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Effects of salicylate on RNA polymerase activity and on the incorporation of orotic acid and thymidine into the nucleic acids of rat foetuses *in vitro*

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Salicylate (10 mM) significantly inhibited the activity of RNA polymerase and the incorporation of radioactivity from orotic acid-5-^{3}H and thymidine-6-^{3}H into whole 13 day and 16 day rat foetuses. The polymerase activity and the incorporation of the labelled orotate were significantly inhibited by 2 mM salicylate in the 16 day foetus only. The inhibitory action of salicylate on RNA biosynthesis may be related to its effects in causing an increased incidence of foetal death and congenital malformation in pregnant rodents.

The chronic administration of acetylsalicylic acid to pregnant rats and mice causes a high incidence of foetal death (Obbink & Dalderup, 1964). A single injection of either methyl or sodium salicylate during gestation in these species not only produces foetal death and premature birth (Eriksson & Larsson, 1968) but also several congenital malformations of the litters carried to full term (Warkany & Takacs, 1959; Larsson, Ericson & Bostrom, 1963). The major foetal abnormalities comprise skeletal anomalies and disturbances of vascular and neural development. The day or days of pregnancy on which the salicylates are given appear to determine which type of abnormality predominates. Thus, the highest incidence of skeletal anomalies in mice were observed after injection on the 9th day of gestation whereas vessel anomalies were the most prominent after injections on the 15th day (Larsson & Eriksson, 1966). These findings suggest that each organ or system may have a critical period when it is most susceptible to the teratogenic action of salicylate.

Salicylate could be teratogenic either by interfering with placental function or by directly affecting metabolic reactions in the foetal tissues. The drug uncouples oxidative phosphorylation reactions (Brody, 1956), interferes with the biosynthesis of mucopolysaccharides (Larsson & Bostrom, 1965), inhibits the activities of dehydrogenase, decarboxylase and aminotransferase enzymes (Smith, 1968) and impairs protein synthesis (Dawkins, Gould & Smith, 1966) in animal tissues. A further, and perhaps more relevant, action is that salicylate inhibits the activity of nucleic acid polymerases prepared from rat liver (Janakidevi & Smith, 1969) and interferes with the biosynthesis of nucleic acids in adult mice (Janakidevi & Smith, 1970). We have, therefore, studied the effects of sodium salicylate on the activity of RNA polymerase and on the incorporation of radioactivity from labelled orotic acid and thymidine into the nucleic acids of rat foetuses at varying stages of development.

EXPERIMENTAL

Animals

Female rats of the Wistar strain, 300 to 400 g, were mated and the day in which sperm was found in vaginal smears was considered to be the first day of pregnancy.

Groups, each of six animals, were killed by stunning and cervical fracture on the 13th, 15th, 16th and 19th day of pregnancy and the foetuses, freed from the foetal membranes, were placed in an ice-cold solution containing 0·1M tris-HCl, pH 7·5, 0·01M $MgCl_2$ and 0·25M sucrose (TMS medium).

Materials

Orotic acid-5-^{3}H (specific activity 1Ci/mmol), thymidine-6-^{3}H (specific activity 5Ci/m mol) and UTP-5-^{3}H (specific activity 1·5 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks. Calf thymus DNA, UTP, CTP, GTP, ATP and orcinol were obtained from the Sigma Chemical Co., St. Louis, RNA from the Boehringer Corporation (London) Ltd. and diphenylamine from Hopkins and Williams Ltd., Chadwell Heath, Essex. Sodium salicylate was B.P. grade, all other chemicals were of analytical grade and glass distilled water was used throughout.

Measurement of RNA polymerase activity

Between 30 and 40 foetuses were homogenized in 10 volumes of ice-cold 0·32M sucrose, containing 3 mM $MgCl_2$, using an all-glass homogenizer. The nuclei were isolated and purified as described previously for adult rat liver. RNA polymerase activity was estimated at 17°, to minimize interference from ribonuclease, by measuring the incorporation of radioactivity from UTP-5-^{3}H into RNA in a Beckman LS 200B liquid scintillation system, using GF/A (2·1 cm) glass fibre discs (Janakidevi & Smith, 1969). The RNA product from the incubation mixtures had a DNA-like base composition.

Incorporation experiments

Single foetuses were each placed in 1 ml of the TMS medium containing 5 μCi of either the labelled orotic acid or thymidine plus either enough sodium salicylate to give a final salicylate concentration of either 2 or 10 mM or sufficient sodium chloride to produce the same final concentration of sodium. The mixture was incubated, with shaking, for 2 h at 37°. At the end of the incubation period the foetus was removed, washed with ice-cold TMS medium and homogenized in 3 ml of 6% (v/v) perchloric acid. The homogenate was centrifuged at 3000 *g* for 15 min and the residue re-extracted with 2 ml of the perchloric acid followed by 1 ml of water. The final residue was washed with two quantities of ethanol–ether mixture (3:1) to remove lipid material and the RNA and DNA extracted according to the directions of Widnell & Tata (1964; 1966). The specific activities of the extracted nucleic acids were measured by estimating the RNA content by the orcinol method (Hurlbert, Schmitz & others, 1954), the DNA content by the diphenylamine technique (Burton, 1952) and the radioactivity, in aliquots of 0·1 ml, using the Beckman liquid scintillation system and glass fibre discs.

RESULTS

The incorporation of radioactivity from the labelled orotic acid and thymidine into the whole foetuses obtained between the 13th and 19th day are given in Fig. 1. It was not possible to obtain adequate material to perform similar incorporation experiments on whole foetuses from earlier stages of pregnancy. In these and subsequent experiments an incubation period of 2 h was chosen because preliminary work showed that the incorporation of radioactivity from the orotic acid and thymidine into the nucleic acids of whole rat foetuses was linear over a period of 4 h.

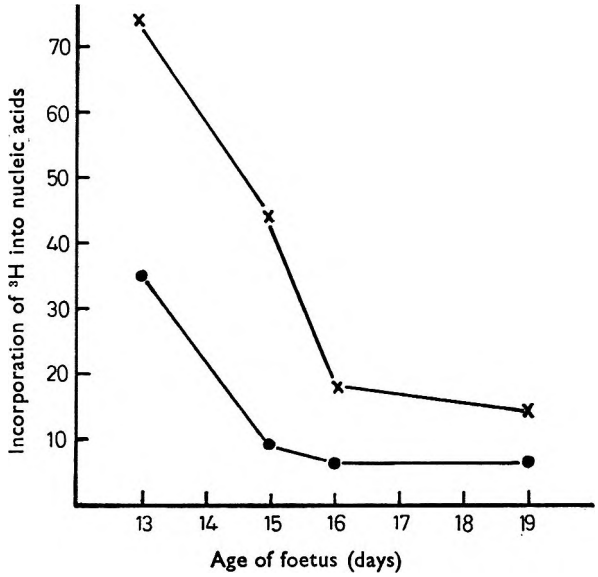

FIG. 1. Incorporation of orotic acid-5-^{3}H and thymidine-6-^{3}H into the nucleic acids of whole rat foetuses varying from 13 to 19 days old. Individual results represent the mean values from three or four experiments: ●, from orotic acid, expressed as counts/min × 10^{-2}/mg RNA isolated; ×, from thymidine, expressed as counts/min × 10^{-3}/mg DNA isolated.

The results in Fig. 1 show that the incorporation of the labelled precursors was highest in the 13 day foetus, had markedly decreased in the 16 day foetus and did not apparently change with increasing age of the foetus. The 13th and 16th day foetuses were therefore chosen to investigate the effects of salicylate.

The results, given in Table 1, show that the 16 day foetus was more sensitive than the 13 day foetus to the effects of salicylate. The high concentration of the drug (10 mM) significantly inhibited RNA polymerase activity and the incorporation of tritium from both orotic acid and thymidine in both foetuses where 2 mM salicylate caused significant inhibition of the polymerase and the orotate incorporation in the 16 day foetus only.

Table 1. *Effects of sodium salicylate on RNA polymerase activity and on the incorporation of orotic acid and thymidine into rat foetuses* in vitro. Each value is given as the mean ± standard deviation, the number of whole foetuses used in each experiment being given in parentheses. The results have been analysed by the *t*-test and * indicates a statistically significant decrease ($P < 0.05$) between the control and salicylate values.

Age of foetus (days)	Salicylate concentration (mM)	RNA polymerase activity (pmol/mg DNA)	Radioactivity incorporated From orotate (counts/min mg^{-1} RNA isolated)	Radioactivity incorporated From thymidine (counts/min mg^{-1} DNA isolated)
13	0	867 ± 213 (3)	3511 ± 267 (3)	74377 ± 19947 (3)
	2	908 ± 167 (3)	4675 ± 313 (3)	90684 ± 24696 (3)
	10	*347 ± 69 (3)	*116 ± 29 (3)	*5760 ± 2242 (3)
16	0	1191 ± 244 (6)	578 ± 45 (4)	16712 ± 2993 (4)
	2	*846 ± 207 (6)	*379 ± 44 (4)	14818 ± 3719 (4)
	10	*596 ± 205 (6)	*191 ± 25 (4)	*8789 ± 1756 (4)

DISCUSSION

The results of the present work show that 2 mM salicylate significantly inhibits the activity of RNA polymerase and the incorporation of radioactivity from tritiurated orotic acid, but not from labelled thymidine, into 16 day but not into 13 day whole rat foetuses. Thus the 2 mM salicylate appears to inhibit preferentially the biosynthesis of RNA rather than that of DNA in the 16 day foetus. The formation of both types of nucleic acid in the 13 and 16 day foetuses is inhibited by 10 mM salicylate.

These observations may bear some relevance to the increased incidence of foetal death and congenital malformations found to occur in pregnant rodents treated with large doses of the drug. Warkany & Takacs (1959) used a single subcutaneous injection of sodium salicylate in doses between 300 and 900 mg/kg in pregnant rats and these would be expected to produce tissue salicylate levels about 1 to 3 mM (Sturman, Dawkins & others, 1968). The increased sensitivity of the 16 day foetus to salicylate may be related to the increased incidence of foetal death and resorption in pregnant rodents which occurs as the drug is administered at an increasingly later stage in the pregnancy (Larsson & Eriksson, 1966). In addition, the predominance of vessel anomalies when salicylate was given on or about the 16th day of pregnancy in the mouse may reflect the relative susceptibility of this system to salicylate at this particular stage of gestation. It must be emphasized that the present results were obtained with whole foetuses and it will be necessary to extend the experiments to include foetuses of different ages and also to investigate separately individual organs and systems.

Acknowledgements

We wish to thank Miss M. Sandiford for expert technical assistance and the Nuffield Foundation for generous financial help.

Note added in proof. Since this paper was submitted for publication Richards (1969) has reported the results of a retrospective epidemiological study of congenital malformations in human pregnancy. The results showed that the taking of salicylate preparations in the first trimester of pregnancy is associated with significant increases in abnormalities of the central nervous system and the alimentary tract of the foetus. It was concluded that either salicylates have a teratogenic effect in man or that the conditions for which they are given have such an action.

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Preparation of active, drug-metabolizing, microsomal enzymes under optimal conditions and by iso-electric precipitation

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The influences of homogenization technique and of relative centrifugal force on the enzyme activity of hepatic microsomes have been investigated to establish optimal conditions for preparation. In general, a glass or Ultra Turrax homogenizer provided a satisfactory homogenate having maximal enzyme activity when nuclear and mitochondrial material were removed by centrifugation at >6500 *g* for 20 min. Esterase, UDP-transglucuronylase, reductase and oxidase activities of microsomes prepared from liver breis under optimal conditions from guinea-pig, rabbit and rat have been compared. The enzyme activities in microsomes prepared by iso-electric precipitation at pH 5·0 were similar to those prepared by differential centrifugation except for a 50–75% reduction in glucuronyl transferase activity observed in rabbit and guinea-pig.

Microsomes are currently prepared by modification of the method of Siekevitz (1962), the initial tissue breis being prepared by a variety of homogenization procedures. However, in spite of much published work on microsomes, the effect of the preparative procedure on microsomal yield and enzyme activity is poorly documented.

Lathe & Ricketts (1964), working with neonatal tissue and requiring maximum microsomal yield, established "conditions for maximal recovery of the microsome fraction from rabbit liver homogenates", but investigated only the influence of the time for which the microsomal fraction was centrifuged at 114 400 *g*.

Many enzymes are known to be stable between pH 4–10, and this stability has been exploited in the purification of some enzymes (Strelitz, 1944). Claude (1946) isolated microsomes by precipitation at an acid pH, but pH precipitation for preparing subcellular fractions has subsequently received little attention although the procedure is still occasionally used (Görlich & Heise, 1963).

I have therefore investigated the influence of the homogenization technique and the fractionation procedure (relative centrifugal force) on the microsomal yield and enzyme activity, and compared this with the yield and activity of microsomes prepared by iso-electric precipitation at pH 5·0.

A preliminary report of this work was presented at the Fourth Meeting of the Federation of European Biochemical Societies (Mitchard, 1967).

Subsequently, Karler & Turkans (1968) also investigated the use of precipitation at acid pH for the preparation of microsomes for drug metabolism studies, and they

reported that precipitation at pH 5·0 produces a preparation which metabolizes hexobarbitone at a similar rate to preparations prepared by centrifugation.

EXPERIMENTAL

Materials

Glucose-6-phosphate, NADPH and uridine diphosphoglucuronic acid (C. F. Boehringer and Soehne G.m.b.H., Mannheim, Germany). Acetylsalicylic acid, nicotinamide, nitrobenzoic acid, *o*-aminophenol (technical) and *N*-1-naphthylethylenediamine dihydrochloride (British Drug Houses Limited, Poole, Dorset). Riboflavin (Sigma Chemical Company, St. Louis, Mo.). Benzphetamine (Upjohn Company, Kalamazoo, Mich., U.S.A.). The *o*-aminophenol was purified by recrystallization from aqueous ethanol. Young adult male Wistar albino rats (250–300 g), Hartley albino guinea-pigs (350–400 g) and Dutch rabbits (1·5–2·0 kg), fasted for 24 h, were used. They were killed by decapitation after stunning.

Methods

Preparation of liver sample. The livers were removed immediately after death, rinsed in ice-chilled isotonic KCl and divided into one 20 g and three 10 g (±100 mg) portions. If several livers were required to yield 50 g, three portions of equal and one of double weight were removed from each liver to give a total of three 10 g and one 20 g samples. The three 10 g liver samples were each placed in 20 ml of ice-chilled isotonic KCl and cut into small pieces (*ca* 5 mm^3). All subsequent operations were at 2–4°.

Homogenization procedure. One 10 g sample (A) was homogenized in a Potter-Elvejheim (PE) glass homogenizer with a Teflon pestle (diam. 19 mm) having a 0·10–0·15 mm clearance. Three up and down strokes were used during a period of 60 s whilst the pestle speed increased from an initial 2500 rev/min to the pre-set speed of 4000 rev/min. The second 10 g sample (B) was homogenized by an Ultra-turrax (UT) homogenizer (Janke and Kunkel K.G., Stanfen i. Br., Germany) for two periods of 5 s (5 s pause) in a Potter-Elvejheim glass mortar which had an internal diameter 1·5 mm larger than the external diameter of the homogenizer tube. The third 10 g sample (C) was placed in a 100 ml vortex beaker and homogenized for six 10 s periods (5 s pauses) by an MSE homogenizer rotating at 12000 rev/min. The 20 g sample was forced through a nylon mesh (St. Martin's Bolting Cloth, 142 μm) and a 10 g sample (D) of the homogenate weighed. The volume of each homogenate was adjusted to 50 ml with isotonic KCl.

Subfractionation of microsomes. The relative centrifugal force used to separate the more dense homogenate material was investigated and homogenates (2 × 20 ml) were centrifuged at between 1000 and 12000 *g* for 20 min in a rotor SS34 of a Sorvall Superspeed RC2-B. The decanted supernatant (2 × 15 ml) was (i) centrifuged in an MSE Superspeed 40 at 140 000 *g* for 45 min, or (ii) adjusted to pH 5·0 by the gradual addition of 0·02M acetate buffer pH 4·0 and the precipitate collected by centrifugation in the Sorvall Superspeed at 6500 rev/min for 10 min, to give a microsomal pellet above a small glycogen pellet. The microsomes prepared as in (i) were washed by resuspending in isotonic KCl and recentrifuged. When microsomes were prepared by procedure (ii), the supernatant was divided into two equal parts before centrifugation, and one part was treated as in (i) whilst the other as in (ii). All microsomal

preparations were suspended in 0·05M phosphate buffer at 7·4, the final volume being adjusted to 15 ml.

Enzyme activities

Assay of esterase (EC.3.1.1.6). Activity of a 50-fold dilution of the microsomal preparations in 0·05M phosphate buffer pH 7·4 was assayed by the method of Howes & Hunter (1968) using 2×10^{-3}M acetylsalicylic acid as substrate. The change in absorbance at 295 nm was continuously monitored during the 30 min incubation period at 37° in a Gifford-Unicam model 2000 dual wavelength spectrophotometer.

Assay of UDP-transglucuronylase (EC.2.4.1.17). Activity was assayed essentially by the method of Storey & Dutton (1955). Reaction mixtures contained 1·5 μmol of UDPGA, 10 μmol of $MgCl_2$, 1·5 μmol *o*-aminophenol and 6 μmol of ascorbic acid in a total volume of 3·0 ml of 0·05M phosphate buffer pH 7·4. One ml of microsomal preparation was added to start the reaction, which was incubated at 37° for 30 min in a Mickle shaking incubator. The reaction was stopped by the addition of 1 ml of 7·5% trichloroacetic acid. Controls consisted of the above system in which the microsomal preparation was replaced by water or in which the trichloroacetic acid was added to the reaction mixture before the microsomal preparation. The precipitated protein was removed by centrifugation.

Assay of nitroreductase (EC.1.6.99.1). Activity was assayed in an atmosphere of N_2 at 37° by the procedure described by Umar & Mitchard (1968), using *p*-nitrobenzoic acid as substrate. *Assay of* N-*demethylating ability*. The assay system contained in a total volume of 5·0 ml: 0·05M phosphate buffer, pH 7·41, 20 μmol of NADPH, 4 μmol of semicarbazide (dissolved in 0·05M phosphate buffer pH 7·4 and neutralized with dilute ammonia to pH 7·4 before adding to incubate), 5 μmol of benzamphetamine, 2 ml of washed microsomes. Incubation was in an atmosphere of air at 37° for 60 min in a Mickle shaking incubator. The reaction was stopped by addition of 2 ml of $ZnSO_4$ and 2 ml of saturated solution of $Ba(OH)_2$, and the precipitate separated by centrifugation. The amount of formaldehyde semicarbazone produced was determined by the method of Nash (1953); 3 ml of Nash reagent being added to 3 ml of supernatant. Controls were prepared by adding the $ZnSO_4/Ba(OH)_2$ protein precipitant *before* the washed microsomes, or consisted of the above system minus the benzamphetamine.

Determination of protein. Microsomal protein concentrations were determined on samples diluted 20-fold by the method of Lowry, Rosebrough & others (1951).

In all assays involving spectrophotometry, a Unicam SP.800 (Unicam Instruments Ltd., Cambridge) was used.

Weight of freeze-dried material. To determine the amount of solid material in each microsomal preparation, samples (2 ml) of washed microsomes were freeze-dried in tared tubes on an Edwards freeze dryer, Model 10P (Edwards High Vacuum Ltd., Crawley).

RESULTS

These are presented from typical experiments in which values are compared only with others obtained during the same experiment from the same homogenate.

Influence of homogenization procedure on microsomal yield and enzyme activity. Table 1 shows the enzyme activities, protein concentrations and microsomal yields

Table 1. *Influence of technique used for homogenizing rabbit, rat and guinea-pig livers on protein yield and enzyme activity of microsomal preparations*

Method of homogenization	Esterase		Trans glucuronylase		Nitro-reductase		Demethylating activity		Protein mg/ml	Weight of freeze-dried material mg/ml
	Total activity μmol/ml h^{-1}	Specific activity μmol/mg protein	Total activity μmol/mg in 30 min	Specific activity μmol/mg protein	Total activity μmol/ml h^{-1}	Specific activity μmol/mg protein	Total activity μmol/ml h^{-1}	Specific activity μmol/mg protein		
Rabbit liver										
Ultra-Turrax	21·8	2·4	0·39	0·043	0·43	0·048	1·65	0·18	9·0	23·3
Potter-Elvejheim	11·1	2·1	0·16	0·030	0·22	0·042	1·48	0·28	5·2	13·9
MSE homogenizer	18·8	2·0	0·40	0·043	0·42	0·045	1·23	0·13	9·2	25·0
Nylon mesh (142 μmesh) ..	9·4	2·1	0·15	0·033	0·15	0·033	1·27	0·28	4·5	16·1
Rat liver										
Ultra-Turrax	59·8	5·0	0·108	0·009	0·31	0·016	0·34	0·028	11·9	31·4
Potter-Elvejheim	49·1	5·5	0·052	0·006	0·10	0·011	0·57	0·065	8·8	26·5
MSE homogenizer	53·0	4·9	0·108	0·010	0·27	0·025	0·23	0·021	10·8	30·6
Nylon mesh (142 μmesh) ..	49·1	5·7	0·032	0·004	0·13	0·015	0·67	0·078	8·6	26·2
Guinea-pig liver										
Ultra-Turrax	70·9	6·2	0·64	0·056	0·19	0·016	0·22	0·019	11·4	23·9
Potter-Elvejheim	53·8	6·9	0·26	0·033	0·06	0·007	0·30	0·038	7·8	21·3
MSE homogenizer	59·8	5·5	0·92	0·085	0·17	0·015	0·20	0·020	10·8	24·7
Nylon mesh (142 μmesh) ..	53·4	6·5	0·50	0·061	0·03	0·003	0·30	0·036	8·2	20·2

(in terms of weight of freeze-dried material) of rabbit, rat and guinea-pig liver microsomal suspensions respectively, as determined by the procedures described in this paper. In all cases there was a close correlation between protein concentration and

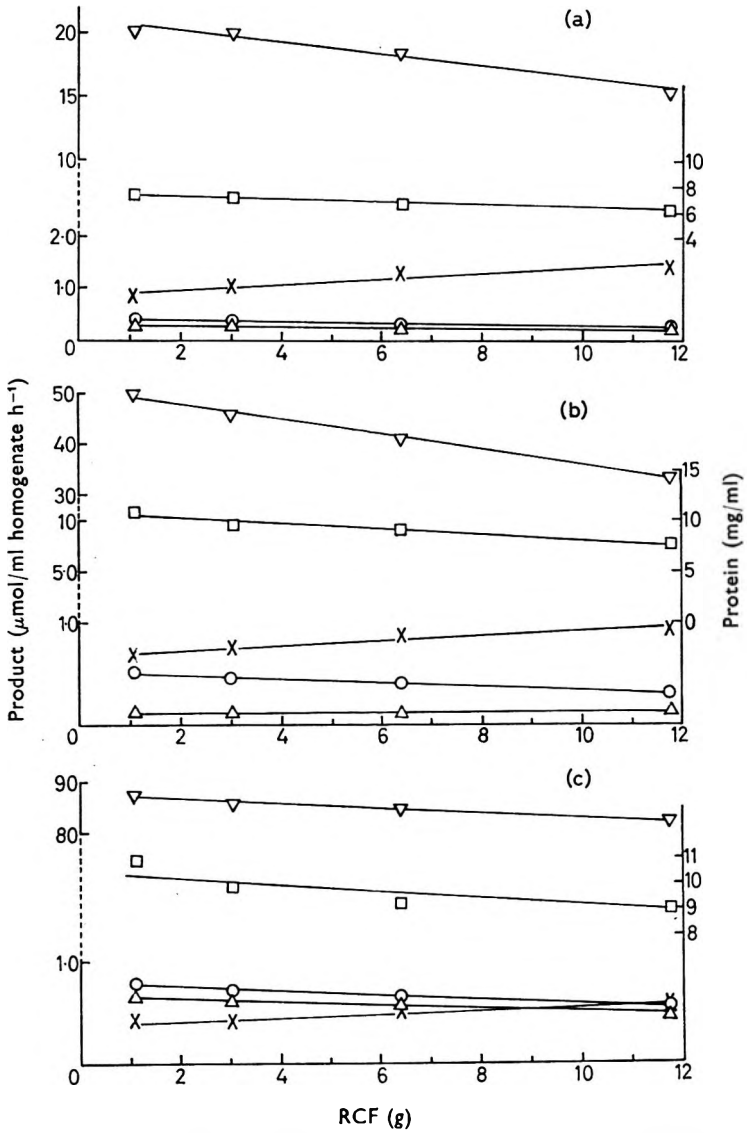

FIG. 1. Influence of the relative centrifugal force which was used to fractionate microsomes from rabbit (a), rat (b) and guinea-pig (c) livers on microsomal protein yield □—□ and microsomal esterase ▽—▽, transglucuronylase △—△,* nitro-reductase ○—○ and *N*-demethylating X—X activities. Experimental details are given in the text.

