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The influence of 1-hexadecanol on the acid-catalysed hydrolysis of sodium dodecyl sulphate*

B. W. BARRY† AND E. SHOTTON

The kinetics at 60° of the acid-catalysed hydrolysis of sodium dodecyl sulphate in the presence of 1-hexadecanol above the critical micelle concentration showed the hydrolysis to be first order and the rate to depend on the molar ratio of alcohol to alkyl sulphate. Up to a ratio of 0·75 the rate increases; above this there is a steady decrease. The changes in rate have been correlated with phase changes and explained on the basis of the effect on the charge density on the micelle, and on the dielectric constant of the associated medium, in passing from a mixed spherical micelle to the lamellar structure and then to the expanded lamellar structure prevailing in liquid crystals.

SODIUM dodecyl sulphate in aqueous solution will hydrolyse to produce sufficient dodecanol to give a minimum in the surface tension curve after as short a period as 24 hr (Harrold, 1960). The hydrolysis of the lower alkyl sulphates in alkaline solution has been reported to follow a second order reaction (Green & Kenyon, 1950), a first order reaction (Burwell, 1952) and, for certain alkyl sulphates, first and second order reactions simultaneously (Calhoun & Burwell, 1955). Kurz (1962) showed that the rate of hydrolysis of sodium alkyl sulphates catalysed by hydrogen ions was strongly accelerated by the aggregation of the ester anions into micelles, the corresponding hydroxide ion catalysed rate was strongly suppressed and in neutral solution the rate was unchanged. Motsavage & Kostenbauder (1963) found that the hydrogen ion catalysed hydrolysis of sodium dodecyl sulphate was first order from pH 1 to 3·25 and from 30 to 70°. Solutions of the sulphate at concentrations above the critical micelle concentration (CMC) underwent hydrolysis at rates more than thirty times that for solutions below the CMC and the non-ionic agents dodecanol and Pluronic F-38 further increased the rate of hydrolysis. Nogami, Awazu & Kanakubo (1963) have shown that the rate of hydrolysis of sodium dodecyl sulphate in acid solution at high temperature increased markedly at the CMC whilst in alkaline solution the rate decreased. Nogami & Kanakubo (1963) studied the acid catalysed hydrolysis of long-chain alkyl sulphates, assuming a first order reaction to hold, and found that they were relatively stable below their CMC's but less stable above; the longer the chain length of the alkyl sulphate the more unstable the surfactant became.

Experimental and results

Materials. As used by Barry & Shotton (1967). The perchloric acid (71–73%) was Analar grade.

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* This work forms part of a thesis accepted for the degree of Ph.D. in the University of London.

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PREPARATION AND ANALYSIS OF SYSTEMS

The systems were of the general formula: sodium dodecyl sulphate 12 g; 1-hexadecanol varied; perchloric acid 5 ml; water 1200 g.

The 1-hexadecanol concentration was varied from a molar ratio (alcohol to sulphate) of 0 to 1·75. Water, heated to 70–75°, was weighed (1200 g) and the sodium dodecyl sulphate, perchloric acid and 1-hexadecanol added and vigorous mixing accomplished using a Silverson mixer fitted with an axial flow head. The systems were rapidly cooled to room temperature with running water, a pump head attachment fitted to the mixer and previously washed and dried 20 ml ampoules were filled with the mixture using a tube fitted with a wide bore canula. The pH of the mixture was 1·7, as determined by a glass electrode.

The ampoules were sealed and stored at 60° $\pm$ 0·01° in a thermostat bath. At set time intervals two ampoules were removed, rapidly cooled, the contents bulked and two 20 ml quantities titrated against 0·1N NaOH to pH 7·0, using an E.I.L. Model 24 Automatic Titrimeter, and the average titre taken.

MICROSCOPY

Microscopic observations were made using a microscope fitted with a Kofler micro hot stage. Systems were stored for not less than 12 hr at 60° and transferred after mixing to a slide with fused-on glass cell, which was maintained at 60° on the hot stage. Examination was carried out between crossed polars at a magnification of $\times$200. Systems were clear up to a molar ratio in the region of 0·26–0·28 when they became opalescent and increased in opacity with increased molar ratio. Characteristic anisotropic spherulites showing the well-formed extinction cross of uniaxial crystals were visible in this region. This new phase was considered to be liquid crystal composed of sodium dodecyl sulphate–1-hexadecanol–water (containing perchloric acid). By the time the molar ratio had reached two, many small isotropic globules were present, presumably emulsified globules of excess alcohol in equilibrium with the liquid crystal phase. Further increase in the molar ratio led to an increase in the number of isotropic globules in the field of view, and a reduction in the number of spherulites.

DETERMINATION OF CRITICAL MICELLE CONCENTRATION

The determination of the CMC of sodium dodecyl sulphate using the conductivity method was not possible in the presence of the strongly ionized perchloric acid. The dye method of Corrin & Harkins (1947) was used with the cationic dyes rhodamine 6G and acriflavine at a concentration of 10^{-5}M (Shinoda, Nakagawa & others, 1963). Both dyes gave the CMC of sodium dodecyl sulphate at 60° as 0·076–0·078% w/w in the presence of a molal concentration of perchloric acid of 0·0508; the CMC at 60° in the presence of perchloric acid and an equimolecular ratio of 1-hexadecanol to sodium dodecyl sulphate was 0·035% w/w. These results were used to ensure that the experimental work was at concentrations above the CMC.

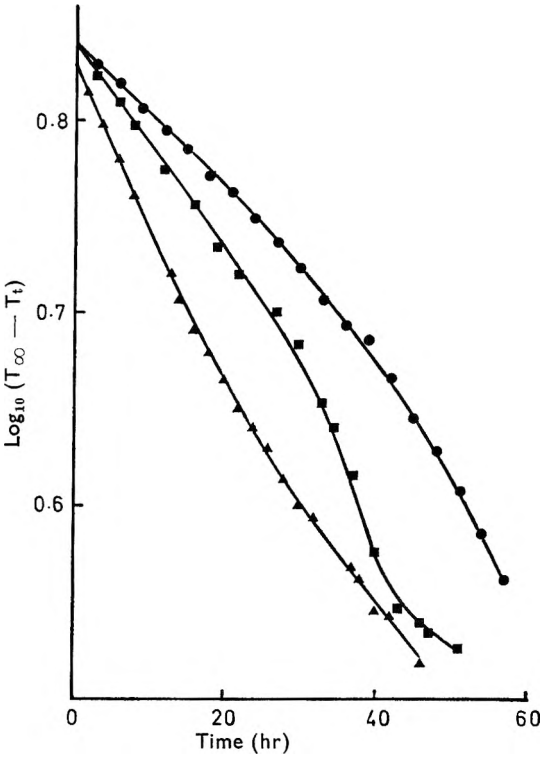

FIG. 1. $Log_{10}(T_\infty - T_t)$ versus time for systems of molar ratio (1-hexadecanol to sodium dodecyl sulphate) of 0 (—●—), 0·25 (—■—) and 0·75 (—▲—). Temperature 60° C.

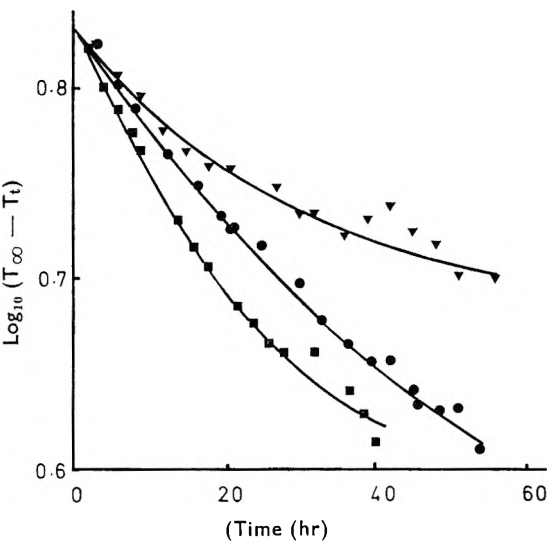

FIG. 2. $Log_{10}(T_\infty - T_t)$ versus time for systems of molar ratio (1-hexadecanol to sodium dodecyl sulphate) of 1 (— ■—), 1·5 (—●—) and 1·75 (—▼—). Temperature 60° C.

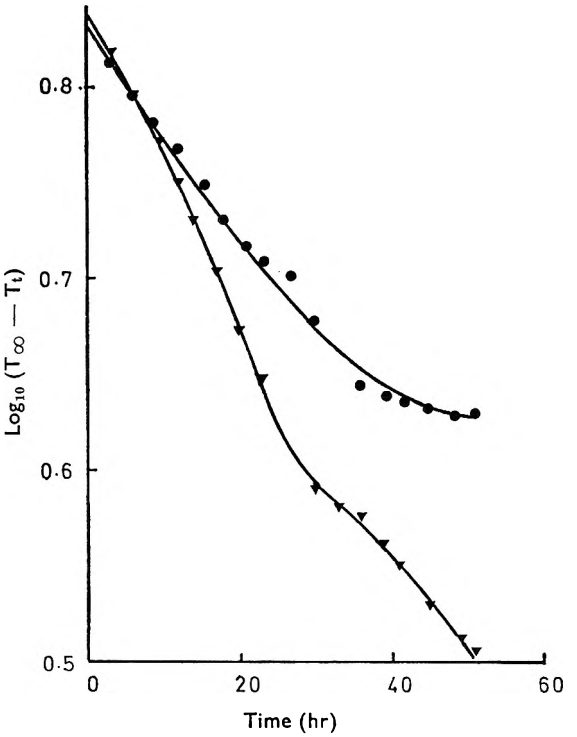

FIG. 3. $Log_{10}(T_\infty - T_t)$ versus time for systems of molar ratio (1-hexadecanol to sodium dodecyl sulphate) of 0·5 (—▼—) and 1·25 (—●—). Temperature 60° C.

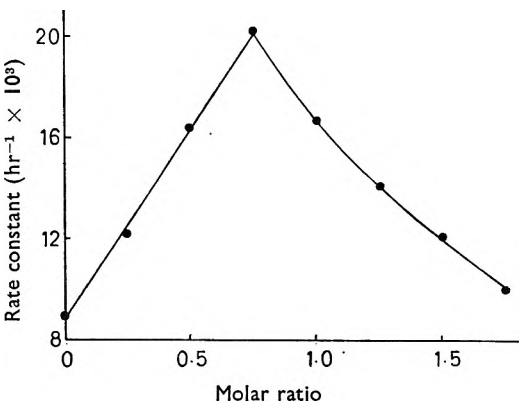

FIG. 4. Rate constant versus molar ratio (1-hexadecanol to sodium dodecyl sulphate) Temperature 60° C.

Figs 1, 2 and 3 are plots of $\log_{10}(T_\infty - T_t)$ against time, where T_∞ is the theoretical titre after infinite time of hydrolysis and T_t the titre at successive time intervals (Glasstone & Lewis, 1960). In all systems examined, some deviation from an initial straight line takes place after approximately 18 hr hydrolysis; the linear relationship confirms that at least in the first hours of hydrolysis the reaction is first order. Rate constants derived from the linear region of the graphs are plotted against molar ratio 1-hexadecanol to sodium dodecyl sulphate in Fig. 4. This graph shows a pronounced maximum in the rate of reaction at a molar ratio of 0·75.

Discussion

In making a kinetic study of this nature, it is desirable to keep the environmental conditions constant. The temperature was kept uniform and the pH low and constant using perchloric acid, which in concentrations greater than 0·01M depresses the ionization of the bisulphate ion produced by the hydrolysis of sodium dodecyl sulphate (Kurz, 1962). However, during hydrolysis the ratio of alcohol to alkyl sulphate increases. The rate of hydrolysis was dependent on this ratio and the rate will therefore change during an experiment. In addition, the hydrolysis of the alkyl sulphate results in the production of dodecanol, which will have a qualitatively similar effect to the 1-hexadecanol on the hydrolysis rate. With systems which have a molar ratio less than the maximum in Fig. 4, the rate constant will increase as hydrolysis proceeds, due to the molar ratio increasing. The rate constants of systems which have a ratio equal to or greater than 0·75 will decrease, due to the same cause. These changes in rate become apparent in Figs 1, 2 and 3 where the deviation from linearity of the plots for systems of molar ratio 0·25 and 0·5 results in these plots becoming convex to the time axis, whereas the plots for systems of molar ratio 0·75, 1, 1·25, 1·5 and 1·75 become concave to this axis. The plot for the system of molar ratio zero becomes convex as the sodium dodecyl sulphate hydrolyses and produces dodecanol, showing that dodecanol has a similar effect to 1-hexadecanol on the hydrolysis rate of the alkyl sulphate. The experimental points for the system of molar ratio 1·75 show much scatter. It was difficult to obtain consistent end-points in the titrations for this system, and experiments at higher molar ratios were therefore not attempted.

Attempts to correlate the maximum in Fig. 4 with any phase changes observed microscopically or macroscopically were unsuccessful, and it must be assumed in the absence of evidence to the contrary that no new phase appeared at molar ratio 0·75.

The complication of the changing molar ratio due to hydrolysis does not appear to affect significantly the rate of decomposition during the initial hours of reaction. Thus rate constants have been calculated for all eight experimental systems by assuming that first order reaction kinetics hold, at least during the first 18 hr hydrolysis. Nogami & Kanakubo (1963) obtained initial linear periods of hydrolysis for sodium

octyl, myristyl, cetyl and pentadecane-8 sulphates of 24, 10, 8 and 10 hr respectively. The value of 18 hr for dodecyl sulphate falls between the octyl and myristyl values.

The actual mechanism of the acid-catalysed hydrolysis of monoalkyl sulphates was considered by Kurz (1962) to proceed through the equilibrium protonation of the alkyl sulphate followed by attack of water on the sulphur atom.

$$RSO_4^- + H^+ \rightleftharpoons RSO_4H \quad .. \quad .. \quad .. \quad .. \quad (1)$$

$$RSO_4H + H_2O \rightarrow ROH + HSO_4^- + H^+ \quad .. \quad (2)$$

Kurz (1962) considered that the sharp increase in the rate constant which occurred when micelles were formed was due to the electrostatic potential between the micelle and the bulk solution. The sulphate groups became stronger bases due to the presence of a negative potential on the micelle and there was a resulting shift to the right in the prior protonation equilibrium (eqn 1). The reaction of the protonated ester molecules (eqn 2) then occurred with a specific rate essentially unchanged from its value in bulk solution, that is, where the ester molecules are not aggregated into micelles.

Motsavage & Kostenbauder (1963) explained the further increase in rates of hydrolysis consequent on the incorporation of non-ionic molecules in the micelle as follows. Although the charge density of the micellar surface was reduced by the polar heads of the alcohol molecules and hence, on Kurz's theory, the sulphate groups should now become weaker bases, the heads of the alkyl sulphate ions may now be regarded as being adjacent to a region of lower dielectric constant, i.e. the alcohol has lowered the dielectric constant along the surface of the micelle. Electrostatic interactions in this region should be strengthened and the rate of hydrolysis increased. However, it would seem probable that charge density did not fall as Motsavage & Kostenbauder (1963) assumed. An examination of the results quoted by Osipow (1962) shows that in a micellar solution of an alkyl sulphate only about 20% of the surfactant molecules are ionized. Introduction of a nonionic molecule would dilute the micellar surface concentration of alkyl sulphate and also allow increased ionization. The charge density would be restored and the rate of hydrolysis maintained. Any effect of lower dielectric constant would then be in addition to this.

The above considerations may explain the increase in rates of hydrolysis with increase in molar ratio in the systems examined, up to the appearance of a liquid crystal phase, i.e. up to a molar ratio of 0·26–0·28. The continuation of the increase into the liquid crystalline region may be ascribed to a radical change in the packing of the molecules. They are now held in a lamellar structure, and are closer together than in the original spherical micelle. A further decrease in dielectric constant at the surface of the micelle causes an increase in hydrolysis rate. To explain the fall in the rate of hydrolysis after a molar ratio of 0·75 it is postulated that the increased number of alcohol molecules forces the

alkyl sulphate heads further apart than they were in aqueous solution, i.e. before the appearance of smectic phase.

In addition, 1-hexadecanol has an alkyl chain which is four carbon atoms longer than the dodecyl sulphate chain, and this will modify the packing in the micelles. This effect will become more pronounced the higher the alcohol concentration. The hydroxyl group of the alcohol will protrude further into the aqueous environment than the sulphate group. In systems of alcohol content greater than molar ratio 0·75 the result may be a partial shielding of the sulphate ion, resulting in a lowering of the rate of hydrolysis.

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Abortifacient and toxic actions of the glycoside 'albitocin' extracted from some *Albizia* species

A. LIPTON

Conscious intact mice, rats, guinea-pigs, rabbits and monkeys were dosed with "albitocin", an active glycoside extracted from plants of certain *Albizia* species used by East African native doctors to accelerate labour and procure abortion. In pregnant animals abortion usually occurred within 12 hr at dose levels characteristic for each species irrespective of stage of gestation. In larger doses the drug was toxic, and with lethal doses animals survived 12–170 hr, with increasing apathy and anorexia, conscious but moribund as death approached. Toxicity in the orally dosed animals was lower than in those dosed intraperiotoneally or intravenously. The changes observed which could account for the mortality are discussed.

THE isolation of an oxytocic glycoside, albitocin, in the form of an amorphous white powder, from some members of the plant genus *Albizia*, its partial chemical characterization (Lipton, 1959, 1960, 1963), its actions on uterine strips *in vitro* (Lipton, 1963), and its actions on the blood pressure and uterus of anaesthetized mammals (Lipton, 1964) have been described previously. Some of its actions on conscious intact mammals, with details of attempts to determine the nature of its toxicity are now described.

Experimental

Mice, rats, guinea-pigs, rabbits and vervet monkeys (*Cercopithecus aethiops*) were dosed by one of four routes: gastric, via a semi-stiff polythene tube of 2 mm external diameter, lubricated with glycerol; intraperitoneal (ventral abdomen); intravenous (ear vein in rabbits, short saphenous vein in monkeys); intramuscular (semitendinosus muscle in guinea-pigs).

All doses and injections were given in 0·9% saline solution with full sterile precautions in conscious immobilized animals and were controlled by administration of equivalent volumes of the saline solution to comparable animals in each series.

As in previous work, the assumption has been made that albitocin is the only new active principle present in the plant extracts. Potency was determined by assay on the gravid guinea-pig uterus *in vitro* with appropriate controls. Animals were fed *ad lib.* on suitable green food and balanced diet pellets enriched with vitamins. They were marked and weighed daily, some had rectal temperature recorded, and those that died or were killed after dosing were examined post mortem for gross or microscopic changes.

The gravid state was detected by palpation, confirmed where necessary by X-ray photography, and abortion was only recorded if conception products were seen in the cage, or if a significant fall in weight occurred overnight and the uterus was clearly post-partum when the animal died or

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was killed. (In such instances foetuses, etc. were assumed to have been eaten.) The aborted foetus weights and crown-to-rump lengths were recorded where possible.

HAEMATOLOGY

Serial blood samples were taken from 8 rabbits before and at 6 hr intervals after lethal doses of albitocin, continuing to the time of death; immediately after the heart stopped a further sample was taken direct from the ventricles by thoracic puncture. Standard techniques were used with the blood samples from some of the rabbits for haemoglobin, haematocrit, specific gravity, erythrocyte count, and plasma protein, glucose, sodium and potassium concentrations, alkali reserve and pH.

Blood bacteriology. Samples were: incubated under hydrogen by the method of Mackie & McCartney (1953); cultured on blood agar, McConkey's bile-salt agar and incubated for 24 hr; examined microscopically as a smear stained with Leishman and Giemsa stains.

Results

GASTRIC TUBE, INTRAVENOUS AND INTRAPERITONEAL ADMINISTRATION

The ED50 (abortion) and LD50 doses by these routes were calculated by the method of Litchfield & Wilcoxon (1949) and are summarized in Table 1. Only approximations were possible for the rabbits and monkeys owing to the relatively short series available. No data were obtained for abortifacient activity by albitocin in very early gestation, but above a foetal crown-rump length of 0·5 cm abortions were produced at all stages of gestation. The crown-rump (C–R) length of aborted mice foetuses ranged from 0·6–2·3 cm; aborted rat foetuses ranged from 1·0 cm C–R length, wt 0·48 g up to 5·0 cm C–R length, wt 5·8 g; guinea-pig foetuses ranged from 0·8 cm C–R length, wt 0·45 g to 10 cm C–R length, wt 70 g.

TABLE 1. EFFECTIVE DOSES FOR ABORTION (ED50) AND LETHAL DOSE (LD50) IN VARIOUS SPECIES AFTER ALBITOCIN

Species	No.	Route	ED50 mg/kg	LD50 mg/kg
Mouse ..	355 120	i.p. i.v.	3·2 ± 0·6 —	5·9 ± 0·6 6·0 ± 0·3
Rat ..	54	i.p.	0·5 ± 0·1	0·8 ± 0·1
Guinea-pig ..	75 84	Gastric i.p.	11·0 ± 2·5 0·7 ± 0·2	19·0 ± 5·0 1·0 ± 0·2
Rabbit ..	42	i.v.	1·0 ± 0·3	1·8 ± 0·5
Monkey ..	17	i.v.	Approx. 1·0	Approx. 2·5

Aborted foetuses observed soon enough after expulsion were alive and normal in appearance and movements. Several guinea-pig foetuses aborted near term, even by females given lethal dosage, were sufficiently mature to survive with foster mothers and showed no subsequent abnormalities. It was concluded that the drug either possessed low foetal toxicity or did not traverse the placenta.

Plant extracts used by native doctors are taken by mouth, so the abortifacient and lethal dosage was also determined by this route in guinea-pigs. Results (Table 1) indicate that both abortifacient action and toxicity were much less in these animals, supporting earlier observations that gastric tube administration of up to 5·0 mg/kg albitocin failed to increase contractions in uteri of anaesthetized gravid guinea-pigs (Lipton, 1964).

INTRAMUSCULAR ADMINISTRATION

Seventeen gravid, 18 non-gravid female and 16 adult male guinea-pigs were injected with single doses in the range 0·6–2·0 mg/kg albitocin in one semitendinosus muscle. Saline-dosed control animals showed no effects. Within a few hr, the injected muscle in all the dosed animals was swollen and hard, and the leg was dragged, with no attempt to use it. All reflexes were present, but weak where the injected muscle was involved. Direct stimulation with needle electrodes showed that all the muscles in the limb distal to that injected were normal when stimulated directly or via the motor nerves. The injected muscle was sluggish and weak in response to such stimulation, but still capable of contracting. Squeezing or stretching it did not appear to be painful to the animals. The body weights were maintained, the animals were otherwise normally active and no abortions or deaths occurred. From this absence of systemic effects it was concluded that the drug injected intramuscularly did not escape, or escaped only slowly into the general circulation.

