Journal of Pharmacy and Pharmacology

Published by
The Pharmaceutical Society
of Great Britain

Volume 19 No. 11
November 1967

Journal of Pharmacy and Pharmacology

Published by THE PHARMACEUTICAL SOCIETY OF GREAT BRITAIN

17 Bloomsbury Square, London, W.C.1. Telephone: 01-405 8967

Notice to Contributors

GENERAL. 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 previously published in whole or in part in any other journal.

PREPARATION OF 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 1½ inch (4 cm) margin. 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 according 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.

ILLUSTRATIONS. Illustrations are usually limited to those considered necessary to the proper understanding of the subject matter. They need not be duplicated if foolscap size or less. With larger illustrations there should be included for ease in sending through the post a set of photocopies or rough drawings suitable for submission to a referee. Line illustrations such as graphs or apparatus should be clearly and boldly drawn at least twice the size of the final reproduction, which will usually have a baseline (width) of not more than 4 inches, in Indian ink on white paper, Bristol Board, faintly blue-lined graph paper or tracing cloth or paper. Kymograph records and photographs should be selected to allow for reductions of similar dimensions. Lettering and numbering should be reduced to the minimum and 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 on the back of each illustration. The approximate position of each illustration should be marked in the text. All illustrations should be understandable without reference to 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

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

J. Pharm. Pharmac., 1967, **19**, 705–708 Received June 16, 1967

On the disposition of [^{3}H]metaraminol in the rat salivary gland*

O. ALMGREN AND B. WALDECK

The left salivary glands of rats were sympathetically denervated or decentralized. In some experiments the excretory ducts of the left submaxillary and sublingual glands were ligated to produce atrophy of the acinar cells. The rats received [^{3}H]metaraminol (^{3}H-MA) intravenously and were killed at various time intervals thereafter. The amount of ^{3}H-MA in the salivary glands was determined. ^{3}H-MA was taken up and retained in the intact gland, but disappeared rapidly from the denervated one, indicating that ^{3}H-MA is taken up and stored in the adrenergic neuron. Decentralization resulted in a decreased turnover of the amine, especially during the first 18 hr, which supports the view that metaraminol is released by nerve activity. The ability of the salivary gland to take up ^{3}H-MA was diminished by glandular atrophy, and the disappearance of the ^{3}H-MA so taken up was delayed.

METARAMINOL is an analogue of noradrenaline and it is similarly taken up by the adrenergic neuron and released by nerve stimulation and by drugs (Andén, 1964; Crout, Alpers & others, 1964; Shore, Busfield & Alpers, 1964) but it is not attacked by monoamine oxidase and catechol-*O*-methyl transferase.

By using [^{3}H]-labelled metaraminol, its fate in tracer amounts has been studied (Carlsson & Waldeck, 1965). We have now examined the fate of [^{3}H]metaraminol (^{3}H-MA) in the rat salivary gland after denervation, decentralization or ligation of the excretory ducts, to gain further insight into the mechanisms of amine uptake and release in adrenergic nerves.

The submaxillary gland of the rat has a relatively rich supply of adrenergic nerves and is easily available for surgical procedures. Ligation of the excretory duct of the gland causes a weight reduction of about 65% due mainly to a reduction of the cytoplasm in the acinar cells. Fluorescent microscopic pictures indicate that the network of noradrenaline nerve terminals is much denser after duct ligation while the adrenergic ground plexus of the blood vessels is not noticeably different from the innervated organ. The noradrenaline content of the atrophied gland, per unit weight, is almost double that found in the intact organ (Andén, Norberg & Olson, 1966). It has been suggested that extraneuronal binding sites might be of importance for the efficient uptake of monoamines (e.g. tyramine and noradrenaline) in the adrenergic nerves of the rat salivary gland, and that glandular atrophy might result in the loss of such binding sites leading to a reduced uptake capacity (Almgren, Andén & Waldeck, 1965).

From the Department of Pharmacology, University of Göteborg, Sweden.

* Part of this report was presented in a discussion remark at the "International Symposium on Mechanisms of Release of Biogenic Amines" in Stockholm, February 21–24, 1965 (Almgren, 1966).

Experimental

MATERIALS AND METHODS

Sprague-Dawley rats of either sex were used. In one group the left submaxillary and sublingual glands were denervated by excising the superior cervical ganglion. In a second group decentralization was effected by removing about 1 cm of the cervical sympathetic trunk proximal to the superior ganglion, and in a third group the excretory ducts were ligated causing the glands to atrophy. In all animals the operations were performed on the left side, the right side serving as a control. In some experiments both ligation of the excretory ducts and decentralization were performed.

The ganglionectomized animals were left 10 days to allow adrenergic nerves to degenerate. After decentralization the animals had 5 days to recover from the operation. After duct ligation, maximal atrophy developed in 14 days. At the end of the above time intervals the rats were given 0·01 mg/kg of (±)-^{3}H-MA via a tail vein and maintained in an environmental temperature of 30°. At different time intervals following drug administration the animals were killed by a blow on the head. The submaxillary and sublingual glands of each side were removed, weighed and extracted in perchloric acid. After ion-exchange chromatography the ^{3}H-MA content of the eluates was determined in a liquid scintillation counter (Carlsson & Waldeck, 1965).

Results

The results are presented on a semilogarithmic graph (Fig. 1). In the intact gland ^{3}H-MA was rapidly taken up and then disappeared in two distinct phases, both following an exponential course. Ten min after the injection of the amine about 8 ng of ^{3}H-MA per gland was found. Eighteen hr later about 2·5 ng was left, the half-life of the amine during this initial phase being about 12 hr. Between 18 and 144 hr the release was much slower. During this period the half-life was approximately 2·5 days.

In the postganglionically denervated gland about 2 ng of ^{3}H-MA was found 10 min after its injection; it then disappeared rapidly at a single exponential rate. The half-life was only about 3 hr and after 18 hr the amine was almost completely lost.

The level of ^{3}H-MA found in the decentralized gland 10 min after the injection of the amine, 6 ng/gland, was not significantly lower than in the intact gland. The amine disappeared at a single exponential rate with a half-life of about 4 days.

The atrophied gland showed a markedly reduced capacity to take up ^{3}H-MA. Only about 3 ng was found after 10 min, i.e. one third of the corresponding control value. As in the control gland the amine disappeared exponentially in two phases. It may also be noted that in both cases the change in the rate of turnover occurred at about 18 hr after the injection of the amine. The half-life of the amine during the first 18 hr

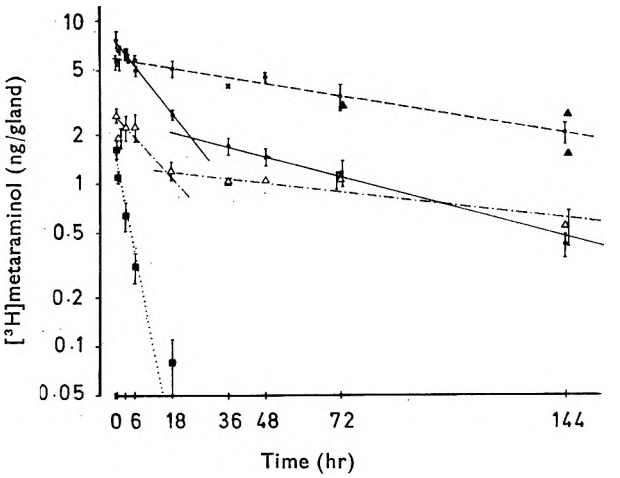

FIG. 1. Disposition of [³H]metaraminol in the rat salivary gland. [³H]-Metaraminol 0·01 mg/kg was given i.v. to rats whose left submaxillary and sublingual glands had been either denervated (■ - - - - ■), decentralized (○ — — ○) or atrophied by ligation of the excretory ducts (△ — · — · — △). ▲ Denotes decentralized and atrophied. The right glands served as controls (●——●). At various time intervals after the injection the animals were killed and the amount of [³H]metaraminol in the salivary glands was determined. The symbols denote the means of 6–18 (controls), 3–4 (denervated), 4–8 (decentralized), and 2–5 (atrophied) experiments. Symbols without indication of the s.e.m. denote single values.

was approximately 12 hr, while the half-life after that time was about 6 days, which is much longer than in the control gland. When the atrophied gland was decentralized the ^{3}H-MA level 72 and 144 hr after the injection was of the same order of magnitude as in a gland only decentralized.

Discussion

The submaxillary gland of the rat was able to take up and concentrate ^{3}H-MA. The ability to retain the ^{3}H-MA taken up was lost after sympathetic denervation indicating that adrenergic nerves are responsible for the storage of this amine, thus giving further support to the conclusions reached by Andén (1964) and Shore & others (1964). During the first 18 hr the disappearance of ^{3}H-MA from the intact salivary gland was more rapid than that previously observed in the mouse heart, although not as rapid as from the femoral muscle of this animal (Carlsson & Waldeck, 1965).

When the nerve impulse flow to the gland was blocked by decentralization, the rapid decrease of ^{3}H-MA during the first 18 hr after its injection was markedly reduced, whereas the difference in turnover rate between the decentralized and the intact gland after that time was less marked. Crout & others (1964) found that metaraminol was more easily released by nerve stimulation 2 hr rather than 17–20 hr after its injection. It would

thus appear that the early decrease of ^{3}H-MA in the intact gland is due mainly to nerve activity. After about 18 hr, however, the amine seems to be less available to nerve impulses, possibly due to a movement from an "available" to a "less available" pool. It is hard to evaluate to what extent loss of the (+)-form of the amine may contribute to the rapid initial decrease (Shore & others, 1964).

In the atrophied gland the accumulation of ^{3}H-MA was markedly reduced. One possible cause of this reduced uptake could be a decreased blood flow through the atrophied gland. This possibility, however, does not seem likely since studies on the distribution of ^{22}Na in the atrophied salivary gland made earlier (Almgren & others, 1965), indicate that alterations in the blood flow cannot be of major importance. Moreover, as seen in the microscope, there seem to be no visible changes in the distribution of the blood vessels after glandular atrophy. A more likely reason for the reduced uptake capacity would be loss of extraneuronal binding sites facilitating the uptake of amines by the adrenergic nerve fibres (Almgren & others, 1965). The turnover rate in the atrophied gland during the first 18 hr did not appreciably differ from that of the intact gland, while after that time it was markedly lower. Since both curves show the same rapid, initial decrease in contrast to that of the decentralized gland, it is difficult to believe that the nerve impulse flow could be changed. This is also supported by the fact that decentralization of an atrophied gland causes a large increase of the ^{3}H-MA level 72 and 144 hr after the injection. The data, however, do not permit a conclusion whether this is due to an increased uptake of the amine or to a delayed disappearance.

No certain explanation can be offered at present for the markedly reduced turnover rate of ^{3}H-MA in the atrophied gland from 18–144 hr after the injection. It is possible that the increased density of nerve terminals seen after glandular atrophy results in a more efficient recapture of the amine.

Acknowledgements. This research has been supported by grants from the Swedish State Medical Research Council (B67-14X-155-03A) and from the Medical Faculty, University of Göteborg. For skilful technical assistance we are indebted to Mrs. Inger Börjesson, Miss Ingrid Weigner and Mrs. Evelin Öst.

References

Almgren, O. (1966). In *Mechanisms of Release of Biogenic Amines*, editors Euler, U. S. von, Rosell, S. & Uvnäs, B. Oxford: Pergamon.
Almgren, O., Andén, N.-E. & Waldeck, B. (1965). *Life Sci.*, **4**, 121–126.
Andén, N.-E. (1964). *Acta pharmac. tox.*, **21**, 260–271.
Andén, N.-E., Norberg, K.-A. & Olson, L. (1966). *Acta physiol. scand.*, **66**, 501–507.
Carlsson, A. & Waldeck, B. (1965). *Acta pharmac. tox.*, **22**, 293–300.
Crout, J. R., Alpers, H. S., Tatum, E. L. & Shore, P. A. (1964). *Science, N.Y.*, **145**, 828–829.
Shore, P. A., Busfield, D. & Alpers, H. S. (1964). *J. Pharmac. exp. Ther.*, **146**, 194–199.

J. Pharm. Pharmac., 1967, **19**, 709–713 Received April 14, 1967

The effect of reserpine and α-methyldopa on the analgesic action of morphine in the mouse

JANET W. ROSS AND A. ASHFORD

In the tail clip test, reserpine inhibited the analgesic action of morphine, and this action of reserpine was prevented by pretreatment with α-methyldopa. In the hot plate test reserpine potentiated the action of morphine, and α-methyldopa pretreatment had no inhibitory action on reserpine. α-Methyldopa alone, and in combination with reserpine, showed an analgesic action in the hot plate test.

THE effect of reserpine on morphine analgesia has been widely studied; some reports describe an inhibitory action of reserpine, others a potentiating effect. Antagonism in mice was demonstrated by Schneider (1954), Schaumann (1958), Sigg, Caprio & Schneider (1958), Tsou Kong & Tu Zeng-Hong (1963), Medakovic & Banic (1964) and Takagi, Takashima & Kimura (1964). On the other hand, Tripod & Gross (1957), Garcia Leme & Rocha e Silva (1961) and Dandiya & Menon (1963) reported that reserpine enhanced morphine analgesia.

In our hands, the nature of the effect of reserpine on morphine in mice was dependent on the method used to demonstrate analgesia. Whereas reserpine antagonized the action of morphine in the tail clip test it potentiated morphine when thermal stimulation of the paw was used. These results are now reported.

Experimental

Male Schneider mice, 18–22 g, were housed in groups of 5. Tail clip and hot plate tests were made on the same animals, with additional mice occasionally being used in either test.

TAIL CLIP TEST (Bianchi & Franceschini, 1954)

An artery clip covered in plastic tubing was applied to the base of the tail for a period not exceeding 10 sec. Mice were tested at intervals of 10 min after the injection of morphine, for a period of 50 min. Those reacting by biting the clip or by rapid backward movements, were regarded as showing a positive response. A negative response was shown by a characteristic state of immobility when the clip was in position.

HOT PLATE TEST (based on the method of Eddy & Leimbach, 1953)

This was done in a copper histological embedding bath in which the water temperature was held at 55°. Mice were placed on the hot plate at 30 min intervals after the injection of morphine during a period of 2 hr. Those that reacted by licking the front paws or by jumping out of the bath during a 15 sec period, were classed as positive.

From the Pharmacology Department, Twyford Laboratories Ltd., 309 Elveden Road, London, N.W.10.

DRUGS

L-α-*Methyldopa.* 100 or 400 mg/kg, intraperitoneally, was administered 2 hr before reserpine and 4 hr before morphine. *Reserpine.* Serpasil, 2·5 mg/kg subcutaneously, was injected at either 15 min, 2, 24 or 48 hr before morphine. Alternatively a dose of 1·0 mg/kg, subcutaneously, was given 2 hr before morphine. *Morphine hydrochloride* was administered intraperitoneally at doses of 20, 10 or 5 mg/kg. All drugs were made up in saline with the exception of reserpine which was diluted with water. The dose volume was 0·2 ml/20 g. Controls received saline at the appropriate times. P values were calculated by the χ^2 test.

Results

Effect of reserpine, α-methyldopa alone or α-methyldopa followed by reserpine (Table 1). In the tail clip test, neither reserpine nor α-methyldopa caused analgesia and the action of α-methyldopa was not modified by a subsequent injection of reserpine.

TABLE 1. EFFECT OF RESERPINE, α-METHYLDOPA ALONE OR FOLLOWED BY RESERPINE

Pretreatment and time before analgesic test	Tail clip % analgesia						Hot plate % analgesia				
	No. mice	Time (min) 10	20	30	40	50	No. mice	Time (min) 30	60	90	120
Saline s.c.	70	0	0	0	0	0	70	3	16	13	14
Reserpine 2·5 mg/kg, s.c., 15 min	20	5	0	0	0	0	20	0	5	5	5
,, ,, 2 hr	35	0	0	0	0	0	35	0	9	9	14
,, ,, 24 hr	20	0	0	0	0	0	20	35[1]	20	30	30
,, ,, 48 hr	25	0	0	0	0	0	25	4	4	20	36[3]
Saline i.p. 4 hr. Saline s.c. 2 hr	20	0	0	0	0	0	20	0	10	5	15
α-Methyldopa 100 mg/kg, i.p. 4 hr. ,, ,, ,,	20	0	0	0	0	0	20	5	10	0	10
α-Methyldopa 400 mg/kg, i.p. 4 hr ,, ,, ,,	20	10	10	5	0	5	20	0	20	60[1]	55[1]
α-Methyldopa 100 mg/kg, i.p. 4 hr. Reserpine 2·5 mg/kg, s.c. 2 hr	20	5	0	0	0	0	20	20[2]	35	65[1]	90[1]
α-Methyldopa 400 mg/kg, i.p. 4 hr. Reserpine 2·5 mg/kg, s.c. 2 hr	20	5	5	15	5	5	20	20[2]	55[1]	65[1]	80[1]

P values refer to comparison with saline control. [1] <0·001; [2] <0·01; [3] <0·05.

In the hot plate test, reserpine showed a slight analgesic action when given 24 or 48 hr before the test but none at shorter pretreatment times. α-Methyldopa, however, although inactive at 100 mg/kg, had a strong analgesic action at 400 mg/kg. This activity was evident only at the 90 and 120 min test periods, that is at 5·5 and 6 hr after injection. When α-methyldopa at either dose level was followed by reserpine, a marked analgesic effect was obtained.

Effect of reserpine or α-methyldopa on morphine (Table 2). In the tail clip test, reserpine either abolished or much reduced the action of morphine at all pretreatment times, whereas α-methyldopa had no significant effect.

In the hot plate test reserpine greatly enhanced the effect of the 20 or 10 mg/kg dose of morphine. Potentiation was evident at all pretreatment times from 15 min to 48 hr. α-Methyldopa did not affect the action of morphine.

TABLE 2. EFFECT OF RESERPINE OR α-METHYLDOPA ON MORPHINE

Pretreatment and time before morphine	Morphine mg/kg, i.p.	Tail clip						Hot plate				
		No. mice	% showing analgesia					No. mice	% showing analgesia			
			Time (min)						Time (min)			
			10	20	30	40	50		30	60	90	120
Saline s.c., 15 min	20	20	40	55	50	25	25	25	36	24	12	4
	10	20	20	30	30	20	5	20	15	5	0	7
	5	20	5	10	5	5	0	20	0	0	0	5
Reserpine 2·5 mg/kg, s.c., 15 min	20	20	15[4]	10[3]	5[2]	5	0[3]	25	36	68[2]	48[2]	52[1]
	10	20	0[4]	0[2]	0[2]	0[4]	0	20	25	15	30[3]	27
	5	20	0	0	0	0	0	20	5	15	15	5
Saline s.c., 2 hr	20	110	43	52	52	52	45	105	46	38	26	22
	10	55	14	22	24	20	7	55	13	24	13	16
	5	30	7	3	3	0	0	20	10	15	15	25
Reserpine 2·5 mg/kg, s.c., 2 hr	20	75	4[1]	7[1]	4[1]	1[1]	0[1]	80	65[3]	72[1]	75[1]	67[1]
	10	35	0[3]	0[2]	0[2]	0[2]	0	45	64[1]	56[2]	53[1]	60[3]
	5	20	0	0	0	0	0	20	15	40	40	35
Reserpine 1·0 mg/kg, s.c., 2 hr	20	15	7[3]	7[1]	0[1]	0[1]	0[1]	15	53	40	47	13
	10	20	0	0[4]	0[3]	0[4]	0	20	30	35	50[1]	67[1]
Saline s.c., 24 hr	20	10	50	40	50	40	30	10	50	20	30	40
Reserpine 2·5 mg/kg, s.c., 24 hr	20	20	0[1]	0[2]	0[1]	0[2]	0[2]	20	75	65[3]	80[2]	75
Saline s.c., 48 hr	20	10	70	40	50	50	50	10	20	30	10	10
Reserpine 2·5 mg/kg, s.c., 48 hr	20	20	10[1]	5[3]	5[2]	15[4]	5[3]	20	60[4]	80[2]	75[1]	70[2]
Saline i.p., 4 hr	20	20	45	65	65	50	60	20	30	30	45	25
	10	30	27	40	30	27	27	50	14	36	24	30
α-Methyldopa 100 mg/kg, i.p. 4 hr	20	20	70	55	65	70	65	20	35	30	30	15
	10	20	55[4]	45	40	45	30	20	35	35	30	35
α-Methyldopa 400 mg/kg, i.p. 4 hr	20	20	50	55	50	60	55	20	40	35	50	35

P values refer to a comparison with the saline pretreated morphine control. [1] <0·001; [2] <0·01; [3] <0·02; [4] <0·05.

Effect of α-methyldopa followed by reserpine on morphine (Table 3). In the tail clip test, α-methyldopa prevented the inhibitory effect of reserpine on morphine, while in the hot plate test, morphine preceded by α-methyldopa and reserpine caused pronounced analgesia. This can be attributed to an additive effect between morphine on the one hand and α-methyldopa and reserpine on the other.

TABLE 3. EFFECT OF α-METHYLDOPA, FOLLOWED BY RESERPINE, ON MORPHINE

Pretreatment			Tail clip test						Hot plate test				
				% showing analgesia						% showing analgesia			
				Time (min)						Time (min)			
−4 hr, i.p.	−2 hr, s.c.	Morphine mg/kg, i.p.	No. mice	10	20	30	40	50	No. mice	30	60	90	120
Saline	Saline	20	20	45[3]	65[1]	65[1]	50[2]	60[2]	20	30[3]	30[4]	45	25[4]
Saline	Saline	10	30	27[2]	40[1]	30[2]	27[2]	27[2]	50	14[1]	36	24	30
Saline	Reserpine	20	10	0	0	0	0	0	20	70	65	65	60
Saline	Reserpine	10	30	0	0	0	0	0	50	52	48	42	28
α-Methyldopa 100 mg/kg	Reserpine	20	20	65[1]	40[3]	60[2]	80[1]	80[1]	30	70	87	93[3]	87[4]
α-Methyldopa 400 mg/kg	Reserpine	20	20	45[3]	45[3]	45[3]	50[2]	45[3]	20	65	65	90	90[4]
α-Methyldopa 100 mg/kg	Reserpine	10	20	20[3]	20[3]	20[3]	20[3]	15[4]	20	60	65	75[3]	85[1]

Reserpine 2·5 mg/kg s/c.
P values refer to a comparison with saline, reserpine, morphine pretreated mice. [1] <0·001; [2] <0·001; [3] <0·02; [4] <0·05.

Discussion

In the same mice, reserpine showed a marked antagonism to morphine in the tail clip test and potentiation in the hot plate test. Antagonism in the former test confirms the results of Schaumann (1958), Tsou Kong & Tu Zeng-Hong (1963) and Takagi & others (1964), and potentiation in the hot plate test is in agreement with the results of Garcia Leme & Rocha e Silva (1961—hot plate) and Dandiya & Menon (1963—hot wire) but not with those of Medakovic & Banic (1964—hot plate), Sigg & others (1958—hot wire) and Schneider (1954—heat on tail). It is interesting to note that whereas there is unanimous agreement between different workers on the interaction of reserpine and morphine in the tail clip test, results differ when methods based on heat are used.

It would appear from these results that different mechanisms are involved in the nociceptive response to mechanical compression of the tail and thermal stimulation of the paw, and this conclusion is supported by the action of α-methyldopa in antagonizing reserpine in the former test and being synergistic with it in the latter. Inhibition of morphine by reserpine is probably a central effect since tetrabenazine, which also reduces the action of morphine in the tail clip test (Takagi & others, 1964) has little peripheral action (Pletscher, 1957; Quinn, Shore & Brodie, 1959). In addition reserpine is known to inhibit another central effect of morphine, namely psychomotor stimulation (Tripod, Bein & Meier, 1954; Tripod & Gross, 1957).

Potentiation could also result from a central effect if different receptors are involved and certainly the complex coordinated behaviour that serves as an endpoint in both tests is susceptible to modification at several points. However, a peripheral action of the drugs, as an explanation of the hot plate results, cannot be ruled out. A heat stimulus may be more readily antagonized at the periphery than mechanical compression since, with the former, a release of chemical mediators has been described.

There is evidence that bradykinin is released in response to heat injury of the rat paw (Rocha e Silva & Antonio, 1960) and that kinins may be released in response to a 20 sec burn of the guinea-pig foot at 55° (Davies & Lowe, 1966). The ability of this substance to cause pain is well known (Armstrong, Jepson & others, 1957; Elliott, Horton & Lewis, 1960), and it is not inconceivable that the nociceptive response to heat is initiated by release of a bradykinin-like substance. Rocha e Silva (1962) speculated that liberation of catecholamines from peripheral sites preceded the activation of bradykininogen and formation of bradykinin after local heating, and showed that pretreatment with reserpine inhibited the resulting inflammatory response. Winder's (1959) suggestion that an analgesic effect could result from interference with preinflammatory pain-producing substances at the site of injury may explain how the interaction of reserpine and morphine and of reserpine and α-methyldopa produces results in the hot plate test that are opposed to those obtained by the tail clip method.

References

Armstrong, D., Jepson, J. B., Keele, C. A. & Stewart, J. W. (1957). *J. Physiol., Lond.*, **135**, 350–370.
Bianchi, C. & Franceschini, J. (1954). *Br. J. Pharmac. Chemother.*, **9**, 280–284.
Dandiya, P. C. & Menon, M. K. (1963). *Archs int. Pharmacodyn. Thér*, **141**, 223–232.
Davies, G. E. & Lowe, J. S. (1966). *Br. J. Pharmac. Chemother.*, **27**, 107–113.
Eddy, N. B. & Leimbach, D. (1953). *J. Pharmac. exp. Ther.*, **107**, 385–393.
Elliott, D. F., Horton, E. W. & Lewis, G. P. (1960). *J. Physiol., Lond.*, **153**, 473–480.
Garcia Leme, J. & Rocha e Silva, M. (1961). *J. Pharm. Pharmac.*, **13**, 734–742.
Medakovic, M. & Banic, B. (1964). *Ibid.*, **16**, 198–206.
Pletscher, A. (1957). *Science, N.Y.*, **126**, 507.
Quinn, G. P., Shore, P. A. & Brodie, B. B. (1959). *J. Pharmac. exp. Ther.*, **127**, 103–109.
Rocha e Silva, M. (1962). *Biochem. Pharmac.*, **10**, 3–23.
Rocha e Silva, M. & Antonio, A. (1960). *Medna exp.*, **3**, 371–382.
Schaumann, W. (1958). *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.*, **235**, 1–9.
Schneider, J. A. (1954). *Proc. Soc. exp. Biol. Med.*, **87**, 614–615.
Sigg, E. B., Caprio, G. & Schneider, J. A. (1958). *Ibid.*, **97**, 97–100.
Takagi, H., Takashima, T. & Kimura, K. (1964). *Archs int. Pharmacodyn. Thér.*, **149**, 484–492.
Tripod, J., Bein, H. J. & Meier, R. (1954). *Ibid.*, **96**, 406–425.
Tripod, J. & Gross, F. (1957). *Helv. physiol. pharmac. Acta*, **15**, 105–115.
Tsou Kong & Tu Zeng-Hong (1963). *Acta physiol. sin.*, **26**, 360–366.
Winder, C. V. (1959). *Nature, Lond.*, **184**, 494–497.

J. Pharm. Pharmac., 1967, **19**, 714–719 Received April 11, 1967

A quantitative comparison of the antagonism of tubocurarine and diallylnortoxiferine by four anticurare agents

BARBARA J. PLEUVRY AND A. R. HUNTER

The antagonism of tubocurarine and diallylnortoxiferine by neostigmine, physostigmine, edrophonium and ambenonium, has been examined on the rat phrenic nerve diaphragm preparation. Ambenonium showed the greatest activity and physostigmine and edrophonium were the least potent and equally active. This order applied to both neuromuscular blocking agents, but all four antagonists show significantly greater activity against diallylnortoxiferine. The possibility that neostigmine and physostigmine have some qualitative differences in anticurare mechanism compared with edrophonium and ambenonium, on this preparation, is discussed.

THE anticurare actions of physostigmine, neostigmine (Bülbring & Chou, 1947), edrophonium (Randall, 1950) and ambenonium (Lands, Karczmar & others, 1955) are well known. However, their curare antagonism has been attributed to anticholinesterase activity alone (Hobbiger, 1952; Nastuk & Alexander, 1954), to presynaptic activity (Riker, Werner & others, 1959) and to a combination of these two mechanisms together with some direct activity on the motor end-plate (Blaber & Bowman, 1963).