* Transglucuronylase activity expressed as μmol product/ml in 30 min.

microsomal yield, but there was a significant difference between values obtained for samples prepared with the UT or MSE homogenizers and those prepared by the PE and nylon mesh techniques. Although there were differences in microsomal yield and protein concentrations of samples prepared from different species, the UT and

MSE homogenizers always gave homogenates from which microsomal fractions having similar values of total enzyme activity were obtained and which were consistently higher than values obtained from samples prepared by the other procedures. In all three species the specific activity of *N*-demethylation (i.e. enzyme activity/mg protein) was much higher (2–3-fold), and the specific activity of esterase slightly higher, in samples prepared by the PE or nylon mesh techniques.

Influence of the relative centrifugal force (RCF), used to fractionate the homogenate, on microsomal yield and enzyme activity. Fig. 1 illustrates the effect of RCF, which was used to separate the more dense subcellular materials, on microsomal yield as measured by protein concentration and enzyme activities of rabbit, rat and guinea-pig preparations. The initial homogenate was prepared by the UT. Except for *N*-demethylating ability there was a relation between decrease in enzyme activity and decrease in microsomal yield. Interestingly, the activity of the enzyme responsible for *N*-demethylation increased as the microsomal content (as measured by the protein concentration of the preparation) decreased.

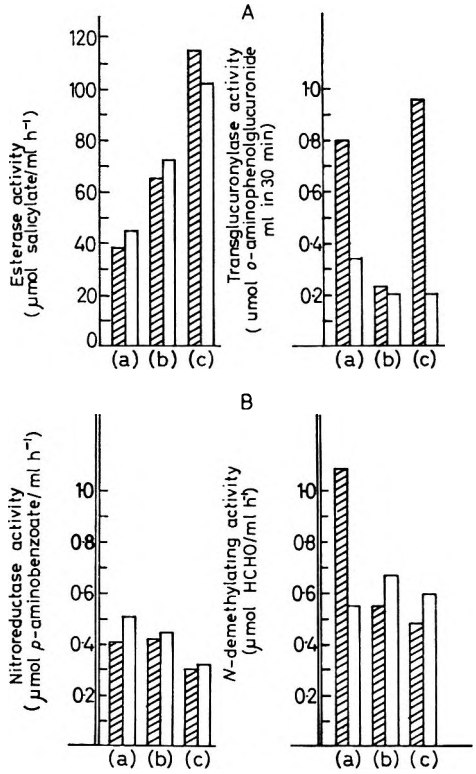

FIG. 2. Effect of precipitating microsomes obtained from rabbit (a), rat (b) and guinea-pig (c) livers at pH 5·0 on esterase and transglucuronylase (A), and nitro-reductase and *N*-demethylating activities (B) (open columns), compared with the activities of these enzymes in microsomes obtained by centrifugation at 140 000 *g* (hatched columns).

Preparation of microsomal fractions by iso-electric precipitation at pH 5·0. Fig. 2 shows the effect on microsomal enzyme activities when microsomes obtained from rabbit, rat and guinea-pig were prepared by iso-electric precipitation. Esterase and nitro-reductase activities were similar in each species to the activities of a microsomal

fraction obtained by centrifugation at 140 000 *g*. Transglucuronylase activity was much reduced in both rabbit and guinea-pig (about 2 and 5-fold respectively) and the *N*-demethylating activity of the rabbit preparation was reduced (about 2-fold).

DISCUSSION

In the present study, liver microsomal enzyme activities have been shown to be influenced both by the procedure used to prepare the initial tissue breis and by the RCF used to subfractionate the homogenate.

As the microsomal preparations were suspensions of insoluble washed subcellular particles in a buffer, it was assumed that the microsomal content was equivalent to the protein concentration or weight of freeze-dried material obtained for each preparation. Table 1 shows that homogenization by UT or MSE homogenizers produced microsomal preparations which had high microsomal content and high enzymic activity related to protein concentration or weight of freeze-dried product.

Microsomal preparations isolated from homogenates prepared by the use of violent procedures (i.e. UT or MSE homogenizers) did not appear to contain a high proportion of inactive protein, because specific activities calculated with respect to the protein concentration for esterase, transglucuronylase and nitroreductase were similar to, or higher than, the corresponding values obtained for comparable preparations prepared from homogenates obtained by the PE or nylon mesh techniques (Table 1). These latter techniques which utilize shearing forces to disrupt tissues, yielded microsomal preparations in which the *N*-demethylating activity was higher, whether calculated as total or specific activity, than activities measured in preparations obtained from UT or MSE homogenates. There would therefore appear to be a high proportion of inactive (devoid of *N*-demethylating activity) material in microsomal fractions obtained from homogenates prepared by the use of violent mechanical techniques.

When the RCF used to subfractionate microsomes was varied, the enzymic activities were again shown to parallel protein concentrations. However, in all three species, ability to demethylate increased as protein concentration (and presumably microsome content) decreased. It is also significant that a high demethylating activity was obtained in preparations isolated from homogenates obtained by the PE or nylon mesh techniques, preparations that also had a low protein concentration, and it would therefore appear probable that a membrane-associated protein component was inhibiting this process. This possibility is at present being investigated.

McLaren (1957) emphasized that extremes of pH do not kill living cells, and suggested that a wide variation in pH occurs in the micro environment of the cell. Leone & Redstone (1962) established that the pH of lowest solubility of cellular components was between 3·5 and 6·5, which supports the view of Morton (1954) that the particulate fraction precipitated at pH 5·0 during his studies on alkaline phosphatase was the microsomal fraction. The microsomal fraction obtained during this investigation by precipitation at pH 5·0 gave a preparation having similar enzymic activity to the microsomal preparations obtained by centrifugation, and, with the exception of transglucuronylase, the enzymes studied were stable at this pH; only rabbit liver microsomes showed an impaired ability to demethylate. Both rabbit and guinea-pig transglucuronylase activities were much decreased but although rat microsomal transglucuronylase is very unstable its activity was unaffected by the change in pH.

The present investigation demonstrates that microsomes retaining a high level of the enzymic activities involved in drug metabolism can be prepared by precipitation at pH 5·0 as an alternative to high speed centrifugation.

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The relation between the uptake of cetyltrimethylammonium bromide by *Escherichia coli* and its effects on cell growth and viability

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The effects of CTAB uptake on cells of *E. coli* have been examined in terms of growth inhibition and decrease in cell viability. Different thresholds of CTAB uptake exist below which no effect on cell growth or viability could be detected and there was a distinct separation of the two effects. It is suggested that both reflect all or none responses by individual cells. A mechanism for the mode of antibacterial action of CTAB against *E. coli* is postulated.

Salt & Wiseman (1968) have previously described a diphasic pattern of uptake of cetyltrimethylammonium bromide (CTAB) by *Escherichia coli* and the concurrent release of phosphorus compounds from the metabolic pool of the cells. This paper describes studies into the effects of CTAB on cell growth and viability in relation to its uptake by *E. coli*.

EXPERIMENTAL

Cetyl-*NNN*-trimethylammonium bromide was kindly prepared by J. E. Adderson, using the method of Adderson & Taylor (1964). The organism used was *Escherichia coli* NCTC 1093; the culture and suspending media, conditions of cultivation and methods of measuring absorbance were as described by Rye & Wiseman (1966). The preparation of cell suspensions and the methods of measuring CTAB uptake and of the leakage of metabolic pool ^{32}P-labelled compounds were as described by Salt & Wiseman (1968).

Growth inhibition. Equal volumes (10 ml) of suspensions of *E. coli* in glucose-free medium, cell concentration 0·25 mg/ml, and of solutions of CTAB in glucose-free medium or of medium alone were mixed in 50 ml conical flasks and maintained at 25° for 15 min. Glucose solution (20% w/v, 0·1 ml) was then added to each reaction mixture which was then incubated at 25° with aeration by shaking at 120 throws/min. The absorbance (650 nm) of each culture was measured at approximately 15 min intervals using a Unicam SP 500 series II spectrophotometer, after 90 min incubation the percentage growth in CTAB-treated cultures was calculated from the ratio of their increase in absorbance to the increase in absorbance of the untreated cultures. Percentage inhibition of growth was then determined by difference. This method is based on the observation of Rye & Wiseman (1968) that the decrease in overall growth rate of cultures of *E. coli* treated with sub-inhibitory concentrations of CTAB results from the complete inhibition of some of the cells whilst the remainder grow at an unimpeded rate. This technique has been discussed in detail by Wiseman (1969).

Viable counts. The numbers of viable cells in suspensions was estimated using the pour plate method. Samples of cells were diluted 1 in 100 in tryptone soya broth

containing 1% v/v polysorbate (Tween) 80 and allowed to stand for 10 min. Further dilutions were then made in tryptone soya broth and samples for counting plated in tryptone soya agar. Colonies were counted after incubation at 37° for 24 h.

RESULTS AND DISCUSSION

Fig. 1 shows the relation between the amount of CTAB taken up by cells of *E. coli* and the percentage inhibition of growth, cell viability and the release of metabolic pool phosphorus. From these results it is apparent that a threshold uptake of CTAB exists below which growth inhibition does not occur. Above this threshold uptake the percentage inhibition increases greatly for a small increase in the amount of CTAB taken up and reaches 100% at an uptake of about 2·3 μg CTAB/ml suspension, an uptake that is equivalent to the formation of a theoretical close packed monolayer of CTAB molecules around every cell (Salt & Wiseman, 1968).

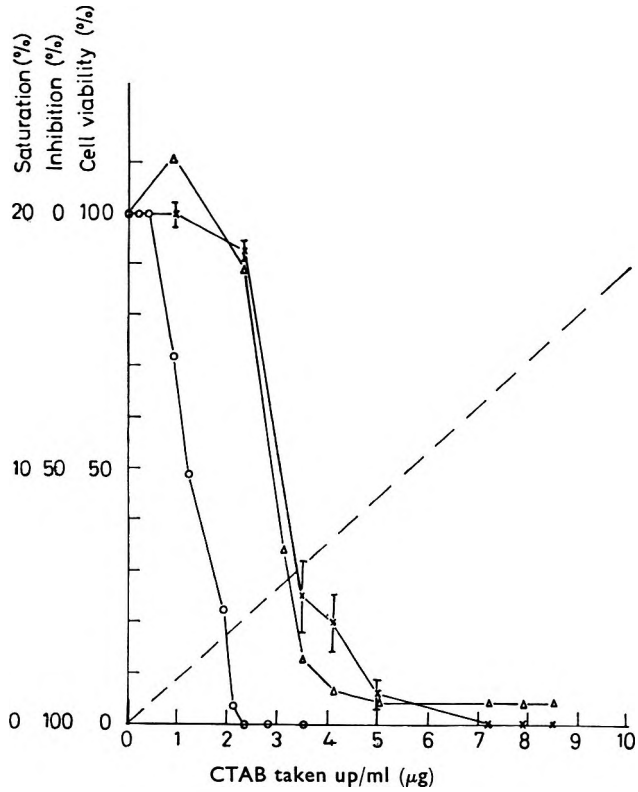

FIG. 1. The relation between uptake of CTAB per ml of *E. coli* suspension in glucose free medium and % increase in growth inhibition (○–○), % release of ^{32}P-labelled compounds (△–△), % decrease in cell viability (×–×) and % CTAB saturation of the cells (----). Cell concentration 0·125 mg/ml. Temp. 25°. Contact time 15 min.

Decrease in cell viability (Fig. 1) also exhibits a threshold uptake of CTAB below which little or no cell death occurs, an uptake of CTAB just causing 100% inhibition of growth having little or no effect on cell viability, or on the release of metabolic pool compounds. At an uptake of 2·3 μg CTAB/ml the cell suspension is still more

than 90% viable but above this uptake there is again a large increase in effect for a small increase in CTAB uptake, reductions in cell viability of more than 90% being observed at an uptake of about 5 μg/ml. This latter uptake approximates to that equivalent to a theoretical double layer of CTAB molecules around every cell. It is interesting to note that the percentage release of ^{32}P-labelled material from the CTAB-treated cells is similar to the percentage decrease in viability, agreeing with the observations of Salton (1951).

Thus, whether the antibacterial effect of CTAB on suspensions of *E. coli* is bacteriostatic or bactericidal depends on the amount of antibacterial agent taken up by the cells, and therefore on the ratio of CTAB to the cells, not on the total CTAB concentration present.

Fig. 1 also shows as a broken line the uptake of CTAB as a percentage of the uptake required to saturate the cells (Salt & Wiseman, 1968). From this it can be seen that inhibition of growth, release of metabolic pool material and cell death are all complete when less than 15% of the total possible uptake of CTAB by the cells has occurred.

The uptake isotherm of CTAB by suspensions of *E. coli* has been shown to exhibit two distinct phases and it has been suggested that the form of this isotherm results partly from an initial non-uniform distribution of CTAB molecules amongst the cells (Salt & Wiseman, 1968). Non-uniform responses of *E. coli* cells treated with CTAB have been reported by McQuillen (1950) in a study of electrophoretic mobilities and by Rye & Wiseman (1968) in studies of growth inhibition. It seems likely that the results reported by these workers and in this paper reflect all or non responses of individual cells in a culture rather than a uniform response of all the cells.

The existence of two cell membranes in *E. coli* with one forming the outermost surface of the cell has been suggested by Salton (1967) and demonstrated by de Petris (1967). In the light of this work it is possible to postulate a mechanism for the results reported herein.

In cell suspensions of *E. coli* treated with increasing concentrations of CTAB, individual cells take up different amounts of the agent until a single close packed monolayer has built up on the surface of the outermost cell membrane. On completion of this monolayer, cell growth ceases but the integrity of the cell is not altered. Increased leakage of cellular constituents does not therefore occur and in fact the reverse effect is detectable due to mechanical blockage of natural exchange processes. This surface adsorption will be reversible and cell death will not occur. In the presence of higher concentrations of CTAB more molecules are taken up by the cells by the formation of a second monolayer. Preliminary electrophoretic mobility studies (unpublished data) do not suggest the formation of a surface double layer and it is possible that the second monolayer is built up at the inner cell membrane. After completion of this second layer further uptake by an individual cell results in penetration of CTAB molecules through the inner cell membrane following disruption of its more or less ordered structure, resulting in the leakage of cellular constituents and cell death.

This mechanism would suggest the existence of different thresholds of CTAB uptake for the inhibition of growth and for the leakage of metabolic pool material and death in an individual cell.

The results reported in this paper indicate that such thresholds do exist and correspond closely to uptakes equivalent to the formation of theoretical close packed single and double monolayers at the cell surface.

Acknowledgements

We wish to thank Dr. R. M. Rye for valuable advice and one of us (W.G.S.) is indebted to the Science Research Council for a research studentship.

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Simultaneous determination of the effect of a non-ionic surfactant on the dissolution rate and biological activity of tablets of chlorpromazine hydrochloride

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The effect of a non-ionic surfactant, polysorbate 80, on the biological activity of chlorpromazine hydrochloride in solution was investigated using the goldfish, *Carassius auratus*. Below a certain critical concentration the activity was enhanced in unbuffered drug solutions, but above this concentration the activity was diminished, possibly due to some association between surfactant micelles and drug molecules. The rate of solution of chlorpromazine hydrochloride from coated tablets was increased by 2% polysorbate 80, but the activity was decreased when compared with that in a simple aqueous dissolution medium. This is direct evidence of the erroneous conclusions that can be obtained if dissolution measurements alone are used in assessing the effect of additives on drug performance.

It is now well established that the presence of surfactants can drastically alter both the physical and biological behaviour of drugs in solution (Elworthy, Florence & Macfarlane, 1968). The rates of dissolution of a number of compounds, including griseofulvin (Elworthy & Lipscomb, 1968), hexoestrol (Bates, Gibaldi & Kanig, 1966) and prednisolone (Taylor & Wurster, 1965) are increased by the presence of surfactants in the dissolution medium. The biological activity of penicillins (Ullmann, 1961) and secobarbitone (Levy, Miller & Reuning, 1966) is increased below the critical micellar concentration (CMC) of the added surfactants and reduced above the CMC, the latter effect probably being due to the solubilization of the drug in the surfactant micelles.

It appears that no work has been done using simple systems in which both physical and biological parameters are measured in the same experiment.

The purpose of this paper is to present results which illustrate the necessity of making both biological and physical determinations to assess the effects of additives such as surfactants on the behaviour of organic biologically active substances. In this work goldfish are used as the test animals as described by Levy & Gucinski (1964), and the time of death noted as the end point of the experiment. The dosage form was placed in a beaker of surfactant solution containing the fish, the amount of drug released was measured spectrophotometrically at intervals and the biological effect on the fish determined by noting the time of death.

Chlorpromazine hydrochloride was chosen because it is surface active and because its solution properties are being studied in detail in this laboratory.

EXPERIMENTAL

Materials

Chlorpromazine hydrochloride was a commercial sample (Largactil, May & Baker) used without further purification. 100 mg chlorpromazine hydrochloride tablets were also of this brand. Polysorbate 80 (Tween 80), a polyoxyethylene sorbitan monooleate (Atlas Chemical Company), was used as received. Water was once distilled. Goldfish (*Carassius auratus*) were 2·5–3·5 g in weight.

Methods

Biological activity. The time of death of at least four goldfish immersed each in 200 ml (pH 5·74–5·76) of test solution at room temperature was noted, the end point being the cessation of mouth and gill movements and the lack of response of the fish to stimulation. The overturn point used by Gibaldi & Nightingale (1968) was unreliable at the concentrations of drug used in these experiments (up to 0·1%). The reciprocal of the death time (min^{-1}) was the index of activity used. A calibration curve of reciprocal death time versus concentration of chlorpromazine was obtained and found to be linear up to 0·1% (Fig. 1).

Drug release was measured by removing samples (1 ml) from the beaker at intervals, diluting 1 in 100 with water and determining the extinction at 255 nm using an SP500 (Unicam) spectrophotometer, using water or suitably diluted surfactant solution as blank. No extraneous material absorbing at this wavelength was released from the tablet coat nor did fish excretions interfere. In these experiments there was no stirring of the solution, except that caused by the movement of the fish, which, though random, was often vigorous in the small amount of solution in which the fish swam.

RESULTS

The reciprocal death time of goldfish as a function of the concentration of chlorpromazine HCl is shown in Fig. 1. Each point is the mean of at least four determinations. The effect of polysorbate 80 concentrations on the reciprocal death time

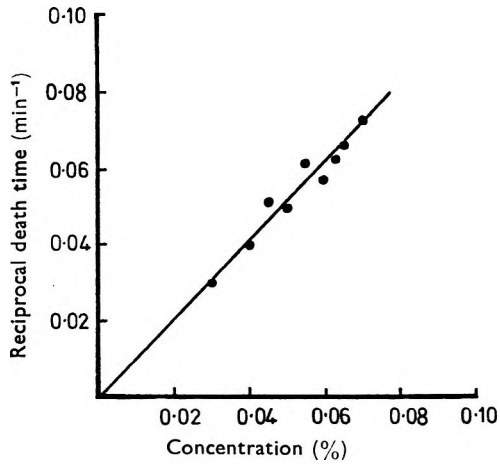

FIG. 1. Reciprocal death time of goldfish immersed in 200 ml of chlorpromazine hydrochloride solution as a function of the percentage concentration of drug. Each point is the mean of at least 4 results.

of the fish when immersed in 0·05, 0·065 and 0·1% chlorpromazine hydrochloride solutions (pH 5·75) is shown in Fig. 2. These results show the typical behaviour described by e.g. Levy, Miller & Reuning (1966); that is an increased activity at low concentrations of surfactant and a reduction in activity on increasing the surfactant concentrations to 1 and 2% which are greatly in excess of the CMC.

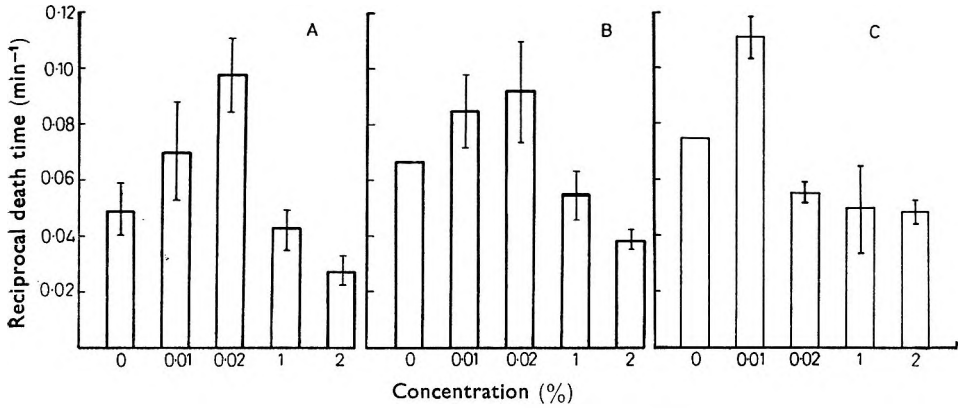

FIG. 2. The effect of polysorbate 80 on the time of death of goldfish immersed in chlorpromazine hydrochloride solutions (unbuffered) ± 1 standard deviation shown as vertical bars. A 0·05%, B 0·065%, C 0·10% chlorpromazine hydrochloride.

The significance of these results is shown by the representative figures presented in Fig. 3A for the rate of release of chlorpromazine from two 100 mg sugar coated tablets in the presence of polysorbate 80. In these experiments an increase in chlorpromazine release rate was observed with increase in surfactant concentration yet the biological activity of the solution in the 2% polysorbate is almost identical with that in water, although the fish has been in contact with about three times the amount of drug in the surfactant solution. Because drugs which are reasonably soluble in water are not thought to be significantly solubilized by surfactant micelles, it is perhaps unlikely that the reduction in activity noted is a true solubilization effect although there is possibly some association between the surfactant and the chlorpromazine, resulting if not in a decreased molecular mobility in solution then in a reduced rate of diffusion across the fish membrane. The nature of the interaction between phenothiazine derivatives and non-ionic surfactants is being investigated by nuclear magnetic resonance and pH measurements to differentiate between solubilization effects and the effect on the ionization of the chlorpromazine. The addition of non-ionic surfactants such as cetomacrogol lowers the pH of chlorpromazine HCl solutions suggesting a greater dissociation which is reminiscent of the micellar form of the chlorpromazine. The same experiments were made in phosphate buffer at pH 6·0 to determine whether bulk changes were responsible for the observed effects. However, the same general results were obtained (Table 1) although there was no evidence for the enhancement of activity at any of the concentrations of polysorbate used. Drug release and activity in stirred buffered solutions are shown in Fig. 3B.

In Fig. 2 it can be seen that the enhancement of activity at 0·02% polysorbate 80 is more pronounced in the more dilute drug solution. As phosphate buffer at pH 6 appears to enhance the activity of the 1·0% chlorpromazine (reciprocal death time

0·128 min^{-1} compared with 0·078 min^{-1} in unbuffered solution) the results in the phosphate are perhaps a continuation of the trend in the enhancement of activity by the polysorbate.

