and ulceration was scored against transmitted light according to the scheme used previously (Anderson & Soman, 1965b). The results are in Table 1.

TABLE 1. ULCERATION IN THE GUINEA-PIG AFTER DEPOT INJECTIONS OF HISTAMINE, HISTAMINE PLUS ATROPINE, AND ATROPINE

No. of animals	Medication mg/ml, intramuscularly	Average ulceration (± s.e.)	
		gastric	duodenal
12	Histamine 10	1·4 ± 0·30	3·7 ± 0·11 } $P < 0.05$
22	Histamine 10 + atropine sulphate 1	1·3 ± 0·29	2·8 ± 0·26 }
12	Atropine sulphate 1	0	0

Atropine allowed the accumulation in the stomach of the gastric juice secreted under the influence of the non-lethal dose of depot histamine. Atropine is unlikely to confuse the present results because neither atropine nor vagotomy prevent the experimental histamine ulcer (Merkel, 1942; Baronofsky, Friesen, Sanchez-Palomera, Cole & Wangensteen, 1946), also the secretion-inhibitory effect of atropine in ulcer patients is controversial (Stein & Meyer, 1948; Kirsner, Levin & Palmer, 1948, Wyllie & Smith, 1965). Paton & Vane (1963) showed that atropine reduced histamine-induced motility of the guinea-pig stomach; Watt (1956) observed an increase in peristalsis in the guinea-pig stomach after histamine and also (Watt, 1963) greater duodenal damage following repeated administration of water which encouraged increased stomach emptying.

At the end of the present experiment, the stomachs were full of gastric juice and significant amounts of food, in spite of fasting, indicating delay in gastric emptying caused by atropine.

Table 1 shows decreased duodenal ulceration ($P < 0.05$) but unchanged gastric ulceration in the histamine plus atropine group. Duodenal ulceration is believed to be associated with the secretion of large volumes of acid gastric juice, and the decrease in duodenal ulceration observed could therefore be attributed to the less vigorous exposure to acid gastric juice occasioned by the decreased stomach motility. Increased damage to the stomach did not occur even when large volumes of juice were being retained for longer periods of time in contact with the gastric mucosa, supporting the view that, contrary to the supposed aetiology of the duodenal ulcer, the gastric lesion is not caused in the first instance by exposure to acid gastric juice. The severity of duodenal ulceration, which is not included in the ulcer score, was greater in the histamine group than in histamine plus atropine group, emphasising the association of the duodenal lesion with the acid gastric juice.

The acute gastric toxicity and subsequent devitalisation of the gastric mucosa produced by a sufficiently high, rapidly released, dose of histamine must precede the action of the gastric juice if ulceration is to follow (Anderson & Soman 1965a). This, taken with the fact that the relatively low, slowly released, histamine dose of 10 mg/kg i.m. in the present experiment caused ulceration primarily in the duodenum, indirectly supports Hakkinen's (1960) hypothesis.

This concept extends the belief (Dragstedt 1956, 1965) that gastric ulcer could be caused by hypersecretion following excessive antral activity. Further experimental examination of this point might be rewarding.

The direct approach of higher doses of depot histamine has been explored but antihistamine cover is required to protect the animals from the rapidly lethal effect of the histamine and a consistent gastric ulcer picture of 4+ grade cannot be obtained (Soman, 1963). We believe that this is due to interference by antihistamines with the gastric response to histamine (Watt, 1964; Anderson & Soman, 1965a) together with rapid removal of the juice as a result of histamine-induced hypermotility of the stomach.

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November 10, 1965

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Histochemical amd biochemical effects of diethyldithiocarbamate on tissue catecholamines

SIR,—Recently diethyldithiocarbamate, an inhibitor of dopamine-β-oxidase, has been found to cause a decrease in noradrenaline and an increase in dopamine in rat ileum (Collins, 1965). The present study was planned to elucidate this effect further by means of a combined histochemical and biochemical approach.

