Journal of Pharmacy and Pharmacology

The Pharmaceutical Society of Great Britain

Volume 22 Number 10 October 1970

Journal of Pharmacy and Pharmacology

Published by THE PHARMACEUTICAL SOCIETY OF GREAT BRITAIN
17 Bloomsbury Square, London, W.C.1. Telephone: 01-405 8967
Volume 22 Number 10 **October 1970**

Notice to Contributors

THE JOURNAL OF PHARMACY AND PHARMACOLOGY reviews and reports original research in those sciences which contribute to the development and evaluation of medicinal substances, covering *inter alia* biochemistry, chemistry, microbiology, pharmaceutics, pharmacognosy and pharmacology. Short papers of immediate interest are published as 'Letters to the Editor.'

Original research papers or review articles are accepted on the understanding that they are subject to editorial revision and that their content has not been published in whole or in part elsewhere.

Text. Authors should consult a CURRENT issue of the Journal and conform to the typographical conventions, use of headings, lay-out of tables, and citation of references. Texts must be typewritten in double spacing on sheets not larger than 9 × 13 inches (22 × 32 cm) with a minimum margin of 1½ inch (3 cm). The top copy and one carbon copy should be sent. The name(s) of the contributor(s), the name and address of the laboratory where the work was done and a shortened title (not more than a total of 50 letters and spaces) should accompany the typescript. The presentation adopted should be that best suited to the clear exposition of the subject matter. A summary should be included, giving results and conclusions in the form of an abstract suitable for use as such by abstracting journals.

References. References should be arranged to the HARVARD system. In the text the surname of the author(s) and the date of publication are given thus: Lewis & Train (1965) described . . . . or . . . . has been described (Lewis & Train, 1965). The list of references is in alphabetical order of first authors and each reference is arranged as follows: LEWIS, C. J. & TRAIN, D. (1965). *J. Pharm. Pharmac.*, **17**, 33–41. The title of publication is underlined and abbreviated as in *World List of Scientific Periodicals* (4th edn, 1963–1965 and supplements) and is followed by the volume number and first and last page numbers. References to books should be as follows: GOODMAN, L. S. & GILMAN, A. (1965). *The Pharmacological Basis of Therapeutics*, 3rd edn, p. 464, London: Collier-Macmillan.

Tables (for each copy of the text) should be typed on separate sheets, their headings should describe their content and they should be understandable without reference to the text. They should not be ruled.

Illustrations. Should be kept to the minimum number necessary to the proper understanding of the subject matter. Two of each are required, one prepared as described below the other a photo copy suitable for submission to a referee. Line illustrations such as graphs or apparatus diagrams should be clearly and boldly drawn in Indian ink on tracing cloth or paper, white paper, faintly blue-lined graph paper or Bristol board. The initial dimensions should be chosen to allow for a reduction to at least one-half and not more than one-quarter of the original size. (Most illustrations in the Journal are 2-4 inches, in width.) Kymograph records and photographs should be selected to allow for reduction to a similar extent. Lettering and numbering should be inserted lightly and clearly *in pencil*. Curves based on experimental data should carry clear and bold indications of the experimentally determined points, which should be marked by using, preferably, circles, crosses, triangles or squares. Legends for illustrations should be typed on separate sheets of paper and appended to the typescript of the paper or to the individual figures. The author's name, the title of the paper and the number of the figure should be written lightly in pencil on the back of each illustration. The approximate position of each illustration should be marked in the text.

Reprints. 50 reprints are supplied free of charge to the author. A further 10 reprints are supplied free to each co-author. Additional reprints may be purchased.

Copyright

Annual subscription (including postage) £9, (U.S.A. $25). Single copies £1, (U.S.A. $3).

Claims for missing copies cannot be considered unless received within 3 months of publication

J. Pharm. Pharmac., 1970, **22**, 729–732 *Received June* 19, 1970

Structural specificity for inhibition of [^{14}C]-5-hydroxytryptamine uptake by cerebral slices

A. CARLSSON

Department of Pharmacology, University of Göteborg, Göteborg, Sweden

Cerebral slices were prepared from mice pretreated with reserpine and nialamide. The slices were incubated for 40 min at 37° in a Krebs-Henseleit solution equilibrated with 5% carbon dioxide in oxygen and containing [^{14}C]-5-hydroxytryptamine. An uptake, dependent on energy metabolism and temperature, was observed. The uptake was blocked by tricyclic antidepressants, the order of activity being chlorimipramine > imipramine > desipramine. For 50% inhibition 0·03 μg/ml of chlorimipramine was required, when added to the suspension medium, or 1 mg/kg, when injected intraperitoneally 20 min beforehand. Comparison with earlier experiments using different experimental techniques appears to justify the conclusion that the evidence obtained represents the uptake mechanism of cerebral 5-hydroxytryptamine neurons.

In earlier experiments we have investigated the reserpine-resistant "membrane pumps" by means of which monoamine-carrying neurons take up and concentrate amines from the extracellular fluid. To identify the neurons under investigation we have concentrated on the influence of membrane pump blockade on the endogenous monoamines–noradrenaline, dopamine, and 5-hydroxytryptamine (5-HT). This has necessitated the use of indirect methods like the blockade of the monoamine-displacing action of certain synthetic analogues, and the release of monoamines after membrane pump blockade (Carlsson, Corrodi, & others, 1969a, b; Carlsson & Lindqvist, 1969; Carlsson, Jonason & Lindqvist, 1969; Carlsson, Jonason & others, 1969).

Using these techniques clear-cut differences in the structural requirements for inhibition of the membrane pumps of the different monoamine carrying neurons of the brain have been detected. In the present preliminary report this problem is further examined using *in vitro* uptake of ^{14}C-5-HT by cerebral slices.

EXPERIMENTAL

White female mice (NMRI), 17–25 g, were injected intraperitoneally with reserpine, 25 mg/kg, 17 h before death. After 14·5 h the animals were placed at an ambient temperature of +30°. Nialamide (250 mg/kg i.p.) was given 40 min before decapitation of the mice. In some experiments test drugs were injected 20 min before death.

Brain hemispheres (except the corpus striatum) were dissected in a petri dish filled with ice and then quickly rinsed twice with Krebs-Henseleit solution (equilibrated with 5% carbon dioxide in oxygen at room temperature, 23–25°) to remove blood on the surface. Each hemisphere was divided into 0·8 mm slices by means of a miniature "household egg slicer": the tissue was supported in a shallow depression whilst a grid of longitudinal wires (0·05 mm diameter) was pressed through it.

The slices of 3 hemispheres from 3 different brains (about 200 mg) were transferred into a glass tube containing 10 ml Krebs-Henseleit solution equilibrated with 5% carbon dioxide in oxygen and with the test drug added to the medium. The interval between killing an animal and the introduction of its second sliced hemisphere into the

Krebs-Henseleit solution was about 90 s. Preincubation was made at 37° for 10–25 min. In some experiments 5% carbon dioxide in nitrogen was used and the glucose in the Krebs-Henseleit solution was omitted.

After the preincubation, 10 ng of 5-HT-2-^{14}C (18 mCi/mmol) were added and the incubation was continued for additional 40 min. The incubation was terminated by transferring slices and incubation medium to plastic centrifuge tubes and the slices were immediately spun down in a refrigerated centrifuge at 14 000 *g* for 5 min. The supernatant was decanted and the slices were washed for 10 min by shaking in 10 ml of 0·9% saline at +4°. After centrifugation and decantation the slices were extracted twice in 5 + 4 ml 0·4N perchloric acid containing totally 0·2 ml of 10% EDTA (disodium ethylenediamine tetra-acetate) and 0·1 ml of 2% ascorbic acid.

Three ml aliquots of the supernatants of incubation medium, saline and extract were each taken and mixed with 3 ml Insta-Gel. Radioactivity was measured by liquid scintillation (Packard Tri Carb). Quenching was checked by internal standards. The sum of ^{14}C-5-HT recovered from the 3 supernatants was approximately 100% of the amount added to the incubation medium. The extract activity values, corrected for background, were used for calculating ^{14}C-5-HT uptake.

RESULTS

When added to the incubation medium, chlorimipramine caused a pronounced inhibition of ^{14}C-5-HT uptake. At maximum inhibition the uptake was only about 20% of the control value (Fig. 1). This remaining chlorimipramine-resistant uptake probably represents a true uptake mechanism and not an unspecific adsorption phenomenon. This assumption is supported by the fact that the ^{14}C-5-HT taken up had evidently not been removed by the washing procedure routinely employed.

The concentration of chlorimipramine required for 50% inhibition (EC50) was 0·027 μg/ml, corresponding to 10^{-7} M. Imipramine was some 5 times weaker than chlorimipramine but 10 times stronger than desipramine as an inhibitor of 5-HT uptake.

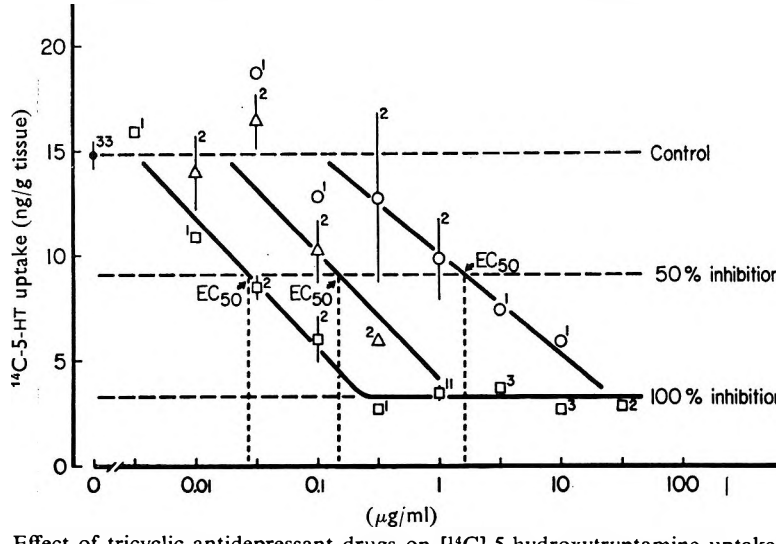


FIG. 1. Effect of tricyclic antidepressant drugs on [^{14}C]-5-hydroxytryptamine uptake by mouse cerebral slices. Addition of inhibitors *in vitro*. For estimation of EC50 the 100% inhibition level is obtained from the chlorimipramine data as shown in the figure. ○—○, desipramine (EC50 = 1·6 μg/ml); △—△, imipramine (EC50 = 0·15 μg/ml); □—□, chlorimipramine (EC50 = 0·27 μg/ml).

Fig. 2 shows the results obtained when the inhibitors had been administered intraperitoneally 20 min before killing the animals and preparing the cerebral slices for the *in vitro* uptake experiments. Again, chlorimipramine proved more active than imipramine, whereas desipramine was devoid of activity in the doses employed.

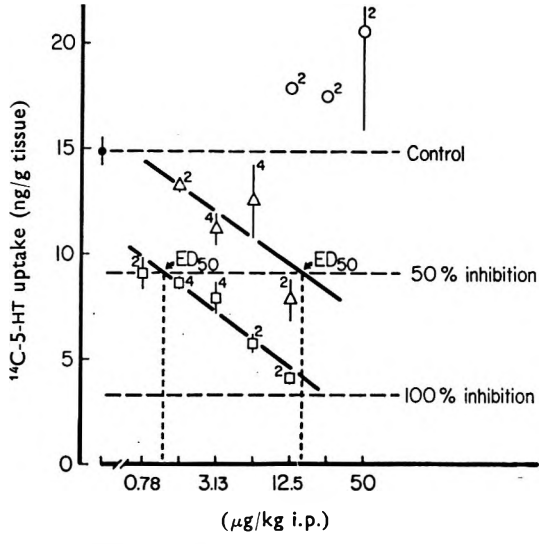


FIG. 2. Effect of tricyclic antidepressant drugs on [^{14}C]-5-hydroxytryptamine uptake by mouse cerebral slices. Intraperitoneal injection of inhibitors. The 100% inhibition level is obtained from the chlorimipramine *in vitro* data. ○—○, desipramine; △—△, imipramine (ED50 = 15·6 mg/kg); □—□, chlorimipramine (ED50 = 1·1 mg/kg).

Table 1 summarizes some preliminary results with other inhibitors of ^{14}C-5-HT uptake. Among the most potent "inhibitors" found thus far 5-HT itself has a comparably high affinity for the membrane pump. 5-HT is equally or closely followed by chlorimipramine and dexbrompheniramine. Other fairly potent inhibitors are dexchlorpheniramine, H75/12 (4-methyl-α-ethyl-*m*-tyramine), tryptamine and its *N*-dimethyl and α-ethyl derivatives. Ouabain was active in a relatively low concentration ($3 \cdot 10^{-6}$M).

When glucose was omitted from the suspension medium and oxygen was replaced by nitrogen, ^{14}C-5-HT uptake dropped to approximately the same level as after a high concentration of chlorimipramine.

Table 1. *The inhibition of* ^{14}C-5-HT *uptake in cerebral slices by drugs.* Shown are approximate concentrations necessary for 50% inhibition.

	μg/ml	M
5-hydroxytryptamine	0·02	10^{-7}
Tryptamine HCl	0·06	3×10^{-7}
NN-Dimethyltryptamine	0·06	3×10^{-7}
α-Ethyltryptamine acetate	0·06	2×10^{-7}
H75/12 HCl	0·10	5×10^{-7}
p-Chloromethamphetamine HCl	0·03	10^{-7}
Chlorimipramine HCl	0·03	10^{-7}
Imipramine HCl	0·15	5×10^{-7}
Desipramine HCl	1·6	5×10^{-6}
Dexbrompheniramine maleate	0·07	2×10^{-7}
Dexchlorpheniramine maleate	0·20	5×10^{-7}
Ouabain octahydrate	2	3×10^{-6}
Iodoacetic acid	20	10^{-4}

At an incubation temperature of 0° the uptake of ^{14}C-5-HT was approximately the same as after addition of chlorimipramine in a high concentration at 37°.

DISCUSSION

Our experiments indicate a relatively high structural specificity of the 5-HT uptake mechanism of mouse cerebral slices. The observations are in fairly close agreement with our earlier experiments using indirect methods for measuring inhibition of the membrane pump of cerebral 5-HT neurons. Thus the order of potency chlorimipramine > imipramine > desipramine is the same for all the various techniques we have used so far for studying 5-HT uptake. This order is reversed for the membrane pump of central noradrenaline neurons.

The ED50 of chlorimipramine found in the present investigation after intraperitoneal injection was 1 mg/kg. In our earlier study using H75/12 the ED50 observed was 7 mg/kg (Carlsson & others, 1969a). This discrepancy may be apparent rather than real. In the H 75/12 test the inhibitor probably has to compete for the uptake sites with H 75/12 which has a relatively strong affinity for these sites, as indicated by the present data. This should result in a right-hand shift of the dose-response curve, compared to the present conditions where little or no competition will occur. Moreover, a very close agreement between the two sets of data cannot be expected for another reason; in the 4-h test with H 75/12 the duration of action of the inhibitor may be a more important factor than in the present experiments.

Part of the present experiments confirms earlier work on ^{14}C-5-HT uptake by cerebral slices (Blackburn, French & Merrills 1967, Ross & Renyi 1967, 1969). For example, Ross & Renyi found imipramine and desipramine to inhibit 5-HT uptake in approximately the same concentrations as in the present investigation. On the other hand about 10 times higher concentrations of *NN*-dimethyltryptamine were required for 50% inhibition in their investigation compared to ours. The most likely explanation of this difference appears to be that our animals, but not theirs, had been pretreated with reserpine and nialamide.

Acknowledgements

This research was supported by the Swedish Medical Research Council (Grant no B70-14X-155-06B). For generous gifts of drugs I am indebted to the Swedish Ciba Ltd. (reserpine), J. R. Geigy Ltd. (imipramine, desipramine, chlorimipramine), Hoffmann-La Roche & Co., Ltd., through Dr. A. Pletscher (p-chloromethamphetamine), Hässle Ltd. (H 75/12), the Swedish Pfizer (nialamide) and Schering Ltd. (dexchlorpheniramine, dexbrompheniramine). The expert technical assistance of Mrs. Ingrid Bergh and Mrs. Birgitta Nyström is gratefully acknowledged.

REFERENCES

BLACKBURN, K. J., FRENCH, P. C. & MERRILLS, R. J. (1967). *Life Sci.*, **6**, 1655–1663.
CARLSSON, A., CORRODI, H., FUXE, K. & HÖKFELT, T. (1969a). *Europ. J. Pharmac.*, **5**, 357–366.
CARLSSON, A., CORRODI, H., FUXE, K. & HÖKFELT, T. (1969b). *Ibid.*, **5**, 367–373.
CARLSSON, A. & LINDQVIST, M. (1969). *J. Pharm. Pharmac.*, **21**, 460–464.
CARLSSON, A., JONASON, J., LINDQVIST, M. & FUXE, K. (1969). *Brain Res.*, **12**, 456–460.
CARLSSON, A., JONASON, J. & LINDQVIST, M. (1969). *J. Pharm. Pharmac.*, **21**, 769–773.
ROSS, S. B. & RENYI, A. L. (1967). *Life Sci.*, **6**, 1407–1415.
ROSS, S. B. & RENYI, A. L. (1969). *Europ. J. Pharmac.*, **7**, 270–277.

J. Pharm. Pharmac., 1970, **22**, 733–737 *Received May* 15, 1970

Effect of some antiparkinsonian drugs on catecholamine neurons

LARS-OVE FARNEBO, KJELL FUXE, BERTIL HAMBERGER AND HÅKE LJUNGDAHL

Department of Histology, Karolinska Institutet, S–104 01 Stockholm, Sweden

The inhibitory effect of some antiparkinsonian drugs on the accumulation of catecholamines into rat dopamine and noradrenaline neurons was studied with isotope and histochemical fluorescence methods *in vivo* and *in vitro*. Benztropine was the most potent drug hitherto tested to inhibit accumulation of catecholamines into dopamine neurons and was effective down to a dose of 10 mg/kg. Also ethybenztropine, brompheniramine, diphenylpyraline, chlorpheniramine and methixene, in doses of 50 mg/kg, inhibited accumulation of catecholamines. Atropine, scopolamine, benzhexol, diphenhydramine and many other antiparkinsonian agents were ineffective.

The dopamine content in the neostriatum and the substantia nigra is reduced in Parkinson's disease due to degeneration of the nigro-neostriatal dopamine neuron system (see Gillingham & Donaldson, 1969). This leads to a decreased neostriatal dopamine neuro-transmission which probably results in an imbalance between α- and γ-motoneuron activity (Steg, 1969) and in the symptoms rigidity and akinesia found in Parkinsonism. It is conceivable that the decreased dopamine neurotransmission could partially be counteracted by inhibition of re-uptake of released dopamine into the dopamine nerve terminals. Therefore, in the present experiments some antiparkinsonian drugs of the anticholinergic and antihistamine type (see Yahr & Duvoisin, 1968) were tested for their ability to inhibit dopamine uptake in central dopamine nerve terminals *in vivo* and *in vitro* using isotope and histochemical fluorescence methods. The available evidence suggest that such a site of action may exist only for certain antiparkinsonian agents.

EXPERIMENTAL

Isotope methods

Sprague-Dawley rats, 180–200 g, were killed and neostriatal slices with a thickness of about 0·5 mm and a diameter of 3 mm were prepared. The slices were preincubated for 15 min in a Krebs–Ringer bicarbonate medium, pH 7·4, containing the drug to be tested. Tritiated dopamine (^{3}H-DA) was then added to the incubation medium (final concentration of ^{3}H-DA: 2×10^{-8}M; specific activity 8 Ci/mmol). The slices were incubated for another 30 min, and then rinsed in ^{3}H-DA-free buffer for 10 min, after which they were dissolved in Soluene and the radioactivity determined by liquid scintillation counting and the activity/slice used for calculations of uptake inhibition. This was possible because early experiments had shown that ^{3}H-uptake/slice gave reproducible results. Variation in thickness of the slice, which changes the weight, did not affect the uptake of tritiated noradrenaline (^{3}H-NA) proportionally. This is probably due to the poor penetration of the catecholamines into the slices (Hamberger, 1967). The effect of the same drugs on

the uptake of [^{3}H]noradrenaline (^{3}H-NA) (final concentration of ^{3}H-NA: 10^{-7}M; specific activity 10 Ci/mmol) in the isolated rat iris was determined analogously (Jonsson, Hamberger & others, 1969). In other experiments drugs were injected intraperitoneally in a dose of 50 mg/kg 1 h before the animals were killed. After the animals had been killed we measured the uptake of ^{3}H-DA or ^{3}H-NA in slices of neostriatum or in the isolated iris respectively as described above.

Fluorescence histochemistry

Studies were made on the dopamine nerve terminals in the median eminence which lie outside the blood brain barrier (see Fuxe, Hamberger & Malmfors,1967). The rats were pretreated with reserpine (5 mg/kg, i.p. 18–24 h before killing) to deplete the endogenous catecholamine stores and with the α-adrenergic receptor blocking agent, azapetine phosphate (20 mg/kg, i.p., 20 min before the amine injection; Ilidar, Roche) to protect the rat from the peripheral actions of α-methyl-noradrenaline (α-methyl-NA) (0·5 mg/kg) given intravenously 30 min before killing. 30 min before the α-methyl-NA injection, the drugs mentioned above were injected intraperitoneally in doses of 25–50 mg/kg. The effects of benztropine and ethybenztropine were also examined in doses of 5 and 10 mg/kg. At least 5 and at most 10 rats were used in each single experiment with the different compounds. The rats were killed by decapitation and the hypothalamus was taken for the histochemical fluorescence analysis of catecholamines (Falck, Hillarp & others, 1962; Corrodi & Jonsson, 1967).

Drugs. The following drugs were tested: (belladonna alkaloids) atropine sulphate, scopolamine hydrobromide; (synthetic tropane derivatives) benztropine mesylate (Cogentin, MSD), ethybenztropine (Ponalide, Sandoz); (piperidyl compounds) benzhexol hydrochloride (Pargitan, Kabi); procyclidine hydrochloride (Kemadrin, B.W. & Co.); (antihistamines) diphenhydramine hydrochloride (Desentol, LEO), orphenadrine hydrochloride (Disipal, Brocades), chlorpheniramine maleate (Allergisan, Pharmacia), brompheniramine maleate, diphenylpyraline hydrochloride (Histyn, Hässle), diethylaminoethylbenzhydryl ether hydrochloride (Rigidyl, Dumex); (phenothiazine derivatives) profenamine hydrochloride (Lysivane, Rhodia); (a derivative of thioxantene) methixene hydrochloride (Tremoquil, Tika); desipramine hydrochloride (Pertofran, Geigy). We are grateful to the drug companies for the supply of their respective compounds. The doses given refer to the form shown.

RESULTS

Isotope measurements

Benztropine was the most potent drug tested in inhibiting the uptake of ^{3}H-DA in neostriatal slices (Table 1). Several other drugs with anticholinergic or antihistamine properties were also able to inhibit ^{3}H-DA uptake. However, both atropine and scopolamine were without effect. Benztropine, brompheniramine and methixene inhibited the uptake of ^{3}H-NA in the isolated iris. The inhibitory effects of these drugs on the uptake of ^{3}H-NA by the iris are, however, far weaker than those of the tricyclic antidepressants such as desipramine (see Hamberger, 1967). This drug is more than a 1000 times more potent on noradrenaline nerve terminals than on dopamine nerve terminals.

Table 1. *Effect of certain antiparkinsonian drugs on the uptake of* ^{3}H-DA *and* ^{3}H-NA *in neostriatal slices and isolated irides respectively of untreated rats*

Drug*	Drug *in vitro*		Drug *in vivo*	
	Neostriatum	Iris	Neostriatum	Iris
Atropine	88† ± 6·9 (6)	130 ± 14 (4)	96 ± 2·2 (12)	82 ± 7·6 (8)
Scopolamine	93 ± 3·2 (6)	120 ± 13 (4)	76 ± 5·9 (6)	111 ± 5·9 (4)
Benztropine	33 ± 2·0 (12)	17 ± 0·6 (8)	64 ± 5·1 (11)	63 ± 2·6 (8)
Ethybenztropine	41 ± 1·0 (6)	35 ± 2·1 (4)	83 ± 6·9 (6)	115 ± 2·9 (4)
Procyclidine	80 ± 2·0 (6)	104 ± 14·1 (4)	76 ± 4·9 (6)	78 ± 4·6 (4)
Diphenhydramine	87 ± 5·2 (5)	46 ± 6·3 (4)	92 ± 2·2 (6)	77 ± 6·0 (3)
Brompheniramine	55 ± 4·0 (6)	32 (2)	69 ± 4·8 (6)	43 ± 5·2 (3)
Diphenylpyraline	59 ± 1·1 (6)	32 (2)	78 ± 2·8 (12)	84 ± 8·5 (4)
Methixene	48 ± 5·6 (12)	34 ± 1·5 (8)	73 ± 4·4 (12)	58 ± 7·0 (8)
Cocaine	53 ± 1·4 (6)	17 ± 1·1 (4)		
Desipramine	86 ± 4·6 (6)	‡	85 ± 2·8 (9)	6·1 ± 1·1 (8)

* The drugs were either added *in vitro* in a concentration of 10^{-5}M or the rats were pretreated with the drug in a dose of 50 mg/kg i.p. 1 h except for ethybenztropine, where 25 mg/kg 1 h was given.

† The values are expressed as % of control radioactivity without drug and are the means ± s.e. ( ) Number of experiments. In each experimental run at least 6 neostriatal and 4 iris controls were included.

‡ The concentration of desipramine giving a 50% reduction in the noradrenaline uptake in the isolated rat iris is 6×10^{-9}M.

Fluorescence microscopy

Benztropine in doses of 25–50 mg/kg caused a strong blockade of the accumulation of α-methyl-NA in the dopamine nerve terminals of the median eminence (Table 2). A clear effect was observed in a dose of 10 but not of 5 mg/kg. Ethybenztropine reduced the amine accumulation to a moderate degree in a dose of 25 mg/kg. In doses of 50 mg/kg diphenylpyraline, brompheniramine, chlorpheniramine and methixene also had moderate blocking actions. Diphenylpyraline and brompheniramine were studied also at a dose of 25 mg/kg and blocking effects were still observed. The belladonna alkaloids, the piperidyl compounds, and profenamine were without effects. Furthermore, the other antihistamine compounds tested had no clear-cut blocking actions.

DISCUSSION

It is obvious from the present results that most of the antiparkinsonian agents used clinically today do not owe their therapeutic effects to blockade of dopamine uptake in the central dopamine neurons. Thus, atropine, scopolamine, benzhexol and procyclidine probably act by way of their anticholinergic activities which are clearly apparent with regard to behaviour in doses below those used in the present study (Carlton, 1961, 1963; Arnfred & Randrup, 1968; Scheel-Krüger, 1970). However, the diphenylester of tropanol, benztropine, was a potent blocker of dopamine uptake in doses of 25–50 mg/kg both in the nigro-neostriatal dopamine neurons and the tubero-infundibular dopamine neurons. This drug is known to be a potent synthetic anticholinergic agent. However, obviously, as shown in the present paper, at higher doses an additional site of action exists, i.e. blockade of dopamine uptake, which also may be of functional importance (Fuxe, Goldstein & Ljungdahl, 1970). Thus, it seems reasonable that in combination with dopa treatment in parkinsonian patients (Cotzias, van Woert & Schiffer, 1967; Cotzias, Papavasiliou

Table 2. *Effect of some antiparkinsonian agents on the accumulation of α-methyl-noradrenaline in dopamine nerve terminals of the rat median eminence*

Drug	mg/kg	Fluorescence intensity	Effect on accumulation of α-methyl-NA
No drug		1+ (4)—2+ (8)	
Atropine	50	1+ (1)—2+ (4)	
Atropine	25	2+ (3)	
Scopolamine	50	2+ (4)	
Benztropine	50	½+ (4)—1+ (1)	diminished
Benztropine	25	½+ (3)—1+ (1)	diminished
Benztropine	10	1+ (4)—2+ (1)	diminished
Benztropine	5	1+ (2)—2+ (5)	
Ethybenztropine	25	1+ (4)—2+ (2)	diminished
Benzhexol	50	2+ (4)	
Procyclidine	50	1+ (2)—2+ (4)	
Diethylaminbenzhydrylether	50	1+ (2)—2+ (6)	
Diphenhydramine	50	1+ (1)—2+ (4)	
Chlorpheniramine	50	1+ (4)	diminished
Chlorpheniramine	25	1+ (3)—2+ (3)	
Brompheniramine	50	1+ (4)	diminished
Brompheniramine	25	1+ (4)	diminished
Brompheniramine	10	1+ (1)—2+ (4)	
Diphenylpyraline	50	1+ (4)	diminished
Diphenylpyraline	25	1+ (4)—2+ (1)	diminished
Diphenylpyraline	10	2+ (3)	
Orphenadrine	50	1+ (3)—2+ (4)	
Profenamine	50	2+ (3)	
Methixine	50	1+ (4)—2+ (2)	diminished

All rats were treated with reserpine (5 mg/kg, i.p., 18–24 h), azapetine (20 mg/kg, i.p., 50 min) and α-methyl-NA (0·5 mg/kg, i.v., 30 min). The drugs to be tested were injected i.p. 30 min before the α-methyl-NA injection. A semiquantitative estimation of fluorescence intensity has been made: moderate = 2+; weak = 1+; very week = 1/2+. When the fluorescence intensity of an individual brain preparation could not be definitely determined as belonging to one intensity grade or the next, this was indicated by adding one half to the lower grade. The range in the experiments are given. Number of rats within parentheses.