If the four drugs mentioned antagonize tubocurarine by the same mechanism, they ought to show a similar relation between the concentration of antagonist and the degree of antagonism. The assessment of this relation was made [within the framework of the limitations set out by Rees (1966)] by the determination of pA_2 values (Schild, 1947) and by the application of a test for competitive antagonism (Arunlakshana & Schild, 1959).

There is some conflict in the literature about the ability of anticurare agents to antagonize a new neuromuscular blocking agent, diallylnortoxiferine (Hunter, 1964; Lund & Stovner, 1962; Foldes, Brown & others, 1963; Venn, 1965). Since no *in vitro* work on this subject has been published, the antagonistic potency of the four anticurare drugs against diallylnortoxiferine was also examined.

Experimental

METHODS

The rat phrenic nerve diaphragm preparation (Bulbring, 1946) and the apparatus described by Starmer & Thomas (1961) were used. The experimental procedure of Rees (1966) was followed except that both male and female rats, 200–300 g, were used and rectangular electric pulses of 0·8 msec duration were applied to the phrenic nerve at a frequency of 7/min.

From the Departments of Pharmacology and Anaesthesia, University of Manchester.

The neuromuscular blocking agents used were tubocurarine chloride and diallylnortoxiferine dichloride, and the antagonists were neostigmine methyl sulphate, physostigmine salicylate, ambenonium chloride and edrophonium chloride. All concentrations are expressed as final molar concentration in the bath.

The experiments were designed according to the method of Bulbring & Chou (1947). It was necessary, however, to extend the wash-out period, especially for ambenonium, to ensure adequate removal of the drug. Repeated additions of tubocurarine to a preparation, after a 15 min wash-out period, gave quantitatively similar responses, but such a preparation was found to be unexpectedly sensitive to diallylnortoxiferine. Similarly, the response to repeated applications of diallylnortoxiferine was constant when a 15 min wash out period was used, but the preparation was subsequently more sensitive to tubocurarine. This increase in sensitivity was, however, much less than when tubocurarine preceded diallylnortoxiferine.

A log concentration effect curve for tubocurarine alone was first determined, and the determination was then repeated in the presence of various concentrations of either neostigmine, physostigmine, edrophonium or ambenonium added to the bath 1 min before each dose of tubocurarine. The same procedure was repeated for diallylnortoxiferine. Three or four different concentrations of each antagonist were used, the ranges of which are shown in Table 1.

TABLE 1. RANGE OF MOLAR CONCENTRATIONS OF ANTICURARE AGENTS USED

Anticurare agent	Tubocurarine	Diallylnortoxiferine
Neostigmine	$3{\cdot}33$–$13{\cdot}3 \times 10^{-7}$	$1{\cdot}66$–$6{\cdot}67 \times 10^{-7}$
Physostigmine	$1{\cdot}66$–$6{\cdot}67 \times 10^{-6}$	$0{\cdot}83$–$3{\cdot}33 \times 10^{-6}$
Edrophonium	$1{\cdot}66$–$13{\cdot}3 \times 10^{-6}$	$1{\cdot}66$–$6{\cdot}67 \times 10^{-6}$
Ambenonium	$1{\cdot}66$–$6{\cdot}67 \times 10^{-7}$	$6{\cdot}67$–$26{\cdot}6 \times 10^{-8}$

Using these results, dose ratios for each antagonist were determined and graphs were drawn of log (dose ratio -1) plotted against the negative logarithm of the molar concentration of antagonist (Arunlakshana & Schild, 1959). The slopes of the resulting lines were measured and pA_2 values were derived from the intercept of this plot with the abscissa. For competitive antagonism this plot should result in a straight line of slope $= 1$.

It seemed that ambenonium and edrophonium might antagonize diallylnortoxiferine in a qualitatively different manner to tubocurarine on this preparation. This was investigated by examination of the effect of the duration of pretreatment contact time on the efficiency of the anticurare action of the four antagonists. A 3 min contact time with the neuromuscular blocking agent was allowed and the antagonists were added to the rat phrenic nerve diaphragm preparation 8 min before, 4 min before, and simultaneously with the blocking agent. The doses of antagonist against each neuromuscular blocking drug were selected to give mean responses, at 8 min, between 30 and 70%.

Each mean with its standard error (s.e.) was derived from six experiments. Significance levels were all $P = 0.05$ and all statistical calculations were based on the methods of Saunders & Fleming (1957).

Results

Table 2 shows the pA_2 values obtained for each anticurare agent and the slopes of the Arunlakshana & Schild plots against both tubocurarine and diallylnortoxiferine.

TABLE 2. THE POTENCY OF THE ANTICURARE AGENTS EXAMINED

Anticurare agent	pA_2 values (±s.e.)*		A & S** slopes (±s.e.)	
	Tubocurarine	Diallylnor-toxiferine	Tubocurarine	Diallylnor-toxiferine
Neostigmine	5·80 (±0·07)	6·16 (±0·03)	0·80 (±0·09)	0·86 (±0·06)
Physostigmine	4·97 (±0·05)	5·33 (±0·05)	0·74 (±0·08)	0·87 (±0·05)
Edrophonium	4·84 (±0·14)	5·36 (±0·05)	0·47 (±0·11)	0·56 (±0·05)
Ambenonium	5·95 (±0·10)	6·52 (±0·08)	0·49 (±0·07)	0·95 (±0·11)

* All means and standard errors (s.e.) were based on six experiments.
** A & S—Graph of log (dose ratio − 1) plotted against the negative logarithm of the molar concentration of anticurare agent (Arunlakshana & Schild, 1959).

The relative order of potency of the antagonists was the same for tubocurarine and diallylnortoxiferine, ambenonium being most potent, neostigmine second and edrophonium and physostigmine (having no significant difference in their potencies) equal third. All four drugs, however, showed significantly greater antagonistic potency against diallylnortoxiferine than against tubocurarine.

The Arunlakshana & Schild plots gave straight lines in all experiments over the limited range of concentrations used. No experiments were made to determine the range over which this linearity extended, as the necessary pA_2 values and slopes could be obtained from the results plotted. In 25% of experiments some extrapolation was necessary to obtain the pA_2 value but the extrapolation was never more than 10% of the plotted line.

The results of the investigation of the effect of the duration of pretreatment with the antagonists are shown in Figs 1 and 2. It can be seen that, in the presence of both neuromuscular blocking agents, increased time of pretreatment greatly increases the degree of antagonism by neostigmine and physostigmine. This is also true for edrophonium and ambenonium in the presence of diallylnortoxiferine, but in the presence of tubocurarine there is no significant increase in antagonistic potency with time of pretreatment.

The different concentrations of antagonists used against the two neuromuscular blocking agents reflects the increased activity of the antagonists against diallylnortoxiferine as compared with tubocurarine.

Discussion

All the anticurare drugs examined antagonized diallylnortoxiferine more than tubocurarine. This correlates with the clinical findings of Venn (1965) and Foldes & others (1962), but not with those of Hunter

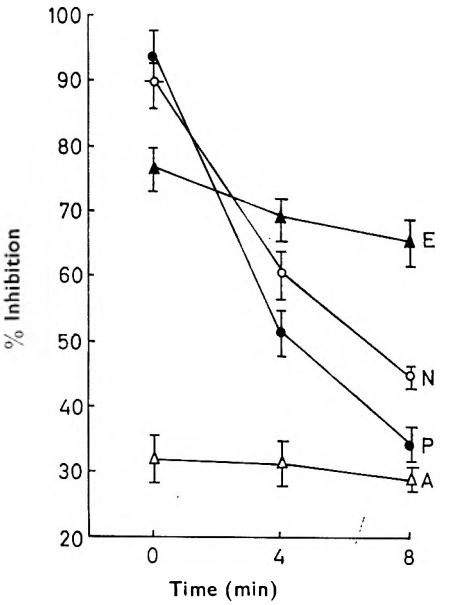

FIG. 1. The effect of the time of pretreatment on the efficiency of tubocurarine antagonism by four anticurare drugs. E = $1{\cdot}0 \times 10^{-6}$M edrophonium. N = $8{\cdot}45 \times 10^{-8}$M neostigmine. P = $6{\cdot}67 \times 10^{-7}$M physostigmine. A = $6{\cdot}67 \times 10^{-8}$M ambenonium. Ordinate represents % inhibition relative to tubocurarine $3{\cdot}33 \times 10^{-6}$M = 100%.

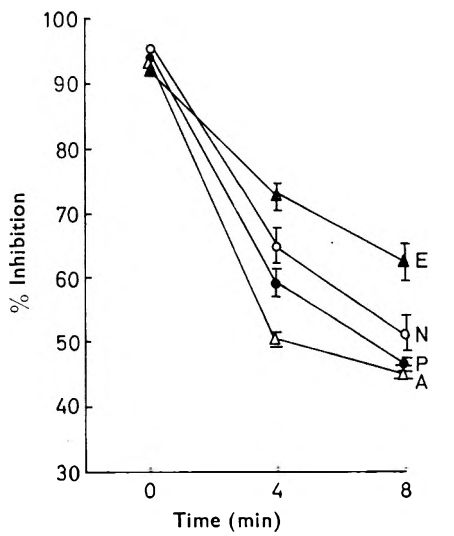

FIG. 2. The effect of the time of pretreatment on the efficiency of diallylnortoxiferine antagonism by four anticurare drugs with s.e. at time 0. E = $6{\cdot}67 \times 10^{-7}$M edrophonium ($\pm 2{\cdot}77$); N = $6{\cdot}67 \times 10^{-8}$M neostigmine ($\pm 2{\cdot}32$); P = 5×10^{-7}M physostigmine ($\pm 3{\cdot}375$); A = $3{\cdot}33 \times 10^{-8}$M ambenonium ($\pm 2{\cdot}46$). Ordinate represents % inhibition relative to tubocurarine $3{\cdot}33 \times 10^{-6}$M = 100%.

(1964) (who found reversal uncertain with diallylnortoxiferine), or of Lund & Stovner (1962) (who found no difference in the reversibility of the two relaxants). It must be remembered, however, that post-treatment with the antagonist is used clinically, not pretreatment as was used in the present work.

The relative anticurare potencies of the four antagonists do not correlate with any recorded value for relative anticholinesterase potencies (Table 3). But if figures could be obtained for the activity of these drugs against the cholinesterase present in rat diaphragm, more meaningful comparisons might be made.

TABLE 3. THE RELATIVE ANTICHOLINESTERASE POTENCIES OF THE DRUGS EXAMINED

Agonist	Potency ratio				References
	Neostigmine	Physostigmine	Edrophonium	Ambenonium	
Tubocurarine	1	0·145	0·089	1·3	
Diallylnortoxiferine	1	0·155	0·165	2·34	
Cholinesterase of cat CNS	1	0·5			Bhattacharya & Feldberg (1958)
Red blood corpuscles	1	0·58	<0·01	5·8	Lands, Hoppe & others (1958)
Bovine red blood corpuscles	1	0·88	0·02		Smith, Cohen, & others (1952)
Red blood corpuscles	1		0·009		Hobbiger (1952)
Cat anterior tibialis homogenate	1	0·5	0·01	35	Blaber (1963)
Dog caudate nucleus	1	0·5			Blaschko, Bulbring & Chou (1949)

When tubocurarine was the agonist, the slopes obtained from the Arunlakshana & Schild plots suggested a division of the four anticurare drugs into two groups. The slopes of the plots for neostigmine and physostigmine were not significantly different and the slope for neostigmine was not significantly different from 1. Similarly the slopes for edrophonium and ambenonium were not significantly different from each other, but were significantly different from those of physostigmine and neostigmine. Thus neostigmine and physostigmine almost fulfil the requirements for competitive antagonism, but edrophonium and ambenonium do not. This difference is not so marked for antagonism to diallylnortoxiferine, only edrophonium producing an Arunlakshana & Schild slope significantly different from 1. However, edrophonium and ambenonium do not have the same quantitative relations between concentration of anticurare drug and potency as physostigmine and neostigmine. There may therefore be a qualitative difference in their anticurare mechanism at least against tubocurarine.

The differences in the time course of tubocurarine antagonism by neostigmine and edrophonium have been investigated by Smith, Mead & Unna (1957). They postulated that, in the intact animal, differences in the time effect curve obtained for the antagonism to curare produced by these compounds are causally related to the differences in the kinetics of the inhibitor-cholinesterase combinations and dissociations. Thus, if it is accepted that anticholinesterase activity plays at least a part in the

anticurare mechanism of the four anticurare drugs examined, this hypothesis could equally well apply to the time course differences between physostigmine and neostigmine on the one hand and edrophonium and ambenonium on the other.

However, there is no obvious explanation of why the time courses for all four drugs are similar against diallylnortoxiferine, but not against tubocurarine. This may indicate a qualitative, as well as quantitative difference between the relaxant drugs.

The nature of this possible difference and the differences between the two groups of anticurare drugs has not been revealed.

An attempt was made to demonstrate anticurare activity with the four antagonists in a preparation in which all the cholinesterase had previously been inactivated by contact with di-isopropylfluorophosphonate (DFP) 20 μg/ml for 1 hr. After application of this drug it was not possible to demonstrate antagonism of tubocurarine by any of the antagonists even when concentrations were increased. This would at first sight suggest that all the antagonists were simply anticholinesterase agents. However, Webb (1948) showed that DFP also inhibited a number of other enzyme systems; Burgen, Keele & Slome (1949) demonstrated the direct action of DFP on the motor end-plate and Riker, Roberts & others (1957) suggested that DFP may have some presynaptic activity, and so this argument is not wholly conclusive.

References

Arunlakshana, O. & Schild, H. O. (1959). *Br. J. Pharmac. Chemother.*, **14**, 48–58.
Bhattacharya, B. K. & Feldberg, W. (1958). *Ibid.*, **13**, 151–155.
Baber, L. C. (1963). *Ibid.*, **20**, 63–73.
Baber, L. C. & Bowman, W. C. (1963). *Ibid.*, **20**, 326–344.
Blaschko, H., Bulbring, E. & Chou, T. C. (1949). *Ibid.*, **4**, 29–32.
Burgen, A. S., Keele, C. A. & Slome, D. (1949). *J. Pharmac. exp. Ther.*, **96**, 396–409.
Bulbring, E. (1946). *Br. J. Pharmac. Chemother.*, **1**, 38–61.
Bulbring, E. & Chou, T. C. (1947). *Ibid.*, **2**, 8–22.
Foldes, F. F., Brown, I. M., Lunn, J. N., Moore, J. & Duncalf, D. (1963). *Curr. Res. Anesth. Analg.*, **42**, 177–187.
Hobbiger, F. (1952). *Br. J. Pharmac. Chemother.*, **7**, 223–236.
Hunter, A. R. (1964). *Br. J. Anaesth.*, **36**, 466–470.
Lands, A. M., Hoppe, J. O., Arnold, A. & Kirchner, F. K. (1958). *J. Pharmac. exp. Ther.*, **123**, 121–127.
Lands, A. M., Karczmar, A. G., Howard, J. W. & Arnold, A. (1955). *Ibid.*, **115**, 185–198.
Lund, I. & Stovner, J. (1962). *Acta anaesth. scand.*, **6**, 161–170.
Nastuk, W. L. & Alexander, J. T. (1954). *J. Pharmac. exp. Ther.* **111**, 302–328.
Randall, L. O. (1950). *Ibid.*, **100**, 83–93.
Rees, J. M. H. (1966). *J. Pharm. Pharmac.*, **18**, 289–293.
Riker, W. F., Roberts, J., Standaert, F. G. & Fujimori, H. (1957). *J. Pharmac. exp. Ther.*, **121**, 286–312.
Riker, W. F., Werner, G., Roberts, J. & Kuperman, A. (1959). *Ann. N.Y. Acad., Sci.*, **81**, 328–344.
Saunders, L. & Fleming, R. (1957). *Mathematics and Statistics for use in Pharmacy, Biology and Chemistry*, London: The Pharmaceutical Press.
Schild, H. O. (1947). *Br. J. Pharmac. Chemother.*, **2**, 189–206.
Smith, C. M., Cohen, H. L., Pelikan, E. W. & Unna, K. R. (1952). *J. Pharmac. exp. Ther.*, **105**, 391–399.
Smith, C. M., Mead, J. C. & Unna, K. R. (1957). *Ibid.*, **120**, 215–228.
Starmer, G. A. & Thomas, J. (1961). *J. Pharm. Pharmac.*, **13**, 752–758.
Venn, P. H. (1965). *Br. J. Anaesth.*, **37**, 213.
Webb, E. C. (1948). *Biochem. J.*, **42**, 96–98.

J. Pharm. Pharmac., 1967, **19**, 720–728 Received April 17, 1967

Carrageenans and the proteolytic activity of human gastric secretion

W. ANDERSON AND A. J. BAILLIE

The inhibition of the peptic activity of human gastric secretion by undegraded and degraded carrageenans of similar sulphate content has been examined over the pH range 1·5–3·75. Inhibition by degraded carrageenan is constant throughout this range, but inhibition by undegraded carrageenan decreases between pH 2·5 and 3·25, when a lower level is established. The inhibition by both types of carrageenan is caused by substrate-inhibitor interaction. The differences in degree of inhibition and the effect of pH on the inhibition by undegraded carrageenan appear to originate in the differing natures of the substrate-inhibitor complexes formed by degraded and undegraded carrageenans.

CARRAGEENANS inhibit the peptic activity of gastric juice but quantitative differences in antipeptic activity are to be found amongst carrageenans from different seaweeds and even from the same species of seaweed harvested in different seas. There are also differences in activity between the κ- and λ-components of the same carrageenans and between undegraded and degraded carrageenans of similar bound sulphate content from the same source (Anderson & Harthill, 1967).

For one degraded carrageenan it has been shown (Anderson, 1961) that interaction with substrate with consequent substrate occlusion, or depletion, is responsible for the observed antipeptic activity. Hence the factors affecting such interaction would be expected to influence antipeptic activity.

It was therefore of interest to study, over a pH range, carrageenans of different molecular weight and of similar bound sulphate content, to determine whether there is a common type and mechanism of inhibition of proteolysis for carrageenans, and to show whether known aspects of structure which would influence protein interaction do in fact give rise to differences in antipeptic activity. The effect of pH has added interest because it is now known that gastric juice contains several proteases with different pH optima for proteolysis (Tang, Wolf & others, 1959; Taylor, 1962; Seijffers, Miller & Segal, 1964).

We here report on the nature of the antipeptic activity displayed over a pH range by undegraded and degraded carrageenans.

Experimental

MATERIALS AND METHODS

Carrageenans. Undegraded carrageenan was the λ-carrageenan from *Chondrus crispus* (CY-λ) containing 37·3% bound sulphate (SO_3Na); the degraded carrageenan was a degraded κ-like carrageenan, derived from the κ-like carrageenan of *Eucheuma spinosum* by mineral acid degradation.

From the Department of Pharmacy, University of Strathclyde, Glasgow.

under mild conditions and containing 36·1% SO_3Na. These carrageenans have been described and their antipeptic activity compared under standardized but necessarily restricted conditions (Black, Blakemore & others, 1965; Anderson & Harthill, 1967). Undegraded λ-carrageenan was chosen as an example of undegraded carrageenans because of the greater activity of λ-carrageenans compared with κ-carrageenans. Degraded *Eucheuma* (κ-like) carrageenan was chosen as an example of a degraded carrageenan because of the relative ease of extensive degradation of this type of carrageenan under mild conditions, and its consequent stability compared with degraded λ-carrageenans. The κ-λ differentiation in this respect is less important than the state of degradation.

Solutions of undegraded carrageenan were made by hydrating the substance by shaking in the appropriate buffer for up to 18 hr before adjusting volume; degraded carrageenan dissolved immediately.

Gastric secretion. Secretions obtained during augmented histamine tests were pooled and used after centrifugation to remove insoluble matter. Acid-free, blood- and bile-stained secretions were not used. They were obtained from ulcer patients through the courtesy of Dr. A. I. M. Glen, Western Infirmary, Glasgow.

ANTIPEPTIC ACTIVITY

(*a*) *Carrageenan added to enzyme (gastric juice) before digestion.* Digestion was by a method similar to that of Hunt (1948) at pH values between 1·5 and 3·75 using appropriate glycine buffers (Long, 1961). The substrate solution was 5% w/v whole human plasma protein dissolved in buffer at the appropriate pH. Any necessary adjustment of pH was by addition of dilute hydrochloric acid. The centrifuged gastric juice was diluted with an equal volume of buffer (control) or buffer containing the inhibitor (carrageenan). All solutions were at 37°. 1 ml of diluted gastric secretion or solution of carrageenan in gastric juice was added to 5 ml substrate solution and digestion allowed to proceed at 37° for 15 min, at the end of which trichloroacetic acid (10 ml, 0·35 N) was added. After 4 min the digests were filtered (Whatman No. 1) and to 2 ml filtrate, sodium hydroxide (20 ml, 0·25 N) was added, followed by 1 ml of Folin-Ciocalteu reagent, mixing being effected by swirling. Colour development required 15 min standing and the extinction was then read at 680 mμ. Following Hunt (1948), extinction values were converted to units. The differences between inhibited and uninhibited values was expressed as a fraction of the uninhibited after due allowance for blanks, which in addition to those prescribed by the method included one containing only gastric secretion and buffer. This fractional inhibition, i, which equals $1 - V_i/V$ where V_i and V represent digestion with and without inhibitor respectively, is also referred to as "inhibition".

(*b*) *Carrageenan added to substrate before digestion.* Amounts and concentrations of the components of the digestion mixture were as in (*a*) above. The appropriate amount of carrageenan in buffer solution was

added to a solution of substrate and mixed, followed by adjustment of volume and any necessary adjustment of pH. The diluted gastric secretion was then added and digestion was as in (*a*).

In methods (*a*) and (*b*), the digestion mixture (6 ml total) contained 2 mg of the undegraded carrageenan and 30 mg of the degraded carrageenan in solution.

(*c*) *Variation of substrate and inhibitor concentrations. Method* (*a*) was used at pH 2·2 and 3·2.

(i) With carrageenan concentration (I), constant at 1 mg undegraded, and 10 mg degraded, in the 6 ml digest, substrate concentrations (S) ranged from 100–300 mg. The same experiment was also performed in the absence of carrageenan.

(ii) Using two substrate concentrations (mg/6 ml digest), 125 and 250, the following carrageenan concentrations were used (mg/6 ml digest): 0, 0·15, 0·3, 0·6, 0·75, 1·25, 1·5, 2·0 (undegraded); and 0, 2·5, 10, 15, 20, 25, 30 (degraded).

In the digestion systems used, digestion increased linearly with time and substrate inhibition was absent.

SUBSTRATE-INHIBITOR INTERACTION

Increasing amounts of carrageenan in 0·2% sodium chloride solution were added to 1 ml volumes of plasma protein solution (0·25%) and the final volumes adjusted to 5 ml. The appearance of free carrageenan in the supernatant (detected by toluidine blue) indicated saturation of the protein. One series of experiments was made at pH 2·2, another at pH 3·2. The ratio mg protein : mg carrageenan at which free carrageenan first appeared in the supernatant is defined as the "saturation value" and is a measure of the protein-carrageenan interaction.

Results and discussion

Fig. 1 shows that when carrageenan was added to enzyme first, the inhibitions caused by the two carrageenans were similar up to pH 2·5, after which the inhibition caused by the undegraded carrageenan fell to a new level which was reached at pH 3·25, whilst the inhibition caused by degraded carrageenan remained reasonably constant throughout the pH range. It also shows that when the undegraded carrageenan was added to enzyme first, method (*a*), a significantly greater inhibition ($P < 0{\cdot}001$ at the pH of minimum difference in inhibition) always occurred than was obtained when substrate was added first. For degraded carrageenan no such difference occurred.

The marked difference in inhibition pattern between undegraded and degraded carrageenans as inhibitor concentration is increased at a constant pH of 2·0 in both methods (*a*) and (*b*), is shown in Fig. 2a, b; possible explanations of this difference in behaviour will be discussed later.

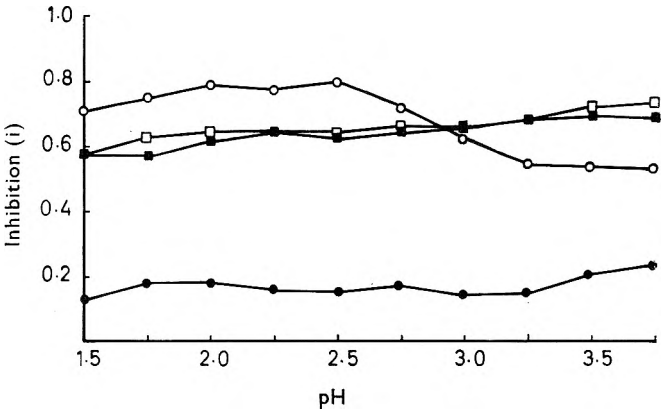

FIG. 1. Inhibition by two carrageenans of the peptic activity of human gastric secretion at various pH values; 6 ml digests contained: 0·5 ml gastric secretion, 250 mg plasma protein substrate, and either 2 mg undegraded, or 30 mg degraded carrageenan. ○, Undegraded carrageenan added to enzyme (gastric secretion) before addition of substrate. Significance of difference of inhibition by undegraded carrageenan at pH 2 and at pH 3·25, $P < 0{\cdot}01$. ●, Undegraded carrageenan added to substrate first. □, Degraded carrageenan added to enzyme first. ■, Degraded carrageenan added to substrate first. For undegraded carrageenan added to enzyme first each point is the mean of 20 determinations (n); added to substrate first, $n = 6$. For degraded carrageenan $n = 4$ in both instances.

Double reciprocal plots (Fig. 3a, b) and plots (Fig. 4a, b) of $1/V_i$ against I (Dixon, 1953) for undegraded and degraded carrageenans at pH 2·2 and pH 3·2 indicate substrate-inhibitor interaction as the mechanism of inhibition. Using the data of Figs 4 and 5, plots of S/V_i against S, V_i against V_i/S, and 1/i against 1/I also yielded curves typical (Webb, 1963) of substrate-inhibitor interaction.

Three mechanisms underlying inhibition of this general type can be distinguished (Reiner, 1959) by plotting I against i/(1—i). (*i*) Substrate depletion. (*ii*) Inhibition of the enzyme by a substrate-inhibitor complex. (*iii*) A combination of both mechanisms. The shape of such a plot for both undegraded and degraded carrageenans at pH 2·2 and pH 3·2 (Fig. 5a, b) is typical of inhibition effected by substrate depletion.

SUBSTRATE-INHIBITOR INTERACTION

In the substrate-inhibitor interaction studies, saturation values for undegraded and degraded carrageenans were approximately 4 for both types, indicating that the plasma substrate can remove one-quarter of its weight of either degraded or undegraded carrageenan, before free carrageenan appears in solution. In digestion studies maximum inhibition by degraded carrageenan is seen when the plasma protein-carrageenan ratio is also around 4, using methods (*a*) and (*b*). It therefore appears that maximum inhibition by degraded carrageenan is seen when the substrate is "saturated". In the case of undegraded carrageenan, however, although the saturation value is also about 4, maximum

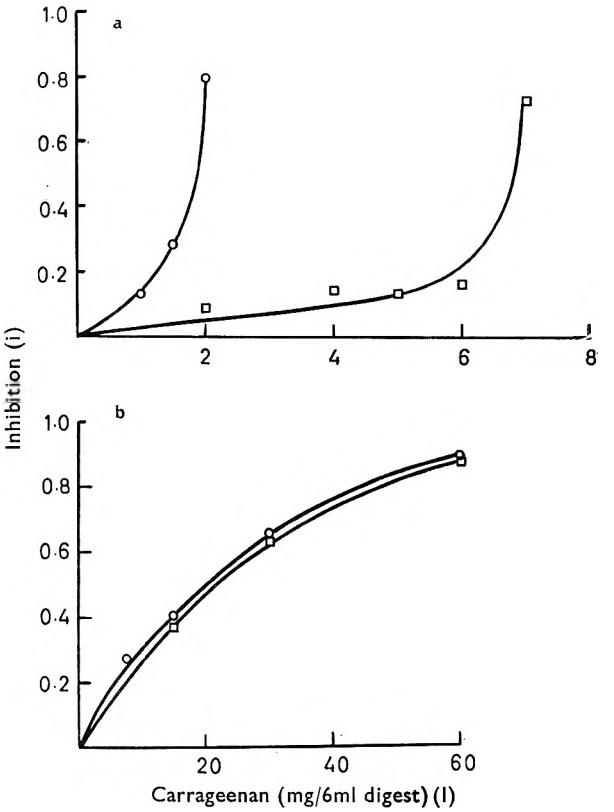

FIG. 2. Effect of increasing concentration of (a) undegraded, and (b) degraded carrageenan (I) on the inhibition (i) of the peptic activity of human gastric secretion. ○, Carrageenan added to enzyme first. □, Carrageenan added to substrate first.

inhibition is seen in method (*a*) when the substrate-inhibitor ratio by weight is about 125; in method (*b*) where the undegraded carrageenan displays lower activity, the ratio is reduced to 35 (Fig. 2a).