Some male, albino, Sprague-Dawley rats (200–300 g) were treated with a single dose of 500 mg/kg s.c. of diethyldithiocarbamate (calculated as the sodium salt with 3 molecules of water of crystallisation) and killed at various intervals after the injection, while others received 2 doses of 500 mg/kg s.c., 7 and 3–4 hr before death. The brain, heart, submaxillary and adrenal glands, small intestine and femoral muscle were examined. Some animals were taken for the biochemical assay of dopamine (Carlsson & Waldeck, 1958; Carlsson & Lindqvist, 1962) and noradrenaline (Bertler, Carlsson & Rosengren, 1958). Others were taken for histochemical analysis (Falck, Hillarp, Thieme & Torp, 1962; Falck, 1962; Hillarp, Fuxe & Dahlström, 1965).

TABLE 1. NORADRENALINE (NA) AND DOPAMINE (DA) IN RAT HEART, BRAIN AND ILEUM AT VARIOUS INTERVALS AFTER SODIUM DIETHYLDITHIOCARBAMATE, 500 MG/KG S.C.

The values are means ± standard errors of the means, expressed in μg/g tissue.
Figures in brackets indicate number of experiments. Each experiment was performed on 2 or 4 pooled organs.

	Heart		Brain		Ileum	
Interval	NA	DA	NA	DA	NA	DA
Normal	0·77 (9) ± 0·029	0·02 (8) ± 0·010	0·41 (9) ± 0·017	0·64 (9) ± 0·028	0·25 (7) ± 0·017	0·03 (8) ± 0·007
2 hr	0·74 (1)	0·06 (1)	0·15 (1)	0·70 (1)	0·12 (1)	0·06 (1)
4 hr	0·72 (2) ± 0·020	0·09 (2) ± 0·040	0·14 (2) ± 0·050	0·77 (2) ± 0·070	0·12 (2) ± 0·010	0·08 (2) ± 0·020
6 hr	0·62 (3) ± 0·093	0·07 (3) ± 0·024	0·14 (3) ± 0·015	0·66 (3) ± 0·033	0·15 (3) ± 0·015	0·08 (3) ± 0·020
24 hr	0·96 (1)	0·12 (1)	0·29 (1)	0·72 (1)	0·17 (1)	0·04 (1)

After the single dose of diethyldithiocarbamate (500 mg/kg; preliminary observations show that much lower doses are active biochemically as well as pharmacologically) the animals appeared sedated but were easily aroused. Biochemical analysis of the brains revealed a decrease to about 30% of the normal value in noradrenaline 2–6 hr after injection (Table 1). After 24 hr the noradrenaline level was possibly still somewhat below normal. Dopamine did not change significantly. Histochemically no certain decrease in fluorescence intensity could be detected in central or peripheral catecholamine neurones at any interval studied. Biochemically a moderate decrease in noradrenaline was seen in the ileum but not in the heart. Dopamine showed an increase in both tissues, but levels sufficient to prove identity were hardly reached.

After two doses of diethyldithiocarbamate (500 mg/kg each) the animals were possibly somewhat more sedated than after a single dose. A marked depletion (to about 10% of normal) was obtained in the brain noradrenaline levels (Table 2), whereas the brain dopamine levels showed no significant change. In 3 experiments the brains were divided into hemispheres, striatum and stem; in the brain stem the dopamine levels were regularly (average 150%) higher in

the treated animals than in the controls; the same tendency was seen in the hemispheres, while no difference was seen in the striatum. The decrease in noradrenaline appeared to be the same in these different parts of the brain. Histochemically the various noradrenaline terminal systems of the brain were markedly depleted of fluorescent substances, whereas the catecholamine nerve terminals of the neostriatum, the tuberculum olfactorium, the nucleus accumbens, the median eminence, the nucleus amygdaloideus centralis and the dorsal part of the nucleus interstitialis striae terminalis showed normal fluorescence intensity. These areas have been found to contain mainly dopamine nerve terminals (see Fuxe, 1965; Carlsson, Dahlström, Fuxe & Hillarp, 1965). All the catecholamine nerve cell groups, however, showed normal fluorescence intensity after this treatment. The amine levels of the 5-hydroxytryptamine neurones were unaffected.