& Gellene, 1969; Yahr, Duvoisin & others, 1969) this action could be of some therapeutic importance. In agreement with our results it has recently been found that benztropine blocks dopamine uptake in synaptosomes (Coyle & Snyder, 1969).

In view of the present results it may be that a compound with an antihistamine-like ring-structure in combination with a side-chain containing a ring nitrogen atom could be a potential blocker of the dopamine uptake mechanism. This could explain why diphenylpyraline was effective whereas many other antihistamine compounds studied (diphenhydramine, orphenadrine, etc.) were relatively ineffective since they all had dimethylamine alkyl side-chains. In agreement with this suggestion, methixene, which was effective in blocking the dopamine uptake, is a thioxanthene derivative having a piperidyl side-chain. One exception, however, is brom- and chlorpheniramine which had a clearcut blocking activity in the high dose studied. This is probably due to the halogen atom in the ring.

The above results suggest that antiparkinsonian drugs of the antihistamine type available at present either exert their therapeutic effects via their anticholinergic effects or via some other unknown action. The fact that Coyle & Snyder (1969) obtained relatively good blocking effects with diphenhydramine, orphenadrine and benzhexol *in vitro* on dopamine uptake in synaptosomes from the neostriatum may not be incompatible with the present results, since the synaptosomes may be more susceptible to the blocking action of drugs than when left undetached in the tissue.

As seen from the results benztropine is not a selective blocker of dopamine uptake, since there is about the same degree of inhibition of noradrenaline uptake in the rat iris as of DA uptake in the neostriatum. However, the difference towards desipramine should be pointed out. As seen methixene is similar to benztropine in this respect. It may be mentioned that Carlsson & Lindqvist (1969) have found that many drugs belonging to the antihistamine group are blockers of the amine membrane pump in the central noradrenaline and 5-hydroxytryptamine neurons.

In conclusion, the results obtained with benztropine, ethybenztropine, diphenylpyraline, brompheniramine, chlorpheniramine and methixene suggest that a new type of antiparkinsonian drug related to the antihistamine compounds may be developed with its main action being a blockade of the membrane pump in the central dopamine neurons.

Acknowledgements

This investigation was supported by grants from the Swedish Medical Research Council (B70-14X-2330-03, B70-14X-1015-05), Magnus Bergwalls Stiftelse, Svenska Livförsäkringsbolags nämnd för medicinsk forskning and Ollie and Elof Ericssons Stiftelse.

REFERENCES

ARNFRED, T. & RANDRUP, A. (1968). *Acta pharmac. tox.*, **25**, 384–394.

CARLSSON, A. & LINDQVIST, M. (1969). *J. Pharm. Pharmac.*, **21**, 460–464.

CARLTON, P. L. (1961). *Psychopharmacologia*, **2**, 377–380.

CARLTON, P. L. (1963). *Psychol. Rev.*, **70**, 19–39.

CORRODI, H. & JONSSON, G. (1967). *J. Histochem. Cytochem.*, **15**, 65–78.

COTZIAS, G. C., PAPAVASILIOU, P. S. & GELLENE, R. (1969). *New Engl. J. Med.*, **280**, 337–345.

COTZIAS, G. C., WOERT, M. H. VAN & SCHIFFER, L. M. (1967). *Ibid.*, **276**, 374–379.

COYLE, J. T. & SNYDER, S. H. (1969). *Science, N.Y.*, **166**, 899–901.

FALCK, B., HILLARP, N.-Å., THIEME, G. & TORP, A. (1962). *J. Histochem. Cytochem.*, **10**, 348–354.

FUXE, K., HAMBERGER, B. & MALMFORS, T. (1967). *Europ. J. Pharmac.*, **1**, 334–341.

FUXE, K., GOLDSTEIN, M. & LJUNGDAHL, Å. (1970). *Life Sci.* In the press.

GILLINGHAM, F. J. & DONALDSON, I. M. L. (1969). *Third Symposium on Parkinson's Disease*, Edinburgh and London: Livingstone.

HAMBERGER, B. (1967). *Acta physiol. scand., Suppl.*, **295**, 1–56.

JONSSON, G., HAMBERGER, B., MALMFORS, T. & SACHS, CH. (1969). *Europ. J. Pharmac.*, **8**, 58–72.

SCHEEL-KRÜGER, J. (1970). *Acta pharmac. tox.*, **28**, 1–16.

STEG, G. (1969). *Third Symposium on Parkinson's Disease*, pp. 26–29, Edinburgh and London: Livingstone.

YAHR, M. D. & DUVOISIN, R. C. (1968). *Mordern Treatment*, **5**, 283–300.

YAHR, M. D., DUVOISIN, R. C., SCHEAR, M. J., BARRETT, R. E. & HOEHN, M. M. (1969). *Arch. Neurol.*, **21**, 343–354.

J. Pharm. Pharmac., 1970, **22**, 738–744 *Received January* 19, 1970

Effects of some phenothiazines, tricyclic antidepressants and antihistamines on red cell agglutination

B. TAIT

Department of Pathology, The Royal Women's Hospital, Carlton, Victoria 3053, *Australia*

The observation that promethazine hydrochloride inhibited red cell agglutination in the ABO blood group system led to the screening of a series of drugs structurally related to promethazine in an attempt to establish a structure activity relation. Standard group O serum agglutinated group A and group B cells. The serum in each test was pretreated with drug and the degree of agglutination on the slide was assessed by both direct and microscopic means. All phenothiazines tested possessed this property except thiopropazate and the cyclic compound MO967C. Several tricyclic antidepressants and antihistamines tested possessed inhibitory activity, the tricyclic compounds were generally as active as the phenothiazines but the antihistamines varied in activity. The structure associated with maximum activity appeared to be a mono- or di-methyl substituted ethylamine or propylamine chain. However, the presence of the phenothiazine nucleus seems to strengthen activity suggesting that the overall shape of the molecule is important. Within the phenothiazine group there appeared to be a relation between basicity and inhibitory activity. It is suggested that the positively charged drug molecules in solution are attracted to the negative acidic groups on the red cell membrane thus inhibiting the formation of antibody-antigen complexes. The cyclization of the phenothiazine and the tricyclic compounds' side-chain appears to abolish the inhibitory action. These findings may be of clinical importance with respect to ABO incompatibilities between mother and foetus and also in laboratory serum antibody studies.

Promethazine inhibits agglutination in the ABO blood group system (Tait, 1968). This finding led to the investigation of other drugs having chemical structures or pharmacological activities related to those of promethazine, to determine the extent to which these factors were related to inhibition of red cell agglutination.

EXPERIMENTAL

Commercial preparations of human group A and group B cells suspended in a medium including inosine and disodium EDTA were supplied by the Ortho Pharmaceutical Company. Aliquots of suspensions of group A or group B cells were mixed separately on a slide with equal volumes of incompatible group O serum containing concentrations of drugs ranging from 5×10^{-11} to 5×10^{-2}M. One drop each of the cell suspension and of the treated serum were delivered from standardized pipettes. The rate and degree of agglutination were assessed by direct inspection and by microscopic examination of the mixtures on the slide, and were compared with control samples in which the drug solution was replaced by an equal volume of

0·9% NaCl solution. The ratio between successive concentrations of each drug was ten. The threshold concentration at which inhibition of agglutination was observed was recorded. Aqueous stock solutions or ampouled preparations of drugs were diluted in 0·9% NaCl solution for use in these tests.

RESULTS

Determination of the threshold concentration for inhibition of agglutination of group A and group B cells by group O serum with promethazine and the other drugs is illustrated in Fig. 1.

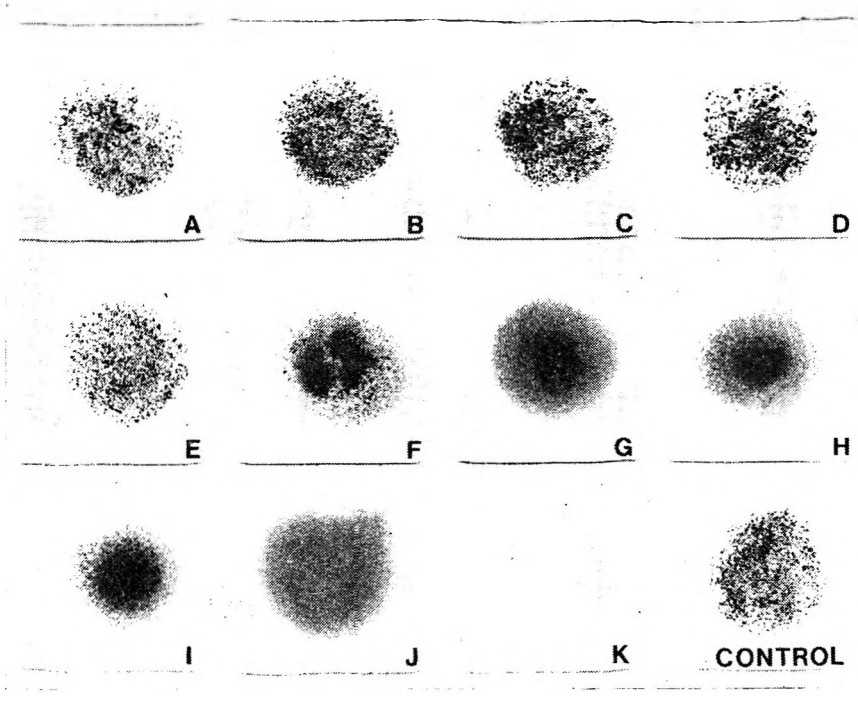
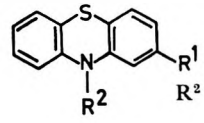
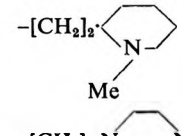
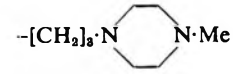
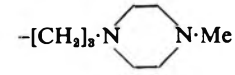
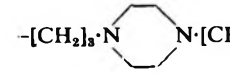
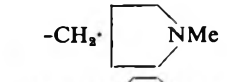
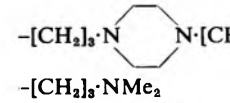
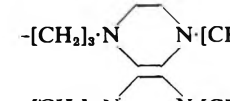
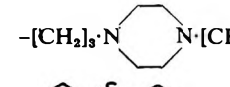
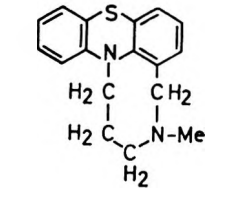


FIG. 1. The effect of a series of concentrations of promethazine hydrochloride on agglutination of a suspension of Group A red cells by Group O serum. The concentrations of promethazine ranged from 5×10^{-11}M in A to 5×10^{-2}M in J in ten-fold steps. In the control panel, the promethazine solution was replaced by saline. The threshold for inhibition of agglutination was 5×10^{-5}M with promethazine (G). The threshold concentration for haemolysis was 5×10^{-2}M (J).

Drugs used may be classified into the following four groups: (i) Phenothiazine derivatives with tranquillizing or sedative properties (Table 1). (ii) Tricyclic antidepressants which are considered to be structurally similar to the phenothiazines (Table 2). (iii) Drugs with antihistamine activity (Table 2). (iv) Drugs with structural similarities to promethazine, such as an ethylamine or propylamine chain and methyl or ethyl substitutions on the terminal nitrogen. The drugs in this group have diverse pharmacological activities.

Phenothiazine derivatives were found to be the most effective inhibitors of agglutination. Two (thioridiazine and thiethylperazine) were as potent as promethazine, but there was a wide range of threshold concentrations for inhibition of agglutination

Table 1. *Inhibition of agglutination by some phenothiazines*

Drug	R^1	 R^2	Threshold concn (mM)
Thioridazine	–S—Me		0·05
Thiethylperazine	$-SO_2-CH_2-Me$		0·05
Promethazine	–H	$-CH_2\cdot CH(Me)\cdot NMe_2$	0·05
Promazine	–H	$-[CH_2]_3\cdot NMe_2$	0·5
Chlorpromazine	–Cl	$-[CH_2]_3\cdot NMe_2$	0·5
Thioperazine	$-SO_2-NMe_2$		0·5
Fluphenazine	$-CF_3$		0·5
Methdilazine	–H		0·5 (weak)
Carphenazine	$-CO-CH_2-Me$		5 (weak)
Trifluprom azine	$-CF_3$	$-[CH_2]_3\cdot NMe_2$	5 (very weak)
Perphenazine	–Cl		5 (very weak)
Thiopropazate	–Cl		N.I.
MO967C			N.I.

N.I. = No inhibition.

in this group of drugs (Table 1). Three phenothiazine drugs tested did not inhibit red cell agglutination: thiopropazate, MO 967C in which the side-chain is formed into a heterocyclic ring (Table 1), and methylene blue which has a phenothiazine nucleus.

The tricyclic antidepressants included three fairly potent inhibitors of agglutination (Table 2). Chlorprothixene was weak, and a cyclic derivative of imipramine was without activity.

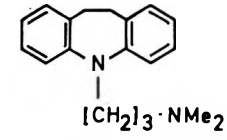
The activity of compounds with a cyclized side-chain, MO 967C and MO 795E differed from that of the respective parent open-chain compounds promazine and imipramine. The open-chain compounds inhibited agglutination with threshold

concentrations of 5×10^{-4} and 2×10^{-4}M respectively but the closed-chain compounds caused haemolysis, MO 967C at 5×10^{-4}M and MO 795E at $2{\cdot}5 \times 10^{-3}$M. This suggests that cyclization of the side-chain leads to loss of potency in inhibiting agglutination, but the extent of the loss could not be determined.

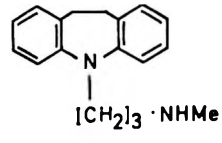
The antihistamines (Table 2), though inhibiting agglutination were generally less effective than the phenothiazine derivatives, many of which also possess antihistamine activity.

Atropine (25), butacaine (100), decamethonium (100), isoprenaline (100), methylene blue (50), neostigmine (100), oxyphencyclimine (25), penthienate (100), procaine (100), procaine amide (400), pronethalol (100), pyridostigmine (100), fenoxazine (50), suxamethonium (100), trimethidinium (100), all failed to inhibit agglutination at the concentrations (mM) shown.

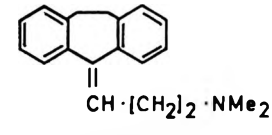
Table 2. *Inhibition of agglutination by some tricyclic antidepressants and antihistamines.* Threshold concentrations are given in parentheses



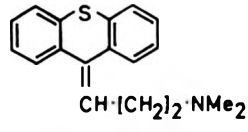
Imipramine
(0·2 mM)



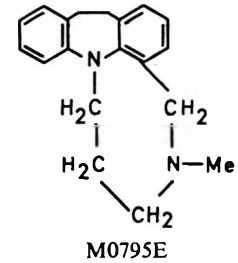
Desipramine
(0·5 mM)



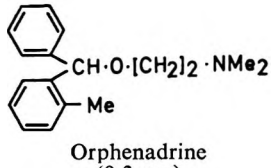
Amitriptyline
(1·5 mM)



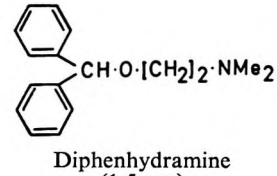
Chlorprothixine
(50 mM)



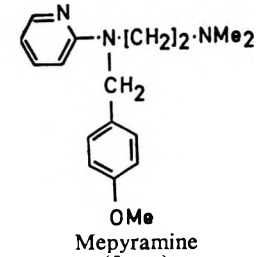
M0795E



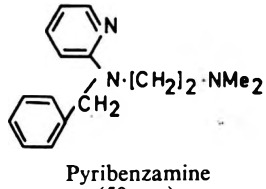
Orphenadrine
(0·3 mM)



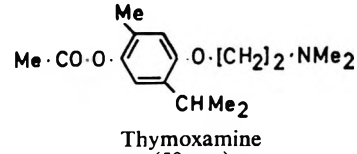
Diphenhydramine
(1·5 mM)



Mepyramine
(5 mM)



Pyribenzamine
(50 mM)



Thymoxamine
(50 mM)

DISCUSSION

The number of phenothiazine derivatives tested is too small to allow firm conclusions about correlation of structural characteristics with activity in inhibiting agglutination. However the nature of the chain attached to the nitrogen atom at the 10 position of the nucleus affects the properties of the drug. Thus thiopropazate had no activity and perphenazine was a weak inhibitor of agglutination whereas the analogous drug with a shorter chain, chlorpromazine, was a potent inhibitor. However trifluprom azine was weaker than fluphenazine although it has a shorter chain. Foster & Fyfe (1966) reported that, as a result of stearic hindrance, long aliphatic side chains attached to the phenothiazine nucleus decrease the stability of electron donor acceptor complexes with 1,4-dinitrobenzene. The nature of the side-chain at the 2-position of the phenothiazine nucleus also influences the activity of the drugs in inhibiting agglutination. For example, thioperazine with a sulphonamide grouping on the phenothiazine nucleus was a weaker inhibitor of agglutination than the analogous compound thiethylperazine with a 2-ethylthio-grouping. There was also a tenfold difference in strength of inhibition between chlorpromazine (–Cl at the 2-position) and trifluprom azine ($-CF_3$) the latter being much weaker. However, fluphenazine ($-CF_3$) was more potent than perphenazine.

Amongst the phenothiazines the weakest inhibitors are weak bases, but there was no consistent relation between pK_a and potency in inhibiting agglutination (Fig. 2). The major structural feature determining pK_a in phenothiazines is the nature of the 10-alkylamine side-chain (Green, 1967). A 2-halo-substituent may effect ionization

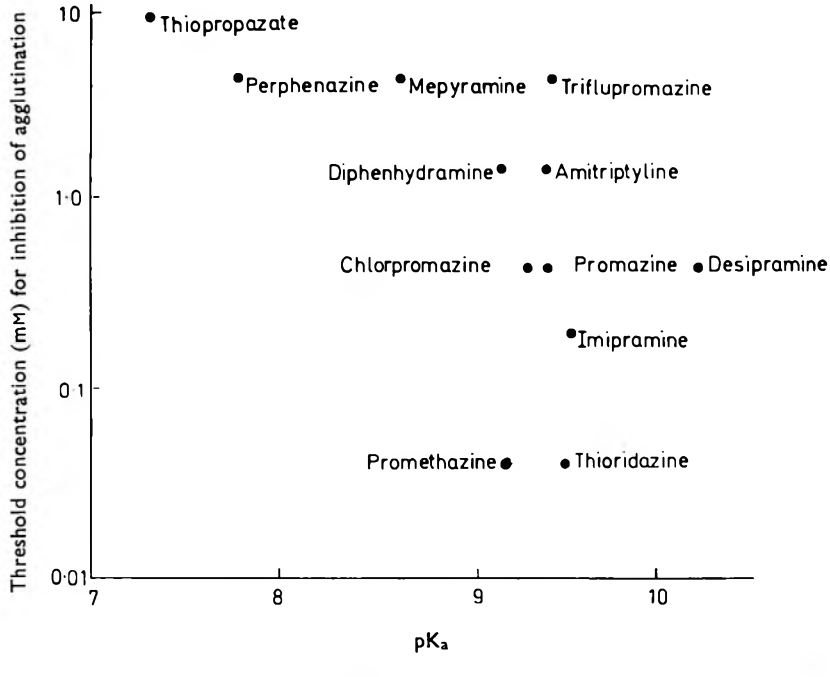


FIG. 2. Relation between potency in inhibiting agglutination and pK_a values for some phenothiazines, tricyclic antidepressants and antihistamines. Minimum concentrations required for inhibition of agglutination are expressed as $\log_{10}$ (100 × mM concentration). Not all the drugs used are included because of the absence of pK_a values of many in the literature.

but the conformation of the alkylamino-chain may remove it from this influence (Chatten & Harris, 1962). Strong bases with a quaternary nitrogen were inactive. The structure of the molecule rather than its degree of ionization appears to determine its ability to inhibit agglutination. However, it is likely that the cationic form of the drug molecule may be responsible for inhibition of agglutination, since acidic groups on the red cell membrane are incorporated in antigenic sites. That the drug attaches to the red cells is supported by the observation that promethazine-treated red cells washed several times in saline do not agglutinate as much as control cells which have not been exposed to the drug (unpublished observations). Irwin, Smith & Trams (1961) reported that gangliosides inhibit the action of chlorpromazine on muscle and suggested that this was due to combination of the drug and the acidic ganglioside rather than to an effect on drug receptors. They also suggested that neuraminic acid, which is present in gangliosides, was concerned in this interaction. Neuraminic acid is also present in the red cell membrane and may form part of the antigenic moiety (Cook, 1968).

The presence of *N*-methyl substituted alkylamine side-chain in phenothiazine, antidepressant and antihistamine drugs with activity in inhibiting agglutination suggests that this group may be important for the effect. The *NN*-dimethyl substituted ethylamine chain is common to most antihistamine drugs and is thought to be involved in binding at histamine receptor sites. Drugs with *NN*-diethyl substituted alkylamine chains such as procaine did not inhibit agglutination, possibly because the larger substituents on the nitrogen atom might prevent a close association of the cation with an anionic site on the red cell membrane.

Phenothiazine derivatives apparently show an inverse correlation of potency in inhibiting agglutination and tranquillizing potency. The most active drugs in inhibiting agglutination, such as thioridazine and promazine, are given in relatively large doses (75–200 mg in 24 h to ambulatory patients and 200–600 mg in 24 h to hospital patients). The weaker inhibitors of agglutination such as perphenazine are given in smaller doses (1–3 and 2–20 mg in 24 h). It is possible that the weaker inhibitors of agglutination are less firmly bound to red cell membranes and may be absorbed more readily into the central nervous system.

There are clinical implications to the observed inhibition of red cell agglutination by a number of drugs. Since the phenothiazine drugs are placenta permeable their administration during pregnancy in the case of foeto-maternal incompatibilities may help to lessen erythroblastosis at least when it is due to immune anti-A or anti-B antibodies. Bierme & Bierme (1967) observed that administration of promethazine in conjunction with intraperitoneal transfusions proved of benefit in cases of rhesus immunization. However promethazine *in vitro* has proved much less effective in inhibiting red cell agglutination due to Rh antibodies than that observed with the ABO system (unpublished observations).

Routine ABO grouping should come under closer scrutiny if the patients have been administered drugs which are potent in inhibiting agglutination. Although it is unlikely that a wrong ABO group determination will be obtained, the avidity of the reaction may be reduced. The use of some antihistamines in treating cases of transfusion reaction could be responsible for negative antibody tests on the slide, particularly if the antibody is weak, and could mask a genuine incompatibility reaction. The findings indicate the importance of taking blood samples for cross-matching and grouping, where practical, before drugs are given.

Acknowledgements

The author wishes to thank Professor M. J. Rand, Professor of Pharmacology, University of Melbourne for his supervision of this work. The author is also indebted to the following drug companies for the generous supply of drugs: Abbot Labs. Pty. Ltd., Amido Labs. Pty. Ltd., Bayer Pharmaceuticals Pty. Ltd., Geigy Aust. Pty. Ltd., ICI Ltd., May & Baker Pty. Ltd., Mead Johnson Pty. Ltd., Merck Sharp & Dohme Pty. Ltd., Parke Davis & Co., Pfizer Pty. Ltd., Riker Labs. (Aust.) Pty. Ltd., Roche Products Pty. Ltd., Sandoz Aust. Pty. Ltd., Schering Pty. Ltd., Searle, G. D. (Aust.) Pty. Ltd., Siquil Pty. Ltd., Smith, Kline & French Pty. Ltd., Squibb, E. R. & Sons Pty. Ltd., Winthrop Labs., Wyeth Pharmaceuticals Pty. Ltd.

REFERENCES

BIERME, A. & BIERME, R. (1967). *Lancet*, **1**, 574.
CHATTEN, L. G. & HARRIS, L. E. (1962). *J. analyt. Chem.*, **34**, No. 11, 1495–1501.
COOK, G. M. W. (1968). *Br. med. Bull.*, **24**, No. 2, 118–123.
FOSTER, R. & FYFE, C. A. (1966). *Biochem. Biophys. Acta*, **112**, 490–495.
GREEN, A. L. (1967). *J. Pharm. Pharmac.*, **19**, 10–16.
IRWIN, R. L., SMITH, H. J., TRAMS, E. G. (1961). *The Pharmacologist*, **3**, No. 2, 54.
TAIT, B. D. (1968). *J. Pharm. Pharmac.*, **20**, 479–481.

J. Pharm. Pharmac., 1970, **22**, 745–752 *Received March* 26, 1970

Corticosteroid-collagen interaction *in vitro:* fibril formation from collagen solutions

D. R. COOPER, I. KANFER* AND C. H. PRICE*

Leather Industries Research Institute, Rhodes University, Grahamstown

**Pharmacy Department, Rhodes University, Grahamstown,*

Republic of South Africa

The disodium phosphate ester salts of betamethasone, prednisolone and hydrocortisone dissolved in a fibril initiating buffer accelerated the precipitation of fibrils from saline solutions of acid-soluble collagen at pH 7·0, when compared with the rate for the initiating buffer alone at the same pH. Without the initiating buffer these corticosteroids also precipitated collagen fibrils, but at a slower rate than when the initiating buffer was present. A relation has been found between the substitution of active groups on the basic steroid nucleus and the rate of collagen fibril formation from solution. Electron microscope examination of the fibrils precipitated by these corticosteroids showed that they corresponded to normal collagen fibrils in appearance and in having the characteristic repeat period of 60–70 nm.

Many factors such as pH, ionic strength, temperature, the method of preparing the collagen, and the addition of complex molecules influence the formation *in vitro* of fibrils from solutions of soluble collagen (Gross & Kirk, 1958; Bensusan & Hoyt, 1958; Gross, 1958b; Bensusan, 1960; Bensusan & Scanu, 1960; Convy & Wynn, 1967; Bowden, Chapman & Wynn, 1968; Wasteson & Obrink, 1968). The precipitation of fibrils occurs in two consecutive steps, a lag period or nucleation step, in which soluble collagen particles aggregate to form nuclei, followed by a growth step represented by a sigmoid precipitation curve in which the nuclei grow into fibrils by accretion of further soluble collagen particles (Bensusan & Hoyt, 1958; Wood & Keech, 1960; Wood, 1960a).

We have examined the effect *in vitro* of several water-soluble corticosteroids on fibril formation from solutions of soluble collagen. The relatively minor changes in active groups substituted on the basic steroid nucleus in these compounds allows a systematic correlation of the effect of this substitution on fibril formation.

EXPERIMENTAL

Preparation of acid-soluble collagen

Two preparations of acid-soluble collagen from calf-skin were used. The first (P1) was obtained according to Cooper & Davidson (1965), the second (P4) by the method of Piez, Eigner & Lewis (1963). The acid-soluble collagen was purified by phosphate precipitation (Gross, 1958a), and the purity checked by amino-acid analysis, chromatography and ultracentrifugation (Cooper & Davidson, 1965; Davidson & Cooper, 1967).

Preparation of collagen solutions

The freeze-dried acid-soluble collagen was dissolved in physiological saline to give a final concentration of 1·2 to 1·4 mg/ml and a pH of 4·2. Clarification of collagen solutions was by centrifugation at 5° for 1 h in an M.S.E. centrifuge at a maximum of 32 000 *g*, and in one case at 5° for 1 h in a Spinco model L2–65B ultracentrifuge at a maximum of 107 000 *g*.

Corticosteroids

Prednisolone disodium phosphate [batch EPY (C) 7/7; 11β,17α,21-trihydroxy-pregna-1,4-diene 3,20-dione 21-(disodium phosphate)] and hydrocortisone disodium phosphate [batch EPY (C) 7/6; 11β,17α,21-trihydroxypregna-4-ene-3,20-dione 21-(disodium phosphate)] were obtained from Glaxo Laboratories Limited, England, and betamethasone disodium phosphate [batch DOH–M–13–1; 9α-fluoro-11β,17α, 21-trihydroxy-16β-methyl-pregna-1,4-diene-3,20-dione 21-(disodium phosphate)] from the Schering Corporation, South Africa. All these compounds were of B.P. purity.

Methods of following fibril precipitation

Fibril precipitation was followed by modifications of the turbidity methods of Bensusan & Hoyt (1958) (Method 1) and Wood & Keech (1960) (Method 2). The development of turbidity was monitored at 400 nm using a recording Beckman DB spectrophotometer fitted with a constant temperature cell holder.

Modified Bensusan and Hoyt Method (*Method* 1). Two matched cuvettes (1 cm path length) were placed for 30 min in the sample and reference beams of the spectrophotometer with the cell-housing being kept at 25°. The collagen solution was removed from a refrigerator at 4° and kept in a water bath at 25° for 30 min. The "initiating buffer", consisting of 0·04M-KH_2PO_4-NaOH buffer (pH 6·95, I 0·23), was also placed in a water bath for 15 min at 25°. At zero time, 1 min after their removal from the water bath, 3 ml of the collagen solution was added to 3·4 ml of the initiating buffer in a glass stoppered test tube which was inverted ten times during 1 min. The mixture was then placed in a water bath at 25° for 4 min and an aliquot of 3·2 ml then transferred to the sample cuvette kept in the spectrophotometer, this operation being done in 1 min. The recorder connected to the spectrophotometer was started exactly 2 min after removal of the reaction mixture from the water bath, the reference cell with its contents of the original collagen solution having previously been allowed to equilibrate at 25°. The change in extinction with time was recorded. All the operations were done in an air-conditioned room at 25°.