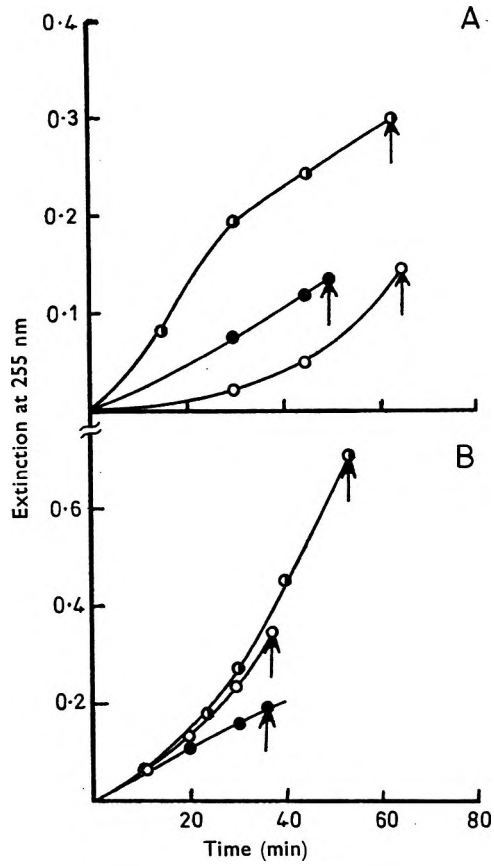

FIG. 3. Two representative results of the effect of polysorbate 80 concentration on the dissolution of drug from commercial chlorpromazine hydrochloride tablets and the biological activity in the goldfish. Death time in each denoted by an arrow. A in water (unstirred), B in phosphate buffer (stirred) at pH 6·0. Ordinate: extinction at 255 nm of a 1 in 100 dilution of sampled solution. ○ Water or buffer; ●, 0·01% polysorbate 80; ◑, 2·0% polysorbate 80. The experiments in the mechanically stirred solution (B) show the same overall effects as those in the unstirred solution.

Table 1. *Effect of polysorbate* 80 *on reciprocal death times of goldfish immersed in chlorpromazine hydrochloride solutions buffered at pH* 6·0

Polysorbate concn (%)	Chlorpromazine concn (%)	Reciprocal death time* ± standard deviation (min^{-1})
0	0·05	0·0847 ± 0·0107
0·02	0·05	0·0854 ± 0·0098
0	0·1	0·128 ± 0·039
0·01	0·1	0·104 ± 0·010
0·02	0·1	0·077 ± 0·051
1·0	0·1	0·072 ± 0·006
2·0	0·1	0·061 ± 0·009

* Results of four fish at each concentration.

DISCUSSION

In many published investigations an increased rate of solution of drug from a dosage form has been presumed to lead to more rapid absorption. Such an assumption would be erroneous in this study, which indicates that high concentrations of surfactant, that increase dissolution rates, can retard the biological effects. The tablet–water–goldfish system does not immediately appear to approximate to the human *in vivo* situation. However, Levy & his co-workers (1966) have claimed that as far as passive diffusion characteristics are concerned the fish membrane bears a similarity to human membranes.

There is a need for a simple laboratory test which has relevance to the *in vivo* performance of a formulation. Goldfish can be used with many drugs other than the phenothiazine tranquillizers: the barbiturates (Levy, Miller & Reuning, 1966), alcohol (Gibaldi & Nightingale, 1968) and an analgesic (Flanagan, Broad & others, 1969) are recent examples. The system described here has the advantage of great simplicity and goes one stage toward the development of a relevant routine test. That pretreatment of fish with polysorbate does not affect the subsequent activity of chlorpromazine by having a direct effect on the membrane requires investigation. Low concentrations of polysorbate may make the membrane more permeable whilst high concentrations might make it less permeable. This could explain the results.

Acknowledgement

I thank Mrs. Christine Selkirk for technical assistance.

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Stereochemical influences upon antihistamine activity. Further studies of isomeric 4-amino-1,2-diarylbutenes*

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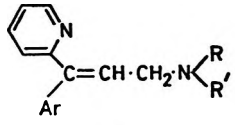
The acid-catalysed dehydration of some 4-amino-1,2-diarylbutan-2-ols yields all four possible 4-amino-1,2-diarylbutenes. The structure and configuration of pure isomers have been established from spectroscopic data, and the ability of these compounds to anatagonize histamine-induced contractions of the guinea-pig ileum is reported. The most potent derivatives are all *cis*(H/Ar)-4-amino-1,2-diarylbut-2-enes. These results together with other data are discussed in terms of the possible structural and conformational requirements of histamine antagonists.

Geometrical configuration influences the antihistamine properties of 3-amino-1-aryl-1-pyrid-2′-ylprop-1-enes (I), the *cis*(H/pyrid-2-yl)-isomers being the more potent (Adamson, Barrett & others, 1957). The same dependence of activity upon stereochemistry is now reported for some antihistamine 4-amino-1,2-diarylbut-2-enes (II). The formation of 4-aminobutenes from the tertiary alcohols (III) by dehydration was first reported by Stoll, Morel & Frey (1950), but the products were not satisfactorily characterized either to double-bond position or to configuration (four isomers, two but-1-enes and two but-2-enes may result from the dehydration). The elimination of water from 4-dimethylamino-1,2-diphenylbutan-2-ol (III; R = R′ = Ph, X = NMe_2) was later investigated in detail (Casy & Pocha, 1967) and the most active antihistamine butene of Stoll & others (1950) was shown to be a ternary mixture, the *cis*(H/Ph)-but-2-ene (IV) being the most active component (Casy & Parulkar, 1969). In this paper, pharmacological data upon a further series of isomerically pure 4-aminobutenes are given and tentative structure-activity relations drawn from the results.



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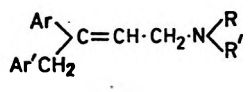

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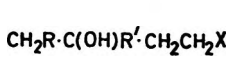

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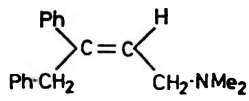


IV

* Presented in part at the 4th International Congress on Pharmacology, Basle, 1969.

CHEMISTRY

All the compounds tested as antihistamine agents were obtained by fractional crystallization of 4-aminobutene hydrohalide mixtures derived by dehydration of the corresponding tertiary alcohols (III); these mixtures comprised all four possible isomers, namely *cis*- and *trans*-but-1- (V) and -2-enes (VI), as was evident from the presence of four vinylic signals in the pmr spectrum of the total products of elimination. Amongst these isomers, but-1-enes were differentiated from but-2-enes by the multiplicities of their vinylic pmr signals (but-1-ene signals were singlets and but-2-ene signals were triplets, $J \sim 7$ Hz), whilst configurational assignments rested upon association of the lower field vinylic chemical shift with the more planar of the two isomers and upon ultraviolet absorption data (Casy & Parulkar, 1969); diagnostic spectral characteristics of the 4-amino-1,2-diarylbutenes are given in Table 1.

Table 1. *Spectral data of* 4-*amino*-1,2-*diarylbutenes*

$CHR{:}CR'{\cdot}CH_2{\cdot}CH_2{\cdot}X$ (V) $\qquad$ $CH_2R{\cdot}CR'{:}CH{\cdot}CH_2{\cdot}X$ (VI)

Compound No.	Isomer*	Alkene type	Structure R	R′	X	Chemical shift of vinylic signal† C-1	C-3	λ(ε)‡
2	*cis*	VI	Ph	Ph	NMe_2		378	241 (11,500)
3	*trans*	VI	Ph	Ph	NMe_2		358	end absorption
		V and VI mixture§	Ph	Ph	piperidine	408	371	—
						391	341	—
4	*cis*	V	Ph	Ph	piperidino	395	—	255 (17,100)
5	*trans*	V	Ph	Ph	piperidino	406	—	263 (20,000)
6	*cis*	VI	Ph	Ph	piperidino	—	382	241 (15,400)
		V and VI mixture§	Ph	Ph	pyrrolidin-1-yl	403	367·5	—
						386	337	—
7	*trans*	V	Ph	Ph	pyrrolidin-1-yl	415	—	261 (20,400)
8	*cis*	VI	Ph	Ph	pyrrolidin-1-yl	—	377	240 (12,700)
		V and VI mixture§	*p*-Cl·C_6H_4	Ph	pyrrolidin-1-yl	403·5	371	—
						388	341	—
	cis	V	*p*-Cl·C_6H_4	Ph	pyrrolidin-1-yl	397·5	—	266 (15,800)
	trans	V	*p*-Cl·C_6H_4	Ph	pyrrolidin-1-yl	407·5	—	268 (19,200)
	cis	VI	*p*-Cl·C_6H_4	Ph	pyrrolidin-1-yl	—	368	241 (12,500)
	trans	VI	*p*-Cl·C_6H_4	Ph	pyrrolidin-1-yl	—	364	end absorption

* Configurational reference groups: R and R′ for V; R′ and $H_{(3)}$ for VI.
† Chemical shifts in Hz from tetramethylsilane (60 MHz operating frequency, Varian A-60 spectrometer), solvent $CDCl_3$; C-1 signal a singlet, C-3 a triplet $J \sim 7$ Hz. Pure isomers examined as hydrohalides.
‡ Solvent water. λ is wavelength in nm; extinction coefficient ε shown in parentheses.
§ Total base from corresponding t-alcohol III.

The *cis*-4-dimethylamino-1,2-diphenylbut-2-ene (IV; compound No. 2), obtained previously as a hydrochloride mixed with other isomers (Casy & Pocha, 1967) was isolated pure as a hydrobromide. Three isomerically pure butenes were separated from the mixture derived from the 4-piperidino-alcohol (III; R = R′ = Ph, X = piperidino) and two from the 1,2-diphenyl-4-pyrrolidin-1′-ylbutene mixture.

Although the antihistamine pyrrobutamine (1-*p*-chlorophenyl-2-phenyl-4-pyrrolidin-1′-ylbut-2-ene) is described in the patent literature as a 4-aminobut-2-ene (Lilly Patent, 1954), no evidence is given either for its double-bond position or configuration, hence its formation from the corresponding tertiary alcohol (III; R = *p*-Cl-C_6H_4, R′ = Ph, X = pyrrolidin-1-yl) has been included in the present study. The initial crop deposited from the dehydration mixture after acidification with ethanolic hydrogen chloride proved to be the pure *cis*(H/Ph)-but-2-ene hydrochloride. The pmr spectrum of this butene, as free base, was identical with that of the base derived from pyrrobutamine (marketed as a phosphate). Thus, pyrrobutamine is a *cis*-(H/Ph)-but-2-ene. The remaining isomers were isolated from the mother liquors

as hydrobromide salts; the pharmacological evaluation of all four isomers is in progress.

PHARMACOLOGICAL RESULTS

The antihistamine potencies of a series of 4-amino-1,2-diphenylbutenes, as measured by their ability to antagonize the histamine-induced contraction of the guinea-pig ileum are given in Table 2. Thanks are due to Drs. R. T. Brittain and R. G. W. Spickett of Allen and Hanburys, Ware, Herts, for arranging these tests. These data only allow semi-quantitative activity comparisons but it is clear that the three most effective compounds (No. 2, 6 and 8) are all *cis*(H/Ph)-4-amino-1,2-diphenylbut-2-enes. These are all active at a concentration of 0·01 μg/ml and cause 90% or greater inhibition 3 min after application and over 50% six min later. Both the *cis*-4-dimethylamino- and the *cis*-4-piperidino-but-2-enes (compound No. 2 and 6 respectively) are more potent than related isomers (*cf.* compound No. 1–3 and 4–6), while the 4-pyrrolidinyl isomers (compound No. 7 and 8), although initially of like potency, differ in duration of action, that of the *cis*-but-2-ene (compound No. 8) being the more prolonged. The *cis*-4-piperidino-but-2-ene (compound No. 6), the most active member of the series, has a pA_2 value of 8·76 comparable with that of antihistamines in clinical use (*cf.* chlorpheniramine pA_2 8·1 and mepyramine pA_2 8·71). Its *in vivo* specificity of action was demonstrated by the fact that it antagonized the effects of histamine but not those of acetylcholine, 4-hydroxytryptamine and bradykinin upon the guinea-pig lungs (Konzett & Rossler, 1940) (these spasmogens increase the resistance of the lungs to inflation). It potentiated hexobarbitone-induced sleeping times in mice and hence has undesirable CNS depressant properties in common with other antihistamine drugs such as mepyramine and diphenhydramine.

Table 2. *Inhibition of histamine-induced contractions of isolated guinea-pig ileum by some 4-amino-1-phenylbutenes*

$CHR{:}CR'{\cdot}CH_2{\cdot}CH_2{\cdot}X$ (V) $\qquad$ $CH_2R{\cdot}CR'{:}CH{\cdot}CH_2{\cdot}X$ (VI)

Compound No.	Alkene type (R = Ph)	Structure R′	X	Concn μg/ml	% inhibition at time (in min) :3	:6	:9	:12	:15	:18	:21	:24	:27
1	V and VI mixture*	Ph	NMe_2	0·10	90	71	62	56	50	29	8	—	—
2	*cis*-VI	Ph	NMe_2	0·01	89	84	79	62	52	28	—	—	—
3	*trans*-VI	Ph	NMe_2	0·10	87	62	45	20	—	—	—	—	—
4	*cis*-V	Ph	piperidino	0·10	88	83	77	52	32	20	5	—	—
5	*trans*-V	Ph	piperidino	0·10	88	75	51	37	21	—	—	—	—
6	*cis*-VI	Ph	piperidino	0·01	100	99	97	90	76	72	64	42	26
7	*trans*-V	Ph	pyrrolidin-1-yl	0·10	92	87	76	76	70	62	44	30	24
				0·01	100	60	37	22	5	—	—	—	—
8	*cis*-VI	Ph	pyrrolidin-1-yl	0·01	96	74	52	37	26	14	10	—	—
9†	*trans*-V	But	NMe_2	0·10	81	50	20	5	—	—	—	—	—
10†	*cis*-VI	But	NMe_2	0·01	4	—	—	—	—	—	—	—	—
11†	*trans*-VI	But	NMe_2	0·10	91	81	64	36	26	24	7	—	—
12‡	*cis*-3-dimethylamino-1-phenyl-1-t-butylprop-1-ene			0·01	4	—	—	—	—	—	—	—	—
13§	1,2,5,6-tetrahydro-1-methyl-4-phenylpiperidine (XXI)			0·10	55	7	—	—	—	—	—	—	—
	mepyramine			0·001	81	50	20	5	—	—	—	—	—

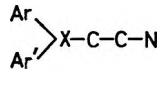
* Ternary mixture: *cis*- and *trans*-V (major) and *cis*-VI (minor component).
† Casy & Ison (1969).
§ Schmidle & Mansfield (1955).

These results emphasize the influence of steric factors upon activity among antihistaminic aminobutenes and show that the disposition of functions about the double bond in a *cis*(H/Ar)-1,2-diarylbut-2-ene are optimal for activity, in confirmation of

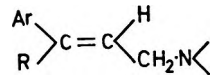
previous data upon isomeric 4-dimethylamino-2-*p*-methoxyphenyl-1-phenylbut-2-enes (Casy & Parulkar, 1969). It is further significant that pyrrobutamine (VI; R = *p*-Cl·C_6H_4, R′ = Ph, X = pyrrolidin-1-yl), a but-2-ene already marketed as an antihistamine agent, also has a *cis*(H/Ar)-configuration (see chemistry section).

DISCUSSION

Most compounds which effectively antagonize histamine at low dose levels may be described by the general structure VII, where Ar is aryl (including heteroaryl), Ar′ is aryl or arylmethyl, and X is N, C–O or saturated C; X–C in VII may be replaced by C:C (Burger, 1960; Wilson & Gisvold, 1962; Barlow, 1964). The terminal nitrogen atom is part of a tertiary acyclic or alicylic basic grouping. If it be assumed that the aryl function(s) and the basic group are the essential pharmacodynamic moieties of these molecules, it is probable that their relative dispositions at the histamine receptor are similar in all potent derivatives if these antagonists have related modes of action. Some ideas upon the optimal arrangement may be gained



VII

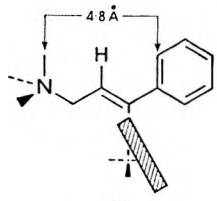


VIII

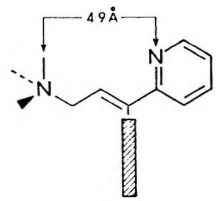
by examination of antihistamines in which the key features are attached to a C:C grouping. In these alkenes, the relative dispositions of molecular components are greatly restricted, especially in comparison with derivatives based on the general Structure VII itself. There are two groups of alkene derivatives with antihistamine properties, namely 3-aminopropenes such as triprolidine (I; Ar = *p*-Me·C_6H_4, NRR′ = pyrrolidin-1-yl) and the 4-aminobutenes presently described; the most active isomers of both groups possess the common structure VIII rigidly orientated in the same manner and it is proposed, in view of the high potency of these compounds and of the activity fall which follows a configurational change, that this unit is an optimal conformation for activity in antihistamine agents*. The overall shape of the more potent aminoalkene antihistamines will now be considered in terms of probable conformations.

In *cis*(H/Ar)-4-aminobut-2-enes of structure VIII (R = Ar′-CH_2), the Ar ring and the carbon–carbon double bond are approximately coplanar (physical evidence); to minimize non-bonded interactions, the plane of the second aryl group (Ar′) attached to the methylene group must lie at about 70° to the Ar–(C=C) plane and the net shape of the molecule in its preferred conformation, as indicated from examination of Catalin and Framework Molecular (FMM) models, is shown diagrammatically in IX. The planes of the two aromatic rings are thus nearly at right-angles, while all components of the Ar·C:CH·CH_2·N< grouping lie close to a mean plane. The distance between the nitrogen atom and an *ortho*-aromatic carbon atom shown in IX and X relates to the work of Kier (1968) on histamine, discussed later. In *trans* (H/Ar)-analogues of VIII (R = Ar′·CH_2), of much lower antihistamine potencies the Ar and carbon–carbon double-bond planes greatly diverge; conformers are

* In 3-aminoprop-1-enes (I), the structure VIII exists in either isomer (since, in VIII, R = Ar′ as well), and hence specificity in regard to the nature of the aryl group *cis* to $H_{(a)}$ must also obtain. This point will be discussed elsewhere.



IX



X

IX. Shaded rectangle represents the end-on view of aromatic ring in this, and other drawings; single lines represent features close to the plane of the paper, dotted lines, those below, and heavy lines those above the same plane.

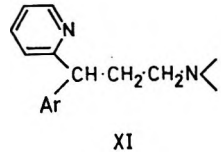
probable in which the benzyl aromatic ring lies in a similar plane to th $C:CH\cdot CH_2\cdot N<$ grouping, but the comparable distance between the nitrogen atom and the aromatic carbon atom is at least 1 Å greater than that of a *cis*-isomer.

In isomeric 3-aminopropenes of the triprolidine type (I), only one of the aryl groups may be coplanar with the alkenic double bond. Models indicate that the aryl group *cis* to the hydrogen atom is more probable as the coplanar group and this is confirmed from physical data (Adamson & others, 1957, and unpublished pmr data). The preferred conformation of compounds containing structure X (see also Barlow, 1964) is thus very similar to that of the 4-aminobut-2-enes, the only difference between IX and X being the absence of an arylmethylene group in the latter. Pharmacological results upon the triprolidine isomers and related compounds show that a pyrid-2-yl group is preferred to phenyl and substituted phenyl groups as the aromatic member of the planar $Ar\cdot C:CH\cdot CH_2:N<$ grouping.

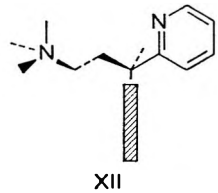
Formally less rigid antihistamine agents will now be examined to establish whether or not they are likely to adopt conformations in which their aryl group and $CH_2\cdot N<$ grouping are disposed in a similar fashion to the aminoalkene conformations IX and X.

Pheniramines

These compounds have the general formula XI and may be regarded as alkane analogues of the 3-aminopropenes I. Models of XI of shape close to that of the 4-aminobutenes IX seem to be very reasonable conformations, as they appear to entail no serious non-bonded interactions (see XII). In these models, the pyrid-2-yl group is made the aryl group most nearly coplanar with the alkylamine chain by analogy with the 3-aminopropene results. In XII the dihedral angle* between the



XI



XII

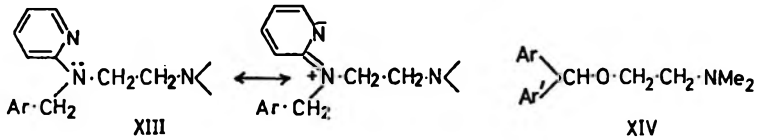
aromatic rings is not far removed from 90°, as in IX. A model in which the near-planar $Ar\cdot CH\cdot CH_2\cdot CH_2\cdot N<$ grouping is maintained may also be made when the

* Defined as the angle between planes containing (*a*) C–1 and C–2 of one aryl group and (*b* C–1 and C–2 of the other aryl group, with the quaternary carbon atom common to both.

inter-aryl dihedral angle is greater than 90° ("butterfly" arrangement of Ar_2CH), but non-bonded interactions are judged to be greater in this conformation.

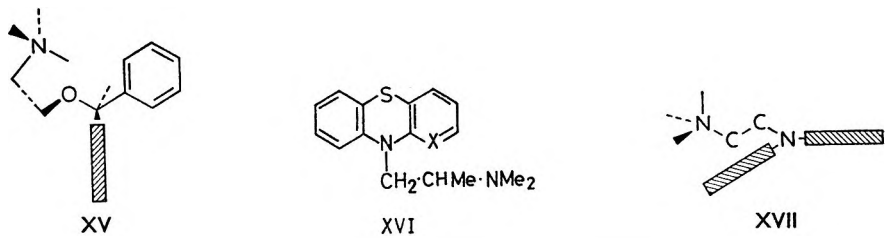
Ethylene diamines

The compounds, general formula XIII (the pyrid-2-yl group is replaced by a phenyl group in some derivatives, e.g., Antergan), have an aryl group and an aryl-methyl group linked to one of the diamine nitrogen atoms; restricted rotation about the N–Ar bond is probable as a result of resonance interactions as shown. Once again it is possible to construct a model of XIII that is very similar to the 4-amino-but-2-ene conformation IX except that $CH_2{\cdot}N<$ (planar nitrogen) replaces C=C. This form is probable on the grounds of minimum steric interactions and also maximum operation of N–Ar resonance effects.



Diphenhydramines

The variant of the general structure VII in which X is C–O and the terminal carbon atom carries two aromatic groups (e.g., diphenhydramine (XIV; Ar = Ar′ = Ph) similarly yields a strain-free model XV, akin to the 4-aminobutenes IX, in which the ether-oxygen bonds lie close to the plane of one of the aromatic rings with an inter-aryl dihedral angle near 90°. Nauta, Rekker & Harms (1966) have proposed an "active" diphenhydramine conformation similar to XV and have infrared and ultraviolet spectroscopic evidence for the coplanarity of the oxygen atom and the *p*-tolyl group in *p*-methyldiphenhydramine (XIV, Ar = Ph, Ar′ = *p*-$Me{\cdot}C_6H_4$).



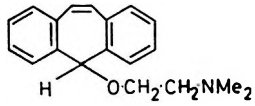
XVII. Molecule viewed along a line joining the hetero-atoms of the phenothiazine nucleus; the sulphur atom is obscured by the nitrogen atom.

Cyclic derivatives

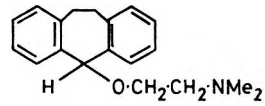
These are formed when the two aryl groups of VII are fused at *o*-positions to a sulphur or carbon atom. In phenothiazine derivatives, e.g., promethazine (XVI; X = CH) a model can be made in which the 2-aminoethyl side-chain attached to ring nitrogen atom and one of the aromatic rings lie more or less in one plane with the second aromatic ring removed from this dimension (XVII). However, the angle between the two aromatic rings is obtuse rather than acute as in the 4-aminobutenes

IX, as a result of the rings being constrained to a "butterfly" conformation by their linkages to the heterocyclic atoms (the alternative "butterfly" conformation is unlikely on account of steric interactions between the 2-aminoethyl side-chain and adjacent aromatic hydrogen atoms). Similar considerations of molecular shape apply to isothipendyl (XVI; X = N).

In the fused-ring derivatives XVIII and XIX, the angle between the two aromatic planes is less than that in the phenothiazines and models of these compounds closely resemble IX in having a nearly planar Ph·C·O·CH_2·CH_2·N< grouping with the second ring approximately at right-angles to this feature (Nauta & others, 1966). These derivatives may be regarded as analogues of diphenhydramine and both are significantly more active than the parent compound in which the two rings are unconstrained. The recently introduced antihistamine cyproheptadine XX has a molecular shape similar to XVIII and XIX, except that the planes of the aminoalkyl group and one aromatic ring do not coincide so closely, as they are held apart by the double-bond linkage.