After 2–4 weeks the swelling subsided and the animals began to use the limbs again, and by 6 weeks after the injection appeared normal.

NATURE OF TOXICITY

Mice dosed intraperitoneally with 8·0 mg/kg, rabbits intravenously with 3·5–7·0 mg/kg and monkeys intravenously with 4·0 mg/kg were examined for effects of the drug during survival and immediately after death and in some cases after killing at intervals following the dose.

These animals and all others receiving lethal dosage showed similar symptoms, a gradual development of anorexia, and a concomitant decline in weight, a sleepy appearance and apathy to handling. There was no diuresis but some showed signs of diarrhoea. Respiration sometimes slowed slightly and changed to deep gasping during the last 30 min before death. Rectal temperature was constant.

Examination of the blood samples from the rabbits showed small falls in haemoglobin and haematocrit values of comparable size in both dosed and control animals, proportional to the volume of the sample removed. No haemolysis occurred at these dose levels. Specific gravity was constant at 1·055 throughout and pH was unchanged at 7·4. The alkali reserve fell significantly more in the dosed than in the controls (from 21 to 13 m-equiv./litre serum HCO_3^-) then rose before death to 18 m-equiv./litre. Plasma albumin fell significantly from 3·4 ± 0·2 g/100 g plasma to 2·8 ± 0·2 g/100 g but in some animals this was partly restored before death.

No consistent change in plasma sodium concentration occurred but plasma potassium concentration rose from 5·0 ± 0·3 m-equiv./litre to 8 ± 1·2 m-equiv./litre. One animal, however, showed no change in plasma potassium concentration up to the time of death.

On the first day the dosed animals' blood glucose showed a rise from 130 ± 15 to 205 ± 11 mg/100 ml which did not occur in the controls, but this fell to 185 ± 16 mg/100 ml before death.

Erythrocytes showed no change in shape or size or sedimentation rate (6·5 ± 2·5 mm/min). Leucocyte count rose from 4200 ± 480 to 5400 ± 650/mm^3. Control leucocyte counts remained constant.

At no time was there any sign of an infective process to account for death in the dosed animals; cultures of the blood, intraperitoneal fluid and nasal mucus showed only normal flora. Common ecto- and endoparasites were present, but the only pathogens detected in the blood were microfilaria, which were present in similar numbers in the control animals.

POST-MORTEM AND HISTOLOGICAL EXAMINATIONS

Apart from the increased leucocyte count referred to above, the blood picture remained unchanged up to death. Smears of the intraperitoneal fluid also showed no abnormalities subsequent to dosing by any route.

None of the earlier studies showed abnormality in frozen or paraffin wax sections of the heart, lungs, liver, kidneys, skeletal muscle, brain, intestinal and gastric mucosae, adrenal glands, bladder, ovaries and testes using standard staining techniques.

When this work was repeated later, however, necroses were observed in the renal tubules in rabbits dosed with a total of 8 mg/kg albitocin, and Kerr & Pound (1966) reported liver necroses in mice and rats dosed with albitocin (see Discussion).

Discussion

ABORTIFACIENT ACTION OF ALBITOCIN

In addition to its spasmogenic action on the isolated uterus and the uteri of anaesthetized animals *in situ*, albitocin in adequate dosage produced a high abortion rate in intact mammals of all species tried at any stage of gestation. This gives strong support to the use of the plant by African witch-doctors for acceleration of labour and abortion. There are superstitious reasons for a desire for rapid parturition among women of African Bantu tribes beyond the normal wish to reduce pain and these are discussed elsewhere in detail (Lipton, 1960). Powerful uterine spasmodics are known and are used in most births, often even where these take place in modern hospitals.

It is probable that the exceptionally high incidence of uterine rupture in Uganda (Rendle-Short, 1960) is due in part to this practice. Where such spasmodics are taken in large doses or too early in labour, especially if disproportion is present or there is any other obstruction to normal birth, a high incidence of uterine rupture is not surprising.

THE TOXICITY OF THE ACTIVE PRINCIPLE

The cause of death in those animals overdosed with albitocin has not yet been determined in all the species but evidence of necrosis in renal tubules was observed in rabbits, and Kerr & Pound (1966) showed that albitocin intraperitoneally produced an elaborate pattern of necrosis in the livers of mice and rats well correlated with the outward symptoms of toxicity and death, commencing with enlargement of parenchymal cell nuclei and peripheral zone fatty infiltration and progressing to coagulative necrosis in the intermediate zone, with eventual irreversible cytoplasmic and nuclear damage in the outer two-thirds of the lobules. These effects were not modified by dietary enrichment with tocopherol or by administration of promethazine hydrochloride. Oral dosing produced much less necrosis. Kerr later observed some centrilobular necrosis in mice about 14 days pregnant, after similar dosage (Dr. J. F. R. Kerr, personal communication). There is no information on the means by which the necrosis is produced by the drug, nor on how subsequent symptoms leading to death are produced, but these do fit a hepatic-coma type death.

Changes in plasma sodium were slight and variable and increase in plasma potassium concentration suggests increase in cell membrane permeability, but the electrical activity of the heart did not show any consistent changes (Lipton, 1963), and death from the drug was not rapid even when massive doses were given, but always took at least 12 hr, sometimes up to 7 days.

Many dosed pregnant animals aborted the uterine contents and died subsequently, but, given suitable dosage, many aborted and survived for the normal life span. This suggests mild or reversible actions of the drug in these instances. It also raises the possibility that, in addition to the previously reported uterotonic property, actions on the liver might also be involved in the abortifacient action.

It is possible that the human uterus *in situ* is more sensitive to the drug than that of lower animals, and that oral doses well below the toxic level are effective in accelerating labour. This seems likely if albitocin is the effective constituent of the native medicine.

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The role of the adrenal glands and of α- and β-adrenergic receptors in bronchodilatation of guinea-pig lungs *in vivo*

G. W. LYNN JAMES

Adrenaline, aminophylline, isoprenaline, noradrenaline, papaverine and phenylephrine exerted non-specific bronchodilator effects in guinea-pig lungs *in vivo*. The descending order of bronchodilator potency was isoprenaline > adrenaline > noradrenaline > phenylephrine. These agents also exerted a bronchoconstrictor effect, the descending order of bronchoconstrictor potency being phenylephrine > noradrenaline > adrenaline > isoprenaline. Bronchodilator activity could be antagonized by either α- or β-adrenergic receptor antagonists. The bronchodilator action of aminophylline, papaverine, phenylephrine and noradrenaline was partly mediated via the adrenal gland.

BRONCHODILATATION induced by sympathomimetic substances can be antagonized by β-adrenergic receptor antagonists (Nagasaka, De Schaepdryver & Heymans, 1964; Römer & Weidmann, 1964; Farmer & Lehrer, 1966). Such bronchodilatation, however, is not antagonized by α-antagonists (Römer & Weidmann, 1964; Bianchi & De Vleeschouwer, 1960). Although no evidence for the presence of α-constrictor receptors could be found in guinea-pig isolated trachea (Foster, 1966), the presence of such receptors has been reported in guinea-pig isolated lung (Nagasaka, Bouckaert & others, 1964), in dog lung *in vivo* (Castro de la Mata, Penna & Aviado, 1962) and possibly in cat isolated tracheal muscle (Türker & & Kiran, 1965). These findings imply that the adrenergic system has a dual effect on bronchial muscle, the dilator effect being dominant.

The present experiments were undertaken to determine the mode of action on guinea-pig lungs *in vivo* of some well known bronchodilator drugs. By the use of selective α- and β-adrenergic receptor antagonists and by removal of the adrenal glands, the bronchodilator action of these drugs was investigated.

Experimental

MATERIALS AND METHODS

The bronchodilator drugs used were adrenaline tartrate, aminophylline, isoprenaline sulphate, noradrenaline bitartrate, papaverine sulphate and phenylephrine hydrochloride. To block α- and β-adrenergic receptors, tolazoline hydrochloride and pronethalol hydrochloride respectively were used. As bronchoconstricting agents, acetylcholine bromide, bradykinin, histamine acid phosphate and 5-hydroxytryptamine creatinine sulphate (5-HT) were used. Bradykinin was synthesized according to Nicolaides & De Wald (1961). Vasopressin was used in experiments on blood pressure. All drugs were administered intravenously in solution in 0·9% w/v saline.

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Guinea-pigs of either sex weighing 200–400 g were anaesthetized with urethane (1·25–2·5 g/kg) intraperitoneally, the dose being adjusted to suppress spontaneous respiration. Animals were prepared for intravenous administration of substances and for recording air overflow volume by the method of Konzett & Rössler (1940). Bronchodilator activity was assessed from the reduction of the increased air overflow volume after administration of acetylcholine (5–20 μg/pig), bradykinin (0·5–2 μg/pig), histamine (1–4 μg/pig) or 5-HT (1–4 μg/pig). Acetylcholine or histamine was administered at 5 min and bradykinin or 5-HT at 10 min intervals. Acetylcholine was used in experiments to determine the antagonism of bronchodilatation by tolazoline (5 mg/kg) or by pronethalol (5 mg/kg) or by bilateral adrenalectomy. Acetylcholine was also used in all dose-ratio experiments. Doses of bronchodilating drug were adjusted to give submaximal antagonism of the acetylcholine response. This antagonism was measured at its peak effect, usually 30 sec.

Bilateral adrenalectomy was performed after midline incision 15 min before test. Dose ratios were obtained from the ratio of the dose of agonist (bronchodilator) given after antagonist to that given before antagonist that gave a similar quantitative response. Blood pressure was measured from an indwelling cannula in the carotid artery connected to a Statham pressure transducer. In these experiments, bronchoconstriction was assessed from the increase in tracheal pressure measured by a "Greer" differential micromanometer connected to the side arm of the tracheal cannula. The results were recorded on an electronic multichannel recorder.

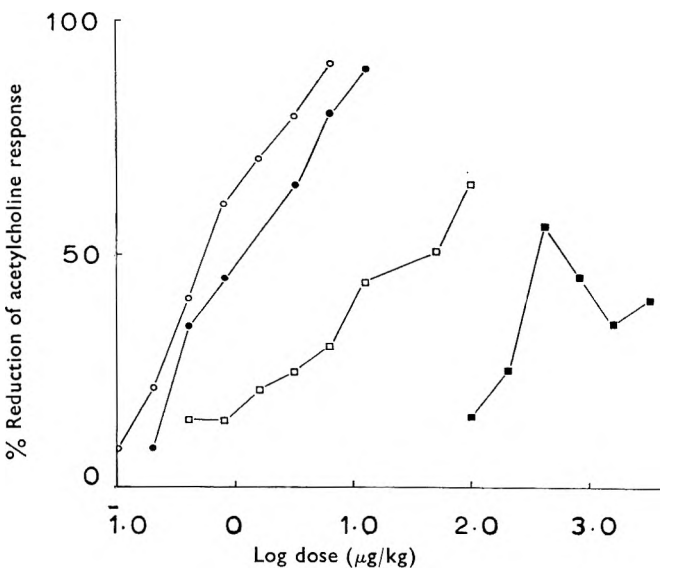

FIG. 1. Dose response curves of isoprenaline (○), adrenaline (●), noradrenaline (□) and phenylephrine (■), all given intravenously, assessed on guinea-pig lung *in vivo*. The response was assessed as the % reduction of the bronchoconstrictor effect of acetylcholine given 30 sec after the bronchodilating agent. All curves are the mean of at least 5 experiments.

Results

All the bronchodilator drugs tested antagonized bronchoconstriction induced by acetylcholine, bradykinin, histamine or 5-HT, indicating a non-specific effect. Fig. 1 gives the mean dose-response curves for four bronchodilator compounds, selected for their ability to stimulate predominantly β-(isoprenaline) or α-(phenylephrine) or both β- and α-(adrenaline and noradrenaline) adrenergic receptors. Each point is the mean of at least five observations. With the exception of isoprenaline, these compounds also induced bronchoconstriction.

Table 1 gives the percentage antagonism by tolazoline, by pronethalol or by adrenalectomy of the bronchodilator activity of adrenaline, aminophylline, isoprenaline, noradrenaline, papaverine and phenylephrine. For reasons discussed below, the percentage antagonism by pronethalol or by tolazoline of bronchodilatation induced by noradrenaline was determined in animals in which both adrenal glands had been removed. After adrenalectomy, pronethalol or tolazoline still exerted an approximately equal and almost complete antagonism of noradrenaline (Table 1).

TABLE 1. ANTAGONISM BY α- AND β-ADRENERGIC BLOCKING AGENTS AND BY BILATERAL ADRENALECTOMY OF BRONCHODILATOR ACTIVITY OF SOME DRUGS ON GUINEA-PIG LUNGS *in vivo*. All substances were given intravenously. Acetylcholine (5–20 μg/pig) was used as bronchoconstricting agent. Tolazoline and pronethalol were given at 5 mg/kg. An interval of 15 min was allowed after bilateral adrenalectomy; N.O., not obtainable.

Bronchodilator		Tolazoline		Pronethalol		Bilateral adrenalectomy % antagonism (90% limits)
Name	Dose (mg/kg i.v.)	% antagonism (90% limits)	Dose-ratio (range)	% antagonism (90% limits)	Dose-ratio (range)	
Adrenaline	0·002	22 (12–31)	18 (16–64)	70 (64–76)	108 (64–128)	3 (−23 to 29)
Aminophylline	5	10 (−1 to 21)	2·6 (2–4)	60 (32–89)	5·6 (4–16)	66 (41–116)
Isoprenaline	0·001	6 (0–12)	4 (nil)	99 (83–115)	56 (32–128)	28 (11–44)
Noradrenaline	0·02	91 (76–105)	6 (4–8)	92 (81–103)	11 (4–16)	44 (8–80)
	0·02	84 (67–102)		81 (65–96)		
	0·008	58 (43–73)		59 (35–84)		58 (30–86)
Papaverine	5	−6 (−14 to 3)	2·6 (1–8)	33 (25–44)	9 (4–32)	52 (31–73)
Phenylephrine	0·5	77 (41–113)	N.O.	−14 (−44 to 15)	N.O.	62 (30–93)
After bilateral adrenalectomy						
Noradrenaline	0·02	73 (55–92)	N.O.	81 (64–98)	N.O.	

The effect of tolazoline and pronethalol on the potency of the bronchodilator compounds in Table 1 has also been estimated as a dose ratio. The results obtained by the dose ratio method were similar to those obtained as a percentage antagonism. The values given in the Table are the mean of at least 5 observations. Dose ratios for noradrenaline after adrenalectomy and for phenylephrine could not be obtained, since the high doses needed to obtain the ratio caused intense bronchoconstriction.

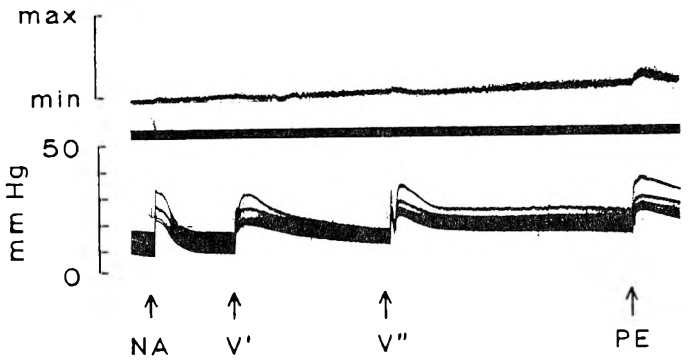

FIG. 2. Effect of noradrenaline, vasopressin and phenylephrine on the tracheal pressure (upper trace) and carotid arterial blood pressure (lower trace) of the guinea-pig. NA, 1 μg/kg of noradrenaline; V′, 1 unit/kg and V″, 5 units/kg of vasopressin; PE, 1 mg/kg of phenylephrine, all given intravenously; min, 10 cm H_2O; max, 23·5 cm H_2O.

Fig. 2 shows that phenylephrine induced rises in both blood pressure and tracheal pressure, whereas doses of noradrenaline and vasopressin induced a comparable rise in blood pressure but did not appreciably raise tracheal pressure.

Discussion

The relative bronchodilator potencies of the compounds illustrated in Fig. 1 was in descending order, isoprenaline > adrenaline > noradrenaline > phenylephrine. This confirms previous results obtained in guinea-pig lung (Carminati & Cattorini, 1966; Foster, 1966). This order is similar to that of these compounds in stimulating β-receptors (Ahlquist & Levy, 1959; Furchgott, 1960). The bronchoconstrictor potencies of the compounds of Fig. 1 were in reverse order of their bronchodilator potencies, i.e. phenylephrine > noradrenaline > adrenaline > isoprenaline. This, in turn, is similar to their relative potencies in stimulating α-receptors (Ahlquist & Levy, 1959; Furchgott, 1960). These correlations suggest that bronchodilator activity is due to the stimulation of β-receptors and bronchoconstrictor activity to stimulation of α-receptors. This presupposes that α-receptors are present in guinea-pig lung, a view held by Nagasaka & others (1964). The bronchoconstriction obtained, particularly with higher doses of phenylephrine, is unlikely to be due to an increase in arterial blood pressure, since equi-vasopressor doses of vasopressin did not cause bronchoconstriction (Fig. 2). The presence of α-constrictor receptors in guinea-pig lung would explain why some of the dose-response curves in Fig. 1 did not approach 100% irrespective of the dose, and why the curve for phenylephrine was "bell shaped."

In the case of adrenaline, aminophylline, isoprenaline and papaverine, but not of noradrenaline and phenylephrine, pronethalol was a more potent antagonist of bronchodilatation than was tolazoline (Table 1). Isoprenaline, a substance known to act predominantly on β-receptors, was

antagonized completely by a β- but not by an α-receptor blocking agent. This result agrees with those of other authors (Nagasaka & others, 1964; Römer & Weidman, 1964; Farmer & Lehrer, 1966). Adrenaline, a substance acting on both β- and α-receptors, was antagonized by both β- and α-receptor antagonists, whereas phenylephrine, an α-receptor stimulant, was antagonized only by an α-receptor antagonist. The effect of α- and β-receptor antagonists on bronchodilatation due to sympathomimetic substances was therefore more dependent on whether the bronchodilating agent was an α- or β-receptor stimulant than on the relative number of α- or β-receptors in the lung (Nagasaka & others, 1964). That an α-receptor blocking drug antagonized bronchodilatation is contrary to the results of Bianchi & De Vleeschouwer (1960). At least three explanations are possible: (1) that tolazoline acts on β- as well as α-receptors; (2) that tolazoline has sympathomimetic effects; (3) that α-dilator receptors are also present in guinea-pig lung. The first two possibilities seem unlikely since, if tolazoline acted on β-receptors or had sympathomimetic effects, its efficacy against all bronchodilators would be similar. The present results therefore support the third possibility.

Noradrenaline was antagonized equally by pronethalol or tolazoline. At high doses of noradrenaline (20 μg/kg), the antagonism was almost complete, although at lower doses, an approximate 50% reduction of response was obtained with either antagonist. This result could be explained if high doses of noradrenaline mediated its bronchodilator effect on either β- or α-receptors indirectly, i.e. via the adrenal glands. The result obtained (Table 1) showed that pronethalol and tolazoline still exerted an approximately equal and almost complete antagonism in adrenalectomized animals, indicating that noradrenaline did not mediate an indirect effect on either β- or α-receptors, through the adrenal glands. The result that two different types of receptor antagonist almost completely antagonized the noradrenaline response is paralleled by the antagonism by morphine and phenoxybenzamine of the contraction of guinea-pig isolated ileum induced by 5-HT (Day & Vane, 1963). The authors concluded that either the antagonism by morphine or by phenoxybenzamine was not specific. Since pronethalol has weak α-receptor blocking activity (Gulati, Gokhale & Udwadia, 1965), this may explain the present results.

The bronchodilator actions of aminophylline, papaverine, phenylephrine and noradrenaline are partly mediated via the adrenal glands, since removal of the adrenal glands much reduced the activity of these compounds (Table 1). This result is consistent with the view that some sympathomimetic drugs act via the adrenal medulla (Rubin & Jaanus, 1966; Schümann & Philippu, 1962). The findings of Farmer & Chick (1967) confirm that the bronchodilator activity of papaverine is partly mediated via the adrenal glands. The sum of the percentage antagonism of the bronchodilator activity of papaverine and aminophylline due to α- and β-receptor blockade was approximately 50%, indicating a dual action, i.e. sympathetic and probably a direct action. The activity of the remaining compounds appears to be mainly sympathetically mediated.

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The influence of anaesthetic agents on the formation of methaemoglobin induced by aniline in cats

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Administration of anaesthetic agents modifies the methaemoglobin formation induced by aniline in cats. The maximum amount of methaemoglobin is reduced, so too is the rate at which the methaemoglobin disappears. Studies with phenylhydroxylamine indicate that these changes are due to anaesthetics modifying the metabolism of aniline to phenylhydroxylamine rather than modifying the action of phenylhydroxylamine on systems within the red blood cell. The microsomal metabolism inhibitor SKF 525A has no effect on methaemoglobin formation induced by aniline.

IN examining the ability of aromatic amines and amides to induce the formation of methaemoglobin, we have used cats which were not anaesthetized (McLean, Murphy & others, 1967), although most other workers have used anaesthetized animals. Kiese (1963) used chloralose (0·05 g/kg) and urethane (0·5 g/kg) in both cats and dogs. He reported that the maximum methaemoglobin level was attained 4 hr after the intravenous administration of *m*-toluidine and 6 hr after the intravenous administration of *o*-toluidine. We have observed that the maximum methaemoglobin level was reached after 2 hr with *o*-toluidine and after 4 hr with *m*-toluidine in cats when no anaesthetic was used. Because of these differences and others of a similar nature between anaesthetized and conscious animals, we decided to examine the effects of various anaesthetic procedures on the formation of methaemoglobin induced by aniline in cats.

Experimental

CHEMICAL

All the anaesthetic agents were obtained from commercial sources. Aniline hydrochloride was recrystallized until analytically pure. Phenylhydroxylamine was prepared by the method of Vogel (1959) and had m.p. 80–81°.

BIOLOGICAL

The methaemoglobin of the cats was determined by the cyanmethaemoglobin method as previously described (McLean & others, 1967).