Modified Wood and Keech Method (*Method* 2). An aliquot of 1·5 ml of the collagen solution was placed in a cuvette (1 cm path length) in the spectrophotometer and allowed to stand for 30 min at 20° to attain temperature equilibrium. At the same time another matched cuvette was filled with the identical collagen solution and allowed to equilibrate as the blank. At zero time 1·7 ml of the "initiating buffer" kept at 20° for 30 min was added to the cuvette containing 1·5 ml of collagen solution which was then inverted three times and replaced in the spectrophotometer. A recording of extinction against time was made. All operations were done in an air-conditioned room at 20°.

The influence of the corticosteroids on the fibril precipitation was followed either by dissolving these in the initiating buffer, or by dissolving them in physiological saline when no initiating buffer was used. Except where otherwise stated, 2 mg of corticosteroid was dissolved in 1·7 ml of the initiating buffer or physiological saline and added to 1·5 ml of collagen solution containing 1·2 to 1·3 mg/ml.

Electron microscopy

Samples of the fibrils obtained from the precipitation experiments were prepared for electron microscopy by drying on formvar-covered copper grids (200 mesh). These were stained by immersion for 1 min in 0·1% phosphotungstic acid pH 6·1, followed by washing in distilled water, before final drying. The specimens were examined in a Hitachi HU 11B electron microscope.

RESULTS

Standardization of the procedure for turbidity measurements

The procedure generally adopted to maintain reproducibility was to bracket duplicate runs in the presence of the corticosteroids with blank runs using initiating buffer alone, all on the same or consecutive days. It was thus necessary to have the fibril formation complete within a few hours. A typical example of the reproducibility obtained with strict adherence to procedure is given in Fig. 1a. All subsequent

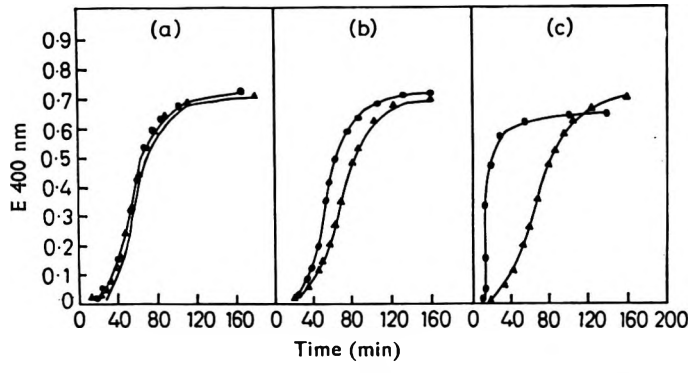


FIG. 1. Effect of varying experimental conditions on fibril precipitation from solutions of acid-soluble collagen in physiological saline using initating buffer at pH 7·04. (a) Duplicate determinations at 25° using filtered solutions, method 1 and preparation P1; (b) Determinations on a filtered solution (●) and a solution centrifuged at 32 000 *g* (▲) using preparation P1 and method 1 at 25°; (c) Determinations on solutions of preparation P1 (▲) and P4 (●) centrifuged at 32 000 *g* and using method 1 at 25°.

graphs or data given in the Tables represent the average of duplicate or triplicate determinations. When solutions of preparation P4 were used, method 1 gave too rapid a reaction compared with preparation P1 (Fig. 1c and Run 3, Table 1) and therefore the modified Wood & Keech method was developed (method 2).

Fig. 1b, corresponding to Run 2 of Table 1, shows that clarification of the collagen solution, after making sure that the collagen had dissolved, had an important bearing on the reaction rate, which corresponds to the findings of Wood & Keech (1960). Centrifugation of the solutions presumably removes collagen molecular aggregates

Table 1. *Rate of precipitation of collagen from physiological saline by initiating buffer.* Experimental conditions: pH of reaction mixture 7·04, I 0·23, 25°

Run No.	Pretreatment of collagen solution	Turbidity method	Collagen preparation	$E\infty$	$t_{0\cdot01}$ (min)	$t_{0\cdot5}$ (min)	S ($\times 10^2$)
1	filtered	1	P1	0·72	19	60	1·54
2A	filtered	1	P1	0·74	19	56	1·54
2B	× 32 000 *g*	1	P1	0·72	21	68	1·22
3A	× 32 000 *g*	1	P1	0·73	21	70	1·13
3B	× 32 000 *g*	1	P4	0·66	11	17	11·92

For definition of $E\infty$, $t_{0\cdot01}$, $t_{0\cdot5}$ and S, see text.

which would act as nucleating centres for fibril formation, hence the reaction in the centrifuged solution was slower than in the filtered solution. This point is illustrated again in later experiments (Table 2, Fig. 2).

All the precipitation curves had a similar sigmoid shape, consisting of a lag period, during which no precipitation was recorded, and a sigmoid portion or growth phase. It was difficult to determine the lag period due to the shape of the curves, but a comparable estimate of this is given by $t_{0\cdot01}$, the time taken for the extinction to rise to the value 0·01. The rate of growth is given by the half-growth time ($t_{0\cdot5}$)being the time taken for the extinction to rise to one-half of its final value. The reactions are also compared by the slope (S) of the linear portion of the sigmoid curve, which is equivalent to the reaction rate (Bensusan & Scanu, 1960). The extinction at the end of the reaction is given by $E\infty$.

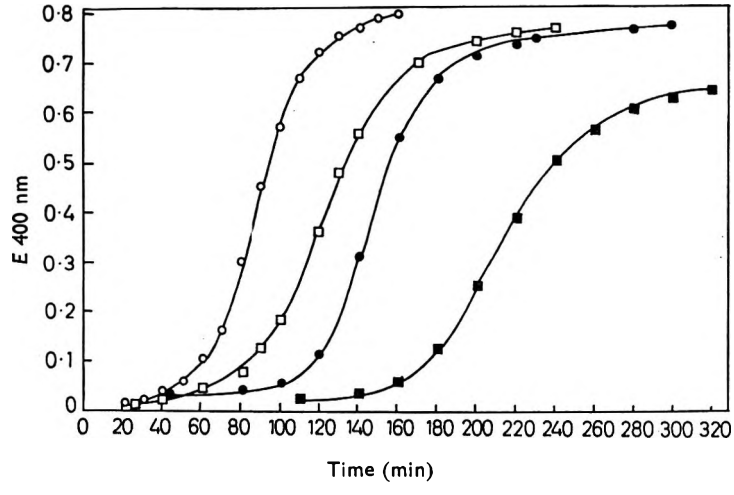
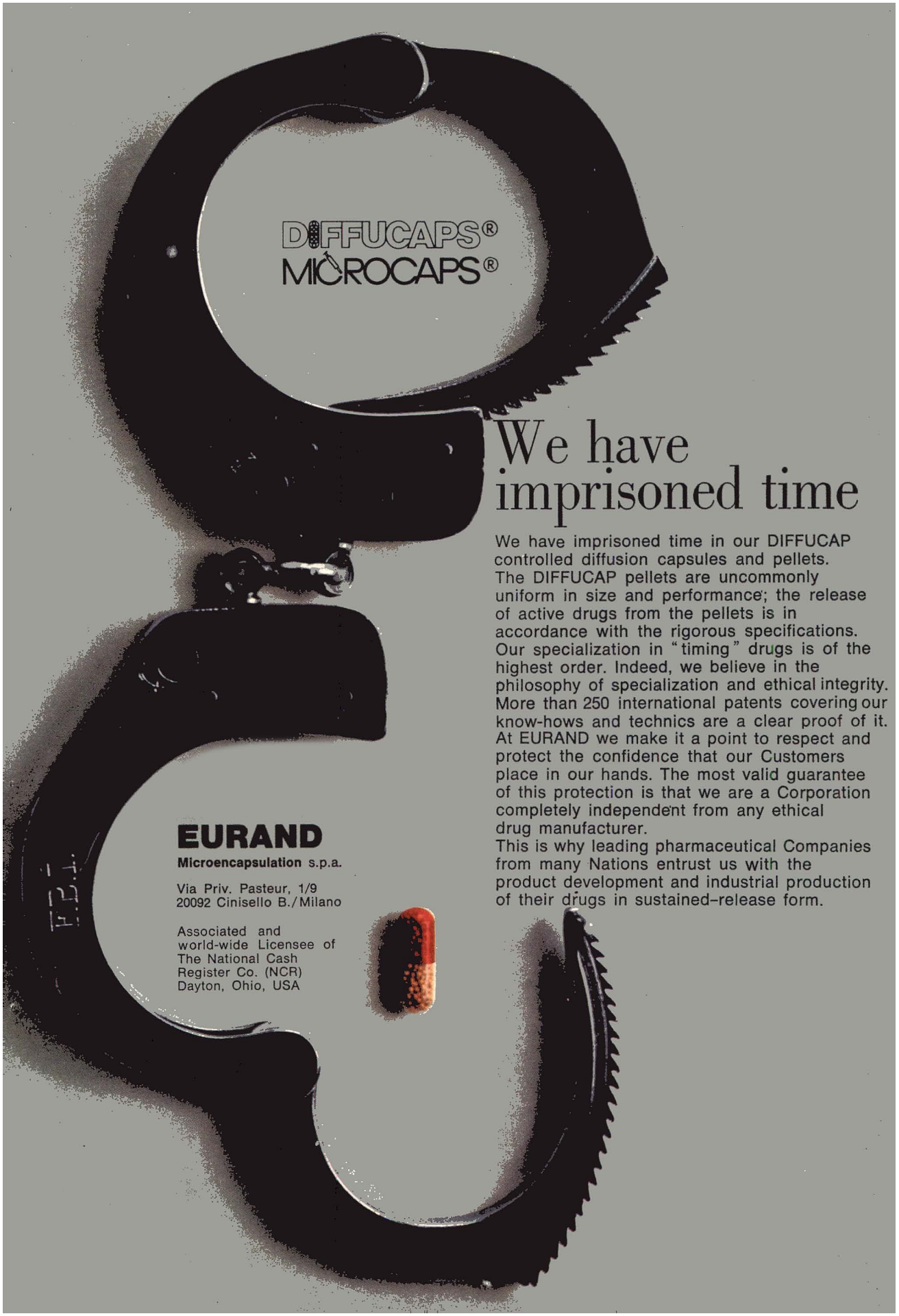


FIG. 2. Fibril precipitation from solutions of acid-soluble collagen (P1) in physiological saline using initiating buffer alone (pH 7·04) and initiating buffer containing betamethasone disodium phosphate (pH 6·95) and method 1 at 25°; □, solution centrifuged at 32 000 *g* and precipitated with initiating buffer alone; ○, solution centrifuged at 32 000 *g* and precipitated with initiating buffer containing betamethasone disodium phosphate; ■, solution centrifuged at 107 000 *g* and precipitated with initiating buffer alone; ●, solution centrifuged at 107 000 *g* and precipitated with initiating buffer containing betamethasone disodium phosphate.


DIFFUCAPS®
MICROCAPS®
We have imprisoned time
We have imprisoned time in our DIFFUCAP controlled diffusion capsules and pellets. The DIFFUCAP pellets are uncommonly uniform in size and performance; the release of active drugs from the pellets is in accordance with the rigorous specifications. Our specialization in "timing" drugs is of the highest order. Indeed, we believe in the philosophy of specialization and ethical integrity. More than 250 international patents covering our know-hows and technics are a clear proof of it. At EURAND we make it a point to respect and protect the confidence that our Customers place in our hands. The most valid guarantee of this protection is that we are a Corporation completely independent from any ethical drug manufacturer.
This is why leading pharmaceutical Companies from many Nations entrust us with the product development and industrial production of their drugs in sustained-release form.
EURAND
Microencapsulation s.p.a.
Via Priv. Pasteur, 1/9
20092 Cinisello B./Milano
Associated and world-wide Licensee of The National Cash Register Co. (NCR) Dayton, Ohio, USA

DIFFUCAPS®
MICROCAPS®

Timed release drugs is our job

Our specialization in this field is of the highest order. Our technics and know-hows are covered by more than 250 international patents. Indeed, we believe in the philosophy of specialization and ethical integrity.
At EURAND we make it a point to respect and protect the confidence that our Customers place in our hands. The most valid guarantee of this protection is that we are a Corporation completely independent from any ethical drug manufacturer.
This is why leading pharmaceutical Companies from many Nations entrust us with the product development and industrial production of their drugs in sustained release form.
DIFFUCAP pellets are uncommonly uniform in size and performance; the release of active drug from the pellets is in accordance with rigorous specifications.

EURAND
Microencapsulation s.p.a.

Via Priv. Pasteur, 1/9
20092 Cinisello B./Milano

Associated and world-wide Licensee of The National Cash Register Co. (NCR) Dayton, Ohio, USA

G&R ASSOCIATI

Table 2. *Rate of precipitation of collagen from physiological saline by corticosteroids in initiating buffer.* Experimental conditions: pH of reaction mixture containing initiating buffer alone 7·04; pH of reaction mixture containing initiating buffer and corticosteroid 6·95; I 0·23; Method 1, collagen preparation P1, reaction temperature 25°

Run No.	Initiating solution	Pretreatment of collagen solution	$E\infty$	$t_{0\cdot01}$ (min)	$t_{0\cdot5}$ (min)	S ($\times 10^2$)
4A	IB	filtered	0·84	23	69	1·45
4B	IB + BDP	filtered	0·94	18	56	2·05
5A	IB	filtered	0·74	19	56	1·51
5B	IB + BDP at half conc.	filtered	0·82	18	52	2·12
6A	IB	× 32 000 *g*	0·78	37	124	0·96
6B	IB + BDP	× 32 000 *g*	0·86	33	98	1·48
6C	IB	× 107 000 *g*	0·66	114	228	0·77
6D	IB + BDP	× 107 000 *g*	0·86	50	152	1·43

For definition of $E\infty$, $t_{0\cdot01}$, $t_{0\cdot5}$ and S, see text. Abbreviations: IB, initiating buffer, BDP, betamethasone disodium phosphate.

The action of corticosteroids in initiating buffer on fibril formation

In the initial study of the effect on fibril formation of the corticosteroids, the drugs were dissolved in the initiating buffer, and the rate of fibril formation compared with that for initiating buffer alone. The action of betamethasone disodium phosphate on fibrillogenesis is clearly illustrated in Fig. 2, accelerated formation of fibrils occurring after addition of the corticosteroid to the initiating buffer. The reaction constants (Table 2), also show an acceleration of fibril formation, while the extinction value $E\infty$ was greater in the presence of betamethasone disodium phosphate. Similar results were obtained using half the concentration of the corticosteroid although the differences in the reaction constants were not as great (Run 5, Table 2). Betamethasone disodium phosphate also accelerated fibril formation with a different collagen preparation (P4) and the second method of following fibril formation (Run 7, Table 3). In all the above studies the pH of the reaction mixture was 7·04 (initiating buffer only) and 6·95 (betamethasone disodium phosphate dissolved in initiating buffer).

Prednisolone disodium phosphate dissolved in the initiating buffer (Run 8, Table 3) also accelerated fibril formation. The pH of the reaction mixture being 6·95. To confirm that the three corticosteroids used had different effects on fibril formation when dissolved in the initiating buffer, duplicate determinations on each were made with the same collagen solution on successive days. These were reproducible and the reaction constants (Run 9, Table 3) illustrate the differences.

Effect of corticosteroids in physiological saline on fibril formation

To show that corticosteroids can initiate fibril formation on their own, the disodium phosphate derivatives of betamethasone, prednisolone and hydrocortisone were dissolved in physiological saline, instead of initiating buffer, and added to the collagen dissolved in the same solvent. The control for each of these reactions was similar initiating buffer to that used previously but made up to give a pH in the reaction mixture of 6·2 which is close to the pH of the reaction mixtures containing the

Table 3. *Rate of precipitation of collagen from physiological saline by corticosteroids in initiating buffer.* Experimental conditions: pH of reaction mixture containing initiating buffer alone 7·04; pH of reaction mixture containing initiating buffer and corticosteroid 6·95; I 0·23; Method 2; collagen preparation P4; reaction temperature 20°; collagen solutions clarified by centrifugation at 32 000 *g*

Run No.	Initiating solution	$E\infty$	$t_{0\cdot01}$ (min)	$t_{0\cdot5}$ (min)	S ($\times 10^2$)
7A	IB	0·75	12	22	6·13
7B	IB + BDP	0·79	10	18	7·08
8A	IB	0·73	13	24	5·60
8B	IB + PDP	0·79	13	20	7·60
9A	IB	0·93	32	46	1·55
9B	IB + BDP	1·00	26	50	2·47
9C	IB + HDP	0·96	32	48	1·80
9D	IB + PDP	1·01	27	50	2·32

For definition of $E\infty$, $t_{0\cdot01}$, $t_{0\cdot5}$ and S, see text. Abbreviations: IB, initiating buffer; BDP, betamethasone disodium phosphate; PDP, prednisolone disodium phosphate; HDP, hydrocortisone disodium phosphate.

corticosteroids (Table 4). In each case the blank solution placed in the reference cell of the spectrophotometer was collagen dissolved in physiological saline, which showed no precipitation of fibrils. Table 4 and Fig. 3 show that in the absence of the initiating buffer the three corticosteroids induced fibril formation at different rates, all of which were slower than that for initiating buffer alone. Further, the final extinction values ($E\infty$) in the presence of all three of the corticosteroids were significantly greater than the value obtained with the initiating buffer.

Fibril precipitation was also obtained at a tenth of the concentration of betamethasone disodium phosphate used above, though at a very much slower rate ($t_{0\cdot01}$ 180 min, $t_{0\cdot5}$ 1250 min and S 0·0319. Preliminary results indicate that fibril formation can be obtained at very much lower concentrations of corticosteroids by raising the temperature above the relatively low values used in the current experiments.

Electron microscopy

The fibrils precipitated in the presence of initiating buffer or the corticosteroids resembled native collagen in appearance and in having a repeat period of about 64 nm.

Table 4. *Rate of precipitation of collagen from physiological saline by corticosteroids in physiological saline.* Experimental conditions: Collagen preparation P4; turbidity method 2; collagen solutions clarified by centrifugation at 32 000 *g*; 20°

Run No.	Initiating solution	pH of steroid in saline	pH of reaction mixture	$E\infty$	$t_{0\cdot01}$ (min)	$t_{0\cdot5}$ (min)	S ($\times 10^2$)
10A	IB	—	6·2	0·70	20	48	3·08
10B	BDP	7·1	6·3	1·08	41	90	1·76
10C	PDP	7·2	6·3	1·02	54	111	1·33
10D	HDP	7·3	6·4	1·01	55	114	1·33

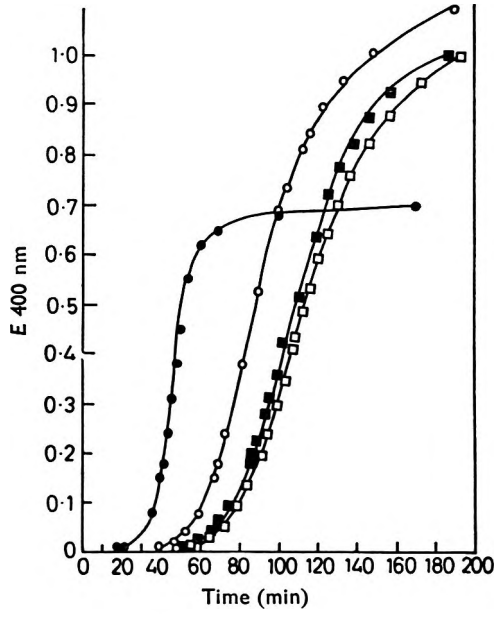


FIG. 3. Fibril precipitation from solutions of acid-soluble collagen (P4) in physiological saline using method 2 at 20°; ●, initiating buffer alone (pH 6·2); ○, betamethasone disodium phosphate in physiological saline (pH 6·3); ■, prednisolone disodium phosphate in physiological saline (pH 6·3); □, hydrocortisone disodium phosphate in physiological saline (pH 6·4).

DISCUSSION

The results in Tables 2 and 3, and Fig. 2 show conclusively that an increase in fibril precipitation rate was obtained when the disodium phosphates of betamethasone, prednisolone or hydrocortisone were dissolved in the initiating buffer before adding this to the collagen solution. This was independent of the method used to clarify the collagen solution, although centrifugation with increasing centrifugal force reduced the general rate in comparison with filtration, presumably because of the removal of collagen molecular aggregates which would assist the nucleation rate. Wood (1960b) concluded from studies with polyanions that those which accelerated precipitation lowered $E\infty$ whereas in the present case the acceleration of precipitation with the disodium phosphate ester salts of the three steroids was accompanied by a significant increase in $E\infty$. This effect does not appear to be due to a pH factor since the pH of the reaction mixtures containing the corticosteroid (pH 6·92) was similar to that for the initiating buffer alone (pH 7·04), while differences in reaction curves and constants are greater than would be expected for the small differences in the pH of the reaction mixtures (Wood & Keech, 1960). This was particularly evident when the reaction rate was slowed down by centrifuging the collagen solution at 107 000 *g* (Table 2 and Fig. 2). Further, Wood & Keech (1960) found that increasing the ionic strength above I 0·13 decreased the rate of fibril precipitation at pH 7·1. The addition of ionizable corticosteroids to the initiating buffer should have increased the ionic strength slightly, but nevertheless the precipitation rate also increased.

The ability of corticosteroids to induce fibril precipitation was confirmed by experiments in which these were added to the collagen solution after dissolving in physiological saline and not the initiating buffer. These experiments (Fig. 3, Table 4)

involved the phosphate derivatives of betamethasone, prednisolone and hydrocortisone which have relatively slight structural variations. These precipitated fibrils with longer lag and growth phases compared with fibril formation at the same pH using initiating buffer alone. The final extinction value ($E\infty$), which Wood (1960b) has related directly to fibril width, were much increased in the presence of corticosteroid.

The order of increasing anti-inflammatory activity of these compounds *in vivo* is hydrocortisone, prednisolone and betamethasone (Liddle & Fox, 1961; Sarett, Patchett & Steelman, 1963) which corresponds to the substitution of active groups on the basic steroid nucleus, and in turn to the order of increasing fibril precipitation rate found experimentally *in vitro*.

Acknowledgements

One of us (I.K.) wishes to acknowledge research grants from the Council for Scientific and Industrial Research and Rhodes University, South Africa. The authors are grateful to Glaxo Laboratories Ltd., England, Glaxo-Allenburys (S.A.) (Pty) Ltd., South Africa, and the Schering Corporation, South Africa, for supplying the corticosteroids, and to Dr. A. E. Russell for discussion.

REFERENCES

BENSUSAN, H. B. & HOYT, B. L. (1958). *J. Am. Chem. Soc.*, **80**, 719–724.
BENSUSAN, H. B. (1960). *Ibid.*, **82**, 4995–4998.
BENSUSAN, H. B. & SCANU, A. (1960). *Ibid.*, **82**, 4990–4995.
BOWDEN, J. K., CHAPMAN, J. A. & WYNN, C. H. (1968). *Biochim. biophys. Acta*, **154**, 190–195.
CONVY, S. & WYNN, C. H. (1967). *Biochem. J.*, **103**, 413–419.
COOPER, D. R. & DAVIDSON, R. J. (1965). *Ibid.*, **97**, 139–147.
DAVIDSON, R. J. & COOPER, D. R. (1967). *Ibid.*, **105**, 965–969.
GROSS, J. (1958a). *J. exp. Med.*, **107**, 247–258.
GROSS, J. (1958b). *Ibid.*, **108**, 215–226.
GROSS, J. & KIRK, D. (1958). *J. biol. Chem.*, **233**, 355–360.
LIDDLE, G. W. & FOX, M. (1961). *Inflammation and Diseases of Connective Tissue*, pp. 302–327. Editors: Mills, L. C. & Moyer, J. H. Philadelphia & London: W. B. Saunders Co.
PIEZ, K. A., EIGNER, E. A. & LEWIS, M. S. (1963). *Biochemistry*, **2**, 58–66.
SARETT, L. H., PATCHETT, A. A. & STEELMAN, S. L. (1963). *Progress in Drug Research*, **5**, pp. 13–153. Editor: Jucker, E. Basel: Birkhauser Verlag.
WASTESON, A. & OBRINK, B. (1968). *Biochim. biophys. Acta*, **170**, 201–204.
WOOD, G. C. & KEECH, M. K. (1960). *Biochem. J.*, **75**, 588–598.
WOOD, G. C. (1960a). *Ibid.*, **75**, 598–605.
WOOD, G. C. (1960b). *Ibid.*, **75**, 605–612.

J. Pharm. Pharmac., 1970, 22, 753–757 *Received April* 30, 1970

Theoretical changes in drug distribution resulting from changes in binding to plasma proteins and to tissues

STEPHEN H. CURRY

Department of Pharmacology and Therapeutics, London Hospital Medical College, Turner Street, London, E.1, U.K.

Concentrations of chlorpromazine fluctuate in the plasma of dogs and man after intravenous doses. The possibility that the fluctuations could arise from movement of the drug between tissue and plasma stores is examined theoretically. Calculations show that small changes in protein binding of drugs in plasma and tissues could cause redistribution of highly bound drugs between tissues and plasma. Redistribution would be greatest after changes in tissue binding of highly bound drugs. Fluctuations in chlorpromazine concentrations could be caused in this way.

Fluctuations were recently observed in concentrations of chlorpromazine in plasma of dogs and man after intravenous doses (Curry, Marshall & others, 1970; Curry, Derr & others, 1970). For example, concentrations were sometimes seen to increase by 50% within 10 min, and similar decreases could also occur.

A constant concentration of a drug in plasma could be considered to be maintained in a theoretical situation in which input and output were balanced. Increases could usually arise only as a result of absorption of previously unabsorbed drug; decreases would usually result from removal of drug by metabolism and excretion. However, after an intravenous injection there are no opportunities for changes in the rate of input of drug. Thus fluctuations can occur only by movement of the drug backwards and forwards between plasma and other tissues. The possibility that redistribution between tissue and plasma stores, caused by changes in binding, could be sufficient to cause fluctuations in concentrations of chlorpromazine in plasma was therefore investigated from a theoretical point of view.

The findings are in agreement with, but more extensive than, previous reports concerning the significance of binding in drug distribution (Brodie, 1966; Martin, 1965; Meyer & Guttman, 1968).

EXPERIMENTAL

Relations describing protein binding of drugs are derivable from the Law of Mass Action. The constant, Kap, for each set of binding sites is determined from the equation:

$$\text{Kap} = \frac{[\text{Db}]}{[\text{Df}]\,[\text{Pf}]}$$

in which: [Db] is the molar equilibrium concentration of bound drug; [Df] is the molar equilibrium concentration of unbound drug; and [Pf] is the molar equilibrium concentration of protein not associated with drug molecules. At equilibrium in the

body, a drug is distributed between plasma water, plasma protein, and tissues (including blood cells), largely by reversible processes. The total amount of drug in the body D, consists of the sum of drug in plasma water, [Df] V, drug bound to plasma proteins [Db] Vp, and drug in tissues, [Dt] Vt, where [Dt] is the concentration of the drug in tissues, and V, Vp and Vt are the values of the volume of distribution of the drug in plasma water, plasma, and tissues respectively. The concentration of drug in plasma [Dp] = [Df] + [Db].

In this theoretical study 10 μg of drug were distributed through 1 g of biological material (tissue volume 10 × plasma volume) with various degrees of binding. Excess albumin at a molar concentration of 6×10^{-4} was the plasma protein. Calculations were made of the distribution of a drug in this system in four theoretical sets of conditions, as defined in the figure legends.

RESULTS

The results of the calculations are shown in Figs 1–4. As an example, in Fig. 1, at each value of fraction bound, the points on the lines, multiplied by V, Vp or Vt, as applicable, add up to 10 μg. (The calibrations of the axes should be noted.) The different values of [Df], [Db] and [Dt] in different binding conditions result from defining one binding ratio, and varying another; changes in all three concentrations are necessary if equilibrium is to be preserved.

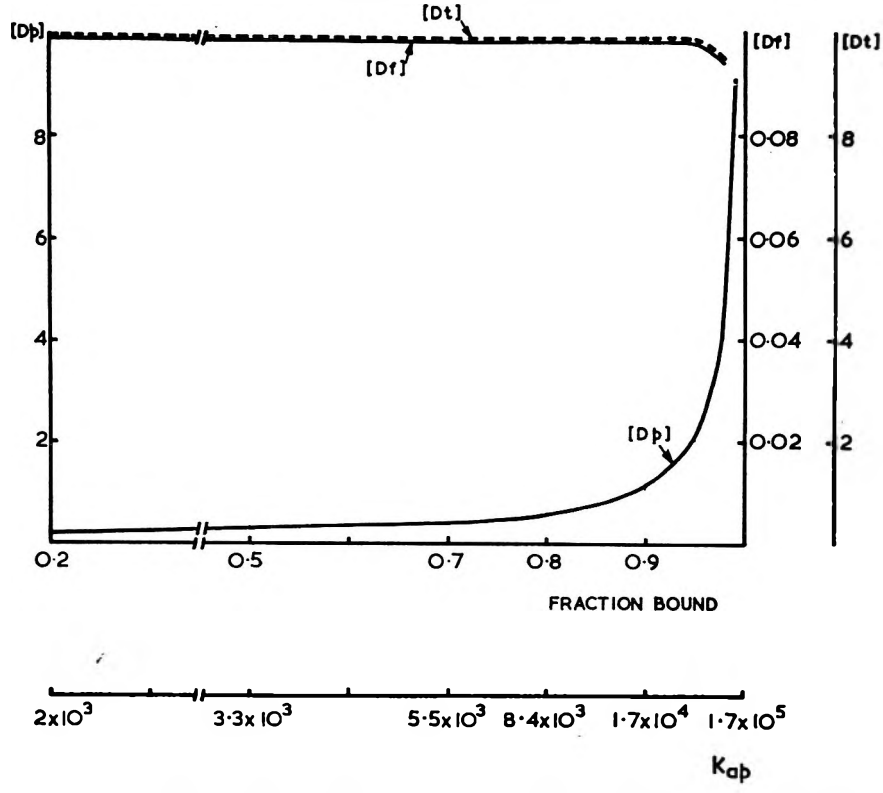


FIG. 1. Concentrations (μg/ml) of drug in plasma [Dp] and plasma water [Df], and mean concentration (μg/g) in tissues [Dt] for a model system (tissue volume ten times plasma volume), in which 10 μg of drug is distributed through 1 g of tissue and plasma, with a high degree of binding to tissues ([Dt] / [Df] = 100), and with varying degrees of binding to plasma protein.