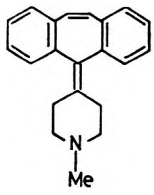


XVIII

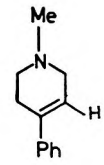


XIX

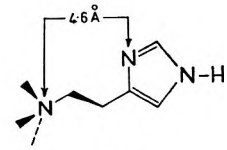
The preceding account has demonstrated that many antihistamine drugs in clinical use may adopt conformations similar to those of the model aminoalkene antihistamine IX and X without serious non-bonded interactions being generated. If the overall molecular arrangement typified in IX and X is accepted as specially conducive to the blockade of histamine receptors, it appears most reasonable to postulate that the near-planar aminoalkyl-aryl moiety of the antagonist molecule occupies the histamine receptor itself, as this unit has a similar shape to, and dimensions of, the histamine molecule (see below). It is assumed that histamine sites occupied by the protonated NH_2 group and the imidazole ring of the agonist probably interact with the protonated side-chain nitrogen atom and the aryl groups respectively of the antagonist. The second aromatic feature of the antagonist (antiplanar



XX



XXI



XXII

with the rest of the molecule) is assumed to occupy an additional receptor area which is not implicated in the uptake of histamine itself (the concept of antagonists utilizing more receptor sites than the molecules which they antagonize has been discussed by Ariëns & Simonis, 1964). Speculations along these lines have previously been made by Nauta & others (1966) about *p*-methyldiphenhydramine. The importance of

having two aromatic groups in an antihistamine agent is emphasized by the low activity of the tetrahydropyridine XXI (Table 2, compound No. 13), a molecule which contains the near-planar Ar·C:CH·CH_2·N< grouping of the potent amino-alkenes, while results upon the t-butyl derivatives (Table 2, compounds No. 9–12) emphasize the importance of the planar feature itself. In these derivatives, all of low potency, coplanarity of the aromatic and double-bond planes is seriously distributed by the bulky t-butyl group.

Kier (1968) has proposed favoured *anti-* and *gauche-*($\overset{+}{N}H_3$/Ar)-conformations for histamine based on molecular orbital calculations and has suggested that the similar N–N interatomic distances of the *anti*-conformer XXII and of triprolidine X may be of significance regarding the latter compound's antagonistic properties. It is of interest that the distances between the side-chain nitrogen atom and an aromatic *ortho*-atom (N or C) comprising the near-planar feature of conformations proposed for the antihistamine agents of structure I, *cis* VI, XI, XIII and XIV are all close to the histamine N–N distance specified above and this observation shows how the shape and dimensions of the histamine molecule may be reproduced in anti-histamine agents.

EXPERIMENTAL

4-Amino-1,2-diarylbutenes. The butan-2-ol III hydrochlorides were dehydrated with a mixture of acetic acid and concentrated hydrochloric acid by the previously reported method (Casy, Myers & Pocha, 1966), and the isomers in the dehydration mixture separated by fractional crystallization from ethanol-ether of the hydrohalides. Isomers are listed in their order of separation.

Dehydration of 4-dimethylamino-1,2-diphenylbutan-2-ol (15 g) gave cis-*4-dimethylamino-1,2-diphenylbut-2-ene* (compound No. 2) *hydrobromide* (2·3 g), m.p. 179–180° (Found: C, 64·7; H, 6·6; N, 3·9. $C_{18}H_{22}BrN$ requires: C, 65·0; H, 6·7; N, 4·2%). The other isomers have been previously reported (Casy & Pocha, 1967).

Dehydration of *1,2-diphenyl-4-pyrrolidin-1'ylbutan-2-ol hydrochloride* (13·5 g), m.p. 163° from ethanol-ether (Found: C, 72·0; H, 7·8; N, 4·2. $C_{20}H_{26}ClNO$ requires: C, 72·3; H, 7·9; N, 4·2%) gave cis-1,2-*diphenyl-4-pyrrolidin-1'-ylbut-2-ene* (compound No. 8) *hydrochloride* (0·77 g), m.p. 198° (Found: C,76·6; H, 7·5. $C_{20}H_{24}ClN$ requires: C, 76·3; H, 7·7%) and the corresponding trans-*but-1-ene hydrochloride* (1·53 g), m.p. 160–162° (Found: C, 76·3; H, 7·8%).

Dehydration of 1-*p*-chlorophenyl-2-phenyl-4-pyrrolidin-1'-ylbutan-2-ol (Lilly Patent, 1954) (12 g) gave the *cis*-1-*p*-chlorophenyl-2-phenyl-4-pyrrolidin-1'-ylbut-2-ene hydrochloride (1·73 g), m.p. 227–228°, reported m.p. 227–228° (Lilly Patent, 1954); the base formed a *methiodide*, m.p. 117°, from acetone-ether (Found: C, 55·6; H, 5·65. $C_{21}H_{25}ClIN$ requires: C, 55·6; H, 5·55%). Acidification of the residual bases in the dehydration mixture with ethanolic hydrogen bromide gave the corresponding cis-*but-1-ene hydrobromide* (1·3 g), m.p. 184–185° (Found: C, 61·4; H, 6·2; N, 3·3. $C_{20}H_{23}BrClN$ requires: C, 61·15; H, 5·9; N, 3·6%), the corresponding trans-*but-1-ene hydrobromide* (2·01 g), m.p. 191° (Found: C, 61·2; H, 6·0; N, 3·7%), and the corresponding trans-*but-2-ene hydrobromide* (0·44 g), m.p. 152–153° (Found: C, 61·0; H, 6·0; N, 3·7%).

Dehydration of 1,2-diphenyl-4-piperidinobutan-2-ol (Pohland & Sullivan, 1953) (10 g) gave the cis-1,2-*diphenyl-4-piperidinobut-2-ene* (compound No. 6) *hydrochloride* (1·54 g), m.p. 248–249° (Found: C, 76·6; H, 8·1. $C_{21}H_{26}ClN$ requires:

C, 76·9; H, 8·0%), the corresponding trans-*but*-1-*ene* (compound No. 5) *hydrochloride* (1·42 g), m.p. 195° (Found: C, 76·7; H, 8·0%) and the corresponding cis-*but*-1-*ene* (compound No. 4) *hydrochloride* (0·67 g), m.p. 160–161° (Found: C, 77·0; H, 8·0%). The *cis*-but-2-ene gave a *methiodide*, m.p. 163–164° from acetone-ether (Found: C, 61·2; H, 6·8. $C_{22}H_{28}IN$ requires: C, 60·95; H, 6·5%).

Acknowledgements

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Phosphocholine 2, 6-xylyl ether bromide: proton-phosphorus coupling constants and preliminary pharmacological assessment

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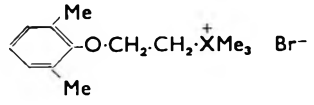
Phosphocholine 2,6-xylyl ether bromide [*PPP*-trimethyl-2-(2,6-xylyloxy)ethylphosphonium bromide], has been synthesized by reacting 2-(2,6-xylyloxy)ethyl bromide with a solution of trimethylphosphine in phenol, but ethylenebis(trimethylphosphonium bromide), 1,2-di(2,6-xylyloxy)ethane and 2,6-xylenol were the only reaction products identified when ether was used as solvent. The $^2J_{PH}$ and $^3J_{PH}$ coupling constants for these phosphonium salts have been determined. Although the phosphocholine xylyl ether blocks the Finkleman preparation in concentrations of $2–3 \times 10^{-5}$ g/ml, this blockade does not have all the characteristics of the adrenergic neuron blockade produced by xylocholine.

The substitution of a phosphorus atom for a nitrogen atom in quaternary salts usually leads to a reduction in agonist activity. Thus Hunt & Renshaw (1925) demonstrated that tetramethylphosphonium iodide is less active than tetramethylammonium iodide in both the atropinized and non-atropinized anaesthetized cat, and Holton & Ing (1949) showed that acetylphosphocholine possesses only 8% of the potency of acetylcholine when tested on cat blood pressure, the rabbit intestine and the frog heart. A similar fall in potency on substituting a phosphorus atom for the nitrogen atom in xylocholine (I) could lend support to the postulated involvement of acetylcholine in adrenergic transmission. Phosphocholine 2,6-xylyl ether bromide [PPP-trimethyl-2-(2,6-xylyloxy)ethylphosphonium bromide] (II) has therefore been synthesized and tested for adrenergic neuron-blocking action on the Finkleman rabbit intestine preparation.



I, X = N; II, X = P

EXPERIMENTAL

Chemistry

Infrared spectra were recorded on a Perkin-Elmer 257 grating spectrophotometer. The mass spectrum of 1,2-di(2,6-xylyloxy)ethane was recorded on an A.E.I. MS902 spectrometer and its nmr spectrum on a Varian A60 spectrometer. Nmr spectra of the quaternary phosphonium salts were recorded on a Varian HA-100D spectrometer at the Physicochemical Measurements Unit, Harwell, using sweep widths of 1000 and 100 Hz, enabling coupling constants to be measured to an accuracy of 0·1 Hz.

Preparation of trimethylphosphine. A solution of phosphorus trichloride (0·4 mol) in dry ether (1000 ml) was added slowly to a vigorously stirred solution of methylmagnesium iodide (2·5 mol) in ether (1000 ml) at −40° under nitrogen. When the addition was complete the reaction mixture was allowed to warm to room temperature and stirred for a further hour. The trimethylphosphine and ether were distilled from the reaction mixture by gentle warming and condensed at −70°. When the bulk of the ether had distilled and a thick syrup remained in the reaction vessel, distillation was terminated. The distillate was warmed to 0° and extracted with 5 × 100 ml of air-free cold 2·5N sulphuric acid.

Trimethyphosphine was isolated as an ethereal solution by neutralizing the acid extract with 20–30% aqueous potassium hydroxide solution under nitrogen, warming gently and drying the ether–trimethylphosphine vapours by passage up a potassium hydroxide tower before condensing at −70°.

When the trimethylphosphine was to be reacted subsequently in phenol, the acid extract was first heated to 70° to remove the bulk of the dissolved ether and then cooled before liberation and isolation of the trimethylphosphine as described above.

Reaction of trimethylphosphine with 2-(2,6-xylyloxy)ethyl bromide. 2-(2,6-Xylyloxy)ethyl bromide (0·25 mol) (prepared from sodium 2,6-xylyl oxide and 1,2-dibromoethane in t-butanol) was reacted with trimethylphosphine, prepared as above, either in 500 ml of dry ether or in 200 g of phenol. Both reaction mixtures were allowed to stand (protected from moisture) for 7 days at room temperature.

(*a*) In the ethereal reaction mixture, a white precipitate was produced (22·4 g) which was recrystallized from 95 pts methanol–5 pts light petroleum (60–80°) to give white crystals, m.p. above 340° ν_{max} (KCl disc—assignments are derived by comparison with spectrum of Me_4PI and are tentative): 1434 s (CH_3 def.); 1398 (CH_2 def.); 1300 s, 1295 s (CH_3-P); 1228 s; 1144 s; 980 vs (C–P); 983 s; 885 s; 783 s, 762 m (PCH_3); 688 m. Found: C, 28·15; H, 6·45; Br, 47·2; P, 17·9. Ethylenebis(trimethylphosphonium bromide, $C_8H_{22}Br_2P_2$, requires C, 28·25; H, 6·5; Br, 47·0; P, 18·2%. Extraction of the ethereal mother-liquor from the reaction mixture with 10% sodium hydroxide solution followed by acidification of the alkaline extract, extraction of the acid mixture with ether, evaporation of the ether and distillation of the residue under reduced pressure yielded a material (1·8 g) whose infrared spectrum, b.p., m.p., and mixed m.p. were identical with an authentic sample of 2,6-xylenol. Removal of the ether from the ethereal phase remaining from the alkaline extraction and distillation of the residue under reduced pressure yielded 10·1 g of unreacted starting material and 7·4 g of a colourless liquid, b.p. 158–162° at 1 mm, nmr (CCl_4, TMS): *ca* τ 3·1 (intensity *ca* 6, m, ArH), τ 5·96 (intensity *ca* 4, s, CH_2), τ 7·70 (intensity 12, s, $ArCH_3$). ν_{max} (liquid film): 1605w, 1593m, 1512m (aromatic ring); 1298 vs (aralkyl ether); 768 s (ArH). Mass spectrum (m/e values; % intensity in parentheses): 270 (80), 149 (100), 135 (19), 121 (41), 105 (98), 28 (15). Found: C, 79·65; H, 8·4. 1,2-Di(2,6-xylyloxy)ethane, $C_{18}H_{22}O_2$ requires C, 79·95; H, 8·2%.

(*b*) On pouring the phenolic reaction mixture into dry ether (1000 ml), an oil was formed which quickly gave a white solid (44·8 g) on agitation. The solid was dissolved in a small amount of dry ethanol and poured into dry ether (1000 ml). White crystals formed which were recrystallized from 98 pts dry acetone–2 pts dry ethanol, m.p. 185–187°. ν_{max} (KCl disc): 1593 w, 1478 s (aromatic ring); 1427 m (CH_3 def.); 1297 s (CH_3–P); 1200 s, 1192 vs (aralkyl ether); 976 vs (C–P); 806 s (ArH). Found:

C, 51·35; H, 7·25; Br, 26·5; P, 10·25. *PPP*-Trimethyl-2-(2,6-xylyloxy)ethylphosphonium bromide, $C_{13}H_{22}BrOP$ requires C, 51·15; H, 7·25; Br, 26·2; P, 10·15%.

Pharmacology

Rabbit intestine preparation. Short pieces of ileum or duodenum were taken from freshly killed rabbits and prepared according to the method of Finkleman (1930) using Tyrode solution (35°) gassed with 5% carbon dioxide in oxygen. Supramaximal stimulation of the periarterial nerves was carried out with rectilinear pulses (supramaximal voltage: 0·5 ms duration; 20/s for 30 s in every 7½ min) and longitudinal contractions of the preparation were recorded with a frontal writing lever on smoked paper.

RESULTS AND DISCUSSION

Chemistry

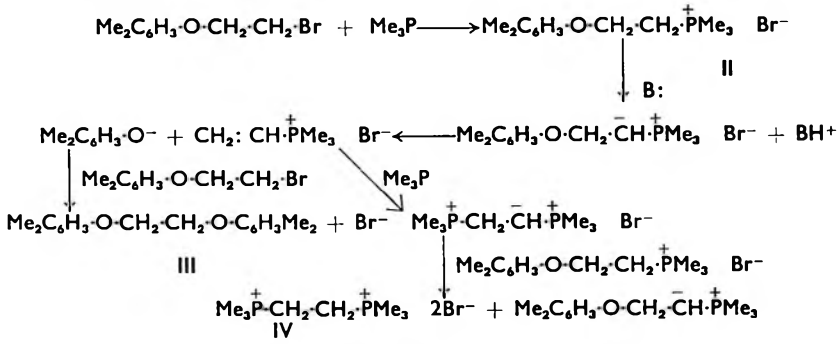
The synthesis of phosphocholine 2,6-xylyl ether bromide (II) was first attempted by the reaction of 2-(2,6-xylyloxy)ethyl bromide with trimethylphosphine in ethereal solution. Ether was chosen as the solvent because an ethereal solution of trimethylphosphine resulted from its method of preparation. With ether as the solvent, however, none of the required phosphonium salt was obtained, 1,2-di(2,6-xylyloxy)-ethane (III), ethylenebis(trimethylphosphonium bromide) (IV) and 2,6-xylenol being the only identifiable products. The same result, except for the isolation of the bis-phosphonium salt as the iodide, was obtained when 2-(2,6-xylyloxy)ethyl iodide was used instead of the analogous bromide.

The structure of the di-ether III was established by infrared, nmr, and mass spectrometry while the infrared spectrum, boiling point, melting point and mixed melting point of the isolated 2,6-xylenol were identical with those of an authentic sample.

The proton magnetic resonance spectrum of the bisphosphonium compound IV in D_2O, with TMS as external reference, is similar to that described by Carty & Harris (1967) for 1,2-bis(diphenylphosphino)ethane bismethiodide. The CH_2 resonance is a doublet around a much less intense and unresolved region of absorption, the mid-point of the signal occurring at τ 7·35. Separation of the outer lines gives $|^2J_{PH} + {}^3J_{PH}| = 6{\cdot}4$ Hz. [The coupling constant notation is essentially that of Musher & Corey (1962), as modified by Carty & Harris (1967).] The three peaks of the methyl resonance signal are in the approximate ratio 2 : 1 : 2 and are centred at τ 8·03. The separation of the outer lines gives $|^2J_{PMe} + {}^5J_{PMe}| = 14{\cdot}2$ Hz, and if ${}^5J_{PMe} = 0$ (Hendrickson, Maddox & others, 1964), $|^2J_{PMe}| = 14{\cdot}2$ Hz. This is similar to the value of $|^2J_{PMe}| = 14{\cdot}8$ Hz found for tetramethylphosphonium iodide. Carty & Harris (1967) give ${}^2J_{PMe} = -13{\cdot}0$ Hz for 1,2-bis(diphenylphosphino)ethane bismethiodide and −14·4 Hz for tetramethylphosphonium iodide.

The formation of the bisphosphonium salt and 2,6-xylenol from the reaction of 2-(2,6-xylyloxy)ethyl bromide and trimethylphosphine in ethereal solution is analogous to the isolation of ethylenebis(triphenylphosphonium bromide) and phenol by Schweizer & Bach (1964) from reaction of triphenylphosphine and 2-phenoxyethyl bromide in non-protonic solvents. Schweizer and Bach did not isolate or identify any 1,2-diphenoxyethane. They established a mechanism involving triphenyl-vinylphosphonium bromide and excluded the participation of phenyl vinyl ether. It seems probable that an analogous mechanism will be involved in the formation of

ethylenebis(trimethylphosphonium bromide) and that the di-ether III is formed by the attack of a xylyloxy-anion on the 2-(2,6-xylyloxy)ethyl bromide.



The failure of Schweizer and Bach to identify 1,2-diphenoxyethane amongst the products of their reaction may be due to their use of vapour-phase chromatography to analyse their reaction solution and to the very high boiling point of the di-ether, rather than to the absence of this material.

The required phosphocholine 2,6-xylyl ether bromide (II) was obtained as the sole identified product by reacting 2-(2,6-xylyloxy)ethyl bromide with trimethylphosphine in phenol. The proton magnetic resonance spectrum of this monophosphonium salt dissolved in D_2O, and with TMS as external reference, showed a complex signal for the aromatic protons at approximately τ 3·0 (intensity 3), a doublet (intensity 9) at τ 8·07 due to the methyl group attached to the phosphorus atom ($^2J_{PMe}$ = 14·5 Hz), and a singlet at τ7·80 (intensity 6) due to the aryl methyl groups. The two methylene groups occur as a well-resolved doublet of triplets (intensity 2) at τ 5·93 ($^3J_{PH}$ = 17·7 Hz) assigned to the $O.CH_2$ protons and a less well-resolved doublet of triplets (intensity 2) centred at τ 7·28 ($^2J_{PH}$ = 13·7 Hz) assigned to the $CH_2.P$ protons. In this latter signal the two innermost bands are almost coincident and in a lower resolution spectrum the signal appears as five peaks. The mean J_{HH} for the methylene groups is 6·25 Hz. Manatt, Juvinall & Elleman (1963) deduce that $^3J_{PH}$ is always positive and that $^2J_{PH}$ may be positive or negative. By using the $^2J_{PH}$ coupling constant for the monophosphonium compound (II), which is less likely to be influenced by the proximity of the ether linkage than is $^3J_{PH}$, and substituting into the relation $|^2J_{PH} + {}^3J_{PH}| = 6{\cdot}4$ Hz, we obtain $^3J_{PH} = +7{\cdot}3$ Hz or $+20{\cdot}1$ Hz for the bisphosphonium compound (IV). Since $^3J_{PH}$ for the monophosphonium compound is 17·7 Hz, we favour the larger of these two values. Carty & Harris (1967) suggested two values, $+11{\cdot}0$ or $+22{\cdot}2$ Hz for $^3J_{PH}$ for 1,2-bis-(diphenylphosphino)ethane bismethiodide, and favoured, from a comparison with other coupling constants, the larger value, but had no direct evidence for their choice. Our values for $^3J_{PH}$ support their belief that the larger value is correct.

Pharmacology

Phosphocholine 2,6-xylyl ether bromide in concentrations of 2–3 $\times$ 10^{-5} g/ml blocks the effects of stimulation of the periarterial adrenergic nerves in the Finkleman (1930) preparation. The blockade, which can be observed shortly after administration of the phosphonium compound, deepens slowly over 1 h, and is difficult to reverse by washing. Amphetamine sulphate (0·3–1 $\times$ 10^{-5} g/ml) does not prevent

the establishment of the blockade and has little effect on the intensity of the blockade once it is established, though these concentrations are effective in preventing and reversing the blocking action of xylocholine (Fig. 1).

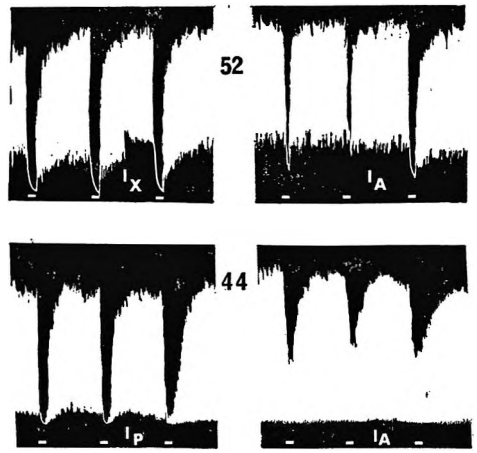


FIG. 1. Rabbit intestine suspended in Tyrode solution at 35°. Showing the effect of xylocholine (4×10^{-6} at X) and phosphocholine 2,6-xylyl ether bromide (3×10^{-5} at P) on the response to supramaximal stimulation of the periarterial nerves (40 V, 0·5 ms duration, 20/s for 30 s in every 7½ min) at the white bars. Amphetamine sulphate (7×10^{-6}) was present in the bath from A. The figures show the time elapsed (min) between the two parts of each record and all concentrations are expressed in g/ml final bath concentration.

Although the slow onset and difficulty in reversing the blockade produced by the phosphonium compound by washing are compatible with a specific adrenergic neuron blocking action such as that possessed by xylocholine, the inability of amphetamine to reverse the blockade suggests that the blocking action of phosphocholine 2,6-xylyl ether might be attributable to mechanisms other than adrenergic neuron blockade.

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A comparison of the local anaesthetic-, "quinidine-like"- and adrenergic β-blocking-activities of five β-receptor antagonists

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Propranolol, pronethalol, INPEA, MJ1999 and MJ1998 exhibit local anaesthetic activity when assessed by infiltration anaesthesia and at motor nerve endings but only propranolol, pronethalol and INPEA exhibit local anaesthetic activity on the phrenic motor nerve. All the β-adrenergic blocking agents exhibit a "quinidine-like" activity and there appears to be a close correlation between local anaesthetic and "quinidine-like" activities, which cannot be extended to include adrenergic β-receptor antagonism.

Dawes (1946) observed a close correlation of the local anaesthetic and "quinidine-like" activities of a series of secondary and tertiary amines. The secondary amine β-adrenergic receptor antagonists, propranolol (Morales Aguilera & Vaughan Williams, 1965) and pronethalol (Gill & Vaughan Williams, 1964) are reported to exhibit local anaesthetic activity, whilst INPEA (Somani & Lum, 1965; Schmild & Hanna, 1967; Levy 1968), MJ1999 (Lish, Weikel & Dungan, 1965; Levy, 1968) and MJ1998 (Lish & others, 1965) are reported to be devoid of such activity. The local anaesthetic, "quinidine-like" and sympathetic β-receptor blocking activities of the β-receptor antagonists were measured in order to relate their local anaesthetic and "quinidine-like" actions and to consider whether this correlation itself could be related to adrenergic β-receptor antagonism.

EXPERIMENTAL

Local anaesthetic activity

Local anaesthetic action was measured by the intradermal weal method (Bülbring & Wajda, 1945) and by nerve conduction in the phrenic nerve (Matthews, 1961) and also at the neuromuscular junction of the isolated rat phrenic nerve diaphragm preparation (Bülbring, 1946; Straughan, 1961).