PROCEDURE

Food was withheld from the animals for 16 hr before the start of an experiment. For ease of handling, the cats, anaesthetized or conscious, were restrained in jackets made from strong cloth. The anaesthetic agents were given intraperitoneally, except ethyl chloride which was administered by the open drop method. Chloralose was injected as a solution in

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propylene glycol or in aqueous suspension; urethane and sodium pentobarbitone were given as aqueous solutions.

Various degrees of central nervous system (CNS) depression were produced by the procedures adopted. Surgical anaesthesia was induced by chloralose 75 mg/kg, chloralose 50 mg/kg with urethane 500 mg/kg, and pentobarbitone 40 mg/kg. Unconsciousness without loss of corneal reflex was induced by chloralose 50 mg/kg and deep sedation by chloralose 35 mg/kg. Ethyl chloride and oxygen 95%–carbon dioxide 5% mixture were used to produce brief periods of unconsciousness. As soon as muscular relaxation occurred, administration of ethyl chloride was stopped; full consciousness returned after 1–2 min. The cats were given ethyl chloride while the aniline hydrochloride solutions were being injected and then every hour when blood samples were taken. Aniline hydrochloride solutions for intravenous injection were adjusted to pH 5·5 and made isotonic with sodium chloride. Injections were made slowly into the femoral vein with a dose volume of up to 10 ml. A single dose level of aniline (0·0625mmole/kg) was used in all animals. Phenylhydroxylamine solution was administered by intravenous injection into the femoral vein at a dose of 0·5 mg/kg (0·00459 mmole/kg) and an injection volume of 1 ml. The phenylhydroxylamine was dissolved in 0·9% sodium chloride solution adjusted to pH 8 with sodium bicarbonate. Because of the instability of phenylhydroxylamine solution, which became cloudy on standing for 30 sec, the dose for each cat was made up under nitrogen and administered immediately. The saline was previously boiled, and bubbled with nitrogen while cooling. Phenylhydroxylamine was given to five conscious cats and also to five cats which had been anaesthetized with chloralose 50 mg/kg with urethane 500 mg/kg.

Blood samples were taken from the femoral vein just before administration of aniline and at each hr afterwards until the methaemoglobin level began to fall. Blood samples were also taken in a similar manner just before the administration of phenylhydroxylamine, and then every hr for 5 hr.

Methaemoglobin estimations were made in duplicate on each sample. Dilution of 0·1 ml quantities of heparinized blood in 10 ml of M/60 phosphate buffer pH 6·6 was made immediately after withdrawal of the samples since it has been shown that methaemoglobin is reduced to haemoglobin when whole blood is allowed to stand at room temperature (Climie, McLean & others, 1967). At least five cats were used for each anaesthetic treatment.

In some of the experiments body temperatures of the cats were monitored by means of rectal thermocouples inserted to a depth of 6 cm. All experiments were made in a thermostatically controlled air-conditioned laboratory.

Results and discussion

The results obtained with aniline are given in Table 1 and the statistical treatment is given in the appendix and Tables 3 and 4. A number of conclusions may be drawn from these. The first relates to the effect of the

TABLE 1. TYPICAL RESULTS FOR METHAEMOGLOBIN (% OF TOTAL HAEM PIGMENTS) FORMED IN CATS AFTER INTRAVENOUS INJECTION OF ANILINE. DOSE 0·0625 MM/KG. THE METHODS OF ADMINISTERING THE ANAESTHETICS ARE GIVEN IN THE TEXT. The values for the first 5 hr only were taken for the statistical treatment of the results, data for which were also drawn from results not shown here in the interests of brevity.

Treatment given to cats	Cat No.	Methaemoglobin formed % — Time after administration of aniline (hr) 1	2	3	4	5	6	7	8
No anaesthetic	1	31·4	17·9	7·8	3·4	1·1	—	—	—
	2	36·9	30·6	19·9	14·1	11·7	—	—	—
	3	25·2	32·3	33·7	32·3	27·6	—	—	—
Chloralose (35 mg/kg)	1	19·1	28·5	25·0	20·0	11·4	7·7	—	—
	2	21·3	25·7	32·4	37·9	26·8	16·2	—	—
	3	13·4	24·6	27·2	23·7	22·0	19·4	—	—
Chloralose (50 mg/kg) + propylene glycol (1 ml/kg)	1	22·7	28·9	34·5	36·0	39·8	37·8	33·9	29·8
	2	36·2	40·0	35·2	31·9	25·2	18·5	—	—
	3	21·8	27·4	25·2	19·1	10·4	5·2	—	—
Chloralose 50 mg/kg in aqueous solution	1	2·3	12·6	12·6	17·7	—	—	—	—
	2	19·7	38·5	32·4	44·5	—	—	—	—
	3	14·2	28·8	25·2	16·4	—	—	—	—
Chloralose (75 mg/kg)	1	19·0	24·7	22·7	26·2	22·7	23·7	—	—
	2	15·9	15·2	15·9	15·2	13·4	8·3	—	—
	3	14·2	19·2	18·2	20·9	15·3	14·9	—	—
Chloralose (50 mg/kg) + urethane (500 mg/kg)	1	13·0	20·1	27·6	25·9	27·2	28·4	25·6	25·9
	2	20·1	16·5	15·8	11·9	10·4	7·2	4·3	—
	3	16·4	17·1	16·8	10·1	11·1	7·4	4·0	—
Ethyl chloride	1	25·6	27·4	16·7	12·6	4·2	—	—	—
	2	15·3	20·1	15·3	14·9	8·7	—	—	—
	3	18·8	18·8	15·6	10·9	—	—	—	—
Sodium pentobarbitone (40 mg/kg)	1	11·8	22·2	13·3	8·8	4·8	4·4	—	—
	2	17·0	21·0	23·2	22·3	18·3	14·3	—	—
	3	0	0	Cat died					

anaesthetic treatments on the methaemoglobin formed after the administration of aniline. From a consideration of the "mean effect" column in Table 4 it may be seen that there was no significant difference in the methaemoglobin produced when aniline was given to cats which were either unanaesthetized, or which had received chloralose at doses of 35 or 50 mg/kg. However, the methaemoglobin produced was significantly reduced after the administration of chloralose 75 mg/kg, chloralose 50 mg/kg with urethane 500 mg/kg, ethyl chloride or sodium pentobarbitone 40 mg/kg. There was no significant difference among these four anaesthetic procedures in their effectiveness in depressing methaemoglobin formation and the effect appears to be related to CNS depression itself. Perhaps the most surprising finding is that ethyl chloride had such a marked effect, since the animals were only rendered unconscious for short periods (less than 2 min) at the time of administration of aniline hydrochloride solution and at each hr when blood samples were taken. The second conclusion about the effect of anaesthetic treatments on methaemoglobin formation induced by aniline can be obtained from the "linear effect" column in Table 4. This effect relates to the rate at which methaemoglobin levels fell from hr 1 to hr 5 after the administration of aniline. For the unanaesthetized animals it can be seen that the level of methaemoglobin fell relatively rapidly with time, but with all the

anaesthetic treatments the rate of fall was significantly reduced. Again it is evident that this effect is a function of the animal being under CNS depression rather than the chemical nature of any anaesthetic agent. With the exception of the ethyl chloride treatment, there was no significant difference in the response obtained with any of the anaesthetic procedures used. This effect is more sensitive to anaesthetics than the reduction in the amount of methaemoglobin formed since even the low doses of chloralose (35 mg/kg and 50 mg/kg) have the same effect as the other anaesthetics. The ethyl chloride treatment was the only one which produced a less pronounced change in the time course of methaemoglobin removal than any of the other anaesthetics, but even this treatment produced a response which was significantly different from the results with conscious animals.

Under anaesthesia with chloralose 50 mg/kg with urethane 500 mg/kg the rectal temperature fell by approximately 1°/hr. However, the effects of anaesthesia on methaemoglobin formation and removal cannot be explained simply as a result of hypothermia since after chloralose 35 mg/kg and ethyl chloride there was no measurable fall in body temperature during the course of the experiment.

TABLE 2. METHAEMOGLOBIN (% TOTAL HAEM PIGMENTS) FORMED IN CATS AFTER INTRAVENOUS INJECTION OF PHENYLHYDROXYLAMINE (0·00459 MMOLE/KG)

Time after phenylhydroxylamine administered (hr)	Methaemoglobin formed %									
	Unanaesthetized cats					Cats treated with chloralose 50 mg/kg + urethane 500 mg/kg				
	1	2	3	4	5	1	2	3	4	5
1	32·3	38·5	32·2	47·8	33·9	37·9	25·9	40·9	32·5	47·6
2	28·5	29·5	9·8	42·9	19·4	23·9	18·9	30·2	21·4	34·9
3	23·8	16·6	8·3	28·2	12·7	18·3	13·6	22·9	20·1	33·1
4	20·3	10·6	3·8	26·9	5·7	—	10·4	13·1	22·2	24·8
5	18·0	9·3	4·2	23·3	5·7	—	6·3	6·8	10·7	17·9

The appearance of methaemoglobin following the administration of aniline to cats is the result of at least three processes. One is the metabolism of aniline to the active methaemoglobin-forming species and a second is the action of this metabolite on the haemoglobin in the intact erythrocytes. The third process involved is the erythrocyte reductase systems of the red blood cell which reduce methaemoglobin back to haemoglobin. Kiese (1963) considered that the active metabolite of aniline is phenylhydroxylamine and there is much evidence to support this view. The effects observed after administration of anaesthetics can therefore be due to the anaesthetic either reducing the rate of metabolism of aniline to phenylhydroxylamine, reducing its effect on haemoglobin, or affecting the methaemoglobin reductase systems in the erythrocyte.

To see if the effect of anaesthetics on methaemoglobin formation is due to effects on metabolism or due to effects in the erythrocytes, experiments were made using phenylhydroxylamine in place of aniline. The results of these are in Table 2 and the statistical treatment is given in the appendix (Table 5), from which it can be seen that there was no

significant difference in the amount of methaemoglobin formed and the rate at which it is removed in unanaesthetized cats or in cats treated with chloralose 50 mg/kg with urethane 500 mg/kg. This result indicates that the anaesthetic agents are exerting their effects on the metabolism of aniline to phenylhydroxylamine rather than on the events which occur in the erythrocytes.

Since the effects of anaesthetics seemed to be on the metabolism of aniline, the effect of SKF 525A, a potent inhibitor of microsomal metabolism, on the formation of methaemoglobin by aniline was examined. Cats were pretreated with SKF 525A at doses of 30 and 60 mg/kg intraperitoneally and then given aniline hydrochloride (0·0625 mmole/kg) intravenously after 45 min. The amount of methaemoglobin formed in the cats treated with SKF 525A was not significantly different from that in untreated cats. The metabolic step of aniline which is essential for methaemoglobin formation was not blocked by SKF 525A.

The effect of anaesthetics on cats has been investigated by Berglund, Nylen & Wallentin (1965), who found that chloralose 50 mg/kg with urethane 100 mg/kg caused metabolic acidosis, whereas sodium pentobarbitone 30 mg/kg, chloralose 50 mg/kg and urethane 1 g/kg did not. Propylene glycol, when used as a solvent, has been shown to reduce the amount of methaemoglobin produced following the administration of *p*-chloracetanilide in rats and guinea-pigs compared with the amount produced when the same dose of *p*-chloracetanilide was given in aqueous solution (Glocklin, 1954). It has now been found that there is no significant difference in the amount of methaemoglobin produced by aniline in animals pretreated with chloralose 50 mg/kg whether the chloralose was given as an aqueous suspension or in propylene glycol. However, the dose of propylene glycol (mg/kg) used by Glocklin (1954) was four times greater than the dose used in the present work.

The fact that anaesthetic procedures have such significant effects on methaemoglobin formation induced by aniline suggests that structure-action studies on methaemoglobin formation induced by aromatic amines should preferably be made on unanaesthetized animals.

The effect of the various anaesthetic procedures on the formation of methaemoglobin induced by other aromatic amines has been examined. Amines used included *o*, *m* and *p*-toluidine as well as 2,3-, 2,4- and 3,4-dimethylaniline, with and without chloralose 50 mg/kg and ethyl chloride. In all instances the effects of the anaesthetic procedures were essentially the same as their effects with aniline. However, in one experiment, sodium

TABLE 3. ANALYSIS OF DISPERSION FOR MEAN AND LINEAR EFFECTS (ANILINE)

Source of variation	d.f.	s.s. mean	s.s. linear	s. products
Between treatments	6	4,948·7	3,749·7	−2,438·0
Within treatments	41	12,944·4	2,341·8	2,587·0
Total	47	17,893·1	6,091·5	149·0

$$\Lambda = 0{\cdot}217$$

$$\frac{1 - \sqrt{\Lambda}}{\sqrt{\Lambda}} \cdot \frac{40}{6} = 7{\cdot}65^{**}$$

TABLE 4. TREATMENT MEANS FOR THE MEAN EFFECT AND LINEAR EFFECT WITH S.E.'S OF DIFFERENCES FOR ANILINE

Treatment	Mean effect	Linear effect
1. No anaesthetic	24·89	−5·74
2. Chloralose 35 mg/kg	22·10	0·23
3. Chloralose 50 mg/kg	26·66	0·49
4. Chloralose 75 mg/kg	14·77	−0·06
5. Chloralose 50 mg/kg + urethane 500 mg/kg	15·40	0·05
6. Ethyl chloride	17·46	−2·78
7. Sodium pentobarbitone 40 mg/kg	15·42	0·31
s.e. of difference for treatments—		
(1,2); . . . ; (1,6)	3·99	1·20
(1,7)	4·37	1·32
(2,3); . . . ; (5,6)	5·03	1·51
(2,7); . . . ; (6,7)	5·33	1·60

TABLE 5. TREATMENT MEANS FOR THE TOTAL EFFECT AND LINEAR EFFECT WITH S.E.'S OF DIFFERENCES USING PHENYLHYDROXYLAMINE

Treatment	Total effect	Linear effect
Phenylhydroxylamine	21·25	−6·22
Phenylhydroxylamine + chloralose and urethane	22·70	−6·13
s.e. of difference	5·26	1·17

pentobarbitone was given intravenously at a dose level of 50 mg/kg to five cats which then received aniline (0·0625 mmole/kg i.v.). The methaemoglobin percentages produced were significantly lower than control values for the first 2 hr and all the animals died before the 3rd hr.

Amount of aniline converted to phenylhydroxylamine. The metabolism of aniline is a complex process producing a series of metabolites, of which ring hydroxylation and *N*-hydroxylation are considered to be important. If it is assumed that phenylhydroxylamine is overridingly the most important methaemoglobin forming metabolite of aniline, as is suggested by Kiese (1963), then from the results now obtained with aniline and phenylhydroxylamine it is possible to estimate approximately the proportion of aniline metabolized to phenylhydroxylamine. It can be seen from Tables 4 and 5 that aniline at a dose of 0·0625 mmole/kg and phenylhydroxylamine at a dose of 0·00459 mmole/kg produce the same effects, both with regard to the amount of methaemoglobin produced (total or mean effect) and to the rate at which the methaemoglobin disappears (linear effect) 1 hr after the administration of aniline and phenylhydroxylamine. This indicates that about 7% of aniline is metabolized to phenylhydroxylamine. The fact that the linear effects of aniline and phenylhydroxylamine are close supports the view that phenylhydroxylamine is the methaemoglobin-forming compound derived from aniline. Since if the methaemoglobin-forming metabolite from aniline is not phenylhydroxylamine, it might be expected that the rate of removal of methaemoglobin would be different from the rate with phenylhydroxylamine.

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Appendix

The five hourly observations on each cat make up a set of correlated observations which present a problem of interpretation. A transformation was made to new variates on which meaningful comparisons can be made. The new variates chosen were an average effect, a linear effect and quadratic, cubic and quartic effects. If h_1, h_2, h_3, h_4, h_5 are the observations then the new variates are

$$\begin{aligned} y_1 &= h_1 + h_2 + h_3 + h_4 + h_5, \\ y_2 &= -2h_1 - h_2 + h_4 + 2h_5, \\ y_3 &= 2h_1 - h_2 - 2h_3 - h_4 + 2h_5, \\ y_4 &= -h_1 + 2h_2 - 2h_4 + h_5, \\ y_5 &= h_1 - 4h_2 + 6h_3 - 4h_4 + h_5, \end{aligned}$$

where y_1, y_2, y_3, y_4, y_5 are the mean, linear, quadratic, cubic and quartic effects respectively.

Standard analysis of dispersion techniques may be used on the new variables to test hypotheses concerning treatment differences (Rao, 1965). It is desirable to reduce the number of variables considered as much as possible to simplify the interpretation, so a test of significance was made to determine the additional information on treatment differences supplied by the quadratic, cubic and quartic effects. This was performed by the method of Rao (1965) and gave non-significant results. Consequently, further analysis was carried out using only mean and linear effects.

The analysis of dispersion for these effects is given in Table 3 where Λ is the test statistic for a test of the hypothesis of equality of treatments and $\frac{40}{6}\frac{1-\sqrt{\Lambda}}{\sqrt{\Lambda}}$ may be tested as an F variate with 12 and 80 degrees of freedom (Rao, 1965). This test showed that significant differences between the treatments existed. A detailed comparison of the treatments may be made from Table 4 which sets out the means of the mean effects and the linear effects for each treatment and the standard errors of comparisons to be considered.

It may be noted that a quadratic effect existed in the treatments but no differences between the treatments were evident in this effect.

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The effect of various environmental factors on cocaine and ephedrine toxicity†

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The effect of environmental factors on the toxicity of ephedrine and cocaine has been examined and the results compared with those obtained with amphetamine. Ambient temperatures were identified at which these drugs have an "aggregation effect", and isolated mice treated with ephedrine or cocaine were then forcibly exercised and the effect of increased motor activity on body temperature and mortality compared with the results obtained by grouping the animals together. In general, the toxicity of either drug is increased by a rise in ambient temperature. Both drugs produce an aggregation effect and forced exercise increases the toxicity of each drug as much as does grouping. In all parameters tested the effects of ephedrine and cocaine resemble qualitatively those of amphetamine, but are less potent. In mice given certain central nervous system stimulants the aggregation effect may be due to hyperpyrexia associated with increased spontaneous motor activity resulting from a greater response to external stimuli.

COCAINE will raise the body temperature of some animals (Barbour & Gilman, 1934), so too will ephedrine (Chen & Schmidt, 1930). Cocaine causes an increase in spontaneous motor activity (Dews, 1953). Lal & Chessick (1965) found that more cocaine-treated mice subjected to aggregation or electric shock died than did controls. The effect was prevented by pretreatment with chlorpromazine or reserpine. Ephedrine is similar to amphetamine in that it produces in mice an "aggregation effect" (Chance, 1946) characterized by increase in drug toxicity in grouped animals. Increase in spontaneous motor activity (Greenblatt & Osterberg, 1961) and elevation of body temperature (Askew, 1962) have been reported to be associated with the amphetamine aggregation effect. Forced exercise of isolated mice treated with amphetamine causes as great an increase in body temperature and mortality as does grouping (Hardinge & Peterson, 1964) while restriction of movement decreases amphetamine toxicity (Hardinge & Peterson, 1963).

Moore (1963) reported the aggregation effect to be associated with depletion of catecholamines. Beauvallet & Solier (1964) found a decreased level of noradrenaline in brains of aggregated mice and also mice treated with amphetamine and forced to exercise.

To determine if forced exercise increases the toxicity of other sympathomimetic agents and to further evaluate the relation between motor activity and aggregation effect, mice treated with either ephedrine or cocaine were subjected to forced activity. The results were then compared with those from grouped mice and from isolated unexercised mice.

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Methods

Groups of 10 Swiss-Webster male mice each weighing 18–21 g were placed in a predetermined environment for acclimatization 1 hr before the initial measurement of body temperature and administration of one of the test drugs. Environmental temperatures maintained at $\pm 0{\cdot}5°$ of 18, 22, 26, 30 and 34° were used. Air movement was constant. Test drugs were given intraperitoneally in saline, 0·01 ml/g body wt.

The LD50 for each drug was determined in isolated and grouped mice at each environmental temperature. The LD50 for isolated animals forced to exercise was determined for each drug at a temperature in which grouping significantly increased toxicity. The mortalities occurring in 4 hr after drug treatment were recorded. The 95% fiducial limits for the LD50 were calculated by the method of Litchfield & Wilcoxon (1949). In comparing the median lethal doses, the differences between means were considered to be statistically significant if the 95% confidence limits did not overlap.

The effect of ephedrine in an environmental temperature of 30° and of cocaine at 34° was examined by measuring rectally the body temperatures of isolated, grouped and forcibly exercised mice. The body temperature was measured initially immediately before the drug was given, and subsequently every 40 min for 2 hr.

Isolated animals that were not exercised and aggregated mice in groups of 10 were placed in 7 × 13 in stainless steel cages. Isolated animals that were exercised were placed on a continuous belt exerciser (Hardinge & Peterson, 1963), travelling at a rate of 9·6 m/min for periods of 20 min alternating with 5 min rest periods for 2 hr.

Results

Effect of change of environmental temperature. Raising the environmental temperature increased the toxicity of both ephedrine and cocaine significantly (Table 1). This effect was much greater for ephedrine, the LD50 of which increased more than 20 times while that of cocaine approximately doubled when the temperature was raised from 26 to 34°.

Effect of grouping. Both drugs produced an aggregation effect. Grouping increased cocaine toxicity (Table 1) at the three highest temperatures (26°, 30° and 34°) but the effect was not great at any temperature. The effect of grouping on ephedrine toxicity (Table 2) was much greater and was most apparent at a temperature of 30°. The LD50 reduction at 30° is 27% for cocaine and 80% for ephedrine.

Effects of exercise. Forced exercise increased body temperature in both control and drug-treated isolated mice (Table 2). Exercise also increased drug toxicity (Table 1). Body temperature and drug toxicity in isolated exercised mice were similar to those in grouped animals and in most instances were greater than in isolated unexercised animals or in untreated controls.