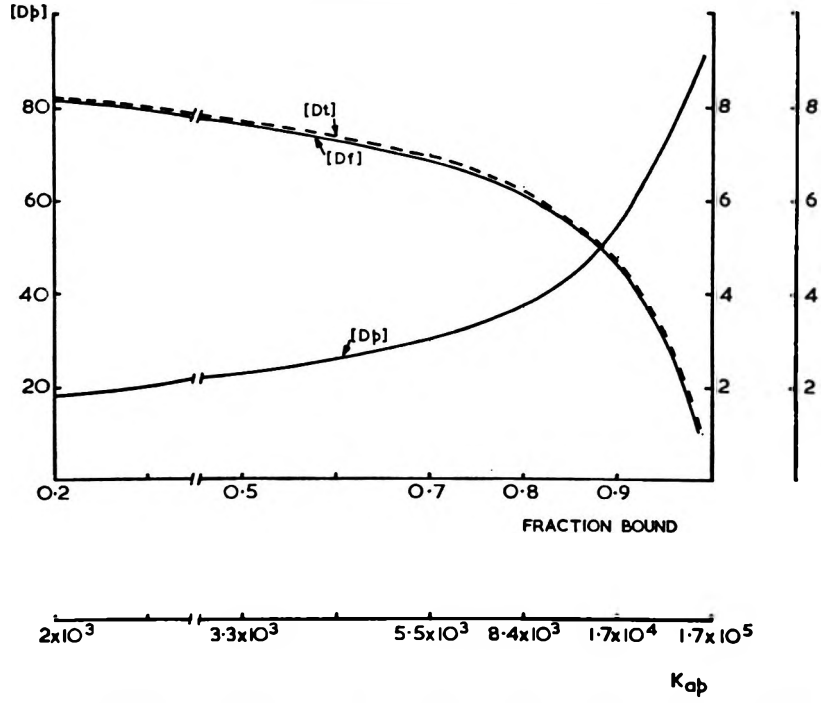


FIG. 2. Concentrations of drug in plasma, plasma water and tissues, for the model system of Fig. 1 with a low degree of binding to tissues ([Dt] / [Df] = 1), and with varying degrees of binding to plasma protein.

For drugs highly bound to plasma protein (fraction bound = 0·95) and highly bound to tissues ([Dt] / [Df]) = 100, a small change (± 0·01) in fraction bound could cause much redistribution between tissues and plasma (Fig. 1). However,

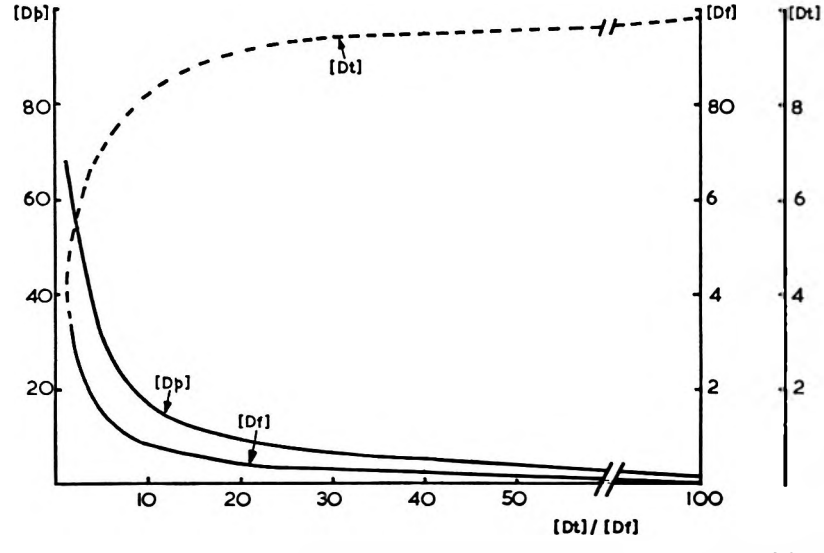


FIG. 3. Concentrations of drug in plasma, plasma water and tissues, for the model system of Fig. 1, with a high degree of binding to plasma protein (fraction bound = 0·95; Kap = 3·3 × 10^{-4}) and with varying degrees of binding to tissues.

because the tissue compartment is relatively large, the concentrations in tissues and plasma water would change less than might be expected. Thus drug concentrations at active sites in tissues might be relatively unchanged, in spite of large changes in concentrations in plasma. With drugs with lower binding to tissues ([Dt] / [Df]) = 1, concentrations in tissue and plasma water would change more with small changes in plasma protein binding (Fig. 2). Concentration changes in plasma would be correspondingly less. After changes in tissue binding, the redistribution would be greatest at lower tissue to plasma water concentration ratios (Figs 3 and 4). Considering all four situations, redistribution would be most marked after small changes in *tissue* binding of drugs *highly* bound to plasma protein.

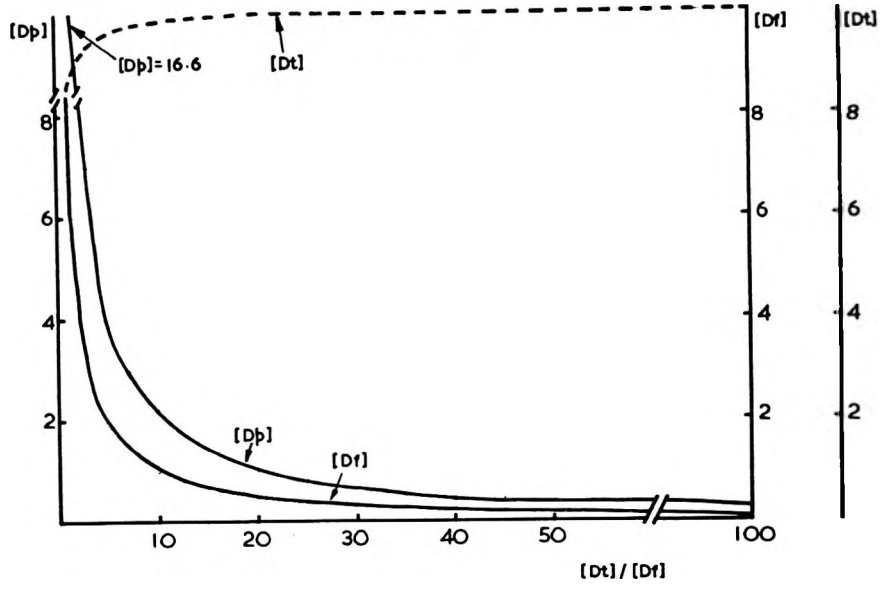


FIG. 4. Concentrations of drug in plasma, plasma water and tissues, for the model system of Fig. 1, with a degree of binding to plasma protein lower than in Fig. 3 (fraction bound = 0·5; Kap = $3{\cdot}3 \times 10^{-3}$), and with varying degrees of binding to tissues.

DISCUSSION

Pharmacological effects are sometimes related to concentrations of drugs in plasma or plasma water (Brodie, 1967). Redistribution of the type discussed theoretically, would affect concentrations of drugs in plasma, and would affect the amount of drug in plasma as a proportion of the amount in the whole body. The result would be an increased complexity of the relation between concentrations in plasma and at receptor sites on the one hand, and between concentrations in plasma and pharmacological effects on the other.

The model used can be criticized as being an oversimplification of what must be in nature a highly complicated system. For example, the chosen figure of 10 × Vp for Vt is, at best, an estimate of the mean value for the volume of tissue which a drug penetrates. Equally, different tissues can carry different concentrations of drugs and the use of a mean value for [Dt] is undoubtedly an over-simplification. An infinite number of permutations of tissue and plasma binding is theoretically possible, yet only four combinations were considered in the present study. Finally plasma protein is not entirely albumin. However, the model was used to facilitate the

examination of the possibility of redistribution. To have considered each tissue individually and to have considered a large number of combinations of binding would have been a task of theoretically infinite proportions and the results would have made little difference to the conclusions.

The model was for no particular drug, as it represented a theoretical situation. Nevertheless, the diagrams were designed so that in a situation in which [Dp], [Df] and [Dt] are known for a chosen drug, it will be possible to estimate the likely influence of small changes in binding on distribution of the compound. The model could be used in the determination of changes in the distribution of one drug resulting from a change in binding, and for comparison of two drugs with different binding.

Changes in binding with concentration have been observed with phenylbutazone (Brodie & Hogben, 1957). Interspecies variations in binding have been observed with acidic drugs (Anton, 1960; Sturman & Smith, 1967), and with basic drugs (Borgå, Azarnoff & Sjöqvist, 1968; Curry, 1970). Differences between individuals have been recorded with thiopentone (Dayton, Perel & others, 1967), and with chlorpromazine (Curry, 1970). The possibility of variation within individuals appears not to have been explored.

Binding can be affected by minor changes in pH of plasma, and of intracellular fluid, by changes in concentrations of interfering substances, or by physical changes in the proteins or protein complexes to which drugs bind. Additionally, outside the limits of the model discussed in this report, differences in fraction bound can follow from changes in protein concentrations.

Chlorpromazine is a highly bound drug (the fraction bound to plasma protein is as high as 0·98 in man; the value for the ratio of average concentration in tissues to concentration in plasma water is 140) (Curry, 1970; Curry, Derr & others, 1970). Fluctuations in concentrations in plasma could apparently be caused by redistribution resulting from small changes in binding.

Acknowledgements

This work was supported (in part) by contract number PH 43–66–1167 with the United States National Institute of General Medical Sciences; a preliminary report was presented to a Discussion Gathering at the Fourth International Congress on Pharmacology, Basel, Switzerland, in July, 1969.

REFERENCES

ANTON, A. H. (1960). *J. Pharmac. exp. Ther.*, **129**, 282–290.

BORGÅ, O., AZARNOFF, D. L. & SJÖQVIST, F. (1968). *J. Pharm. Pharmac.*, **20**, 571–572.

BRODIE, B. B. (1966). In *Transport Function of Plasma Proteins*, pp. 137–145. Editors: Desgrez, P. & Traverse, P. M. Amsterdam: Elsevier.

BRODIE, B. B. (1967). *J. Am. med. Ass.*, **202**, 600–609.

BRODIE, B. B. & HOGBEN, C. A. M. (1957). *J. Pharm. Pharmac.*, **9**, 345–380.

CURRY, S. H. (1970). *Ibid.*, **22**, 193–197.

CURRY, S. H., DERR, J. E., MALING, H. M. & WILLIAMS, M. A. (1970). *Proc. Soc. exp. Biol. Med.* In the press.

CURRY, S. H., MARSHALL, J. H. L., DAVIS, J. M. & JANOWSKY, D. S. (1970). *Arch. Gen. Psychiat.*, **22**, 209–215.

DAYTON, P. G., PEREL, J. M., LANDRAU, M. A., BRAND, L. & MARK, L. C. (1967). *Biochem. Pharmac.*, **16**, 2321–2826.

MARTIN, B. K. (1965). *Nature, Lond.*, **207**, 274–276.

MEYER, M. C. & GUTTMAN, D. E. (1968). *J. pharm. Sci.*, **57**, 895–918.

STURMAN, J. A. & SMITH, M. J. H. (1967). *J. Pharm. Pharmac.*, **19**, 621–623.

J. Pharm. Pharmac., 1970, **22**, 758–762 *Received April* 29, 1970

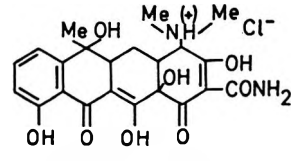
The effect of the anion on the absorption of tetracycline from the rat stomach

J. H. PERRIN AND J. J. VALLNER

School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53706, *U.S.A.*

The absorption of tetracycline from the rat stomach has been investigated at acid pH values. The absorption is dependent on the anion of the buffer and appears to be related to the surface activity of the buffer.

As a result of the "pH partition hypothesis" (Brodie, 1964) it is widely believed that amines and certain other compounds can only be absorbed in appreciable quantities from the gut when the pH is such that a significant fraction of the drug is in the non-ionized and therefore lipid-soluble form. In such cases the absorption process is thought to be that of passive diffusion. Some drugs are absorbed as charged species; for example, Levine (1959) has suggested an active transport process for the absorption of quaternary ammonium amines. Fiese & Perrin (1968–1969) have shown that dextromethorphan can be absorbed from the rat stomach as the protonated species in an apparently passive diffusion process. Here the absorption was linked to the surface



Tetracycline HCl

$pK_{a1} = 3.30$
$pK_{a2} = 7.68$
$pK_{a3} = 9.69$

(Stepenhs, Murai & others, 1956)

activity of the species rather than the lipid solubilities. Tetracycline is known to exist as the charged species at all pH's of the alimentary tract; it is also therapeutically active when given orally, and so it must be absorbed as a charged species. In the investigations reported here, the effect of the anion on the absorption from acid conditions is reported, using the rat as the test animal.

EXPERIMENTAL

Materials

Tetracycline HCl and tetracycline base were supplied by Lederle (Lederle Labs. Division, American Cyanamid Co., Pearl River, N.Y.), the hydrochloride being for parenteral use and the base being 92·42% pure. Trichloroacetic acid was Baker Analyzed reagent grade (J. T. Baker Chemical Co., Phillipsburg, N.J.). Radioactive tetracyline was tetracycline-7-^{3}H (Amersham-Searle Corp., Des Plaines, Ill.) and the

TCA was trichloroacetic acid-1-^{14}C (Amersham-Searle Corp., Des Plaines, Ill.). PPO and Dimethyl POPOP (Packard Instrument Co., Downer's Grove, Ill.) were used in the Bray solution (Bray, 1960) for scintillation counting. The dioxane used in the Bray solution was redistilled over sodium metal. Naphthalene, sodium chloride, sodium nitrate, and sodium carbonate were all analytical reagent grade. Perfluoropropionic acid and perfluorobutyric acid were obtained from the Pierce Chemical Company (Rockford, Ill.). All aqueous solutions were prepared in deionized water, made isotonic, and adjusted to the pH required.

Investigation of absorption of tetracycline from various buffers

Isotonic hydrochloride, trichloroacetate (TCA), nitrate, perfluoropropionate (PFP), and perfluorobutyrate (PFB) buffers at pH 2·0, each containing 400 mg/litre tetracycline base, were used. Female Holtzman (Madison, Wis.) rats, 200 to 250 g, were fasted 18 to 24 h before the experiments but water was freely allowed. The animals were anaesthetized with urethane (1·25 g/kg) given intraperitoneally. The stomach was then exposed, tied off, care being taken not to injure or occlude major blood vessels, and then washed with distilled water or stock drug solution warmed to approximately 37°. Finally, 4 ml of labelled drug solution (previously warmed to 37°) was introduced by a blunt needle into the stomach. The ligature was tightened to prevent any backflow or leakage. Immediately the stomach was removed from the rat by cutting anterior to the ligature on the oesophagus and posterior to that on the small intestine. It was then briefly rinsed in warmed physiological phosphate buffer and subsequently put into the *in vitro* apparatus. This consisted of a large, jacketed, test tube-like container which allowed oxygen in from the bottom, and a constant temperature of 37° was maintained by circulation from a heater pump. All stomachs were surrounded by 50 ml of physiological phosphate buffer. At 30-min intervals two 0·5 ml samples were withdrawn for assay in a Tri-Carb Liquid Scintillation Spectrometer (Model 3002, Packard Instrument Co., Downer's Grove, Ill.). This 1 ml of buffer which was removed for assay purposes was replaced by 1 ml of fresh buffer, and the necessary adjustments were made to subsequent concentration determinations. At least four animals were used for each determination. After each set of experiments the stomach was examined both macroscopically and microscopically for gross effects; however, no untoward effects were seen on the membrane.

Investigation of absorption of trichloroacetate

It was also necessary to examine the absorption of TCA alone from the isotonic buffers. Isotonic TCA buffers of pH 2·0 and pH 3·0 were prepared and labelled with active TCA. The same assay procedure as above was used except that the assay was for radioactive carbon instead of tritium. In one set of experiments a dual analysis of the absorption from the stomach of both tetracycline-7-^{3}H and trichloroacetic acid-1-^{14}C was made. This was possible since the ratio of the beta energies for the nuclides ^{14}C and ^{3}H does not exceed four [the method and necessary equations can be found in the Packard Operation Manual (Gibbs, 1967) or Liquid Scintillation Counting (Nuclear Chicago Corporation, 1966)].

Surface tension measurements

Surface tensions of the drug solutions were determined with a du Nouy tensiometer at 30 $\pm$ 0·5°. Care was taken to keep the surfaces clean, and new solutions were prepared and the readings repeated four times.

RESULTS AND DISCUSSION

Tetracycline studies in which appearance of the drug in the physiological phosphate buffer surrounding the stomach containing chloride or other anionic buffers at various drug concentrations and pH's indicate that the drug is absorbed by a passive diffusional process (Table 1). The low absorption from chloride buffer (approximately 1%) in a 150 min test period and the passive diffusion process are in agreement with the observations of Pindell, Cull & others (1959). Using vascularly intact intestinal loops in dogs and assaying according to effluent blood from each segment, Pindell & others found absorption to be greater from the ileum and duodenum than from the stomach; however, only 3% of an administered dose of 334 mg was absorbed from the small intestine in an 80 min test period. The results of Table 1 show that the amount of tetracycline absorbed is directly dependent upon the amount in the stomach at any one time, suggesting a passive diffusional process; however, the role for the anion is not clearly determined.

Table 1. *Absorption of tetracycline in isotonic chloride buffers at pH* 2·0 *from the rat stomach*

Initial dose (mg)	Rate constant $\times 10^5$/min	Drug absorbed after 150 min (mg)	Drug absorbed %
0·80	7·21	0·0085	1·06
1·60	7·22	0·0165	1·03
2·40	6·74	0·0226	0·94
3·20	6·81	0·0293	0·91

To determine the effect of the anion on the absorption process, isotonic buffers containing only the anion under test were placed in the rat stomach, and the absorption of a constant dose of tetracycline from the various buffers determined. The use of buffers containing a single anion avoids any complex equilibria problem. The results of these experiments are seen in Table 2 and Fig. 1A, with the absorption data being treated as a first-order process. It can be seen that the anion exerts a definite influence on the amount of tetracycline absorbed, but does not show whether or not the anion is absorbed in association with the drug. To check whether or not the anion actually was involved in the absorption process, isotonic trichloroacetate buffers were made as before but were labelled with TCA-1-^{14}C, which enabled easy assay of the TCA transported. Experiments were made using two different pH's and two different solutions at each pH, one containing only TCA, the other TCA and tetracycline. Since TCA (pK_a 0·66) is probably absorbed as the undissociated acid, then significant quantities should be absorbed at pH's 2 and 3, with more being

Table 2. *Absorption of tetracycline* (1·6 *mg dose*) *in various pH* 2·0 *isotonic buffers from the rat stomach*

Buffer	% Drug absorbed (after 150 min)	Rate constant $\times 10^5$/min	Surface tension*
Hydrochloride	1·03	7·22	61·8
Trichloroacetate	1·39	9·83	56·0
Perfluoropropionate	2·10	14·43	52·9
Perfluorobutyrate	2·57	17·04	37·1
Nitrate	1·00	6·60	70·7

* Dyne cm^{-1} or mN m^{-1}.

absorbed at pH 2; this is confirmed by Fig. 1B. It can also be seen from this Figure that less TCA is absorbed in the presence of drug at the pH's involved. If tetracycline is being absorbed as an ion pair (in combination with an anion) then addition of it should have resulted in an increased amount of TCA absorption. Since there is no increase in TCA absorption (in fact, a slight decrease is seen), the conclusion may be made that tetracycline is not being absorbed in conjunction with trichloroacetate. The reason TCA was chosen as an appropriate anion for these investigations is that it is reported to have no physiological effect and it has an extended plasma half-life (Butler, 1948).

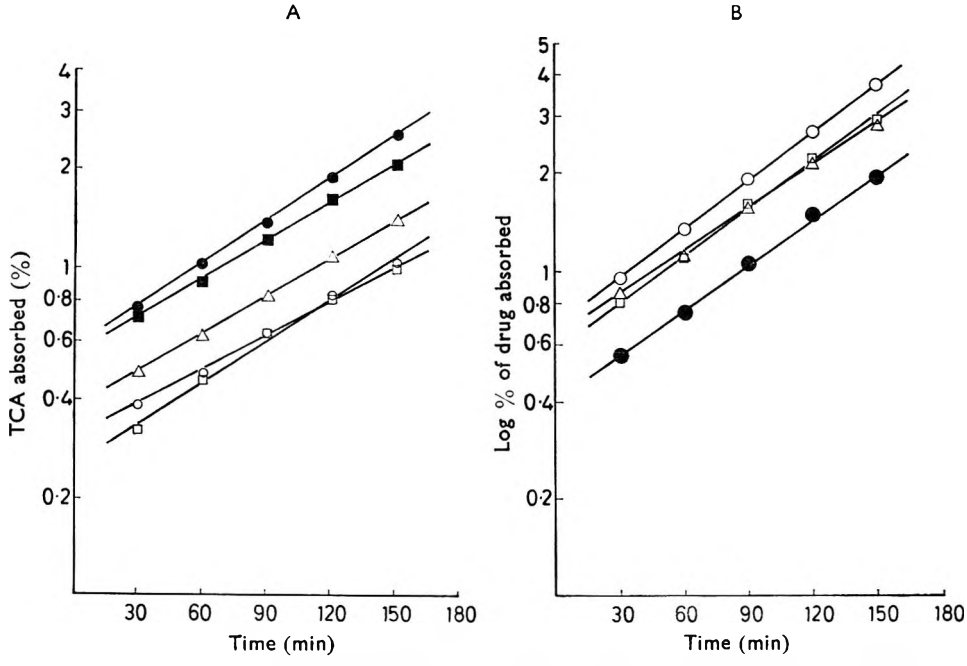


FIG. 1. A. Absorption of tetracycline (400 mg/litre) at pH 2·0 from various buffers in the rat stomach. ● Perfluorobutyrate buffer. ■ Perfluoroproprionate buffer. △ TCA buffer. ○ Chloride buffer. □ Nitrate buffer.

B. Absorption from the stomach of labelled trichloroacetate with and without tetracycline. ○ pH 2·0 no drug. △ pH 2·0 with drug. □ pH 3·0 no drug. ● pH 3·0 with drug.

These results suggest that the lipid solubility of the tetracycline-anion pair is not the dominant factor in the absorption process, but the data of Fig. 1A and Table 2 show the absorption to be anion dependent. Fiese & Perrin (1969) have shown that the absorption of protonated dextromethorphan was related to the surface activity of the various salts. To check whether or not these same factors are involved in the tetracycline absorption process, the surface tensions of all the buffers used (at a fixed tetracycline concentration of 400 mg/litre) were measured and are shown in Table 2. A plot of absorption rate against surface tension for the various anions is shown in Fig. 2, and, as was the case with dextromethorphan, a reasonable correlation between surface activity and absorption was found.

These results suggest that positively charged tetracycline is transported across the gut wall by combining with some non-specific anionic site on the membrane, the non-specific nature of the site being suggested by the apparent passive diffusional process involved.

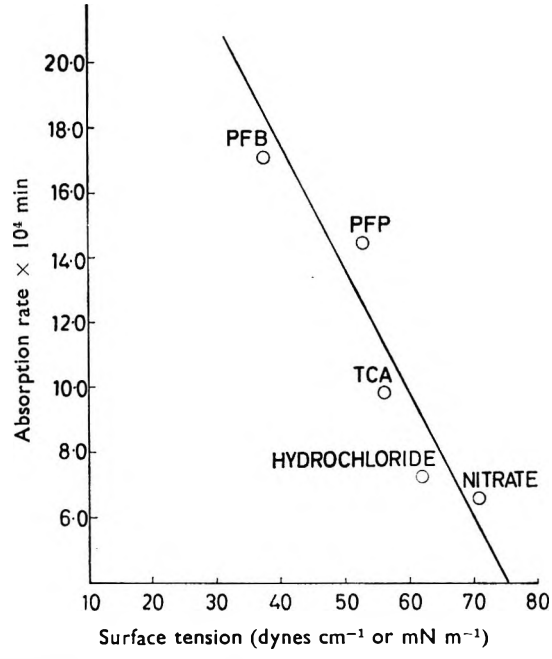


FIG. 2. Plot of surface tensions against absorption rate for various isotonic pH 2·0 buffers each containing 400 mg/litre of tetracycline base.

The measured surface activity of some of the salts indicates that their concentration is increased at the interface and therefore more drug is available for transfer at the absorption site. This is in agreement with the prediction of Ling (1964, 1965) that the absorption of a solute will be determined by its surface concentration and interaction at the membrane with fixed ionic and hydrogen bonding sites.

Preliminary investigations into the absorption of tetracycline from segments of the small intestine have shown that there is considerable absorption as the pH's are increased to 5·0 while keeping the anion constant. However, over the pH range from 2·0 to 5·0 the absorption from the small intestine is also anion dependent.

REFERENCES

BRAY, G. A. (1960). *Analyt. Biochem.*, **1**, 279–285.

BRODIE, B. B. (1964). In *Absorption and Distribution of Drugs*. Editor: Binns, T. B. Edinburgh: Livingston.

BUTLER, T. (1948). *J. Pharmac. exp. Ther.*, **92**, 49–58.

FIESE, G. & PERRIN, J. (1968). *J. Pharm. Pharmac.*, **20**, 98–101.

FIESE, G. & PERRIN, J. (1969). *J. pharm. Sci.*, **58**, 599–601.

GIBBS, J. A. (1967). In *Packard Operation Manual—Simultaneous Assay Procedure (for Two-Channel Spectrometers)*.

LEVINE, R. M. (1959). *Archs int. Pharmacodyn. Ther.*, **121**, 146–149.

LING, G. N. (1964). *Texas Rept. Biol. Med.*, **22**, 244–265.

LING, G. N. (1965). *Perspect. Biol. Med.*, **9**, 87–106.

Liquid Scintillation Counting (1966). Nuclear Chicago Corporation.

PINDELL, M. H., CULL, K. M., DORAN, K. M. & DICKISON, H. L. (1959). *J. Pharmac. exp. Ther.*, **125**, 287–294.

STEPHENS, C. R., MURAI, K., BRUNINGS, K. J. & WOODWARD, R. B. (1956). *J. Am. chem. Soc.*, **78**, 4155–4159.

J. Pharm. Pharmac., 1970, **22**, 763–766 *Received January* 9, 1970

The effects of drugs on barbiturate withdrawal convulsions in the rat

P. R. E. NORTON*

School of Pharmacy, Nottingham University, Nottingham, U.K.

The effects of drugs on barbitone withdrawal convulsions in rats have been examined. Morphine and mebanazine had no effect on audiogenically induced barbiturate withdrawal seizures. Alcohol, although suppressing the seizures, did not maintain drug dependence. Chlorpromazine prolonged the recovery period after the induction of the convulsions. Meprobamate, chlordiazepoxide and primidone substituted for the barbiturate and maintained drug dependence. 5-Hydroxytryptamine, when administered intraventricularly, tryptophan, α-methyl-*p*-tyrosine and ethosuximide reduced the severity of the withdrawal seizures. Reserpine and *p*-chlorophenylalanine greatly increased the severity of the seizures. Anxiolytic sedatives substituted for barbitone in dependent animals, other drugs studied affected barbiturate withdrawal convulsions in a way similar to other convulsive processes.

Crossland & Leonard (1963) have shown that rats can become physically dependent on barbiturates. The characteristics of the barbiturate withdrawal syndrome are similar to those in other animals and man (Turnbull, 1966). The withdrawal syndrome is characterized by a loss of body weight and a susceptibility to sound-induced convulsions; spontaneous convulsions are also sometimes seen. The effect of some centrally active drugs on the barbiturate withdrawal convulsions has been assessed in the hope that some indication could be obtained of the nature of the central nervous system changes induced by barbiturate dependence.