TABLE 2. EFFECT OF SODIUM DIETHYLDITHIOCARBAMATE (TWO SUBCUTANEOUS INJECTIONS OF 500 MG/KG 7 AND 4 HR BEFORE KILLING) ON RAT TISSUE CATECHOLAMINES

The values are means ± standard errors of the means. For the adrenals dopamine and the sum of adrenaline and noradrenaline are given in μg/2 adrenals. The other values are expressed in μg/g tissue.
Figures in brackets indicate number of experiments. Each experiment was performed on 2 or 4 pooled organs.

	Heart	Brain	Ileum	Femoral muscle	Adrenals
Noradrenaline					
Normal	0·77 (9) ± 0·029	0·41 (9) ± 0·017	0·25 (7) ± 0·017	0·12 (5) ± 0·014	28·7 (5) ± 3·390
Treated	0·80 (4) ± 0·049	0·05 (4) ± 0·011	0·10 (3) ± 0·025	0·07 (3) ± 0·006	18·4 (3) ± 2·322
Dopamine					
Normal	0·02 (8) ± 0·010	0·64 (9) ± 0·028	0·03 (8) ± 0·007	0·01 (5) ± 0·002	0·23 (4) ± 0·043
Treated	0·08 (4) ± 0·023	0·73 (4) ± 0·052	0·06 (4) ± 0·006	0·03 (3) ± 0·004	1·48 (3) ± 0·145

Biochemical analyses revealed a significant decrease in the noradrenaline levels of the ileum and femoral muscle, but not of the heart and adrenals. These effects were not large enough to be detected histochemically. The dopamine levels behaved as after a single dose; in the adrenals a considerable increase in dopamine was observed. In this case there is no doubt about the identity.

Histochemical observations on brains of rats treated with two doses of diethyldithiocarbamate as above and, in addition, nialamide (500 mg/kg intraperitoneally 6 hr before killing) revealed a normal fluorescence microscopic picture with normal fluorescence intensity in all parts of the catecholamine neurones of the brain. The controls receiving no nialamide showed marked noradrenaline depletion as described above. The nialamide-treated animals were less sedated than the controls.

The present data support the view that diethyldithiocarbamate inhibits dopamine-β-oxidase *in vivo*. The concomitant decrease in noradrenaline and increase in dopamine—except in dopamine neurones—supports this view. Although the percentage increase in dopamine after diethyldithiocarbamate treatment was probably large in the various noradrenaline-containing tissues examined, it reached levels which permitted identification only in the adrenals

and in the brain stem. We have not been able to confirm the high dopamine levels reported by Collins to occur even in normal ileum. We have examined various parts of normal rat ileum and obtained very low, hardly significant values for dopamine.

The present results reveal beyond doubt that a marked and selective depletion of the amine content of the brain noradrenaline nerve terminals occurred after treatment with diethyldithiocarbamate. The amine content of the central dopamine nerve terminals, on the other hand, was unchanged. By using diethyldithiocarbamate in this way it now seems possible to separate the dopamine and noradrenaline nerve terminals from each other. The histochemical experiments with diethyldithiocarbamate in combination with nialamide may be interpreted to show either, that noradrenaline is preserved by monoamine oxidase inhibition, or that it is replaced by dopamine. Biochemical studies are necessary to elucidate this point.

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Some geometrical considerations concerning the design of tablets: corrigenda

SIR,—My attention has been drawn to several small errors in my paper entitled "Some geometrical considerations concerning the design of tablets" (Cleave, 1965). These are:

p. 700, 6 lines from bottom: Suppose $l \geqslant d$. . . .
p. 701, equation (6): $-24\sigma^3t^2$ . . .
p. 701, equation (8): $+d(3G + 2D - 6d)$
p. 701, 10 lines from bottom: $(3G + 2D)/d = 12 + \mu$
p. 702, 3 lines from bottom: 3/31, 12/77, 3/7 . . .