Intradermal weal method

Groups of six albino male guinea-pigs were used for the intradermal weal tests. The hair was removed from the test area on the back with a depilator of composition: 200 g barium sulphide in a 200 ml suspension containing 10% Teepol and 10% glycerin. 24 h after depilation, the local anaesthetic activities of the adrenergic β-receptor antagonists were compared with those of procaine using a 2 × 2 double blind, latin square assay design. Drugs were dissolved in saline, adjusted to pH

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7·10, and injected intradermally in volumes of 0·2 ml. Using a needle, the minimal force required to elicit a response was found and this stimulus was applied in a series of six tests on each test area at zero time and at intervals of 10 min for a 40 min period. A positive score was noted each time the guinea-pig did not respond.

Conduction studies in the isolated phrenic nerve diaphragm preparation

A triangular segment of the left hemidiaphragm with attached rib segments, intercostal tissue and phrenic nerve of a decapitated rat was attached by its intercostal margin to a steel holder, which acted as an electrode for direct muscle stimulation, and by a fine steel wire to the central tendon, which served to attach the diaphragm to a semi-isotonic writing lever. The preparation was bathed in Krebs solution at 37·0° gassed with 5% carbon dioxide in oxygen at pH 7·32. For studies on nerve conduction, the cut end of the phrenic nerve was drawn into a nerve bath 0·3 ml capacity, through a small perforation in a rubber dam closure at one end, thus separating the fluid in the nerve bath from that bathing the diaphragm. The nerve was applied to two platinum electrodes in the nerve bath. To study the effects of drugs on transmission at the neuromuscular junction, the phrenic nerve was placed around electrodes in the diaphragm bath.

The local anaesthetic activities of the adrenergic β-receptor antagonists were compared with those of procaine using a 2 × 2 latin square design. The drug contact time was 3 min, direct muscle stimulation was applied for 20 s at zero, one and 3 min and the cycle time was 20 min. The diaphragms were stimulated indirectly through the phrenic nerve at a frequency of 15 impulses/min with rectangular pulse width of 0·1 ms at a supramaximal voltage and directly with rectangular pulse width of 1·0 ms at a supramaximal voltage. Before an assay, a control dose of procaine was repeatedly administered, using the experimental contact and cycle times, until the responses were constant.

Mode of action at the neuromuscular junction

The actions of the adrenergic β-receptor antagonists were compared with those of procaine and tubocurarine using a method devized by Straughan (1961). The diaphragm was stimulated indirectly through the phrenic nerve with rectangular pulses of 0·1 ms duration at a supramaximal voltage at a frequency of 15 impulses/min. The drug contact time was 3 min and the cycle time 20 min. A concentration of drug was found which produced a 5–20% inhibition of the indirectly elicited diaphragm response. The procedure was then repeated with a lower concentration of drug to find the highest concentration which had no effect on the response of the diaphragm. The preparation was then equilibrated for 10 min with $2{\cdot}5 \times 10^{-7}$ g/ml eserine sulphate and the subeffective dose was then added to the bath and its effect noted.

"Quinidine-like activity"

The "quinidine-like" activity of the adrenergic β-receptor antagonists was determined as a measure of the reduction in the maximal drive rate, using the isolated atria preparation (Dawes, 1946).

Guinea-pig atria were suspended in an organ bath containing Locke solution at 29° aerated with 5% carbon dioxide in oxygen at pH 6·8. The gas mixture passed

into the organ bath up a Perspex tube, in the upper end of which were two silver stimulating electrodes. The tip of the right atrium was pulled into the tube to make contact with the electrodes and the left atrium was connected to a strong spring lever which wrote on a smoked kymograph.

Rectangular pulse waves of 3·0 ms duration at double the voltage threshold determined at 240 beats/min were used. The frequency of stimulation was variable over a range of 120–414 beats/min. The atria were stimulated for 10 s and rested for 15 s periods. The frequency of stimulation was increased in steps of 6 impulses/min until the frequency at which the atria could not follow was ascertained. The maximal drive rate of the atria was taken as the highest frequency that the atria could follow. When the maximal drive rate had been confirmed, the test drug was added to the bath for 10 min and then the maximal drive rate of the atria was again measured.

Adrenergic β-receptor antagonism

The adrenergic β-blocking activities of the drugs were assessed by their antagonism of the response of the rabbit isolated ileum preparation to isoprenaline (Lockett & Bartlet, 1956).

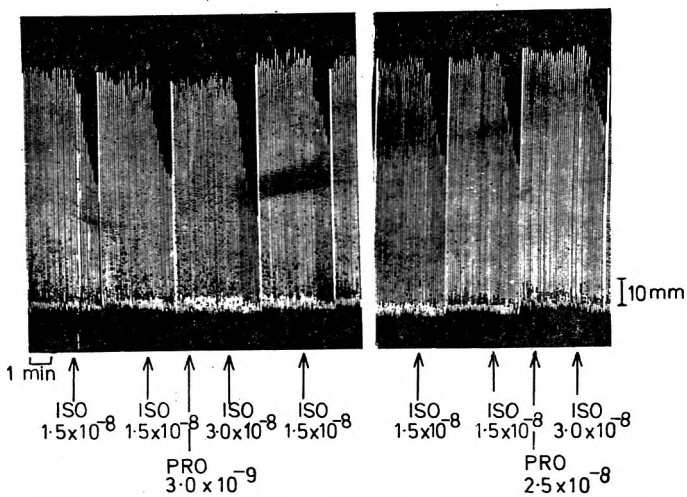

FIG. 1. Rabbit isolated ileum preparation. The tracing shows the effects of isoprenaline sulphate (ISO), on the spontaneous contractions of the ileum, and the antagonism of the isoprenaline response with propranolol hydrochloride (PRO). The tracing illustrates the method of determining pA_2 values. See text for explanation. The preparation was bathed in Krebs solution at 38·0° containing phentolamine ($5·0 \times 10^{-8}$ g/ml). All concentrations are expressed as the salt in g/ml.

Approximately 1 inch of proximal ileum was suspended in an organ bath containing Krebs solution oxygenated with 5% carbon dioxide in oxygen of pH 7·32. Phentolamine ($5·0 \times 10^{-8}$ g/ml) was included in the Krebs solution to prevent possible inhibition of spontaneous contractions due to α-receptor stimulation. This concentration of phentolamine was that found to completely abolish a 50% reduction in response produced by phenylephrine. Spontaneous contractions were recorded on a smoked kymograph with a frontal writing lever which magnified 8 times.

Control responses to a concentration of isoprenaline which produced approximately a 50% reduction (K) of the spontaneous contractions, were obtained using

a 1 min contact time for isoprenaline and a 5 min cycle time. The adrenergic β-receptor antagonist was then added to the bath and acted for 2 min, at which time twice the previous dose of isoprenaline was added to the bath and the response (2K) noted (Fig. 1).

RESULTS

Local anaesthetic activity

Propranolol, pronethalol, INPEA, MJ1999, MJ1998 and procaine showed local anaesthetic activity by the intradermal weal method. The potency ratios and confidence limits (Finney 1952), are shown in Table 1.

Propranolol, pronethalol, INPEA, MJ1999, MJ1998 and procaine also block conduction at the neuromuscular junction of the phrenic nerve diaphragm. The mode of action of the drugs was ascertained using an anticholinesterase-treated preparation. A normally sub-effective concentration of tubocurarine had no effect, but a normally subeffective concentration of procaine reduced the indirectly-elicited responses of the eserinized phrenic nerve diaphragm to the pre-eserinization level of response. Normally sub-effective concentrations of the adrenergic β-receptor antagonists acted in a similar manner to procaine, demonstrating their procaine-like nerve blocking action. The potencies of the drugs in producing nerve blockade at the neuromuscular junction are of the same order as their potency when assessed by the intradermal weal method, but the concentrations required at the neuromuscular junction are much smaller than those required for local anaesthesia by the intradermal weal method (Table 1).

Table 1. *Local anaesthetic activity assessed by the intradermal weal method and at motor nerve terminals*

	Intradermal weal method			At motor nerve terminals	
Drug	Effective concentration g/ml	Potency expressed against procaine (= 100)	Confidence limits $P = 95{\cdot}0\%$	Effective concentration g/ml	Potency expressed against procaine (= 100)
Propranolol hydrochloride	$2{\cdot}5 \times 10^{-3}$ to $5{\cdot}0 \times 10^{-3}$	198·0	112·0 to 240·0	$2{\cdot}5 \times 10^{-5}$ to $1{\cdot}0 \times 10^{-4}$	217·0
Pronethalol hydrochloride	$2{\cdot}5 \times 10^{-3}$ to $5{\cdot}0 \times 10^{-3}$	162·0	107·0 to 229·0	$2{\cdot}5 \times 10^{-5}$ to $1{\cdot}0 \times 10^{-4}$	168·0
INPEA hydrochloride	$2{\cdot}0 \times 10^{-2}$ to $4{\cdot}0 \times 10^{-2}$	21·6	18·5 to 26·4	$1{\cdot}0 \times 10^{-4}$ to $2{\cdot}5 \times 10^{-4}$	31·0
MJ 1999 hydrochloride	$5{\cdot}0 \times 10^{-2}$ to $1{\cdot}0 \times 10^{-1}$	12·7	7·6 to 17·4	$2{\cdot}5 \times 10^{-4}$ to $5{\cdot}0 \times 10^{-4}$	13·6
MJ 1998 hydrochloride	$5{\cdot}0 \times 10^{-2}$ to $1{\cdot}0 \times 10^{-1}$	6·3	4·1 to 8·4	$5{\cdot}0 \times 10^{-4}$ to $1{\cdot}0 \times 10^{-3}$	7·6

* INPEA; *N*-isopropyl-*p*-nitrophenylethanolamine. MJ 1999; 4-(2-isopropylamino-1-hydroxyethyl)methanesulphonanilide. MJ 1998, 4-(2-methylamino-1-hydroxypropyl)methanesulphonanilide.

Propranolol (minimal effective concentration $1{\cdot}0 \times 10^{-4}$ g/ml), pronethalol ($1{\cdot}0 \times 10^{-4}$ g/ml), INPEA ($5{\cdot}0 \times 10^{-4}$ g/ml) and procaine ($1{\cdot}0 \times 10^{-4}$ g/ml) block conduction along the phrenic nerve of the phrenic nerve diaphragm preparation, but MJ1999 and MJ1998 are devoid of such local anaesthetic activity in concentrations up to $5{\cdot}0 \times 10^{-2}$ g/ml, even when the contact time is extended to 15 min.

"Quinidine-like" activity

All the adrenergic β-receptor antagonists exhibited "quinidine-like" activity on the electrically driven isolated atria preparation. The "quinidine-like" activity was measured as a % reduction of the maximal drive rate of the atria which was determined as:

$$\frac{\text{The difference in the maximal drive rate before and after the drug} \times 100}{\text{The last determined maximal drive rate before the drug action}}$$

Four determinations were made at each of four doses and the relative activities assessed graphically, the point of comparison being taken where the maximal drive rate was reduced by 20%. The relative activities of the adrenergic β-receptor antagonists compared with procaine as "quinidine-like" agents were similar to their potencies as local anaesthetic agents when assessed by the intradermal weal method and as nerve blocking agents at the neuromuscular junction. The concentrations required to produce "quinidine-like" activity were similar to those required to produce nerve block at the neuromuscular junction (Table 2).

Table 2. *The "quinidine-like" activity of the adrenergic β-receptor antagonists assessed on the isolated guinea-pig atrial preparation*

Drug	Effective concentration g/ml	Potency of drugs expressed against procaine (= 100)
Propranolol hydrochloride	$2{\cdot}5 \times 10^{-6}$ to $1{\cdot}0 \times 10^{-5}$	206·0
Pronethalol hydrochloride	$2{\cdot}5 \times 10^{-6}$ to $1{\cdot}0 \times 10^{-5}$	170·0
INPEA hydrochloride	$2{\cdot}0 \times 10^{-5}$ to $4{\cdot}0 \times 10^{-5}$	45·5
MJ 1999 hydrochloride	$5{\cdot}0 \times 10^{-5}$ to $1{\cdot}0 \times 10^{-4}$	15·6
MJ 1998 hydrochloride	$1{\cdot}4 \times 10^{-4}$ to $2{\cdot}0 \times 10^{-4}$	6·8

Adrenergic β-receptor antagonism

All the adrenergic β-receptor antagonists inhibit the effects of isoprenaline on the rabbit isolated ileum preparation. Using four determinations at five doses for each drug, the relative potencies were measured graphically from a log dose against response $(2K-K)/K \times 100$, where 2K and K were measured as a % reduction in the spontaneous contractions of the ileum produced by isoprenaline. The point

of comparison was taken where $(2K - K)/K \times 100 =$ zero, at which point $2K = K$. The potencies of the drugs were expressed against propranolol (= 100) and also as pA_2 values (Table 3). The concentrations of adrenergic β-receptor antagonists required to produce adrenergic β-blockade are some 1000 times smaller than the concentrations required to produce a "quinidine-like" action on the atria and blockade at motor nerve endings.

Table 3. *Adrenergic β-receptor antagonism assessed by inhibition of the response of rabbit ileum to isoprenaline*

Drug	Effective concentrations g/ml	Potency expressed against propranolol (= 100)	pA_2 values
Propranolol hydrochloride	$3{\cdot}125 \times 10^{-9}$ to $5{\cdot}0 \times 10^{-8}$	100·0	7·27
Pronethalol hydrochloride	$2{\cdot}5 \times 10^{-8}$ to $2{\cdot}0 \times 10^{-7}$	19·6	6·58
INPEA hydrochloride	$2{\cdot}5 \times 10^{-8}$ to $8{\cdot}0 \times 10^{-7}$	7·7	6·11
MJ 1999 hydrochloride	$1{\cdot}25 \times 10^{-8}$ to $2{\cdot}0 \times 10^{-7}$	19·6	6·70
MJ 1998 hydrochloride	$2{\cdot}0 \times 10^{-7}$ to $1{\cdot}6 \times 10^{-6}$	1·40	5·34

DISCUSSION

All the adrenergic β-receptor antagonists examined here exhibited local anaesthesia by the intradermal weal (infiltration anaesthesia) method. The ratio of activities of propranolol : procaine 1·98 : 1·00, and pronethalol : procaine 1·65 : 1·00, as local anaesthetics are similar to those reported for propranolol : procaine 2·30 : 1·00 (Morales Aguilera & Vaughan Williams, 1965), and pronethalol : procaine 1·80 : 1·00 (Gill & Vaughan Williams, 1964), using the same method and animal species. The demonstration that MJ1999 and MJ1998 exhibit local anaesthesia in concentrations of $5{\cdot}0 \times 10^{-2}$ g/ml and greater, are not in agreement with the observations of Lish & others (1965) who reported that MJ1999 and MJ1998 were devoid of local anaesthetic activity in doses up to $6{\cdot}2 \times 10^{-2}$ g/ml when assessed by the intradermal weal method, or with the findings of Levy (1968), who used the rabbit corneal method, or Schmild & Hanna (1967) who used earthworms as the diagnostic agent for assessing local anaesthetic activity. Contrary to the findings of Somani & Lum (1965) and Levy (1968) who used the corneal method and Schmild & Hanna (1967), who used earthworms, and who reported INPEA to be devoid of local anaesthetic activity, the intradermal weal method showed INPEA to have one-third the activity of procaine. These differences, not unexpected, are to be attributed to the known influence of routes of absorption.

The ratio of activities of the adrenergic β-receptor antagonists ascertained by blockade of neuromuscular transmission, which was demonstrated to be a procaine-like local anaesthetic action, are very similar to the values obtained by the intra-

dermal weal method. The *in vitro* nerve experiments show propranolol, pronethalol and INPEA to block conduction in the phrenic nerve whilst MJ1999 and MJ1998 did not. Calculated from the partition coefficients obtained for a water–chloroform suspension, propranolol (97%), pronethalol (93%) and INPEA (56%) have high lipid solubilities, while MJ1999 (3·0%) and MJ1998 (3·5%) (Levy, 1968, Larsson, personal communication), have low lipid solubilities. The lack of effect of MJ1999 and MJ1998 on nerve conduction in the phrenic nerve may be explained in terms of the low lipid solubilities of these compounds, rendering them unable to penetrate the highly lipid nerve and myelin sheaths. At the fine diameter nerve terminals of sensory and motor nerves, MJ1999 and MJ1998 are able to reach the cell membrane and produce their local anaesthetic effects.

All the adrenergic β-receptor antagonists exhibit a "quinidine-like" action on the electrically driven atria preparation. A comparison of the local anaesthetic and "quinidine-like" activities of the adrenergic β-receptor antagonists with those of procaine hydrochloride showed their relative potencies as local anaesthetic and "quinidine-like" agents to be similar. Dawes (1946) also found the amines with the most potent local anaesthetic activity were most potent as "quinidine-like" agents. Levy (1968), who introduced the additional factor of corneal penetration with the rabbit corneal method, could not demonstrate a correlation between the local anaesthetic and "quinidine-like" activities of some adrenergic β-receptor antagonists.

The potency of the drugs as β-receptor antagonists was propranolol $>$ pronethalol $=$ MJ1999 $>$ INPEA $>$ MJ1998. The correlation of local anaesthetic and "quinidine-like" activity cannot be extended to include adrenergic β-receptor antagonism because the relative activities of the drugs as β-receptor antagonists differ from their activities as local anaesthetic and "quinidine-like" agents, and the concentrations of these drugs required to produce their local anaesthetic and "quinidine-like" activities are some 1000 times greater than those required to produce adrenergic β-blockade.

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The mode of action of insulin potentiation by mebanazine

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Chronic oral treatment of rats with mebanazine potentiated the hypoglycaemic response to insulin both in intensity and duration. A similar potentiation of intensity but not of duration was observed in pair-fed animals. In consequence, mebanazine-treated rats subjected to a 21 h fast before insulin died in hypoglycaemic coma whereas control and pair-fed animals did not. Intravenous glucose brought about a rapid return to consciousness in moribund mebanazine-treated rats in insulin coma but hyperglycaemic catecholamines did not. The observed hypoglycaemic unresponsiveness was not solely due to depletion of liver glycogen since both control and treated rats had undetectable levels of glycogen after fasting, yet only the treated animals died. Tissue levels of noradrenaline were raised by mebanazine treatment in brain, heart and liver but these enlarged stores were readily depleted by reserpine. A similar potentiation of hypoglycaemia was observed following chronic treatment of intact rats with tranylcypromine and of adreno-demedullated rats with guanethidine and bethanidine. No potentiation was observed with reserpine or α-methyldopa in either type of animal. Blood glucose was significantly raised by adrenaline and α-methylnoradrenaline, but not by octopamine. The observations support the hypothesis that hypoglycaemic unresponsiveness following chronic inhibition of monoamine oxidase is due to a replacement of noradrenaline in adrenergic neurons by octopamine. This substance is an ineffective hyperglycaemic agent and its physiological release in response to hypoglycaemia does not bring about a return to normoglycaemia.

Increased sensitivity to insulin hypoglycaemia in rats pretreated with mebanazine was first reported in 1965 (Barrett, 1965; Zor, Mishkinsky & Sulman, 1965). Attempts to define the mechanism of this effect have not been conclusive.

A specific inhibition of growth hormone activity, rather than monoamine oxidase inhibition, was considered by Zor, Dikstein & Sulman (1965a,b). Subsequent experiments (Adnitt, 1968a; Barrett, 1966, 1969) clearly showed that mebanazine possessed an anorexic action and that paired-feeding could produce very similar results in terms of weight gain, pituitary growth hormone content and the width of the tibial epiphysial cartilage. The increased insulin sensitivity was shown to be well correlated with inhibition of monoamine oxidase by Adnitt (1968a) after prolonged mebanazine-treatment and to occur both with hydrazine (mebanazine) and non-hydrazine (tranylcypromine) types of enzyme inhibitor. A single dose of mebanazine sufficient to produce a 98% inhibition of monoamine oxidase did not increase insulin sensitivity. Two pieces of evidence supported the hypothesis that the potentiation of insulin was related to the accumulation of a false neurochemical transmitter, β-hydroxytyramine (octopamine) after monoamine oxidase inhibition

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(Kopin, Fischer & others, 1964). First, the duration of hypoglycaemia was significantly prolonged, implying a failure of the normal response to low blood sugar levels. Second, the effect of mebanazine only occurred after chronic treatment (10–21 days). The present investigation was undertaken to help clarify the mechanism of increased insulin sensitivity in mebanazine-treated rats.

EXPERIMENTAL

The animals were male albino rats (190–230 g) from the specific pathogen-free strain bred at Alderley Park. They were maintained on a cubed diet and water *ad libitum* except where food intake was restricted for purposes of pair-fed controls. Adreno-demedullated rats were maintained on 0·9% saline in place of drinking water for 4 weeks and used in experiments 6 to 8 weeks after operation. Blood samples were obtained from the abdominal aorta after intraperitoneal pentobarbitone sodium anaesthesia, or by cardiac puncture under ether anaesthesia where indicated. There were five animals used in each experimental group and the rats were weighed daily, food and water consumption being recorded for groups of 5.

Blood glucose was determined by a kit glucose-oxidase method and glycogen by the method of Krisman (1962). The extraction of noradrenaline from tissues and its subsequent fluorimetric estimation was carried out by modifications of literature methods described by Iversen (1963). Octopamine does not interfere in the fluorimetric assay of noradrenaline since it is a phenol derivative.

RESULTS

The mean fall in blood glucose, 90 min after insulin (0·5 U/100 g, s.c.) was 37% in a group of control rats. Animals which had received mebanazine orally (15 mg/kg daily) for 3 weeks showed a significantly greater hypoglycaemic response, 61%, when given the same dose of insulin. A third group of rats which had been pair-fed with the mebanazine group also exhibited a potentiation of insulin hypoglycaemia (67%) which was not statistically different from the treated group. These results are summarized in Table 1.

Table 1. *The effect of insulin* (0·5 U/100 g, s.c.) *on blood glucose levels* (mg %) *in control rats, rats receiving mebanazine orally* (15 mg/kg daily) *for* 21 *days and rats pair-fed with those receiving mebanazine* (*blood sampled* 90 *min after insulin; means* ± *s.e.*, $n = 5$)

Treatment group	Initial value	90 min after insulin	Change	% fall	*P* value
Control	98·3 ± 6·4	64·7 ± 10·1	—37·5 ± 7·7	36·8 ± 8·4	—
Mebanazine	107·1 ± 5·5	41·2 ± 4·0	—65·9 ± 5·2	61·0 ± 4·3	<0·05
Pair-fed	92·5 ± 10·1	30·8 ± 5·2	—61·6 ± 5·8	67·0 ± 2·7	<0·01

From these results it might have been concluded that the potentiation of insulin hypoglycaemia was secondary to the reduction in food intake by mebanazine (Barrett, 1969). However, the pattern of eating behaviour in the three groups was quite different. The control group exhibited mainly nocturnal eating whereas the mebanazine-treated rats appeared to nibble their food more or less continuously with a lower over-all intake. The pair-fed animals consumed all their allowance within

3 h and thus spent 21 h daily without food. In an attempt to equalize these differences the experiment was repeated, fasting all three groups for 21 h before giving insulin. The results, summarized in Table 2, showed that fasting *per se* increased hypoglycaemic sensitivity in control rats. In the mebanazine-treated group the degree of hypoglycaemia was greater but the difference did not achieve statistical significance. Pair-fed animals were also more sensitive to insulin than were the controls, but again not significantly so. The results might be construed as showing an influence on food

Table 2. *The effect of insulin* (0·5U/100 g, s.c.) *on blood glucose levels* (mg %) *in control rats, rats receiving mebanazine orally* (15 mg/kg daily) *for* 21 *days and rats pair-fed with those receiving mebanazine, after* 21 *h of fasting* (*blood sampled* 90 *min after insulin; means* ± *s.e.*, $n = 5$)

Treatment group	Initial value	90 min after insulin	Change	% fall	*P* value
Control	74·4 ± 9·8	32·8 ± 4·8	—41·6 ± 5·0	57·2 ± 3·4	—
Mebanazine	69·0 ± 4·6	20·6 ± 3·4	—48·4 ± 8·0	69·0 ± 6·6	N.S.
Pair-fed	78·8 ± 8·0	26·0 ± 5·6	—52·8 ± 6·7	67·2 ± 5·4	N.S.

intake alone and that mebanazine had no specific effect on the sensitivity to insulin. However, in the afternoon following this experiment (blood samples having been obtained by cardiac puncture in the morning) the mebanazine-treated rats were moribund or dead. One pair-fed animal was dead, but all controls were alive and active. Post-mortem examination of the animals showed cardiac tamponade in the dead pair-fed animal but no damage in those rats receiving mebanazine. A blood glucose analysis on one of the moribund rats gave a value of 7%. This experience suggested that it would be worthwhile studying the time course of the response to insulin in the fasting state. The results of such an experiment are summarized in Table 3. In this case the maximum hypoglycaemic effect was significantly greater in the mebanazine and pair-fed groups compared with control rats, 90 min after insulin. Whereas at 3 h both the control group and the pair-fed values had returned to pre-insulin glucose levels, that of the mebanazine-treated group had dropped to

Table 3. *Time course of blood glucose response to insulin* (0·5 U/100 g, s.c.) *in control rats, rats receiving mebanazine orally* (15 mg/kg daily) *for* 21 *days and rats pair-fed with those receiving mebanazine after* 21 *h of fasting* (*means* ± *s.e.*, $n = 5$)

Treatment group	Time after insulin injection (min)				
	0	60	90	120	180
Control	80 ± 6·1	61 ± 4·9	41 ± 3·7	60 ± 4·9	85 ± 8·1
Mebanazine	74 ± 6·2	43 ± 3·1	20 ± 4·1	18 ± 5·3	10 ± 4·1
Pair-fed	72 ± 8·4	45 ± 4·9	22 ± 3·9	41 ± 6·1	70 ± 6·4

10 mg % and the animals were unconscious. Intravenous injection of glucose (2 ml of 10% solution) brought about a dramatic return to consciousness and the animals survived for a period of 2 weeks after which they were killed.