EXPERIMENTAL

Young adult female Wistar rats, not susceptible to audiogenic seizures, were used. Barbitone dependence was induced according to the method of Crossland & Leonard (1963) and Leonard (1967). In this method barbitone is dissolved in the drinking water in increasing concentration so that rats receive 400 mg/kg after 4 weeks. The taste of the barbitone is disguised with saccharin. The animals are dependent on barbitone after 5 weeks. Groups of five dependent rats were used for both experimental and control tests. Morphine and chlorpromazine were given subcutaneously, 5-hydroxytryptamine (5-HT) was given intraventricularly; the other drugs were administered by stomach tube as 2 ml of a suspension in a 5% mucilage of compound tragacanth powder B.P. Dosing was normally twice daily. Rats not receiving the drug served as controls and were always given the test vehicle. Miniature intraventricular cannulae were prepared (Norton, 1968) for the administration of 5-HT from 18–24 h after barbiturate withdrawal in a total dose of 140 μg, the last dose (40 μg) being given 15 min before the animals were tested for audiogenic seizures. In those experiments made to assess whether the drug would substitute for the

* Present address: B.D.H. Research Ltd., Godalming, Surrey, U.K.

barbiturate and maintain drug dependence, the drug was administered for about 5 days. In the other experiments the drugs were administered for 2 days.

At the time of barbiturate withdrawal and on each subsequent day the animals were weighed and placed in an experimental chamber and subjected to an auditory stimulus (bell intensity 100 dB for 1 min) to determine their susceptibility to seizures. Four types of convulsion were distinguished, in descending order of severity these were: an extensor tonic spasm in both front and hind legs, an extensor tonic spasm in the front legs only, a front leg flexor tonic spasm and generalized clonic convulsive activity. Spontaneous convulsive activity was recorded and was considered more severe than the convulsions induced by a bell. The significance of the drug effect on the convulsions was tested by ranking the convulsions in the order of severity. Convulsions within any one of these groups were then ranked according to the time taken to induce them.

RESULTS

The effects of drugs on the audiogenically induced barbiturate withdrawal convulsions are presented in Table 1.

Table 1. *The effect of drugs on the barbiturate withdrawal convulsion*

Drug	Daily dose mg/kg	Effect on withdrawal convulsions	Effect on ceasing drug administration
Morphine	20	0	
Meprobamate	2000	−−	++
Chlordiazepoxide ..	200	−−	++
Ethanol	see text	−−	
Chlorpromazine ..	10	0	
Reserpine	4	++	
Primidone	50	−−	++
Ethosuximide	400	−	
p-Chlorophenylalanine	300	+	
5-Hydroxytryptamine ..	see text	−	
Tryptophan	2000	−	
Mebanazine	10	0	
	20	0	
α-methyl-*p*-tyrosine ..	100	−	

− significantly less severe convulsion ($P < 0{\cdot}05$).
−− significantly less severe convulsion ($P < 0{\cdot}01$)
+ significantly more severe convulsion ($P < 0{\cdot}05$)
++ significantly more severe convulsion ($P < 0{\cdot}01$).

Morphine did not affect the barbiturate withdrawal syndrome but meprobamate and chlordiazepoxide prevented any withdrawal seizures occurring. When these drugs were withheld a syndrome developed identical to that of barbiturate withdrawal. The syndrome when chlordiazepoxide was withheld did not appear until 3 days later. In additional experiments, meprobamate (1 g/kg daily for 5 days) was found to cause dependence in rats that had received barbitone previously, but chlordiazepoxide (200 mg/kg daily for 5 days) did not. Similar meprobamate administration to previously untreated rats did not induce dependence. Primidone suppressed the barbiturate withdrawal seizures. After 6 days administration primidone was withheld and the animals became susceptible to audiogenic seizures. However this substitution appeared to be partial since the weight loss normally seen at barbiturate withdrawal occurred under primidone and the animals lost no weight

when primidone was no longer given. Ethanol suppressed the seizures but only at a dose that kept the animals markedly sedated (approximately 4 ml of a 75% solution daily). This continued sedation resulted in the death of most of the animals after 6 days. Chlorpromazine did not significantly affect the extent of the convulsions but recovery was slower and one animal died. Reserpine potentiated the withdrawal seizures, all animals having front and back leg extensor tonic convulsions, whereas none of the animals not receiving reserpine had convulsions of this severity, this effect was greater the longer the animals received the drug. *p*-Chlorophenylalanine increased the severity of the convulsions, most animals exhibiting extensor tonic spasm of both front and hind legs as opposed to the untreated animals showing only flexor tonic spasm of front legs. Five days after barbiturate withdrawal, tonic convulsions were seen in all animals which had received *p*-chlorophenylalanine whereas withdrawn animals not receiving the drug exhibited no convulsions at this time. Additional experiments were made with this drug and it was found that it did not make untreated animals susceptible to audiogenic seizures. In addition *p*-chlorophenylalanine had an anticonvulsant action on the seizures caused by a convulsant barbiturate (5-(1,3-dimethylbutyl)-5-ethyl barbituric acid (DMBEB), 10 mg/kg) when given 24 h previously.

Intraventricularly administered 5-HT, tryptophan, α-methyl-*p*-tyrosine and ethosuximide reduced the severity of the barbiturate withdrawal convulsions to a small extent. In all these groups some overlap was seen in the severity of the convulsions and separation of drugged and non-drugged groups ($P < 0{\cdot}05$) occurred due to the somewhat longer time taken to induce the convulsions in the drugged animals. The monoamine oxidase inhibitor mebanazine did not have any effect on the withdrawal seizures.

DISCUSSION

Cross dependence exists between barbiturate dependence and that induced by other depressant drugs (Weiss, 1964; Essig, 1966). However the present work has shown that the non-specific depressant drug ethanol, was not able to maintain drug dependence; this agrees with clinical observations (Fraser, Wikler & others, 1957). Depressants with a more specific action, meprobamate and chlordiazepoxide, substituted for the barbiturate and maintained drug dependence. Of the anticonvulsant drugs used, primidone suppressed the barbiturate withdrawal syndrome and also partially substituted for the drug. Ethosuximide reduced the severity of the seizures. Turnbull (1966) previously noted that diphenylhydantoin could suppress the barbiturate withdrawal seizures in the rat. These drug effects confirm the grand mal nature of the withdrawal seizures.

The neocortex plays very little part in the genesis of the barbiturate withdrawal seizure (Essig, 1962; Sharpless & Jaffe, 1966) and it has been concluded that the seizure arises subcortically (Essig & Flanary, 1961). A more specific effect than mere neuronal rebound hyperexcitability (Wikler, Fraser & others, 1955; Jaffe & Sharpless, 1965) is therefore likely and many general theories of drug dependence involving putative central synaptic transmitters have been proposed (Crossland, 1957; Collier, 1966). However Turnbull (1966) studied the whole brain concentrations of acetylcholine, γ-aminobutyric acid, noradrenaline, dopamine, 5-HT and histamine in barbiturate-dependent and withdrawn rats and concluded that his experiments provided no support for the hypothesis that barbiturate withdrawal convulsions in

the rat are caused by a dearrangement of any of the transmitter substances investigated.

Substances considered to have reasonably specific effects on the synaptic transmitters 5-HT and noradrenaline, such as reserpine, tryptophan (Paasonen & Giarman, 1958), *p*-chlorophenylalanine (Koe & Weissman, 1968) and α-methyl-*p*-tyrosine (Spector, Sjoerdsma & Udenfriend, 1965), affected the withdrawal convulsions. Particularly striking was the increased severity of the barbiturate withdrawal seizures when reserpine or *p*-chlorophenylalanine were administered; both substances cause a decrease in whole brain content of 5-HT. Reserpine potentiates other forms of convulsions (Jones & Roberts, 1968), and *p*-chlorophenylalanine potentiates electroshock convulsions (Koe & Weissman, 1968) and therefore specificity of effect of these substances on the withdrawal syndrome is unlikely. It appears that the convulsions arising from barbiturate withdrawal are affected by drugs in a way similar to other convulsive processes.

Acknowledgements

This work was supported by Educational Grants and the Redwood Scholarship of the Pharmaceutical Society of Great Britain.

The following companies are thanked for gifts of drugs: meprobamate (Wyeth), chlordiazepoxide (Roche), primidone and mebanazine (ICI), α-methyl-*p*-tyrosine (Merck, Sharp & Dohme), *p*-chlorophenylalanine (Pfizer), ethosuximide (Parke Davis), DMBEB (Lilly). This work was carried out as part of a thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy. The supervision of the work by Dr. J. Crossland and Dr. B. E. Leonard and operative help of Dr. J. Crossland is gratefully acknowledged.

REFERENCES

COLLIER, A. O. S. (1966). *Advances in Drug Research.* Editors: Harper, N. J. and Siminonds, A. B. **3**, 171–188. London: Academic Press.

CROSSLAND, J. (1957), cited by Wolff, M. H. (1959). *Electroenceph. clin. Neurophysiol.*, **11**, suppl. 14.

CROSSLAND, J. & LEONARD, B. E. (1963). *Biochem. Pharmac.*, **12**, *Suppl.* 103.

ESSIG, C. F. & FLANARY, H. G. (1961). *Exp. Neurol.*, **3**, 149–159.

ESSIG, C. F. (1962). *Arch. Neurol.*, **7**, 471–475.

ESSIG, C. F. (1966). *J. Am. med. Ass.*, **196**, 714–717.

FRASER, H. F., WIKLER, A., ISBELL, H. & JOHNSON, N. K. (1957). *Q. J. Stud. Alcohol*, **18**, 541–551.

JAFFE, J. H. & SHARPLESS, S. K. (1965). *J. Pharmac. exp. Ther.*, **150**, 140–145.

JONES, B. J. & ROBERTS, D. J. (1968). *Br. J. Pharmac.*, **34**, 27–31.

KOE, B. K. & WEISSMAN, A. (1968). *Advances in Pharmacology.* Editors: Garattini, S. and Shore, P. A. **6B**, 29–47. New York: Academic Press.

LEONARD, B. E. (1967). *Int. J. Neuropharmac.*, **6**, 63–70.

NORTON, P. R. E. (1968). Ph.D. Thesis, Univ. of Nottingham.

PAASONEN, M. K. & GIARMAN, N. J. (1958). *Archs Int. Pharmacodyn. Thér.*, **114**, 189–200.

SHARPLESS, S. K. & JAFFE, J. H. (1966). *J. Pharmac. exp. Ther.*, **151**, 321–329.

SPECTOR, S., SJOERDSMA, H. & UDENFRIEND, S. (1965). *Ibid.*, **147**, 86–95.

TURNBULL, M. J. (1966). Ph.D. Thesis, Univ. of Nottingham.

WEISS, S. (1964), cited by Deneau, C. A. In *Pharmacological Techniques for Drug Evaluation*, 406–410. Editors: Nodine, J. H. and Siegler, P. E. Chicago: Year Book Med.

WIKLER, A., FRASER, H. I., ISBELL, A. & PRESCOR, F. T. (1955). *Electroenceph. clin. Neurophysiol.*, **7**, 1–13.

J. Pharm. Pharmac., 1970, 22, 767–773 *Received March* 18, 1970

The effect of magnesium ions and Tris buffer on the uptake of cetyl trimethyl ammonium bromide by *Escherichia coli*

W. G. SALT AND DAVID WISEMAN

School of Studies in Pharmacy, University of Bradford, Bradford 7, *Yorks, U.K.*

The uptake of CTAB by *E. coli* from a glucose-free mineral salts medium occurs in two distinct phases. Increasing the magnesium ion content of the suspending medium emphasizes the diphasic nature of the isotherm. The primary phase is extended and the height of the "saturation" plateau of the secondary phase, as a function of CTAB uptake, is reduced. The CTAB uptake isotherm for *E. coli* suspended in water is not obviously diphasic and the maximum amount of CTAB taken up by the cells at "saturation" is less than that in medium. The height of this plateau is influenced by Tris buffer, an increase in the Tris concentration increasing the maximum CTAB uptake. A method for the rapid screening of uptake isotherms based on turbidity changes is also discussed.

Isotherms for the uptake of CTAB by *E. coli* have been described by Salton (1951) and by Salt & Wiseman (1968). Salt & Wiseman showed that uptake occurs in two distinct phases and suggested that the first phase was predominantly a surface phenomenon, whilst the second phase resulted from penetration into the cells. These studies were of uptake of CTAB from a buffered medium containing large numbers of inorganic and organic ions. The results reported here describe the effects of changes in the concentration of magnesium ions and the buffer tris (hydroxymethyl)aminomethane on the shape of the uptake isotherm.

The use of changes in turbidity of cell suspensions treated with CTAB for the rapid screening of uptake isotherms is also discussed.

EXPERIMENTAL

NNN-Trimethyl[cetyl-1-^{14}C]ammonium bromide (^{14}C-CTAB) was obtained from the Radiochemical Centre, Amersham, Bucks, England and *NNN*-trimethylcetylammonium bromide (CTAB) was kindly prepared by J. E. Adderson using the method of Adderson & Taylor (1964). Tris (hydroxymethyl)aminomethane (Tris), was obtained from Mann Research Laboratories, New York, U.S.A.

The organism was *Escherichia coli* NCTC 1093; the culture and suspending media, conditions of cultivation and method of measuring uptake of ^{14}C-CTAB were, unless otherwise stated, as described by Salt & Wiseman (1968).

Turbidity changes. Equal volumes of suspensions of *E. coli* and solutions of CTAB in various media were mixed and maintained at 25° for 15 min. The absorbance of these mixtures was measured at 650 nm using a 1 cm path length in a Unicam SP500 series 2 spectrophotometer.

Magnesium release. CTAB-treated suspensions were prepared and maintained as above after which the cells were removed by centrifuging at 5000 rev/min for 10 min.

Samples of the supernatant fluids were assayed for magnesium content using a calibrated Unicam SP900 flame photometer correcting for surface tension effects by the addition of 0·1 ml of 1% CTAB solution to each sample before assay.

Dye solubilization. The solubilization of waxoline yellow IS by solutions of CTAB in either water of glucose free medium (GFM) was studied by equilibrating the solutions with an excess of solid dye for 24 h at 25°, centrifuging the mixtures at 5000 rev/min for 15 min to remove the undissolved dye, and assaying the solubilized waxoline yellow spectrophotometrically.

RESULTS

Cells of *E. coli* suspended in GFM of different magnesium ion concentrations exhibit different isotherms for the uptake of ^{14}C-CTAB (Fig. 1b). As the magnesium ion concentration of the suspending medium is increased, the maximum amount of CTAB taken up by the cells is reduced, by an amount that is a function of the magnesium ion concentration (Fig. 2). In addition to this decrease in the height of the saturation plateau, the equilibrium concentration of CTAB required to complete the primary phase of uptake increases, accentuating the diphasic nature of the isotherm. The second phase of uptake still commences, however, after the same amount of CTAB has been taken up by the cells.

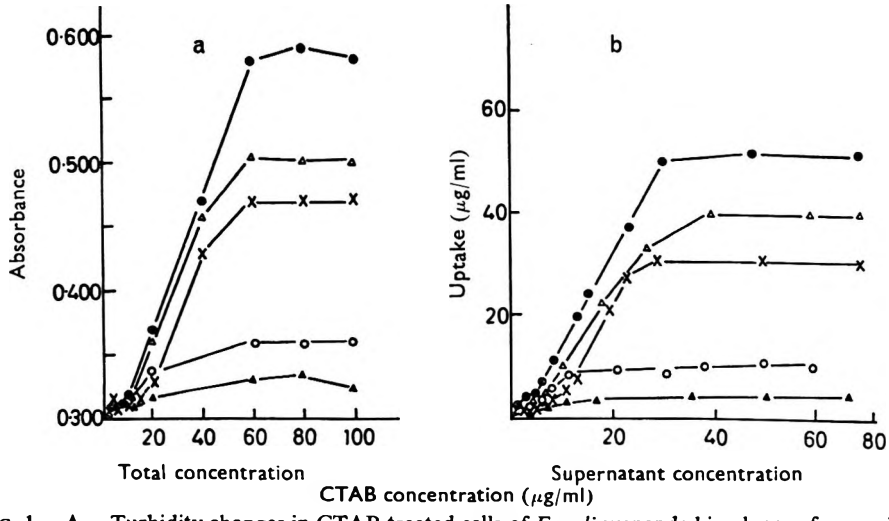


FIG. 1. A. Turbidity changes in CTAB-treated cells of *E. coli* suspended in glucose-free medium (pH 7·7) containing different concentrations of magnesium ions.

B. Uptake of ^{14}C-CTAB by *E. coli* suspended in glucose-free medium (pH 7·7) containing different concentrations of magnesium ions, plotted as a function of the ^{14}C-CTAB concentration in the supernatant fluid. Cell concentration 0·125 mg/ml, 3·2 × 10^8 cells/ml. Temperature 25°. Contact time 15 min. ●—● GFM (M/2000 Mg^{++}), △—△ M/100 Mg^{++}, ×—× M/50 Mg^{++}, ○—○ M/20 Mg^{++}, ▲—▲ M/10 Mg^{++} in GFM.

Cells treated with CTAB in water release magnesium into the suspending medium. Magnesium release occurs even at CTAB concentrations as low as 2 μg/ml and the extent of the release increases with increase in the CTAB concentrations.

The release of magnesium (in μg/ml) from CTAB-treated cells of *E. coli* suspended in water at a cell concentration 3·2 × 10^8 cells/ml (0·125 mg/ml) at 25°; contact time 15 min, is 0·01, 0·04, 0·06, 0·08, 0·10, 0·15 and 0·29 for CTAB concentrations of 1, 2, 3, 4, 6, 10, 20 μg/ml respectively.

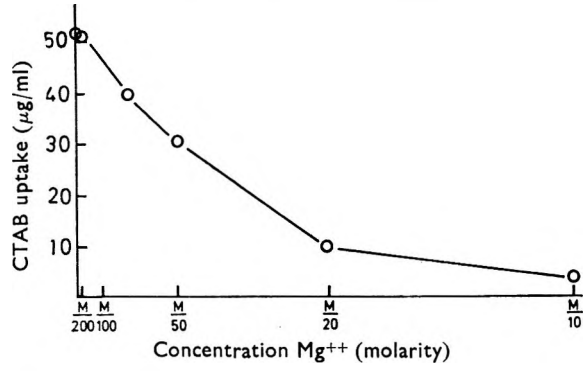


FIG. 2. The effect of increasing magnesium ion concentration on the maximum uptake of ^{14}C-CTAB by cells of *E. coli* suspended in glucose-free medium (pH 7·7). Cell concentration 0·125 mg/ml, 3·2 × 10^8 cells/ml. Temperature 25°. Contact time 15 min.

Fig. 1a shows the effect of different CTAB concentrations on the turbidity of suspensions of *E. coli* in GFM of various magnesium ion concentrations. The curves show two distinct regions. A first region where little change in absorbance occurs, as the CTAB concentration is increased, followed by a second region in which the absorbance increases approximately linearly with CTAB concentration, finally flattening into a plateau where little further change in turbidity occurs. The height of this plateau is decreased by increasing the magnesium ion concentration whilst the length of the first region, in terms of range of CTAB concentration, is increased. In all cases the concentration of CTAB equivalent to the beginning of the plateau in the turbidity curves approximates to the concentration just required to "saturate" the cells with CTAB. In addition, the threshold concentration of CTAB required to cause a marked increase in turbidity corresponds to the concentration at which the secondary phase of uptake commences.

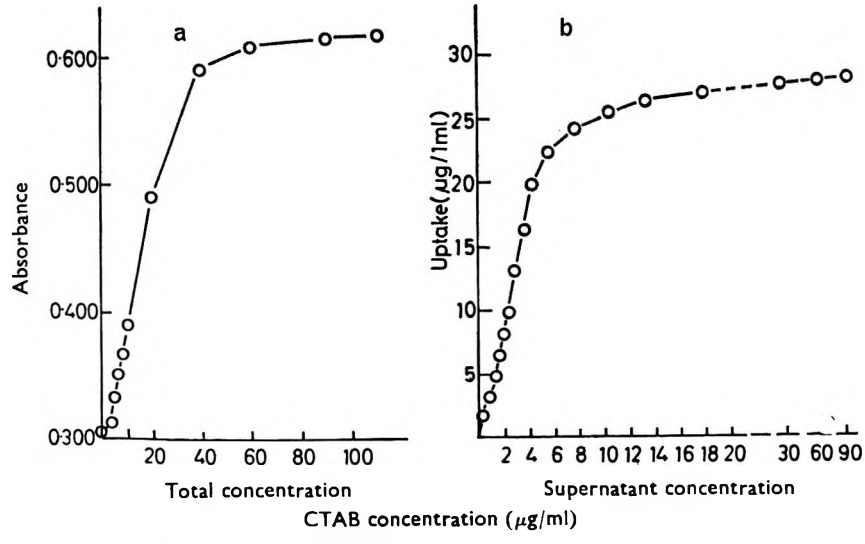


FIG. 3. A. Turbidity changes in CTAB-treated cells of *E. coli* suspended in water. B. Uptake of ^{14}C-CTAB by *E. coli* suspended in water plotted as a function of the ^{14}C-CTAB concentration in the supernatant fluid. Cell concentration 0·125 mg/ml, 3·2 × 10^8 cells/ml. Temperature 25°. Contact time 15 min.

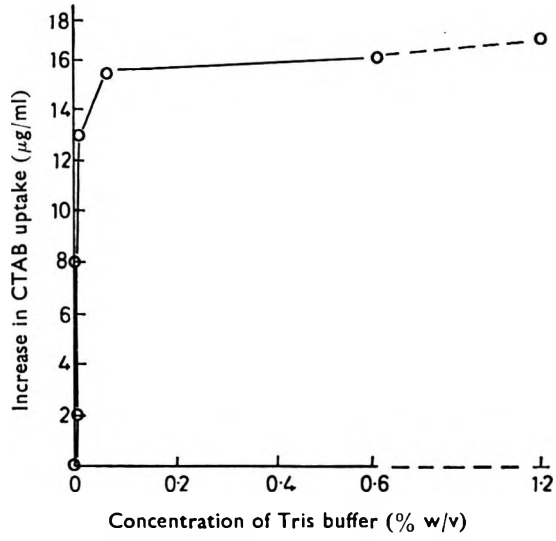


FIG. 4. The effect of Tris buffer concentration (pH 7·7) on the maximum uptake of ^{14}C-CTAB by *E. coli* suspended in water. Cell concentration 0·125 mg/ml, 3·2 × 10^8 cells/ml. Temperature 25°. Contact time 15 min.

Fig. 3a shows the changes in turbidity of suspensions of *E. coli* treated with different concentrations of CTAB in water. Although the curve is not obviously diphasic (cf. Fig. 1a) it is S shaped being initially convex to the CTAB concentration axis, becoming linear and finally flattening off in a plateau at a CTAB concentration of about 60 μg/ml. Fig. 3b is the uptake isotherm from water for CTAB by *E. coli* corresponding to the results shown in Fig. 3a. As in Fig. 3a the curve is not obviously diphasic although there is a change in slope at a CTAB uptake of about

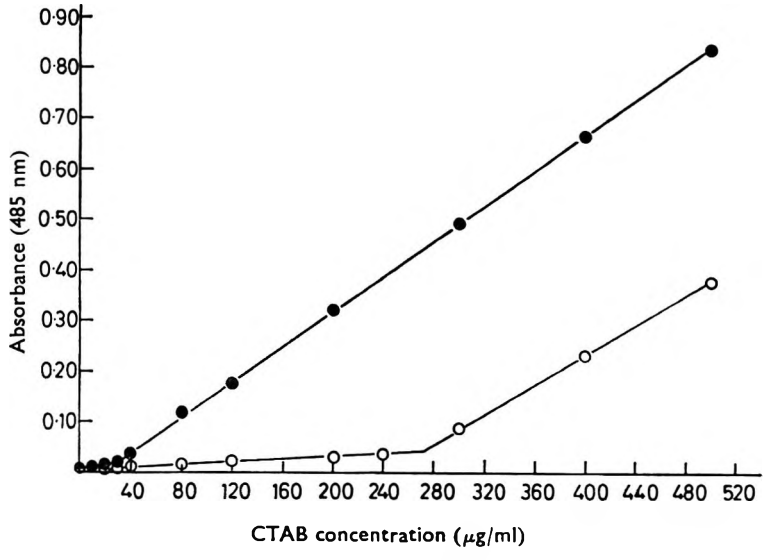


FIG. 5. Solubilization of waxoline yellow IS by CTAB at 25° in ●—● glucose free medium and ○—○ water.

3 μg/ml. Above this uptake the curve is approximately linear flattening into a plateau at an uptake of about 30 μg/ml from an original concentration of 60 μg/ml.

Cells of *E. coli* suspended in GFM have a greater saturation capacity for CTAB than cells suspended in water. Fig. 4 shows the effect of changes in the concentration of Tris (pH 7·7) in water on the height of the uptake saturation plateau. Increasing the concentration from zero to 0·06% w/v causes a 50% increase in the amount of CTAB taken up by the cells at "saturation" but further increases in Tris concentration has little or no effect on uptake. The Tris concentration in GFM is 1·2% w/v.

Fig. 5 shows the solubilization of waxoline yellow IS by solutions of CTAB in water and GFM. The results suggest critical micelle concentrations for CTAB of 280 μg/ml and 30 μg/ml in water and GFM respectively.

DISCUSSION

The diphasic uptake of CTAB by cells of *E. coli* was first reported by Salt & Wiseman (1968). They suggested that CTAB uptake occurred initially as a surface phenomenon, individual cells building up a critical amount of CTAB before the second phase of uptake, penetration into the cells, commences.

Cells suspended in a medium containing an excess of a magnesium salt have a high concentration of magnesium ions loosely bound at their surface (Strange & Shon, 1964; Tempest, Dicks & Meers, 1967). Our results suggest that the appearance and shape of the first phase of CTAB uptake by bacterial cells is due to competition with such ions for sites at the cell surface and that CTAB uptake is primarily an ion exchange process. This is analogous to the uptake of basic stains by bacteria reported by McCalla (1941) who studied the release of hydrogen ions and magnesium ions from bacterial cells treated with crystal violet and methylene blue. The release of magnesium from CTAB-treated cells further supports this analogy, increases in the magnesium content of the suspending medium being detected even in low CTAB concentrations (less than 4 μg/ml). Cations, such as magnesium ions, play a major role in the stabilization of cell membranes. and their removal and replacement by CTAB could well cause phase changes in the cell phospholipids, such as the change from one micellar form to another as suggested by Seufert (1965) who studied the effect of surfactants on spread lipid bilayers. The presence of excess magnesium ions would oppose such an exchange by simple competition.

The diphasic nature of the CTAB uptake isotherm is further emphasized in this paper, and the significance in growth and viability studies of the point of inflection, corresponding to the start of the second phase of uptake, has been demonstrated by Salt & Wiseman (1970).

Tris buffer has a marked effect on the uptake of CTAB by bacteria. The shape of the second region of the uptake isotherm, similar to the C type of Giles, MacEwan & others (1960), together with the increase in the height of the saturation plateau, suggests that Tris may be acting as an indirect carrier for CTAB, penetrating into the cell revealing more sites for uptake than are readily available in its absence. Tris has been shown to alter cellular permeability barriers (Voss, 1967; Mclean, Poland & others, 1967) and its ability to penetrate into intracellular membraned compartments has been reported (Humphreys & Garrard, 1969) tempting the suggestion that the increase in CTAB uptake in its presence is due to binding to the proteins and nucleic acids in normally inaccessible regions of the cell.

The critical micelle concentration (CMC) of CTAB in GFM is reduced to one tenth of its normal value in water (Fig. 5). The saturation plateaus reported for the GFM systems (Fig. 1b) may not therefore relate directly to cellular saturation but to competition between the micelles and the cells for the CTAB still in solution. This also suggests that CTAB uptake does not occur as the uptake of aggregates, since uptake might otherwise be expected to continue after the equilibrium concentration had exceeded the CMC. The decrease in the total amount of CTAB taken up by the cells as the magnesium ion concentration is increased, may be a reflection of this CMC effect on plateau height. Alternatively it may be explained on a competition basis, though whether the magnesium ions are competing with the Tris or the CTAB cannot be directly interpreted.

Turbidity changes of cell suspensions treated with quaternary ammonium compounds have been observed by other workers (Avi-Dor, Kuczynski & others, 1956; Gilby & Few, 1960; Hugo & Frier, 1969) but no real attempt has been made to put this phenomenon to use by relating it to the uptake isotherm of the antibacterial agent in question. Figs 1 and 3 show that the CTAB-induced turbidity changes in cells of *E. coli* are qualitatively similar to the CTAB uptake isotherms for that system and the effect of varying the ionic content of the suspending medium produces turbidity changes that can be related to the shape of the later determined isotherms.

It has been suggested that such changes in turbidity may result from shrinkage of the cells due to osmotic effects (Mager, Kuczysnki & others, 1956; Bernheim, 1963) or to changes in the reflective characteristics or refractive index of the cells due to the action of the quaternary salt (Hugo & Frier, 1969), though the latter two possibilities could be a consequence of the former. It can be shown that the large change in turbidity of the cells could be produced by a very small change, about 2%, in their refractive index. Such a small change could be expected in cells taking up large amounts of CTAB, as for example occurs during the second phase of CTAB uptake in GFM (Fig. 1b), especially if such uptake is taking place by penetration into the cells and by combination with their constituents.

Thus the effects of various ions on the uptake of CTAB by *E. coli* can be qualitatively predetermined. The technique is relatively quick, involving none of the centrifuging and sampling of the normal methods available for studying uptake and the effects of several different ions or concentrations of ions on CTAB uptake can be indicated in the time normally required for one experiment.

Acknowledgement

We would like 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.

REFERENCES

ADDERSON, J. E. & TAYLOR, H. (1964). 4th International Congress of Surface Activity, 613–620.