Body temperature elevation and mortality. In general, mortality rate increased as mean body temperature increased (Table 2). Nearly all mice whose body temperature reached 42° or above died. More than half the

TABLE 1. THE LD50 OF COCAINE HYDROCHLORIDE AND EPHEDRINE SULPHATE FOR ISOLATED AND GROUPED MICE* IN SEVERAL ENVIRONMENTAL TEMPERATURES AND FOR ISOLATED EXERCISED MICE AT A TEMPERATURE AT WHICH AN AGGREGATION EFFECT WAS APPARENT

Drug	Category	18° mg/kg	18° % change	22° mg/kg	22° % change	26° mg/kg	26° % change	30° mg/kg	30° % change	34° mg/kg	34° % change
Cocaine	Isolated	88 (77·2–100·3)†	—	92 (81·4–104·0)	—	92 (81·5–104·0)	—	63 (52·0–76·6)	—	51 (44·4–58·7)	—
	Grouped	86 (76·1–97·2)	2	81 (74·4–88·4)	12	76 (73·8–78·2)	17	46 (37·5–56·5)	27	36 (31·8–40·7)	29
	Isolated exercised									31 (26·3–36·6)	39
Ephedrine	Isolated	375 (350·5–401·3)	—	385 (367·0–405·0)	—	380 (355·0–407·0)	—	273 (227·0–328·0)	—	14 (7·8–25·2)	—
	Grouped	360 (339·6–381·6)	4	350 (297·0–413·0)	9	257 (228·0–291·0)	32	55 (45·8–76·0)	80	13·5 (10·4–17·5)	4
	Isolated exercised							28 (19·3–40·4)	90		

* 10 animals were used in each group to determine each point on LD50 curve for each category.
† 95% fiducial limits determined by method of Litchfield & Wilcoxon (1949).

TABLE 2. GREATEST RISE IN BODY TEMPERATURE AND MORTALITY RATE IN CONTROL MICE AND MICE TREATED WITH COCAINE HYDROCHLORIDE IN AN ENVIRONMENTAL TEMPERATURE OF 30° AND WITH EPHEDRINE SULPHATE IN AN ENVIRONMENTAL TEMPERATURE OF 30°

Category	Cocaine Dose mg/kg	Cocaine Mortality %	Cocaine Greatest rise Mean s.d.	Ephedrine Dose mg/kg	Ephedrine Mortality %	Ephedrine Greatest rise Mean s.d.
Isolated	—	0	0·7 ± 1·3	—	0	0·8 ± 0·5
Grouped	—	0	1·8 ± 0·6	—	0	1·2 ± 0·4
Isolated exercised	—	0	2·5 ± 0·4	—	0	2·3 ± 0·8
Isolated	42·0	20	2·4 ± 1·6	50	0	2·7 ± 0·5
Isolated	63·0	80	3·2 ± 1·4	200	20	3·3 ± 0·7
Isolated	—	—	—	300	60	2·4 ± 0·6
Grouped	31·5	10	2·7 ± 0·9	50	40	3·9 ± 0·9
Grouped	42·0	90	4·5 ± 1·3	75	80	4·4 ± 0·9
Isolated exercised	21·0	20	3·0 ± 1·8	15	20	3·5 ± 0·9
Isolated exercised	31·0	40	3·1 ± 1·3	25	50	4·2 ± 1·1
Isolated exercised	42·0	90	4·7 ± 0·5	50	70	4·5 ± 1·1
Isolated exercised	—	—	—	75	90	5·3 ± 1·1

isolated mice given higher doses of ephedrine died within 15 to 20 min and their temperatures did not reach such high levels as those of aggregated animals which were given lower doses and which lived longer.

Appearance of mice. Grouped mice given ephedrine became hyperactive. They often assumed a "defensive position" similar to that described for amphetamine-treated mice (Chance, 1946). Later they appeared weak and exhausted and partially paralysed before death. These animals died without convulsing after smaller doses of ephedrine, in contrast to isolated unexercised mice which did convulse if given doses of drug large enough to produce death. When the exercised animals were near death they were removed from the exerciser. At this time their appearance was similar to that of grouped animals just before death. Nearly all cocaine-treated mice that did not survive convulsed. Shortly after the onset of convulsions they became weak and incoordinated and those on the exerciser were removed.

Survival time. Survival time was relatively short in mice given a lethal dose of cocaine. Of those that survived for at least 45 min most recovered. Ephedrine-treated mice often died as long as two, but rarely more than 3 hr after the drug.

Discussion

Effect of environmental temperature. The toxicity to mice of ephedrine or cocaine was significantly greater at the higher than at the lower temperatures used, both drugs producing an effect similar to that reported for amphetamine (Warren & Werner, 1946; Höhn & Lasagna, 1960). Askew (1962) found that both isolated and grouped mice dying of amphetamine poisoning usually experienced a rise in body temperature to nearly 42°. This temperature is often lethal to rodents that have not received any drug (Adolph, 1947). Of the animals whose body temperatures were monitored, most of those that died had a temperature of nearly 42° before death, irrespective of the ambient temperature. It is likely that the increase in toxicity of the test drugs to mice in higher environmental temperatures is due to less rapid heat loss.

Effect of grouping. Grouping increased the toxicity of ephedrine and of cocaine but the aggregation effect due to ephedrine was most apparent at an ambient temperature of 30° rather than at 25 or 26° as with amphetamine (Höhn & Lasagna, 1960; Hardinge & Peterson, 1963). The grouped animals treated with ephedrine and cocaine were much more active than isolated animals. This is similar to amphetamine-treated mice (Chance, 1946; Greenblatt & Osterberg, 1961). Increased spontaneous motor activity in these animals is probably due to additional sensory stimuli in the grouped situation in the presence of increased excitability caused by central nervous system-stimulating drugs.

Effect of exercise. At temperatures where an aggregation effect was noticed, forced exercise caused an increase in body temperature and an increase in toxicity of either drug. This was similar to the effects caused by grouping.

Body temperature elevation and mortality. Regardless of the environment of the animal there appeared to be a direct relation between body temperature elevation and mortality unless the dose of the drug was so large that the animal died within a few min. Most of the grouped animals and isolated animals forced to exercise apparently died of hyperthermia, while many of the isolated animals that had to be given larger drug dosages to cause death appeared to die of convulsions. Whether actual cause of death was due to respiratory failure due to muscle spasm was not established. Death in cocaine-treated animals appeared to be related to convulsions more frequently than in those animals treated with ephedrine. The effect of cocaine was of shorter duration than that of ephedrine. Since cocaine-treated animals often either died or recovered within a relatively short time, there was less time for body temperature rise to affect mortality. This may explain why the effect of grouping, exercise and environmental temperature on ephedrine toxicity was greater than it was on cocaine toxicity. It is likely that the increased toxicity of these central nervous system stimulating drugs to grouped animals is due primarily to an increase in body temperature resulting from an increase in spontaneous motor activity; impaired temperature regulation may also be a factor (Hardinge & Peterson, 1964).

The body temperatures of the isolated mice given 300 mg/kg ephedrine was never higher than 40°. These animals probably died of some cause other than hyperpyrexia; this also appears to be true in amphetamine poisoning when high dosages of the drug are given (Peterson & Hardinge, 1964).

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Effects of reserpine on the sensitivity of rat isolated uteri preparations to oxytocic drugs

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Stimulant activity of the isolated uteri of female virgin rats to acetylcholine, 5-hydroxytryptamine, and oxytocin was examined quantitatively (4-point assay method) in anoestrus, induced oestrus (diethylstilboestrol 10 μg/100 g body weight injected subcutaneously), reserpinized and stilboestrol-treated reserpinized animals. Each group had 30 animals. Reserpine was administered intraperitoneally in two doses each of 1 mg/kg body weight on the first and third days of dioestrus. The sensitivity to acetylcholine, 5-hydroxytryptamine and oxytocin of the uteri in induced oestrus was increased by 20, 86 and 730 times respectively. In the group of animals receiving reserpine alone the experiment could not be made due to persistant spontaneous activity (even up to 2 hr) of the uterine tissue. In the stilboestrol-treated reserpinized group, the sensitivity to all the oxytocic drugs was as low as in anoestrous tissue. Thus, stilboestrol injection failed to increase the sensitivity of the rat uteri to oxytocic drugs or to produce changes in the vaginal smear pattern. The possibility of progesterone being present in the tissue due to persistance of corpora lutea after reserpine is put forward.

IN 1927, Krause (cited by Henry, Browne & Venning, 1950) observed an increase with oestrogen and a decrease with progesterone in the sensitivity of isolated uterus preparations of rabbits and guinea-pigs to posterior pituitary extract added to the organ bath. Stewart (1949) (cited by Krantz & Carr, 1961) also found an increase in sensitivity to oxytocin during oestrus and a decrease during dioestrus. Marshall (1962) observed a marked increase in the frequency and amplitude of action potentials set up by oxytocin in rat isolated uterus preparations removed from oestrogen-dominated animals. The rat-uterus preparation has been used for assay of oxytocic drugs (Pennefather, 1961).

Erspamer (1940) observed that rat isolated uteri in induced oestrus were sensitive to 5-hydroxytryptamine, (5-HT) and Erspamer (1953) found this to be 50 times more potent in oestrus than in anoestrus.

Reserpine delayed ovulation and caused amenorrhoea in monkeys (De Feo & Reynolds, 1956), and Barraclough & Sawyer (1959) observed pseudopregnancy following reserpine treatment in female rats.

Sensitivity of the atrium of rabbit to 5-HT was increased significantly after reserpinization (Trendelenburg, 1960).

Although many workers have referred to the changes produced in sensitivity of the uterine preparations to various oxytocic drugs by oestrogen, there is a need to investigate these changes quantitatively as one of the parameters of action of oestrogens. We have selected 5-HT, oxytocin and acetylcholine as stimulants for isolated uteri from virgin rats during anoestrus and after stilboestrol treatment.

Reserpine treatment of an animal modifies the responses of 5-HT and catecholamines on some tissues and we therefore examined the effect of reserpine on the responses of the uteri to 5-HT and the other two oxytocic drugs.

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Experimental

METHOD

Female virgin rats of 2–3 months of age, 200–250 g, from the Jinnah Postgraduate Medical Centre Colony were divided into 4 groups of 30 animals. The first group had animals both untreated and in anoestrus. In the second group, anoestrous animals were treated subcutaneously with stilboestrol (10 μ/100 g) 12 hr before death. Animals in the third group received reserpine intraperitoneally in two doses, each of 1 mg/kg, on the first and third day of dioestrus. The dose was estimated to be the maximum tolerated. Animals in the fourth group received stilboestrol 24 hr after the second dose of reserpine and 12 hr before death.

DRUGS

Doses of acetylcholine chloride and 5-hydroxytryptamine creatinine sulphate are expressed as bases. Oxytocin (Syntocinon) is expressed as 2 μg $\equiv$ 1 unit. Solution of diethylstilboestrol was prepared in sesame oil and further diluted in arachis oil.

PROCEDURE

Stimulant activity of the isolated pieces of uteri was estimated by suspending a 2 cm piece of uterine horn in a 5 ml organ bath, containing de Jalon solution at 30° (similar to that of Barlow & Khan, 1959). The contractions of the uterine tissues were recorded isotonically through a force transducer attached to a polygraph. The drug was left in contact with the tissue for 45 sec and a 2 min cycle was maintained throughout the experiment. Acetylcholine was used as a control.

Dose response curve. After allowing the tissue 30 min in the bath, a dose-response curve to acetylcholine was obtained. The entire dose-response curves (minimum to maximum) of each oxytocic drug were then recorded and compared with that of acetylcholine. If the dose-response curve of an oxytocic drug was found to be parallel to that of acetylcholine, then its potency was estimated by a 4-point assay method.

The 4-point assay was in accordance with the method used by Holton in 1948 (cited by Burn, Finney & Goodwin, 1950), for assay of oxytocin. The potencies were finally expressed as equipotent molar ratios in terms of acetylcholine (similar to Barlow & Khan, 1959).

Vaginal smear examination was by the Papanicolaou method and macroscopic examination of uteri in each group of the animals was also made.

Results

ANOESTROUS TISSUES (Group 1)

Four of 30 tissues used showed spontaneous activity, which subsided after 30 min in the bath. The tissue responded to doses of acetylcholine from 0·25 to 2 μg/5 ml. No tachyphylaxis or spontaneous activity, after washing out the drug, was observed with this drug. The dose-response

curve was linear. The potency of acetylcholine on this tissue was arbitrarily kept as 100 and the other drugs were compared with it.

The sensitivity to 5-HT was from 65 ng to 0·35 μg/5 ml. No spontaneous activity was observed after washing out the drug, but doses larger than these invariably produced tachyphylaxis. The entire dose-response curve of 5-HT was parallel to that of acetylcholine. The equipotent molar ratio of 5-HT as estimated and compared with acetylcholine was 42·0 $\pm$ 5·4 (Table 1); hence 5-HT was 2·4 times more potent than acetylcholine on a molar basis.

The effective dose range of oxytocin was from 0·8 to 8 ng/5 ml. It did not produce tachyphylaxis or spontaneous contractions after washing out the drug. The entire dose-response curve was parallel to that of acetylcholine. Its equipotent molar ratio was 0·11 $\pm$ 0·03 (Table 1). Thus oxytocin was 910 times more potent than acetylcholine.

TISSUES WITH INDUCED OESTRUS (Group 2)

All the 30 tissues used showed spontaneous activity, which subsided after 30 min in the bath. The tissue responded to acetylcholine in a dose range of 5 ng to 1 μg/5 ml. The dose-response curve of acetylcholine on this tissue was similar to the one in anoestrus except that this tissue was 20 times more sensitive to acetylcholine.

The tissue responded to 5-HT in doses varying from 30 to 340 ng/5 ml. Tachyphylaxis developed with higher doses. The entire dose-response curve was parallel to that of acetylcholine. The equipotent molar ratio of 5-HT compared with acetylcholine was 9·4 $\pm$ 0·6; hence 5-HT was 10·6 times more potent than acetylcholine on this tissue.

Oxytocin stimulated this tissue in doses ranging from 1·8 to 16 pg/5 ml. No tachyphylaxis or spontaneous activity was observed. The entire dose-response curve was parallel to that of acetylcholine. Its equipotent molar ratio was 0·003 $\pm$ 0·0002 (Table 1). Oxytocin was found to be 33300 and 3130 times more potent than acetylcholine and 5-HT respectively.

TISSUES AFTER RESERPINIZATION (Group 3)

Tissues from all the 30 animals when suspended in the bath showed persistent spontaneous activity for more than 2 hr. Addition of acetylcholine to the bath did not help. The experiments were therefore discontinued.

TISSUES FROM STILBOESTROL-TREATED RESERPINIZED ANIMALS (Group 4)

Uterine tissue of all the 30 animals showed spontaneous activity to variable degree when suspended in the bath. These movements persisted for up to 30 min. A few small doses of acetylcholine helped in settling the tissue. The sensitivity range of acetylcholine on this tissue was 0·3 to 4 μg/5 ml which is similar to that of anoestrous tissues.

The dose range of 5-HT was from 50 ng to 0·4 μg/5 ml and tachyphylaxis was not observed. No spontaneous activity was noted after washing out the drug. The entire dose-response curve was parallel to that of acetylcholine. Its equipotent molar ratio compared to acetylcholine was

TABLE 1. RAT ISOLATED UTERUS PREPARATION. STIMULANT ACTIVITIES OF 5-HYDROXYTRYPTAMINE (5-HT) AND OXYTOCIN (OX) COMPARED WITH ACETYLCHOLINE (Ach) ON VARIOUS TISSUES

Types of tissues	Range (minimal and maximal) of effective doses of stimulant drugs in a 5 ml bath			Equipotent molar ratios, Ach = 100		Potency ratios Ach = 1	
	Ach	5-HT	OX	5-HT	OX	5-HT	OX
Group 1. Rats in anoestrus	25 ng to 2 μg	65 to 350 ng	0·8 to 8 ng	42·0 ± 5·4 s.e. (10)	0·11 ± 0·03 s.e. (10)	2·4	909
Group 2. Induced oestrus	5 ng to 1 μg	30 to 340 ng	1 to 16 pg	9·4 ± 0·6 s.e. (10)	0·03 ± 0·0002 s.e. (10)	10·6	33300
Group 4. Stilboestrol and reserpine treated	300 ng to 4 μg	50 to 400 ng	0·8 to 6 ng	33·0 ± 3·0 s.e. (10)	0·06 ± 0·002 s.e. (10)	3	1670

Figures in parentheses indicate number of observations. As the sensitivity of uteri in various groups vary for acetylcholine, the comparison is only valid for each group.

TABLE 2. ISOLATED RAT UTERUS PREPARATION (relative equipotent molar ratios of 5-hydroxytryptamine and oxytocin to acetylcholine* on various tissues)

Drugs	Group 1 Anoestrus	Group 2 Induced oestrus	Group 4 Stilboestrol and reserpine-treated
Acetylcholine	100*	5	100
5-Hydroxytryptamine	42	0·5	33·0
Oxytocin	0·11	0·00015	0·06

Value of 100 has been arbitrarily fixed for acetylcholine in Group 1.

33·0 ± 3·0 (Table 1). So 5-HT was 3·0 times more potent than acetylcholine.

Oxytocin stimulated this tissue in doses ranging from 0·8 to 6·0 ng/5 ml. No tachyphylaxis was seen. The sensitivity was the same as on the anoestrous tissue and no spontaneous activity was observed. The entire dose-response curve was parallel to that of acetylcholine. Its equipotent molar ratio compared with acetylcholine was 0·06 ± 0·002 (Table 1); hence oxytocin was 1670 times more potent than acetylcholine.

The increase in sensitivity of the uterine tissue during induced oestrus (after stilboestrol treatment) to acetylcholine, 5-HT and oxytocin was 20, 86 and 730 times respectively (Table 2). The sensitivity of the tissues from the Group 4 to all the oxytocic drugs seemed to be the same as on anoestrous tissues (Tables 1 and 2).

Microscopic examination of vaginal smears. In the group in anoestrus (1), the field was full of leucocytes with a few nucleated epithelial cells, while for the induced oestrus group the field was full of cornified epithelial cells. After reserpine treatment a large number of leucocytes and a few nucleated epithelial cells were seen (Group 3). This picture was similar to dioestrus. Stilboestrol-treated tissue after reserpinization had a large number of leucocytes, a few nucleated epithelial cells, and occasional stratified epithelial cells. This resembled the dioestrous phase except for the presence of occasional epithelial cells.

Macroscopic examination of uteri. In the anoestrus group the uterine horns were 4–5 cm long, pale and quiescent. No fluid was detected inside the lumen, which was slit-like. The uterine horns of the induced oestrus group were 5–6 cm long, congested and swollen with fluid. After reserpine treatment, with and without stilboestrol, the size of the uterine horns were the same as in anoestrus, but were hyperaemic. No fluid was detected inside the lumen.

Discussion

The results with 5-HT on oestrous tissue are qualitatively similar to those of Erspamer & Correale (1953) obtained under similar circumstances. The work also gives quantitatively the comparative effects of 5-HT and oxytocin to acetylcholine on the three types of rat isolated uteri preparations. The sensitivity of tissues in induced oestrus to acetylcholine, 5-HT and oxytocin is 20, 86 and 730 times higher than in the anoestrous tissues.

The findings for the stilboestrol-treated reserpinized rat uteri are most interesting. One would expect an increase in the sensitivity to 5-HT, because smooth muscle of atria, from reserpinized rabbit, were found to have become more sensitive to 5-HT (Trendelenburg, 1960). But on the contrary there was a marked reduction in sensitivity to a level as low as in anoestrous tissue, not only to 5-HT but to the other oxytocic drugs used. This means that diethylstilboestrol failed to produce its sensitizing effects on the uteri in animals already treated with reserpine.

Could progesterone antagonize the action of diethylstilboestrol? Knaus (1927, cited by Henry & others, 1950) observed that addition of

progesterone to the bath decreased the sensitivity of the rabbit isolated uteri to posterior pituitary extract. Marshall (1962) demonstrated that the action potentials set up by oxytocin in progesterone-dominated rat uteri were infrequent, irregular and smaller in amplitude.

Vaginal smear examination of reserpinized animals, in both groups receiving stilboestrol and untreated animals, showed the presence of a large number of leucocytes and a few nucleated epithelial cells. This picture is similar to that of anoestrous phase but animals in the last group showed in addition desquamated epithelial cells. This shows that reserpine therapy has prevented the changes produced by stilboestrol on vaginal smear.

Nikitovitch-Winer & Everett (1958) suggested that reserpine increases secretion of luteotrophic hormone from the adenohypophysis, due to depressive effect of reserpine on the hypothalamus. Normally the hypothalamus has an inhibitory control over the secretion of luteotrophic hormone from the anterior pituitary, and progesterone is secreted from the corpora lutea under influence of luteotrophic hormone. Both during pregnancy and pseudopregnancy corpora lutea persist under the action of increased amount of luteotrophic hormone and secrete more progesterone. Fajer & Barraclough (1966) have produced pseudopregnancy in rats by giving reserpine, 1 mg/kg, during pro-oestrus. They measured the progesterone level in ovarian venous blood and showed a marked increase in its level after reserpine. On the basis of the work of Nikitovitch-Winer & Everett (1958), Barraclough & Saywer (1959) and Fajer & Barraclough (1966), we suggest that there is a possibility that in our work reserpine has made large amounts of progesterone available in the body of the animal. This progesterone has then antagonized the action of diethylstilboestrol on the myometrium of the reserpinized animals. This view has yet to be proved.

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The antibacterial action of crystal violet*

E. ADAMS

Crystal violet has an antibacterial action against *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus faecalis* and *Bacillus subtilis*. The effect of the dye, measured as minimum inhibitory concentration or retardation of growth, increases as the pH rises from 6 to 8. Of the four species *E. coli* is most resistant to the dye; the resistances of the other organisms are similar. The mode of action put forward by Stearn & Stearn (1928) that the action of crystal violet is due to the formation of an unionized complex of bacteria with dye, is supported. Gram-negative organisms, such as *E. coli*, have high isoelectric points and contain less acidic components than Gram-positive bacteria which usually have lower isoelectric points, so the former combine with crystal violet less readily and are more resistant to the dye. In extension of this theory, the negative charge on bacteria is increased as the pH of the medium is increased, and the organisms become more sensitive to dye. Evidence is presented which refutes the theory of a poising of the redox potential by crystal violet suggested by Dubos (1929) and Ingraham (1933).

IN 1923, Churchman stated that the growth of Gram-positive bacteria was inhibited by most triphenylmethane dyes but, except at high concentrations, Gram-negative organisms were unaffected. The bacteriostatic activity of gentian violet (an impure form of crystal violet) towards *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus haemolyticus* was modified by pH, being greater at high pH values (Stearn & Stearn, 1926).

I have made experiments to determine the effect of pH on the minimum inhibitory concentration of crystal violet, and the lag or reduction of growth produced by crystal violet in four species of organisms.