It was possible that treatment with mebanazine altered either the amount of hepatic glycogen available to counteract the hypoglycaemic effect of insulin or the

ability of the treated animals to mobilize an adequate glycogen reserve satisfactorily. Determination of hepatic glycogen content in control rats which had been allowed to feed normally gave a value of 6·0 ± 0·6 mg % in comparison with 5·6 ± 1·0 mg % for rats given mebanazine orally for 21 days. In the pair-fed group the concentration was only 2·7 ± 0·6 mg %. Bearing in mind that pair-fed animals had a 21 h interval between consumption of the previous day's food, liver glycogen determinations were also made on animals in control and mebanazine-treated groups, deprived of food for 21 h. In neither case was there any detectable glycogen concentration. It was apparent, therefore, that both fasting control and pair-fed rats were able to survive a severe hypoglycaemic episode without measurable liver glycogen in the former case, but that mebanazine-treated animals could not do so. Absence of an adequate glycogen reserve could not therefore explain the effect of mebanazine in the present experiments.

Table 4. *Concentrations of noradrenaline* (μg/g) *in brain, heart and liver of control rats and rats receiving mebanazine orally* (15 mg/kg daily) *for* 21 *days* (*means* ± *s.e.*, $n = 5$)

Treatment group	Brain	Heart	Liver
Controls	0·32 ± 0·04	0·89 ± 0·09	0·08 ± 0·002
Mebanazine	0·56 ± 0·03	1·47 ± 0·08	0·10 ± 0·007
P value	<0·01	<0·01	<0·01

The 15-day period of treatment with mebanazine led to a statistically significant increase in the brain, heart and liver concentrations of noradrenaline (Table 4). These levels of noradrenaline were not significantly altered by the administration of insulin (0·5 U/100 g, s.c.). Treatment with mebanazine did not protect the enlarged stores of noradrenaline from depletion by reserpine (Table 5).

Table 5. *Effect of reserpine* (5 mg/kg, i.p.) *on noradrenaline concentrations in heart and liver of control rats and rats receiving mebanazine orally* (15 mg/kg daily) *for* 21 *days* (*samples taken* 24 *h after reserpine; means* ± *s.e.*, $n = 5$)

	Tissue noradrenaline content (μg/g)	
Treatment group	Heart	Liver
Control	0·970 ± 0·010	0·103 ± 0·040
Control + reserpine ..	0·032 ± 0·009	0·001 ± 0·001
Mebanazine	1·214 ± 0·012	0·114 ± 0·036
Mebanazine + reserpine ..	0·054 ± 0·010	0·001 ± 0·001

Attempts were made to determine whether or not other drugs affecting the storage and release of noradrenaline might also potentiate insulin hypoglycaemia and prolong sub-normal glucose levels. The experiments were duplicated in both intact and adreno-demedullated rats because of the insensitivity of medullary stores of catecholamines to depleting agents. The results are summarized in Table 6. After the administration of reserpine, guanethidine, α-methyldopa, bethanidine, tranylcypromine and mebanazine to intact rats only the latter two agents afforded potentiation.

In adreno-demedullated rats in addition to tranylcypromine and mebanazine, bethanidine and guanethidine also produced significant potentiation. The failure of reserpine to produce any potentiation even after de-medullation was most surprising. Although it produced almost complete depletion of tissue catecholamines there was no evidence of hypoglycaemic unresponsiveness despite the fact that the animals were in poor condition and not eating.

Table 6. *Effect of various drugs on the sensitivity of intact and adrenodemedullated rats to insulin hypoglycaemia* (0·5U/100 g, s.c.). A plus sign indicates significant potentiation at the 5% level.

Treatment	Daily dose (mg/kg) oral route	Duration	Potentiation of insulin: Intact rats	Potentiation of insulin: Demedullated rats
Reserpine	5 i.p.	2 days	—	—
Guanethidine	10 oral	10 days	—	+
Bethanidine	7·5 oral	10 days	—	+
α-Methyldopa	10 oral	21 days	—	—
Tranylcypromine	7·5 oral	21 days	+	+
Mebanazine	15 oral	21 days	+	+

It has been suggested that the administration of α-methyldopa or a monoamine oxidase inhibitor leads to the displacement of noradrenaline at adrenergic nerve terminals by α-methylnoradrenaline and octopamine respectively (see Kopin, 1968, for references). Intravenous administration of saline in fed rats had little effect on blood glucose, 15 min afterwards. Adrenaline (10 μg/kg, i.v.) raised the circulating glucose level to 155 mg per 100 ml compared with 168 mg per 100 ml 15 min after α-methylnoradrenaline (50 μg/kg). In contrast, octopamine at 100 μg/kg had no effect on blood glucose levels. These results are summarized in Table 7.

Table 7. *Blood glucose levels in rats given intravenous injection of saline or various drugs, 15 min later (values expressed as* mg/100 ml, *means* ± *s.e., n* = 5)

Treatment	Dose	Blood glucose
Saline	0·1 ml/100 g	116 ± 2·0
Adrenaline	10 μg/kg	155 ± 13·2
α-Methylnoradrenaline	50 μg/kg	168 ± 18·0
Octopamine	100 μg/kg	110 ± 3·2

In a final experiment I tried to revive 21 h fasted rats, which had received mebanazine chronically for 3 weeks, from a fatal insulin hypoglycaemia with adrenaline and α-methylnoradrenaline, but was unsuccessful. Only the intravenous injection of glucose was effective in this condition.

DISCUSSION

Chronic administration of mebanazine has been shown to potentiate the hypoglycaemic response to insulin and to delay the recovery of blood glucose levels to normal. The former effect may well be associated with the reduction in food intake, since pair-fed animals showed a spontaneous recovery to pre-insulin glucose levels

comparable to that in control rats. The failure of the homeostatic response in mebanazine-treated rats could not be attributed to a depletion of liver glycogen or tissue noradrenaline. Mebanazine did not protect the enlarged noradrenaline stores from depletion by reserpine. Depletion of catecholamines by adrenergic neuron blocking agents also prolonged hypoglycaemia in adreno-demedullated rats.

Other workers (Adnitt, 1968a; Cooper & Ashcroft, 1966) have shown that enhanced insulin sensitivity was associated with the monoamine oxidase inhibition rather than any other property of this class of drugs. Further, clinical exploitation of the phenomenon has been reported in several different centres (Adnitt, 1968b; Cooper, 1966; Wickstrom & Pettersson, 1964). The present results help to provide a rational explanation of previously reported clinical findings.

It is well established that chronic inhibition of monoamine oxidase leads to an increase in tissue stores of noradrenaline. Yet at the same time there is a diminished sympathetic responsiveness in relation to neural release of the transmitter (Davey, Farmer & Reinert, 1963). Pre-treatment of rabbits with iproniazid increased the noradrenaline content of brain, heart and liver, but also elevated the octopamine content to a proportionately greater extent (Kakimoto & Armstrong, 1962). Pheniprazine has also been found to raise the level of octopamine in cat heart and spleen (Kopin, Fischer & others, 1965). These authors demonstrated release of octopamine from cat spleen by nerve stimulation and this, taken with other reports reviewed by Kopin (1968), may be taken as evidence of the presence of octopamine as a genuine "false transmitter". Inhibition of monoamine oxidase leads to an accumulation of tyramine; it is taken up by sympathetic nerve endings and β-hydroxylated to octopamine. Octopamine then replaces noradrenaline in the storage vesicles. In the present experiments mebanazine was shown to increase tissue noradrenaline levels, yet to diminish responsiveness to hypoglycaemia. Octopamine was found to have very weak hyperglycaemic activity. Depletion of catecholamines by guanethidine or bethanidine was also shown, in adreno-medullated rats, to produce a similar hypoglycaemic unresponsiveness to that seen with the monoamine oxidase inhibitors. It is logical to conclude that following mebanazine treatment the failure of blood glucose levels to return to normal after insulin is due to replacement of the normal transmitter by one which is inactive in the context of raising blood sugar.

Mebanazine and other monoamine oxidase inhibitors have been found to induce hypotensive effects in man (Pletscher, 1966). Experiments in man have shown equipressor doses of (−)-noradrenaline and (−)-α-methylnoradrenaline to be in the ratio of approximately 1 to 3, noradrenaline being the more potent (Mueller & Horwitz, 1962). Replacement of noradrenaline by its α-methyl analogue has been advanced as the explanation of the hypotensive action of α-methyldopa. Experiments in anaesthetized dogs have shown a 10 to 1 pressor potency ratio for (−)-noradrenaline and (−)-octopamine (Kappe & Armstrong, 1964). In contrast to these observations, Mueller & Horwitz (1962) found α-methylnoradrenaline and noradrenaline to be approximately equipotent in raising the blood sugar level whereas octopamine was virtually inactive both in the hands of Supniewsky (1929) and the present author. In his clinical study of mebanazine, Adnitt (1968b) found evidence of enhanced insulin sensitivity but no effect on pulse rate or blood pressure in response to hypoglycaemia. He considered it unlikely that monoamine oxidase inhibitors selectively antagonized carbohydrate changes without affecting cardiovascular changes but the evidence of the present study would support just such a viewpoint.

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A test for the assessment of the duration of the action of neostigmine and of the relative potencies of various anticholinesterases using the pupil of the mouse

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The miotic effect of anticholinesterases was used to monitor the duration of their activity and to compare the relative potency of different members of this group. Female albino mice were used and the diameter of their pupils measured with the help of a monocular microscope at a magnification of × 60. After a suitable control period, readings were taken at 30 min intervals. This test was found to be superior to previous tests described for the same purpose.

During the search for a suitable indicator substance for the assessment of a new formulation for the slow release of drugs (Collings & Schneider, 1970), it was decided to use the anticholinesterase, neostigmine. Various methods for monitoring the release of neostigmine proved unsuitable. Eventually a test measuring the effect of neostigmine on the diameter of the mouse pupil proved satisfactory. The success of this test in assessing the duration of the drug effect led to an investigation of its usefulness for the evaluation of the biological potency of different anticholinesterases.

This paper describes details of the method.

EXPERIMENTAL

In the early experiments albino mice of the Schofield strain of either sex weighing approximately 20·0 g were used. However, female mice were found to be the more sensitive and also gave more consistent results; therefore in the later work only female animals were used. There was no restriction of food or drink before the experiment. The mice, suitably restrained, were placed on the stage of a monocular microscope and their pupils examined through a 10× objective and a 6 × eyepiece which contained a graticule calibrated in arbitrary units. Control readings were taken at 10 min intervals until they became consistent. Thereupon the drug was administered either as an aqueous control solution or in different slow release formulations. Injections (0·1 ml/20 g) were given subcutaneously into the scruff of the neck and readings were taken every 30 min; lengthening of the time intervals produced unduly large pupils. Readings were expressed as a percentage of the control values. To eliminate observer discrepancies, each set of experiments was made by the same observer. Some mice had pupil diameters outside the usual range and were not used, nor were the mice that had continuously changing pupils so that consistent control values could not be obtained. The reason for this behaviour was not ascertained.

Drugs used were: neostigmine methyl sulphate (Prostigmin, Roche); physostigmine sulphate (BDH); pyridostigmine (Mestinon, Roche); atropine sulphate (Burroughs Wellcome); edrophonium chloride (Tensilon, Roche).

The slow release formulations used are described by Collings & Schneider (1970).

RESULTS

The use of the mouse pupil for the estimation of the slow release of drugs is illustrated in Fig. 1. The effect of an aqueous solution given in a dose of 1·25 μg of neostigmine/20 g, Fig. 1b, was compared with that of 5·0 μg of neostigmine/20 g contained in two types of slow release formulation (A and B), Fig. 1c,d. An experiment with an aqueous solution containing the same dose of neostigmine (5·0 μg/20 g), Fig. 1a, had to be abandoned, as all the animals involved showed violent convulsions. None of the animals that had received the drug at the same level in the slow release formulation convulsed; all survived. The duration of the effect was prolonged from 150 min or under, to 370 min or more than 440 min respectively with the two slow release formulations.

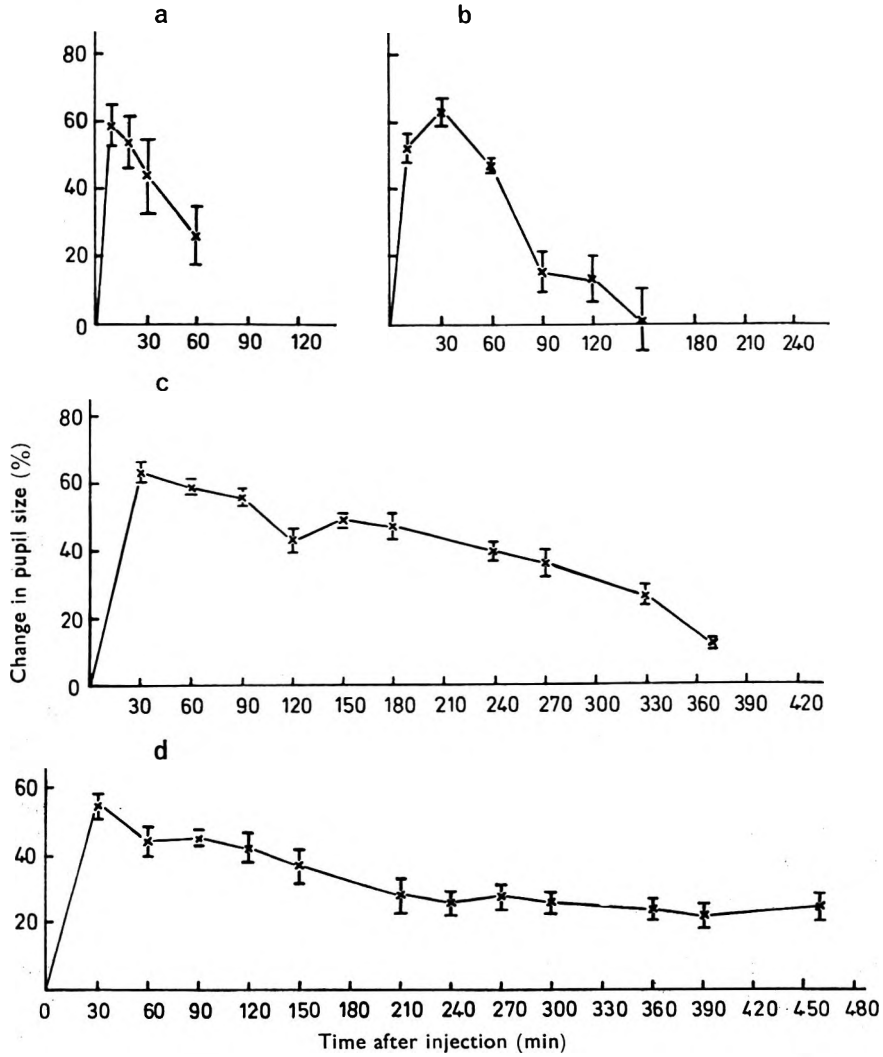

FIG. 1. Duration-action curves for neostigmine, using the mouse pupil test. (a) 5·0 μg/20 g in aqueous solution; (b) 1·25 μg/20 g in aqueous solution; (c) 5·0 μg/20 g in slow release formulation A; (d) 5·0 μg/20 g in slow release formulation B. Each point on the graph represents the mean value ($\pm$ s.e.) of 10 assays. Readings represent maximum change of pupil size (30 min after injection).

Fig. 2a shows the dose-response curves for the four anticholinesterases, neostigmine, physostigmine, pyridostigmine and edrophonium, the corresponding correlation coefficients for which were 0·80, 0·74, 0·88 and 0·82 respectively. The lines in Fig. 2 are calculated regression lines. In all cases there was a significant correlation between the dose of the drug and its effect on pupil size.

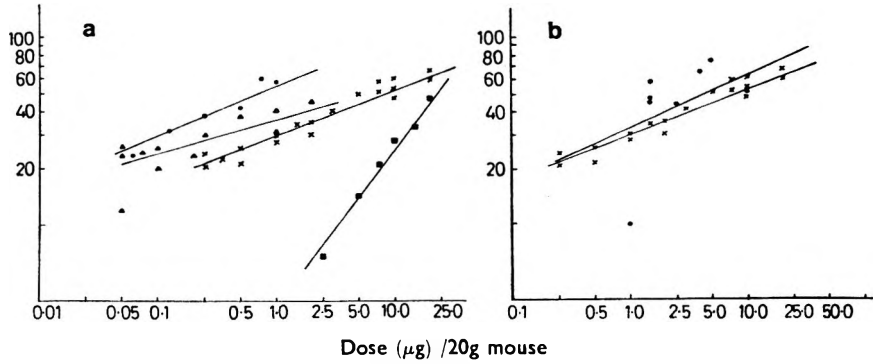

FIG. 2. Concentration-action curves for 4 "reversible" anticholinesterases. (a) Using the mouse pupil test without a mydriatic: (● neostigmine; ▲ physostigmine; × pyridostigmine; ■ edrophonium). (b) Using a similar test with the simultaneous administration of atropine 1·5 μg/20 g (× pyridostigmine; ● pyridostigmine + atropine). Each point on the graph represents the mean value (± s.e.) of 10 assays. Readings represent maximum change of pupil diameter (30 min after injection for neostigmine; 20 min for physostigmine, pyridostigmine and edrophonium. For the test with pyridostigmine + atropine, a set time of 15 min was used) (Grewal, 1951). Ordinate: Change in pupil size (%, log scale).

Fig. 2b illustrates the difference between the test using the antagonistic effect of the miotic pyridostigmine to atropine-induced mydriasis (Grewal, 1951) and the present test using a single drug only. It can be seen that the scatter in the test with atropine is much greater than in the present one, and the values for the correlation coefficients were 0·32 and 0·88 respectively. Further, the threshold value for the pyridostigmine induced miosis without atropine was much lower (125 ng) than that for the test including atropine (1 μg) which made it possible to extend the dose range.

DISCUSSION

In vitro techniques for the measurement of the effect of neostigmine on the serum cholinesterases are regarded as unsatisfactory. Various factors may invalidate the results of such methods, the most important of these being the dilution effect which leads to dissociation of the enzyme-inhibitor system and thereby to unduly high values for enzyme-activity compared with those of the undiluted serum (Krayer, Goldstein & Plachte, 1944). It was therefore decided to use a method which would measure directly a biological effect of the injected anticholinesterase.

The reason why the red tear test (Burgen, 1949) was not successful in our hands remains obscure. It consists in the stimulation of porphyrin secretion from the Harderian glands of the rat by the injection of graded doses of acetylcholine. After determining the threshold value for each rat, a non-toxic effective dose of neostigmine that would lower the threshold value of acetylcholine was given. Acetylcholine injections were then repeated at half-hourly intervals until the control values were regained. In spite of many repeated experiments with various slow release preparations we were unable to demonstrate any delay in the release of neostigmine with this

method. Control levels were invariably regained within 3 h whether aqueous solutions or test emulsions were used. Furthermore the response of individual rats varied from 50–200 μg of acetylcholine from day to day. Even greater variations were found between groups of rats tested at different periods. In August 1966, no response was obtained in some rats with doses exceeding 1000 μg, whereas threshold values were as low as 3–12·5 μg of acetylcholine in January 1967.

No definite reason for these discrepancies was found. The cause may possibly have been environmental, as all the rats were from the same basic stock.

A method for testing the miotic effect of certain drugs after intraperitoneal injection was described by Grewal (1951). This was based on Pulewka's (1932) method of testing mydriatics on mice. The method consisted in assessing the antagonistic effect of the miotic against the mydriatic effect of atropine. With such a test it is impossible to assess unusual responses to either drug and the chances of error are increased. Furthermore it is not possible to use the individual animals as their own controls and to express the results as percentage of the control values. They could only be compared with a separate control group tested for atropine response at a different time. Also the correlation between dose and response became less significant than when only a single drug was used (Fig. 2b).

The test described can be used to assess the relative potencies of anticholinesterases and compared favourably with older tests designed for this purpose. It proved superior to the chromodacryorrhoea test (Burgen, 1949) inasmuch as it was not subject to the tremendous fluctuations in response that made that test unmanageable. It also compared favourably with the test for miotics using their antagonism to atropine mydriasis (Grewal, 1951) in the example studied (pyridostigmine). In this case there was a much closer correlation between dose and response with the present test than with the older one. Indeed the correlation coefficient was not significant (0·32) with the older test, whereas it was highly significant (0·88) with the present one.

The most important advantage however, lay in the fact that the new test proved useful for the assessment of the prolongation of the effect of drugs in a slow release formulation (Collings & Schneider, 1970). Many formulations were tested and the methods proved reliable if the precautions mentioned were observed.

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I should like to thank Mr. J. K. Johnson for pointing out the difficulties associated with the *in vitro* assessment of the activity of "reversible" anticholinesterases, and Dr. J. A. Waterhouse for his invaluable and patient help with the statistics. My thanks are also due to Mrs. V. P. McKay and to Mr. P. G. Dearn without whose expert technical assistance this work would never have been completed. The work was supported by a grant from The National Research Development Corporation.

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LETTERS TO THE EDITOR

Prostaglandins in dog renal medulla

During an investigation into the effects of acute experimental renal ischaemia in dogs (McGiff, Crowshaw & others, 1969), we detected prostaglandin-like substances in the venous blood of both the ischaemic and contralateral kidneys. Prostaglandins (PGE_2, $PGF_2\alpha$ and PGA_2) have been unequivocally identified in rabbit renal medulla (Lee, Crowshaw & others, 1967; Daniels, Hinman & others, 1967). This report concerns the isolation of three biologically-active lipids from dog renal medulla which exhibited the typical chromatographic and pharmacological properties of PGE_2, $PGF_2\alpha$ and PGA_2.

Kidneys from 20 dogs were sectioned and the medulla was separated from the cortex and stored at $-10°$. The pooled medullary tissue (453 g) was homogenized for 2 min in 400 ml water at 4°. Five volumes of ethanol were added and the mixture was vacuum filtered. The residue was washed with a further 500 ml ethanol and the combined filtrates were evaporated *in vacuo* to a small volume, then diluted to 800 ml with water and extracted (3 × 800 ml) with ethyl acetate. The extracts were combined and evaporated to 100 ml. The concentrate was extracted (4 × 50 ml) with 0·1M phosphate buffer (pH 8). The pH of the buffer phase was adjusted to 3 with 2N HCl and the acidic solution extracted (6 × 200 ml) with chloroform. The combined chloroform extracts were evaporated to dryness, the residue dissolved in ethanol (10 ml) and 0·9 ml of the solution was taken for preliminary chromatographic and biological testing. The remainder was evaporated and dissolved in 10 ml of benzene–ethyl acetate (9 : 1, v/v) and applied to a 20 g silicic acid column as described by Lee & others (1967).