AVI-DOR, Y., KUCZYNSKI, M., SCHATZBERG, F. & MAGER, J. (1956). *J. gen. Microbiol.*, **14**, 76–83.

BERNHEIM, F. (1963). *Ibid.*, **30**, 53–58.

GILBY, A. R. & FEW, A. V. (1960). *Ibid.*, **23**, 19–26.

GILES, C. H., MACEWAN, T. H., NAKHWA, S. N. & SMITH, D. (1960). *J. chem. Soc.*, 3973–3993.

HUGO, W. B. & FRIER, M. (1969). *Appl. Microbiol.*, **17**, 118–127.

HUMPHREYS, T. E. & GARRARD, L. A. (1969). *Phytochemistry*, **8**, 1055–1064.

MAGER, J., KUCZYNSKI, M., SCHATSBERG, F. & AVI-DOR, Y. (1956). *J. gen. Microbiol.*, **14**, 69–75.

MCCALLA, T. M. (1941). *Stain Tech.*, **16**, 27–32.

MCLEAN, W. M., POLAND, D. M., COHON, M. S., PENZOTTE, S. C. & MATTOCK, A. M. (1967). *J. pharm. Sci.*, **56**, 1614–1626.

SALT, W. G. & WISEMAN, D. (1968). *J. Pharm. Pharmac.*, **20**, *Suppl.* 145S–175S.

SALT, W. G. & WISEMAN, D. (1970). *Ibid.*, **22**, 261–264.

SALTON, M. R. J. (1951). *J. gen. Microbiol.*, **5**, 391–404.

SEUFERT, W. D. (1965). *Nature, Lond.*, **207**, 174–176.

STRANGE, R. E. & SHON, M. (1964). *J. gen. Microbiol.*, **34**, 99–114.

TEMPEST, D. W., DICKS, J. W. & MEERS, J. C. (1967). *Ibid.*, **49**, 139–147.

VOSS, J. G. (1967). *Ibid.*, **48**, 391–400.

J. Pharm. Pharmac., 1970, **22**, 774–777 *Received April* 20, 1970

Thermodynamics of micellization of some non-ionic surfactants in mixed solvents

C. McDONALD

Department of Pharmacy, The University, Manchester, U.K.

The thermodynamics of micellization of three non-ionic surfactants in mixed solvents are reported. The contribution of the alkyl chains of the surfactant molecules to micellization is estimated from solubility data.

The thermodynamics of micellization are widely used in the examination of properties of surfactant solutions, and herein are presented such, approximate, parameters of three non-ionic surfactants in mixtures of water and formamide, water and *N*-methylformamide (NMF), water and dimethylformamide (DMF) and in water alone, where applicable. The surfactants used in the measurements were dodecyl tetraoxyethylene glycol monoether ($C_{12}E_4$), dodecyl hexaoxyethylene glycol monoether ($C_{12}E_6$) and dodecyl octaoxyethylene glycol monoether ($C_{12}E_8$). The synthesis and purification of these materials have been reported previously (McDonald, 1969).

RESULTS AND DISCUSSION

Thermodynamic parameters are calculated from the critical micelle concentrations (CMC) of the surfactants, in the various solvents, which have been reported previously (McDonald, 1967, 1969, 1970a). The hypothetical state for the monomers is chosen as unit mol fraction, with individual molecules behaving as at infinite dilution, and the final state is considered to be that of the micelle itself. It can be shown (Corkill, Goodman & Harrold, 1964; Molyneux, Rhodes & Swarbrick, 1965) that, provided the concentration of free surfactant molecules, i.e. the CMC, is low, the standard free energy, ΔG°_m, for the transfer of 1 mol of the surfactant from solvent to micelle can be represented by equation 1.

$$\Delta G^\circ_m = RT \ln \text{CMC (mol fraction)} \quad \ldots \quad (1)$$

It is assumed that the CMC values are small enough for equation 1 to be valid, although this may be only true, approximately, in solvents where the concentrations of amides are high. The partial enthalpy of micellization, ΔH°_m, may be calculated from the variation of CMC with temperature, equation 2. From the usual relation between free energy, enthalpy and entropy, the entropy of micellization, ΔS°_m, may be obtained. The thermodynamic parameters, at 25°, are shown in Table 1.

$$\Delta H^\circ_m = -RT^2 \frac{d \ln \text{CMC}}{dT} \quad \ldots \quad (2)$$

It should be noted from Table 1 that no thermodynamic parameters are available for $C_{12}E_4$ in water, since the cloud point of this material is 9° (McDonald, 1969). Table 1 shows that, on a mol fraction basis, formamide is least effective in decreasing the spontaneity of micelle formation, NMF and DMF have approximately equivalent effects at lower concentrations, and DMF may be most effective at higher concentrations. For all, but two, of the mixed systems studied, the negative free energy

values are accompanied by favourable enthalpy and entropy changes. From the available data, ΔH_m° values appear to tend towards zero as the concentrations of water increase. Indeed, for $C_{12}E_6$ and $C_{12}E_8$ in 0·33 mol fraction formamide, and, of course, in water, endothermic heat changes are observed for such compounds.

Table 1. *Thermodynamics of micellization at 25°C*

$C_{12}E_4$	Solvent % w/w	mol fraction	ΔG_m° kJ mol^{-1}	ΔH_m° kJ mol^{-1}	ΔS_m° Jmol^{-1} deg^{-1}
Formamide ..	100	1·0	—17·0	—2·3	+49
	90·0	0·78	—19·8	—1·3	+62
	*55·0	0·33	—26·1	—	—
NMF ..	50·0	0·24	—20·5	—10·7	+33
	40·0	0·17	—23·4	—9·0	+48
DMF ..	60·0	0·27	—19·5	—11·5	+27
$C_{12}E_6$					
Formamide ..	100	1·0	—16·6	—3·6	+43
	90·0	0·78	—19·7	—1·0	+63
	55·0	0·33	—25·2	+1·5	+90
	42·0	0·22	—26·3	—	
	25·0	0·12	—28·3	—	
NMF ..	76·0	0·49	—15·7	—	
	50·0	0·24	—20·8	—13·2	+25
	25·0	0·09	—27·0	—7·4	+66
DMF ..	75·0	0·42	—15·1	—	
	65·0	0·31	—18·0	—	
	60·0	0·26	—19·7	—11·5	+27
	44·0	0·16	—22·8	—	
	30·0	0·10	—26·1	—9·2	+56
†H_2O ..			—33·0	+16·3	+155
$C_{12}E_8$					
Formamide ..	100	1	—16·2	—3·1	+44
	90·0	0·78	—19·5	—1·2	+61
	55·0	0·33	—24·3	+2·1	+89
NMF ..	49·0	0·23	—20·5	—13·5	+23·4
DMF ..	60·0	0·27	—19·6	—13·0	+19·2
	45·0	0·17	—22·7	—	—
	32·0	0·10	—25·1	—	—
H_2O ..			—32·0	+18·6	+170

For aqueous solutions of non-ionic surfactants large positive enthalpies and entropies of micellization have been attributed to partial desolvation of the monomers, on transfer to the micelle (Corkill, Goodman & Tait, 1964). If this is assumed to be so, the addition of amides to water in some way decreases or modifies the solvation of surfactant monomers by water. The observed enthalpies may represent a balance between desolvation of the monomers on one hand and possible hydrogen bond effects due to solvent–solvent, solvent–solute and solute–solute interactions. Presumably, in water the desolvation effects predominate whereas, in most systems studied here, one or other of the latter possibilities predominates.

Similarly, the overall entropy effects may be composed of two opposing entities; a desolvating effect and an aggregation effect. In the present systems the former effect appears to predominate, although the numerical entropy values are all much lower than corresponding values in water. This suggests a decrease in importance of the desolvating effect. Another factor which could affect the thermodynamic parameters is the variation of aggregation numbers as the solvent constituents change, but nothing is known of this for the present systems.

It is possible to divide the free energy of micellization into two components, one being due to the hydrocarbon part of the surfactant molecule which favours micellization, ΔG_h, and the second being due to the glycol chain, ΔG_e, which might be expected to oppose micellization. An individual knowledge of ΔG_h and ΔG_e would enable a comparison to be made with ΔG_m^o. Although solubility measurements of the hydrocarbon component, in the relevant solvents, may give approximate values for ΔG_h, it is not usually possible to measure directly ΔG_e, which may only be calculated from the difference between ΔG_m^o and ΔG_h. The values for ΔG_h used here have been obtained from solubility measurements of dodecane in H_2O–amide mixtures (McDonald, 1970b). Since the transfer of hydrocarbon from solvent to the hydrocarbon interior in the micelle is being considered, the values of free energies of solution of dodecane in the various solvents are used with the signs changed. Since the processes involved are not exactly analogous the limitations involved in using such data must be remembered. Values of ΔG_h and ΔG_e are shown in Table 2.

Table 2. *Solvophobic and solvophilic contributions to thermodynamics of micellization at 25°C*

$C_{12}E_4$	Solvent % w/w	mol fraction	ΔG_h kJ mol^{-1}	ΔG_e kJ mol^{-1}
Formamide ..	100	1·0	—26·1	+9·1
	90·0	0·78	—28·0	+8·2
	*55·0	0·33	—33·9	+7·8
NMF	50·0	0·24	—29·7	+9·2
DMF	60·0	0·27	—27·9	+8·4
$C_{12}E_6$				
Formamide ..	100	1·0	—26·1	+9·5
	90·0	0·78	—28·0	+8·3
	55·0	·033	—33·9	+8·7
NMF	50·0	0·24	—29·7	+8·9
	25·0	0·12	—31·5	+4·5
DMF	60·0	0·26	—27·9	+8·2
	30·0	0·10	—31·0	+4·9
$C_{12}E_8$				
Formamide ..	100	1·0	—26·1	+9·9
	90·0	0·78	—28·0	+8·5
	55·0	0·33	—33·9	+9·6
NMF	50·0	0·24	—29·7	+9·2
DMF	60·0	0·26	—27·9	+8·4
	30·0	0·10	—31·0	+5·9

* 21°. † Taken from Corkill, Goodman & Harrold, 1964.

In all systems it may be seen that the hydrocarbon chain is the driving force for micellization. Free energies of micellization decrease or increase as do the free energies of transfer of hydrocarbon from solvent to micelle interior. In the H_2O–formamide systems it can be seen that for any concentration of formamide ΔG_e increases as does the length of the ethylene oxide chain, i.e. micellization is less spontaneous. An individual ethylene oxide unit has only a small effect in opposing micelle formation (about +0·2 kJ mol^{-1}). This is not surprising since each ethylene oxide unit consists of one oxygen atom which presumably opposes micellization and two methylene groups which would favour micellization. For the surfactants

considered here the hydroxyl group of the glycol chain appears to be the dominant factor in opposing micelle formation (about $+8$ kJ mol^{-1}).

The direct effects of the solvents on both the hydrocarbon and glycol chains of the surfactant molecules must also be considered in the micellization process. The equation of the negative value of the free energy of solution with the transfer of a hydrocarbon chain of a surfactant from solvent to micelle may be true, in water, where a complete desolvation of the hydrocarbon in passing to the micelle is envisaged. It may not be so in other solvents, where it is possible that the hydrocarbon chain may "carry" some of the solvent into the hydrophobic centre of the micelle (see later). Hence the calculation of ΔG_e from ΔG_h and ΔG_m° may also be in doubt.

The exact nature of the effects of additives on micellization in water cannot be explained solely from thermodynamic parameters. However, comparison of such parameters can give information on the effects of additives on the micellization process.

For the present systems NMF and DMF are more effective than formamide in altering the thermodynamics of micellization, although the changes in the parameters are quantitative rather than qualitative. In a study of the effects of additives on the micellization of ionic surfactants Emmerson & Holtzer (1967), divide the additives into those which penetrate the micelle and those which do not. Low concentrations of penetrating additives were less effective in increasing CMC's than similar concentrations of non-penetrating additives. At high additive concentrations, when micelles may be still expected in solutions containing non-penetrating additives, no micelles were present in solutions containing the penetrative type. Penetrative additives might be expected to be molecules which contain a certain hydrophobic content, although they may be miscible, or partially miscible with water. Confirmation of this may be shown from the effects of aliphatic alcohols on the micellization of a non-ionic surfactant (Hermann & Benjamin, 1967). As the hydrocarbon chain length of the alcohols increased, changes in thermodynamics of micellization were attributed to complex formation between the longer chain length alcohols and the micelles.

For the amide systems considered here CMC's increased regularly with increasing concentrations of amides (McDonald, 1969) and there were no obvious manifestations of complex formation between the amides and the micelles. It is unlikely however that micelles form in pure DMF. For example the CMC of $C_{12}E_6$ in such a solvent would be about 0·81 mol litre^{-1}, i.e. 36% w/v. The amides used would appear to be non-penetrative in character.

Acknowledgements

I should like to thank Professor K. Bullock for his interest in this work and Professor P. H. Elworthy for his helpful criticisms.

REFERENCES

Corkill, J. M., Goodman, J. F. & Harrold, S. P. (1964). *Trans. Faraday Soc.*, **60**, 202–207.
Corkill, J. M., Goodman, J. F. & Tate, J. R. (1964). *Ibid.*, **60**, 986–995.
Emmerson, M. F. & Holtzer, A. (1967). *J. phys. Chem. Ithaca*, **71**, 3320.
Hermann, K. W. & Benjamin, L. (1967). *J. Colloid Inter. Sci.*, **23**, 478–486.
McDonald, C. (1969). Ph.D. Thesis, University of Manchester.
McDonald, C. (1967). *J. Pharm. Pharmac.*, **19**, 411.
McDonald, C. (1970a). *Ibid.*, **22**, 148–150.
McDonald, C. (1970b). *Ibid.*, **22**, 10–14.
Molyneux, P., Rhodes, C. T. & Swarbrick, J. (1965). *Trans. Faraday Soc.*, **61**, 1043–1052.

J. Pharm. Pharmac., 1970, **22**, 778–780 *Received April* 27, 1970

A new dry extract of cascara (*Rhamnus purshiana* D.C. bark)

J. W. FAIRBAIRN AND S. SIMIC

*Department of Pharmacognosy, The School of Pharmacy, University of London, Brunswick Square, London, W.C.*1, *U.K.*

The B.P. method of preparing a dry extract of cascara leads to significant breakdown of *O*-glycoside links in the active compounds. This is shown to be due to enzyme activity during percolation. A dry extract, containing most of the active glycosides originally present in the bark with no change in glycosylation status, can be prepared using boiling water as extracting solvent. A modification of the chemical assay process is recommended as well as the use of an official standardized extract with *upper* and lower limits for glycoside content.

Analysis of samples of cascara bark and of dry extract of cascara B.P. (Fairbairn & Simic, 1964) showed that the extracts contained only about half of the theoretical amount of anthraquinone glycosides and that there had been significant breakdown of *O*-glycosides. These conclusions were confirmed (*Analyst*, 1968). As Fairbairn & Simic found ethanol (70%) to be a good extracting solvent, an extract using this solvent has been made and compared with one using the cold water extraction method of the B.P. 1968 and one made with boiling water because enzyme action during extraction proved to be influencing the yield of the constituents.

EXPERIMENTAL AND RESULTS

Powdered bark (100 g) was percolated with 70% ethanol to give 500 ml of percolate which was evaporated to dryness in a vacuum. A further 100 g of the same bark sample was percolated with water to exhaustion (1310 ml percolate) and evaporated to dryness (B.P. 1968). The original bark, the percolates and the final extracts were analysed (Fairbairn & Simic, 1964) and the results recorded in Table 1. Unexpectedly, the main destruction of the *O*-glycoside link (conversion of cascarosides to aloins as well as loss of *O*-glycosides) was found to occur during percolation, possibly because glucosidases of the bark pass into the percolate and bring about hydrolysis during the 2 to 3 day process.

Enzymatic studies

Ten g of the same bark sample was percolated with cold water (100 ml) and to the percolate increasing quantities of ammonium sulphate were added and dissolved. The precipitate formed after each addition was centrifuged, re-dissolved in water and dialysed. The dialysed solution was extracted with ethyl acetate to remove traces of anthraquinone compounds and the glucosidase activity assessed by determining the hydrolytic effect on pure cascarosides. The precipitate formed at 30% saturation with ammonium sulphate was the most active and a solution of it was prepared and 0·5 ml quantities were mixed separately with known quantities of cascaroside A and cascaroside B and allowed to stand at room temperature (20°) and pH about 5. Samples were taken at intervals, diluted with water and extracted

Table 1. *Comparative analyses of dried extracts from the same sample of Bark* (100 g), *made by the B.P.* 1968 *process, percolation with* 70% *ethanol and boiling water.* Glycosides calculated as cascaroside A; each figure is the mean of two assays

Bark	Cascarosides	Aloins	*O*-glycosides: Ethyl acetate insoluble	*O*-glycosides: Ethyl acetate soluble	Total glycosides	Free compounds (as emodin)
100 g	4·46	1·86	0·43	0·99	7·74	0·83
B.P. Method						
Percolate 1310 ml	2·24	3·39	0·17	0·21	6·01	0·23
Dry extract 28·5 g	2·16	3·09	0·14	0·17	5·56*	0·22
70% *Ethanol*						
Percolate 500 ml	3·56	2·96	0·28	0·56	7·36	1·07
Dry extract 31·2 g	3·40	2·91	0·30	0·48	7·09†	1·07
Boiling water						
Decoction 954 ml	5·08	1·21	0·35	1·62	8·26	0·19
Dry extract 30·8 g	4·80	1·30	0·27	1·80	8·17‡	0·17
Assay of bark .. (new method)	4·98	1·55	0·33	1·79	8·65	0·20

* Equivalent to 19·5%, † 22·7%, ‡ 26·5% of the dry extract.

with ethyl acetate to remove barbaloin and other hydrolytic products of the cascarosides. The amount of unchanged cascaroside was determined from the absorbance at 325–327 nm, a diluted solution of the enzyme being used as a blank and boiled enzyme solution, similarly treated, as a control.

The results in Table 2 show that there was significant hydrolysis of cascaroside B compared with the control but little hydrolysis of cascaroside A. This specificity was checked by repeated experiments and also by TLC examination of the enzyme treated glycosides on Silica gel G plates using methyl ethyl ketone–ethyl acetate–methanol–water (10:5:2:2). As a further control, aqueous solutions of cascarosides A and B at pH about 5 were stored at room temperature but no significant hydrolysis took place after 3 days. Similarly, treatment with boiling water for 15 to 30 min produced little hydrolysis, indicating that the glycosides are not so thermolabile as previously thought (*Analyst*, 1968).

Table 2. *Effect of an enzyme preparation and of other conditions on the stability of the cascarosides.* (t_0 = at zero time)

Conditions	Cascaroside A: Amount	Cascaroside A: Loss	Cascaroside B: Amount	Cascaroside B: Loss
Enzyme t_0	3·14 mg		2·93 mg	
Enzyme t_0 + 3 days	2·93 mg	6·7%	2·32 mg	20·8%
Enzyme boiled t_0 + 3 days	3·20	0%	2·74	6·5%
Water alone, t_0 + 3 days	—	—	2·82	3·7%
Boiling water, 15 min, then cooled ..	—	—	2·93	0·0%

Boiling water extract

As boiling water destroyed enzyme activity and had little effect on the cascarosides, we prepared an extract by adding to 100 ml of boiling water 10 g of the same sample of bark slowly with stirring. After being boiled for a further 5 min, the decoction was cooled, made up to 100 ml, filtered and a suitable aliquot evaporated to dryness

in vacuo and then analysed. From the weight of the decoction and the weight per ml of the filtrate the water-insoluble fraction of the bark was estimated to be 4·6 ml of the 100 ml of decoction and this was used to correct the analysis results in Table 1 which have also been adjusted to give figures for 100 g of bark for comparison with other figures in the Table. Much less breakdown of *O*-glycosides occurred than with the other extracts and the overall yield of glycosides is practically 100%.

Modified assay procedure

As the results in Table 1 also show that the boiling water extract contains more glycosides than the original bark assayed using the 70% ethanol extract, the sample of bark was re-assayed by the previous *Analyst* (1968) method modified by adding 1 g of powdered bark to 100 ml of boiling water and boiling for 5 min, cooling and making up to 100 ml, 10 ml of the filtrate being used for assay. The results are recorded in Table 1.

DISCUSSION

A potent and standardized dry extract of cascara

Cold percolation with water, or 70% ethanol, extracts sufficient enzymes from the bark to produce significant destruction of the *O*-glycoside link during percolation. This seems mainly responsible for the diminished yield of total glycosides and the conversion of cascarosides to aloins in official dry extracts. Boiling water prevents these losses and changes and a potent extract containing nearly all the anthraquinone glycosides originally present in the bark, with only slight changes in their glycosylation status, has been prepared. The U.S.P. XIV (1950) used boiling water for the preparation of cascara sagrada extract and the B.P. 1968 uses boiling water for the preparation of cascara elixir but not for the dry or liquid extract.

Published figures (Fairbairn & Simic, 1964; *Analyst*, 1968) show that the glycoside content of 10 samples of bark varied from 8 to 10·3%; the amount of boiling water extractive would also be expected to vary significantly so that the percentage glycosides in the final extract would also vary widely, therefore we suggest that official extracts should be standardized chemically with both *upper* and lower limits of glycosides, together with a minimum proportion of cascarosides.

Modification of the chemical assay process

The use of boiling water gives a slightly higher yield of glycosides in the assay process and is quicker and more convenient and we therefore recommend its adoption in the pharmacopoeial monograph.

The "shortened" assay process recommended in the *Analyst* Report (1968) estimates only the "cascarosides" (water soluble glycosides) and the "aloins" (ethyl acetate soluble glycosides) but the figures in Table 1 are based on the "long" assay process (Fairbairn & Simic, 1964). However, as they are all calculated as cascaroside A, they can be readily converted into "cascarosides" (water soluble glycosides) by adding together the figures in column 2 and 4, and into "aloins" (ethyl acetate soluble glycosides) by adding together the figures in columns 3 and 5.

REFERENCES

FAIRBAIRN, J. W. & SIMIC, S. S. (1964). *J. Pharm. Pharmac.*, **16**, 450–454.
Analyst (1968). **93**, 749–755.

J. Pharm. Pharmac., 1970, 22, 781

LETTERS TO THE EDITOR

The frog as a subject for screening thymoleptic drugs

Lapin, Osipova & others (1968) observed in frogs pretreated with the monoamine-oxidase inhibitor phenelzine that the tricyclic antidepressants imipramine and desipramine enhanced reserpine or 5-hydroxytryptophan effects–loss of the righting reflex (sedative action) and the appearance of the typical twitches of the extremities. The 5-hydroxytryptamine (5-HT) antagonist bromolysergide (BOL-148) prevented this potentiating effect. Oxenkrug, Osipova & Uskova (1970) reported that potentiation of reserpine effects by desipramine occurs only when the concentration of brain 5-HT was 3 or more times greater than that in the control. The concentration of adrenaline was unaffected. It was suggested that enhancement of reserpine effects in the frog reflects the central 5-HT potentiation of desipramine (or another agent with the similar action in this test).

This phenomenon was then used to examine the mechanism of action of the tricyclic antidepressants and agents with similar pharmacological action and chemical structure: neuroleptics, anticholinergic drugs and stimulants. All drugs, as aqueous solutions, were injected into thigh or mandibular lymph sacks. Frogs (*Rana temporaria*) were treated with phenelzine (25 mg/kg), 1·5 h later with desipramine (or test compound), and after another 30 min with reserpine (10 mg/kg). Four h after injection of reserpine the righting reflex was recorded, the number of successful attempts (from 10 trials) being counted. The Student *t*-test was used for statistical treatment of data. At the same time twitches of the extremities were tested as positive or negative, and the number of animals presenting twitches was recorded.

The antidepressants of the imipramine group: imipramine (5 and 10 mg/kg), desipramine (10 mg/kg), amitriptyline (10 mg/kg), chlorimipramine (10 mg/kg), prothiaden (10 mg/kg) and dibenzepin (10 mg/kg) enhanced the sedative action of reserpine. The twitches of extremities appeared in frogs treated with all these drugs except dibenzepin.

Chlorpromazine (10 mg/kg) and benactyzine (20 mg/kg) also enhanced the sedative effect of reserpine, but did not produce twitches. Chlorpromazine (5 mg/kg), promazine (10 mg/kg), haloperidol (2 mg/kg), trifluoperazine (2 mg/kg), atropine (20 mg/kg), amphetamine (5 and 10 mg/kg) and imidazol derivative AW-151129* (10 and 20 mg/kg) did not enhance the two effects of reserpine. AW-151129 appeared to be a compound with the pharmacological profile of tricyclic antidepressants, but it did not exert a thymoleptic effect in depressive patients (Stille, Lauener & others, 1968).

The data accord with the hypothesis that the thymoleptic effect is mediated through the activation of the central 5-HT processes (Carlsson, Corrodi & others, 1969; Lapin & Oxenkrug, 1969).

The phenomenon of potentiation of the reserpine effects in the frog may prove useful in the screening of thymoleptics.

* 5-(*p*-Chlorophenyl)-2,3,5,6-tetrahydro-imidazo (1,2-c) quinazoline.

Laboratory of Psychopharmacology,
Bekhterev Psychoneurological
Research Institute,
Leningrad, U.S.S.R.

I. P. LAPIN
G. F. OXENKRUG

The Department of Pharmacology,
Leningrad Pediatric Medical Institute,
Leningrad, U.S.S.R.

S. V. OSIPOVA
N. V. USKOVA

June 5, 1970

REFERENCES

CARLSSON, A., CORRODI, H., FUXE, K. HÖKFELT, T. (1969). *Europ. J. Pharmac.*, **5**, 357–366.

LAPIN, I. P., OSIPOVA, S. V., USKOVA, N. V. & STABROVSKI, E. M. (1968). *Archs int. pharmacodyn. Thér.*, **174**, 37–49.

LAPIN, I. P. & OXENKRUG, G. F. (1969). *Lancet*, **1**, 132–136.

OXENKRUG, G. F., OSIPOVA, S. V. & USKOVA, N. V. (1970). In *Serotoninergic Processes in Action of Psychotropic Drugs.* Editor: Lapin, I. P., pp. 45–57. Leningrad.

STILLE, G., LAUENER, H., EICHENBERGER, E., MATUSSEK, N. & PÖLDINGER, W. (1968). *Pharmakopsychiatrie neuropsychopharmakologie*, **2**, 123–135.

Molecular complexation of morphine and indol-3-yl sulphuric acid in the dog

During the course of investigation on morphine conjugates in the dog (Misra, Yeh & Woods, 1970) it was found that the methanolic eluate from Amberlite XAD-2 resin column on which urinary metabolites of morphine-*N*-methyl[^{14}C] had been adsorbed, on descending paper chromatography with the solvent systems n-butanol–acetic acid–water (I, 4:1:5 v/v organic phase; II, 35:3:10 v/v; III 100:4:24 v/v) consistently showed the presence of another iodoplatinate-positive radioactive spot having an Rf higher than that of free morphine and morphine conjugates. The Rf values of free morphine and this unknown radioactive spot in systems I-III were 0·53, 0·63; 0·43, 0·52; 0·38, 0·55 respectively. Paper and thin-layer chromatography using n-butanol-mineral acid or ammonia systems however showed the morphine spot only. Washing the methanolic residue of unknown product from Whatman 3 MM paper chromatograms with 4% K_2HPO_4 solution, saturated bicarbonate or ammonia solutions, and subsequent extraction with ethylene dichloride containing 30% n-amyl alcohol, gave an extract which showed the presence of morphine only. Similarly autoclaving the residue of unknown product with 2·4 N hydrochloric acid at 15 p.s.i. for 1 h, basification to pH 9, and solvent extraction showed a light indigo blue colour in the organic phase which on evaporation and paper chromatography showed the presence of morphine only.

A positive colour test for indol-3-yl sulphate with Ehrlich reagent (Decker, 1955; Rodnight, 1956) and morphine on acid or alkaline treatment suggested that the unknown radioactive product was a molecular complex of morphine and indol-3-yl sulphuric acid. Co-chromatography of the eluted unknown product with a synthetic morphine-indol-3-yl sulphuric acid complex prepared as described below substantiated this point. A single spot (Rf 0·52), positive to iodoplatinate and Ehrlich reagents, with a single peak of radioactivity coincidental to this spot was obtained using system III (Rf non-labelled morphine, 0·36). The complex isolated from Whatman 3 MM paper chromatograms with methanol and purified on neutral alumina column was a brownish hygroscopic powder of ill-defined melting point softening at 155–160° and melting at 175–178° (decomp.).

Molecular complexes of morphine, nalorphine, normorphine and tryptamine bases 1:1 with indol-3-yl potassium sulphate (indican) were prepared by the method of Boyland, Sims & Williams, 1956. Morphine complex: turns blue at 150°, m.p. 167–170° (decomp.), $C_{25}H_{26}N_2O_7S$, $\frac{1}{2}H_2O$, yield 55%. Nalorphine complex: m.p. 186–187° (decomp.), $C_{27}H_{28}N_2O_7S$, $\frac{1}{2}H_2O$, yield 80%. Normorphine complex: sinters 167°,

m.p. 173–175° (decomp.), $C_{24}H_{24}N_2O_7S$, yield 58%. Tryptamine complex: m.p. 160–161° (decomp.), $C_{18}H_{19}N_3SO_4$, yield 50%. Analyses for C, H and N were within the usual limits. The melting points of the morphine and normorphine complexes were ill-defined.