Experimental

Materials

Crystal violet B.P. re-crystallized from ethanol [E (1% 1 cm) = 2059 at 591 mμ] was used to prepare a 0·01 M aqueous solution.

Nutrient broth was prepared from granules (Oxoid CM1) and 1% potassium dihydrogen phosphate (Analar) added, and the pH adjusted using a freshly prepared solution of potassium hydroxide.

Organisms

Cultures were prepared from freeze-dried specimens of *E. coli* 1 NCTC 8196, *Staph. aureus* NCTC 7447, *Str. faecalis* NCTC 370, *Bacillus subtilis* NCTC 3610. A suspension was prepared in quarter-strength Ringer solution (Wilson, 1922) from the 24-hr growth on a nutrient agar slope (overnight for *B. subtilis*), followed by filtration through sterile Whatman No. 1 filter paper to remove clumps of bacteria and particles of agar. The extinction of the suspension was checked using

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an EEL nephelometer, and the suspension diluted to give a viable count of about 1×10^7 organisms/ml, determined by the drop-plate method of Miles & Misra (1938).

Methods

10 ml amounts of pH-adjusted broth containing 1 to 6×10^{-7} M crystal violet were maintained at 37° for 24 hr to allow equilibrium to be reached between the coloured and the carbinol form of the dye (Goldacre & Phillips, 1949), and bacterial suspension (0·1 ml) prepared as above was then added. The pH-adjusted broth containing no dye was similarly treated to show the effect of pH alone on growth. The dye-broth mixtures were made in triplicate and the controls in duplicate, and average values taken of the extinction. The addition of dye to broth had a negligible effect on extinction both in the presence and absence of growth.

Oxidation-reduction potentials were measured with a platinum electrode against a calomel reference and expressed as E_h (Hewitt, 1950).

Results

Inhibition of growth at extremes of pH

The limiting values of pH for growth in broth at 37° were: *E. coli*, range pH 4·5–9·0, optimum 5·9–7·1; *Staph. aureus*, range 5·6–9·5, optimum 6·7; *Str. faecalis*, range 5·7–9·5, optimum 6·8; *B. subtilis*, range 5·1–9·5, optimum 6·0–6·8.

Inhibition of growth by pH-adjusted crystal violet

At pH values above pH 7·5, concentrations of dye below 1×10^{-6}M prevented growth of *Staph. aureus*, *Str. faecalis* and *B. subtilis* for 48 hr or more; about 1×10^{-5}M was necessary to prevent growth of *E. coli*. On the other hand, at pH 6·0 nearly 1×10^{-5}M was required to prevent growth of the first three species, and more than 1×10^{-3}M for *E. coli*. Fig. 1 shows the effect of pH on the minimum inhibitory concentration of

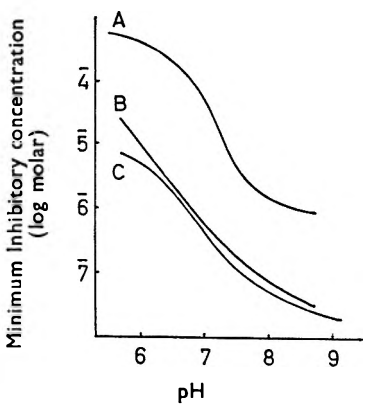

Fig. 1. Effect of pH on minimum inhibitory concentration of crystal violet in nutrient broth at 37°. A. *E. coli*. B. *B. subtilis*. C. *Staph. aureus*.

crystal violet. The curve for *Str. faecalis* (not shown) was similar to those for *Staph. aureus* and *B. subtilis*. At high pH values *Str. faecalis* was more resistant to the dye than *Staph. aureus* or *B. subtilis*.

EFFECT OF pH-ADJUSTED CRYSTAL VIOLET ON GROWTH

The effect of pH on the antibacterial action of crystal violet was determined by plotting extinction against log time in the presence of dye (test) and in the absence of dye (control) at different pH values. Fig. 2 shows the graphs for *E. coli* at pH 6·7.

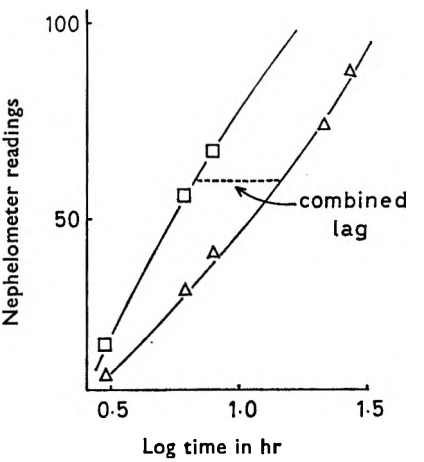

FIG. 2. Effect of $5{\cdot}7 \times 10^{-7}$M crystal violet on the growth of *E. coli* (△) in broth at pH 6·7. □ Control, in absence of dye.

When very low concentrations of dye were used there was little change in the antibacterial effect with pH at pH values below 7, but the effect was much greater at high pH values. For instance, *B. subtilis* in the presence of $1{\cdot}03 \times 10^{-7}$M crystal violet below pH 7 grew as quickly as in the control, but with $1{\cdot}29 \times 10^{-7}$M dye, growth was prevented at pH 8 and above.

Somewhat higher concentrations of crystal violet (2–5×) caused a prolonged lag phase followed by normal growth with *Str. faecalis* and *B. subtilis* at low pH values, while *E. coli* and *Staph. aureus* showed both a prolonged lag phase and an increased generation time. At high pH values growth was prevented.

A method of evaluating the antibacterial effect of crystal violet to include both prolonged lag and reduced growth was introduced. Extinction was plotted against log time for the test and control, and the difference in time between the two at a given arbitrary density I have termed "combined lag".

The Berry & Parkinson method (1955) required a rapidly growing culture giving an opacity in 3 or 4 hr even in the presence of a bacteriostat. The "combined lag" method is not confined to a stated time; it has the advantage that evaluation of bacteriostasis may be assessed in more

severe conditions, in this case at higher pH values and with higher concentrations of dye. A nephelometer reading of 60 was chosen as indicative of appreciable growth, but similar results were obtained if other readings were chosen for comparison of the test and control media.

The combined lag was plotted against pH for each organism (Fig. 3). The increase in combined lag with rise in pH corroborates the results shown in Fig. 1. The curve for *Staph. aureus* was similar to that for *Str. faecalis*.

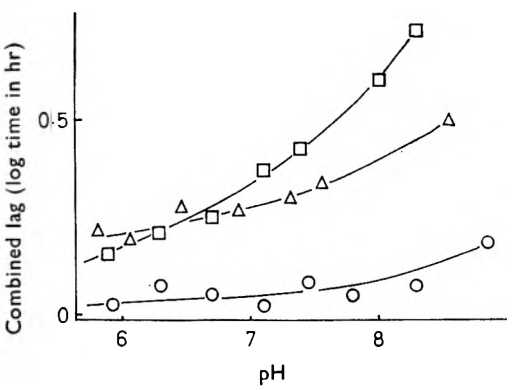

FIG. 3. Effect of pH of broth-dye medium on combined lag produced by crystal violet. △ *Str. faecalis* with $0{\cdot}80 \times 10^{-7}$M dye, □ *E. coli* with $3{\cdot}8 \times 10^{-7}$M dye, ○ *B. subtilis* with $1{\cdot}03 \times 10^{-7}$M dye.

Although buffer was present, during growth of the organisms the pH of the system, particularly when at high initial values, tended towards neutrality. This change was as great as a fall of 0·7 unit from pH 9 with *Str. faecalis*. McCalla (1941) found that crystal violet displaced hydrogen ions from bacteria, which would cause a slight lowering of pH. A reduction in pH would partially antagonize inhibition by crystal violet, and this would necessitate a higher concentration to cause the same inhibitory effect (Fig. 1).

B. subtilis spores germinated normally in nutrient broth containing crystal violet, but the vegetative cells formed from the spores were then inhibited or killed.

ANTAGONISM OF ANTIBACTERIAL ACTION OF CRYSTAL VIOLET BY OXIDIZING AND REDUCING AGENTS

Small amounts of hydrogen peroxide, thioglycollic acid, or methylene blue were added to nutrient broth containing $3{\cdot}5 \times 10^{-5}$M crystal violet. The tubes were inoculated with *E. coli* and observed for growth at 37°. Table 1 shows that no growth occurred in the presence of the dye alone, but when hydrogen peroxide, thioglycollic acid or methylene blue were added the organisms were able to grow. Larger concentrations of hydrogen peroxide or thioglycollic acid prevented growth. Similar results were found with *B. subtilis* using a lower concentration of dye.

TABLE 1. THE EFFECT OF OXIDIZING AND REDUCING AGENTS ON THE ANTIBACTERIAL ACTION OF CRYSTAL VIOLET TOWARDS *E. coli*, AND ON THE REDOX POTENTIALS

Medium	Growth*	Redox potential (V)
Nutrient broth	+	0·320
Broth + $3·5 \times 10^{-5}$M crystal violet ..	−	0·310
Broth + $3·2 \times 10^{-4}$ H_2O_2	+	0·400
Broth + dye + H_2O_2	+	0·405
Broth + $5·7 \times 10^{-4}$M thioglycollic acid	+	0·120
Broth + dye + thioglycollic acid ..	+	0·125
Broth + $9·4 \times 10^{-6}$M methylene blue	+	0·305
Broth + dye + methylene blue ..	+	0·315

* After incubation at 37°, 48 hr: + growth, − no growth.

Discussion

The extreme susceptibility of the antibacterial activity of crystal violet to pH change made evaluation difficult. At low pH values growth was virtually unaffected, while at high values growth was prevented, e.g. the concentration required to prevent growth of *B. subtilis* at pH 5·6 was 800 times that required at pH 8·7.

The pH of the medium affects the degree of ionization of crystal violet, but because it is more than 90% ionized ($pK_a = 9·3$) over the range 5·6–8·3 (Albert, 1965b) the increase in inhibitory activity with rise in pH is not due to the increase in the number of dye cations.

E. coli was the most resistant organism examined. The other species were similar in their susceptibility, but *Staph. aureus* was the least resistant at the highest and lowest pH values used (Fig. 1). Gram-positive organisms such as staphylococci possess more acidic components than Gram-negative bacteria such as *E. coli*, so that the former would combine more readily than the latter with basic dyes. This gave rise to the hypothesis of Stearn & Stearn (1928) that the antibacterial action of the dye was due to the formation of an unionized complex of a cell constituent with the dye.

Those bacteria with a higher isoelectric point, e.g. *E. coli* (5·5), should therefore be more resistant than organisms with lower isoelectric points, e.g. the other three species (1·8–3·0), which is so. This theory can be extended to include the effect of pH. As the pH of the medium is increased the negative charge on bacteria is increased, and this would result in an increase in sensitivity towards crystal violet. Albert (1965a) supposed that *E. coli* possessed anionic groups on its surface with a pK_a of 9 or higher. At pH 6 the anionic groups would be so weakly ionized that combination with crystal violet cations would be negligible, but at pH 8 the anionic groups would be 10% ionized and combination with dye cations would readily occur. This would explain the relative antibacterial effect of crystal violet at these two pH values shown above.

Bacterial growth in broth results in a lowering of the oxidation-reduction potential; this occurred at all pH values, the potential falling by as much as 0·21 V. Dubos (1929) and Ingraham (1933) supposed that dyes

produced bacteriostasis by poising the medium at a potential unsuitable for growth. This is unlikely because crystal violet is bacteriostatic at very low concentrations, and when small amounts of thioglycollic acid or hydrogen peroxide are present in broth the addition of bacteriostatic concentrations of crystal violet causes very little change in potential (Table 1). According to Dubos the potential caused by hydrogen peroxide (0·400 V) should be reduced in the presence of dye, but it was 0·405 V; the potential caused by thioglycollic acid (0·120 V) should be raised in the presence of dye, but it was only 0·125 V.

The antagonistic action of methylene blue towards crystal violet may be explained by supposing that crystal violet interferes with the uptake of hydrogen ions by a coenzyme in the bacterial cell which normally accepts hydrogen ions from a substrate. Methylene blue acts as an alternative hydrogen acceptor and so permits oxidation of the substrate and growth of the bacteria. Fischer & Munoz (1947) suggested that crystal violet might block important biological mechanisms, possibly connected with oxidation processes.

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Micelles as simple models of drug receptors

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Some interactions of optically active *N*-alkyl-*NN*-dimethylalanine hydrobromide (alkyl betaine) micelles, representing model flexible drug receptors, with simple "drug" molecules, represented by a number of optically active amino-acids, choline derivatives and a dipeptide have been examined. Comparisons of these "drug-receptor" interactions have been made using a refractive index technique and an explanation of the difference in the degree of adsorption of different "drug" molecules at identical micelle surfaces is advanced.

STEREOSELECTIVE adsorbents (Beckett & Anderson, 1957, 1959, 1960) in which active sites or "footprints" are formed during their preparation may be regarded as elementary models of rigid drug receptors incapable of adaptation to meet the requirements of a substrate (Beckett & Youssef, 1963). Koshland (1958) on the other hand has stressed the importance of flexibility in drug-receptor interactions. We now report some interactions of model flexible drug receptors with "drug" molecules represented by a number of pairs of enantiomorphic amino-acids and related compounds. This work emphasizes the importance of steric factors between the amino- and carboxyl-groups, changes in the size of the cationic head and stereochemical features.

Experimental

DIFFERENTIAL REFRACTIVE INDEX MEASUREMENTS

Materials. *N*-Alkyl-*NN*-dimethylalanine hydrobromides (Beckett, Kirk & Virji, 1967). L-Alanyl-L-alanine (Sigma Chemical Company), and α-amino-acids (Koch-Lights). Dimethylalanine hydrochlorides and dimethylvaline hydrochlorides (Bowman & Stroud, 1950). α- and β-Methylacetylcholine iodides (Beckett, Harper & Clitherow, 1963).

Apparatus. A Hilger Rayleigh Interference Refractometer for liquids (Model M.154) fitted with constant temperature water jacket and tungsten lamp. Cells had path lengths of 1 and 10 cm.

Preparation of betaine-"drug" solutions. Filtered solutions of betaines of the highest required concentration were prepared using double distilled water; from these, dilutions were made as required. All these solutions were mixed with similarly prepared "drug" solutions to give solutions containing betaine and "drug" in the required molar concentrations.

Measurements of differential refractive index. All measurements were made at 20 ± 1°. The zero of the instrument was checked before and at the end of each experimental run. For each measurement, when using a 1 cm cell, a 20 min period was allowed for equilibration of cell and contents; when a 10 cm cell was used, 35 min was allowed. All measurements were made on duplicate solutions and an average of 3 to 4 readings was taken on each.

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CONSTRUCTION OF MOLECULAR MODELS

Catalin models of the "drug" molecules and a portion of the betaine micelle were constructed in the light of the following considerations.

1. The long-chain alkyl group on the betaine nitrogen atom will be orientated away from the aqueous phase. Its conformation will be such that non-bonded interactions are at a minimum and the closest possible "fit" with the long-chain alkyl group of a neighbouring betaine molecule is obtained.

2. The charged carboxyl group and nitrogen atom of the betaine molecule will be orientated towards the aqueous phase. Maximum stability will result when the nitrogen atom of one betaine molecule lies in the closest possible proximity to the carboxyl group of a neighbouring betaine molecule. Carboxyl groups by reason of electrostatic repulsion will be as far as possible from each other on the micelle surface.

3. The methyl group on the betaine asymmetric carbon atom will be orientated in the same direction as the long-chain alkyl group and the hydrogen atom on the same carbon atom will be in the micelle-water interface. The choice of this conformation is supported by critical micelle concentrations (CMC) of *N*-alkyl-*NN*-dimethylglycines and the corresponding *N*-alkyl-*NN*-dimethylalanines. An increase in the length of the long-chain alkyl group of a glycyl betaine by one methylene group results in a decrease in CMC. A decrease in CMC is also observed between a glycyl betaine and its corresponding alanyl betaine (Beckett & others, 1967). It is therefore logical to assume that the decrease in the CMC of the alanyl betaine is due to the methyl group being orientated in the same direction as the long-chain alkyl group.

As a result of the above restrictions, one of the *N*-methyl groups and the carboxyl group of a betaine molecule lie in close proximity at the surface of the micelle while the other *N*-methyl group points away from the carboxyl group leaving a *cavity* which is partially overlapped by the hydrogen atom on the betaine asymmetric carbon atom.

4. The conformation of "drug" molecules will be such as to allow the closest possible "fit" at the betaine micelle surface to bring together charges of opposite character, and the fit will be such that the "drug" carboxyl group and nitrogen atom are directed towards the aqueous phase.

Results and discussion

INTERACTIONS OF ALANINE ENANTIOMORPHS WITH D-BETAINES

Differential refractive index measurements show that solutions containing D-betaine and D-alanine have higher refractive indices than the corresponding solutions of D-betaine and L-alanine. Refractive index is a function of molecular density and polarizability. The difference in interaction of the enantiomorphs with an asymmetric betaine micelle are

considered to arise from differences in density in the packing of the molecules rather than differences in polarizability: the reasons are as follows.

(*a*) When the concentration of a betaine is increased from a value below its CMC to a value above this there is an increase in the slope of the refractive index–concentration curve. Thus when betaine molecules aggregate there is an increase in refractive index. This is more likely to be a density effect than a polarizability effect because the interaction of ionized groups on the surface of the micelle would be expected to make the electron cloud less polarizable than in the discrete betaine molecules.

(*b*) A betaine solution above its CMC in the presence of an amino-acid gives a higher refractive index than the sum of the refractive indices of the separate components in the appropriate concentrations. It is reasonable to predict that the amino-acid adsorbed at the highly polar surface of the micelle would increase the density but reduce polarizability.

(*c*) The fact that stereoselectivity is shown by the betaine micelle for the amino-acid enantiomorphs indicates that the amino-acids must come into close contact with the micelle surface. Any explanation of the refractive index results based upon differences in organization of water molecules rather than interaction of the betaine surface with the amino-acid molecules would not account for the differences obtained with enantiomorphs.

It would appear to follow that a D-betaine-D-alanine complex has a smaller volume to mass ratio than the corresponding D-betaine-L-alanine complex. Thus at the surface of a D-betaine micelle, D-alanine is better adsorbed than is L-alanine. Examination of molecular models supports this conclusion. The hydrogen atom attached to the asymmetric carbon atom of D-alanine can "fit" into the *cavity* between one of the *N*-methyl groups and the carboxyl group of a betaine molecule at the surface of a D-betaine micelle (see Fig. 1 (a) R = Me). With L-alanine the hydrogen atom is now on the opposite side of the molecule and comes into contact with the other *N*-methyl group of the D-betaine molecule, thus preventing

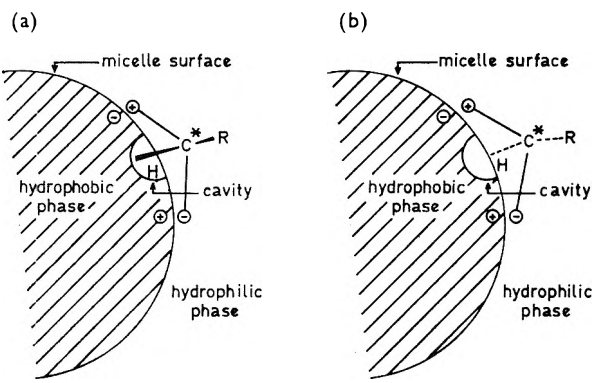
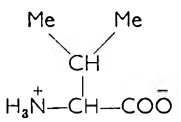


FIG. 1. Diagrammatic representation of amino-acid-betaine interactions. (a) D-Betaine/D-drug. (b) D-Betaine/L-drug. Shaded portion is an equatorial section through two polar groups of a portion of the betaine micelle. C* is the drug asymmetric carbon atom, with thick lines representing bonds above the plane of the paper and dotted lines below.

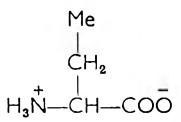
the L-amino-acid from fitting as closely as its enantiomorph at the surface of the micelle (see Fig. 1 (b) R = Me). Refractive index results for other pairs of optically active amino-acids (see Table 1) indicate that D-amino-acids are better adsorbed than their enantiomorphs at the surface of a D-betaine micelle. Examination of molecular models also supports this conclusion.

EFFECT OF INCREASING THE SIZE OF THE "DRUG" α-ALKYL SUBSTITUENT

The difference in refractive index between solutions of D-betaine-D-valine and solutions of D-betaine-L-valine is 127 refractometer units for dodecyl and 131 units for tetradecyl betaine compared with values of 10 and 32 units for the corresponding betaine-alanine solutions (see Table 1). If the difference in volume between a D-betaine-D-valine complex and a D-betaine-L-valine complex is the same as that between a D-betaine-D-alanine complex and a D-betaine-L-alanine complex, then the greater difference in refractive index between betaine-valine complexes could be due to the higher molecular weight of valine compared with that of alanine. Examination of molecular models indicates that a second and probably more important factor is the larger difference in the degree of adsorption of D- and L-valine at a D-betaine micelle surface. In the D-betaine-D-valine complex, the two methyl groups of the isopropyl group of the "drug" molecule can come into close contact with the micelle surface and thus help in binding the "drug" molecule to the micelle surface.



Valine

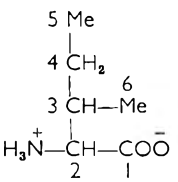


α-Aminobutyric acid

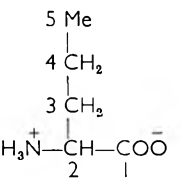
With the D-betaine-L-valine complex the two methyl groups cannot come into contact with the micelle surface. This feature of additional binding of one enantiomorph to the micelle surface is absent in the alanine enantiomorphs. Added support for these conclusions is obtained by examining the adsorption of D- and L-α-aminobutyric acid at the surface of a D-micelle. With these enantiomorphs, the difference in the degree of "fit" at the surface of a micelle would appear to be similar to that obtained with alanine and valine enantiomorphs, but the difference in refractive index between a D-betaine-D-α-aminobutyric acid solution and the corresponding D-betaine-L-α-aminobutyric acid solution is intermediate between the values obtained for alanine and valine enantiomorphs (see Table 1). This may be explained as being due partly to the fact that the molecular weight of α-aminobutyric acid is intermediate between that of alanine and valine and partly to the fact that the terminal methyl group of the ethyl group on the "drug" asymmetric carbon atom of D-α-amino-butyric acid can come into contact with the surface of the micelle thus

assisting in the binding to the surface of a D-betaine micelle. With L-α-aminobutyric acid no such binding is possible.