From Fig. 2a, b it can be calculated that, for i = 0·2 to 0·8 the ratio of weights of carrageenans degraded/undegraded varied between 5 and 23, method (*a*), and between 1 and 7, method (*b*).

THE DIFFERENCE BETWEEN UNDEGRADED AND DEGRADED CARRAGEENANS

Although undegraded and degraded carrageenans inhibit peptic activity by the same mechanisms, Figs 1 and 2 reveal points of difference. Since inhibition by carrageenan reflects interaction with protein substrate it would be remarkable if two carrageenans with such markedly different molecular weights (even though sulphate contents are similar) interacted with substrate protein similarly, to give identical inhibitions.

The results of the substrate-inhibitor interaction studies indicated that for undegraded carrageenan, saturation values were the same at pH 2·5

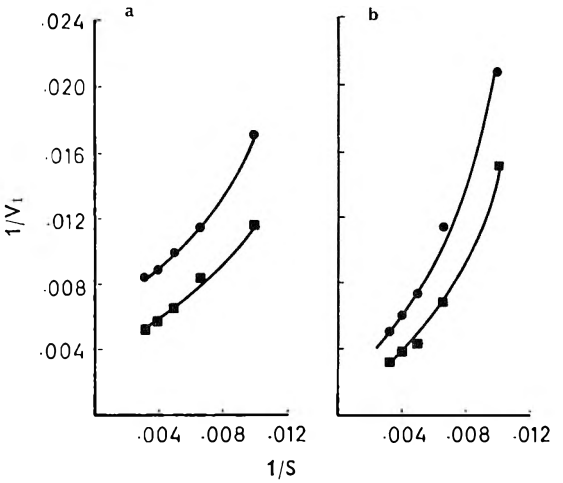

FIG. 3. Plot of $1/V_1$ against $1/S$ for (a) undegraded, and (b) degraded carrageenan at two pH values. 6 ml digest contained either 1 mg undegraded or 10 mg degraded carrageenan. ■, pH 2·2; ●, pH 3·2.

and pH 3·25, suggesting that the decrease in inhibition with pH (Fig. 1) was not due to quantitative difference in substrate-inhibitor interaction at the two pH levels. This suggests the possibility of qualitative differences at the two pH values in the interaction between undegraded carrageenan and protein responsible for the depletion or occlusion of substrate, one being more efficient in preventing access of enzyme than the other. Even allowing that at pH 3·25, peak gastricsin activity will occur, the possibility that, as an explanation of the results in Fig. 1, undegraded carrageenan acts principally on the proteolytic activity seen at pH 2·2 (pepsin) and not on that seen at pH 3·25 (gastricsin) is discounted by the similarity of the results in Figs 3–5 which do not point to a pH-dependent qualitative difference in inhibition type. With degraded carrageenan inhibition is unaffected by pH and there is therefore no reason to suspect that the structure of the protein-polysaccharide complex varies with pH in this range, at least insofar as it affects access of enzyme to protein.

With undegraded carrageenan the order of mixing of enzyme-substrate-inhibitor affects the level of inhibition and the appearances of the digests. Thus, when undegraded carrageenan is mixed with gastric secretion first, subsequent addition to substrate is followed by formation of large curds; the greater the amount of undegraded carrageenan the more colourless the supernatant becomes, suggesting greater depletion of protein from solution. On the other hand, when undegraded carrageenan is added to the substrate first, the appearance of the mixture after addition of gastric juice is similar to that seen when degraded carrageenan is used, a coarse precipitate of uniformly sized floccules being formed which are quite distinct in appearance from the curds. With the degraded carrageenan, order of addition affects neither the appearance of the digest nor the level of inhibition (Fig. 1).

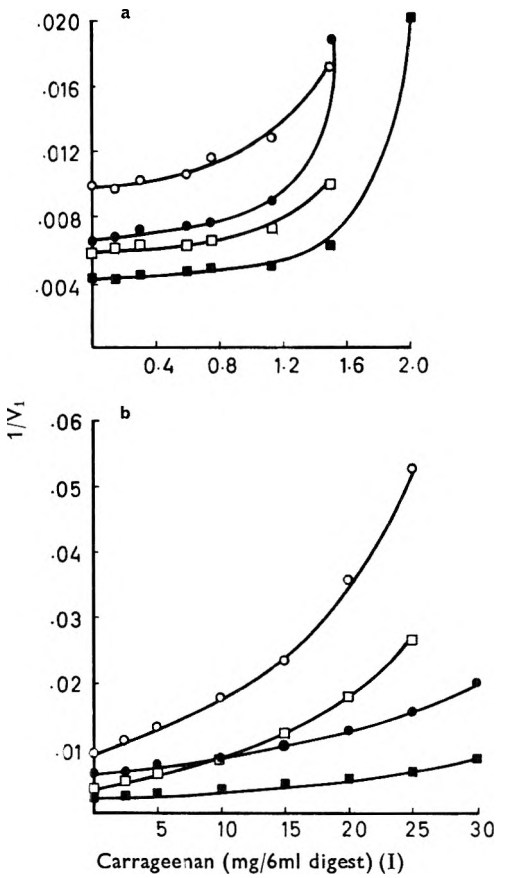

FIG. 4. Plot of $1/V_1$ against I for (a) undegraded, and (b) degraded carrageenan at two pH values and two substrate concentrations (S) (mg/6 ml digest). ○ = S,125; pH 3·2. ● = S,250; pH 3·2. □ = S,125; pH 2·2. ■ = S,250; pH 2·2.

Both types of carrageenan act by substrate depletion (Figs 3–5), but Fig. 2a, b reveal differences between the two carrageenans in rate of approach to complete inhibition with increasing inhibitor concentration. To examine this point a control experiment was made using decreasing amounts of substrate without inhibitor (to correspond to substrate depletion caused by inhibitor). A curve similar to Fig. 2a (undegraded carrageenan) was obtained when fractional reduction in digestion was plotted against decreasing substrate concentration (Fig. 6). Fractional reduction in digestion, r, equals $1 - v/V$ where V is the digestion at maximum substrate concentration and v is the digestion at various lower substrate concentrations.

The difference between Fig. 2a (undegraded carrageenan) and Fig. 2b (degraded carrageenan) suggests that substrate depletion (or occlusion) by degraded carrageenan from the enzyme system is not complete.

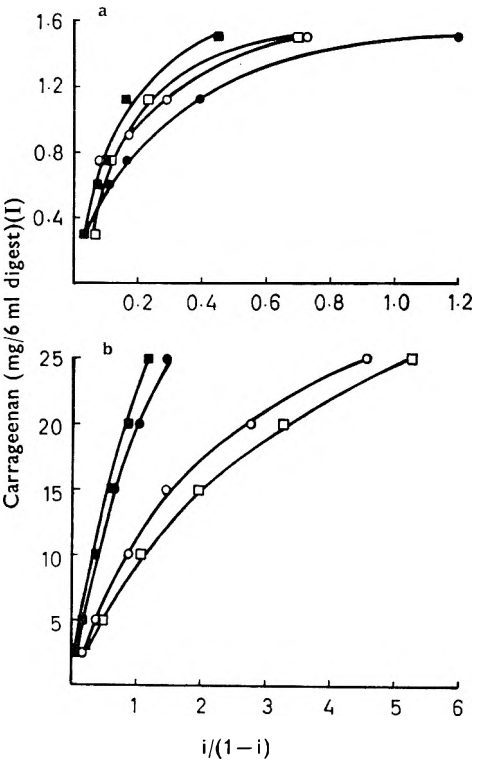

FIG. 5. Plot of I against i/1-i for (a) undegraded, and (b) degraded carrageenan at two pH values and two substrate concentrations (S)(mg/6 ml digest). ○ = S,125; pH 3·2 ● = S,250; pH 3·2. □ = S,125; pH 2·2. ■ = S,250; pH 2·2.

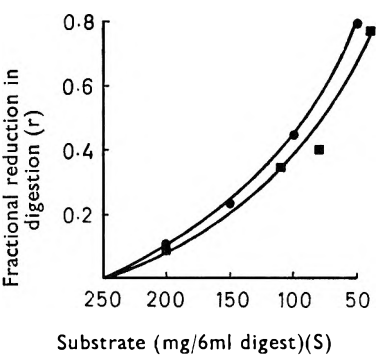

FIG. 6. The effect of decreasing substrate concentration, S, on fractional reduction (r) in digestion. ■ = pH 2·2; ● = pH 3·2.

These differences in inhibitory pattern between the two types of carrageenan could be explained by the differing molecular sizes. The larger carrageenan molecule with a large number of charged sites on the chains, more effectively binds greater amounts of protein substrate with the formation of a compact polysaccharide-protein barrier separating enzyme and substrate. The smaller, degraded carrageenan molecules on the other hand will interact with small numbers of protein molecules tending to form smaller complexes covered with a polysaccharide envelope which is less compact than that formed by the undegraded molecule, leaving some access for the pepsin to the protein unless high concentrations are used. This suggestion is in accord with the differences in appearance between the different protein-polysaccharide precipitates in the digests to which attention has already been drawn, and also with the additional amount of degraded carrageenan, compared with undegraded carrageenan, which is necessary for protection (i = 0·8) of the substrate. In terms of molarities (molecular weight of undegraded carrageen = 800,000; degraded carrageenan = 25,000), the difference (at i = 0·8) is around 200, method (*b*) and 700, method (*a*).

The similarity of the protein : degraded carrageenan ratio at saturation value and in the digest for maximum inhibition, supports the idea of a system wherein the degraded carrageenan and protein molecules react in small units. The marked difference, on the other hand, between the corresponding protein : undegraded carrageenan ratios suggests that one undegraded carrageenan molecule can effectively cover and protect from digestion many protein molecules.

It must be pointed out that although undegraded carrageenan can, in the present type of experiment, be shown to inhibit peptic activity more effectively, such a difference may not be realized clinically. The much more rapid and easy dissolution and greater solubility, coupled with the possibility of using greater dosage of degraded carrageenan would here assume greater importance. Indeed the time taken for the undegraded molecule to dissolve in gastric juice would result in a large amount of its potential activity being unavailable.

Acknowledgement. We thank Miss J. E. Harthill for technical assistance.

References

Anderson, W. (1961). *J. Pharm. Pharmac.*, **13**, 139–147.
Anderson, W. & Harthill, J. E. (1967). *Ibid.*, **19**, 460–467.
Black, W. A. P., Blakemore, W. R., Colquhoun, J. A. & Dewar, E. T. (1965). *J. Sci. Fd Agric.*, **16**, 573–585.
Dixon, M. (1953). *Biochem. J.*, **55**, 170–171.
Hunt, J. N. (1948). *Ibid.*, **42**, 104–109.
Long, C. (1961). *Biochemists' Handbook*, p. 30, London: Spon.
Reiner, J. M. (1959). *Behaviour of Enzyme Systems*, p. 156, Minneapolis: Burgess.
Seijffers, M. J., Miller, L. L. & Segal, H. L. (1964). *Biochemistry*, **3**, 1203–1209.
Tang, J., Wolf, S., Caputto, R., Trucco, R. E. (1959). *J. biol. Chem.*, **234**, 1174–1178.
Taylor, W. H. (1962). *Physiol. Rev.*, **42**, 519–553.
Webb, J. L. (1963). *Enzyme and Metabolic Inhibitors*, Vol. 1, London: Academic Press.

J. Pharm. Pharmac., 1967, **19**, 729–734 Received April 21, 1967

The dissolution of aspirin and aspirin tablets*

A. G. MITCHELL** AND DOROTHY J. SAVILLE

An attempt has been made to standardize the "beaker" method of measuring *in vitro* dissolution rates of tablets against published data for aspirin. An unexpected problem arose when it was found that samples of commercial aspirin have different intrinsic dissolution rates. The form of aspirin used in tablet manufacture is likely therefore to be of significance from the viewpoint of *in vivo* dissolution and drug availability.

FOR many drugs, dissolution is the rate-limiting factor in their absorption from the gastrointestinal tract. It has become increasingly apparent that the standards for a pharmaceutical product should include some measure of the availability of the drug and simple, convenient *in vitro* methods, previously correlated with *in vivo* studies are required for adequate quality control. Levy, Leonards & Procknal (1965) have said that the development of *in vitro* dissolution rate tests capable of reflecting absorption rate in man is one of the most important tasks in biopharmaceutics. The difficulties involved in the design and operation of such a test have been discussed by Morrison & Campbell (1965).

Aspirin is rapidly absorbed and is therefore a convenient drug to use in the design of an *in vitro* dissolution test. Levy has made an extensive study of the dissolution of aspirin and aspirin tablets using a "beaker" method (Levy & Hayes, 1960; Levy & Hollister, 1964). Excellent correlation was found between *in vitro* dissolution rates and *in vivo* absorption rates determined from measurements of plasma salicylate (Levy, 1961; Levy & Hollister, 1964; Levy & others, 1965). It is probable that the beaker method will prove satisfactory as a standard method of testing for drug availability. To establish this, however, further work is necessary using other drugs.

Dissolution rates determined by the beaker method depend on the dimensions of the stirrer and beaker, and on the stirring rate. It would be theoretically possible to duplicate the apparatus and the conditions used by Levy, but a more reliable approach would be to determine the stirring rate giving conditions of agitation equivalent to those used by Levy. Having established these conditions, the apparatus should be suitable, in principle, for evaluating the dissolution behaviour of other tablets.

We describe an attempt to "standardize" the beaker method against published data for aspirin, as a preliminary step to its application to other tablets. However, it became apparent that the intrinsic dissolution rate depended on the crystalline form of aspirin used.

From the Pharmacy Department, University of Sydney, Australia.

* From a paper presented to the 39th A.N.Z.A.A.S. Congress, Melbourne, January, 1967.

** Present address: Faculty of Pharmacy, University of British Columbia, B.C., Canada.

Experimental

Aspirin. Aspirin is available in a number of grades and crystalline forms. Some of these are suitable for tablet compression without preliminary treatment (British patent 810,050, U.S. patent 2,890,240).

Preliminary examination of samples of commercial aspirin showed them to have marked differences in intrinsic dissolution rate. The purity of each sample was established spectrophotometrically using recrystallized aspirin as a standard. Two samples showing the maximum difference in intrinsic dissolution rate were selected for more detailed investigation. These are designated form I* and form II**.

Aspirin tablets. Five brands of plain aspirin tablets were examined.

MEASUREMENT OF INTRINSIC DISSOLUTION RATE

(i) "*Beaker*" *method* (Levy & Procknal, 1964). Discs of 400 mg and 1·3 cm diameter were prepared by compression of finely ground aspirin at about 5,000 kg/cm² in a potassium bromide punch-die assembly. Each disc was mounted on a microscope cover slip with a suitable water-insoluble adhesive such as flexible collodion or hard paraffin, so that only one face remained exposed.

(ii) "*Rotating disc*" *method.* About 300 mg finely ground aspirin was compressed at about 3,500 kg/cm² in a compression punch and die assembly constructed according to the specifications of Wood, Syarto & Letterman (1965). The assembly was then used as the rotating disc holder at 430 rev/min.

Intrinsic dissolution rates were independent of pressure over the compression range 2,000 to 13,000 kg/cm². The intrinsic dissolution rate was also independent of the particle size of aspirin used in preparing the compressed discs.† Hence no attempt was made to standardize the particle size although normally each sample was ground in an agate mortar before compression. At least two replicate determinations were made and the results averaged. The reproducibility was within ±2% for form I and ±3% for form II.

Dissolution of tablets. The dissolution behaviour of aspirin tablets was investigated using the "beaker" method of Levy & Hayes (1960) with the modifications described by Levy & Hollister (1964). On completion of each dissolution experiment the remaining aspirin was dissolved to enable calculation of the percentage of drug dissolved. Up to 7 replicates were made on each brand of tablet and the results were averaged.

All dissolution experiments were at 37° in 0·1N hydrochloric acid. 5 ml samples were withdrawn at suitable intervals, by pipette, using a glass-wool filter when necessary. The samples were diluted appropriately with 0·1N hydrochloric acid and analyzed spectrophotometrically at 278 and 305 mμ for aspirin and salicylic acid respectively. The total amount of aspirin dissolved was calculated using simultaneous equations (Willard, Merritt & Dean, 1958).

* Aspirin No. 3 special. ** Asagran 4D (Monsanto, Chemicals, Australia).

† Micronized aspirin, sample J, supplied by Nicholas Pty. Ltd. showed the same intrinsic dissolution rate as the same material not subjected to the micronizing process (Table 1).

Results and discussion

Intrinsic dissolution rates were calculated from the slope of the dissolution curve as shown in Fig. 1, and the surface area of the compressed disc of aspirin.

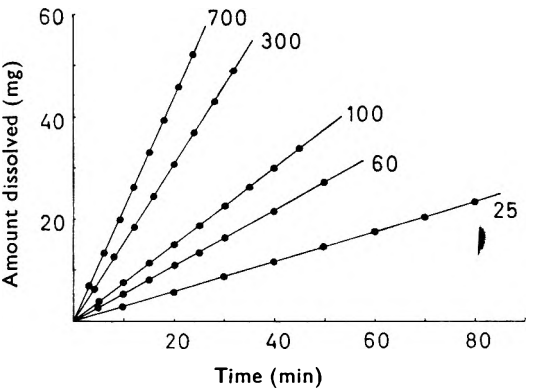

FIG. 1. The dissolution of aspirin at various stirring rates (rpm). Beaker method using compressed discs (diameter 1·3 cm) of aspirin (form I) in 0·1N HCl at 37°.

Levich (through Cooper & Kingery, 1962) derived an equation, relating intrinsic dissolution rate, DR, and rotation rate, RR. Levy & Procknal (1964) expressed this in the form

$$\log DR = a \log RR + C \qquad (1)$$

where a and C are constants.

Fig. 2 shows the intrinsic dissolution rates of aspirin form I and II, plotted as a function of stirring rate over the range 25–700 rev/min. If the dissolution process is purely transport controlled the plot of log DR

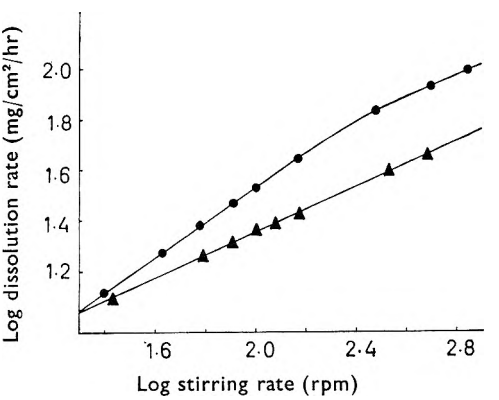

FIG. 2. Variation of intrinsic dissolution rate of aspirin with stirring rate. Beaker method using compressed discs of aspirin: form I, ●; form II, ▲; in 0·1N HCl at 37°.

versus log RR should be a straight line where the slope, a, is approximately 0·5 (Riddiford & Bircumshaw, 1952). If there is a change in control with RR the plot is non-linear with a maximum slope $<0{\cdot}5$ (Bircumshaw & Riddiford, 1952). In the present work the dissolution of form II appears to be transport controlled since $a = 0{\cdot}45$. Form I, however, shows a non-linear plot with a maximum slope of 0·65 which indicates that some additional process is involved. Work is continuing on this point. At high stirring rates the DR of form I is 75% greater than that of form II. The difference becomes smaller as the stirring rate is reduced and disappears at about 20 rev/min.

Using the beaker method at 59 rev/min, Levy & Procknal (1964) found that DR for aspirin was 24·6 mg/cm²/hr. On the assumption that Levy used form I the rate of stirring necessary to produce the same DR is 62 rev/min.

The dissolution of several commercial aspirin tablets was followed at 62 rev/min, and the results are shown in Fig. 3. Differences are apparent between the behaviour of the different brands, particularly between

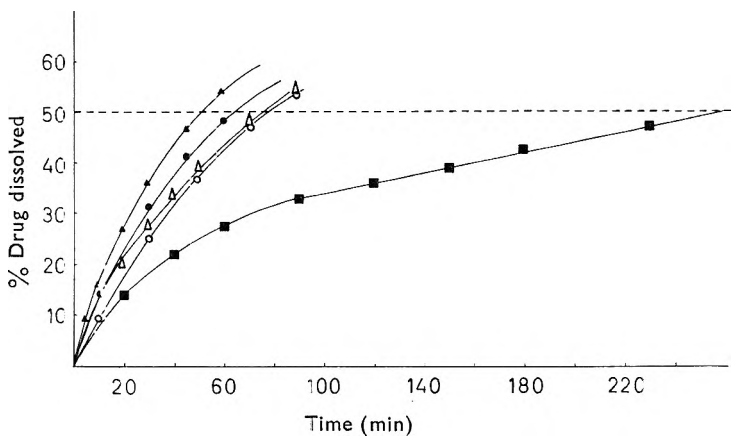

FIG. 3. *In vitro* dissolution of commercial aspirin tablets, P ▲, Q ●, R △, S ○, T ■. Beaker method at 62 rpm in 0·1N HCl at 37°.

brand T and the remaining brands. In each instance, however, dissolution is rapid at first and then decreases in a non-exponential manner. Dissolution behaviour was compared on the basis of the time required for 50% of the aspirin to dissolve, t50%. Values of t50% range from 51 to 267 min and are much longer than times reported by Levy & Hayes (1960). The discrepancy suggests that either there is a marked difference between the tablets tested by Levy and those tested in the present work, or a rotation rate of 62 rev/min does not provide conditions of agitation equivalent to those used by Levy. The variation of t50% with stirring rate for two of the brands of aspirin tablets shown in Fig. 4 emphasizes the importance of the correct choice of stirring rate.

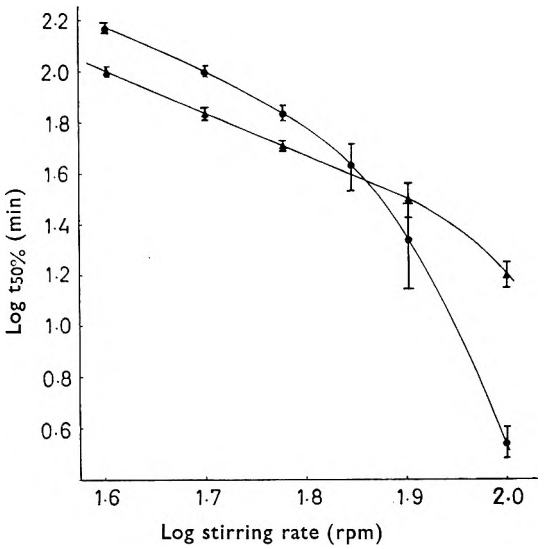

FIG. 4. Variation of *in vitro* dissolution half-lives, t50% of aspirin tablets P ▲, and Q ●, with stirring rate. Beaker method using 0·1N HCl at 37°. Vertical bars show ± 1 s.d.

If we assume that Levy used form II, then from Fig. 2 the stirring rate necessary to produce DR equal to 24·6 mg/cm²/hr is 120 rev/min. This stirring rate would bring the values of t50% for tablets closer to the values reported by Levy & Hayes but one of the conditions of the test is that the disintegrated tablet must remain as an aggregate on the bottom of the beaker. At 120 rev/min in our apparatus, particles of the disintegrated tablet circulate in the dissolution medium.

It is probable that the aspirin used by Levy was not the same as either form I or form II. Intrinsic dissolution rates depend on the conditions of agitation and it is difficult therefore to make comparisons with other published data. However, Wood & others (1965) have determined a value for aspirin using a rotating disc method. Values of DR were therefore measured using the dissolution apparatus of Wood. The results are shown in Table 1. The intrinsic dissolution rate of form I

TABLE 1. INTRINSIC DISSOLUTION RATES OF COMMERCIAL ASPIRIN. ROTATING DISC METHOD IN 0·1N HCL AT 37°

Aspirin sample	Intrinsic dissolution rate (mg/cm²/min)
Wood, Syarto & Letterman (1965)	1·75
B (form I), C and G	1·75
J (before and after micronizing)	1·70
D	1·54
H	1·54
E	1·25
A (form II)	0·995

agrees with the value reported by Wood, and is 75% greater than form II in agreement with the results found using the beaker method at high stirring rates. The intrinsic dissolution rates of a number of the other aspirin samples tested lie between the values found for form I and form II and are included in Table 1.

Variations in intrinsic dissolution rate of crystalline aspirin make it difficult to standardize the beaker method against published data. Moreover it is apparent that the dissolution rate, or t50%, of compressed aspirin tablets will depend not only on the formulation and factors such as particle size but also on the form of aspirin used. The effect on dissolution rate of differences in polymorphic form has been studied by Hamlin, Nelson & others (1962), Levy & Procknal (1964), Wurster & Taylor (1965) and Higuchi, Bernado & Mehta (1967). Preliminary crystallographic studies on aspirin, however, indicate that the differences in intrinsic dissolution rate are not a result of polymorphism.

Acknowledgements. We are grateful to Nicholas Pty. Ltd., Burroughs Wellcome Co. (Aust.) Ltd. and Reckitt and Colman Pty. Ltd. for samples of aspirin and aspirin tablets.

References

Bircumshaw, L. L. & Riddiford, A. C. (1952). *Q. Rev.*, **6**, 157–185.
Cooper, A. R. & Kingery, W. D. (1962). *J. phys. Chem., Ithaca*, **66**, 665–669.
Hamlin, W. E., Nelson, E., Ballard, B. E. & Wagner, J. G. (1962). *J. pharm. Sci.*, **51**, 432–435.
Higuchi, W. I., Bernado, P. D. & Mehta, S. C. (1967). *Ibid.*, **56**, 200–207.
Levy, G. (1961). *Ibid.*, **50**, 388–392.
Levy, G. & Hayes, B. A. (1960). *New Engl. J. Med.*, **262**, 1053–1058.
Levy, G. & Hollister, L. E. (1964). *J. pharm. Sci.*, **53**, 1446–1452.
Levy, G. & Procknal, J. A. (1964). *Ibid.*, **53**, 656–658.
Levy, G., Leonards, J. R. & Procknal, J. A. (1965). *Ibid.*, **54**, 1719–1722.
Morrison, A. B. & Campbell, J. A. (1965). *Ibid.*, **54**, 1–8.
Riddiford, A. C. & Bircumshaw, L. L. (1952). *J. chem. Soc.*, **59**, 698–701.
Willard, H. H., Merritt, L. L. & Dean, J. A. (1958). *Instrumental Methods of Analysis*, 3rd edn, p. 120, Princeton, New Jersey: Van Nostrand.
Wood, J. H., Syarto, J. E. & Letterman, H. (1965). *J. pharm. Sci.*, **54**, 1068.
Wurster, D. E. & Taylor, P. W. (1965). *Ibid.*, **54**, 670–676.

J. Pharm. Pharmac., 1967, **19**, 735–739 Received March 1, 1967

The effect of concentration, time and temperature on the viability of *Penicillium notatum* spores exposed to phenols

N. M. CHAUHAN* AND V. WALTERS†

Spores of *Penicillium notatum* exposed to resorcinol, *p*-chlorophenol, *m*-cresol, chlorocresol and hexylresorcinol, give values for the concentration exponent, n, from 7·3 to 3·7 when calculated from first order reaction velocities; higher values are obtained from the times for fixed mortality levels. Values of the temperature coefficient, θ, vary from 1·045–1·331. The log survivor-time curves are convex to the axes for resorcinol and increasingly concave in the order of decreasing water solubility for the other phenols. Equitoxic (99% mortality in 20 min) molar concentrations of the phenols have about a 680-fold range, but Ferguson values, except for resorcinol, vary only from 0·10 to 0·26.

THE viability of *Penicillium notatum* spores exposed to phenol has been investigated by Chauhan & Walters (1961, 1962). We now describe the change in activity resulting from the introduction of various substituents into the phenol molecule.

Experimental

Aqueous solutions of *m*-cresol (Laboratory Reagent) 2%, resorcinol B.P. 10%, *p*-chlorophenol (Laboratory Reagent) 1%, chlorocresol B.P. 0·25%, hexylresorcinol B.P. 0·4%, were stored at 4° and diluted as required. The activity of these compounds at 25° was examined in the same way as that of phenol (Chauhan & Walters, 1961, 1962), using suspensions containing $6{\cdot}25 \times 10^6$ ml^{-1} *P. notatum* spores. Counts were made after incubation at 28° for 12 hr, and, when hyphal growth was not too dense to permit accurate counting, at intervals up to 48 hr, to ensure maximum recovery.