To minimize losses of prostaglandins during silicic acid chromatography, small volumes of solvents (200 ml) were used in the elution sequence (benzene-ethyl acetate 7 : 3; 2 : 3; 1 : 9; methanol) so that the time during which prostaglandins were on the silicic acid was relatively short and the eluates were quickly evaporated. Fractions containing prostaglandins were eluted with more polar solvent mixtures than usually used (Samuelsson, 1963), but we consistently obtained a reproducible sequence of column separations; PGA compounds were eluted with benzene–ethyl acetate (2 : 3), PGE compounds with benzene–ethyl acetate (1 : 9) and PGF compounds with methanol. The eluates were evaporated to dryness, dissolved in ethanol (1·0 ml) and portions taken for bioassay, those with biological activity being stored at 4° for further assay and thin-layer chromatographic (TLC) characterization as described previously (Lee & others, 1967). For separation of those prostaglandins differing only in their degree of unsaturation, e.g., PGE_1 and PGE_2, $AgNO_3$/silica gel G (1 : 30) was used (Green & Samuelsson, 1964). Solvent systems were "AI", benzene–dioxan–acetic acid (20 : 20 : 1, v/v); B, chloroform–methanol–acetic acid (18 : 1 : 1, v/v); C, chloroform–methanol–acetic acid (18 : 2 : 1, v/v). After separation, 1 cm wide zones were scraped off the plates and eluted with organic solvents.

Purified samples were sonified in saline for testing. Smooth-muscle stimulating activity was determined using rat stomach strip, rat colon and chick rectum continuously superfused in series by Krebs solution at 37·5° (Ferreira & Vane, 1967). Both PGE_2 and $PGF_2\alpha$ contract all three tissues. The effects of PGE_2 were usually distinguishable from $PGF_2\alpha$ because of the differential magnitude of their effects on the assay organs. The rat blood pressure bioassay was used to detect PGA-like material and to characterize further the PGE and PGF fractions obtained from column and thin-layer chromatography (McGiff, Terragno & others, 1969).

The biologically active material was isolated and estimated after each stage of the purification procedures as shown in Fig. 1. The extraction of 453 g of dog renal

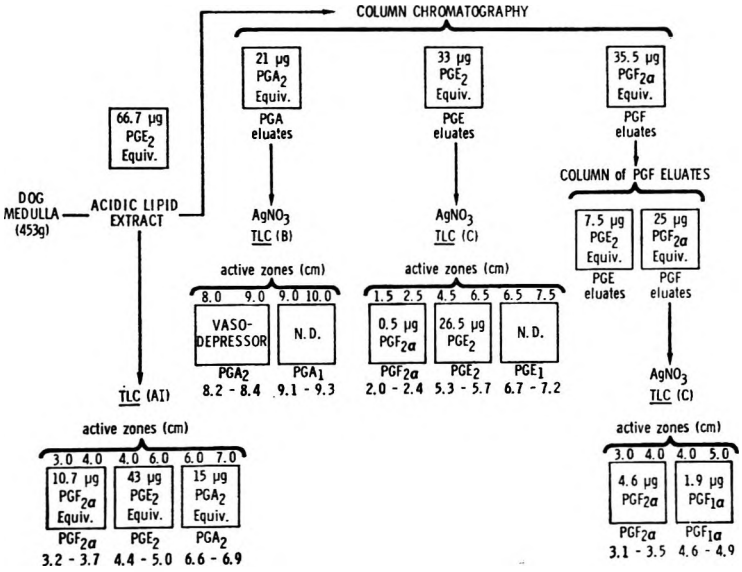

FIG. 1. Extraction and chromatographic separation of prostaglandins in 453 g of dog renal medulla. The prostaglandin-like activity isolated after each step of these procedures is shown in boxes. Silica gel TLC of a portion of the acidic lipid extract using solvent system AI (not equilibrated) yielded 3 biologically active zones. Silicic acid chromatography of the bulk of the extract also yielded a comparable separation, although re-chromatography of the PGF eluates was necessary to remove some PGE-contamination. Final TLC separation of the PGA, PGE and PGF eluates was effected on layers of silica gel containing $AgNO_3$ and using solvent systems B, C and C respectively (see text for solvent composition; solvent front 15 cm). Prostaglandin-like substances were extracted from certain zones characterized by their distance from the origin. Prostaglandin standards, run on the edge of each plate, were visualized after the unknowns were removed. Comparison of these zones with the position of prostaglandin standards (the mobilities are shown under each box in the figure) provides a tentative identification of these compounds. Important zones in which activity was not detected are indicated N.D.

medulla and the subsequent purification of the extract by column chromatography resulted in the recovery of 21 μg PGA_2 (0·033 μg/g), 40·5 μg PGE_2 (0·090 μg/g) and 25 μg $PGF_2\alpha$ (0·055 μg/g). These three prostaglandins have also been identified in rabbit renal medulla (Lee & others, 1967) although much higher prostaglandin concentrations were reported in the rabbit. The demonstration that prostaglandins are present in the kidney medulla of two species suggests that these biologically active compounds may be important renal hormones. The demonstration of PGE-like material in the renal venous blood of hypertensive humans (Edwards, Strong & Hunt, 1969) and in renal venous blood of dogs with experimentally induced ischaemia (McGiff & others, 1969) supports this possibility. Furthermore, in both cases, evidence indicated that the major prostaglandin constituent was PGE_2.

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Response of female mice to anticonvulsants after pretreatment with sex steroids

We have examined in female mice the effects of lynestrenol (progestin) and mestranol (oestrogen) on the intensity and duration of activity of a series of anticonvulsant drugs.

Rumke & Noordhoek (1969) had shown previously that pretreatment of mice with large doses of lynestrenol (20 and 200 mg/kg) 48 h before receiving either phenytoin or phenobarbitone resulted in decreased protection against bemegride convulsions, and increased metabolism of the drugs. Banziger (1965), and Swinyard & Castellion (1966) have demonstrated that the minor tranquillizers chlordiazepoxide (Librium) and diazepam (Valium) in high doses protect mice from tonic extensor seizure produced by maximal electroshock. Chlordiazepoxide is rapidly and extensively metabolized in mice (Coutinho, Cheripko & Carbone, 1968) and its anticonvulsant activity pattern follows closely the disappearance rate of the parent drug and its major metabolites from the brain and plasma. Since both lynestrenol and mestranol have marked and opposite effects upon the metabolism of certain barbiturates (Blackham & Spencer, 1969) it occurred to us that these effects may also occur with phenobarbitone, phenytoin, chlordiazepoxide, and diazepam.

For each anticonvulsant studied 5 groups of 10 female TO mice, weighing 20–25 g, were pretreated with progestin (lynestrenol, 10 mg/kg), or oestrogen (mestranol, 1·0 mg/kg), or their oily vehicle, daily, for four days. On the fifth day, each of the 5 groups received an intraperitoneal injection of one of the following anticonvulsant drugs; phenytoin sodium, 20 mg/kg; phenobarbitone sodium, 40 mg/kg; chlordiazepoxide hydrochloride, 80 mg/kg; or diazepam, 20 mg/kg. At various times afterwards, they were subjected to maximum electroshock, using a shock of 70 V at 100 pulses/s (pulse-width 0·2 ms), for a duration of 0·3 s. This was delivered through silver ear electrodes, according to the method of Cashin & Jackson (1962). The current was supplied by a Scientific Research Instruments' square-wave stimulator. This shock produced tonic extensor convulsions in 100% of all animals not receiving anticonvulsant drugs; each mouse was tested once only.

Our results are summarized in Fig. 1. Lynestrenol produced a reduction in intensity and duration of activity with all of the anticonvulsant drugs examined, whilst mestranol had the reverse effect. Administration of the microsomal enzyme inhibitor SKF 525A mimicked the effect of mestranol, increasing the protection of mice receiving these anticonvulsant drugs. The observed changes in activity of the above anticonvulsant drugs may be due therefore to alterations in their rate of metabolism.

There may be however an alternative explanation. We have already shown that marginal but opposite changes in brain 5-hydroxytryptamine levels occur in female

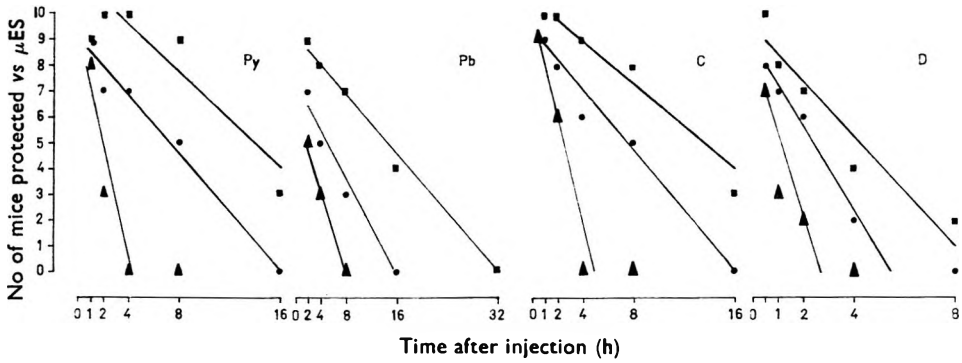

FIG. 1. Duration of action of four anticonvulsant drugs in female mice pretreated with oestrogen (■—■; mestranol, 1·0 mg/kg), progestin (▲—▲; lynestrenol, 10 mg/kg), or vehicle (●—●; controls, 0·1 ml/20 g) daily, subcutaneously, for four days. The anticonvulsant activity of phenobarbitone sodium (Pb; 40 mg/kg), phenytoin sodium (Py; 20 mg/kg), chlordiazepoxide HCl (C; 80 mg/kg) or diazepam (D; 20 mg/kg) was examined by maximal electroshock at various times after intraperitoneal injection.

mice pretreated with mestranol or lynestrenol (Blackham & Spencer, 1969). Schlesinger, Boggan & Griek (1968) showed that 5-hydroxytryptamine protected mice from electroshock seizures while reserpine pretreatment increased the animals' susceptibility to these seizures. Thus, whilst the changes in the response of these mice to anticonvulsant drugs may arise mainly from differences in their rates of metabolism, the changes possibly may be further influenced by differences in the susceptibility of the mice to the electroshock procedure, owing to changes in brain amine metabolism.

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3-Ethyl-8-methyl-1,3,8-triazabicyclo[4,4,0]decan-2-one: a new antifilarial agent*

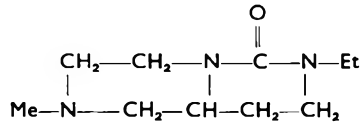
Earlier studies on the antifilarial activity of some open chain (Sewell & Hawking, 1950; Wadia, Asthana & others, 1958) and cyclic (Reinertson & Thomas, 1955; Wadia & Anand 1958a, b; Brookes, Terry & Walker, 1957; Nagpal & Dhar, 1965) analogues of diethylcarbamazine (Hetrazan) have shown that any modifications to the piperazine ring led to a lowering or loss of antifilarial activity. In further exploration in this field 3-ethyl-8-methyl-1,3,8-triazabicyclo[4,4,0]decan-2-one (I) has been synthesized† and found to possess high microfilaricidal activity in cotton-rats

Table 1. *Comparative efficacy of Compound I and diethylcarbamazine against* L. carinii *infection in cotton rat*

Compounds as base	ED90* (mg/kg) × 6 days, cotton rats i.p.	oral	LD50 (mg/kg) in mice i.p.	oral	Microfilarial count as % of pre-treatment level after stopping treatment on days: 1	8	Therapeutic index
Diethylcarbamazine	6	–	240	–	10	80–95	40
I	1	2	300	600	8	27–30	300

* The dose which when administered would clear 90% of the pretreatment circulating microfilariae.

infected with *Litomosoides carinii*. Screening was by the technique of Hawking & Sewell (1948). The comparative efficacy of I and of diethylcarbamazine is described in Table 1. Compound I thus seems to be significantly more effective than diethylcarbamazine in reducing the microfilariae in cotton rats. Its activity also seems to persist longer than diethylcarbamazine. Like diethylcarbamazine it was without significant action against the adult worms of *L. carinii*. Prolonged treatment for 21 days at 25 mg/kg intraperitoneally twice daily killed only about 10–20% of the adult worms.



I

Compound I was effective against the infective larvae of *L. carinii* in cotton rats. When cotton rats were treated with 25 mg/kg of the compound daily for three consecutive days commencing immediately after exposure to infected mites (*L. bacoti*) in desiccators for 24 h they failed to show any microfilariae or adult worms up to 90 days after exposure. Untreated controls invariably had adult worms and microfilariae.

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The effect of ovariectomy on the γ-aminobutyric acid content in the cerebral hemispheres of young rats

The removal of some endocrine glands affects the γ-aminobutyric acid (GABA) content in the rat brain. The GABA content is decreased after castration, but returns to normal after injection of testosterone propionate (Tzu-Yu Li & Chang-Hua Wu, 1964). Adrenalectomy results in decreased GABA content of rat brain (Rindi & Ventura, 1961), cerebral cortex (Vernadakis & Woodbury, 1959; Pandolfo & Macaione, 1964), and subcortex (Sutherland & Rikimaru, 1964). Thyroidectomy also decreased the brain GABA content (Nishioka, 1960). I have examined the effect of ovariectomy on the GABA content in the cerebral hemispheres of young rats.

Young female rats, 40–48 g, were divided into 4 equal groups of 9 each. One group received no treatment. The other animals were ovariectomized and left for 2, 15 and 30 days before being killed by guillotine for the estimation of GABA content in their cerebral hemispheres. At that time the rats weighed 60–70 g. For the analysis the cerebral hemispheres of three animals were pooled.

GABA was quantitatively determined using a chromatographic and colorimetric method. Within one min of death the brain was isolated and the cerebral hemispheres were separated and frozen to −4 to −6°. The frozen pooled sample was quickly weighed, triturated to a homogeneous mixture, and 60 ml of ethanol 75% was used as a solvent. The mixture was centrifuged and the supernatant fluid was evaporated to dryness. The residue was cooled and dissolved in distilled water. This extract was centrifuged and an amount of cerebral hemisphere extract equivalent to 60 mg of the original wet tissue was applied by an Agla micrometer syringe to a 20 × 46 cm band of chromatographic paper.* The chromatogram was developed with the descending technique by the one dimensional method using the solvent phenol–water (4 : 1 v/v) for 18 to 20 h, after which the solvent was removed. The paper was sprayed on both sides with 0·1% ninhydrin in butanol and left suspended for 30 min at 93° for maximal colour development (Roberts & Frankel, 1950). The GABA spots were eluted by distilled water, and the extinction read in a Unicam SP1300 colorimeter using filter No. 4. The relation between the extinction and the concentration of pure GABA† was determined under the experimental conditions. The formula of the curve was obtained by the least square method (Waugh, 1952) and used to convert the colorimetric readings into the equivalent concentrations of GABA.

The relation between different amounts of an authentic sample of GABA when used in between 5 and 35 μg, with the extinction of the ninhydrin-stained chromatographic spots had the general formula $Y = 0{\cdot}011 + 0{\cdot}0051\ X$.

Table 1. *GABA content in the cerebral hemispheres of* 2, 15 *and* 30 *days ovariectomized young female rats compared with the normal content*

Sample number and statistical data	GABA content (mg/100 g wet tissue) of cerebral hemispheres			
		Ovariectomy		
	Control	2 days	15 days	30 days
1	16·5	16·5	18·0	18·4
2	17·6	18·4	18·4	19·5
3	18·0	18·5	18·5	19·7
$\bar{x}$*	17·4	17·8	18·3	19·2 ($P < 0{\cdot}025 > 0001$)†
± s e.	0·5	0·7	0·2	0·4

* Each mean is the average GABA content obtained from 3 pooled samples each of 3 rats.
† By *t*-test.

The results obtained for GABA contents in the cerebral hemispheres of young female rats and in similar animals 2, 15 and 30 days after ovariectomy are in Table 1.

The GABA content did not change significantly in the cerebral hemispheres of young female rats after their ovariectomy by 2 and 15 days but there was a significant increase in GABA content 30 days after ovariectomy amounting to 10·6% from the control value. This appears to be reverse of the effect produced after castration (Tzu-Yu Li & Chang-Hua Wu, 1964).

The induced change in GABA content in the cerebral hemispheres is of importance since GABA appears to be the main inhibitory transmitter in cerebral cortex (Iwama & Jasper, 1957; Krnjević, Randic & Straughan, 1966; Krnjević & Schwartz, 1967).

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* Selecta-Filter Paper No. 2316, Carl Schleicher and Schull Dassel K. R. Einbeck, Pachtbetrieb der Bütenpapierfabrik Habnemühle GMBH, West Germany.
† γ-Aminobutyric acid, m.p. 202–204°, L. Light and Co. Ltd., Colnbrook, Bucks., England.

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An isotonic transducer for general use

The increasing use of electronic recording techniques in pharmacology has meant also an increase in the cost of equipping large numbers of students with modern apparatus. In Fig. 1 the circuit details of an inexpensive, stable and reliable isotonic transducer are shown.

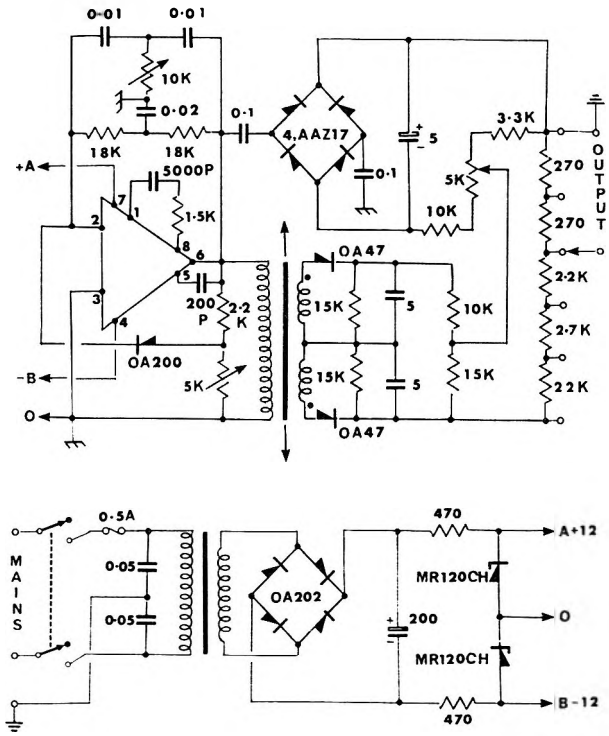

FIG. 1. Circuit diagrams for the isotonic transducer (upper) and power supply (lower) interconnected as indicated by the arrows. The operational amplifier is type μA7709C (SGS Fairchild Ltd.) and all potentiometers are 16 turn Painton mini-flatpots. Values of resistors and capacitors (tolerance ±5%) are in ohms and microfarads respectively unless otherwise stated.

An oscillator (900 Hz), driven by a mains operated semi-stabilized power supply, energizes the primary windings of a linear voltage differential transformer (LVDT) the moving core of which is connected to the tissue. Movement of the core of the LVDT (produced by contraction or relaxation of the tissue) gives rise to a change in the output from the secondary windings of the transformer which is fed to a phase discriminator, filters and attenuator and provides the output of the transducer. The unattenuated d.c. output (500 mV: 9 KΩ source resistance) is sufficient to drive most pen recorders but an attenuator is provided which will reduce the output in 5 steps to a minimum of 5 mV (270 Ω source resistance) which may be more suitable for high gain recorders with a low input resistance.

The core of the LVDT has a moving range of 1·5 cm; the output linearity of the transducer is better than 1% of full scale over the entire range. The zero stability and gain stability are better than 0·05% and 0·1% of full scale respectively, both of these measurements being made over a period of 2 h. Incorporation of the semi-stabilized power supply and constant amplitude integrated-circuit oscillator ensures that the transducer is insensitive to fluctuations in mains voltage, changes of 15%

producing a change in the output of the transducer which was indistinguishable from variations due to gain stability and zero stability.

Eight of these isotonic transducers have been used for teaching and research in this department for 3 months and no breakdowns have yet occurred. The tissue can be connected directly to the moving core of the LVDT (weight 5 g), counterbalance being provided by a weighted pulley system above the core, or more usually through a conventional isotonic lever system which can provide the necessary demagnification of the response from the tissue where this is greater than 1·5 cm This system has been used successfully on a variety of tissues including rabbit, rat and guinea-pig intestine (for conventional and also cumulative dose-response curves), transmurally stimulated guinea-pig vas deferens, rabbit and rat uteri, phrenic nerve-diaphragm and guinea-pig atria. With the latter two preparations "bounce" and the harmonics of the recording system can cause problems as they do with any isotonic recording from these tissues.

The total cost of the components and case for the complete transducer and power supply is £19 15s 0d, the most expensive item being the LVDT (type E300D; cost £13) which was obtained from Electromechanisms Ltd., of Slough.

This isotonic transducer will drive satisfactorily most pen recorders and provides an effective and inexpensive way of replacing the smoked drum.

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Effects of a marihuana homologue (Pyrahexyl) on avoidance learning in the gerbil

Pyrahexyl (synhexyl, 3-hexyl-7,8,9,10-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[*b*,*d*] pyran-1-ol) is a synthetic cannabis compound having behavioural effects similar to tetrahydrocannabinol (THC) one of the active principles of *Cannabis sativa* L (Hollister, Richards & Gillespie, 1968). Although pyrahexyl has been studied in man (Stockings, 1947; Parker & Wrigley, 1950; Thompson & Procter, 1953; Hollister & others, 1968), little is known of its specific behavioural effects except that it seems to have euphoriant properties. Recently, Abel (1969) found in rats that pyrahexyl markedly reduced the amount of time required to resume lever pressing for water after this activity had been suppressed by a fear-producing stimulus and Abel & Schiff (1969) reported that pyrahexyl increased "curiosity" in rats as measured by the time they spent observing other animals. We now report its effect in an avoidance learning situation. The particular testing procedure chosen assessed the effect of pyrahexyl on the acquisition of new behaviour rather than its effect upon a previously learned response as examined by Abel (1969).

Six adult male Mongolian gerbils (*Meriones Unguiculatus*), 80–90 g, were injected intraperitoneally with 0·2 ml of a solution of pyrahexyl (2·3 mg/kg) in olive oil; six control animals received only oil injections. After 2 h animals were placed individually into a standard two-compartment automated shuttle box (Lehigh Valley Electronics, Model 146–04) in which they could avoid being shocked through the grid floor by jumping over a barrier dividing the apparatus. An auditory signal was the conditioned stimulus and a 0·8 mA constant current electric shock the unconditioned stimulus. The conditioned stimulus preceded the onset of shock by

5 s. If an avoidance response was not made during this period, shock came on and remained on until the animal jumped over the barrier. Each presentation of the conditioned stimulus and the performance of a jumping response constituted a single trial; 50 such trials were given each day with a 25 s interval between trials. A total of 250 trials was given over a 5-day test period. The test apparatus and recording equipment were completely automated and the animals were not disturbed once the daily test period had begun. Records were kept of the number of successful trials; the time taken to jump the barrier after each conditioned stimulus onset (jump latency); and the number of between trial jumps which were not associated with the conditioned stimulus.

As shown in Fig. 1A, pyrahexyl-injected animals made more avoidance responses on the first day of testing than did control animals; this difference was statistically significant (*t*-test, Edwards, 1964; $t = 1{\cdot}89$, $P<0{\cdot}05$). By the second test day all animals had achieved high performance levels and no differences in acquisition of the avoidance response were found between groups on this or any subsequent day. However, analysis of variance (Edwards, 1964) showed that, compared to controls pyrahexyl-injected animals had significantly lower response latencies to the conditioned stimulus throughout the five day test period ($F = 11, 21$, df, 1, 10, $P<0{\cdot}01$, see Fig. 1B). There was no effect of the drug on responding independent of presentation of the conditioned stimulus ("between-trial" jumps). The mean daily between-trial jump rates were: pyrahexyl animals, $\bar{X} = 12{\cdot}19$, range 2·8–47; control animals, $\bar{X} = 14{\cdot}32$, range 4·0–50.