The difference in the degree of "fit" of the enantiomorphs of isoleucine and of norvaline would appear to be similar to that shown by the isomers of alanine, but the difference in the degree of adsorption in both cases as indicated by refractive index measurements (see Table 1) is less than with alanine isomers. With isoleucine and norvaline, the flexibility of the longer α-alkyl chain allows the terminal methyl group to assist in the binding of both isomers at the micelle surface. With D-isoleucine and D-norvaline, additional binding at the surface of a D-betaine micelle is provided by the 4-methylene group.

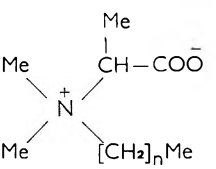


Isoleucine



Norvaline

TABLE 1. INTERACTION OF *N*-ALKYL *NN*-DIMETHYLALANINE HYDROBROMIDES (ALKYL BETAINES) WITH α-AMINO-ACIDS: RESULTS OF DIFFERENTIAL REFRACTIVE INDEX MEASUREMENTS (ΔR)*



Alkyl betaine

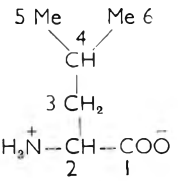
Betaine	Amino-acid	ΔR in scale divisions
Dodecyl (n = 11)	Alanine	10
Tetradecyl- (n = 13)	,,	32
Dodecyl- (n = 11)	Valine	127
Tetradecyl- (n = 13)	,,	131
Dodecyl- (n = 11)	α-Aminobutyric acid	11
Tetradecyl- (n = 13)	,,	82
Dodecyl (n = 11)	Isoleucine	3
Tetradecyl (n = 13)	,,	9
Dodecyl (n = 11)	Norvaline	4
Tetradecyl (n = 13)	,,	5
Dodecyl (n = 11)	Leucine	1
Tetradecyl (n = 13)	,,	4

All solutions contained 0·05 molar concentration of both "drug" and betaine.

Cell path length 1 cm.

ΔR = Refractive index of D-betaine -D-"drug" solution minus the refractive index of D-betaine: L-"drug" solution.

Differential refractive index measurements (see Table 1) indicate little or no difference in the degree of adsorption of D- and L-leucine at a D-betaine micelle surface. This conclusion is supported by examination of molecular models.

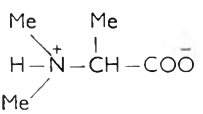


Leucine

In both D- and L-leucine the 5- and 6-methyl groups can assist in binding the "drug" molecule to the micelle surface. The D-isomer receives additional binding from the 4-methine group, but this appears to be the only difference in the adsorption of D- and L-leucine at a D-micelle surface.

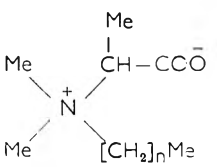
EFFECT OF INCREASING THE SIZE OF THE DRUG CATIONIC HEAD

Solutions containing D-betaine-D-dimethylalanine have a higher refractive index than the corresponding solutions of D-betaine-L-dimethylalanine, the difference in arbitrary units being 4 for dodecyl betaine and 12 for tetradecyl betaine (see Table 2). The corresponding values for D- and L-alanine were respectively 10 and 32.



Dimethylalanine

TABLE 2. INTERACTIONS OF *N*-SUBSTITUTED-α-AMINO-ACIDS WITH *N*-ALKYL *NN*-DIMETHYLALANINE HYDROBROMIDES: RESULTS OF DIFFERENTIAL REFRACTIVE INDEX MEASUREMENTS (ΔR)



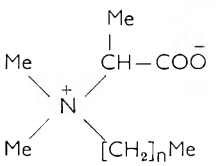
Alkyl betaine

Betaine	Amino-acid derivative	ΔR in scale divisions
Dodecyl- (n = 11)	Dimethylalanine HCl	4
Tetradecyl- (n = 13)	" "	12
Dodecyl- (n = 11)	Dimethylvaline HCl	21
Tetradecyl- (n = 13)	" "	37
Dodecyl (n = 11)	Dimethylalanine methiodide	0
Tetradecyl (n = 13)	" "	0

See footnote to Table 1.

The molecular weight of alanine is less than that of dimethylalanine. A larger difference in refractive index would be expected between solutions of D-betaine-D-dimethylalanine and the corresponding solutions of D-betaine-L-dimethylalanine provided the degree of "fit" of these molecules at D-betaine micelle surfaces is the same as that of alanine enantiomorphs. Since however the differences are smaller with the dimethylalanine enantiomorphs, it is concluded that the "fit" of dimethylalanine is not so close as that obtained with alanine at identical betaine micelle surfaces. Solutions of D-betaine-L-alanine have higher refractive indices than the corresponding solutions of D-betaine-L-dimethylalanine, the difference with the tetradecyl betaine being 60 units (see Table 3). These results indicate that

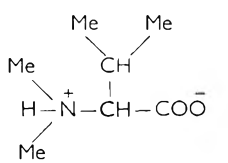
TABLE 3. INTERACTION OF α-AMINO-ACIDS AND *NN*-DIMETHYL DERIVATIVES WITH ALKYL BETAINES: RESULTS OF DIFFERENTIAL REFRACTIVE INDEX MEASUREMENTS (ΔR)



Contents of left hand cell	Contents of right hand cell	ΔR
D-(+)-Betaine (n = 13): L-(+)-alanine	D-(+)-Betaine (n = 13): L-(+)-dimethylalanine	60
D-(+)-Betaine (n = 13): L-(+)-valine	D-(+)-Betaine (n = 13): L-(+)-dimethylvaline	20
L-(−)-Betaine (n = 13): D-(-)-valine	L-(−)-Betaine (n = 13): D-(−)-dimethylvaline	20

All solutions contained 0·05 molar concentration of both "drug" and betaine.
Cell path length 1 cm.

the D-betaine-L-dimethylalanine complex is less dense than the corresponding D-betaine-L-alanine complex. Molecular models show that the two *N*-methyl groups of dimethylalanine prevent a close "fit" between the positively charged nitrogen of the "drug" molecule and the negatively charged carboxyl group at the surface of the betaine micelle. As a result, neither the D- nor the L-forms of dimethylalanine can "fit" closely at the micelle surface and hence there is little difference in the volumes of the drug-betaine complexes. A similar explanation may account for the small differences in refractive index between solutions of D-betaine-D-dimethylvaline and solutions of D-betaine-L-dimethylvaline (see Table 2). The differences shown are higher than those obtained for the corresponding



Dimethylvaline

betaine-dimethylalanine solutions and lower than the values for betaine-valine solutions (see Table 1). The view that neither of the dimethylvaline enantiomers fit closely is supported by refractive index measurements which show that a solution of L-betaine-D-valine has a higher refractive index than the corresponding solution of L-betaine-L-dimethylvaline (see Table 3). The L-betaine-D-valine complex is therefore more dense than the corresponding L-betaine-D-dimethylvaline complex.

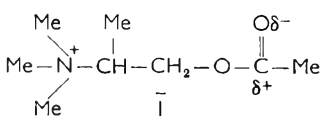
In the case of the quaternary dimethylalanine methiodide enantiomers the refractive index of a solution of D-betaine-D-isomer is the same as that of a corresponding solution of D-betaine-L-isomer (see Table 2).

A number of contributing factors may be involved in the failure of enantiomorphs of quaternary derivatives to show any difference in adsorption at the surface of the micelle.

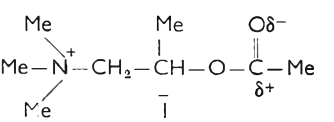
1. The increase in bulk of the group on the nitrogen will prevent it from coming close to the carboxyl group of the betaine micelle. Molecular models show no difference for either enantiomer in the degree of "fit" at the betaine micelle surface.

2. Quaternary amines can form ionic bonds with anions, but there is no possibility of such bonds being reinforced by hydrogen bonding. Apart from quaternary compounds, cations of all amines are known to form hydrogen bonds and ionic bonds simultaneously with the anions of carboxylic acids. The resulting bond has double the strength of a purely ionic bond and has greatly increased permanence (Albert, 1965). With dimethylalanine methiodide the strength of bonding between the postively charged nitrogen of the "drug" molecule and the negatively charged carboxyl group at the betaine micelle surface will be much less, for example, than that for alanine. Furthermore any bond which does form will be less permanent.

No difference can likewise be observed in refractive index between the enantiomorphs of α- and β-methylacetylcholine iodides in the presence of D-betaine micelles, and this may be explained in similar terms. An additional factor for the choline derivatives may be that the very weak negative charge on the oxygen of the acetyl carbonyl group may not be sufficient, in the absence of other reinforcing factors, to bind this end of the molecule to a positively charged nitrogen atom on the micelle surface.



α-Methylacetylcholine iodide



β-Methylacetylcholine iodide

INTERACTIONS OF A DIPEPTIDE WITH BETAINE MICELLES

Solutions containing L-alanyl-L-alanine and a D-betaine each present in 0·05 molar concentration have a lower refractive index than corresponding solutions of L-alanyl-L-alanine and L-betaine, the difference in arbitrary

units being 15 for dodecyl and 28 for tetradecyl betaine with a cell of path length 1 cm. These results indicate that the dipeptide "fits" better at the surface of an L-betaine than at the surface of a D-betaine micelle. Molecular models of the betaine molecule were constructed as previously described. The conformation of the "drug" molecule was arranged in such a manner that the $-\overset{+}{N}H_3$, the — CONH and the $-\overset{-}{C}O\overset{-}{O}$ groups were all orientated in the same general direction, so that all three groups pointed in the direction of the aqueous phase when the peptide was adsorbed at the micelle surface. The two methyl groups were orientated in the same general direction, but for steric reasons pointed away from each other. As a result of these restrictions the two hydrogen atoms on the asymmetric carbon atoms are orientated almost parallel to each other and the $-\overset{-}{C}O\overset{-}{O}$ and $-\overset{+}{N}H_3$ groups point towards each other. Fig. 2 represents that conformation of L-alanyl-L-alanine most stable at the betaine surface.

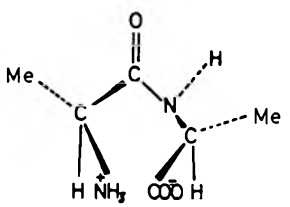


FIG. 2. L-Alanyl-L-alanine.

In fitting the dipeptide model to that of a portion of the betaine micelle it is found that when the carboxyl group of the "drug" molecule is brought close to the positively charged nitrogen of a betaine molecule, then the $-\overset{+}{N}H_3$ group of the dipeptide can bind only to the carboxyl group of a

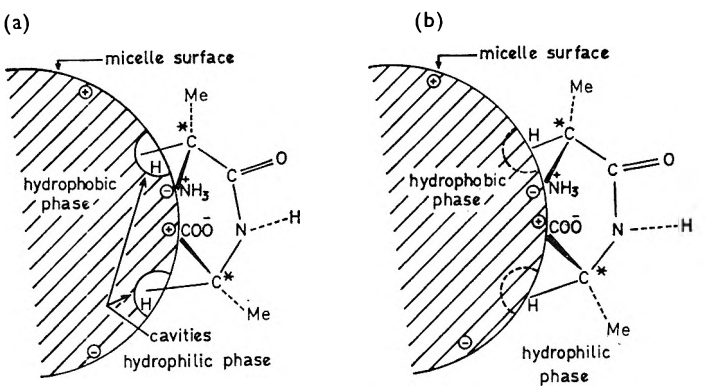


FIG. 3. Diagrammatic representation of dipeptide-betaine interactions. (a) L-Alanyl-L-alanine/L-betaine. (b) L-Alanyl-L-alanine/D-betaine. Shaded portion is an equatorial section through polar groups of a portion of betaine micelle. C* is a drug asymmetric carbon atom, with thick, broken, and unbroken lines for bonds above, below, and in the plane of the paper respectively.

neighbouring betaine molecule in the micelle. With L-alanyl-L-alanine and L-betaine the hydrogens attached to the "drug" asymmetric carbon atoms are able to "fit" into two cavities at the surface of the micelle [see Fig. 3 (b)]. Each of these cavities lies between one of the *N*-methyl groups and the carboxyl group of a betaine molecule. When models representing a portion of the D-betaine micelle surface are used it is found that the two hydrogens on the asymmetric carbon atoms of the drug molecule prevent a close "fit" with the micelle surface by coming into contact with the other *N*-methyl groups on the two betaine molecules involved in the adsorption of the drug molecule [see Fig. 3 (b)].

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A method for measurement and statistical analysis of large numbers of experimental tracings

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An accurate and rapid method of digitizing large numbers of experimental tracings is possible using a Pencil Follower (D-Mac). Its use in conjunction with a computer has meant that data, hitherto virtually unmanageable, can be subjected to full statistical analysis. An example of its application is cited.

THE acquisition of large numbers of experimental tracings, as from a multi-channel electronic recorder, presents difficulties in the measurement and analysis of the results. Experimental records can be digitized in one of three ways. (1) Manually, by selecting the required results and measuring with a ruler. (2) Automatically, where all the results obtained are converted by electronic apparatus. (3) Semi-automatically, where the experimenter selects results and then converts by electronic apparatus.

The pencil and ruler method of digitizing experimental tracings is often adequate for small amounts of data, such as those from smoked drums, but with larger quantities this is time-consuming, tedious and prone to error.

Automatic conversion is often unsuitable since some form of selection of the results is often required, to eliminate artifacts and other unwanted responses, and to reduce the amount of data collected to manageable proportions.

To overcome these problems we have adopted the semi-automatic method with the use of a Pencil Follower (D-Mac Ltd., Glasgow). To illustrate the principles involved, the method employed in a particular pharmacological experiment is cited.

APPARATUS

The pencil follower provides a way of digitizing data presented in pictorial form, such as pen traces from a multi-channel recorder. There are three main components: a "reading table," approximately $2\frac{1}{2}$ ft $\times$ $4\frac{1}{2}$ ft; a "pencil" which can take one of a variety of forms, the commonest being a small magnifying glass with cross-hairs in its centre; and an "electronic console" which is approximately $1\frac{1}{2}$ ft $\times$ $1\frac{1}{2}$ ft $\times$ 3 ft high.

The graph or tracing to be digitized is placed on the reading table. When the pencil is placed on the table, its position, in terms of its x,y co-ordinates relative to the table, is displayed on the console. This visual display is in the form of two four digit numbers, giving the co-ordinates of the pencil's position to an accuracy of 0·1 mm. A push button on the pencil, or a foot-switch, permanently records the co-ordinates on paper tape or punched cards, or alternatively these co-ordinates may be typewritten, by means of an auxiliary output device. The format of the

From the Department of Pharmacological Research, Parke-Davis & Co., Staines Road, Hounslow, Middlesex.

permanent record may be varied by adjusting a plug-board in the console and may include the observation serial number which is recorded automatically. Any titles or comments on the data may be included by the use of a small keyboard which rests on the table top.

METHOD

With care, the baselines of the experimental tracing can be aligned with those of the reading table, a task which is simplified by drawing permanent guide lines on the table. Measurements can then be read directly on the visual display, or typed automatically.

However, the pencil follower is best used with a computer, whether it be on-site or a bureau machine, which has been programmed to make all the subsequent analysis. When so used there is no need for accurate alignment of the graph. It is easier to take two or three baseline points and programme the computer to transform the co-ordinates (see Appendix).

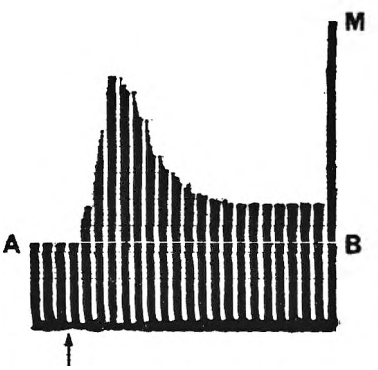

FIG. 1. Intratracheal pressure recording from guinea-pig lung *in vivo*. Guinea-pig, 350 g, anaesthetized with 60 mg/kg i.p. pentobarbitone sodium. Intratracheal pressure was measured on a "Greer" differential micromanometer connected to a Device M8 Polygraph. Recordings were made for 20 sec in each 30 sec. ↑, intravenous administration of a bronchoconstricting agent. A, B, baseline x axis; M, maximum possible response.

Fig. 1 shows an experimental tracing in which the intratracheal pressure of a guinea-pig was recorded continuously for 20 sec periods in every 30 sec. Three control periods were recorded to establish a baseline (AB). Recordings were then taken over 10 min, after which the maximum possible response (BM) was obtained.

In a typical experiment, about 12 animals on each of four treatments are used. For each tracing, the baseline points A and B are first plotted by the pencil follower, and then the maximum possible response M, followed by the twenty maximal responses obtained every 30 sec.

Table 1 (A) shows the data output produced by the pencil follower when the tracing in Fig. 1 was digitized. The title, which represents the animal's number and the experimenter's initials, and the asterisks, which terminate the data from a particular animal or treatment, were typed on the keyboard. The output format in this case has been arranged to

TABLE 1 (A). DATA OUTPUT PRODUCED BY THE PENCIL FOLLOWER IN DIGITIZING FIG. 1. (B) OUTPUT PRODUCED BY THE COMPUTER FROM THE DATA IN TABLE 1A

A			B		
B_3 GWLJ			B_3 GWLJ		
*					
0000	235·9	148·1			
0001	289·1	167·9			
0000	275·3	207·1	MAX	41·55	
**					
0001	244·1	151·4	1	0·56	
0002	244·6	158·8	2	16·83	
0003	242·1	172·9	3	50·73	
0004	240·5	183·3	4	75·53	
0005	243·0	182·6	5	71·85	
0006	245·1	182·0	6	68·74	
0007	249·1	177·7	7	55·68	
0008	253·8	172·3	8	39·56	
0009	256·7	170·3	9	32·61	
0010	260·7	169·1	10	26·55	
0011	262·8	168·0	11	22·30	
0012	265·7	168·0	12	19·87	
0013	267·8	168·7	13	19·68	
0014	270·6	169·1	14	18·24	
0015	273·6	170·2	15	18·20	
0016	275·2	170·7	16	17·98	
0017	276·9	171·4	17	18·14	
0018	279·8	172·4	18	17·96	
0019	281·6	173·1	19	18·02	
0020	284·3	174·1	20	18·01	
***					
			AREA	618·03	SQRT AREA 24·86

Table 1 (A) comprises an animal identification (B3) and an operator identification (GWLJ). *, End of title; **, end of baseline and maximum possible co-ordinates; ***, end of data for animal B3. Left hand column, serial number of operation; middle column, X co-ordinate; right hand column, Y co-ordinate.

In Table 1 (B), the animal and operator identifications (B3 GWLJ) are reprinted. MAX is the absolute value of the maximum possible response. Left hand column, serial number of operation; right hand column, y co-ordinate as a percentage of MAX. AREA is the estimate of the area under the % response curve. SQRT AREA is the square root of the area.

include the automatic counting device, which is reset to zero for each animal before and after the two baseline points are digitized. All the data are punched directly onto paper tape. A print-out of this tape is then obtained from a teleprinter-tape reproducer set and the tape can be corrected if necessary before being sent to a computer.

Table 1 (B) shows the results produced by the computer from the data in Table 1 (A). The animal title is printed at the top followed by the absolute value of the maximum response and then the twenty readings, which are expressed as percentages of the maximum possible response (M). For each treatment the mean % response is also calculated. In addition, the area under each % response curve is estimated by means of the Trapezium rule. An analysis of Variance model (Davies, 1954) is used to test for differences in mean areas between treatments. To obtain minimal residual variance, the areas are transformed before analysis to square root areas (Bartlett, 1947).

DISCUSSION

We have found that laboratory technicians learn to use the system readily, basic instruction taking less than one day. Complete analysis of an experiment of the type illustrated can be made in about 2 hr, compared with the two or three days it took with pencil and ruler methods.

The cost of the computer analysis is more than offset by this saving in technicians' time, since they are freed from much tedious and repetitive work. The system has also proved less prone to error, since the technicians do not, at any stage, have to write down any results or make any calculations.

APPENDIX: TRANSFORMATION OF CO-ORDINATES

If X,Y represent the co-ordinate axes of the reading table and x,y those of the tracing to be digitized, then if two baseline points on the tracing are taken (Fig. 2).

(1) A (X_1,Y_1) at the origin of the graph,

(2) B (X_2,Y_2) at any other point on the graph's x-axis,

then the co-ordinates of any point C (X_3,Y_3) plotted on the table, with respect to the origin of the graph are:

$$x = \frac{(X_3 - X_1)(X_2 - X_1) + (Y_3 - Y_1)(Y_2 - Y_1)}{\sqrt{(X_2 - X_1)^2 + (Y_2 - Y_1)^2}}$$

and

$$y = \frac{(Y_3 - Y_1)(X_2 - X_1) - (X_3 - X_1)(Y_2 - Y_1)}{\sqrt{(X_2 - X_1)^2 + (Y_2 - Y_1)^2}}$$

In the particular example cited, we were not directly concerned with the x co-ordinate and therefore A was placed at any convenient point on the x-axis of the graph.

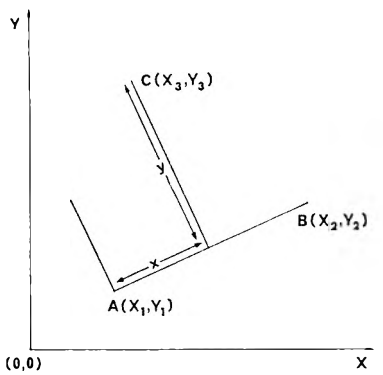

FIG. 2. The transformation of the co-ordinates of a point, C, from those of the reading table to those of the trace. X, Y, the co-ordinate axes of the reading table. AB, the baseline of the trace. x, y, the co-ordinates of the point C relative to the trace.

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Stimulus-dependent antagonism of the α-methyltyrosine-induced lowering of brain catecholamines by (+)-amphetamine in intact mice

SIR,—Littleton (1967) reported that amphetamine retards the decline of brain catecholamines that follows inhibition of their biosynthesis. We wish to confirm his observation and extend it to demonstrate that this antagonism of α-methyltyrosine by (+)-amphetamine is stimulus-dependent.