Results

Spores remaining viable after exposure to resorcinol and phenol, germinated within 12 hr, but 14 hr were necessary with the other phenols. When there were few survivors, germination occurred up to 20 hr after exposure. Untreated spores germinated within 10 hr. Spore mortality increased with the concentration of phenols, reaction time and temperature (Figs 1 and 2). The shape of the log survivor curves for spores exposed to 0·08% chlorocresol at 5° increments in temperature from 15–40°, was the same as for the effect of concentration in Fig. 1.

Discussion

The values of the concentration exponent, n, calculated from reaction velocities, are less than those calculated from the times for particular mortalities (Table 1). This discrepancy occurs because of the greater

From the Department of Pharmacy, University of Ife, Ibadan Branch, Ibadan, Nigeria.

* School of Pharmacy, Haile Sellassie I University, Addis Ababa. † The School of Pharmacy, University of London, 29–39, Brunswick Square, W.C.1.

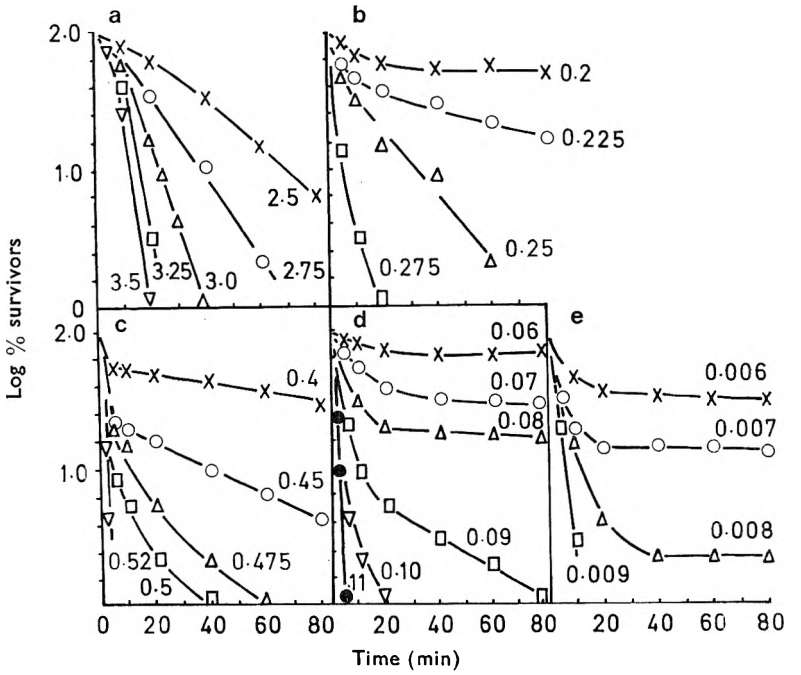

FIG. 1. Log % survivor-time curves for *P. notatum* spores exposed to (a) resorcinol, (b) *p*-chlorophenol, (c) *m*-cresol, (d) chlorocresol and (e) hexylresorcinol. Figures on curves are % concentrations.

divergence of the rate of fungicidal reaction from first order kinetics, over the wider concentration range used in the former method of calculation.

Comparable values of n and the temperature coefficient, θ (Table 2), for phenols have been obtained with bacteria (Chick, 1908; Tilley, 1939, 1942; Withell, 1942; Jordan & Jacobs, 1945, 1946) and bacteriophage (Brown, Cook & Oduro-Yeboah, 1965). The values of n increased with temperature for chlorocresol and hexylresorcinol, but showed little change

TABLE 1. CONCENTRATION EXPONENTS (n) FOR THE ACTIVITY OF PHENOLS ON *P. notatum* SPORES

Compound	n	
	a	b
Phenol	6·9 (0·5–1·25)*	11·6 (1·0–1·25)
Chlorocresol	5·6 (0·06–0·11)	13·8 (0·09–0·11)
m-Cresol	7·0 (0·4–0·5)	37·0 (0·475–0·52)
p-Chlorophenol	7·3 (0·2–0·275)	13·7 (0·25–0·275)
Resorcinol	6·2 (2·5–3·5)	5·2 (2·75–3·5)
Hexylresorcinol	3·7 (0·006–0·009)	7·1 (0·007–0·009)

Mean values of n calculated using Watson's (1908) equation:

(*a*) from first order reaction velocities, k min^{-1}, for each concentration;

(*b*) from times for 99% mortality (75% for hexylresorcinol) read from survivor curves. Values of n from the calculated slopes of log time for 75% mortality—log concentration regressions are similar to (b).

* Range of concentrations.

TABLE 2. TEMPERATURE COEFFICIENTS (θ) FOR THE ACTIVITY OF PHENOLS ON *P. notatum* SPORES

Compound	Concentration %	Temperature range ° C	θ
Chlorocresol ..	0·07	15–40	1·045
	0·08	15–40	1·060
	0·09	15–35	1·081
m-Cresol ..	0·4	15–40	1·075
	0·45	15–35	1·045
p-Chlorophenol	0·25	15–30	1·120
Resorcinol ..	3·25	15–30	1·331
	3·50	15–25	1·276
Hexylresorcinol	0·005		1·055
	0·006	15–40	1·055
	0·007		1·095

θ calculated by substitution of reaction velocities in equation of Phelps (1911).

TABLE 3. EFFECT OF TEMPERATURE ON CONCENTRATION EXPONENTS OF PHENOLS ON *P. notatum* SPORES

Compound	Concentration range %	n					
		Temperature ° C					
		15	20	25	30	35	40
Chlorocresol ..	0·07–0·09	3·1	2·7	4·0	5·6	5·5	6·4
m-Cresol	0·4–0·45	9·6	6·9	6·9	6·7	6·9	—
Resorcinol ..	3·25–3·5	11·2	2·9	6·4	—	—	—
Hexylresorcinol ..	0·005–0·007	2·5	2·9	4·2	5·5	5·4	5·5

θ calculated from reaction velocities.
Experiment not done with *p*-chlorophenol.

for *m*-cresol, and a variable response for resorcinol (Table 3). With bacteria, Berry & Michaels (1950) found a decrease with ethylene glycol ethers, whereas Tilley (1939) noted both an increase and a decrease with phenols. The activity of the phenols used was not modified by ionization, since their pK_a values are about 9–10 (Kortum, Vogel & Andrussow, 1961) and the pH values of the reaction systems were about 5·5. The effect of substituting a *m*-hydroxy group in the phenol molecule was to decrease its relative per cent activity about threefold, but a *m*- Me, *p*- Cl, *m*- Me and *p*- Cl, or *p*- $CH_2(CH_2)_4Me$, increased it about 2, 4, 11 and 125 times respectively (Table 4). The order of molar solubility of the phenols in water correlates with this increase in activity and approximates to the change in shape of the survivor curves, that is, convex to the axes for the very soluble resorcinol, linear for phenol and concave to the axes for the other phenols. The distal portions of the curves for the latter phenols become increasingly flatter in the order of decreasing solubility. The lipid solubility of phenol is increased by a substituent -Cl or alkyl group whereas an -OH decreases it. The cell membrane being of a lipoprotein is penetrated more readily by neutral molecules possessing lipophilic groups (Davson & Danielli, 1952; Albert, 1963, 1965). It is likely, therefore, that the initial rate of reaction, and hence the shape of the proximal portion of the log survivor-time curve, is determined by the lipid solubility of the compound. Thus Richardson & Reid (1940), Fogg & Lodge (1945) and Bean, Heman-Ackah & Thomas (1965) have related the

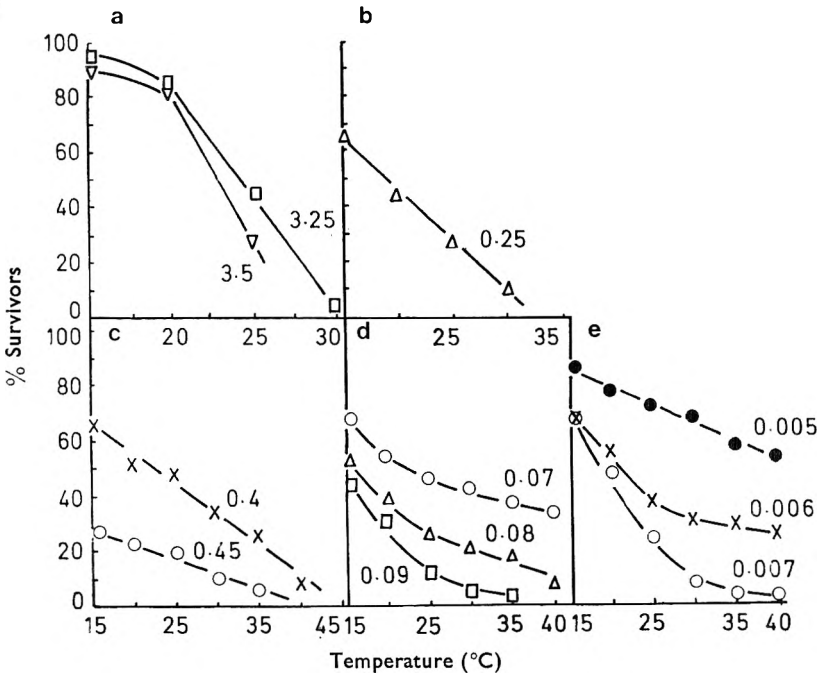

FIG. 2. % Survivor-temperature curves for *P. notatum* spores exposed for 10 min to phenols as in Fig. 1.

antibacterial activity of phenols to their o/w partition coefficients. Presumably, with the highly polar compound, resorcinol, penetration of the lipid layer of the cell membrane occurs slowly with a consequent lag in action. With phenol there was no evident lag while the more lipid soluble compounds act rapidly initially, because adsorption and penetration of the lipid barrier to reaction sites can occur more readily. It is likely that an equilibrium distribution, as reported by Bean & Das (1966), is set up in the systems. Unless more cell receptor sites can be occupied

TABLE 4. RELATIVE ACTIVITIES OF PHENOLS ON *P. notatum* SPORES

	Resorcinol	Phenol	*m*-Cresol	*p*-Chlorophenol	Chlorocresol	Hexylresorcinol
Conc (%) for 99% mortality in 20 min ..	3·5	1·125	0·515	0·275	0·1	0·009
Molarity (St)	0·318	0·119	0·0476	0·0214	0·007	0·00046
Relative activity (%) ..	0·32	1	2·2	4·1	11·3	125
Relative molar activity ..	0·38	1	2·5	5·6	17	258
Approx. solubility in water 1 in	1	12	42	37	260	2000
Solubility in mole/litre (S_o)	9·083	0·885	0·22	0·21	0·027	0·0026
Ferguson value $\frac{St}{So}$ ..	0·035	0·14	0·22	0·1	0·26	0·18

by partitioning of phenol, through increasing the concentration of the aqueous phase, mortality ceases or at least is much reduced, as, for example, with hexylresorcinol. Thus the apparent resistance of the spore population would appear to be relative to the toxic agent, giving rise to survivor curves of different shape.

Table 4 shows that when equitoxic molar concentrations are compared, there is about a 680-fold range in activity. When the thermodynamic activity is calculated (Ferguson, 1939) the variation is only about 7·4-fold, and, except for resorcinol, the aqueous phase is toxic at about 1/10 to 1/4 phenol saturation. The latter values indicate that the lipid solubility of the phenol molecule could be increased, by substitution, beyond that of hexylresorcinol, to produce equitoxic compounds active in greater dilution, since the aqueous phase saturation of the toxic concentration of hexylresorcinol is only 0·18.

References

Albert, A. (1963). *Adv. appl. Microbiol.*, **5**, p. 28, London: Academic Press.
Albert, A. (1965). *Selective Toxicity*, 3rd edn, pp. 178–221, London: Methuen.
Bean, H. S. & Das, A. (1966). *J. Pharm. Pharmac.*, **18**, *Suppl.*, 107S–113S.
Bean, H. S., Heman-Ackah, S. M. & Thomas, J. (1965). *J. Soc. Cosmet. Chem.* (Br. Edn), **16**, 15–30.
Berry, H. & Michaels, I. (1950). *J. Pharm. Pharmac.*, **2**, 105–114.
Brown, W. R. L., Cook, A. M. & Oduro-Yeboah, J. (1965). *Ibid.*, **17**, *Suppl.*, 28S–32S.
Chauhan, N. M. & Walters, V. (1961). *Ibid.*, **13**, 470–478.
Chauhan, N. M. & Walters, V. (1962). *Ibid.*, **14**, 605–610.
Chick, H. (1908). *J. Hyg., Camb.*, **8**, 92–158.
Davson, H. & Danielli, J. (1952). *The permeability of natural membranes*, 2nd edn, pp. 61–63, Cambridge: University Press.
Ferguson, J. (1939). *Proc. R. Soc.*, **127B**, 387–404.
Fogg, A. H. & Lodge, R. M. (1945). *Trans. Faraday Soc.*, **41**, 359–365.
Jordan, R. C. & Jacobs, S. E. (1945). *J. Hyg., Camb.*, **44**, 210–220.
Jordan, R. C. & Jacobs, S. E. (1946). *Ibid.*, **44**, 243–248.
Kortum, G., Vogel, W. & Andrussow, K. (1961). *I.U.P.A.C., Dissociation constants of organic acids in aqueous solution*, London: Butterworths.
Phelps, E. B. (1911). *J. infect. Dis.*, **8**, 27–38.
Richardson, E. M. & Reid, E. E. (1940). *J. Am. chem. Soc.*, **62**, 413–415.
Tilley, F. W. (1939). *J. Bact.*, **38**, 499–510.
Tilley, F. W. (1942). *Ibid.*, **43**, 521–525.
Watson, H. E. (1908). *J. Hyg., Camb.*, **8**, 536–542.
Withell, E. R. (1942). *Q. Jl Pharm. Pharmac.*, **15**, 301–313.

J. Pharm. Pharmac., 1967, **19**, 740–743 Received April 7, 1967

A note on some changes in the physical properties of *Escherichia coli* after heat treatment

DIANN HARRIES AND A. D. RUSSELL

Suspensions of *Escherichia coli* in water at 50, 55, 60 and 100° showed an increase in extinction and nephelometer readings, the rate of increase depending on the temperature. This is considered to represent varying degrees of alteration in bacterial cytoplasmic protein. No such increases at any temperature occurred with cells held in 0·9% w/v sodium chloride. Lysozyme induced no lysis in cells which had been heated in water or in saline.

IN 1967, Russell & Harries observed that suspensions of *Escherichia coli* which were held at temperatures of 50–60° and at 100° leaked intracellular constituents into the surrounding medium, the rate of leakage depending on the temperature used. Leakage was not prevented by 0·33 M sucrose. These authors also found that heated suspensions showed an increase in extinction. Heated and unheated suspensions of *E. coli* have been examined further for this effect; the results are now reported.

Experimental

Details of the organism (*E. coli* Type 1), its growth and the preparation of suspensions containing about 10^9 viable cells/ml in water and in 0·33 M sucrose, and the heating procedure have been described previously (Harries & Russell, 1966; Russell & Harries, 1967). In some experiments, the suspending medium in which the cells were heated consisted of 0·9% w/v sodium chloride.

The temperatures were 50°, 55°, 60° and 100°. Samples of suspensions held at these temperatures were removed at intervals, and the extinction at 500 mμ read in the SP 600 spectrophotometer, using 1 mm cells and the appropriate blank (suspension medium only); further samples were used for measuring turbidity with the EEL nephelometer.

Effect of lysozyme on heated and unheated suspensions. Concentrated solutions of egg-white lysozyme (British Drug Houses, Ltd., London) were prepared and sterilized by filtration. Unheated suspensions, and suspensions held at the desired temperature for 30 min in water or 0·9% w/v sodium chloride, were centrifuged at 2000 rev/min for 40 min, the supernatant fluids removed, and the pellets resuspended in M/15 phosphate buffer, pH 7·4. Each suspension was divided into 2 × 9 ml aliquots: to one was added 1 ml of a lysozyme solution, to give the desired final concentration; to the other was added 1 ml of sterile water. All aliquots were incubated at 37°. Samples were removed at intervals, diluted 1 in 5 with phosphate buffer, pH 7·4, and the extinction at 500 mμ determined, as previously described, using the appropriate blank. Samples were also examined microscopically (phase-contrast, ×400). Experiments were also made in which water replaced the phosphate buffer.

From the Welsh School of Pharmacy, Welsh College of Advanced Technology, Cardiff.

Results and discussion

Both the extinction and nephelometer readings of aqueous suspensions of *E. coli* increased after being maintained at various temperatures, the rate of increase depending on the temperature (Fig. 1). An increase in extinction may represent either gross changes in bacterial cytoplasmic protein (Beckett, Patki & Robinson, 1959; Gilby & Few, 1960), or a decrease in bacterial cell volume (Mager, Kuczynski & others, 1956; Avi-Dor, Kuczynski & others, 1956; Brock, 1958; Bernheim, 1963),

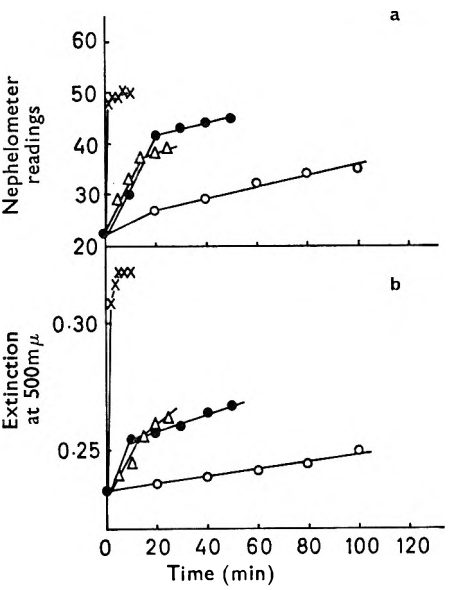

FIG. 1. Changes in (a) nephelometer readings, and (b) extinction, of aqueous suspensions of *E. coli* held at various temperatures. 50°, ○——○; 55°, △——△; 60°, ●——●; 100°, ×——×.

whereas an increase in nephelometer readings, in the absence of cell multiplication, may be associated with an increase in bacterial cell size, although such increased turbidity would also occur on coagulation of proteins. Despite the lack of adequate experimentation in this field to date (Hansen & Riemann, 1963), the coagulation of proteins has been put forward as the reason for the death of bacteria by moist heat (see for example, Sykes, 1965). However, when suspensions of *E. coli* were held in saline and exposed to temperatures of 50°–60° and 100°, nephelometer readings did not increase, and there was a small, but detectable and reproducible, decrease in extinction (Fig. 2). The reasons for these findings are not apparent, particularly as it is known that the protoplasm of *E. coli* cells becomes coarsely granular during their heating in saline (Heden & Wyckoff, 1949; Hansen & Riemann, 1963).

Because sucrose has been used to stabilize spheroplasts of this strain of *E. coli* (Barnett & Russell, 1967), and is thus relatively non-penetrating

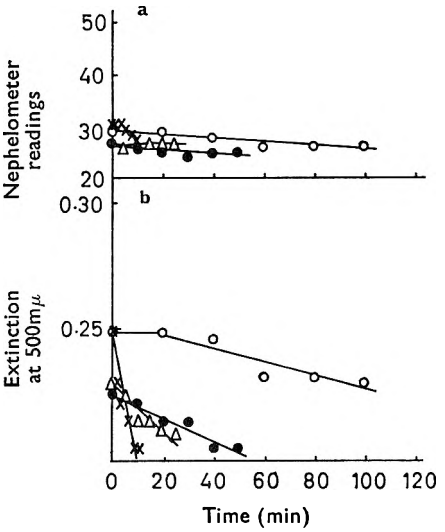

FIG. 2. Changes in (a) nephelometer readings, and (b) extinction, of suspensions of *E. coli* held in 0·9% w/v sodium chloride at various temperatures. 50°, ○——○; 55°, △——△; 60°, ●——●; 100°, ×——×.

into this organism, its effect, at a concentration of 0·33 M, on the extinction of heated cells was investigated. The results indicated that there was a similar response to that demonstrated by cells suspended in water.

The leakage of intracellular constituents from heated suspensions of this organism is not prevented by sucrose (Russell & Harries, 1967). Thus, one of the main effects of moist heat is on the cytoplasmic membrane. It is of interest, in this context, to consider the effect of heat in the Ziehl-Neelsen staining method for *Mycobacterium tuberculosis*; the effect of heat here is to render the waxy material more permeable to aqueous dyes, and this could depend on the existence of a semi-permeable membrane around the organisms which allows fuchsin to diffuse into the cell, but prevents acid fuchsin from diffusing out (Wilson & Miles, 1964).

The effect of lysozyme on heated and unheated (control) suspensions was examined because (*a*) it had previously been found (Russell & Harries, 1967) that the total counts of suspensions heated in water remained constant, which suggested that there was no significant effect on the cell wall of the organism; (*b*) it had been shown by Hoffman, Valinda & Frank (1966) that cells of *E. coli* which had been grown at 45° became swollen when suspended in distilled water, but not in 0·8% w/v sodium chloride, and were lysed when treated with egg-white lysozyme; (*c*) it was hoped that, by treating with lysozyme cells that had been heated in water and saline, information would be obtained on the nature of the differences observed earlier. Accordingly, the effects of lysozyme in phosphate buffer at 37° on cells previously held for 30 min in water or saline at 20°, 50°, 55° and 60° were investigated; the enzyme was used at concentrations of 0, 2·5, 25 and 250 μg/ml. In no instance was there any evidence

of lysis, as determined at 500 mμ or microscopically, over a period of 3½–4½ hr. It must also be pointed out that at the highest lysozyme concentration used, some precipitation (agglutination of cells) occurred; this readily dispersed on shaking to give an even turbidity. A similar finding was made by Salton (1953). The replacement of phosphate buffer with water or with buffer pH 6·2 had no effect on the action of lysozyme.

Heating of suspensions at 100° for more than 10 min resulted in clumping and precipitation of the cells, and such suspensions were therefore not used for studying subsequent lysozyme action.

Thus, the results suggest that moist heat at temperatures of 50–60° does not damage the outer layers of the cell wall of *E. coli* sufficiently to permit the lysozyme molecule to reach its mucopeptide substrate (Salton, 1957, 1964), although lysozyme might not induce lysis in cells in which the protein had been coagulated (Salton, 1953). However, Carson & Eagon (1966) showed that with *Pseudomonas aeruginosa*, the mucopeptide component is not solely responsible for the structural integrity of the cell wall, and this may be so also for the wall of other Gram-negative organisms.

The overall result of our experiments is to indicate that moist heat causes an alteration in the cytoplasmic protein of *E. coli* cells suspended in water, but thermal injury cannot be reconciled with damage to the cell wall. Although protein coagulation could be put forward as being the primary reason for thermal damage, this is not necessarily so, as there is also a release of intracellular constituents (Russell & Harries, 1967). Moreover, in at least some bacterial species (Strange & Shon, 1964; Iandolo & Ordal, 1966) there is a breakdown of ribonucleic acid in heated cells. It is interesting to note that until recently, no really critical experiments had been made to investigate the nature of thermal death in non-sporing bacteria (Wood, 1956; Hansen & Riemann, 1963).

References

Avi-Dor, Y., Kuczynski, M., Schatzberg, G. & Mager, J. (1956). *J. gen. Microbiol.*, **14**, 76–83.

Barnett, M. I. & Russell, A. D. (1967). *J. Med. Lab. Tech.*, **24**, 113–118.

Beckett, A. H., Patki, S. J. & Robinson, A. E. (1959). *J. Pharm. Pharmac.*, **11**, 367–373.

Bernheim, F. (1963). *J. gen. Microbiol.*, **30**, 53–58.

Brock, T. D. (1958). *Can. J. Microbiol.*, **4**, 65–71.

Carson, K. J. & Eagon, R. G. (1966). *Ibid.*, **12**, 105–109.

Gilby, A. R. & Few, A. V. (1960). *J. gen. Microbiol.*, **23**, 19–26.

Hansen, N. H. & Riemann, H. (1963). *J. appl. Bact.*, **26**, 314–333.

Harries, D. & Russell, A. D. (1966). *Experientia*, **22**, 803–804.

Heden, C. G. & Wyckoff, R. W. G. (1949). *J. Bact.*, **58**, 153–160.

Hoffman, H., Valinda, J. & Frank, M. E. (1966). *Ibid.*, **91**, 1635–1636.

Iandolo, J. J. & Ordal, Z. J. (1966). *Ibid.*, **91**, 134–142.

Mager, J., Kuczynski, M., Schatzberg, G. & Avi-Dor, Y. (1956). *J. gen. Microbiol.*, **14**, 69–75.

Russell, A. D. & Harries, D. (1967). *Appl. Microbiol.*, **15**, 407–410.

Salton, M. R. J. (1953). *J. gen. Microbiol.*, **9**, 512–523.

Salton, M. R. J. (1957). *Bact. Rev.*, **21**, 82–100.

Salton, M. R. J. (1964). *The Bacterial Cell Wall*, Amsterdam: Elsevier.

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

Sykes, G. (1965). *Disinfection & Sterilization*, 2nd edn, London: Spon.

Wilson, G. S. & Miles, A. A. (1964). Topley & Wilson's *Principles of Bacteriology & Immunity*, vol. 1, 5th edn, London: Arnold.

Wood, T. H. (1956). *Adv. biol. med. Phys.*, **4**, 119–165.

J. Pharm. Pharmac., 1967, **19**, 744–759 Received March 8, 1967

The morphology and anatomy of the flowers of *Mitragyna ciliata* Aubr. et Pellegr. and *Mitragyna stipulosa* (D.C.) O. Kuntze

E. J. SHELLARD AND A. WADE

From the Department of Pharmacy, Chelsea College of Science and Technology (University of London), London, S.W.3

The morphology and detailed anatomy of the flowers of *Mitragyna ciliata* Aubr. et Pellegr. and *Mitragyna stipulosa* (D.C.) O. Kuntze have been described. Although the histological features are almost identical, the two species can be differentiated by the morphology of their calices.

IN 1963, Shellard & Shadan described the morphology and anatomy of the leaves of *Mitragyna stipulosa* and *Mitragyna ciliata* (family Rubiaceae) and drew attention to the similarity between them, the only noticeable difference being in the number of rows of palisade cells, which indicated *M. stipulosa* to be typical of a shade plant and *M. ciliata* to be typical of a sun plant. The alkaloidal content of the leaves of the two plants differed substantially (Beckett, Shellard & Tackie, 1963) and the question arose as to whether the plants were chemical races of the same species, whether they were geographical variants or whether they were two distinct species. Until 1936 they were considered to be one species, *Mitragyna macrophylla*, but in that year Aubréville & Pellegrin drew attention to slight differences between the flowers on the trees growing in the closed rain forests and those on the trees growing in the savannah.

We have now completed a detailed comparative examination of the flowers from trees growing in these two different regions of Ghana.

Materials

The flowering tops of both species were obtained during the period January 1961–June 1962. Details of the location in which the trees were growing and of their authentication were given by Shellard & Shadan (1963).

The flower of *M. ciliata* is now described; details of *M. stipulosa* are given only where they differ from those of *M. ciliata*.

Macroscopical features

M. ciliata. The *inflorescence* is a number of globose flower heads, each growing to about 20 mm in diameter, arranged in a dichasial cyme. The 65 to 85 individual florets of each flower head are closely packed on a spherical receptacle. Each floret in the mature flower head is surrounded by 20 to 25 closely packed overlapping paleaceous bracteoles. In the flower bud stage the bracteoles completely cover and protect the developing florets which as they develop to maturity gradually push their way through the centre of the group of bracteoles. The first part of the

floret to become visible during this development is the corolla, the tube of which is closed by the infolding of the petal lobes so that the margins of each lobe are adjacent in a valvate arrangement. When the floret is mature, the combined length of the calyx and ovary is *shorter* than the length of the bracteoles (Table 1) so that the calyx is not readily visible on the fully grown flower head (Fig. 1A).

TABLE 1. RELATIVE LENGTHS OF THE CALICES AND BRACTEOLES OF *M. ciliata* AND *M. stipulosa*

	Calyx with ovary range of lengths (mm)	Average length (mm)	Bracteoles range of lengths (mm)	Average length (mm)	Ratio of lengths: calyx with ovary / bracteole
Mitragyna ciliata					
Bud	2·77–3·16	2·99	2·61–3·81	3·34	0·895
Mature flower	2·61–3·42	3·03	2·47–3·77	3·17	0·949
Old flower			none available		
Mitragyna stipulosa					
Bud	3·06–3·45	3·23	1·97–3·63	2·94	1·098
Mature flower	3·77–4·72	4·09	2·14–4·00	2·88	1·420
Old flower	3·92–4·77	4·43	2·28–4·31	3·62	1·228

M. stipulosa. The *flower heads* grow to about 16 mm in diameter and contain 100 to 120 individual florets. In the mature florets the combined length of the calyx and ovary is *greater* than the length of the bracteoles (Table 1) so that the calyx is readily visible in the fully grown flower head (Fig. 2A).