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Cleave, J. P. (1965). *J. Pharm. Pharmac.*, **17**, 698–702.

Pharmacopoeias and Formularies

BRITISH VETERINARY CODEX 1965. Published by the direction of the Council of the Pharmaceutical Society of Great Britain. Pp. xxv + 843 (including Index). The Pharmaceutical Press, 17 Bloomsbury Square, London, W.C.1, 1965. 105s. (U.K. postage, 3s.).

The period since the publication of the first edition of the *British Veterinary Codex* in 1953 has been marked by the introduction into veterinary practice of many new chemotherapeutic agents, and a supplement to the Codex was published in 1959 in an attempt to keep pace with the spate of drugs. The second edition of the Codex incorporates the established new remedies, and the opportunity has been taken to remove obsolete drugs and vaccines. Fifty-seven drugs and chemicals and 18 vaccines have been introduced, but the additions have been more than balanced by the deletion of over 100 drugs and chemicals and 10 vaccines. The additions include anthelmintics, antiprotozoal agents and insecticides. It is interesting to note how the development of veterinary practice is reflected in many of the inclusions. Space has been found for most of the important new antibiotics, anaesthetics, adjuncts to anaesthesia, anti-inflammatory agents and a number of diagnostic agents.

Part I of the Codex is devoted to monographs on drugs and chemicals, Part II is concerned with vaccines and sera, and Part III with new preparations. Amongst these are Depot-tablets of Cobalt Oxide, the so-called cobalt "bullet", a solid preparation containing cobalt which is given by mouth and remains in the reticulo-rumen for several months releasing small quantities of cobalt for utilisation by the rumino-micro-organism in the manufacture of vitamin B_{12}.

It is difficult to take exception to any of the inclusions. There are, however, one or two drugs now in current use which have been omitted, but no doubt the delays of publication are in part an explanation of these omissions. Although Haloxon, Dioxathion and Metriphonate have been included, other organophosphorus compounds seem to have an equal claim. It is unlikely that all the inclusions and deletions would satisfy everyone.

Most of the deletions are well-justified; it is surprising that many found a place in the first edition. The deletion of certain drugs has involved other

modifications; for example, the deletion of barbitone has caused the main monograph on the barbiturates to be written under Phenobarbitone. It is encouraging to see that strychnine and its salts have at last been deleted, and it is hoped that the employment of this substance, even in the control of so-called pests and vermin, will be ended.

Each monograph takes a standard form. A description of the physical and chemical properties of the drug is followed by accounts of its actions and uses, toxicity, incompatibility, dose and the preparations available. Most of the information included under Actions and Uses is accurate and reasonably comprehensive. There are, however, a few surprising statements, such as that Thiopentone is a satisfactory anaesthetic for use in calves: many would not agree with this. It is also surprising to find no mention of the importance of the high lipid solubility of the thiobarbiturates in governing their action. In the monograph on Tubocurarine, it is stated that decamethonium and Mephenesin have similar actions. This is ambiguous and could well have been omitted, especially as decamethonium is not in the Codex. In the monographs on both Chloroform and Carbon Tetrachloride, little mention is made of the toxic effects of these drugs on the liver. This seems rather an unfortunate omission.

There is a slight inconsistency in the setting out of some of the monographs. For example, the monograph on Dimercaprol includes information about the dose under Actions and Uses. The monograph on Trimeprazine not only includes dosage with Actions and Uses, but in addition gives a separate dose in the section headed "Dose".

The second edition of the Codex contains a great deal of information not available elsewhere which will be of value not only to practising veterinary surgeons and pharmacists, but also to experimental pharmacologists. There has been over the past decade an increasing use in experimental pharmacology of animals other than cats and dogs, such as sheep and pigs, and actions and uses of most of the therapeutic agents employed in these species, as well as the dosages are included.

The second edition of the *British Veterinary Codex* is well-produced, substantially bound, and will serve as a valuable work of reference for all who have to administer drugs to animals or dispense drugs for animal treatment.

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Letters to the Editor

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