The 5-hydroxytryptamine complex, 5-hydroxytryptamine, indol-3-yl sulphate had Rf 0·55, 0·44, 0·45 respectively in system I.

Indol-3-yl potassium sulphate is a normal constituent of blood serum (Townsend, 1938) (26–85 μg %) and cerebrospinal fluid (Tinelli, 1945) (23·7 μg %) and its range of excretion (Bryan, 1965) in man on a normal balanced diet has been reported as 83 ± 36 mg/24 h. Its content and retention in blood and cerebrospinal fluid however markedly increased in various nervous pathological conditions (Mitolo, 1955) (67·5 μg %), morphine poisoning (Brocher, 1931), renal insufficiency (147–180 μg %) and intestinal obstruction (Haas, 1916). It was reportedly not destroyed or retained by human organs and excreted unchanged (Schlierbach, 1937) in man within 48 h of intravenous injection of a 100 mg dose. It was the major product of indole metabolism (King, Parke & Williams, 1966) in various animal species and it has been suggested (Posner, Mitoma & Udenfriend, 1961) that it is formed by liver microsomal hydroxylation of indole rather than by bacterial fermentation in the gut.

Synthetic morphine: indol-3-yl sulphuric acid complex (13·1 mg/kg) injected subcutaneously in male Sprague-Dawley rats showed analgesia comparable to morphine sulphate (10 mg/kg) by the hot plate technique. The demonstration of molecular complexation between morphine and indol-3-yl sulphuric acid does not necessarily imply that such a complex forms *in vivo*. However the possibility of such complexation as an artifact should not be overlooked in experiments on identification of narcotic analgesics (Fujimoto and Wang, 1970) in similar excretion studies.

This work was supported by U.S. Public Health Service Grant (NB-02928).

Department of Pharmacology,
College of Medicine,
University of Iowa,
Iowa City, Iowa 52240, *U.S.A.*

A. L. MISRA
L. A. WOODS

May 18, 1970

REFERENCES

BOYLAND, E., SIMS, P. & WILLIAMS, D. C. (1956). *Biochem. J.*, **62**, 546–550.
BROCHER, J. E. W. (1931). *Klin. Wschr.*, **10**, 294–297.
BRYAN, G. T. (1965). *Analyt. Biochem.*, **10** (1), 120–134.
DECKER, P. (1955). *Z. Physiol. Chem.*, **300**, 245–251.
FUJIMOTO, J. M. & WANG, R. I. H. (1970). *Toxic. appl. Pharmac.*, **16**, 186–193.
HAAS, G. (1916). *Dt. Arch. klin. Med.*, **119**, 177–198.
KING, L. J., PARKE, D. V. & WILLIAMS, R. T. (1966). *Biochem. J.*, **98**, 266–277.
MISRA, A. L., YEH, S. Y. & WOODS, L. A. (1970). *Biochem. Pharmac.*, **19**, 1536–1538.
MITOLO, M. (1955). *Boll. Soc. ital. biol. sper.*, **31**, 729–732.
POSNER, H. S., MITOMA, C. & UDENFRIEND, S. (1961). *Arch. Biochem. Biophys.*, **94**, 269–279.
RODNIGHT, R. (1956). *Biochem. J.*, **64**, 621–626.
SCHLIERBACH, P. (1937). *Dt. Arch. klin. Med.*, **180**, 439–449.
TINELLI, G. (1945). *Boll. Soc. ital. biol. sper.*, **20**. 171–173.
TOWNSEND, S. R. (1938). *J. Lab. clin. Med.*, **23**, 809–818.

Effect of a progestogen and an oestrogen on the γ-aminobutyric acid content in the cerebral hemispheres of ovariectomized rats

Ovariectomy increased the γ-aminobutyric acid (GABA) content in the cerebral hemispheres of young rats (Saad, 1970). I now report how a progestogen and an oestrogen affect the GABA content in the cerebral hemispheres of adult rats of 150 to 200 g. Of four groups each of 6 rats, one group received no treatment. Another group was ovariectomized and left for 30 days, two other groups were ovariectomized, left for 27 days, then given daily for 3 successive days either progesterone (5 mg/kg) or diethyldioxystilben dipropionate (Cyren B; Bayer, 0·5 mg/kg). The animals were then killed and the cerebral hemispheres of 2 rats were pooled and analysed for GABA (Saad, 1970).

The increase in GABA content in the cerebral hemispheres 30 days after ovariectomy of adult female rats amounts to 20% above the normal value. This appears to be the reverse of the effect of castration on GABA content (Tzu Yu Li & Chang Hua Wu, 1964). The intramuscular injection of progesterone, 5 mg/kg, for 3 successive days to the 27 days ovariectomized rats produced a significant decrease of 17·7% in their raised GABA content. The increased concentration of GABA of the 27 days ovariectomized rats returned to normal after the progesterone treatment.

The intramuscular injection of diethyldioxystilben dipropionate for 3 successive days to the 27 days ovariectomized rats did not produce any significant difference in their cerebral hemisphere GABA content.

Table 1. *GABA content in the cerebral hemispheres of* 30 *days ovariectomized adult female rats compared with the normal content, and the effect of* 3 *successive daily intramuscular injections of progesterone* (5 *mg/kg*) *and diethyldioxystilben dipropionate* (0·5 *mg/kg*) *on the* 27 *day ovariectomized rats*

	GABA content (mg/100 g wet tissue)			
		30 days after ovariectomy		
Sample data	Controls	Without treatment	Progesterone (3 days)	Oestrogen (3 days)
1	16·7	21·0	17·4	20·4
2	18·3	23·0	19·1	22·7
3	21·6	23·9	19·4	23·6
x	18·9	22·6*	18·6†	22·2
s.e.	1·45	0·9	0·6	1·0

* Significant increase ($P < 0·05$) compared with control.
† Significant decrease ($P < 0·0125$) compared with the GABA content after 30 days of ovariectomy.

The change in GABA content in the cerebral hemispheres induced by different drugs or treatments is of importance since GABA has a depressant action on both the superficial layers of the cortex (Iwama & Jasper, 1957) and on deeper structures (Rech & Domino, 1960; Krnjevic & Phillis, 1963).

Pharmacology Department,
Faculty of Pharmacy, Cairo University,
Cairo, Egypt.

SAMIR F. SAAD

May 5, 1970

REFERENCES

IWAMA, K. & JASPER, H. H. (1957). *J. Physiol., Lond.*, **138**, 365–380.
KRNJEVIC, K. & PHILLIS, J. W. (1963). *Ibid.*, **165**, 274–304.
RECH, R. H. & DOMINO, E. F. (1960). *J. Pharmac. exp. Ther.*, **130**, 59–67.
SAAD, S. F. (1970). *J. Pharm. Pharmac.*, **22**, 308.
TZU YU LI & CHANG HUA WU (1964). *Sheng Li Hsueh Pao*, **27**, 1–4.

Effects of the marihuana-homologue, pyrahexyl, on open field behaviour in the rat

Four concentrations of pyrahexyl (2·5, 5·0, 10·0 and 15·0 mg/kg) dissolved in olive oil, were administered intraperitoneally to four groups of Long Evans hooded rats 2–3 months old of either sex. There were two males and two females in each group. A fifth control group received only olive oil. The rats were observed $2\frac{1}{2}$ h after injection for 5 min in a 30 × $18\frac{1}{2}$ × 18 inch open field which had been marked off in squares. Four measures of behaviour were recorded: activity as defined by the number of lines a rat crossed with both hind feet; rearing, which was scored whenever the animal stood on its hind legs for any purpose other than grooming; grooming, defined as any response involving the smoothing of the fur or bring the forefeet to the face for the purpose of "washing"; and defaecation.

The evidence was examined by a one-way analysis of variance (Hays, 1965). A dose-dependent effect of pyrahexyl on activity ($F = 7{\cdot}01$, $df = 4{\cdot}15$, $P < 0{\cdot}01$) was found. At 10 mg/kg there was no noticeable difference in activity between the treated and untreated rats. However, at 2·5 and 5 mg/kg of pyrahexyl, activity was increased above the control values whereas at 15 mg/kg, activity was depressed.

Rearing was also affected significantly by pyrahexyl ($F = 26{\cdot}79$, $df = 4, 15$, $P < 0{\cdot}01$), a progressive inhibitory effect being seen as the dosage increased, 10–15 mg/kg of the drug inhibiting rearing by 50%.

Neither grooming nor defaecation was significantly affected.

The relation between dosage level and activity is complex; the change in activity response with dose demonstrates how different conclusions about the effects of pyrahexyl could be reached according to dosage. But as rearing was progressively suppressed as drug dosage was increased, various behavioural responses appear to be differentially affected by the drug.

Walters & Abel (1970) found that while pyrahexyl did not affect jumping behaviour itself, it did reduce the latency in the jumping response of gerbils to a stimulus which signalled the onset of shock. But, with rats, Abel & Schiff (1969) found pyrahexyl increased the amount of time spent in observing other animals. It appears then, that the affects of pyrahexyl are not only dose-dependent but are also response specific.

I would like to thank Dr. A. O. Gieszler of Abbott Laboratories, Chicago, U.S.A., for providing the pyrahexyl.

ERNEST L. ABEL

Department of Psychology,
University of Toronto,
Toronto, Ontario, Canada.

June 10, 1970

REFERENCES

ABEL, E. L. & SCHIFF, B. B. (1969). *Psychon. Sci.*, **16**, 38.
HAYS, W. L. (1965). *Statistics For Psychologists.* New York: Holt, Rinehart & Winston.
WALTERS, G. C. & ABEL, E. L. (1970). *J. Pharm. Pharmac.*, **22**, 311–312.

Cortical stimulation in rabbits by fenfluramine is probably due to an unusual metabolite

Having worked with fenfluramine in several species of laboratory animals (but not rabbits) for several years (Foxwell, Funderburk & Ward, 1969) we were surprised by the report of Mayer, Southgate & Wilson (1970) who found that rabbits showed an alert pattern in their cortical EEG tracings after the intravenous administration of equianorectic doses of (+)-amphetamine sulphate (2 mg/kg) or fenfluramine hydrochloride (8 mg/kg). In other experiments the EEG derived from the cortex was slowed by prior administration of pentobarbitone and then "both anorectic drugs showed a clear-cut alerting action."

We have examined the effects of fenfluramine at several levels of the neuraxis in cats with chronically implanted stainless steel electrodes. These results were compared with those obtained in identical experiments with (+)-amphetamine.

Unlike (+)-amphetamine, fenfluramine slowed cortical electrical waves, blocked cortical after-discharges and reduced the effects of stimulating the ascending activating system. Like (+)-amphetamine, fenfluramine reduced thalamic recruitment and increased the electrical activity in the ventromedial nucleus of the hypothalamus without modifying the activity of the lateral hypothalamus.

Similar results were obtained in recent experiments on cats designed after the methods of Mayer & others (1970). Cortical waves, slowed by pentobarbitone, were speeded with (+)-amphetamine and slowed further by fenfluramine. We have never observed an alerting response in the tracing of a cat after administration of fenfluramine. Large, lethal doses of fenfluramine do not produce convulsions in the cats; rather, the cortex becomes isoelectric just before the heart stops beating.

We have now repeated similar experiments in rabbits and found entirely different results. As reported by Mayer & others (1970), fenfluramine speeded the cortical waves slowed by pentobarbitone very much the same as did (+)-amphetamine.

Recent unpublished studies by Chandler, Dannenburg, Polan & Thompson offer a possible explanation for these divergent results. These observers studied the metabolites of fenfluramine in calves and found de-ethylated fenfluramine (norfenfluramine) to be a major metabolite. Furthermore, fenfluramine appeared to produce CNS stimulation in the calves. Norfenfluramine produces CNS stimulation in mice similar to that produced by (+)-amphetamine. If rabbits metabolize fenfluramine much as calves do, and accumulate norfenfluramine, this would account for the stimulant action of fenfluramine in rabbits.

It is premature, however, to translate this stimulant effect of fenfluramine in rabbits to man as proposed by Mayer & others because Bruce & Maynard (1968) have shown that norfenfluramine is not a major metabolite of fenfluramine in man.

A few cases of CNS stimulation by large overdoses of fenfluramine in man have been reported (see Mayer & others, 1970). It appears possible that this could result from the small amount of norfenfluramine (3%) that is formed from the parent drug or from abnormal metabolism. A report by Richards (1969) suggests that the latter possibility occurs.

Department of Pharmacology,
A. H. Robins Company,
1211 *Sherwood Avenue,*
Richmond, Virginia 23220,
U.S.A.

WILLIAM H. FUNDERBURK
JOHN W. WARD

May 19, 1970

REFERENCES

BRUCE, R. B. & MAYNARD, W. R., Jr. (1968). *J. pharm. Sci.*, **57**, 1173–1176.
FOXWELL, M. H., FUNDERBURK, W. H. & WARD, J. W. (1969). *J. Pharmac. exp. Ther.*, **165**, 60–70.
MAYER, S. R., SOUTHGATE, P. J. & WILSON, A. B. (1970). *J. Pharm. Pharmac.*, **22**, 239–240.
RICHARDS, A. J. (1969). *Lancet*, **2**, 1367.

Adrenergic neuron blocking action of dehydroemetine

In recent years, dehydroemetine appears to have replaced emetine in the treatment of amoebiasis. Dehydroemetine is less toxic than emetine, but like emetine, it produces a fall in blood pressure (see *Extra Pharmacopoeia* 1967). The hypotensive effect of emetine has been ascribed to the interference in the release of noradrenaline at the adrenergic nerve endings (Ng, 1966a; 1966b; Abraham, 1968). It is possible that a similar action may be shared by dehydroemetine.

To investigate the effect of dehydroemetine on adrenergic transmission, observations were made on Finkleman (1930) preparations of the rabbit ileum. Preparations were suspended in 50 ml of McEwen solution maintained at 35° and equilibrated with a gas mixture of 5% carbon dioxide in oxygen. The periarterial sympathetic nerves were stimulated through a bipolar electrode (Burn & Rand, 1960) with supramaximal shocks (10–20 V) of 0·5 ms at 20 to 50 Hz for 20 to 30 s. The movements of the preparations were recorded on a kymograph by an isotonic lever.

Segments of intestine removed from six rabbits all showed spontaneous pendular movements. They were inhibited by noradrenaline (0·05–0·1 μg/ml) or by electrical stimulation of the perivascular nerves. Hexamethonium (50–100 μg/ml) did not abolish the inhibitory response produced by nerve stimulation. It was therefore concluded that the electrical stimuli were applied to post-ganglionic adrenergic nerves. Addition of dehydroemetine dihydrochloride (2–10 μg/ml) to the organ bath did not affect the spontaneous activity of the rabbit ileum. On the other hand, dehydroemetine initially reduced and subsequently abolished the inhibitory effect of nerve stimulation. When the effect of nerve stimulation was abolished by dehydroemetine, added noradrenaline still inhibited the spontaneous movements of the rabbit ileum.

These results show that dehydroemetine has no blocking action on the direct effect of noradrenaline on the rabbit ileum. Blockade of the inhibitory effects of nerve stimulation suggests that the action of dehydroemetine is on the adrenergic nerve endings. This pharmacological property is therefore qualitatively similar to the adrenergic neuron blocking action of emetine (Ng, 1966a, 1966b; Abraham, 1968).

We wish to thank La Roche & Co. Ltd. for the gift of dehydroemetine dihydrochloride.

Department of Pharmacology,
University of Singapore,
Singapore, 3.

K. K. F. NG
Y. T. NG

May 16, 1970

REFERENCES

ABRAHAM, G. J. S. (1968). *Indian J. med. Res.*, **56**, 431–427.
BURN, J. H. & RAND, M. J. (1960). *J. Physiol., Lond.*, **150**, 295–305.
Extra Pharmacopoeia, (1967) 25th edn. p. 579, London: The Pharmaceutical Press.
FINKLEMAN, B. (1930). *Ibid.*, 70, 145–157.
NG, K. K. F. (1966a). *J. Pharm. Pharmac.*, **18**, 255–256.
NG, K. K. F. (1966b). *Br. J. Pharmac. Chemother.*, **28**, 228–237.

Modified technique for recording perfusion rate

Electronic devices to record the perfusion pressure changes are expensive. A simple modification of the mechanical method of Sollman & Hanzlik (1939) has been made. Ringer solution from a reservoir flows from a height of 30–50 cm through pressure tubing to the animal. The reservoir has a Marriot's tube (inner diameter 3 mm) connected through pressure tubing to a deep large Mary's tambour with a diaphragm that is not taut. The only "air-inlet" is a hypodermic needle (no. 26) in the pressure tubing. A light straw recording lever magnifies diaphragm movements 15–20 times. Drugs are injected in volumes of not more than 0·5 ml, into the inflow tubing. The principle of the method is based on recording the degree of relative negative pressure created in the air inlet system using a sensitive tambour. The degree of relative vacuum depends on the rate of perfusion—the more the perfusion, greater the vacuum. The recording lever in the resting position (zero inflow) is at "zero" line. As the perfusion starts, the lever shifts downwards till it is stabilized to give a horizontal base line corresponding to the initial inflow rate. Responses of constrictor drugs are recorded by an upward shift and those of dilator drugs are recorded by a downward shift of recording lever from the control line.

The kymographic record can be calibrated either at the beginning or at the end of the experiment. Use of a screw clip on the perfusion tube allows the free flow of fluid to be varied in steps. At each such step, the rate of flow and the rate of bubbles from the Marriot's tube are recorded along with the shift of the lever on the drum. The number of bubbles from the Marriot's tube can thus be converted to its equivalent in volume of fluid. A graphic plot of the vertical shift from the zero outflow line, of the lever on the drum against the actual rate of inflow has been found to have a linear relation. Therefore, the calibration of the inflow records becomes easy. The calibration scale will always remain the same provided the tambour, its lever (degree of magnification), the air inlet needle and the reservoir with Marriot's tube remain unaltered.

The selection of appropriate size of "air inlet" needle is critical. In blood vessel perfusion experiments on small animals (frogs, albino rats, guinea-pigs, rabbits) a number 26 size hypodermic needle was sensitive enough to record a change of 1·0 ml/min flow by a 4 mm vertical shift of the lever on the drum. For larger animals (dogs, cats) a needle of larger bore is required. The vertical flicker of the lever due to the escape of individual bubbles provides a 1·0–1·5 mm width of the record. Records of the effects of varying doses of a vasoconstrictor and a vasodilator drug on the perfused blood vessels of frogs are shown in Fig. 1.

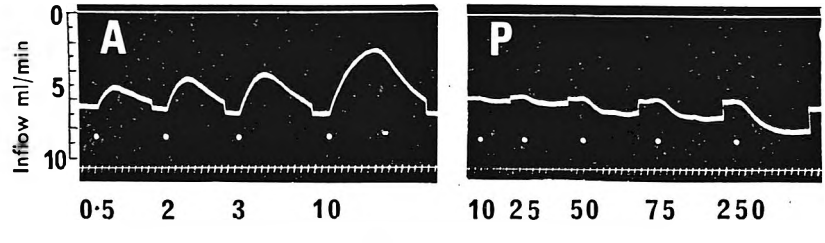


FIG. 1. Recordings of constrictor and dilator responses of perfused systemic blood vessels of a pithed frog. Each record shows from above downwards, the zero line, the perfusion pressure tracing and the time signals every 30 s. A. Constrictor responses of different doses of adrenaline (0·5, 2, 3 and 10 μg). P. Dilator responses of different doses of papaverine (10, 25, 50, 75 and 250 μg) on blood vessels, perfused with frog ringer containing barium chloride (100 μg/ml). A one and a half times increase in dose produces a notable increase in response.

Department of Pharmacology,
College of Medical Sciences,
Banaras Hindu University,
Varanasi-5 (U.P.),
India.

S. S. GAMBHIR
P. K. DAS

July 2, 1970

REFERENCES

SOLLMAN, T. H. & HANZLIK, P. J. (1939). In *Fundamentals of Experimental Pharmacology* 2nd Edition, pp. 159–160. San Francisco: J. W. Stacey, Inc.

Effects of desipramine and cocaine on sympathetic responses in the pithed rat

Desipramine blocks the uptake of noradrenaline into sympathetic nerve endings (Hertting, Axelrod & Whitby, 1961; Iversen, 1965), and this has been accepted as the explanation for enhancement by desipramine of responses to adrenergic nerve stimulation and to exogenous noradrenaline. However, *in vitro* experiments with large doses of desipramine have demonstrated depression of sympathetic responses attributed to α-receptor blockade (Bonaccorsi & Hrdina, 1967; Bassett, Cairncross & others, 1969; Scriabine, 1969). Bonaccorsi & Hrdina (1967) reported that desipramine did not reduce responses to intravenous injections of noradrenaline in pithed rats. We now report the effects of desipramine and cocaine on the pressor responses to both intravenous injections of noradrenaline and sympathetic nerve stimulation in the pithed rat preparation described by Gillespie & Muir (1967). Desipramine was compared with cocaine since the latter drug does not have α-receptor blocking activity, but blocks uptake into sympathetic nerve endings (Muscholl, 1961).

The area of the pressor responses to sympathetic nerve stimulation and noradrenaline were measured by planimetry. The responses after desipramine or cocaine were calculated as a percentage of the mean control response. The results are illustrated in Fig. 1. Desipramine and cocaine in doses up to 2 mg/kg enhanced the pressor responses to sympathetic stimulation and intravenous noradrenaline. With higher doses the effects of the two drugs differed: cocaine continued to enhance the pressor responses whereas maximal enhancement was obtained with 2 mg/kg of desipramine and the enhancement was less with higher doses. The effect of both drugs on noradrenaline released from sympathetic nerve endings was far greater than the effects on injected noradrenaline. However, unlike Bonaccorsi & Hrdina (1967), some potentiation of injected noradrenaline by desipramine was demonstrated: the mean area of the pressor response to injected noradrenaline was 207 $\pm$ 47% of control with 2 mg/kg of desipramine. Unlike cocaine, the sharp cut off in the potentiating action of desipramine above 2 mg/kg can possibly be explained in terms of its dual action, namely: blockade of noradrenaline uptake demonstrated at low doses, and α-receptor blockade demonstrated at higher doses.

Desipramine antagonized calcium-induced contractions in a dose dependent manner (Bonaccorsi & Hrdina, 1967) so its blocking action in high doses could be due to a combination of α-receptor blockade and a membrane stabilizing effect.

The expenses of this research were defrayed by a grant from the National Health and Medical Research Council to Professor M. J. Rand.

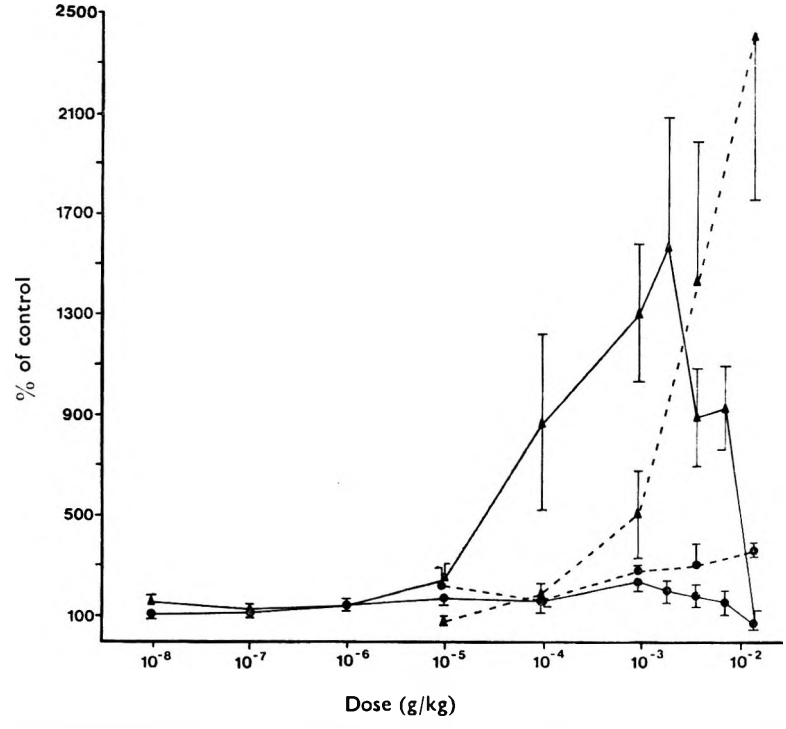


FIG. 1. Pithed rat preparation. The effect of various concentrations of desipramine and cocaine on the area of the pressor response to sympathetic nerve stimulation and to intravenous noradrenaline, expressed as a percentage of the area of the control responses: ●——●, desipramine and noradrenaline; ▲——▲, desipramine and sympathetic stimulation; ●- - -●, cocaine and noradrenaline; ▲- - -▲, cocaine and sympathetic stimulation. The vertical bars represent standard errors.

Department of Pharmacology,
University of Melbourne,
Parkville,
Victoria, 3052,
Australia.

A. B. GLOVER
M. W. MCCULLOCH

June 22, 1970

REFERENCES

BASSETT, J. R., CAIRNCROSS, K. D., HACKETT, N. B. & STORY, M. (1969). *Br. J. Pharmac.*, **37**, 69–78.

BONACCORSI, A. & HRDINA, P. (1967). *First International Symposium on Antidepressant Drugs* Editors: Garattini, S. and Dukes, M., Int. Cong. Series No. 122, pp. 149–157. Amsterdam: Excerpta Medica Foundation.

GILLESPIE, J. S. & MUIR, T. C. (1967). *Br. J. Pharmac. Chemother.*, **30**, 78–87.

HERTTING, G., AXELROD, J. & WHITBY, L. G. (1961). *J. Pharmac. exp. Ther.*, **134**, 146–153.

IVERSEN, L. L. (1965). *J. Pharm. Pharmac.*, **17**, 62–64.

MUSCHOLL, E. (1961). *Br. J. Pharmac. Chemother.*, **16**, 352–259.

SCRIABINE, A. (1969). *Experientia*, **25**, 164–165.

Faecal excretion of degraded and native carrageenan by the young rat

Hawkins & Yaphe (1965) have shown that when native, undegraded carrageenan is fed to young rats, at levels of 2 to 20% in the diet, it is 90–100% excreted in the faeces. No comparable results appear to be available for degraded carrageenan in the 16 000–19 000 number-average molecular weight range, and this report deals with the faecal excretion of both degraded and native iota-carrageenan by the young rat at a concentration of 5% in the diet. We have recently shown (Beattie, Blakemore & others, 1970) that when adult baboons were given a single large dose of 240 mg/kg of degraded carrageenan, which is more than three times the human dose in peptic ulcer treatment, no urinary metachromasia could be detected.

Carrageenans. The degraded carrageenan (code no. C16/L1927) was prepared from *Eucheuma spinosum* seaweed by Laboratoires Glaxo, Paris, and the native iota-carrageenan was a commercial product from Société Auby, Neuilly-sur-Seine, France. The analyses on the anhydrous basis are given in Table 1. The number-average molecular weight of C16 was in the 16 000 to 19 000 range (Blakemore & Dewar, 1970) and the weight-average molecular weight (by light scattering) was between 20 000 and 30 000 (Anderson & Scman, 1966). The molecular weight of the native carrageenan was unknown, but the inherent viscosity (η_{inh} in 0·1M NaCl solution) was about 18 times that of the degraded material.

Table 1. *Analyses of carrageenans*

Carrageenan	Inherent viscosity (η inh) (dl/g)	$[\alpha]_D$ in water	Total sulphate (SO_3 Na), %	Sulphated ash, %	3,6-Anhydro-galactose ($C_6H_8O_4$), %
C16 degraded	0·41	−46·8°	37·9	29·7	18·1
Native	7·43	40	34·8	30·4	21·9

Animal experiments. Fifteen young male albino rats (Charles River C.D. strain) about 115 g, were randomly allocated to three treatment groups: 5% native carrageenan in the diet, 5% degraded carrageenan in the diet, and control diet without carrageenan. Spillers Laboratory Small Animal Diet (SG1/41B) in powdered form was mixed with the powdered degraded or native carrageenan, and 10 g balls were prepared by mixing with a known quantity of water and then drying at less than 60° to remove the water.

The animals were caged singly in suspended metal cages with a wire mesh floor through which scattered food and excreta could fall on to absorbent material beneath. Scatter of food or of faecal pellets between cages was prevented by placing folded sheets of blotting paper beneath each cage. Two balls (20 g) of each diet were placed in the food hopper daily as the sole source of food and the animals had water freely. After each 24 h, food consumption was measured. The feeding experiment lasted ten days, and on the tenth day, the 24 h faecal samples from each animal were collected, dried in a vacuum over phosphoric oxide to constant weight, and analysed for 3,6-anhydro-galactose (Black, Blakemore & others, 1965). The carrageenan content was calculated from this result after correcting for a mean "3,6-anhydrogalactose" value for the control faeces. The validity of the method was confirmed for 2 animals by estimation of total ester sulphate (Black & others, 1965).