M. ciliata and *M. stipulosa.* Each *floret* is pentamerous, complete, perfect, actinomorphic and epigynous. In all these respects the flower and inflorescence are typical of the family Rubiaceae, sub-family Naucleae (Figs 1B and 2B).

The *peduncle* of *M. ciliata* is 30 to 40 mm long, up to 1·5 mm in diameter and woody with a thin brown bark bearing longitudinal ridges (Fig. 1A). The peduncle of *M. stipulosa* is 20 to 30 mm long.

The *receptacle* of *M. ciliata* is spherical and measures about 4 to 5 mm in diameter, the individual florets being attached over its entire surface. It is brown and covered with long stiff trichomes. The surface is rugose because the base of each ovary is attached to a raised portion of the receptacle. The transversely cut surface is white and exhibits numerous vascular strands.

The surface of the receptacle of *M. stipulosa* is much smoother as the bases of the ovaries do not arise from elevated platforms on its surface.

M. ciliata. The *bracteoles* are clavate to spathulate and are 2·5 to 3·8 mm long. They are thickened along a central spine which, on the outer surface, extends over most of the apical region, so that they have a planoconvex transverse section near the apex (Fig. 1F).

The bracteoles of *M. stipulosa* are 2·0 to 4·3 mm long (Fig. 2C).

M. ciliata. The *calyx* is gamosepalous (Fig. 1E) consisting of 5 sepals united for most of their length into a tube and terminating in irregular

undulating lobes 0·2 to 0·3 mm deep. It is about 0·7 to 1·0 mm long and 1·5 to 2·1 mm wide at the open end. The margin of the lobes is distinctly ciliate. The upper portion of the calyx is thin but the basal part is thicker and on the inside has fleshy pads where it is fused to the ovary wall. The lower part of the calyx exhibits a network of prominent anastomosing veins. The calyx is green when fresh but becomes pale cream to brown when dried.

M. stipulosa. The five sepals are united for the whole of their length. The calyx is about 1·4 mm deep and 1·3 to 2·0 mm in diameter at the open end. The margin is entire and glabrous (Fig. 2E).

The appearance of the calyx and its dimensions relative to those of the bracteoles is one of the more important differences between the two species (Table 1).

M. ciliata. The *corolla* is gamopetalous, consisting of five petals which are joined for over half their length. It is from 3·4 to 5·0 mm long, of which the lobes comprise 1·25 to 2·4 mm, the diameter at the open end being 1·5 to 2·8 mm, so that the corolla is salverform (Fig. 1B and D).

The individual petals are linear, but widen slightly near the lobes, which taper to a blunt incurving point at the apex. Valvate closure occurs because at the extreme tips, the lobes are thickened on the inner surface and a narrow strip extends from each margin to fuse and form a "hood." This partially obscures the inner surface of the lobe. On the outer surface the lobes are covered with fine pale golden trichomes except for a semi-circular area at the base of each lobe. The tube of the corolla is glabrous and matt. The corolla is white in the fresh flower but becomes reddish brown on drying.

M. stipulosa. The corolla is from 3·5 to 6·0 mm long, of which the lobes are 1·0 to 2·0 mm long, the diameter at the open end being 1·6 to 2·5 mm. The petals are thus joined to each other for about two-thirds of their length. Externally, the upper third of each lobe surface is covered with fine pale golden trichomes. The remainder of the surface is matt and glabrous (Fig. 2B and F).

M. ciliata and *M. stipulosa.* The *androecium* consists of five epipetalous adnate stamens which alternate with the corolla lobes (Figs 1D and 2F). The filament is absent and the anthers are attached directly to the corolla just below and between the lobes, by means of the connective. The bilobed anthers, which are dorsifixed, are 1·4 to 1·8 mm long and 0·7 to 0·8 mm wide, the lobes being spindle-shaped and joined from their apices for about three-quarters of their length. Dehiscence is introrse through a slit in each lobe. The pale yellow pollen is found on the stigma and entangled in the trichomes of the corolla lobes, all the anthers having dehisced.

M. ciliata. The *gynoecium* consists of a bicarpellary, syncarpous, inferior ovary and a reddish-brown cylindrical style surmounted by a lobed and fleshy stigma, the surface of which is grooved. The ovary, which is 1·5 to 3·0 mm long and 1·5 mm in diameter at the top, is conical with longitudinal ridges. It is inserted on a small mound on the receptacle

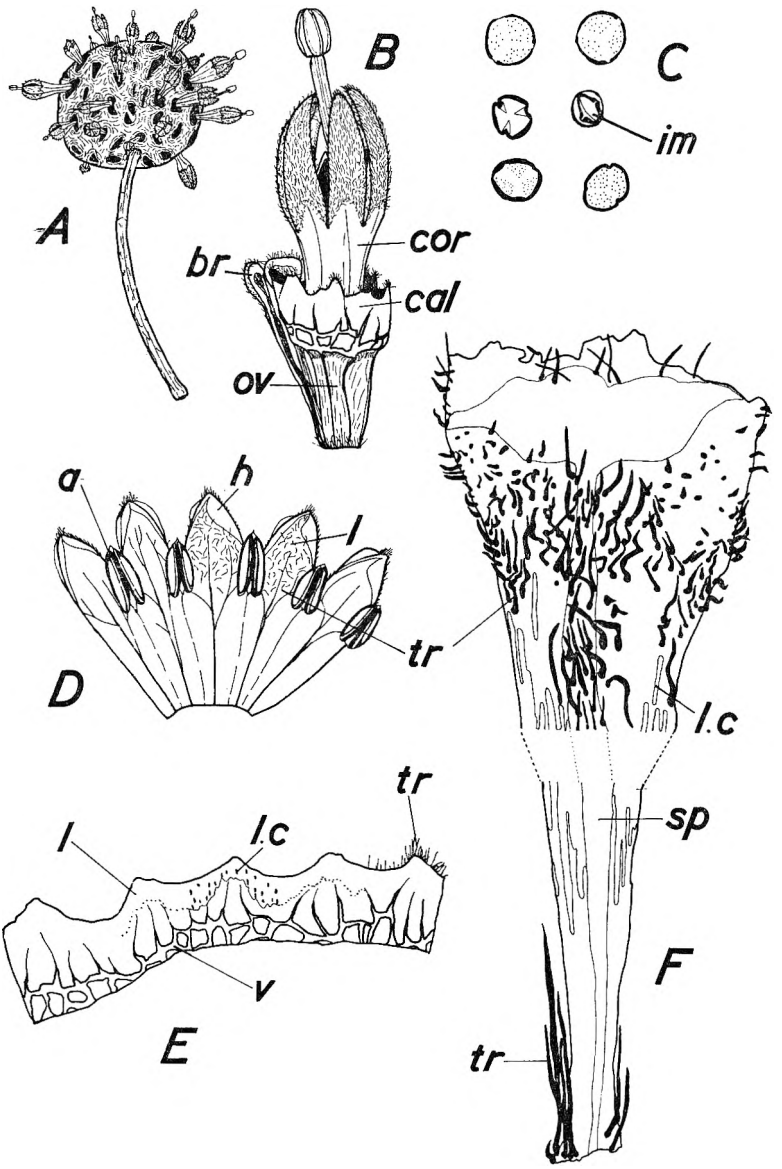

FIG. 1. *Mitragyna ciliata*, A, flower head and peduncle ×2; B, floret ×8; C, pollen ×400; D, corolla, inner epidermis and anthers, surface view ×5; central petals show trichomes; E, calyx, inner epidermis, surface view ×10; second sepal shows outer limit (at dotted line) of lignified cells, central sepal shows region of isolated lignified cells, and right-hand sepal shows lignified trichomes along the margin; F, bracteole, outer epidermis, surface view ×35. a, Anthers; br, bracteole; cal, calyx; cor, corolla; h, hood; im, immature pollen grain; l, lobe; l.c, lignified cells; ov, ovary; sp, spine; tr, trichome; v, vein.

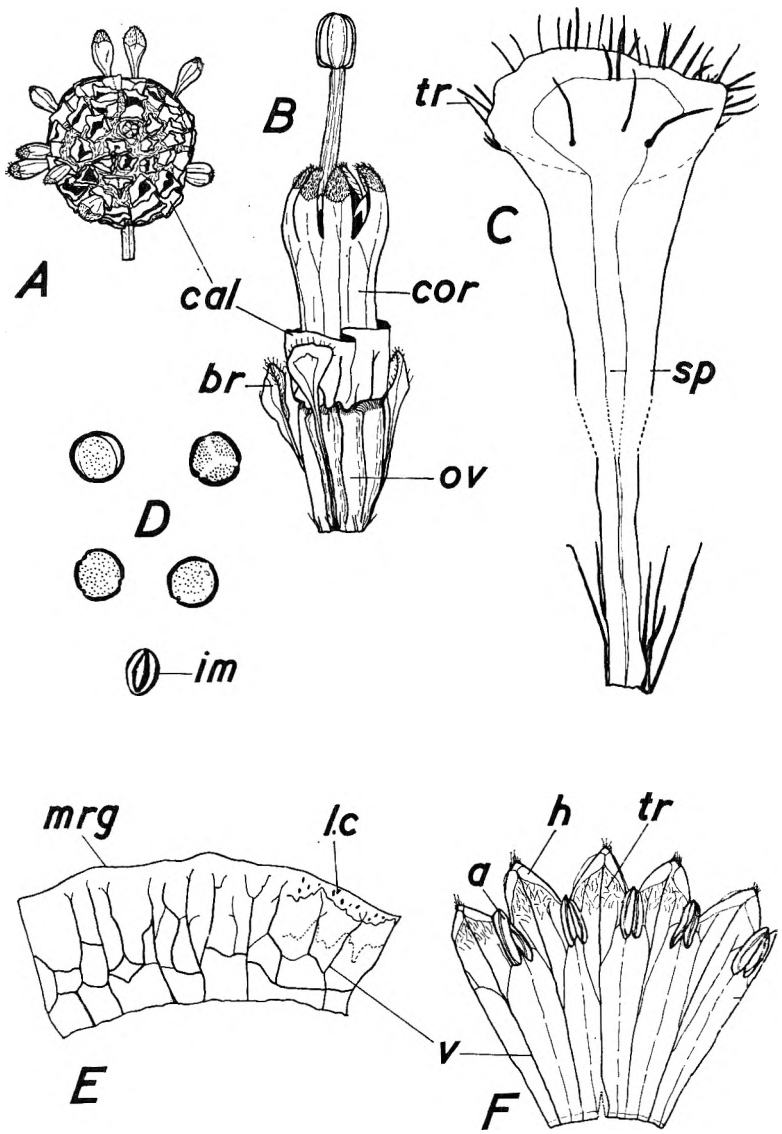

FIG. 2. *M. stipulosa*, A, flower head ×2; B, floret ×8; C, bracteole, outer epidermis, surface view ×35; D, pollen ×400; E, calyx, inner epidermis, surface view ×10; right-hand region shows area of heavily lignified cells (below dotted line) and area of slightly lignified cells (below continuous line); F, corolla, inner epidermis and anthers, surface view ×5. a, Anther; br, bracteole; cal, calyx; cor, corolla; h, "hood"; im, immature pollen grain; lc, lignified cells; mrg, margin; ov, ovary; sp, spine; tr, trichome; v, vein.

and surrounded by the bracteoles. Long pale yellow trichomes arise from the receptacle and pack the space between the ovaries and bracteoles. The ovary is bilocular with axile placentation, numerous minute ovules arranged in an overlapping pattern filling each loculus. The ovules are roughly triangular in section and each is joined to the placenta by a short funicle. In the opened, mature flower the style is 6·0 to 7·0 mm in length, thus extending about 2 mm beyond the corolla (Fig. 1B).

M. stipulosa. The ovary is 2·0 to 3·0 mm long and 1·4 mm in diameter at the top; the style is 5·5 to 8·0 mm long and in the opened mature flower extends about 3·5 mm beyond the corolla (Fig. 2B).

Microscopical features

PEDUNCLE

M. ciliata. The general anatomy is similar to that of the young stem described by Shellard & Shadan (1963) (Fig. 3A). The epidermis consists of thick-walled papillose cells with occasional conical or more elongated trichomes, 70 to 350 μ long. The cortex consists of 4 to 10 rows of collenchymatous cells divided into two regions by a zone of parenchymatous cells. Occasionally up to 4 rows of parenchymatous cells occur immediately below the epidermis. Cluster crystals of calcium oxalate measuring up to 65 μ in diameter occur in the collenchyma but are absent from the parenchymatous cells within the collenchyma.

The primary phloem cannot be distinguished. The secondary phloem consists of 3 to 5 rows of small thin-walled cells associated with sieve tubes and larger collapsed cells. There is a discontinuous ring of phloem fibres in groups of 3 to 8 thick-walled, slightly lignified fibres measuring 700 to 1800 μ long and 6 to 30 μ in diameter (Fig. 3E). Occasional latex cells are present which have deep yellow contents and which measure 30 to 50 μ in diameter.

The secondary xylem consists of groups of 2 to 6 vessels scattered among fibres and xylem parenchyma. The vessels have slightly thickened and lignified pitted walls. The numerous lignified fibres are more angular than the vessels, with thicker walls, and as seen in transverse section, appear in roughly triangular masses of about 25 cells with the base of the triangle along the line of the cambium. Adjacent groups of fibres are divided by radial rows of xylem parenchyma cells; these cells are rounded and lignified. The pith is of parenchymatous cells with thickened and pitted slightly lignified walls.

M. stipulosa. There are no groups of lignified phloem fibres and crystals of calcium oxalate in the collenchymatous cells measure up to 45 μ in diameter.

RECEPTACLE

M. ciliata. The epidermis consists of small polygonal cells from which numerous long straight lignified and thick-walled trichomes arise. They measure 400 to 552 to 620 μ in length and about 30 μ in width; some trichomes contain one or two thin slightly lignified septa.

The internal structure of the receptacle is similar to that of a young stem (Fig. 3B). There are numerous groups of vessels each associated with the individual florets. In section these vascular strands may be cut obliquely, transversely, or longitudinally. Beneath each floret the cells are deep yellow, with thickened cellulosic walls, and this tissue extends to about a third of the distance towards the centre. Slightly lignified spirally thickened vessels (Fig. 3F) are also present. Nearer the centre, about 12 groups of the vessels are arranged radially round the centre of the receptacle. Within this ring of vessels many of the parenchymatous cells contain cluster crystals of calcium oxalate. There are also scattered single lignified fibres, measuring 215 to 370 μ long and 20 to 30 μ wide, mainly near the groups of vessels.

M. stipulosa. The calcium oxalate crystals are more frequent and there are no lignified fibres.

BRACTEOLES

M. ciliata. The *outer epidermis* of the apical region of the non-thickened portion of the clavate or spathulate head consists of polygonal cells with fairly straight thickened cellulosic walls, having a smooth cuticle (Fig. 4A). The cells measure from 15 to 40 μ long, 8 to 25 μ wide and 13 to 26 μ deep. Numerous unicellular trichomes, measuring up to 300 μ in length, with thick lignified cell walls, occasionally with lignified septa, and thick lignified pitted bases, are present on the head and along the thickened central spine of the shaft almost to the base. The longer trichomes are on the margin and along the centre of the shaft. There are some lignified and pitted cells on the surface with either very short conical trichomes or no trichome attached. Anisocytic stomata about 26 μ in diameter occur on the head, mainly near the margin.

Where the head begins to merge with the shaft, the polygonal cells become more elongated and narrower. On the shaft, the epidermal cells are elongated with narrow lumens (Fig. 4B). Near the margin of the shaft the cells have thickened lignified walls with occasional pits. Nearer the centre the rows of cells are shorter with cellulosic walls, and these often contain cluster crystals of calcium oxalate. The lignified cells measure 140 to 280 μ in length and 10 to 20 μ in width, the non-lignified cells 35 to 100 μ in length and 8 to 25 μ in width; the cluster crystals are about 15 μ in diameter and sometimes occur in files of up to 6 crystals. Near the base the lignified cells are absent but there are occasional trichomes on the margin similar to those found on the receptacle.

The thickened part of the bracteole, both on the head and the shaft, is formed of thin-walled cells, with dark brown walls and contents which are slowly bleached by ammoniacal peroxide.

The cells of the *inner epidermis* of the bracteole head are larger than those of the outer epidermis (Fig. 4C). On the shaft, all the cells are elongated, with pitted and lignified walls (Fig. 4D). The *mesophyll* is composed of parenchymatous cells, apart from a single spirally lignified vessel under the thickened spine.

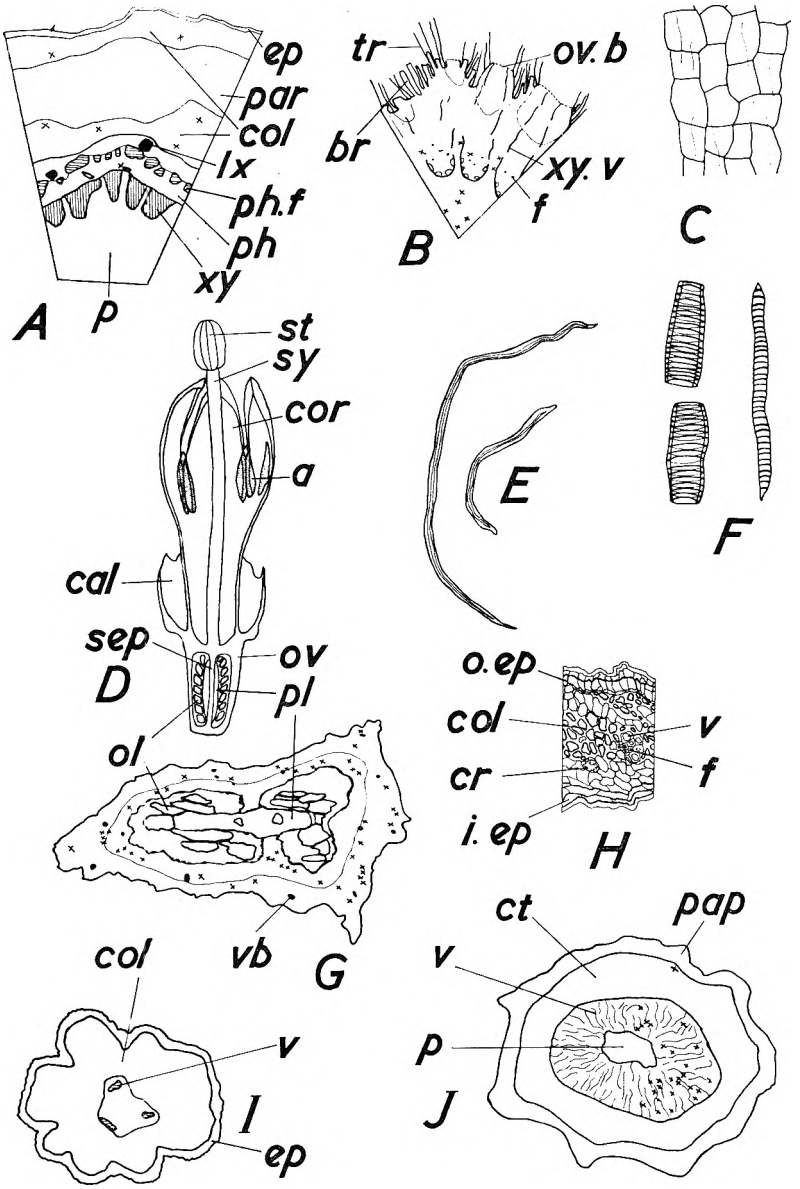

FIG. 3. *M. ciliata*, A, diagram, transverse section of peduncle ×35; B, diagram, transverse section, centre of receptacle ×10; C, ovary, outer epidermis, surface view ×390; D, diagram, vertical section of floret, to show structure ×8; E, isolated phloem fibres from peduncle ×35; F, isolated lignified spiral vessels from receptacle ×300; G, diagram, transverse section of ovary ×35; H, transverse section of ovary wall ×150; I, diagram, transverse section of style ×35; J, diagram, transverse section of stigma ×35. a, Anther; br, bracteole; cal, calyx; col, collenchyma; cor, corolla; cr, crystal of calcium oxalate; ct, cortex; ep, epidermis; f, fibre; i. ep, inner epidermis; lx, latex cell; o. ep, outer epidermis; ol, ovules; ov, ovary; ov. b, ovary base; p, pith; pap, papillose epidermal cells; par, parenchyma; ph, phloem; ph. f, phloem fibres; pl, placenta; sep, septum; st, stigma; sy, style; tr, trichome; v, vessels; vb, vascular bundle; x, cluster crystal of calcium oxalate; xy, xylem; xy. v, xylem vessels.

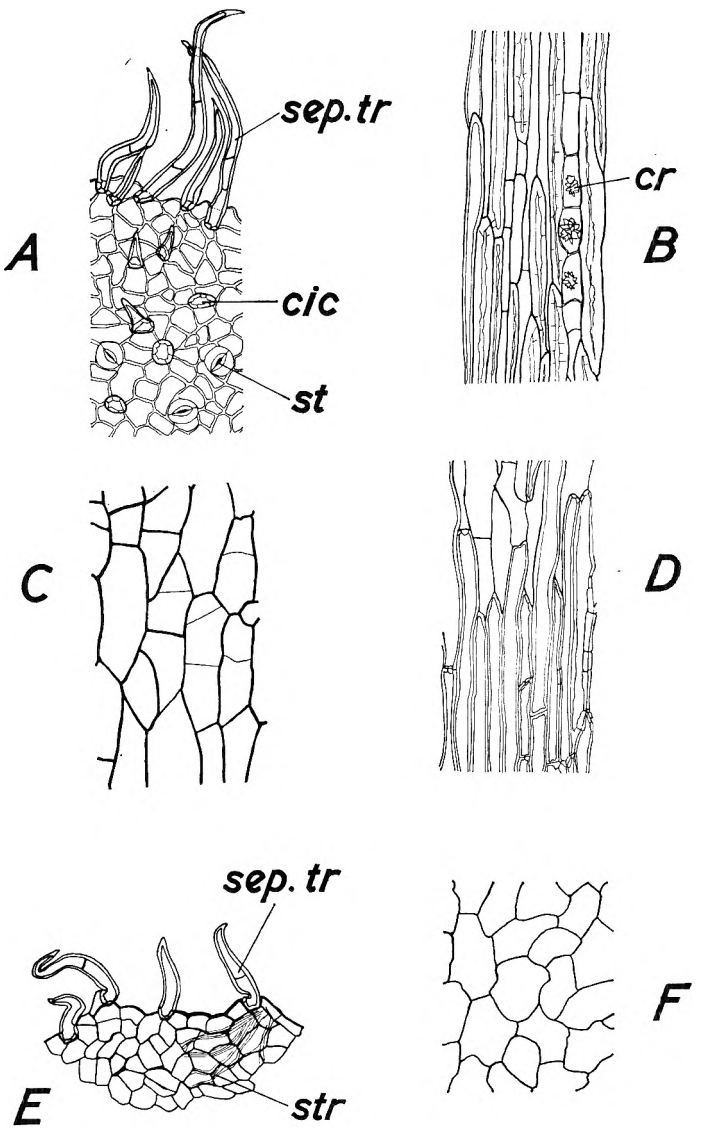

FIG. 4. *M. ciliata*, bracteole surface view, A, outer epidermis of head; B, outer epidermis of shaft; C, inner epidermis of head; D, inner epidermis of shaft; *M. stipulosa*, bracteole surface view, E, outer epidermis of head near margin; F, outer epidermis of head near shaft. All ×150. cic, Cicatrix; cr, crystal of calcium oxalate; sep. tr, septate trichome; st, stoma; str, striations.

M. stipulosa. There are two types of cell on the outer epidermis of the bracteole head. Near the margin of the apex (Fig. 4E) the cells are slightly smaller than in *M. ciliata* (cf. Fig. 4A) and are covered by a faintly striated cuticle. Near the top of the shaft (Fig. 4F) the cells are larger with more sinuous walls; no striations are visible. A more important difference is that there are fewer lignified trichomes on the outer epidermis (cf. Figs 2C and 1F). They are 30 to 280 μ in length and about 15 μ in width. There are no lignified and pitted cells on the outer epidermis similar to those in *M. ciliata.* Also, stomata are uncommon on the outer and absent from the inner surface, and there are fewer calcium oxalate crystals.

CALYX

M. ciliata. The cells of the *outer epidermis* are polygonal with straight or slightly sinuous anticlinal walls, and covered with a thin smooth cuticle (Fig. 5A). The cells are similar over the entire outer surface except that the walls become thicker in the basal region of the calyx. The cells measure 10 to 30 μ long, 8 to 15 μ wide, and 5 to 15 μ deep and are slightly larger on the lobes than in the basal region. Paracytic and anomocytic stomata, about 20 to 25 μ in diameter and slightly raised above the surrounding cells, occur mainly towards the basal region. Trichomes of two types occur near the margin: long straight unicellular trichomes with lignified thickened walls and pitted bases, measuring 100 to **220** to 290 μ long and up to 15 μ wide, are found on the margin; short conical trichomes measuring up to 50 μ in length are numerous on the lobes and have similar walls and bases. Some of the longer trichomes have thin slightly lignified septa. There are occasional pitted lignified cells without trichomes, similar to those on the bracteole.

The *inner epidermis* shows three distinct zones (Fig. 1E). The apical zone consists of cells which are similar to those of the outer epidermis, measuring 13 to 36 μ long, 7 to 20 μ wide and 6 to 14 μ deep. There are no stomata or trichomes. The cells of this zone give way to isolated lignified and pitted cells which become joined in irregular patches of elongated cells each measuring 23 to 90 μ long, 10 to 23 μ wide, and 11 to 23 μ deep (Fig. 5B). In the wide basal zone above the ovary all the cells are heavily lignified, but less pitted. Groups of cells all have their long axes parallel and generally aligned towards the base, but in some groups they are aligned in other directions (Fig. 5C). These cells measure 28 to 88 μ long, 12 to 32 μ wide and 17 to 33 μ deep.

The *mesophyll* has 2 to 4 rows of thin-walled parenchymatous cells which are larger near the ovary (Fig. 5D). Occasional cluster crystals of calcium oxalate, 8 to **14·3** to 22 μ in diameter are present. The small veins consist of spirally thickened vessels, a few phloem cells and occasional lignified fibres. Rather more fibres accompany the larger veins near the ovary.

M. stipulosa. The cells of the outer epidermis, which measure 10 to 55 μ long, 6·5 to 25 μ wide and 6 to 14 μ deep, are similar in shape and

size over the whole surface and are covered with a fairly thin cuticle exhibiting occasional patches of parallel wavy striations (Fig. 5E). The striations are more prominent near the margin. Paracytic and anomocytic stomata measuring 20 to 40 μ in diameter occur frequently. There are no trichomes on either surface. The cells of the inner epidermis are similar to those of *M. ciliata* but have different dimensions, the unlignified cells in the apical region being 15 to 45 μ long, 8 to 20 μ wide and 5 to 14 μ deep, the lignified cells in the middle zone measuring 20 to 90 μ long, 8 to 25 μ wide and 15 to 25 μ deep, while the more pitted cells of the basal zone adjacent to the ovary are 5 to 60 μ long, 8 to 22 μ wide and 15 to 50 μ deep (Fig. 5F). This variation in depth above the ovary makes the surface rugose. The cluster crystals measure 10 to **18** to 25 μ in diameter.

COROLLA

M. ciliata. On the lobes, the cells of the *outer epidermis* are polygonal with thin straight anticlinal walls (Fig. 6A). Near the tube of the corolla the cells become more elongated along the axis of the corolla. The cells, which measure 15 to **29** to 35 μ long and 13 to **19·5** to 25 μ wide, are covered with a thin cuticle which exhibits sinuous parallel striations covering several cells. The trichomes, which cover most of the lobe, are unicellular and elongated with thick lignified walls and pitted bases. They measure 128 to **360** to 700 μ long and up to 30 μ in diameter at the base. The trichomes, some of which bear thin lignified septa, are usually bent over at the base and point toward the apex of the lobe. Towards the edge of the lobes there are fewer trichomes and these are often more contorted, forming a tangled mass; some of the trichomes are short and conical. There are no stomata on the corolla lobes.