Male white Swiss mice born within the same two-day period were weaned at four weeks of age and were individually housed for 8 weeks in a quiet air-conditioned room (27°) containing neither females nor any other species of animal. Such mice become hyperexcitable and after being caged with another male mouse will normally engage in intense fighting within about a minute. Care was taken to cause minimal undesigned disturbance to the animals. Mice were given 80 mg/kg DL-α-methyltyrosine intraperitoneally in 0·2 ml of a 0·9% saline solution at pH 2·5. Exactly 1 hr later they were given either 0·1 ml of a 5 mg/kg solution of (+)-amphetamine sulphate in 0·9% saline at pH 7·0, or vehicle alone. At this time, half of those receiving each treatment were quietly returned to their original cages. All others were placed in a strange cage with another of their kind to fight. The mice were decapitated exactly 30 min after injection. Ten non-fighting and ten fighting mice received (+)-amphetamine only; ten non-fighting and ten fighting mice receiving no drug were killed as baseline controls. Whole brains, exclusive of the *bulbus olfactorius* were removed, weighed, and frozen on dry ice within 2 min of death; they were stored at −20°. Individual brains were analysed for noradrenaline, dopamine and 5-hydroxytryptamine (5-HT) (Welch & Welch, 1967).

Table 1 shows that (+)-amphetamine significantly slowed the decline of whole brain noradrenaline and dopamine which was otherwise induced by (±)-α-methyltyrosine in the fighting mice, but that it did not have this effect in the mice which were not put together to fight. Semi-quantitative behavioural observations supported both the suggestion that α-methyltyrosine prevents supra-normal excitation by (+)-amphetamine (Weissman & Koe, 1965; Weissman, Koe & Tenen, 1966; Mennear & Rudzik, 1966; Dingell, Owens & others, 1967; Hanson, 1967), and the suggestion that (+)-amphetamine may temporarily counteract or delay the onset of the behavioural impairment that otherwise follows administration of α-methyltyrosine (Moore & Rech, 1967a; Poschel & Ninteman, 1966). In non-drug mice, brain amines may be either elevated or lowered by fighting, depending upon its intensity (Welch, 1967; Welch & Welch, 1967). Fighting among non-drug controls slightly lowered all three amines in this experiment (footnote Table 1); nevertheless, brain catecholamines were about the same in fighting and non-fighting mice receiving α-methyltyrosine alone. Fighting mice receiving (+)-amphetamine but no pretreatment were becoming uncoordinated or marginally ataxic (or both) by 30 min and their brain catecholamines were much lowered; on the other hand, those that did not fight were "normal" or were only very mildly activated at the time of death, and their brain dopamine and 5-HT were significantly elevated. 5-HT was significantly higher in all mice receiving amphetamine than in those receiving only α-methyltyrosine.

Weissman & others (1966) explained the ability of α-methyltyrosine to prevent, or rapidly stop, the supra-normal excitation normally seen after (+)-amphetamine by summarizing evidence: that the excitatory action of (+)-amphetamine is dependent upon the release of catecholamines to the outside of the neuron; that the availability of catecholamines for release is dependent upon maintenance of the functional pool; that α-methyltyrosine compromises the

TABLE 1. EFFECTS OF (+)-AMPHETAMINE UPON BRAIN CATECHOLAMINES IN FIGHTING AND NON-FIGHTING MICE PRETREATED WITH (±)-α-METHYLTYROSINE*

	1 hr α-MT, 30 min (+)-Amphet. as % of 1 hr α-MT, 30 min saline (1 hr α-MT, 30 min (+)-Amphet. as % of 1 hr saline, 30 min saline)		
	Non-Fight	Fight	P<
Noradrenaline ..	97 ± 2 (72 ± 2)‡	110 ± 3† (87 ± 4)‡	0·001 (0·01)
Dopamine ..	97 ± 3 (77 ± 1)‡	115 ± 5† (86 ± 1)‡	0·005 (0·001)
5-HT	122 ± 4† (114 ± 2)‡	129 ± 7† (117 ± 6)‡	n.s. (n.s.)

* α-MT was administered at 80 mg/kg, i.p. and (+)-amphetamine at 5 mg/kg, i.p. Each value in the Table represents a mean ± s.e.m. of 9–11 percentages based upon 9–11 pairs of male mice. Data were evaluated by analysis of variance or by the Wilcoxon Two-Sample Test, depending upon their distribution. Ten non-fighting and ten fighting saline controls, respectively, averaged (in ng/g ± s.e.m.): noradrenaline = 374 ± 14, 341 ± 8 ($P < 0·05$); dopamine = 858 ± 40; 811 ± 33 (n.s.); 5-HT = 869 ± 58; 852 ± 49 (n.s.). Ten non-fighting and ten fighting mice which received (+)-amphetamine but no pretreatment, respectively, averaged (as a percentage of their corresponding controls ± s.e.m.): noradrenaline = 95 ± 3; 81 ± 3 ($P < 0·001$); dopamine = 111 ± 4; 88 ± 2 ($P < 0·001$); 5-HT = 131 ± 3, 117 ± 4 ($P < 0·05$); noradrenaline and dopamine were significantly lower than controls in the fighting mice, viz. $P < 0·001$ and $P < 0·01$, respectively; 5-HT was significantly elevated in both the non-fighting and fighting mice, viz. $P < 0·01$ and $P < 0·05$, respectively.

† α-MT, (+)-amphetamine values were significantly different (at least $P < 0·05$) from the α-MT, (+)-amphetamine values of which they are here expressed as a percentage.

‡ α-MT, (+)-amphetamine values were significantly different (at least $P < 0·05$) from the saline, saline controls of which they are here expressed as a percentage.

functional pool by inhibiting biosynthesis. One might reason that *the activating effect of (+)-amphetamine is largely dependent upon nervous stimulus to effect the release of catecholamines.* If this is so, it will help to explain the synergism between nervous activity and (+)-amphetamine that occurs in normal animals (Moore, 1964; Welch & Welch, 1966); and it will explain the seemingly paradoxical observations that although amphetamine easily penetrates into the brain (Axelrod, 1954), it has no effect upon behaviour or upon the electroencephalograph in animals with lesions in the mid-brain reticular formation (*cerveau isolé*) (Bradley & Elkes, 1957).

In both of the published studies in which (+)-amphetamine antagonized the α-methyltyrosine-induced impairment of behavioural performance, the animals were in stimulus situations, e.g. in one, rats were performing at 20% of normal in a conditioned avoidance response situation (Moore & Rech, 1967a); and in the other, rats had just concluded an 8 hr session of hypothalamic self-stimulation (Poschel & Ninteman, 1966). It is probable that the (+)-amphetamine antagonism of α-methyltyrosine-induced behavioural depression reported by these authors was the result of a retarded decline in brain catecholamines such as that observed by Littleton (1967) and by ourselves.

Further, pretreatment with a monamine oxidase inhibitor similarly retards the behavioural depression and the lowering of brain catecholamines caused by α-methyltyrosine (Moore & Rech, 1967b), and enhances the facilitating effect of amphetamine upon hypothalamic self-stimulation (Stein, 1964). We suggest the working hypothesis that *stimulus itself may normally act in some way to continually modulate the degree of inhibition of monoamine oxidase*, thereby exerting a fine control over the amount of neurotransmitter available in the functional pool for release by nerve stimulation. On this basis, our results may be explained by assuming that, in this experiment, (+)-amphetamine slightly enhanced nervous activity by preventing re-uptake (Glowinski & Axelrod, 1965) and, thereby, indirectly increased the degree of natural inhibition of monoamine oxidase; presumably the observed retardation of tissue catecholamine lowering

by α-methyltyrosine was the result of a net savings of neurotransmitter from oxidative deamination in excess of the increased amount released by the enhanced nervous activity. In the mice receiving only (+)-amphetamine, the increased transmitter release from nerve terminals caused by fighting and the increased extraneuronal longevity of the transmitter caused by (+)-amphetamine, acted in positive feedback manner, one upon the other, to produce the intense behavioural activation and the marked lowering of brain amines commonly associated with the effect of (+)-amphetamine. Undoubtedly the rate of release exceeded total biosynthesis, for if this were not the case catecholamine levels would not have been reduced. Even in the mice that were not stimulated by fighting, there was a small tonic release of neurotransmitter, and the (+)-amphetamine prolonged its action, resulting in a mild increase in nervous activation, a slight natural inhibition of monamine oxidase, and an elevation of dopamine and 5-HT. (With smaller doses of amphetamine or with mice that have not been isolated long enough to be quite so responsive to handling, all three amines may be increased; presumably in this experiment the rate of noradrenaline release had just begun to exceed the supply.)

The concept of continuous massive biosynthesis and oxidative deamination of brain biogenic amines as a means of ensuring their availability in amounts in excess of normal needs has previously been suggested by Brodie & Beaven (1963); however, they regarded the rate of synthesis and the rate of breakdown as constant, save that the latter was temporarily diminished when nervous stimulus released transmitter amines from the nerve ending, thus making them unavailable for catabolism within the neuron. Our suggestion differs in that we propose that the level of monoamine oxidase activity may be modulated by stimulus.

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October 2, 1967

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Ionization constants of cholinesterase-reactivating bispyridinium aldoximes

SIR,—Since most pharmacologically active substances contain acidic or basic molecular groups, or both, which are ionized to a different degree at physiological pH, ionization constants are of particular biological importance.

In a number of substances with pK_a values within the physiological range, biological activity has been shown to depend on the pH of the medium, thus reflecting the actual concentrations of the active molecular species (for a review see Albert, 1960).

Quaternary pyridine aldoximes are reactivators of organophosphorus poisoned acetylcholinesterase. Wilson, Ginsburg & Quan (1958) suggested the oxime anion to be the "reactive species". Theoretically, the pK_a should be low enough to yield a sufficient concentration of anions at physiological pH. On the other hand changes in pK_a have been reported to affect reactivating potency to a small extent, giving rise to factors of only 2–3 for one pH unit. These findings have been reported to apply to most reactivations (Wilson & others, 1958).

The pK_a values of a number of mono-oximes have been determined by potentiometric titration (Wilson & others, 1958; Hobbiger, 1963). Wilson & Ginsburg (1958) titrated bisquaternary dibasic oximes for analytical reasons. In their titration curves they observed only one break. This led to the assumption that the pH at one-half neutralization should be equal to the "average" of the two supposed pK_a values. Although such an average value bears only analytical consequences, some authors (Hobbiger, 1963; Engelhard & Erdmann, 1964) refer to it as a true pK_a value.

Engelhard & Erdmann (1964) based their calculations of the percentage ionized on corresponding values. According to their figures the percentage ionization of TMB-4 is 19% and of obidoxime is 28% at pH 7·5. However,

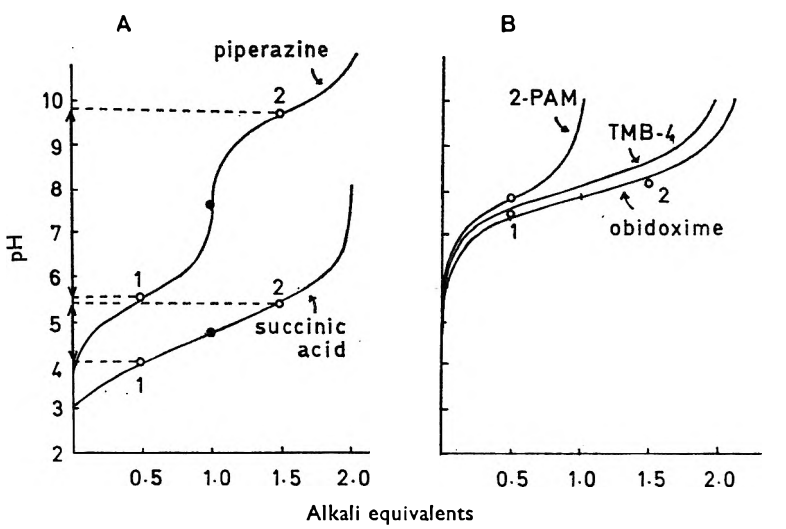


FIG. 1. Titration curves (0·01M, 0·1N KOH, 20° of (A) piperazine diperchlorate ($pK_{a_1} = 5{\cdot}54 \pm 0{\cdot}006$; $pK_{a_2} = 9{\cdot}79 \pm 0{\cdot}01$) and succinic acid ($pK_{a_1} = 4{\cdot}09 \pm 0{\cdot}03$; $pK_{a_2} = 5{\cdot}42 \pm 0{\cdot}03$) (Albert & Serjeant, 1962) and (B) 2-PAM, TMB-4 (1,1′-trimethylenebis(4-formylpyridinium bromide) dioxime and obidoxime (Toxogonin; bis(4-hydroxyiminomethyl pyridinium-(1)-methylether dichloride. (●) One-half neutralization.

the titration midpoint, i.e. the pH at one-half neutralization, may only be set as equal to the pK_a where there is only one ionizing group. In molecules with more than one ionizing group, the less basic may be completely ionized at "one-half neutralization", whereas the more basic group may remain entirely uncharged, as for example in piperazine diperchlorate. (Fig. 1A)

According to the ratio of the respective ionization constants the titration curve of a dibasic electrolyte will either have an inflexion where $K_1 > 16\,K_2$, or yield a straight line where $K_1 = 16\,K_2$, or resemble that of a monobasic compound where $K_1 < 16\,K_2$ (Auerbach & Smolczyk, cited by Britton, 1955) (Fig. 1). From titration curves of quaternary bispyridine aldoximes the presence of two adjacent ionization constants may be inferred. The endpoint of the first stage of ionization is not discernible, because at this instant titration of the second group has already begun (Fig. 1B). In polyvalent electrolytes pK_a values may only be calculated by means of the Henderson-Hasselbalch equation, if they are separated by more than 2·7 pH units ($K_1 > 500\,K_2$). Accurate separation of overlapping pK_a values, however, may be obtained by means of a method which is due to Britton (1955).

As this procedure involves extensive calculations, a programme has been developed for use on an electronic calculator (Bieger, Ehrich & Wassermann, 1967). By this means, ionization constants of a series of dioximes have been determined (Table 1). The two pK_a values of TMB-4, for instance have been found to be 7·78 and 8·61 respectively; for obidoxime these values are 7·54 and 8·24. Such figures permit the calculation of the true proportion of anions to molecules; e.g. in obidoxime the more acidic group is 50% and the less acidic group 17% ionized at pH 7·5. In TMB-4 the corresponding figures are 33% and 7% (Bieger & Wassermann, 1967).

These findings raise the question of the significance of the second oxime group. Comparison with related monoximes shows that the influence of an additional oxime group is consistent with an increase in acidity of the first group. On an

TABLE 1. IONIZATION CONSTANTS OF SOME BISPYRIDINIUM ALDOXIMES

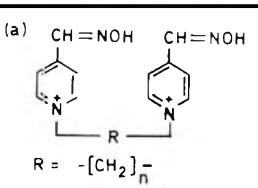
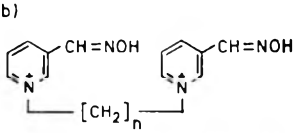
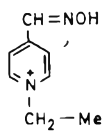
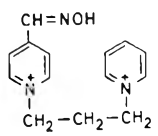
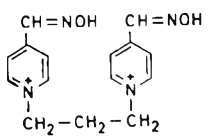
				Percentage ionized at pH 7·4	
		pKa_1	pKa_2	1	2
(a)  R = $-[CH_2]_n-$					
I	n = 2	7·58 ± 0·01	8·34 ± 0·01	39·83	10·33
II	n = 3	7·78 ± 0·01	8·61 ± 0·03	29·47	5·80
III	n = 4	7·93 ± 0·01	8·66 ± 0·01	22·78	5·17
IV	n = 5	7·93 ± 0·01	8·67 ± 0·01	22·69	5·12
V	n = 6	7·98 ± 0·01	8·69 ± 0·05	20·85	2·70
VI	R: $-CH_2-O-CH_2-$	7·54 ± 0·01	8·24 ± 0·02	41·79	12·73
(b) 					
VII	n = 3	8·59 ± 0·01	9·30 ± 0·03	6·08	1·24
VIII	n = 4	8·65 ± 0·01	9·46 ± 0·01	5·34	0·86
IX	n = 6	8·70 ± 0·01	9·50 ± 0·01	4·75	0·78

TABLE 2. REACTIVITY AND DEGREE OF IONIZATION OF A SERIES OF PYRIDINIUM-4-ALDOXIMES

Reactivator	Multiples of reactivation velocity of 2-PAM (diethylphosphoryl-AChE, acc. to Hobbiger & Sadler, 1959)	pKa	Percentage ionized (oxime anion) at pH 7·4
I 	$\frac{1}{33}$	8·2 (Hobbiger & others, 1960)	6%
II 	8	8·0 (Hobbiger, 1963)	20%
III  TMB-4	22	pKa_1 = 7·78 pKa_2 = 8·61	29·5% 5·8%

average, the 4-pyridine aldoximes—favoured by a possible quinonoid structure—are 0·7 pH units more acidic than the corresponding 3-aldoximes. Moreover, acidity may be promoted by a hydrogen bond between the ionized and the uncharged oxime group (Becker, 1965), since extension of the chain linking the two pyridinium nitrogens results in a decrease of the acidity of both oxime groups. For the above reasons, at physiological pH the proportion of active oxime anions will be higher in bispyridinium aldoximes than in corresponding mono-aldoximes.

The sequence of pyridinium aldoximes (I–III) shown in Table 2 is conspicuous for an increasing ability to reactivate acetylcholinesterase inhibited by tetraethyl pyrophosphate (TEPP) or paraoxon *in vitro*. The marked superiority of compound II to compound I (factor 120) may be due not only to an additional binding contribution at an optimal distance, but also to an increase in ionization. Introduction of a second oxime group again enhances the proportion of oxime anions and augments the rate of reactivation by a factor of 2·5. These data, however, do not permit a decisive conclusion, whether TMB-4 owes its superiority exclusively to the increased ionization of the first oxime group or to the presence of a second oxime group, possessing an additional reactivating function.

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Mathematics of three-phase *in vitro* absorption models

SIR,—The use of three-phase *in vitro* models and compartmental kinetics to study the drug absorption process would appear to be of great value to pharmacy. Recent reports by Perrin (1966, 1967), who used such a model, contain an error. Perrin pointed our that if the volumes of the compartments within a system differed, they must be taken into account in any kinetic analysis of data obtained from the system. The example used was for the following set of consecutive first order processes:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

in which A, B and C represent the concentrations in three compartments with volumes V_A, V_B and V_C. The differential equations describing the kinetics were then given as:

$$V_A dA/dt = -V_A k_1 A \quad .. \quad (1)$$

$$V_B dB/dt = V_A k_1 A - V_B k_2 B \quad .. \quad (2)$$

$$V_C dC/dt = V_B k_2 B \quad .. \quad (3)$$

Using the conditions that $V_A = V_C$, and that at time zero, $A = A_0$, $B = C = 0$, the integrated equations were given as:

$$A = A_0 \exp^{-k_1 t} \quad .. \quad (4)$$

$$B = \frac{V_A k_1 A_0}{V_B(k_2 - V_A k_1/V_B)} (\exp^{-V_A k_1 t} - \exp^{-k_2 t}) \quad .. \quad (5)$$

$$C = A_0\left[1 - \frac{1}{k_2 - V_A k_1/V_B}(k_2 \exp^{-k_1 t} - k_1 \exp^{-k_2 t} - \frac{V_A k_1}{V_B}\exp^{-k_1 t} + k_1 \exp^{-V_A k_1 t/V_B})\right] \quad .. \quad (6)$$

Eqn 4 results from a straightforward integration of eqn 1 and is correct as given. Eqns 5 and 6, however, cannot be obtained by integrating the corresponding differential eqns 2 and 3. The volume terms in the integrated equations are inconsistent with the differential equations. This can be shown by inserting eqn 4 into eqn 2 to give:

$$dB/dt = \frac{V_A}{V_B} k_1 A_0 \exp^{-k_1 t} - k_2 B \quad .. \quad (7)$$

$$\text{or } dB + k_2 B dt = \frac{V_A}{V_B} k_1 A_0 \exp^{-k_1 t} dt \quad .. \quad (8)$$

which is a linear differential equation of the first order. It can, therefore be

integrated through use of an integrating factor (Rainville, 1959). The integrating factor is equal to $\exp^{k_2}\int^{dt}$ or $\exp^{k_2 t}$. The equation thus becomes:

$$B \exp^{k_2 t} = \frac{V_A}{V_B} k_1 A_0 \int \exp^{k_2 t} \exp^{-k_1 t} dt + K \quad .. \quad .. \quad .. \quad .. \quad (9)$$

in which K is a constant of integration. Using the condition that at time zero, B = 0, the equation can be integrated readily and re-arranged to give:

$$B = \frac{V_A k_1 A_0}{V_B (k_2 - k_1)} (\exp^{-k_1 t} - \exp^{-k_2 t}) \quad .. \quad .. \quad .. \quad .. \quad .. \quad (10)$$

Consecutive first order reactions involving three species are treated in all standard textbooks of chemical kinetics, including Frost & Pearson (1961). The only difference between reaction kinetics and compartmental kinetics would be that volume terms are included in the differential equations for the latter if the volumes of the compartments differ. Thus, the standard differential equation for reaction kinetics, analogous to eqn 2 would be:

$$dB/dt = k_1 A - k_2 B \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (11)$$

and the solution is generally given as:

$$B = \frac{k_1 A_0}{k_2 - k_1} (\exp^{-k_1 t} - \exp^{-k_2 t}) \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (12)$$

Equations 11 and 2 differ only in the appearance of the V_A/V_B term in eqn 2. Thus it might be logical to assume that the solution to eqn 2 could be obtained by inserting the term $V_A k_1/V_B$ wherever k_1 appears in the solution to eqn 11, which is eqn 12. This would indeed yield eqn 5. Thus, the comparison of apparently analogous equations instead of integrating eqn 2 might yield the incorrect eqn 5, instead of the correct eqn 10.