The food intakes (Table 2) for the first seven and last three days and the weight gains for each animal in the three groups, indicated that there were no meaningful differences between animals receiving native and degraded carrageenan, although the mean weight

gain of the control group was significantly higher than that of the test groups. The mean daily consumption of each rat over the last three days was taken as the mean food intake over the 24 h period of faecal collection. Again (Table 3), there were no significant differences in any parameters between animals receiving native carrageenan and those receiving degraded carrageenan. The presence of carrageenan in the diet was readily detected by the increased weight of faeces compared to that of the control group. The faecal excretions of both native and degraded carrageenans were less than 100%, and the low figures may be partly attributed to small errors in measuring the

Table 2. *Food intake and weight gain in groups of young rats fed native and degraded carrageenan in the diet*

				Over 10 day period on test		
Animal no.	Treatment group	Initial wt (g)	Final wt (g)	Weight gain (g)	Food intake (g/day) over 7 days	Food intake (g/day) over 3 days
1		118	177	59	17·7	18·7
3	5%	105	167	62	16·0	16·9
6	Native	110	178	68	16·3	19·3
13	carrageenan	107	157	50	14·5	14·9
15		117	180	63	16·9	19·5
Mean		111·4	171·8	60·4	16·3	17·9
± s.e.*		2·6	4·3	3·0	0·5	0·9
4		106	175	69	18·2	18·9
7	5%	114	174	60	18·3	19·1
10	Degraded	118	185	67	18·0	19·1
12	carrageenan	120	192	72	19·0	19·8
14		126	162	36	18·6	19·4
Mean		116·8	177·6	60·8	18·4	19·3
± s.e.*		3·3	5·1	6·5	0·2	0·2
2		112	196	84	16·9	19·3
5		112	190	78	15·5	16·8
8	Control	127	196	69	16·5	17·2
9		117	198	81	15·6	19·9
11		120	201	81	16·3	17·2
Mean		117·6	196·2	78·6	16·2	18·1
± s.e.*		2·8	1·8	2·6	0·3	0·6

* s.e. = standard error

Table 3. *Analyses of faeces and excretion of carrageenans over 24 h.*

Animal no.	Treatment group	Mean food intake (g/24 h)	Dry faeces (g)	Faeces, % of food intake	Carrageenan in faeces %	Carrageenan excretion %	Excretion range %	Mean excretion %
1		18·7	4·900	26·2	16·7	88		
3	5% native	16·8	4·631	27·6	15·5	85		
6	carrageenan	19·3	5·366	27·8	13·9	77	68–88	80
13	in diet	14·9	4·545	30·5	13·4	82		±3·5 (s.e.)
15		19·5	4·785	24·5	13·9	68		
4	5%	18·9	4·375	23·1	17·8	82		
7	degraded	19·0	4·513	23·8	18·4	88		
10	carrageenan	19·1	5·054	26·4	17·9	95	82–95	87
12	in diet	19·8	4·398	22·2	18·4	82		±2·4 (s.e.)
14		19·4	4·362	22·5	19·4	87		
2	Control:	19·3	3·145	16·3				
5	rat cake	16·8	2·672	15·9				
8	only	17·2	2·420	14·1				
9	in diet	19·9	4·183	21·0				
11		17·2	2·815	16·3				

daily food intake and to assessing accurately the "3,6-anhydrogalactose" figure for the control faeces blank (0·37% as $C_6H_8O_4$).

The above results show that, when native and partially degraded iota-carrageenans were fed to young rats, at a concentration of 5% in the diet, there were no significant differences in the faecal excretion of the two polymers and the weight gained by the animals.

This work was supported by Laboratoires Glaxo, Paris, and we wish to thank Dr. G. B. Shirlaw, Directeur Général, for his enthusiastic support. We are also indebted to Dr. W. Anderson, University of Strathclyde and Dr. R. E. Lister of this research centre for helpful discussions and advice.

The Biological and Chemical Sciences Divisions,
Inveresk Research International,
Inveresk Gate, Musselburgh,
Midlothian, U.K.

E. T. DEWAR
MORAG L. MADDY

May 28, 1970

REFERENCES

ANDERSON, W. & SOMAN, P. D. (1966). *J. Pharm. Pharmac.*, **18**, 825–827.

BEATTIE, I. A., BLAKEMORE, W. R., DEWAR, E. T. & WARWICK, M. H. (1970). *Fd. Cosmet. Tox.*, **8**. 257–266.

BLACK, W. A. P., BLAKEMORE, W. R., COLQUHOUN, J. A. & DEWAR, E. T., (1965). *J. Sci. Fd Agric.*, **16**, 573–585.

BLAKEMORE, W. R. & DEWAR, E. T., (1970). *Makromolek. Chem.*, **137**, 51-59.

HAWKINS, W. W. & YAPHE, W., (1965). *Can. J. Biochem.*, **43**, 479–484.

Correlation of urinary histamine excretion and 24 h urine volumes in rats and man

Urinary output of free histamine has frequently been used as an indicator of the level of whole body histamine metabolism (Johnston & Kahlson, 1967). The reliability of this measurement has been discussed in relation to the origin of urinary histamine and the very small fraction it represents of the total histamine metabolites (Lindell & Westling, 1966).

We report here a correlation of urinary histamine excretion and the 24 h urine volume in rats and man. This correlation has been the subject of frequent fruitless speculation, being considered unlikely by Anrep (1944) and Duner & Pernow (1956).

Female albino rats, either Wistar (SNR strain) or Sprague Dawley (Charles River strain), 150–250 g, were allowed free access to food and water, the diet being made by mixing Dixon's 41B meal with egg white, casting into sticks and baking. The rats were housed in glass metabolism cages ('Metabowl', Jencon's), and the 24 h urine output collected into 1 ml of 3N hydrochloric acid. Histamine was estimated by the fluorometric method of Oates, Marsh & Sjoerdsma (1962). Successive daily collections of 24 h urines were made. Rats No. 8–11 had 0·1N ammonium chloride solution instead of drinking water, and in addition, on days 4–6 of the experiment, rats 8–11 received 200 mg/kg of histidine hydrochloride subcutaneously.

In 9 of 11 rats there was a good correlation of histamine content and 24 h urine volume (Table 1).

Treatment with ammonium chloride gave a urine of low pH, which would be expected to facilitate the excretion of histamine, but the correlation of histamine excretion and urine volume was not disturbed. Treatment with L-histidine also did not affect the correlation.

Table 1. *Correlation of free histamine output and 24 h urine volumes in rats*

Rat No.	Strain and treatment	No. of samples analysed	Correlation coefficient †	Slope	Intercept	*P* value
1	Wistar	15	0·2	0·323	16·6922	n.s.
2	no treatment	15	0·87	1·6837	−2·8439	<0·001
3		16	0·31	0·3116	14·8033	n.s.
4	Sprague Dawley	9	0·97	2·0271	−7·2074	<0·001
5		7	0·91	1·5978	−3·4863	<0·01
6	no treatment	7	0·71	0·7953	5·8839	<0·1
7		8	0·72	1·0156	0·3683	<0·05
8	Sprague Dawley	6	0·93	1·7846	−2·767	<0·01
9	Ammonium	6	0·72	0·6819	11·0849	<0·1
10	chloride	6	0·73	2·5705	−26·1378	<0·1
11	plus histidine HCl*	6	0·88	1·4127	4·9158	<0·05

† Bravais-Pearson coefficient of linear correlation (γ).
* 0·1N NH_4Cl substituted for drinking water during experiment.
On days 4–6 inclusive these animals received histidine hydrochloride 200 mg/kg, s.c. daily.

In addition we found a good correlation of histamine content and 24 h urine volume (correlation coefficient = 0·70 $P = 0·05$) in a female patient hospitalized with suspected mastocytosis (see Fig. 1).

There are large differences in the slopes for individual histamine content versus urine volume relations (Table 1) and for this reason it would be hard to detect the relation by pooling data from individuals.

If the data we have obtained in rats prove to be similar in man when more subjects have been examined, then similar reasoning would explain the lack of correlation when data from individuals are pooled.

Speculation upon the mode of excretion of histamine in rats and man may be premature using such circumstantial evidence, but filtration and passive tubular reabsorption fit the data fairly well. Some support for this suggestion may be obtained

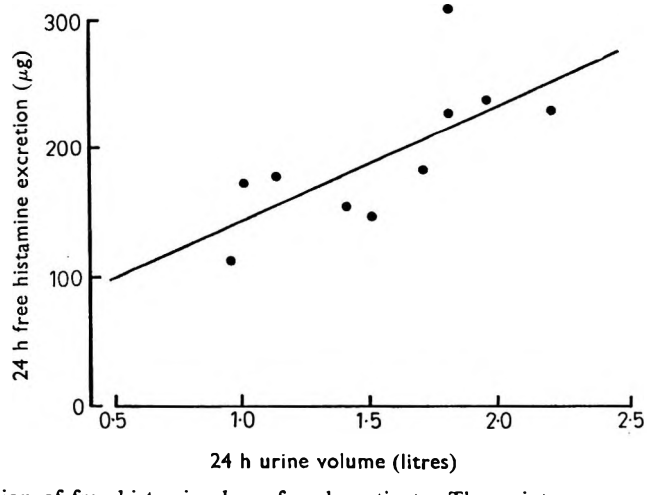


FIG. 1. Excretion of free histamine by a female patient. The points represent free histamine excreted in successive 24 h samples of urine.

from Duner & Pernow (1958), who showed a correlation of blood level of histamine and 24 h urine volume in man given a continuous infusion of histamine. These results cast doubt on the utility of urinary histamine output as a measure of histamine turnover.

Smith and Nephew Research Ltd.,
Gilston Park,
Harlow,
Essex, U.K.

M. HORLINGTON
J. C. KOLTHAMMER
R. LAZARE

June 10, 1970

REFERENCES

ANREP, G. V., AYADI, M. S., BARSOUM, G. S., SMITH, J. R. & TALAAT, M. M. (1944). *J. Physiol, Lond.*, **103**, 155–174.

DUNER, H. & PERNOW, B. (1956). *Scand. J. clin. Lab. Invest.*, **8**, 296–303.

DUNER, H. & PERNOW, B. (1958). *Ibid.*, **10**, 390–393.

JOHNSTON, M. & KAHLSON, G. (1967). *Br. J. Pharmac. Chemother*, **30**, 274–282.

LINDELL, S. E. & WESTLING, H. (1966). *Handbook of experimental pharmacology*, Vol. XVIII/I, pp. 762–3. Berlin: Springer.

OATES, J. A., MARSH, E. & SJOERDSMA, A. (1962). *Clin. chim. Acta*, **7**, 488–497.

Macrocationic enzyme inhibition: pepsin and two polylysines

The strongly acid nature of pepsin is explained by its relatively high content of acidic amino-acids (Taylor, 1968). This property forms the principal basis of association between pepsin and its highly basic natural inhibitor which, together with miscellaneous peptides, constitute the enzymatically inactive pepsinogen (Herriott, 1962). A similar basis has been adduced for the interaction between pepsin and certain basic polyamino-acids, notably poly-L-lysine; and for one polylysine (degree of polymerization, n = 36) inhibition of peptic activity at pH 1·7 and 6 was claimed, digestion of haemoglobin and the clotting of milk being used at the two pH values respectively, to measure the activity of the pepsin (Katchalski, Berger & Neumann, 1954). These authors found that the inhibitory action of polylysine appeared to be instantaneous because pre-incubation of pepsin and polylysine for 5–30 min yielded a constant level of inhibition. It was shown later (Dellert & Stahmann, 1955) that the amount of inhibition of pepsin by a polylysine of mol. wt. 2580 rose to completion when a certain polylysine concentration range was entered, but disappeared as the macrocation concentration was increased to excess. In addition, insoluble complex formation between the polylysine and pepsin did not occur at high macrocation concentrations where inhibition was absent. This appears to be in accord both with knowledge of macroion interaction and the notion that the resulting pepsin inhibition is due to insoluble complex formation involving pepsin and the macrocation. This report deals with the inhibition of pepsin by two polylysines of different molecular weights.

The poly-L-lysines (LY102, LY115) had stated molecular weights of 12 300 and 43 870 (degree of polymerization 59 and 210 respectively), were derived from parent poly ε-carbobenzoxy-L-lysines and were purchased from Miles-Yeda Ltd., Rehovoth, Israel. They were used in solution in acetate buffer, pH 5, I = 0·05 (Long, 1961).

Twice crystallized pig pepsin (Sigma) was used at a concentration of 200 μg/ml in buffer. Gayelord-Hauser dried skim milk was reconstituted by triturating 20 g with water, adding 10 ml buffer, 5 ml 0·2M $CaCl_2$ and diluting to 100 ml with water. Solutions were mixed at 35·5° and milk clotting was carried out at this temperature.

Polylysine solution (or buffer) was mixed in equal volume with pepsin solution and at intervals of time after mixing 0·5 ml of the mixture was added to 5 ml reconstituted

milk and mixed. The time of occurrence of the first granular appearance in the film of milk on the walls, formed by gently tilting the tube, was taken as the end point. This immediately precedes coagulation. The technique is similar to that of Lee & Ryle (1967). Proteolytic units, $[P.U.]^{Rea.}_{ml}$, were calculated (Northrop, Kunitz & Herriott, 1955) and inhibition, i, calculated by expressing the difference in units between the uninhibited control and the inhibited as a percentage of the uninhibited control. Apparent absorbances of the polylysine-pepsin mixtures were read at 400 nm at the same time intervals. Inhibitions are recorded as the maximum (i_{max}) occurring during the 3 h after mixing of polylysine and pepsin and the maximum (i'_{max}) occurring during the first 0·5 h.

Fig. 1 shows that inhibition increases over 3 h particularly for LY115 and that low and high inhibitions conform to this pattern.

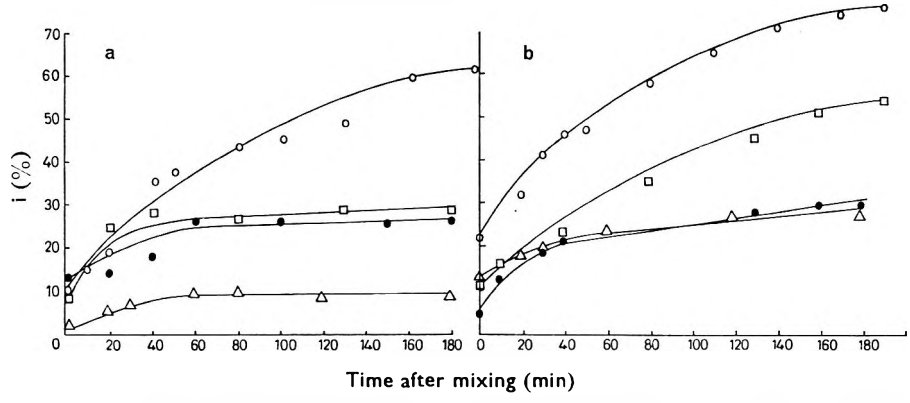


FIG. 1. Pepsin inhibition with time at varying polylsyine concentrations, a. LY102 ○, 70 μg/ml; □, 50 μg/ml; ●, 200 μg/ml; △, 400 μg/ml. b. LY115 ○, 40 μg/ml; □, 30 μg/ml; ●, 50 μg/ml; △, 800 μg/ml.

Table 1 shows that there is a polylysine concentration at which inhibition is a maximum and higher and lower concentrations result in lower, or no, inhibition. Thus for maximum inhibition for LY102 and LY115 concentrations (and polylysine: pepsin mol ratios) were 70 μg/ml (0·99) and 40 μg/ml (0·16) respectively.

Both polylysines follow similar concentration–inhibition patterns, although the higher molecular weight substance (LY115) appears twice as active on a weight basis, probably indicating an easier access to a larger number of interacting sites on the two macromolecules, pepsin and polylysine.

In only one instance was i_{max} observed to occur within 0·5 h (LY115, 40 μg/ml), although even in this instance inhibition increased by about 60% during this period of time. For other concentrations of both polylysines inhibition usually reached a maximum towards 3 h and i_{max} was usually greater than i'_{max}. Maximum absorbances did not usually occur at the same time as maximum inhibition; indeed for the highest inhibitions by both polylysines the maximum absorbance occurred within 40 min after mixing pepsin and polylysine while i_{max} itself usually occurred at around 180 min. Highest inhibition therefore appears to be associated with the most rapid rate of formation of insoluble complex.

It appears that the interaction between pepsin and these polylysines is not completed instantaneously and that some aspect of the interaction proceeds with time, involving the active sites on the pepsin molecule. Whether it is confined to these sites or is an indication of progressive involvement of the whole pepsin molecule in complexed form is not yet clear.

Table 1. *Pepsin inhibition and absorbances resulting from mixing pepsin and polylysine at pH* 5. Pepsin concentration was 200 μg/ml. Mole ratio (polylysine/pepsin) range was LY102 0·29–5·7, LY115 0·08–5·5. Inhibition measured by milk clotting. Concentrations of pepsin and polylysine are quoted before mixing.

Polylysine	Polylysine concentration μg/ml	i_{max} (%) (absorbance at i_{max})	i'_{max} (%) (absorbance at i'_{max})	Maximum absorbance and time (min) of occurrence
LY102	20	12 (0·191)	12 (0)	0·191 (130)
	40	30 (0·284)	29 (0·254)	0·284 (140)
	50	29 (0·246)	24 (0·244)	0·280 (50)
	70	67 (0·341)	41 (0·300)	0·349 (40)
	80	45 (0·345)	14 (0·185)	0·370 (110)
	120	26 (0·210)	0	0·238 (180)
	160	27 (0·209)	11 (0·05)	0·209 (180)
	200	26 (0)	13 (0·03)	0·058 (130)
	320	18 (0)	10 (0)	0
	400	9 (0)	7 (0)	0
LY115	20	15 (0·116)	15 (0·132)	0·146 (60)
	30	54 (0·511)	18 (0·476)	0·731 (60)
	40	79 (0·254)	46 (0·572)	0·366 (40)
	50	29 (0·134)	18 (0·084)	0·136 (70)
	70	24 (0·225)	5 (0·087)	0·225 (180)
	80	21 (0·160)	2 (0·150)	0·155 (120)
	120	25 (0)	3 (0)	0
	200	25 (0)	12 (0)	0
	700	25 (0)	17 (0)	0
	800	27 (0)	20 (0)	0
	1400	36 (0)	30 (0)	0

Earlier reports (Katchalski & others, 1954; Dellert & Stahmann, 1955) dealing with other polylysines have claimed or assumed instantaneous completion of interaction and attainment of maximum inhibition. It is interesting to note that some inhibition can be demonstrated in the absence of insoluble complex formation, although highest inhibitions were always accompanied by precipitation, the precipitate later aggregating and flocculating.

I thank Miss J. E. Harthill for technical assistance.

School of Pharmaceutical Sciences,
University of Strathclyde,
*Glasgow, C.*1, *U.K.*

W. ANDERSON

May 19, 1970

REFERENCES

DELLERT, E. E. & STAHMANN, M.A. (1955). *Nature, Lond.*, **176**, 1028–1029.

HERRIOTT, R. M. (1962). *J. gen. Physiol.*, **45**, 57–75.

KATCHALSKI, E., BERGER, A. & NEUMANN, H. (1954). *Ibid.*, **173**, 998–999.

LEE, D. & RYLE, A. P. (1967). *Biochem. J.*, **104**, 735–741.

LONG, C. (1961). *Biochemists' Handbook.* London: Spon.

NORTHROP, J. H., KUNITZ, M. & HERRIOTT, R. M. (1948). *Crystalline Enzymes* 2nd edn. New York: Columbia University.

TAYLOR, W. H. (1968). In *Handbook of Physiology*, Section 6, Chap. 120, pp. 2567–2587. Editors: Code, C. F. and Heide, W. Washington: American Physiological Society.

The demonstration of a tyramine-like substance in a heart extract (Recosen)

A protein-free total heart extract, available in both tablet and ampoule form, has been reported to be useful in a number of cardiovascular disorders (Karani & Elliott, 1961; Rey & Pattani, 1954). The extract (Recosen) has been reported to contain histamine, adenosine derivatives and various cations (Conway, 1959), while Meyer & Goldberg (1966), using pharmacological techniques, identified a tyramine-like substance. Using chromatography, we have found tyramine in the extract. We have also found that the whole of the cardiotonic activity is accountable for by this amine.

The contents of 30 one ml ampoules were pooled, concentrated and the concentrate dialysed. The dialysate was chromatographed through a column packed with Biogel P2 (Biorad) polyacrylamide gel equilibrated with 0·1 N acetic acid. The fractions eluted from the column were dried below 30° and bioassayed for positive inotropic activity using the isolated papillary muscle of the cat (Thorp & Cobbin, 1967).

The constituents of the extracts were separated by ascending paper chromatography on Whatman No. 1 paper using three solvent systems, and the results visualized by a diazotisation spot test for amines (Dawson, Elliot & others, 1969a), ninhydrin 0·5% in n-butanol, and an alkaline Folin-Ciocalteau reagent for phenols (Dawson, Elliott & others, 1969b). Catecholamines were detected with a potassium ferricyanide spray in phosphate buffer (James, 1948). Some paper chromatograms were cut into segments which were eluted for bioassay. Tyramine, and β-phenethylamine, another indirectly acting sympathomimetic amine occurring in tissue extracts (Jackson & Temple, 1970), were used as controls. Table 1 lists the results.

In each solvent system, two components of the extract were detected by each of the stain techniques used, one having a relatively high and the other a relatively low Rf value, indicating the presence of two phenolic amines. The Rf value for tyramine in all solvent systems was similar to that of the more mobile component in the extract. Moreover, elution and bioassay of the chromatogram in segments indicated that the positive inotropic activity of the extract, which apparently remained a single component in each solvent system, had a similar Rf to tyramine in each solvent system. The intensity of the colour produced with the diazotization or Folin-Ciocalteau reagents by the more mobile spot when 250 μg of extract was chromatographed,

Table 1. *Rf values for components of heart extract compared to tyramine and phenethylamine, using various reagents*

Reagent	Solvent system a	b	c
	Rf values		
Ninhydrin	0·17 MP	0·48 P	0·34 P
		0·63 B	0·57 B
Diazo	0·16 P	0·47 C	0·35 CP
	0·52 C	0·71 C	0·70 P
Folin	0·17	0·44	0·32
	0·50	0·67	0·68
Activity on bioassay of eluted chromatogram	0·27–0·41	0·5–0·7	0·5–0·6
Tyramine (average)*	0·43	0·66	0·64
β-phenylethylamine	0·56	0·85	0·73

a = n-Butanol–water (86:14).
b = n-Butanol–acetic acid–water (12:3:5).
c = n-Butanol–methanol–water (5:5:1).
M = Mauve, P = purple, C = crimson.
* Detected by each reagent and by elution and bioassay.

was comparable to the colour intensity of 0·5 to 1·0 μg tyramine standards under the same conditions. Bioassay of 1 μg of tyramine gave an average value for cardiotonic activity for ten assays on the isolated cat papillary muscle of 203% $\pm$ 40, while the assay of 250 μg of extract before chromatography gave a result of 245% $\pm$ 47 for five assays, the results being expressed as mean $\pm$ standard error. Noradrenaline, adrenaline, dopamine, isoprenaline, histamine, aldosterone, 5-hydroxytryptamine and phenylalanine were also chromatographed in the same systems but none had the same or similar Rf values to the extract in all the systems. These findings suggest that all the cardiotonic activity of the extract is accountable for on the basis of its tyramine content. The extracts were inactive when assayed on muscle preparations from reserpinized cats, confirming the indirectly-acting nature of the extracts. No noradrenaline, adrenaline or dopamine were detected by the ferricyanide spot tests, but a ninhydrin-reacting constituent corresponding in Rf to histamine was detected in the extracts occasionally.

Department of Pharmacology,
University of Sydney,
Sydney, 2006, *Australia.*

D. M. JACKSON
DIANA M. TEMPLE

May 13, 1970

REFERENCES

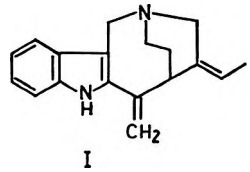
CONWAY, C. M. (1959). *J. Pharm. Pharmac.*, **11**, 477–484.
DAWSON, R. M. C., ELLIOT, D. C., ELLIOT, W. H. & JONES, K. M. (1969a). Data for Biochemical Research, 2nd edn, p. 521. Oxford: Clarendon.
DAWSON, R. M. C., ELLIOT, D. C., ELLIOT, W. H. & JONES, K. M. (1969b). *Ibid.*, p. 577.
JACKSON, D. M. & TEMPLE, D. M. (1970). *Comp. Gen. Pharmac.*, in the press.
JAMES, W. O. (1948). *Nature, Lond.*, **161**, 851–852.
KARANI, S. & ELLIOTT, W. A. (1961). *Br. J. clin. Practice*, **15**, 255–257.
MEYER, M. B. & GOLDBERG, L. I. (1966). *Cardiologia*, **49**, 1–10.
REY, C. H. & PATTANI, F. (1954). *Acta cardiol.*, **9**, 221–234.
THORP, R. H. & COBBIN, L. B. (1967). *Cardiac Stimulant Substances*, p. 19. New York: Academic Press.

The isolation and identification of (–)-apparicine from *Tabernamontana cumminsii*

Conopharyngine, the major alkaloidal component (Thomas & Starmer, 1963; see also Renner, Prins & Stoll, 1959), jollyanine (conopharyngine hydroxyindolenine) (Crooks & Robinson, 1970) and 2-ethyl-3-[2-(3-ethylpiperidino)-ethyl] indole (Crooks, Robinson & Smith, 1968) have already been isolated from the ether-soluble bases obtained from the leaves of *T. cumminsii*. We have now identified a fourth alkaloid, m.p. 188–191°, $[\alpha]_D^{22} = -170 \pm 10°$ (in $CHCl_3$), whose isolation from this source we have already reported (Crooks & Robinson, 1970).

The high resolution mass spectrum of the alkaloid showed a molecular ion at m/e = 264·162387 which indicated a molecular formula $C_{18}H_{20}N_2$ (calculated 264·162641). Apart from the molecular ion, which was also the base peak, the mass spectrum had other significant peaks at m/e = 249, 235, 222, 208, 194, 180, 167, 154, 130 and 128. The ultraviolet spectrum in ethanol had λ_{max} 303–305 nm (log ϵ = 4·65), λ_{infl} 309–312 nm (log ϵ = 4·60), λ_{min} 268 nm (log ϵ = 3·78) which did not change upon acidification.

The above data are in agreement with those reported (Joule, Monteiro & others, 1965) for (−)-apparicine (I). The identity of the alkaloid at present under investigation was confirmed as (−)-apparicine (I) by comparison (TLC behaviour and ultraviolet and mass spectra) with an authentic sample of (−)-apparicine (supplied by Dr. R. T. Brown of the Department of Chemistry, University of Manchester).



I

(−)-Apparicine, the biosynthesis of which from tryptophan via stemmadenine has recently (Kutney, Nelson & Wigfield, 1969) been demonstrated, has previously been isolated from several species of the genus *Aspidosperma* (Arndt & Djerassi, 1965; Gilbert, Duarte & others, 1965) and its enantiomer has been isolated from one such species (Joule, Ohashi & others, 1965). Under the synonyms (Monteiro, 1966) tabernoschizine and pericalline, (−)-apparicine has also been isolated from *Schizozygia caffaeoides* (Renner & Kernweisz, 1963) and from *Catharanthus lanceus* (Blomster, Martello & others, 1964) and *roseus* (Svoboda, 1963) respectively, and the un-named alkaloid, m.p. 186–188° (no rotation given) isolated from *Aspidosperma australe* (Ondetti & Deulofeu, 1961) is also probably apparicine. The present studies represent the first isolation of the alkaloid from the genus *Conopharyngia*.

Department of Pharmacy,
The University,
Manchester 13, *U.K.*

P. A. Crooks
B. Robinson

April 23, 1970

REFERENCES

Arndt, R. R. & Djerassi, C. (1965). *Experientia*, **21**, 566–568.

Blomster, R. N., Martello, R. E., Farnsworth, N. R. & Draus, F. J. (1964). *Lloydia*, 27, 480–485.

Crooks, P. A. & Robinson, B. (1970). *J. Pharm. Pharmac.*, **22**, 471–472.

Crooks, P. A., Robinson, B. & Smith, G. F. (1968). *Chem. Commun.*, 1210.

Gilbert, B., Duarte, A. P., Nakagawa, Y., Joule, J. A., Flores, S. E., Brissolese, J. A., Campello, J., Carrazzoni, E. P., Owellon, R. J., Blossey, E. C., Brown, jun., K. S. & Djerassi, C. (1965). *Tetrahedron*, **21**, 1141–1166.

Joule, J. A., Monteiro, H., Durham, L. J., Gilbert, B. & Djerassi, C. (1965). *J. chem. Soc.*, 4773–4780.

Joule, J. A., Ohashi, M., Gilbert, B. & Djerassi, C. (1965). *Tetrahedron*, **21**, 1717–1731.

Kutney, J. P., Nelson, V. R. & Wigfield, D. C. (1969). *J. Am. chem. Soc.*, **91**, 4278–4279; 4279–4280.

Monteiro, H. J. (1966). Ph.D. Thesis, Stanford University; *Diss. Abstr.*, **27**, B, 1096.

Ondetti, M. A. & Deulofeu, V. (1961). *Tetrahedron*, **15**, 160–166.

Renner, U. & Kernweisz, P. (1963). *Experientia*, **19**, 244–246.

Renner, U., Prins, D. A. & Stoll, W. G. (1959). *Helv. chim. Acta*, **42**, 1572–1581.

Svoboda, G. H. (1963). *J. pharm. Sci.*, **52**, 407–408.

Thomas, J. & Starmer, G. A. (1963). *J. Pharm. Pharmac.*, **15**, 487.

Printed by W Heffer & Sons Ltd Cambridge England

Journal of Pharmacy and Pharmacology

Volume 22 Number 10 October 1970

Original Papers

Letters to the Editor