On the corolla tube, the cells of the outer epidermis are more rectangular, with their long axes orientated along the length of the tube (Fig. 6B). They measure 10 to **29** to 50 μ long and 8 to **15** to 25 μ wide and the entire surface is covered with a thin cuticle bearing long parallel striations which become straighter and less marked towards the base. The cell walls become slightly thicker towards the base. No stomata or trichomes are present on the corolla tube.

The epidermal cells of the *inner epidermis* of the lobes are polygonal and elongated, with fairly straight anticlinal walls (Fig. 6C). The thin cuticle covering the epidermis bears faint longitudinal striations. The cells measure 40 to **73** to 135 μ in length, 12 to **22·1** to 28 μ in width and 12 to **19·2** to 30 μ in depth. There are numerous trichomes which are elongated, unicellular and twisted, with thin cellulosic walls and a thin cuticle bearing faint longitudinal striations. The trichomes measure 200 to **340** to 530 μ in length and up to 35 μ in diameter at the base. Stomata are absent.

The epidermal cells of the tube are narrower than those of the lobes, measuring 60 to **85·7** to 110 μ long, 8 to **14** to 20 μ wide and about 14 μ deep (Fig. 6D). The cell walls become slightly thicker near the base but are not lignified. No stomata or trichomes are present on the tube. The

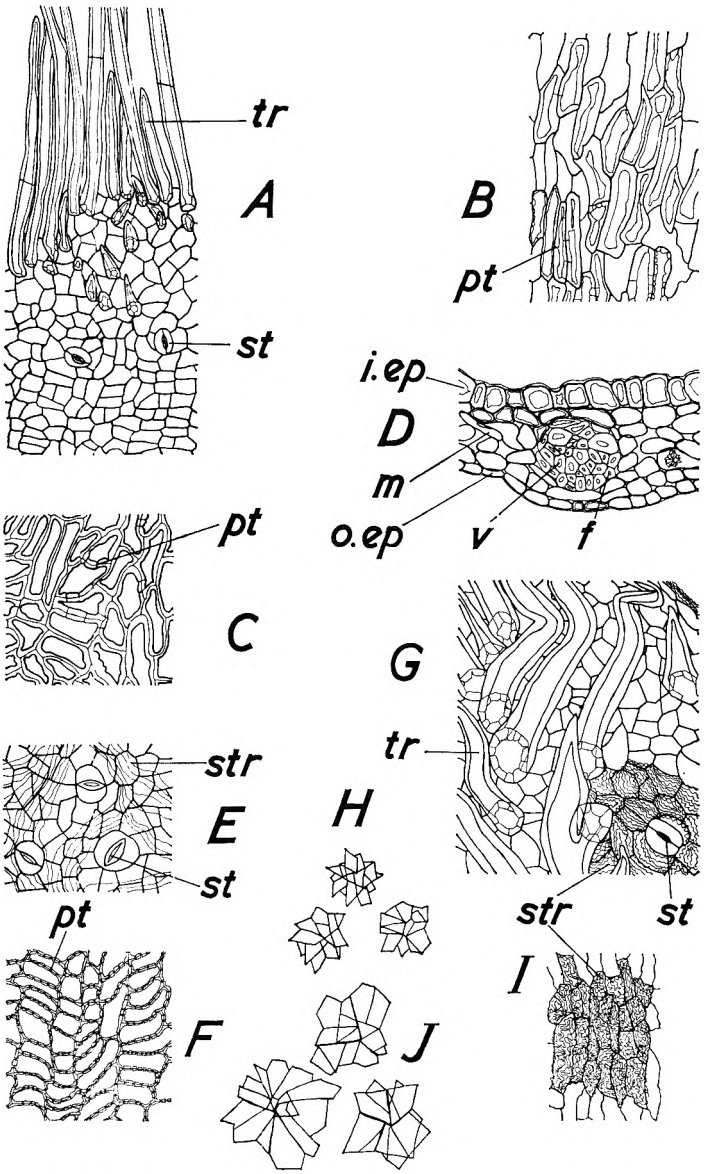

FIG. 5. *M. ciliata*, calyx, A, outer epidermis, margin, surface view; B, inner epidermis, mid-region, surface view; C, inner epidermis, basal region, surface view; D, transverse section, base of calyx; E, *M. stipulosa*, calyx, outer epidermis, surface view; F, calyx, inner epidermis of basal region, surface view; G, corolla, outer epidermis of lobe, surface view; H, cluster crystals of calcium oxalate from anther; I, corolla, epidermis of "hood", surface view; J, *M. ciliata*, cluster crystals of calcium oxalate from ovary. All ×150 except H and J ×950. f, Fibres; i, ep, inner epidermis; m, mesophyll; o. ep, outer epidermis; pt, pit; st, stoma; str, striations; tr, trichomes; v, vessel.

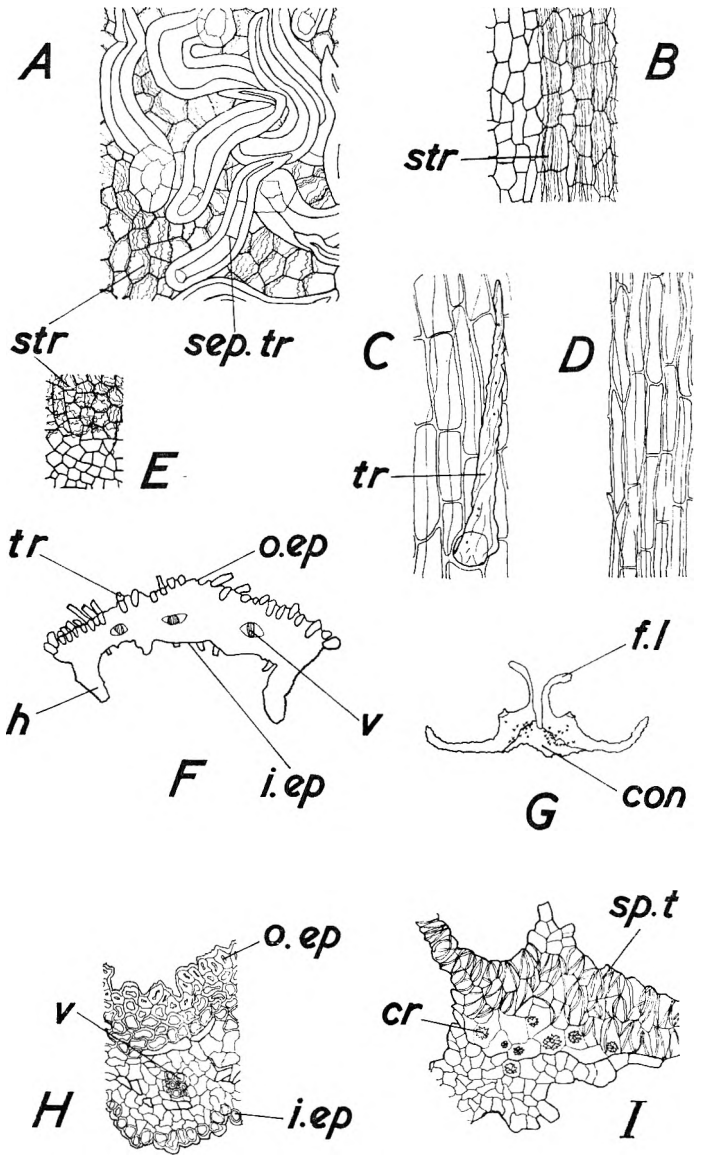

FIG. 6. *M. ciliata*, corolla, A, outer epidermis of lobe, surface view; B, outer epidermis of tube, surface view; C, inner epidermis of lobe, surface view; D, inner epidermis of tube, surface view; E, epidermis of hood, surface view; F, diagram transverse section of lobe; G, anther, diagram transverse section; H, corolla tube, transverse section cut near base; I, anther, transverse section. A, B, C, D, E, H and I ×150; F and G ×35. con, connective; cr, crystal of calcium oxalate; f.l, fibrous layer; h, "hood"; i. ep. inner epidermis; o. ep, outer epidermis; sep. tr, septate trichome; sp. t, spiral thickening; str, striations; tr, trichome; v, vessels; x, cluster crystal of calcium oxalate.

"hood" has very small epidermal cells with thin straight anticlinal walls. The cells are covered with a thick cuticle bearing heavy sinuous striations (Fig. 6E).

The *mesophyll* has mainly undifferentiated, thin walled parenchymatous cells, although immediately below the outer epidermis the cells have thicker cellulosic walls. The depth of this region of thicker-walled cells increases towards the base of the tube (Fig. 6H). Cluster crystals of calcium oxalate measuring 8 to **14·5** to 23 μ in diameter occur occasionally in the cells of the mesophyll of the lobes, but are more frequent in the tube; files of up to 8 crystals are found in adjacent cells. There are numerous cluster crystals in the mesophyll of the "hood". The vascular tissue generally consists of lignified spiral vessels and a group of small tightly-packed phloem cells.

M. stipulosa. The outer epidermal cells on the lobes measure 18 to **28·5** to 42 μ in length and 10 to **17·3** to 25 μ in width, and bear a fairly thick cuticle with well-marked sinuous striations (Fig. 5G). The long lignified trichomes measure 210 to **317** to 400 μ long and up to 35 μ wide at the base, while the shorter conical trichomes near the edges of the lobes measure only 35 to **73** to 125 μ long and up to 32 μ wide at the base. There are occasional paracytic stomata.

Lower down the corolla tube the elongated cells of the outer epidermis measure 13 to **25·7** to 38 μ long and 5 to **16** to 27 μ wide.

The epidermal cells of the inner epidermis measure 33 to **64·2** to 100 μ in length, 17 to **24·2** to 45 μ in width and 12 to **16·7** to 23 μ in depth on the lobes; they are more elongated lower down on the tube, measuring 80 to **126** to 180 μ long, 12 to **18·6** to 33 μ wide, and about 13 μ deep.

The epidermal cells of the "hood" have thin sinuous anticlinal walls and are covered by a thick cuticle with well-marked parallel sinuous striations so that it is difficult to see the exact outline of the cells (Fig. 5I). Cluster crystals of calcium oxalate are mainly found in the "hood" and measure 7 to **10·5** to 14 μ in diameter; small prisms of calcium oxalate are scattered throughout the mesophyll and measure 2 to 6 μ.

ANDROECIUM

M. ciliata. The characteristic tissue of the anther lobe is the "fibrous layer" which has isodiametric cells with a contorted shape in surface view. The cell walls bear spiral lignified thickening linked with the thickening of adjacent cells in an intricate pattern. The cells measure 7 to **14·4** to 22 μ wide and 15 to **26·2** to 42 μ deep (Fig. 6G and I). Epidermal cells were not visible in the material available.

The connective consists of 1 to 7 rows of small thin-walled cells containing numerous cluster crystals of calcium oxalate behind the lobes but absent from the cells adjoining the corolla. The crystals measure 5 to **10·6** to 15 μ in diameter.

The *pollen* grains are subspherical and measure 11 to **15·3** to 19 μ in diameter (Fig. 1C). The exine is covered with minute pits. There are three germinal furrows and pores. In polar view the pollen grains have a

rounded triangular appearance. Immature pollen grains have deeper furrows but no pits are visible. Occasional pollen grains are seen with a thicker contorted exine.

In *M. stipulosa* the cells of the fibrous layer measure 8 to **16·4** to 25 μ wide and 18 to **28·8** to 40 μ deep. The calcium oxalate of the connective measures 5 to **8·8** to 12 μ in diameter (Fig. 5H). The pollen grains are 15 to **17·8** to 19·8 μ in diameter (Fig. 2D).

GYNOECIUM

Stigma

M. ciliata and *M. stipulosa.* The epidermal cells of the stigma are small, polygonal, thin-walled and papillose, with dark yellow contents. The walls are suberized and small globules of fixed oil are present in the cells. The bulk of the tissue is of thin-walled elongated cells, radially arranged. A zone of lignified spirally thickened vessels orientated in various directions occurs around the central pith and is associated with thin-walled isodiametric cells which occasionally contain cluster crystals of calcium oxalate (Fig. 3J).

Style

M. ciliata and *M. stipulosa.* The epidermal cells of the style are polygonal with thickened cellulosic walls, and are covered by a thin striated cuticle. The cortex consists of thickened collenchymatous cells but in the central third of the style the cells are polyhedral with thin walls. There are two vascular strands at the base of the style, which are of lignified spirally thickened vessels, dividing to form 10 strands at the top of the style (Fig. 3I).

Ovary

M. ciliata. The *outer epidermis* has small subrectangular cells with thickened cellulosic walls and yellow contents (Fig. 3C). There is a thin cuticle bearing faint striations. The cells measure 10 to **17·6** to 30 μ in length and 8 to **13·8** to 20 μ in width.

The *inner epidermis* is not readily visible in surface view, but in section is seen to consist of pale thin-walled radially flattened cells.

The *mesophyll* has two regions (Fig. 3G): below the outer epidermis the cells have thickened cellulosic walls and are often coloured dark brown with some vascular bundles; in the region nearer the inner epidermis the cells are paler and thin-walled and may contain cluster crystals (Fig. 5J) of the same size as those in the calyx (Fig. 3H). The crystals are more common near the top of the ovary. Each half of the ovary contains 4 to 5 vascular bundles of lignified spirally thickened vessels.

The septum dividing the two loculi has a structure similar to that of the inner region of the ovary wall. The placenta is similar in structure with a large vascular bundle on each side.

The ovules are immature and are of small closely-packed cells with sinuous walls, the outer cells being darker and papillose but otherwise undifferentiated.

M. stipulosa. The outer epidermal cells of the ovary measure 10 to **15** to 22 μ long and 6 to **10** to 17 μ wide. The cluster crystals of calcium oxalate in the mesophyll are slightly smaller than those in the calyx, measuring 6 to **12·3** to 20 μ in diameter; in addition there are a few scattered prisms up to 5 μ long.

Discussion

There are some differences in the microscopical features of the two species. *M. stipulosa* differs from *M. ciliata* in having: no groups of fibres in the peduncle and receptacle; no trichomes on the calyx and fewer trichomes on the bracteoles; no lignified and pitted cells on the outer epidermis of the bracteoles; only rare stomata on the bracteoles; occasional stomata on the epidermis of the corolla; striated cuticle on the outer epidermis of calyx and bracteoles.

These differences in the microscopical features are not of great value in assessing whether the two plants are from the same or different species. However, the macroscopical features clearly distinguish the two species. In *M. stipulosa* the gamosepalous calyx has an entire margin and is entirely glabrous. The ovary is much longer than the floral bracteoles and is easily visible on the complete inflorescence. In *M. ciliata* the gamosepalous calyx has a lobed margin which is distinctly ciliate. The combined calyx and ovary are much shorter than the floral bracteoles and the calyx is not readily visible in the complete inflorescence. This work confirms the observations of Aubréville and Pellegrin in 1936 and supports their contention that these are two distinct species.

Acknowledgements. Our thanks are due to Mr. E. Allman, (then) Head of the Faculty of Pharmacy, University of Science and Technology, Kumasi, Ghana, for his invaluable assistance in collecting the flowering tops and to Mr. A. Enti for identifying the trees from which the flowers were collected.

References

Aubréville, A. & Pellegrin, F. (1936). *Bull. Soc. bot. Fr.*, **83**, 35–37.
Beckett, A. H., Shellard, E. J. & Tackie, A. N. (1963). *J. Pharm. Pharmac.*, **15**, *Suppl.*, 158*T*–169*T*.
Shellard, E. J. & Shadan, P. (1963). *Ibid.*, **15**, *Suppl.*, 278*T*–291*T*.

J. Pharm. Pharmac., 1967, **19**, 760–766 Received April 24, 1967

Production of cardio-active substances by plant tissue cultures and their screening for cardiovascular activity

BALKRISHENA KAUL, PATRICK WELLS AND E. JOHN STABA

Semi-purified extracts of six plant tissue cultures were examined for their effects on respiration, heart rate, and blood pressure in anaesthetized rabbits. *Ammi visnaga*, *Cheiranthus cheiri*, *Digitalis lanata*, and *Urginea maritima* evoked pronounced vasodilatation and bradycardia which ultimately resulted in the death of the animal.

DIGITALIS-like glycosides (Medora, Tsao & Albert, 1967), the glycosides scopoline (Fritig, Hirth & Ourisson, 1966) and glucovanillin (Goris, 1965), and some triterpenes (Erhardt, Hirth & Ourisson, 1966) have recently been produced by callus plant tissue cultures. *Digitalis* tissue cultures produce cardenolides (Büchner & Staba, 1964), *Ammi visnaga* tissue cultures produce visnagin (Kaul & Staba, 1967), and *Apocynum cannabinum* tissue cultures produce cymarose and glucose but not the cardiac genins normally present in the plant (Carew, Nylund & Harris, 1964). The identification of the cardenolides or related products in *Digitalis* and *Apocynum* tissue cultures has been based entirely upon chromatographic evidence, and by administering *Digitalis mertonensis* tissue culture extracts to guinea-pig isolated heart preparations (Medora, Kosegarten & others, 1967).

Our aim was to determine if semi-purified extracts of *Ammi visnaga* Lam. callus and suspension cultures, *Cheiranthus cheiri* L. (wallflower) suspension cultures, *Cytisus scoparius* Link (broom tops) callus cultures, *Digitalis lanata* Ehrh. suspension cultures, *Digitalis purpurea* L. suspension cultures, and *Urginea maritima* Baker (squill) callus cultures would affect the respiration, heart rate, and blood pressure when administered intravenously to anaesthetized rabbits. The semi-purified extracts were also examined qualitatively for the presence of cardenolides, desoxysugars, and alkaloids by thin-layer chromatography.

Experimental

TISSUE CULTURES AND EXTRACT PREPARATION

Suspension tissue cultures were grown in Erlenmeyer flasks containing modified Murashige's and Skoog's tobacco medium (Lin & Staba, 1961) with 0·1 ppm of 2,4-dichlorophenoxyacetic acid (2,4-D) on a reciprocal shaker (80 strokes/min, 5 cm in length) at approximately 27°. Callus tissue cultures were grown on the same basal medium with 1·0 ppm of 2,4-D and 1·0% agar in 1-oz, square glass vials. The growth period of suspension cultures ranged from 2–6 weeks and for callus tissue from 6–12 weeks, after which they were separately collected and frozen. Collections were made over approximately two years.

From the College of Pharmacy, University of Nebraska, Lincoln, Nebraska. Presented at the Annual Meeting of American Pharmaceutical Association, April 9–14, 1967 at Las Vegas, Nevada, U.S.A.

C. cheiri tissue cultures contained numerous highly developed root structures, *D. lanata* and *U. maritima* cultures were highly organized, containing numerous fasciations (Staba & Lamba, 1963; Carew & Staba, 1965), and from their external appearance *C. scoparius*, *D. purpurea* and *A. visnaga* were undifferentiated.

Frozen tissue cultures of *C. cheiri* (1·5 kg wet wt cells, 9·7 litres medium), *C. scoparius* (1·4 kg wet wt callus), *D. lanata* (3·0 kg wet wt cells, 10 litres medium), *D. purpurea* (0·5 kg wet wt cell, 2·6 litres medium) and *U. maritima* (0·1 kg wet wt callus) were thawed and extracted according to scheme A. Dried *A. visnaga* tissue cultures (3·0 kg wet wt cells, 13 litres medium) were extracted according to scheme B. Media were reduced in volume by evaporation under vacuum and gentle heat before extraction.

Scheme A: tissue and/or media were macerated for 12 hr with methanol (1 : 1 ratio by wet wt and volume respectively). The macerated cells were homogenized for approximately 3 min and filtered. The homogenized cell filtrate or the methanolic-medium mixture were purified with saturated solution of lead subacetate (excess lead removed with 10% Na_2HPO_4). The two semipurified methanolic preparations were extracted with chloroform. The chloroform fractions were combined, evaporated over warm water *in vacuo* to dryness. The residue was redissolved in 75% ethanol (Extract I). The two remaining methanolic preparations were further extracted with a chloroform–ethanol mixture (2 : 1). The chloroform–ethanol fractions were combined, evaporated as previously described, and the residue dissolved in 75% ethanol (Extract II). *U. maritima* Extract III was prepared from media and extracted as described above for Extract I.

Scheme B. This was used for *A. visnaga* Extracts A to D. A dichloroethane extract of dried tissue and media were applied on thick-layer plates (Kaul & Staba, 1967) and the following ultraviolet fluorescent zones removed: visnagin (yellowish green, Rf, 0·53) Band A (bright blue; Rf, 0·25); Band B (deep blue; Rf, 0·63). The remaining adsorbent was eleuted with dichloroethane, evaporated to dryness and redissolved in 75% ethanol (Extract A). The dichloroethane extracted cells and medium were extracted with methanol and purified according to the procedure described for Scheme A (Extract B; trace of visnagin still present). Extract C is similar to Extract B, except that only cells were extracted and all the visnagin removed. Extract D is a chloroform–ethanol extract of the residual methanolic extract from Extract C, and was prepared as described previously for Extract II.

THIN-LAYER CHROMATOGRAPHY

Adsorbosil-1 thin-(200 mμ) and thick-(400 mμ) layer plates were used. The plates were activated for 30 min at 100°. The thin-layer solvent system and spray reagents used are shown in Table 1, and the extract concentrations in Table 2. Approximately 30–50 μl of each extract was applied as a 2 cm band. The plates were examined under ultraviolet light for fluorescent zones and then sprayed with either Raymond, xanthydrol, modified Dragendorff or anisaldehyde reagent.

TABLE 1. THIN-LAYER CHROMATOGRAPHY OF TISSUE CULTURE EXTRACTS

Tissue culture extracts	Rf values*		
	Raymond reagent	Xanthydrol reagent	Modified Dragendorff reagent
C. cheiri			
Ext I ..	0·80, 0·72, 0·53, 0·46, 0·34	0·47, 0·36	0·15, 0·03, 0·00
Ext II ..	0·76, 0·42, 0·31	0·31, 0·19	0·15
C. scoparius			
Ext I ..	0·52	—	0·00
Ext II ..	0·76	—	0·13, 0·00
D. lanata			
Ext I ..	0·76, 0·72, 0·65, 0·61, 0·55, 0·46	0·87, 0·76, 0·71, 0·65, 0·48, 0·20	0·50, 0·04, 0·04, 0·00
Ext II ..	0·69, 0·62, 0·59, 0·53	0·67, 0·58, 0·48, 0·45, 0·21	0·57, 0·48, 0·40, 0·14
D. purpurea			
Ext I ..	0·65, 0·60, 0·50	0·73, 0·63, 0·30	0·00
Ext II ..	0·90, 0·85	—	0·05
U. maritima			
Ext I ..	0·80, 0·70, 0·58	0·75, 0·57, 0·40, 0·23, 0·52, 0·19	—
Ext II ..	—	0·52, 0·19	0·01, 0·00
A. visnaga			
Ext A ..	0·67	0·46, 0·64	0·33
Ext B ..	0·76, 0·72, 0·70, 0·67, 0·53	0·73, 0·52	0·76, 0·66, 0·48, 0·34
Ext C ..	—	—	0·47
Ext D ..	0·50, 0·56	0·67, 0·75	0·46, 0·63

**TLC system*: Benzene–ethanol 95% (3 : 1); Adsorbent: Silica dioxide (Adsorbosil-1, Applied Science Laboratory, State Park, Pa.).

Raymond reagent: Spray A 10% 1,3-dinitrobenzene in benzene. Heat 60° for 3 min. Spray B 6g NaOH dissolved in 25 ml water and 45 ml methanol. *Xanthydrol reagent*: Spray A 0·125% xanthydrol in glacial acetic acid. Spray B conc. HCl acid. Heat 100° for 5 min. *Modified Dragendorff reagent*: 2·6 g bismuth subcarbonate, 7·0 g NaI, 25 ml glacial acetic acid and boil 3–4 min. Set overnight and filter. Stock solution consists of 4 ml filtrate and 16 ml ethyl acetate. Spray consists of 20 ml stock, 40 ml glacial acetic acid, 120 ml ethyl acetate, and 10 ml distilled water. *Anisaldehyde reagent*: 50 ml glacial acetic acid, 0·5 ml anisaldehyde, and 1·0 ml conc. H_2SO_4. To be freshly prepared.

Rf reference compounds: Digitoxin: Raymond, xanthydrol and Dragendorff positive (Rf: 0·57). (b) Digitoxose: xanthydrol positive (Rf: 0·34). (c) Lanatoside ABC: Raymond positive (Rf: 0·18, 0·22, & 0·34). (d) Spartine sulphate: Dragendorff positive (Rf: 0·00). (e) Visnagin: Raymond and Dragendorff positive (Rf: 0·75).

Raymond reagent gives a blue colour with the butenolide ring; xanthydrol reagent gives a reddish colour with desoxysugars such as digitoxose (Wright, 1960). Modified Dragendorff reagent gives false positive tests with at least sixty-five non-nitrogenous compounds, including digitoxin and khellin; the minimal structural feature to give a positive reaction with this reagent appears to be a conjugated carbonyl (ketone or aldehyde) or a lactone function (Farnsworth, Pilowski & Draus, 1962). Anisaldehyde reagent reacts with phenols, terpenes, sugars and steroids to give a variety of colour reactions (Stahl, 1965).

CARDIOVASCULAR EXPERIMENTS

A comparison of the effects of the tissue culture extracts on the respiration, heart rate, and blood pressure was made with rabbits. Fasted rabbits weighing 2–3 kg were anaesthetized with pentobarbitone sodium (35 mg/kg) and a tracheal cannula inserted. The carotid artery was isolated and cannulated for recording blood pressure. Respiration and the electrocardiogram were recorded using the impedance pneumograph with pin chest electrodes. All injections were made intravenously through the marginal ear vein. All extracts containing undissolved particles were

TABLE 2. COMPARISON OF THE TOXIC EFFECTS OF SEVERAL TISSUE CULTURE EXTRACTS ON RESPIRATION, HEART RATE, AND BLOOD PRESSURE IN ANAESTHETIZED RABBITS

Test solution	Extract equivalent*	Amount extract administered to produce death (ml)	Respiratory rate (increase)	Heart rate (decrease)	Blood pressure (decrease)
Controls					
Ethanol 75% ..		10·0	(t)**	(t)	(t)
Tinct. of digitalis NF		3·5	+(t)	+(t)	+(t)
Visnagin 1·0% ..		4·7	+(t)	Increase	+(t)
Extracts					
C. cheiri					
Ext I	100 g + 650 ml	0·75	+++	+++	+++
Ext II	,, ,,	8·0	(t)	(t)	(t)
C. scoparius					
Ext I	97·6 g	1·5	Decrease	+	++(t)
Ext II	,,	2·25	+	+	+
D. lanata					
Ext I	100 g + 333 ml	1·5	+	+	++
Ext II	,, ,,	5·0	+(t)	+	+(t)
D. purpurea					
Ext I	40·7 g + 194 ml	6·0	(t)	+	+(t)
Ext II	,, ,,	4·0	+	+	+(t)
U. maritima					
Ext I	20 g	2·5	++	+	+(t)
Ext II	,,	0·5	+	++	++++
Ext III	735 ml	4·0	+	+	+(t)
A. visnaga					
Ext A	200 g + 866 ml	1·0	+(t)	++	++(t)
Ext B	,, ,,	1·0	+	+++	+++
Ext C	500 g	?	(t)	(t)	(t)
Ext D	,,	?	(t)	(t)	(t)

* Ext equiv.: that amount of cells (wet weight) and medium represented in each ml of extract administered
**(t) Transitory effect.

filtered through a Swinny Hypodermic Adapter containing a Millipore Type HA filter, pore size 0·45 μ. Controls were done using 75% ethanol, tincture of digitalis N.F. (Eli Lilly & Co., Indianapolis, Indiana), and 1·0% visnagin (Memphis Chemical Co., Cairo, Egypt) in 75% ethanol.

Results

THIN-LAYER CHROMATOGRAPHY

The positive Raymond, xanthydrol and modified Dragendorff zones observed in the extracts are summarized in Table 1. The fluorescence and anisaldehyde zones observed are summarized below.

C. cheiri. In Extract I at least 12 fluorescent and 10 anisaldehyde zones were observed. In Extract II 12 fluorescent and 3 anisaldehyde zones were observed.

C. scoparius. In Extract I at least 8 fluorescent and 3 anisaldehyde zones were observed. In Extract II 12 fluorescent and 2 anisaldehyde zones were noted.

D. lanata. There were at least 11 fluorescent and 22 anisaldehyde zones in Extract I. Eleven fluorescent and 11 anisaldehyde zones were observed in Extract II. The anisaldehyde zones were the stronger in Extract I.

D. *purpurea*. There were 12 fluorescent and 9 anisaldehyde zones in Extract I, and 6 fluorescent and 5 anisaldehyde zones in Extract II.