Thus, at the dosage level employed, pyrahexyl affected the acquisition of an active avoidance response in its earliest stages; the effect being confined to the first day of testing. It is likely that the effect was obscured on the remaining four test days because of the rapid learning of the control group during this period. However,

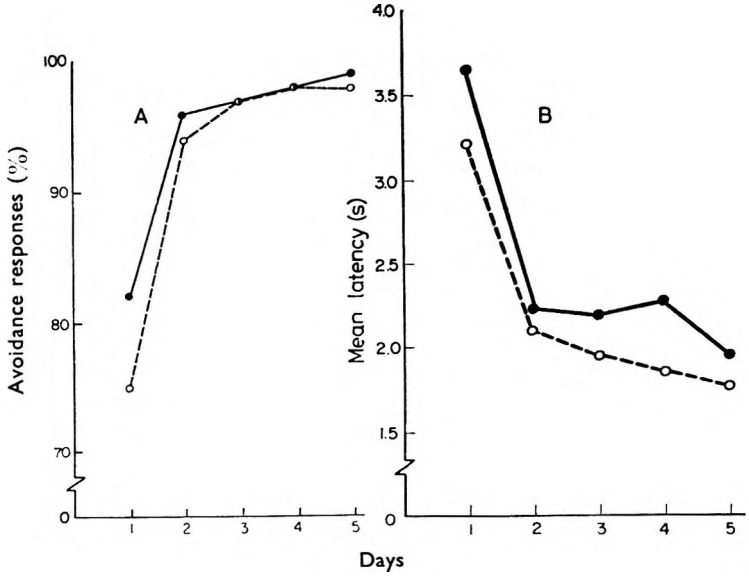

FIG. 1. A. Shock avoidance learning in gerbils given i.p. injections of 2·3 mg/kg pyrahexyl (●—●) and oil injected controls (○—○). Each point represents the mean percent avoidance responses over 50 trials on each of five days.

B. Mean jump latency in response to 5 s auditory warning signal during avoidance conditioning. Each point represents the mean time taken to jump a barrier over 50 trials on each of five days. ●—● Controls. ○—○ Drug.

performance was significantly affected by the drug on all test days since pyrahexyl-injected animals responded to the conditioned stimulus much more quickly than did control animals. This does not appear to be directly attributable to any drug-induced increase in general activity since there were no differences between groups in their between-trial jumping behaviour.

One possible interpretation of these data is that pyrahexyl can serve to increase the probability of a dominant response which would normally occur in a given situation. For example, after a thirsty animal has learned to depress a lever for water, the probability of lever-pressing following water deprivation is very high. However, this behaviour can be inhibited by inducing a conditioned fear reaction in the animal by presenting a tone which has previously been paired with an electric shock. Under such conditions Abel (1969) found that pyrahexyl reduced the time required for thirsty animals to resume lever-pressing for water reward and hypothesized that the drug served to reduce the degree of fear thereby lessening the inhibitory properties of this conditioned fear response.

Another interpretation of these data is that pyrahexyl in some manner facilitates the emission of the dominant response, viz., lever pressing. This has been supported by Abel & Schiff (1969) who found that when rats were placed in a box in which they could either observe other stimulus animals or explore the box, they spent most of their time observing other animals. Thus, the dominant behaviour in this situation was a specific observational response. When pyrahexyl was administered in this situation it was found that drug-injected animals spent even more time observing animals thus suggesting a preservation of a dominant response by the drug.

We found that once the animals had learned to avoid shock by jumping the barrier in the presence of the conditioned stimulus, the dominant response became jumping. Both groups of animals learned this response at about the same rate, but the response was consistently emitted much sooner in the animals given pyrahexyl. This suggests that the probability of making a prepotent response is increased under the influence of pyrahexyl. Therefore, our results may be viewed as offering further experimental support for the dominant response hypothesis.

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L-Dopa induced changes in central monoamine neurons after peripheral decarboxylase inhibition*

The functional and the biochemical effects of L-3,4-dihydroxyphenylalanine (L-dopa) treatment on central monoamine neurons after extracerebral dopa-decarboxylase inhibition have recently been published (see Butcher & Engel, 1969). Small doses of Ro 4–4602, *N*-(DL-seryl)-*N*-(2,3,4-trihydroxybenzyl)hydrazine, an inhibitor of the decarboxylase of aromatic amino-acids, selectively inhibit extracerebral dopa-decarboxylase. At these doses the enzyme in brain is relatively unaffected (Bartholini, Bates & others, 1967). Formation of dopamine in the brain after systemic injection of L-dopa in rats is enhanced by pretreatment with Ro 4–4602 (Bartholini & others, 1967; Butcher & Engel, 1969). At the same time there is a reduction of central 5-hydroxytryptamine (5-HT) concentrations (Bartholini, Da Prada & Pletscher, 1968; Butcher & Engel, 1969) and a slight reduction of noradrenaline (Butcher & Engel, 1969). Furthermore, extracerebral dopa-decarboxylase inhibition by Ro 4–4602 induces an increased formation of catecholamines from L-dopa in the neuropil and a reduced formation of catecholamines in the walls of the capillary cells (Constantinidis, Bartholini & others, 1968). However, no detailed histochemical analyses of the localization and the distribution of the central monoamines after combined Ro 4–4602–L-dopa treatment have been made. We now give a detailed description of L-dopa-induced changes in central monoamine neurons after extra-cerebral decarboxylase inhibition by small doses of Ro 4–4602. We have used a fluorescence method (Falck, Hillarp & others, 1962; Hillarp, Fuxe & Dahlström, 1965).

Adult male Sprague-Dawley rats, 160–180 g, were decapitated after chloroform anaesthesia and all parts of the brain were taken for histochemical fluorescence analyses of catecholamines and 5-HT. In each experiment the brains of 8–10 rats were analysed using coded slides. Ro 4–4602 (50 mg/kg, i.p.) was given 30 min before the L-dopa injection (50; 100; 200 mg/kg, i.p.). The rats were killed 75 min after the L-dopa injection.

Central dopamine neurons. There was a dose-dependent increase in the fluorescence intensity of the catecholamine cell body groups of the mesencephalon (Dahlström & Fuxe, 1964) which are all dopamine cell bodies (Corrodi, Fuxe & others, 1970). A very strong fluorescence intensity was found after 100–200 mg/kg of L-dopa in combination with Ro 4–4602 in the dopamine cell bodies of the zona compacta (lateral part), the pars lateralis and the zona reticulata of the substantia nigra, and also the mesencephalic reticular formation and the arcuate nucleus. The entire pericaryon became fluorescent together with its processes. The nuclei could no longer be observed. After treatment with L-dopa alone in the same doses and at the same time-interval, only a medium to strong fluorescence intensity was observed but this is above normal.

A dose-dependent increase in fluorescence intensity was also observed in the nigroneostriatal dopamine fibres and in the dopamine nerve terminal systems. In the latero-dorsal part of the neostriatum, those nerve terminal systems show more marked increases than those in the medio-ventral part and those in the limbic forebrain. In view of the fact that the dopamine cell bodies in the lateral part of the substantia nigra and in the mesencephalic reticular formation showed the most marked increase after Ro 4–4602 - L-dopa treatment, it may be that these dopamine cell bodies mainly innervate the lateral-dorsal part of the neostriatum (this corresponds mainly to putamen). The existence of a lateral nigroneostriatal dopamine pathway has also been suggested by Poirier, McGeer & others (1969). No increase in fluorescence intensity was observed in the dopamine nerve terminals of the median

eminence. This could be because this area is localized outside the blood-brain barrier. Thus, Ro 4–4602 can reach this area and block the decarboxylation of L-dopa to dopamine.

Central noradrenaline neurons. No increases in fluorescence intensity were observed in the noradrenaline cell bodies of the medulla oblongata and the pons (see Andén, Dahlström & others, 1966), or in the noradrenaline nerve terminal systems. At the highest dose of L-dopa after Ro 4-4602 pretreatment, these terminals appeared indistinct because of the increased background fluorescence in the neuropil, especially in areas having higher densities of the terminals. These changes were probably partly the result of an outflow of newly formed catecholamines, mainly dopamine, (from dopa) over the noradrenaline nerve cell membrane into the extraneuronal space.

Central 5-HT neurons. The 5-HT cell bodies became distinctly greenish when seen in the fluorescence microscope. This fluorescence was mainly localized to a perinuclear ring and was of weak to medium intensity. The 5-HT nerve terminals and fibre bundles exhibited a very weak to weak greenish fluorescence.

Extraneuronal tissue. There was a dose-dependent increase in the diffuse green fluorescence of the neuropil in agreement with Constantinidis & others (1968). Furthermore, the fluorescence in the cells of capillary walls was much reduced compared with that from cells of rats treated with dopa alone. In the highest dose, however, a distinct weak green fluorescence was still seen in the capillary cells in most parts of the brain in spite of Ro 4–4602 pretreatment.

When L-dopa alone is given systemically to rats it is probably mainly decarboxylated extracerebrally because the dopa-decarboxylase activity is much higher in peripheral organs than in the brain (Blaschko & Chruściel, 1960) and also, it has been shown that the dopa-decarboxylase localized in the cells of the brain capillaries constitutes an enzymatic barrier for dopa between blood and brain. Thus, when the extra cerebral dopa-decarboxylase is inhibited by Ro 4–4602 and the blood-brain barrier for dopa is broken there is, as we observed, a high uptake and decarboxylation of dopa in the central monoamine neurons. The result is a marked accumulation of dopamine in these neurons especially in some dopamine nerve cells. At the time-interval studied, these accumulations of dopamine must partly be located extra-granularly since a strong fluorescence also was observed in the fibre bundles which contain only a few dopamine granules. A high reserve capacity of these granules to store dopamine may also contribute to the marked accumulation of dopamine observed.

The results of the present paper have also contributed to the mapping out of central dopamine neurons. Thus, the fact that certain dopamine cell bodies and terminals in the mesencephalon and the lateral neostriatum respectively show very marked accumulation of dopamine after Ro 4–4602 - L-dopa treatment, suggests that these structures belong to the same dopamine nerve cells forming a lateral nigro-neostriatal pathway from the lateral part of the substantia nigra and the mesencephalic reticular formation.

The central noradrenaline neurons do not show any increase or decrease in their fluorescence intensity. In view of the biochemical results (Butcher & Engel, 1969) demonstrating a slight decrease in brain noradrenaline levels after Ro 4–4602 – L-dopa treatment, the histochemical results suggest that the dopamine formed intraneuronally from exogenous dopa in the central noradrenaline neurons can displace noradrenaline from the amine granules in those neurons. These results also underline the view that the storage capacity for dopamine and noradrenaline in the amine granules in the noradrenaline neurons may be more or less saturated already in the physiological state. The results of Corrodi & Fuxe (1967) support this; they found nialamide not

to cause any further increase in the accumulation of noradrenaline in the noradrenaline neurons after dopa injection in rats depleted of their noradrenaline stores by a tyrosine hydroxylase inhibitor. Another operating mechanism may be that the β-hydroxylation of dopamine to noradrenaline is the rate-limiting step in its synthesis in the central noradrenaline neurons, but this has not been proved. The possibility that there is a relatively poor uptake of dopa into the noradrenaline neurons compared with the dopamine neurons must also be considered.

The accumulation of dopamine in the 5-HT neurons we observed suggests that the depletion of 5-HT stores after Ro 4–4602 - L-dopa treatment may be due to a displacement mechanism. The results of Bartholini, Da Prada & Pletscher (1968) support this; they found that the concentrations of 5-hydroxyindoleacetic acid increased after treatment with Ro 4–4602 and L-dopa. A decreased 5-HT synthesis induced by L-dopa may, however, contribute to the depletion of the central 5-HT levels, since the injected L-dopa may compete with the normally available tryptophan and 5-hydroxytryptophan for the entry into the brain and probably also over the 5-HT nerve cell membrane (Bartholini, Da Prada & Pletscher, 1968; unpublished data).

The present results differ from those obtained with L-dopa without extracerebral dopa decarboxylase inhibition where there is a relatively selective uptake and decarboxylation of dopa in central catecholamine neurons. This can be observed when L-dopa is injected to rats treated with a monoamine-oxidase inhibitor or a tyrosine hydroxylase inhibitor (Fuxe, 1965; Corrodi, Fuxe & Hökfelt, 1966; Corrodi & Fuxe, 1967). The present data indicate that this relative degree of specificity is abolished when loading the brain with high amounts of dopa by way of extracerebral decarboxylase inhibition.

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Reversal by trypsin of the action of a 2-haloalkylamine

N-(2-bromo-ethyl)-*N*-ethyl-1-naphthalenemethylamine hydrobromide (SY28) is a potent member of a group of compounds, the 2-haloalkylamines, which are notable for producing an insurmountable antagonism to the motor action of noradrenaline on smooth muscle (Nickerson & Goodman, 1946; Graham, 1962). This blockade has been attributed to alkylation of the specific α-receptor (Harvey & Nickerson, 1954) and the nature of the bonding has been the subject of long research and much speculation (Belleau, 1958, 1959). In 1966 Graham & Katib described how addition of trypsin to a bath containing an isolated vas deferens of the guinea-pig, in which insurmountable antagonism to added noradrenaline had been produced by prior exposure of the tissue to a fully blocking dose of one of three 2-haloalkylamine compounds, reversed the blocking action. An attempt to repeat this work on rabbit vas has been reported (Moran, May & others, 1967) to have given equivocal results. Accordingly, the experiment has been repeated. Vasa from 400 g guinea-pigs were suspended in 10 ml of Hukovic solution at 37°, gassed with 5% carbon dioxide in oxygen, six together at a time, and stimulated 5 times with addition of noradrenaline 10^{-8} g/ml. ^{14}C-SY28 was then added to the bath for 20 min in a concentration of $1{\cdot}34 \times M^{-6}$ containing $3{\cdot}35 \times 10^{-4}$ mCi in the 10 ml. Insurmountable antagonism to noradrenaline was then demonstrated, the tissue washed 12 times at 3 min intervals

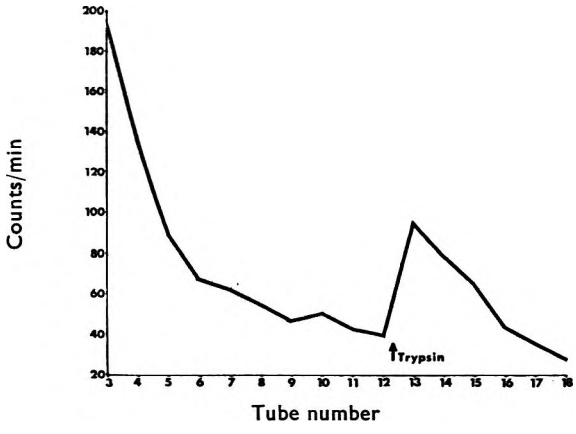

FIG. 1. The scintillation count in counts/min in the fluid in which was suspended guinea-pig vas deferens exposed to ^{14}C-SY28 $1{\cdot}34 M^{-6}$ in 10 ml for 20 min. Each tube no. refers to one 3 min cycle and a change of bath fluid. Trypsin $2{\cdot}5\ 10^{-3}$ BAEE units/ml was added for 2 min at arrow.

and the ^{14}C-activity in aliquots of wash fluid determined by standard scintillation counting technique. The loss of ^{14}C-label from the tissue was by then at a steady and low level. Trypsin (Koch-Light) was added for 2 min at a concentration of $2{\cdot}5 \times 10^3$ BAEE units/ml and washed out. A further six washes were collected and the output of ^{14}C-label determined in each. As may be seen in Fig. 1 the treatment with trypsin caused an increased output of specific activity. This episode coincided in time with the partial restoration of the response of the muscle to noradrenaline. The washes immediately before and after trypsin were concentrated at 50° by passing a warm air draught over the surface and run for 8 h on Whatman 3 MM paper in butanol: glacial acetic acid: water 50: 12: 25 as solvent. The front was marked and the dried paper exposed to Ilford Industrial G X-ray film in the dark for three months. The spot on the paper corresponding to radio activity on the film was ninhydrin positive, Rf 24·2. This result is interpreted to mean that suitable treatment with trypsin removes from guinea-pig vas deferens previously treated with SY28 a small peptide to which the 2-haloalkylamine is bound and that this restores its lost sensitivity to noradrenaline, possibly by uncovering an undamaged noradrenaline - receptor which was previously occluded by the blocking drug. Species differences may well require variation in the detail of enzyme treatment.

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The effects of stress and intravenous 0·9% NaCl injection on concentrations of whole brain 5-hydroxytryptamine in the neonate chick

Alterations in amounts of brain biogenic amines can occur under certain conditions of stress. Barchas & Freedman (1963) reported elevation of 5-hydroxytryptamine (5-HT) and lowering of noradrenaline in the brain of rats subjected to cold and swimming stress. Similarly, Goldberg & Salama (1969) observed an increase in rat brain 5-HT after stress with either electric shock or a revolving drum, although cold exposure alone did not produce this alteration.

The newly hatched chick and the newborn guinea-pig are functionally competent in the first hours of life. These two species do show a significant difference in that the guinea-pig has a well developed blood-brain barrier at birth, whereas the neonate chick does not and is considered to be neurologically immature (Waelsch, 1955; Lajtha, 1957; Key & Marley, 1962). Both however, are capable of surviving some forms of stress.

The basis of our present report occurred, initially, with the observation that one day old chicks entered a continuous roosting state when subjected to the stress of plucking neck feathers, making a small incision in the skin, or injecting 0·9% NaCl into the jugular vein. This was in contrast to the behaviour of untreated birds which

Table 1. *Effect of stress on levels of whole brain* 5-HT *in the neonate chick*

Treatment	5-HT (μg/g)	% change	N
None	1·28	—	25
Stress + 0·9% NaCl	1·53*	+19·72	21

* $P < 0.05$ analysis of variance.

displayed a cyclic pattern that consisted of alternation between states of roosting and arousal. The behaviour of the stressed animals was quite similar, but less pronounced, to that obtained in chicks injected intravenously with 5-HT (Seifter, Rauzzino & Kramer, 1963; Kramer, Kobrin & Seifter, 1963).

To determine whether an alteration in levels of brain 5-HT was occurring, this amine was estimated fluorimetrically in both stressed and non-stressed chicks using the procedure of Bogdanski, Pletscher & others (1956). The results (Table 1) indicate that stress, accompanied by roosting behaviour, does induce a significant increase in whole brain 5-HT of approximately 20% over control animals. This increase is almost identical to that found in rat brain by Barchas & Freedman (1963).

Thierry, Fekete & Glowinski (1968) have shown that stress of electric shock in rats not only causes a small but significant increase in endogenous 5-HT, but also greatly increases its turnover with the rate of amine synthesis being somewhat greater than catabolism. This partially supports the findings of Gál, Heater & Millard (1968) who showed significant increases in cerebral, but not liver, tryptophan-5-hydroxylase during cold exposure without significant increase in endogenous 5-HT. Bliss, Ailion & Zwanziger (1968) demonstrated stress-induced changes in indole metabolism as manifested by a significant increase of 5-hydroxyindoleacetic acid in all major areas of the rat brain, without appreciable change in levels of endogenous 5-HT. It would seem from the work cited that the major effect of stress on the central serotonergic system is stimulation of turnover of 5-HT with sedation as the behavioural correlate. It is questionable, at this point, whether endogenous levels of 5-HT in rat brain are actually altered during this process.

The newly hatched chick with its permeable blood brain barrier does however have the potential for increasing brain levels of 5-HT when this amine becomes elevated in peripheral blood.

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The influence of pulsation on the dissolution rate measurements in column type apparatus

A variety of apparatus and methods have been described for the measurement of dissolution rates *in vitro*. Control procedures require good reproducibility of all physical factors influencing the data obtained. To limit the number of external physical factors different authors (Rippie & Johnson, 1969; Baun & Walker, 1969; Marshall & Brook, 1969; Langenbucher, 1969) have introduced the column type, or flow trough, method. Most of the authors use oscillating or peristaltic methods, i.e. displacement pumps. Experiments we have made have indicated that displacement pumps influence the results.

From one batch of commercial aspirin tablets (500 mg acetylsalicylic acid per tablet) dissolution rates have been measured in a constant circulation column type apparatus. The principle components of the apparatus are shown in Fig. 1A, and consisted of a double-wall dissolution cell (internal cross-sectional area 10 cm^2), a double-wall mixing vessel and one of the pumps under test. The dissolution cell was partly filled with glass spheres, on top of which the dosage form was placed. The flow rate of the circulating test liquid was kept constant on 2 cm^3/s. All experiments were at 37° in 0·1N hydrochloric acid. The samples were pipetted, using a glasswool filter, diluted appropriately with 0·1N hydrochloric acid, and analysed spectrophotometrically at 278 nm for acetylsalicylic acid, checking for the presence of salicylic acid at 303 nm. The data obtained are given in Fig. 1B. All data are the average of 3 runs.

Dissolution curves 1 and 2 have been derived using peristaltic pumps with two rollers and silicone rubber tubing, the pumps being of different manufacture. A peristaltic pump with six rollers was used for dissolution run 3. For dissolution run 4

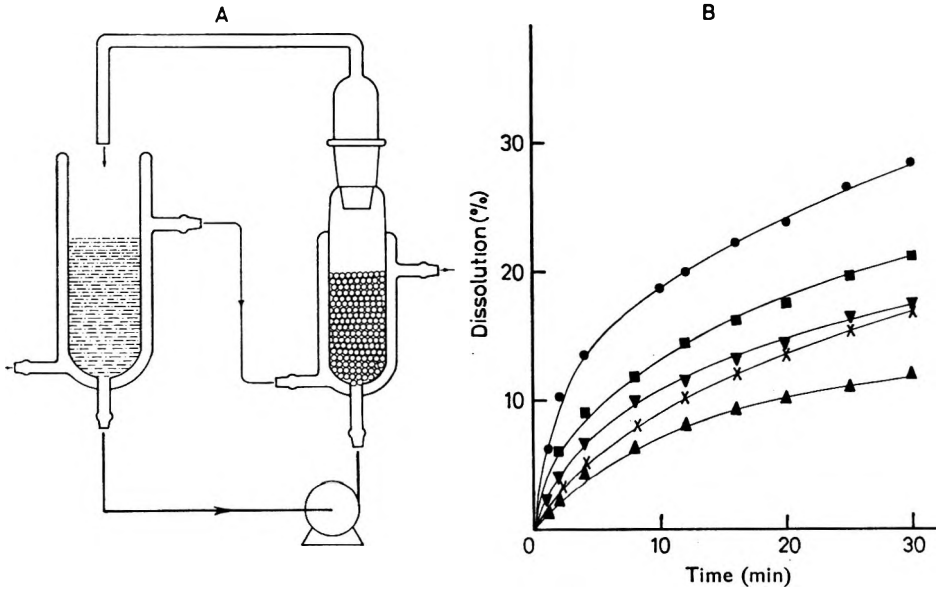

FIG. 1.A. Constant circulation dissolution apparatus. For description see text.

B. Rate of dissolution of one batch of aspirin tablets, containing 500 mg acetylsalicylic acid per tablet. Flow rate of circulation liquid (0·1N HCl) 2·0 cm^3/s. Different pumps: (1) peristaltic pump, two rollers, manufacture a ●; (2) peristaltic pump, two rollers, manufacture b ×; (3) peristaltic pumps, six rollers ▼; (4) plunger pump ■; (5) centrifugal pump ▲.

a plunger pump was used, while dissolution curve 5 was derived applying a centrifugal pump. The results show clearly the influence of the pump used on the dissolution rate, keeping all other variables constant. The serious finding is that peristaltic pumps of the same type but different manufacture give different results. This was attributed to liquid pulsation which caused different hydrodynamic conditions around the dosage form.

Centrifugal pumps, i.e. momentum pumps, show no pulsation. The hydrodynamics of a liquid stream from a momentum pump is therefore determined by the linear liquid velocity only. Consequently centrifugal pumps may be more suitable for reproducing hydrodynamic conditions around a dosage form.

Measurements on acetylsalicylic acid crystals show analogous dependances.

Summarizing, for dissolution tests in flow through methods, it is recommended that momentum pumps are used instead of displacement pumps to allow comparison of results found at different times in different laboratories.

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