Since eqn 3 can only be integrated using the solution to eqn 2, the use of eqn 5, if it is not the correct solution, would indicate that eqn 6 is also not correct. The correct solution to eqn 3 can be found as follows:

$$dC/dt = \frac{V_B}{V_C} k_2 B = \frac{V_A k_2 k_1 A_0}{V_C (k_2 - k_1)} (\exp^{-k_1 t} - \exp^{-k_2 t}) \quad .. \quad .. \quad .. \quad (13)$$

in which the term B was replaced with eqn 10. This equation can be integrated in a straightforward manner under the conditions that $V_A = V_C$, and that at time zero, C = 0 to give:

$$C = A_0 + \frac{k_1 A_0}{(k_2 - k_1)} \exp^{-k_2 t} - \frac{k_2 A_0}{(k_2 - k_1)} \exp^{-k_1 t} \quad .. \quad .. \quad .. \quad (14)$$

which can be re-arranged to give the final equation:

$$C = A_0 \left[1 + \frac{1}{k_1 - k_2} (k_2 \exp^{-k_1 t} - k_1 \exp^{-k_2 t}) \right] \quad .. \quad .. \quad .. \quad (15)$$

There are a number of means whereby the validity of eqns 5 and 10 can be tested. Differentiating them and setting the derivatives equal to zero, will give the time, t_{max}, at which B reaches a maximum. Thus, for eqn 5:

$$t_{max} = \frac{1}{k_2 - V_A k_1/V_B} \ln \frac{k_2}{V_A k_1/V_B} = 2{\cdot}6 \text{ hr} \quad .. \quad .. \quad .. \quad .. \quad (16)$$

and from eqn 10:

$$t_{max} = \frac{1}{k_2 - k_1} \ln \frac{k_2}{k_1} = 3{\cdot}6 \text{ hr} \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (17)$$

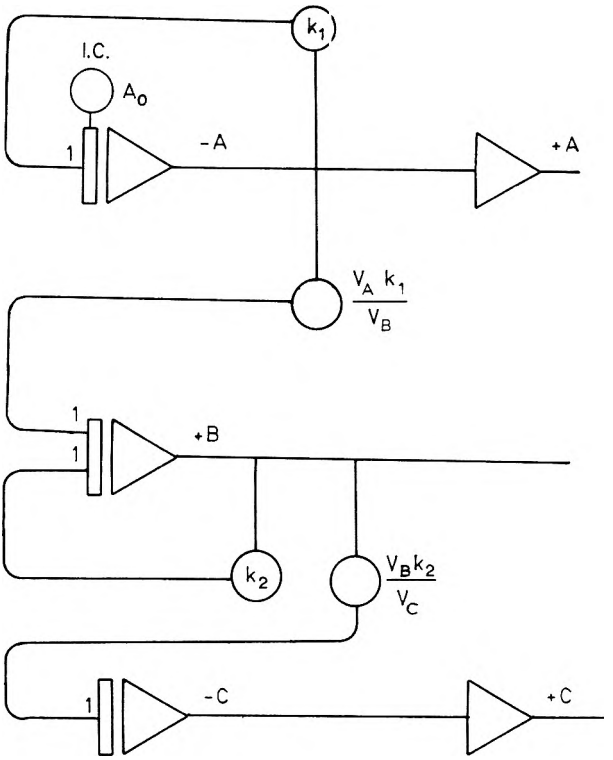

FIG. 1. Analogue computer programme for the solution to eqns 1–3. The time constant was chosen to be 1·00. $A_{max} = B_{max} = C_{max} = 30$ mg/litre.

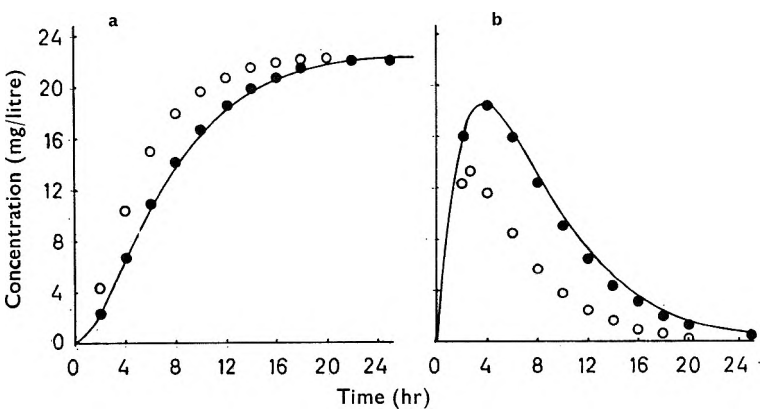

FIG. 2 (a). Plots of C versus time for transfer of salicylic acid from pH 2·0 to pH 7·4. through a layer of 30% decanol in cyclohexane. Constants used were: $k_1 = 0{\cdot}34$ hr^{-1}, $k_2 = 0{\cdot}22$ hr^{-1}, $V_A = 550$ ml, $V_B = 300$ ml, and $V_C = 550$ ml. The line represents the analogue computer solution, ● represents eqn 15 and ○ represents eqn 6. (b) Plots of B versus time for transfer of salicylic acid from pH 2·0 to pH 7·4 through a layer of 30% decanol in cyclohexane. Constants used were: $k_1 = 0{\cdot}34$ hr^{-1}, $k_2 = 0{\cdot}22$ hr^{-1}, $V_A = 550$ ml, $V_B = 300$ ml. The line represents the analogue computer solution, ● represents eqn 10, and ○ represents eqn 5.

We realize that the reduction in size that results from scaling a submitted figure down to publication size would lead to a loss in accuracy in obtaining t_{max} from the published figure. However, Fig. 3 from Perrin (1967) would lead us to believe that the maximum in B is reached much closer to 3·6 hr than 2·6 hr (estimated from the Figure is 3·6 hr). Thus, we can be fairly certain that eqn 10, rather than eqn 5 is correct.

Assuming that eqns 1, 2 and 3 are correct, an analog computer programme can be written and a tracing of B versus time can be plotted on an X-Y recorder using the published values for the rate constants and the volumes (Perrin, 1967). Eqns 5 and 10 can also be solved numerically using the same constants and various values of time to give values for B as a function of time. This was done with the analogue computer (Pace Tr-20) programme being shown in Fig. 1. The various values for B versus time are shown in Fig. 2. Since the computer tracing for B versus time represents the integration of eqn 2, any integrated equation which is claimed to be the solution to eqn 2 must yield data matching the computer tracing for B versus time. Fig. 2 shows clearly that eqn 10 is the correct solution to eqn 2, while eqn 5 cannot be correct. The value for t_{max} from Fig. 2 is found to be 3·6 hr which agrees with the value obtained from eqn 17, as indeed it should. The value of $t_{max} = 3{\cdot}6$ hr can be substituted into eqn 10 to find B_{max}, and $t_{max} = 2{\cdot}6$ hr can be substituted into eqn 5 to find B_{max}. The value of $B_{max} = 18{\cdot}4$ mg/litre from eqn 10 is in perfect agreement with the value of 18·4 mg/litre obtained from the computer drawn line in Fig. 2. The value of $B_{max} = 13{\cdot}4$ mg/litre, from eqn 5, does not agree at all with the result produced by the computer integration of eqn 2.

The same treatment for C versus time is shown in Fig. 3. Here it is obvious that eqn 15, rather than eqn 6 is the correct solution for eqn 3.

If the model described by eqns 1–3 is to be used for drug absorption studies in three-phase *in vitro* systems, and the integrated expressions are to be used for discussion of the results, we suggest that eqns 10 and 15 be used instead of eqns 5 and 6.

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Gas chromatography of Indian hemp (*Cannabis sativa* L.)

SIR,—The use of gas chromatographic analysis for characterizing preparations of *Cannabis sativa* L. has recently been described by Betts & Holloway (1967) and Heaysman, Walker & Lewis (1967). We have also applied this technique to the detection of cannabis preparations as a complement to the thin-layer chromatographic method of Caddy & Fish (1967).

In agreement with Heaysman & others (1967) we found a combination of inert supports and low percentages of polar stationary phases, coupled with moderate oven temperatures to give the most satisfactory resolution. We found, too, that silicone polymers offer worthwhile advantages of stability and column life over the non-silicone polymeric materials.

We used columns of XE-60, a cyanoethyl silicone polymer; either 4% on Chromosorb W-AW-DMCS-100/120 mesh or 1% on Chromosorb G, AW-DMCS-100/120 mesh. These we found superior to those incorporating SE-30 (Betts & Holloway, 1967; Claussen, Borger & Korte, 1966), SE-52 (Claussen & others, 1966) and OV-17 (Lerner & Zeffert, 1966). Under the stated conditions, symmetrical and reproducible peaks are obtained for unmodified phenolic constituents extracted by chloroform maceration or percolation from 50–100 mg amounts of cannabis preparations (Fig. 1A). Peaks were identified by the use of reference compounds kindly supplied by Dr. U. Claussen, Organic Chemistry Institute, Bonn and also with compounds we have separated on thin-layer chromatograms.

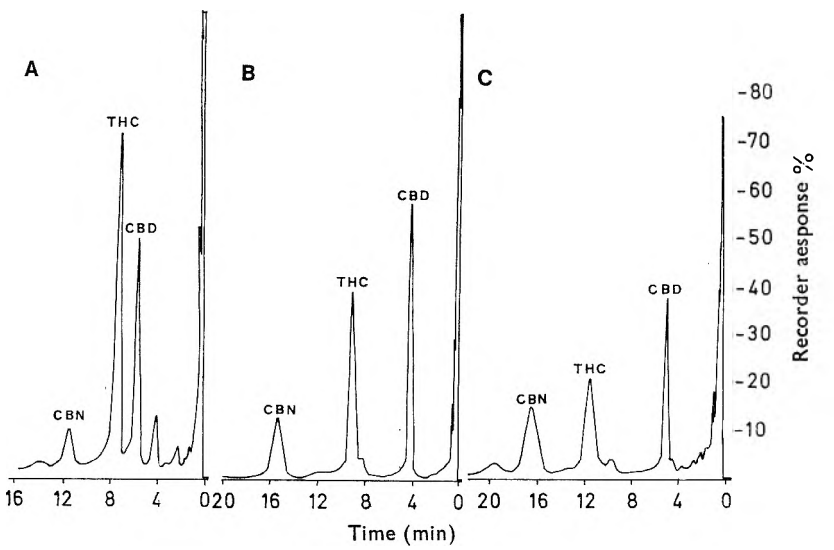

FIG. 1. Typical gas chromatogram of: A. Unmodified constituents of cannabis resin residue from 50 mg in 50 μl chloroform — 1 μl injected). Conditions: Perkin-Elmer F-11 Mk 1 with FID. Spiral glass column 6 ft × 0·3 mm i.d., 4% XE–60 on Chromosorb W. Oven 200°, injection block 250°. Nitrogen (oxygen free) 65 ml/min at Pi 45 psig, hydrogen 18 psig, air 40 psig. Attentuation 20 × 10^2. Chart speed 15 in/hr. B. Trimethylsilyl derivatives of cannabis constituents. Conditions differing from A: 1% XE–60 on Chromosorb G. Oven 165°, injection block 200°. C. Trifluoroacetyl derivatives of cannabis constituents. Conditions differing from A: Oven 150°, injection block 200°. CBD = cannabidiol, THC = tetrahydrocannabinol, CBN = cannabinol.

The resolution is improved by use of either trimethylsilyl ethers (Fig. 1B) or trifluoroacetyl esters (Fig. 1C). The silylation was essentially as described by Makita & Wells (1963), except that we used equal quantities of the two silylating reagents added to the drug residue dissolved in an equal volume of anhydrous pyridine or anhydrous isopropylamine (50–100 μl). Reaction was practically instantaneous, small aliquots being injected after centrifuging to sediment ammonium chloride. There was no difference in the reaction rates of the differing cannabinols.

The repeated use of TMSi-ethers and injection of excess silylating reagents results in contamination of a flame-ionization detector with silicates. Deposition can be delayed appreciably by injection of the isolated ethers in anhydrous hexane or carbon disulphide, but molecules containing silica are still being injected. This led us to the use of trifluoroacetyl esters. These derivatives have been used as adjuncts to the gas chromatography of steroids (van den Heuvel, Sjövall & Horning, 1961) and polar nitrogen containing compounds such as aromatic amines (Dove, 1967). They offer a suitable alternative to the TMSi-ethers and would have the further advantage of being detectable in minute amounts using an electron capture detector. Preparation is effected by refluxing the residue with 0·1 ml trifluoroacetic anhydride and 0·5–1 mg anhydrous sodium acetate, at 70° on a water-bath for 10 min. The excess fluids are driven off with a steady stream of dry air and the residue dissolved in dry acetone for gas chromatography.

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An interaction between the antidepressant drugs desipramine and modaline sulphate

SIR,—Modaline sulphate is a non-hydrazine monoamine oxidase inhibitor, also showing imipramine-like activity and clinical antidepressant effects (Feldman, 1963; Dunlop, De Felice & others, 1964).

This compound, however, requires an initial biotransformation by liver microsomal enzymes, to an active unknown intermediate, before the anti-monoamine oxidase-like (Dubnick, Morgan & Phillips, 1963; Horita, 1966) or the imipramine-like (Jori, Carrara & others, 1965) effects can be demonstrated. On the other hand, tricyclic antidepressant agents, such as imipramine and desipramine, are strong inhibitors of some liver microsomal enzymes important for drug metabolism.

This biochemical activity may be responsible for several interactions of imipramine-like agents with various central nervous system-active drugs. In fact, imipramine potentiates the barbiturate sleeping time by inhibiting the metabolic degradation of pentobarbitone (Kato, Chiesara & Vassanelli, 1963), it shows an anti-tremorine activity by preventing the biotransformation of tremorine to its active metabolite, oxotremorine (Sjöqvist & Gillette, 1965; Hammer & Sjöqvist, 1966) and it potentiates and prolongs the behavioural and hyperthermic effects of amphetamine (Valzelli, Consolo & Morpurgo, 1966) by reducing its hydroxylation to *p*-hydroxyamphetamine (Consolo, Dolfini & others 1967). It was, therefore, interesting to investigate the interaction of desipramine on the pharmacological effects of modaline sulphate.

Experiments *in vivo* and *in vitro* were made to evaluate the potency of the monoamine oxidase inhibition after modaline in rats pretreated with desipramine or SKF 525 A, another compound known to inhibit several liver microsomal enzymes (Brodie, Gillette & La Du, 1958).

Monoamine oxidase inhibition was measured *in vivo* by potentiation of tryptamine symptomatology according to Tedeschi, Tedeschi & Fellows (1959) and the results obtained are in Fig. 1. Fig. 1A represents the effect of desipramine at various dose levels. The activity of modaline is strongly reduced

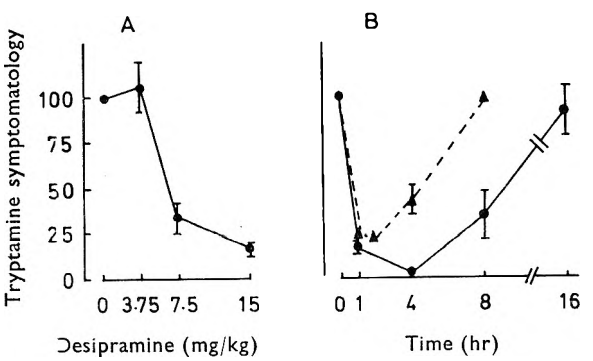

FIG. 1. The tryptamine symptomatology (hunching of the back, backward locomotion, Straub tail, salivation and clonic convulsions of the anterior paws) was scored according to an arbitrary scale. The maximum potentiation induced by modaline was calculated = 100. Vertical bars represent the standard error. A. Desipramine was given intraperitoneally 1 hr before modaline. Modaline (8 mg/kg, i.p.) was injected 30 min before tryptamine (7·5 mg/kg, i.v.). B. ●———● Desipramine (15 mg/kg, i.p.) or ▲ - - - ▲ SKF 525 A (50 mg/kg, oral) was given at various times before modaline and tryptamine.

TABLE 1. MONOAMINE OXIDASE ACTIVITY IN BRAIN HOMOGENATES OF RATS

No. of rats	Treatment	mg/kg	Kynuramine oxidized (μmoles/hr/mg protein) ± s.e.
10	Controls ..		0·033 ± 0·001
10	Desipramine ..	15	0·034 ± 0·001
10	SKF 525 A ..	50	0·034 ± 0·002
15	Modaline ..	8	0·005 ± 0·001*
10	Desipramine ..	15	
	+		
	modaline ..	8	0·012 ± 0·001†
6	SKF 525 A ..	50	
	+		
	modaline ..	8	0·014 ± 0·002†

Desipramine or SKF 525 A was given intraperitoneally and orally, respectively, 1 hr before modaline. Rats were killed 45 min after modaline. Brains were removed, frozen, homogenized and incubated with the substrate kynuramine according to the method of Weisbach & others (1960). Values are expressed as μmoles/hr/mg of protein.

* $P < 0.01$ relative to controls.

† $P < 0.01$ relative to the group receiving modaline only.

by 7·5 and 15 mg/kg of desipramine. The inhibition induced by 15 mg/kg of desipramine is long-lasting and it is still present after 8 hr (Fig. 1B). Desipramine alone did not modify at any dose the response to tryptamine. SKF 525 A is also active at 50 mg/kg, but when the time interval between the injection of SKF 525 A and modaline was more than 4 hr, the interaction disappeared.

The effect of desipramine was also present after repeated treatments (70% inhibition after 7 days of treatment), but it was reversible and completely disappeared 5 days after the last treatment.

The monoamine oxidase inhibition *in vitro* was measured by the Weissbach method (Weissbach, Smith & others, 1960) on brain homogenates, using kynuramine as oxidizable substrate. Table 1 shows that the monoamine oxidase activity of brain of rats treated *in vivo* with modaline is blocked, but it may be partially restored if desipramine or SKF 525 A are given previously. These agents, at the dose used, are unable to affect by themselves the monoamine oxidase activity in brain.

The *in vitro* data corroborate those obtained *in vivo* on the potentiation of tryptamine symptomatology, showing that the decreased activity of modaline sulphate in desipramine-pretreated rats may be dependent on a reduced inhibition of the monoamine oxidase enzymatic system. On the other hand, these data emphasize that a strong inhibition of monoamine oxidase is necessary to elicit potentiation of tryptamine symptomatology in rats, and that a partial recovery of the enzymatic activity is able to prevent it.

As modaline acts on monoamine oxidase by means of its metabolite (Dubnick & others, 1963; Horita, 1966) it can be tentatively concluded that desipramine reduces the activity of modaline by inhibiting its biotransformation at the level of the hepatic microsomes.

These experimental observations may assume some practical importance as desipramine and modaline belong to the same category of antidepressant drugs and might be associated in clinical treatment.

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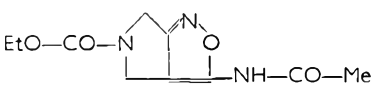
Effects of ethyl 3-acetamido-4*H*-pyrrolo[3, 4-*c*]-isoxazole-5(6*H*)carboxylate on tissue levels of catecholamines and 5-hydroxytryptamine in the rat

SIR,—Various agents have been found which cause a marked lowering of the tissue levels of catecholamines or 5-hydroxytryptamine (5-HT) or both. Some of these depletors, such as α-methyldopa, guanethidine or reserpine, are used in hypertension. The compound, ethyl 3-acetamido-4*H*-pyrrolo-[3,4-*c*]isoxazole-5(6*H*) carboxylate (I;CL-62375), has recently been found to cause hypotension in the rat. We now report that administration of this compound to the rat causes alterations in the tissue levels of catecholamines and 5-HT.



I

Brain catechclamine levels (Lippmann & Wishnick, 1965), brain 5-HT (Bogdanski, Pletscher & others, 1956), heart noradrenaline (Anton & Sayre, 1962) and adrenal catecholamines (Lippmann & Wishnick, 1965), were measured in female rats, Sherman strain, of about 150 g.

CL-62375 was administered intraperitoneally in a single injection (0·5 ml 1% starch, M/15 potassium phosphate buffer, pH 7·0) at 100, 150, 250 or 400 mg/kg and the animals were decapitated 5 hr later. In the heart there was a decline in the noradrenaline level of 70, 42 and 35% at 250, 150 and 100 mg/kg, respectively. In the brain there was a lowering in the catecholamine content of 70% at 250 mg/kg and 40% at 150 mg/kg. The brain 5-HT showed a maximum decline of 30% at the 250 mg/kg level. The 400 mg/kg level was lethal. Thus, there was an appreciable effect on the catecholamine levels in the heart and brain

whereas there was only a slight effect on the 5-HT in the brain. The animals were not sedated and showed only a slight ptosis.

A dose of 50 mg/kg of CL-62375 was administered intraperitoneally three times at 3 hrly intervals and the animals were killed 2 hr after the last injection. A decline of 59% in the noradrenaline level of the heart (μg/g $\pm$ s.e.: control 1·07 $\pm$ 0·06; treated 0·44 $\pm$ 0·04, $P < 0·001$) and a 33% decline in the catecholamine content (μg/g $\pm$ s.e.: control 0·33 $\pm$ 0·03; treated 0·22 $\pm$ 0·006, $P < 0·05$) of the brain were observed. There was a 59% lowering of the brain 5-HT (μg/g $\pm$ s.e.: control 0·83 $\pm$ 0·16; treated 0·34 $\pm$ 0·02, $P < 0·001$). A 31% drop in the catecholamine level of the adrenals also occurred (μg/pair $\pm$ s.e.: control 22·22 $\pm$ 0·96; treated 15·33 $\pm$ 0·27, $P < 0·001$). After the first treatment the animals exhibited a slight ptosis and were not sedated; subsequent treatments did not cause sedation.

To determine the duration of the effects of repeated administration of CL-62375, the animals received 3 injections (50 mg/kg, i.p.) at 3 hrly intervals. The level of noradrenaline in the heart declined 59, 67 and 47% at 2, 8 and 18 hr, respectively, after the last treatment. Thus under these conditions a maximum depletion was observed at 8 hr and the levels were still appreciably lowered after 18 hr. In the brain, the catecholamine levels dropped 33% at 2 hr after the last dose and no significant reduction was observed after 8 or 18 hr. The brain 5-HT was lowered 59, 67 and 40% after 2, 8 and 18 hr.

A decline in the endogenous biogenic amine levels may arise from an alteration in the storage mechanisms; i.e., uptake and release, or an alteration in the synthesis of the amines. The effects of CL-62375 can be compared with compounds exhibiting these activities; i.e., α-methyl-*m*-tyrosine, a releaser, and α-methyltyrosine, a synthesis inhibitor. After a single administration CL-62375, α-methyl-*m*-tyrosine (Hess, Connamacher & others, 1961; Weissmann & Koe, 1965) and α-methyltyrosine (Spector, Sjoerdsma & Udenfriend, 1965) are similar in that they cause an appreciable depletion of both heart and brain catecholamines and cause only a small or no decrease in 5-HT levels. After repeated administration CL-62375 exhibits a decline in catecholamines similar to that produced by α-methyltyrosine (Spector & others, 1965); in contrast, CL-62375 causes a large decrease in the brain 5-HT whereas α-methyltyrosine has no effect (Spector & others, 1965). The noradrenaline-depleting action of CL-62375 might thus be the basis for its hypotensive activity.

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