U. maritima. There were 9 fluorescent and 9 anisaldehyde zones in Extract I, and 5 fluorescent and 7 anisaldehyde zones in Extract II.

A. visnaga. There were at least 3 fluorescent and 6 anisaldehyde zones in Extract A, and 6 fluorescent and 6 anisaldehyde zones in Extract B. But Extract C and D did not show distinct fluorescent or anisaldehyde zones.

CARDIOVASCULAR EXPERIMENTS

Control experiments using 75% ethanol in 1 ml doses given at 5 min interval produced a transient fall in blood pressure. There was no significant change in the respiration, electrocardiogram, and heart rate after five 1 ml doses. In doses which totalled 10 ml, the blood pressure began to decline with a simultaneous increase in heart rate and respiration. The animal died usually due to respiratory arrest and cardiovascular collapse.

Tincture of digitalis N.F. was administered intravenously in doses of 0·5 ml at intervals of 5–10 min. Each dose produced a transient fall in blood pressure with a decrease in cardiac rate. This was usually accompanied by an increase in respiratory rate and decrease in depth. The electrocardiogram remained essentially normal in pattern with some increase in the QRS complex. After the administration of a total of 3·0 ml of the digitalis tincture the ECG pattern showed an inverted T wave and with the seventh dose of 0·5 ml the heart went into ventricular fibrillation and the animal expired.

A 1% visnagin solution in 75% ethanol given in doses of 1·0 ml at 5–10 min intervals caused a fall in blood pressure after each injection with an increase in both cardiac and respiratory rate. In the final doses, the ECG pattern showed alterations associated with anoxia to the cardiac muscle. A comparison of the toxic effects of the controls and the extracts is shown in Table 2.

C. cheiri Extract I. This was the most potent and toxic of the extracts. After an initial dose of 0·25 ml there was a fall in blood pressure with a concurrent increase in heart rate and an increase in both rate and depth of respiration. The ECG remained normal. A second injection of 0·2 ml produced a drastic fall in blood pressure, a twofold increase in rate and depth of respiration, and an ECG pattern associated with myocardial anoxia. Death occurred within 2 min from cessation of respiration and cardiovascular collapse.

Extract II. This produced no significant effects.

C. scoparius Extracts I and II. Injections of 1·5 ml of I and 3·0 ml of II in three equally divided doses produced a decrease in blood pressure, cardiac rate, and respiration. The ECG showed changes in amplitude of the QRS complex, particularly after II. Death resulted from cardiovascular collapse and respiratory arrest.

D. lanata Extract I. One ml produced a fall in blood pressure of 40 mm

Hg which was sustained throughout the experiment. The respiration increased in both rate and depth. The ECG pattern showed an inverted T wave. Heart rate was slightly reduced. A second dose of 0·5 ml caused respiratory arrest with a concurrent fall in blood pressure and cardiac rate. Artificial respiration did not improve blood pressure.

Extract II. Less toxic than I. II, in doses of 1 ml produced a transitory fall in blood pressure with a rise in blood pressure above normal in early doses. The ECG pattern showed increased amplitude of the QRS complex. The heart rate declined with each dose. The rate and depth of respiration increased for several min after each injection and then returned to normal. The animals died after a total dose of 4–5 ml due to cardiovascular collapse and respiratory arrest.

D. purpurea Extracts I and II. In 1 ml doses, these produced a transitory fall in blood pressure and a reduction in cardiac rate. Towards the end of the experiment doses of 3 to 4 ml had been given, there was an increase in rate and depth of respiration and increasing bradycardia. The ECG remained essentially normal after the early doses, but after doses of 3 ml, the T wave was inverted and characteristic patterns of anoxia occurred.

U. maritima Extract I. This, in a 0·5 ml dose evoked a transitory fall in blood pressure with no change in cardiac or respiratory rate. The ECG changed in pattern 1 min after injection with increased amplitude of the QRS complex. Subsequent doses of 0·5 ml after 8 min produced a prolonged fall in blood pressure, bradycardia, and an increase in respiratory rate and depth. The ECG pattern alternated between a normal QRS complex and one of increased amplitude. A total of 2·5 ml produced an inverted T wave with the blood pressure falling to zero.

Extract II. Doses of 0·25 and 0·5 ml produced immediate toxic effects with a complete loss of blood pressure within 1 min and respiratory arrest after 2 min. The ECG showed the characteristic pattern associated with anoxia. Artificial respiration was attempted with no improvement in blood pressure.

Extract III. This produced a decline in blood pressure followed by a rise slightly above normal. There was a decrease in cardiac rate with an increase in respiratory rate and depth. Respiratory failure occurred after the fourth dose of 1·0 ml. Excessive salivary flow was noted before death.

A. visnaga Extract A. This, in a 0·5 ml dose, produced a transitory fall in blood pressure and then a rise slightly above normal. This extract also caused a transitory increase in rate and depth of respiration, and a decrease in cardiac rate which remained stable at this slower rate. The second injection of 0·5 ml produced a dramatic fall in blood pressure with a simultaneous decline in cardiac rate and changes in the ECG pattern. The latter changes occurred in the S wave deflection associated with anoxia to the myocardium. A final dose of 0·5 ml produced cardiovascular collapse and respiratory arrest within 1 min.

Extract B. This, in a dose of 1·0 ml, produced a drastic fall in blood

pressure with a slowing of heart and cardiac failure after 10 min. The respiration showed a transitory increase and then cessation of respiration.

Extracts C and D. These produced no significant effects.

Conclusions

The plant tissue culture extracts that were particularly active pharmacologically were Extract I of *C. cheiri*, *C. scoparius*, and *D. lanata;* Extract II of *U. maritima*; and Extracts A and B of *A. visnaga*. All of these evoked pronounced vasodilatation and bradycardia, ultimately resulting in the death of the animal. Extract I of *C. cheiri*, *D. lanata* and *U. maritima* contained from three to six Raymond positive compounds, and *D. lanata* and *U. maritima* each contained six xanthydrol positive compounds. Although *D. lanata* Extract I contained some of Raymond and xanthydrol positive compounds, it did not evoke the strongest cardiovascular effect. Extract I of *C. cheiri*, Extract II of *U. maritima*, and Extract B of *A. visnaga* were the most potent and toxic. *C. cheiri*, *D. lanata*, and *U. maritima* were very highly organized tissue cultures, and perhaps this is related to the significant pharmacological effects observed.

Acknowledgements. This investigation was supported in part by the Nebraska Heart Association, Omaha, Nebraska, and by grant GM 13440-01 from the National Institute of General Medical Sciences, U.S. Public Health Service, Bethesda, Md.

References

Büchner, S. A. & Staba, E. J. (1964). *J. Pharm. Pharmac.*, **16**, 733–737.
Carew, D. P. & Staba, E. J. (1965). *Lloydia*, **28**, 1–26.
Carew, D. P., Nylund, H. B. & Harris, A. L. (1964). *Ibid.*, **27**, 322–327.
Erhardt, J. D., Hirth, L., & Ourisson, G. (1966). *C.r. hebd. Séanc. Acad. Sci., Paris*, **262**, 809–811.
Farnsworth, N. R., Pilowski, N. A., & Draus, F. J. (1962). *Lloydia*, **25**, 312–319.
Fritig, B., Hirth, L., & Ourisson, G. (1966). *C.r. hebd. Séanc. Acad. Sci., Paris*, **263**, 860–863.
Goris, A. (1965). *Annls pharm. fr.*, **23**, 275–279.
Kaul, B. & Staba, E. J. (1967). *Planta med.*, **15**, 145–156.
Lin, M. & Staba, E. J. (1961). *Lloydia*, **24**, 139–145.
Medora, R. S., Tsao, D. P. N., & Albert, L. S. (1967), *J. pharm. Sci.*, **56**, 67–72.
Medora, R., Kosegarten, D. C., Tsao, D. P. N. & DeFeo, J. J. (1967). *Ibid.*, **56**, 540–541.
Staba, E. J. & Lamba, S. S. (1963). *Lloydia*, **26**, 29–35.
Stahl, E. (1965). *Thin Layer Chromatography*, p. 485. New York: Academic Press Inc.
Wright, S. E. (1960). *The Metabolism of Cardiac Glycosides*, pp. 22–23. Springfield, Ill.: Charles C. Thomas.

Hormonal control of the rat myometrium

SIR,—The influence of the female sex hormones on the myometrium has been most clearly demonstrated in the rabbit (for references see Schofield, 1963). In this species the progesterone-dominated uterus, when compared with the oestrogen-dominated uterus, is insensitive to oxytocin, gives a different staircase effect in response to electrical stimulation and binds calcium more effectively. The present work reports the results of similar studies on the rat myometrium.

A total of 45 Wistar rats, 13–15 weeks old, were ovariectomized under ether anaesthesia and 10 days later two treatments were adopted. (1) The oestrogen-dominated group received 10 μg of oestradiol monobenzoate intramuscularly daily for 10 days. (2) The progesterone-dominated group were treated similarly with oestrogen and, in addition, received 5 mg of progesterone intramuscularly daily for the last 3 days of treatment. A control group were given no hormones.

The animals were stunned, decapitated, and the uteri rapidly dissected into a dish of modified Krebs solution (Knifton, 1966) at 4°. A 25 mm length of uterine horn was cut, transferred to a 10 ml tissue bath and assembled for electrical stimulation and isometric recording as previously described (Knifton, 1966).

After adjusting the tissue to resting length, five procedures were adopted. The threshold voltage and staircase effect were determined and the tissue rested for 10 min before measuring the minimum dose of oxytocin (Syntocinon, Sandoz) causing a uterine contraction (oxytocin threshold). Finally, the tissue was stimulated electrically at 1 min intervals. When the contractions attained a steady state tension, the tissue was washed repeatedly in calcium-free Krebs solution and the time when the tension was reduced to 50% of the steady state tension (T50) was measured. The procedures involving electrical stimulation have been previously described (Knifton, 1966).

The results are summarized in Table 1. In the rat it was not posssible to demonstrate different types of staircase consistent with varying hormone domination of the myometrium as has been shown in the rabbit (Csapo & Corner, 1952), sheep (Bengtsson & Schofield, 1960) and pig (Knifton, 1966). This confirms the work of Schofield (1960).

TABLE 1. THE EFFECT OF FEMALE SEX HORMONES ON THE RESPONSE OF THE MYOMETRIUM TO ELECTRICAL STIMULATION AND OXYTOCIN

	Controls	Oestrogen-dominated	Progesterone-dominated
	Mean values ± s.e.		
Threshold voltage (V/cm resting length) ..	0·63 ± 0·09	0·47 ± 0·07	0·49 ± 0·06
Oxytocin threshold (m.U.)	0·99 ± 0·19	0·03 ± 0·007	0·23 ± 0·04
Steady state tension (g)	1·6 ± 0·4	4·7 ± 0·39	4·7 ± 0·6
T50 (min)		4·2 ± 0·84	5·6 ± 1·09

There was no significant difference in the values for threshold voltage between the 3 groups of uteri, but there were differences in sensitivity to oxytocin. The control uteri were less sensitive ($P < 0.01$) than the hormone treated ones, and the progesterone-dominated uteri were less sensitive ($P < 0.01$) to oxytocin than the oestrogen-dominated. These results are in accord with the concept of "progesterone block" discussed by Schofield (1963).

The steady state tension of the control group was significantly less ($P < 0.01$) than that of the hormone treated uteri, which is to be expected since oestrogens control the synthesis of contractile protein in the myometrium (Csapo, 1950).

A comparison of the hormone treated groups however, shows that progesterone does not reduce the tension in response to electrical stimulation. Similarly, there is no significant difference between the oestrogen- and the progesterone-dominated uteri in the degree of calcium binding. These results contrast with similar studies in the rabbit (Schofield, 1955; Csapo, 1956) and pig (Knifton, 1966).

The only effect of progesterone on the rat myometrium that this study reveals therefore, is a decrease in the sensitivity to oxytocin.

Department of Pharmacology,
The University,
Liverpool 3.

A. KNIFTON

September 12, 1967

References

Bengtsson, L. Ph. & Schofield, B. M. (1960). *J. Reprod. Fert.*, **1**, 402–409.
Csapo, A. (1950). *Am. J. Physiol.*, **162**, 406–410.
Csapo, A. (1956). *Recent Progr. Horm. Res.*, **12**, 405–427.
Csapo, A. & Corner, G. W. (1952). *Endocrinology*, **51**, 378–385.
Knifton, A. (1966). *J. Pharm. Pharmac.*, **18**, *Suppl.*, 151S–159S.
Schofield, B. M. (1955). *J. Physiol., Lond.*, **129**, 289–304.
Schofield, B. M. (1960). *Ibid.*, **154**, 48P–49P.
Schofield, B. M. (1963). In *Recent Advances in Physiology*, editor Cresse, R., pp. 222–251, London: Churchill.

Nature of adrenergic receptors on the skin melanophores of *Rana tigrina*

SIR,—This report describes the experiments conducted to determine the nature of adrenergic receptors on frog melanophores.

Adult *Rana tigrina* (80–350 g) were anaesthetized by injection of pentobarbitone sodium (50 mg/kg) into the abdominal cavity. Drugs, dissolved in 0·6% saline or amphibian Ringer solution, were injected through the cannulated left branch of thoracic aorta (Bhide & Gupta, 1967) or through one of the liver lobes. Skin colour was observed with the naked eye, and melanophores of the web skin were graded by the method of Hogben & Slome (1931). In experiments in conscious frogs, drugs dissolved in distilled water were injected into the abdominal cavity. From 3 to 6 frogs were used for each dose of each drug and the average change in melanophore index recorded (Tables 1 and 2).

Noradrenaline, which acts predominently on α-type adrenergic receptors was more potent in concentrating melanin in melanophores than adrenaline which

TABLE 1. EFFECT OF ADRENERGIC DRUGS ON THE MELANOPHORE INDEX IN *Rana tigrina*

Drug	Anaesthetized frogs		Conscious frogs	
	Dose mg/kg (No. of frogs)	Average change in the melanophore index Decrease (−) Increase (+)	Dose mg/kg (No. of (frogs)	Average change in the melanophore index Decrease (−) Increase (+)
Noradrenaline hydrochloride	0·1 (7)	−1·2	1·0 (4)	−2·0
	0·5 (7)	−1·8	4·0 (9)	−3·5
	1·0 (5)	−2·6		
Adrenaline hydrochloride	0·5 (3)	−0·75	0·3 (5)	−0·7
	1·0 (7)	−1·25	1·0 (3)	−1·33
	2·0 (5)	−1·8	3·0 (3)	−2·33
Isoprenaline sulphate	0·3 (5)	+0·67	0·3 (3)	+0·66
	1·0 (3)	+1·0	1·0 (4)	+1·0
	3·0 (4)	+0·88	3·0 (6)	+1·5

TABLE 2. EFFECT OF ADRENERGIC BLOCKING DRUGS ON NORADRENALINE-INDUCED CHANGE IN MELANOPHORE INDEX IN *Rana tigrina*. Phenoxybenzamine was given 40 min and other blocking drugs 5–10 min before noradrenaline

Blocking agent (dose mg/kg)	Anaesthetized frogs		Conscious frogs	
	Dose of noradrenaline mg/kg (No. of frogs)	Average decrease in melanophore index	Dose of noradrenaline mg/kg (No. of frogs)	Average decrease in melanophore index
None (taken from Table 1) ..	1·0 (5)	2·6	1·0 (4)	2·0
Phenoxybenzamine (25·0)	1·0 (3)	0·0		
	2·0 (4)	0·2	2·0 (4)	0·0
	6·0 (6)	1·25	6·0 (3)	0·0
Dihydroergotamine methanesulphonate (1·0)	1·0 (6)	0·5		
	6·0 (4)	1·6		
Yohimbine hydrochloride (1·5) ..	1·0 (7)	0·6		
Phentolamine methanesulphonate (0·25)	1·0 (4)	0·38		
Pronethalol hydrochloride* (2·0) ..	1·0 (4)	1·0	1·0 (4)	1·9
INPEA hydrochloride* (2·0)	1·0 (4)	1·62	1·0 (5)	1·6

* These drugs themselves caused blanching and decreased melanophore index by 0·5 to 2·0. Further decrease induced by noradrenaline is given here.

acts on both α- and β-receptors (Table 1). Phenoxybenzamine, dihydroergotamine, phentolamine and yohimbine, which block α-adrenergic receptors, blocked or much reduced the action of an effective dose (1 mg/kg) of noradrenaline (Table 2). This confirms previous reports that ergotamine (Lerner, 1959) and phenoxybenzamine (Bhide & Gupta, 1967) block the action of adrenaline and noradrenaline on frog melanophores.

On the other hand, isoprenaline produced dispersion of melanin and this action was blocked by β-adrenergic blocking agents. Pronethalol and INPEA (*N*-isopropyl-*p*-nitrophenylethanolamine), themselves caused some concentration of melanin and did not block further action of noradrenaline on melanophores.

The present work suggests that the adrenergic receptors on skin melanophores of *Rana tigrina* are predominently of the α-type and they are responsible for colour change induced by adrenaline and noradrenaline. It also suggests occurrence of β-type adrenergic receptors on the melanophores.

Acknowledgement. Phenoxybenzamine was kindly supplied by Smith, Kline and French, Philadelphia, dihydroergotamine by CIBA, India and isoprenaline by Burroughs Wellcome, India.

Department of Pharmacology,
All-India Institute of Medical Sciences,
New Delhi-16,
India.

I. GUPTA
N. K. BHIDE

September 14, 1967

References

Bhide, N. K. & Gupta, I. (1967). *J. Pharm. Pharmac.*, **19**, 58–59.
Hogben, L. & Slome, D. (1931). *Proc. R. Soc., Lond.*, **108***B*, 10–53.
Lerner, A. B. (1959). *Nature, Lond.*, **184**, 674–677.

Simple, sensitive, fluorimetric methods for the estimation of physostigmine in tissue samples and solutions

SIR,—Previous methods for the determination of physostigmine in solutions are either insensitive, indirect or otherwise unsuitable for application to tissue samples (Ellis, Plachte & Straus, 1943; Teare & Taylor, 1967). A simple, sensitive, quantitative method for physostigmine in tissue samples utilizing its powerful natural fluorescence (Udenfriend, Duggan & others, 1957) and which can also be applied to solutions, has now been developed.

Approximately 0·5 g of rat brain or other tissue, to which was added 0·1 to 20·0 μg physostigmine, was homogenized in 5 ml 0·01N hydrochloric acid containing 0·05% ascorbic acid and 0·05% EDTA, and 2·5 ml of 1·2N perchloric acid was added. After mixing, the homogenate was centrifuged at 7000 *g* for 5 min. The supernatant was removed to a centrifuge tube to which 5N potassium carbonate solution was added to adjust the pH to 6. After 5 min centrifugation at 7,000 *g*, 1 ml of the supernatant was removed and the fluorescent intensity determined at excitation 290 mμ and emission 350 mμ wavelengths, in an Aminco Bowman fluorimeter. Internal standards and tissue blanks were also determined.

The method can be applied to solutions by removing a 1 ml sample after the appropriate dilution and adjustment to pH 6.

The method can detect 0·1 μg/g (linear range 0–40 μg/g) in tissue samples and 0·05 μg/ml (linear range 0–5 μg) in solutions with 95–100% recovery (14 values).

Samples are adjusted to pH 6 for fluorimetry because: (i) this pH is in the range pH 2–7 over which maximum fluorescence of physostigmine occurs; (ii) it is the pH of maximum stability (Gisvold, 1962); (iii) it allows the precipitation of potassium perchlorate thus reducing the ionic strength of the sample, which reduces quenching of fluorescence.

Decomposition products such as rubreserine, do not interfere with the assay but eseroline, the initial decomposition product, may interfere. However, with the mild, rapid technique little eseroline should be formed (Swallow, 1951).

While salicylate, a commonly used salt in physostigmine solutions, also possesses strong native fluorescence, it was shown not to interfere appreciably with physostigmine estimations as equivalent concentrations of physostigmine as a salicylate or sulphate salt, possessed equal fluorescence. The usual antioxidants, preservatives and other additives present in physostigmine preparations do not interfere with the estimation.

A much more sensitive fluorimetric technique for the estimation of physostigmine involves condensation with ethylenediamine and subsequent extraction of the fluorescent product with isobutanol (Laverty, Michaelson & others, 1963). Ethylenediamine will also condense with physostigmine degradation products, so the fluorescence of the isobutanol extract determined at excitation 420 mμ and emission 510 mμ wavelengths is a measure of physostigmine, eseroline and rubreserine. The method can detect 0·002 μg/ml physostigmine. In tissue samples, catecholamines will seriously interfere, which restricts the application of this reaction to pure solutions and tissue extracts containing little or no catecholamines.

The above methods provide simple, sensitive assay techniques for physostigmine and some of its degradation products in both tissues and solutions.

Wellcome Medical Research Institute,
University of Otago Medical School,
Dunedin, New Zealand.
September 4, 1967

K. M. TAYLOR

References

Ellis, S., Plachte, F. L. & Straus, O. L. (1943). *J. Pharmac. exp. Ther.*, **79**, 295–308
Gisvold, O. (1962). "Amines and other agents having parasympathomimetic activity" in *Textbook of Organic, Medicinal and Pharmaceutical Chemistry*, 4th edn, editors, Wilson, C. O. & Gisvold, O., p. 414. Philadelphia: J. B. Lippincott Co.
Laverty, R., Michaelson, I. A., Sharman, D. F. & Whittaker, V. P. (1963). *Br. J. Pharmac. Chemother.*, **21**, 482–490.
Swallow, W. (1951). *Pharm. J.*, **66**, 11.
Teare, F. W. & Taylor, D. W. (1967). *J. Pharm. Pharmac.*, **19**, 257–261.
Udenfriend, S., Duggan, D. E., Vasta, B. M. & Brodie, B. B. (1957). *J. Pharmac. exp. Ther.*, **120**, 26–32.

α-Methyltyrosine: effects on fixed ratio schedules of reinforcement

SIR,—α-Methyltyrosine is an inhibitor of tyrosine hydroxylase (Nagatsu, Levitt & Udenfriend, 1964) and when administered to guinea-pigs causes a fall in tissue catecholamine levels without affecting 5-hydroxytryptamine (5-HT). α-Methyltyrosine causes a decrease in motor activity, rotorod performance and shuttle-box conditioned avoidance responding (Rech & Moore, 1965) as well as a decrease in conditioned avoidance responding in an operant situation (Hanson, 1965). These effects are attributed to a decrease in catecholamines in that the effects of α-methyltyrosine are decreased by pre-treatment with a monoamine oxidase inhibitor, which does not alter its brain levels (Moore & Rech, 1967a). In addition, the depression of the conditioned avoidance response is reversed by L-dihydroxyphenylalanine (Moore & Rech, 1967b); also, effects of reserpine and α-methyltyrosine on the conditioned avoidance response and motor activity are similar (Smith & Dews, 1962; Seiden & Carlsson, 1963). We now report the effect of α-methyltyrosine on operant behaviour utilizing positive reinforcement.

Six 80-day old, male, albino rats (Holtzman) were trained in a Lehigh Valley operant conditioning chamber on a fixed-ratio schedule of reinforcement (FR-10, i.e., every tenth lever press was reinforced with 0·01 ml of water) (Ferster & Skinner, 1957). Reinforcement contingencies were programmed by means of solid-state logic modules (Massey Dickinson Co.). Training continued until the total number of responses in a 30-min session did not exceed ±10% of the mean total number of responses from the five previous sessions. When animals reached this level of response, α-methyltyrosine (suspended in polyethylene-glycol-200 and saline, 1 : 1) or vehicle was injected (i.p.) 8 and 4 hr before the next daily session. One week later animals given the vehicle were given drug and vice versa.

Lever pressing performance was initially depressed by 36% during the first 4 min period (see Table 1). During subsequent time periods, performance was

TABLE 1. EFFECT OF α-METHYLTYROSINE ON LEVER PRESSING PERFORMANCE. Each value = mean % depression (± s.e.m.) calculated from each animal's previous day's performance.

Schedule	Time (min)						
	4*	8	12	16	20	24	Total
FR-10	36·2 ± 6·6	47·9 ± 6·9	56·9 ± 10·8	72·3 ± 12	77·4 ± 10	72·5 ± 10	62·6 ± 9
FR-20	36·1 ± 7·7	80·2 ± 6·8	92·2 ± 4·1	93·8 ± 4·5	99·4 ± 1	97·9 ± 2	84·6 ± 3

* All time periods significantly different at $P < 0.05$ except 4 min (Wilcoxon Rank Test).

depressed further. The same rats were then conditioned on an FR-20 schedule, and after they had reached the defined response level (3–4 weeks), α-methyltyrosine was again given. During the first 4-min period there was again a 36% depression of lever pressing. In this respect, the effect of the compound on the FR-10 and the FR-20 schedules is the same. However, during the later time periods the rats on the FR-20 schedule showed a greater depression of lever pressing which progressively increased. The rats did not appear sedated and consumed any water obtained by biting and sniffing of the lever. There was no observable effect in the vehicle-treated rats.

Untrained rats killed 6, 8 and 10 hr after the initial dose of α-methyltyrosine showed approximately the same reduction in brain catecholamines. [The animals were given two injections of α-methyltyrosine (2 × 75 mg/kg) 4 hr apart and brain dopamine and noradrenaline levels (μg/g ± s.e.m.) were: for dopamine 0·83 ± 0·06; 0·23 ± 0·02; 0·23 ± 0·01; 0·25 ± 0·05 at 0, 6, 8 and 10 hr after the first injection. For noradrenaline the respective figures were: 0·40 ± 0·03; 0·18 ± 0·02; 0·16 ± 0·01 and 0·17 ± 0·01.] Assay of brain noradrenaline (Bertler, Carlsson & Rosengren, 1958) showed a decrease of between 55 to 60% over the three time intervals studied: assay of brain dopamine (Carlsson & Waldeck, 1958) showed a decrease of between 70 to 72%.

The depression of lever pressing appears not to be a simple function of total brain catecholamine levels, since on both the FR-10 and FR-20 schedules the animals received the same dose of α-methyltyrosine and yet responded differently. Furthermore, the degree of depletion by the compound cannot explain the progressive nature of the depression. There is, therefore, either no involvement of catecholamines in the depression of lever pressing or the involvement is more complex, such as an interaction between the behavioural situation and a critical pool of amines. The progressive depression of the response may then be a function of the degree of interaction of the schedule contingencies and the animal's biochemical status (e.g. during the initial period of responding).

Acknowledgement. This work was supported by grants from the National Institutes of Health (MH-11191-02 and MH-7083). We would like to thank Dr. C. C. Porter of Merck & Co. for supplying the L-α-methyltyrosine.

Department of Pharmacology and Psychiatry,
The University of Chicago,
Chicago, Illinois 60637, U.S.A.

R. SCHOENFELD
L. S. SEIDEN

July 31, 1967

References

Bertler, Å., Carlsson, A. & Rosengren, E. (1958). *Acta physiol. scand.*, **44**, 273–292.
Carlsson, A. & Waldeck, B. (1958). *Ibid.*, **44**, 293–298.
Ferster, C. B. & Skinner, B. F. (1957). *Schedules of Reinforcement*, pp. 39–132. New York: Appleton-Century-Crofts.
Hanson, L. C. F. (1965). *Psychopharmacologia*, **8**, 100–110.
Moore, K. E. & Rech, R. H. (1967a). *J. Pharm. exp. Ther.*, **156**, 70–75.
Moore, K. E. & Rech, R. H. (1967b). *J. Pharm. Pharmac.*, **19**, 405–407.
Nagatsu, T., Levitt, M. & Udenfriend, S. (1964). *J. biol. Chem.*, **239**, 2910–2917.
Rech, R. H., Borys, H. K. & Moore, K. E. (1966). *J. Pharmac. exp. Ther.*, **153**, 412–419.
Seiden, L. S. & Carlsson, A. (1963). *Psychopharmacologia*, **4**, 418–423.
Smith, C. B. & Dews, P. B. (1962). *Ibid.*, **3**, 55–59.

Cation dependence of metaraminol retention by rat uterus

SIR,—The ability of adrenergically innervated organs to retain exogenous noradrenaline is markedly cation dependent; sodium (Iversen & Kravitz, 1966; Gillis & Paton, 1966, 1967), calcium (Titus & Dengler, 1966; Gillis & Paton, 1966, 1967) and potassium (Gillis & Paton, 1966, 1967) are all necessary for optimal retention. However, whether the absence of these cations reduces retention by preventing the uptake of amine across the axonal membrane or by impairing the subsequent binding to intraneuronal granules, or by both mechanisms, has not been definitely established. Certainly the efflux of [^{3}H]noradrenaline from prelabelled tissues is increased in calcium-free (Taylor & Nash, 1966), in potassium-free (Gillis & Paton, 1967), and in low sodium media (Keen, 1967). The ability of peripheral organs to accumulate exogenous [^{3}H]metaraminol is now reported.

Uterine horns from virgin immature Wistar rats (35–55 g body weight) were incubated at 37° and gassed with oxygen 95% and carbon dioxide 5%. One horn from each rat served as a control and was incubated in Krebs Ringer medium; the other horn was used as the test organ and was incubated in either low sodium (22 mM) or potassium-free Krebs Ringer medium. After 30 min pre-incubation, [^{3}H]metaraminol was added to achieve a final concentration of 13·2 ng of the salt per ml and the incubation was continued for a further 45 min. The amine was then extracted from the tissue and measured as described previously for [^{3}H]noradrenaline (Gillis & Paton, 1967). Retention of [^{3}H]metaraminol was expressed as a ratio (R) calculated by dividing the [^{3}H] counts/min/g of uterine horn by [^{3}H] counts/min/ml of medium. Chromatographically pure (±)-metaraminol-7-[^{3}H]-hydrochloride with a specific activity of 6·7 c/mmole was obtained from the New England Nuclear Corporation.

The results obtained are presented in Table 1. Both low sodium and potassium-free Krebs Ringer medium markedly reduced the retention of [^{3}H]metaraminol.

TABLE 1. INFLUENCE OF SODIUM AND POTASSIUM ON THE RETENTION OF [^{3}H]METARAMINOL BY RAT UTERINE HORNS

Medium used	No. of horns in each group	R value mean ± s.e.		*t* test
		Control horns	Test horns	
Low Na^+	8	8·67 ± 0·42	2·78 ± 0·13	$P < 0.001$
K^+-free	9	8·47 ± 0·56	4·28 ± 0·82	$P < 0.01$

Metaraminol appears to be accumulated by adrenergically innervated organs in a manner essentially similar to noradrenaline; however, unlike noradrenaline, it is not a substrate for either monoamine oxidase or catechol-*O*-methyl-transferase (Giachetti & Shore, 1966). The retention by isolated organs of noradrenaline, but not of metaraminol, is much reduced by reserpine pretreatment; to explain these findings Giachetti & Shore (1966) have proposed that reserpine blocks only the intracellular binding of both amines without altering their passage through the axonal membrane. Consequently amines become exposed to degradation by monoamine oxidase in reserpine-pretreated tissues. Since metaraminol is not a substrate for monoamine oxidase, this amine can still accumulate in reserpine-treated organs (Giachetti & Shore, 1966). Consequently, these authors have suggested that any substance inhibiting the accumulation

of metaraminol *in vitro* may be presumed to act on the membrane "pump" (i.e. the uptake phase of retention) and not to be acting by impairing intraneuronal binding (Giachetti & Shore, 1966). Thus the results of the present investigations, using [^{3}H]metaraminol, strongly suggest that both sodium and potassium are required for the optimal functioning of the membrane "pump" or carrier system, which is responsible for the initial uptake of noradrenaline and related amines into adrenergic nerves.

Acknowledgements. This investigation was supported by a grant from the Alberta Heart Foundation and a fellowship from the Canadian Heart Foundation.

Department of Pharmacology,
University of Alberta,
Edmonton, Alberta,
Canada

DAVID M. PATON

July 17, 1967

References

Giachetti, A. & Shore, P. A. (1966). *Biochem. Pharmac.*, **15**, 607–614.
Gillis, C. N. & Paton, D. M. (1966). *Circulation*, **34**, *Suppl.* No. 3, 110.
Gillis, C. N. & Paton, D. M. (1967). *Br. J. Pharmac. Chemother.*, **29**, 309–318.
Iversen, L. L. & Kravitz, E. A. (1966). *Molec. Pharmac.*, **2**, 360–362.
Keen, P. (1967). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **26**, 570.
Taylor, G. S. & Nash, C. W. (1966). *Proc. Can. Fed. Biol. Soc.*, **9**, 37.
Titus, E. & Dengler, H. J. (1966). *Pharmac. Rev.*, **18**, 525–536.

Complexation of penicillins and penicilloic acids by cupric ion

SIR,—We wish to correct two errors in a paper by Cressman, Sugita & others (1966) with the above title. These are: (1) the values given for the logarithmic association constants for penicilloic G and V acids were transposed. The values should read:

penicilloic V acid	$\log K = 4{\cdot}50 \pm 0{\cdot}02$
penicilloic G acid	$\log K = 4{\cdot}20 \pm 0{\cdot}5$

and (2) the value for the logarithmic association constant for penicillin V and cupric ion should be corrected to read:

penicillin V	$\log K = 2{\cdot}24$ (in the absence of ionic strength control)
penicillin V	$\log K = 2{\cdot}09$ (at ionic strength of 0·01 molar).

Department of Pharmaceutics,
Philadelphia College of Pharmacy and Science,
Philadelphia, Pa., U.S.A.

W. A. CRESSMAN
P. J. NIEBERGALL

August 12, 1967

Reference

Cressman, W. A., Sugita, E. T., Doluisio, J. T. & Niebergall, P. J. (1966). *J. Pharm. Pharmac.*, **18**, 801–808.

Blood pressure and noradrenaline levels after treatment with α-methyldopa, α-methyldopamine and α-methyl-*m*-tyrosine

SIR,—An important factor in the depletion of tissue monoamines by α-methyldopa is a displacement of these amines by the metabolites α-methyldopamine or α-methylnoradrenaline, or both, which are formed by decarboxylation and, in the latter case, subsequent β-hydroxylation in analogy with the sympathetic transmitter noradrenaline (Carlsson & Lindqvist, 1962; Carlsson, 1964). The α-methylated amines are stored in the sympathetic neurons and released on stimulation of these nerves (Muscholl & Maître, 1963), acting as substitute or "false" transmitters. The activity of α-methylnoradrenaline on the adrenergic receptors is less than that of noradrenaline (Day & Rand, 1964; Brunner, Hedwall & others, 1966, 1967). Day & Rand (1963, 1964) reported that the sympathetic function was impaired after treatment with α-methyldopa and suggested this impairment to be the cause of the antihypertensive action of the drug.

If this is so, one would expect a relatively close time correlation of the effect on the blood pressure and the depletion of noradrenaline after α-methyldopa. A further implication of the false transmitter concept is that a parallellism should exist between the receptor activity of the assumed transmitter and the magnitude of the hypotensive response. The present investigations were made to evaluate these assumptions. Blood pressure and tissue monoamine levels have been examined in parallel following the administration of a single dose of α-methyldopa to rats. The effect of α-methyl-*m*-tyrosine on blood pressure has been compared with that of α-methyldopa; α-methyl-*m*-tyrosine also depletes noradrenaline from the stores through displacement by its decarboxylated amine products (Carlsson & Lindqvist, 1962) and these amines possess much less receptor activity than those arising from α-methyldopa (Brunner & others, 1966, 1967). Finally, one of the proposed false transmitters of α-methyldopa, α-methyldopamine has been tested for its effect on blood pressure and tissue monoamines in the rat.

Sprague-Dawley rats of either sex weighing 180–250 g were used. Renal hypertension was provoked by surgical removal of the left kidney and ligation of a branch of the right renal artery in young animals (age about 6 weeks). This procedure resulted in a slowly developing hypertension with mean arterial blood pressure values ranging from 150–190 mm Hg (mean 170 mm Hg, s.e.m. = 3·1, n = 23) 4–6 weeks after the operation. The mean arterial blood pressure in normal rats of corresponding age was 116 mm Hg (s.e.m. = 1·7, n = 32). Blood pressure was always recorded in unrestrained conscious animals through in-dwelling polyethylene catheters (Popovic & Popovic, 1960) connected to Statham P23Dc pressure transducers and recorded on a Grass Model 5 Polygraph. Basal values were obtained by continuous recording for 20–40 min 3–4 times daily for at least 2 days before drug administration. L-α-Methyldopa (200 or 400 mg/kg) and DL-α-methyl-*m*-tyrosine (400 mg/kg) were dissolved in saline and injected intraperitoneally. (±)-α-Methyldopamine HBr 25 mg/kg (calculated as the salt) was injected subcutaneously in divided doses at about 2 hr intervals. Rectal temperature was checked frequently and the hypothermia, occurring after α-methyldopa but not after α-methyl-*m*-tyrosine (unpublished observations), was prevented by keeping the treated animals at an ambient temperature of about 29°. For determinations of tissue monoamine levels, normotensive animals of the same age as the hypertensive rats were used. Preliminary observations have indicated that no significant differences seem to exist between the two groups in control monoamine levels in tissues.

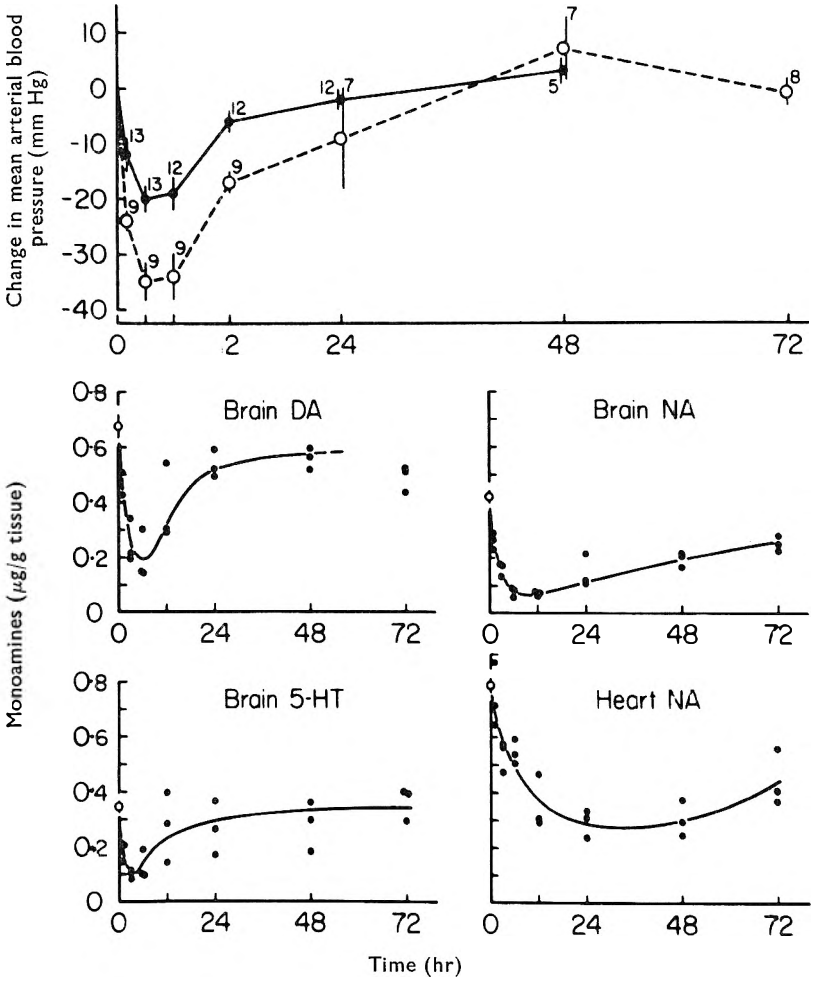

FIG. 1. Decrease in mean arterial blood pressure of conscious normotensive (●—●) and renal hypertensive (○- - - -○) rats (means ± s.e.m., number of experiments indicated by the small figures) and the levels of heart noradrenaline (NA) and brain noradrenaline, dopamine (DA) and 5-hydroxytryptamine (5-HT) following a single dose of L-α-methyldopa 400 mg/kg i.p. Normal blood pressure level of normotensive rats was 116 mm Hg (s.e.m. = 2·1, n = 14), and of hypertensive rats 168 mm Hg (s.e.m. = 5·3, n = 9). Normal amine concentrations: heart noradrenaline 0·79 μg/g (s.e.m. = 0·031, n = 7), brain noradrenaline 0·42 μg/g (s.e.m. = 0·020, n = 7), brain dopamine 0·68 μg/g (s.e.m. = 0·045, n = 6), brain 5-HT 0·34 μg/g (s.e.m. = 0·019, n = 7).

Noradrenaline was determined as described by Bertler, Carlsson & Rosengren (1958), dopamine and 5-hydroxytryptamine (5-HT) by the methods described by Carlsson & Lindqvist (1962) and Andén & Magnusson (1967), respectively.

As shown in Fig. 1, administration of α-methyldopa, 400 mg/kg, lowered the blood pressure of normotensive as well as hypertensive rats. The hypotensive effect appeared to be more pronounced in the latter group, although,

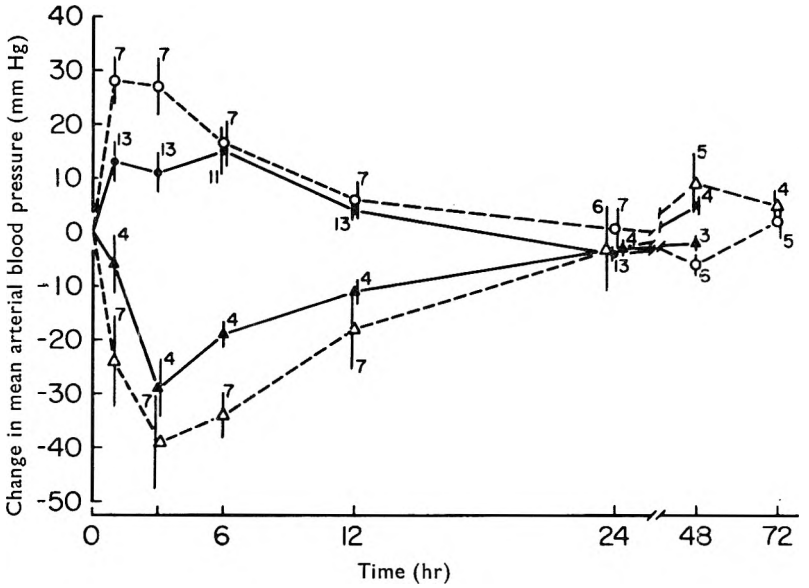

FIG. 2. Change in mean arterial blood pressure of conscious normotensive (solid symbols) and renal hypertensive (open symbols) rats (means ± s.e.m., number of experiments indicated by the small figures) after L-α-methyldopa 200 mg/kg i.p. (triangles) and DL-α-methyl-*m*-tyrosine 400 mg/kg i.p. (circles). Normal blood pressure levels of animals treated with α-methyldopa: normotensive 121 mm Hg (s.e.m. = 3·8, n = 4), hypertensive 171 mm Hg (s.e.m. = 5·9, n = 7); of animals treated with α-methyl-*m*-tyrosine: normotensive 114 mm Hg (s.e.m. = 3·0, n = 14), hypertensive 171 mm Hg (s.e.m. = 5·6, n = 7).

if calculated as % basal values, the response was about the same in the two groups. The effect occurred within 1 hr of the administration and was maximal after 3–6 hr. After 24 hr the blood pressure had returned almost to control values. During the period of blood pressure fall, the animals showed a tendency to develop hypothermia and were sedated.

The levels of monoamines in the brain were lowered by α-methyldopa 400 mg/kg (Fig. 1). Dopamine and 5-HT levels declined to a minimum of about 25% of the normal values after 3–6 hr and then increased to control values after 24 hr. The depletion of brain noradrenaline was slower in onset and longer lasting than that of dopamine and 5-HT. The lowest noradrenaline values were observed after 6–12 hr (about 15% of normal) and control values had not been reached after 72 hr, the depletion still amounting to about 50%. In the heart, maximal depletion, about 70%, was observed after 24–48 hr and the level at 72 hr was about 50% of the normal value.

A comparison between the effect of α-methyldopa on blood pressure and tissue monoamines (Fig. 1) shows that the former effect appeared at a time when there was only a small depletion of brain noradrenaline and an insignificant lowering of heart noradrenaline. The blood pressure had returned to normal values long before the noradrenaline stores were refilled.

Fig. 2 shows the effects of single doses of α-methyldopa (200 mg/kg of the L-form) and of α-methyl-*m*-tyrosine (400 mg/kg of the racemate) on the blood pressure of normotensive and renal hypertensive rats. Since only the L-isomers

of these amino-acids are decarboxylated *in vivo* (for references, see Holtz & Palm, 1966), these doses may be considered roughly equivalent. The hypotensive response of the two groups of rats to α-methyldopa in this dose was in all respects similar to that seen after the higher dose (Fig. 1). α-Methyl-*m*-tyrosine, surprisingly, did not lower the blood pressure in either group of animals for up to 72 hr after the administration; on the contrary, there was a tendency to an initial increase in blood pressure for the first 6–12 hr.

The degree of noradrenaline depletion from the tissues after α-methyldopa 200 mg/kg was nearly as great as that seen after 400 mg/kg (data not presented here). Since α-methyl-*m*-tyrosine seems to be more active than α-methyldopa in depleting catecholamines from the tissues (Porter, Totaro & Leiby, 1961; Udenfriend & Zaltzman-Nirenberg, 1963), it may be inferred that in the present study α-methyl-*m*-tyrosine produced at least the same degree of noradrenaline depletion as α-methyldopa, yet failed to lower the blood pressure.

The effect of α-methyldopamine was studied in normotensive rats receiving 25 mg/kg, s.c., daily for two subsequent days. The blood pressure before the treatment was 110 mm Hg (s.e.m. = 2·8, n = 9) and 16–18 hr after the last injection the blood pressure was 110 mm Hg (s.e.m. = 4·4, n = 9). After 40–42 hr the blood pressure was still within the control range (mean value 113 mm Hg, s.e.m. = 6·0, n = 9). In another series of rats, similarly treated, the heart noradrenaline content was reduced to 0·16 μg/g (s.e.m. = 0·010, n = 6) after 16–18 hr. This reduction was at least as great as that observed after α-methyldopa 400 mg/kg (Fig. 1). The brain noradrenaline was not changed. Apparently, then, depletion of the noradrenaline of peripheral tissues by α-methyldopamine is not accompanied by a reduction of the blood pressure. This may indicate that amine depletion in neurons within the central nervous system is important for the hypotensive effect of α-methyldopa. Also α-methyl-noradrenaline is devoid of blood pressure lowering properties (Henning, unpublished observations, Brunner & others, 1966, 1967).

Thus, after α-methyldopa the time correlation between the noradrenaline depletion and the decrease in blood pressure is poor, the latter effect being much shorter-lasting than the former. This observation is not easily reconciled with the false transmitter concept in its simplest outline. The failure of α-methyl-*m*-tyrosine to lower blood pressure is also difficult to explain assuming a transmitter function of the amine products of this amino-acid.

Acknowledgements. This work was supported by grants from Läkemedelsindustriföreningen, Stockholm, and the Faculty of Medicine, University of Göteborg, Göteborg. The highly competent technical assistance of Miss Inger Pettersson is gratefully acknowledged. For a generous supply of α-methyldopa I am indebted to Merck Sharp & Dohme International.

Department of Pharmacology,
University of Göteborg,
Göteborg SV, Sweden.
August 23, 1967

M. HENNING

References

Andén, N.-E. & Magnusson, T. (1967). *Acta physiol. scand.*, **69**, 87–94.
Bertler, Å., Carlsson, A. & Rosengren, E. (1958). *Ibid.*, **44**, 273–292.
Brunner, H., Hedwall, P. R., Maître, L. & Meier, M. (1966). *Arch. exp. Path. Pharmak.*, **255**, 5–6.
Brunner, H., Hedwall, P. R., Maître, L. & Meier, M. (1967). *Br. J. Pharmac. Chemother*, **30**, 108–122.
Carlsson, A. (1964). *Progr. Brain Res.*, **8**, 9–27.

Carlsson, A. & Lindqvist, M. (1962). *Acta physiol. scand.*, **54**, 87–94.
Day, M. D. & Rand, M. J. (1963). *J. Pharm. Pharmac.*, **15**, 221–224.
Day, M. D. & Rand, M. J. (1964). *Br. J. Pharmac. Chemother*, **22**, 72–86.
Muscholl, E. & Maître, L. (1963). *Experientia*, **19**, 573–575.
Holtz, P. & Palm, D. (1966). *Ergebn. Physiol.*, **58**, 1–580.
Popovic, V. & Popovic, P. (1960). *J. appl. Physiol.*, **15**, 727–728.
Porter, C. C., Totaro, J. A. & Leiby, C. M. (1961). *J. Pharmac. exp. Ther.*, **134**, 139–145.
Udenfriend, S. & Zaltzman-Nirenberg, P. (1963). *Ibid.*, **138**, 194–199.

Enteral absorption of hyoscine *N*-butylbromide

SIR,—In a recent paper, Herxheimer & Haefeli (1966) investigated the oral absorption of hyoscine *N*-butylbromide (Buscopan) in man using the appearance of atropine-like effects as a criterion of oral efficacy. Oral doses of the drug up to 600 mg (approximately 10 mg/kg) failed to produce the effects seen after parenteral injection and it was concluded that the drug was not absorbed from the gastrointestinal tract. However, the results of experiments comparing the toxicity of hyoscine *N*-butylbromide administered by different routes provides evidence for the enteral absorption of the compound.

Three similar groups of Sprague-Dawley rats (females), which had been fasted for the preceeding 12 hr, underwent laparatomy under ether anaesthesia, after which the drug or physiological saline was injected through a fine needle into the lumen of the stomach and duodenum. The operation wound was then sutured, and each animal injected subcutaneously with physiological saline or drug. One group of rats was given the drug subcutaneously, saline being injected into the stomach and duodenum. A second group was given the drug into the stomach, saline being injected subcutaneously and into the duodenum. In the third group of animals, the drug was given into the duodenum and saline was injected subcutaneously and into the stomach. By this experimental design, operative stress and number of injections per animal were evenly distributed throughout the groups. The animals were observed for the following 24 hr and the LD 50 obtained for each route of administration was calculated using the method of Litchfield & Wilcoxon (1949). The values were as follows: LD50 of hyoscine *N*-butylbromide (1) subcutaneously = 510 (386–673) mg/kg, S = 1·365; (2) instilled into the stomach = 1040 (897–1206) mg/kg, S = 1·130; (3) instilled into the duodenum = 180 (154–211) mg/kg, S = 1·195.

In the group of animals given the drug into the duodenum, convulsions started 1 to 2 min after injection and death occurred within 4 to 10 min. When the drug was given subcutaneously or injected into the stomach, the animals did not die for several hr. The drug given into the duodenum was 2·8 times more toxic than when given subcutaneously, which, together with the quick onset of effects, indicates its rapid and good absorption. The possibility that the lower toxicity of hyoscine *N*-butylbromide given into the stomach is caused by decreased gastric peristalsis and slowed passage of the drug to the site of absorption in the small intestine is now being investigated.

Department of Pharmacology,
C. H. Boehringer Sohn,
6507 Ingelheim/Rhine
Germany.
July 13, 1967

H. WICK

References

Herxheimer, A. & Haefeli, L. (1966). *Lancet*, **2**, 418–421.
Litchfield, J. T. & Wilcoxon, F. (1949). *J. Pharmac. exp. Ther.*, **96**, 99–113.

Increased barbiturate sleeping time by simultaneous administration of cardiac glycosides to mice

SIR,—It is well known that cardiac glycosides exert effects on the central nervous system. In addition to stimulant actions, symptoms referable to sedation have been observed.

A central sedative action has been ascribed to adonis glycosides (Bechterew & Pewsner, 1925). Experiments showing the anticonvulsant effect of adonis glycosides against convulsions in rabbits induced by cocaine, camphor and picrotoxin have also been reported (Masslow, 1926).

This sedative effects was ascribed to a special glycosidal fraction of adonis (Fromherz, 1928). We have noted that the hypnotic effect of barbiturates in mice may be potentiated by simultaneous administration of various cardiac glycosides. This may be of interest because cardiac glycosides can be profitably associated with sedatives clinically (Fontana & Portinaro, 1960). The results of our investigations are in Table 1. All drugs were administered orally to male albino mice weighing 20–30 g.

TABLE 1. THE EFFECT OF CARDIAC GLYCOSIDES ON THE PENTOBARBITONE OR QUINALBARBITONE SLEEPING TIMES OF MICE

Drug (mg/kg)	Sleeping time (min) mean ± s.e.	P
Pentobarbitone (100)	196 ± 21	—
Pentobarbitone (100) + desacetyl lanatoside C (3)	448 ± 24	<0·01
Pentobarbitone (100) + G-strophanthin (3)	287 ± 28	<0·05
Pentobarbitone (100) + adonis glycosides (3)	359 ± 30	<0·01
Quinalbarbitone (70)	313 ± 45	—
Quinalbarbitone (70) + desacetyl lanatoside C (3)	375 ± 36	>0·05
Quinalbarbitone (70) + G-strophanthin (3)	444 ± 25	<0·05
Quinalbarbitone (70) + adonis glycosides (3)	564 ± 40	<0·01

It would appear from these results that the sleeping time induced by pentobarbitone and quinalbarbitone was significantly prolonged by simultaneously administering desacetyl lanatoside C, G-strophanthin and adonis glycosides. It is difficult, at present, to provide a satisfactory explanation of these findings. However, direct actions on the brain cells, enzymatic inhibition or modified renal excretion could be involved.

Laboratorio di Farmacologia,
Simes S.p.A., Milan. — R. FERRINI

Cattedra di Farmacologia e
Farmacognosia,
Università di Bologna,
Italy — M. SCUKA

September 8, 1967

References

Bechterew, W. M. & Pewsner, G. A. (1925). *Münch. med. Wschr.*, **72**, 1106
Fontana, G. & Portinaro, A. (1960). *Minerva Med., Roma*, **51**, 2184.
Fromherz, K. (1928). *Münch. med. Wschr.*, **75**, 818.
Masslow, M. (1926). *Arch. exp. Path. Pharmak.*, **111**, 114.

Absence of respiratory depression in the new-born rat after maternal administration of etorphine by the sublingual route

SIR,—Blane, Boura & others (1967) described the general pharmacology of etorphine, a morphine-like analgesic. This drug was found to be remarkably potent by the subcutaneous or intramuscular routes. Nevertheless, respiratory depression in animals was as great, and possibly greater than that seen with equi-analgesic doses of morphine. Campbell, Lister & McNicol (1964) made similar observations in man with the closely related acetorphine (M183 Reckitt). Etorphine was found to be relatively inactive when administered to a variety of species by stomach tube.

More recently the respiratory depressant effect of subcutaneous etorphine was shown again, this time in pregnant rats at term (Blane, 1967) although, surprisingly, the young delivered by Caesarian section from mothers that had received twice the ED80 for analgesia showed significantly less depression of oxygen consumption than did those from mothers receiving an equivalent dose of morphine or methadone. On the other hand, maternal doses of etorphine substantially above the analgesic ED80 resulted in some neonatal mortality—an effect which was seen to a lesser extent with methadone but which did not occur with morphine.

Since etorphine is active by the sublingual route in the rat the respiratory depressant effect in the pregnant animal has now been investigated.

Etorphine hydrochloride was administered in saline solution to 21-day pregnant rats in a dose-volume of 0·001 ml per 10 g rat. The volume placed in the buccal cavity of the largest pregnant animal was thus about 0·03 ml. No spitting occurred and good dose-response lines were obtained for analgesia. Parallel experiments with morphine were impracticable since effective net doses of this drug could not be contained in sufficiently small volumes of saline to

TABLE 1. EFFECTS OF SUBLINGUALLY ADMINISTERED ETORPHINE ON PREGNANT RATS AND THEIR CAESARIAN-DELIVERED OFFSPRING COMPARED WITH SUBCUTANEOUS ETORPHINE AND MORPHINE. Non-pregnant rats were used in the evaluation of analgesic activity. All the values for % reduction of maternal respiratory frequency at analgesic ED80 × 2 differ from each other at significance levels between 1·0 and 0·1%. Subcutaneous etorphine is significantly less depressant on neonatal oxygen consumption at the maternal analgesic dose of ED80 × 2 than morphine ($P = <0{\cdot}001$) and sublingual etorphine has no significant effect at this dose-level.

	Maternal effects		Neonatal effects		
	Analgesic ED80 (mg/kg) 95% confidence limits in parentheses	% reduction of respiratory frequency at analgesic ED80 × 2	Dose reducing O_2 consumption by 20% (mg/kg) 95% confidence limits in parentheses	% reduction of oxygen consumption at maternal analgesic ED80 × 2	Minimum dose at which deaths occurred (mg/kg)
Etorphine sublingual	$38{\cdot}7 \times 10^{-3}$ ($25{\cdot}3 \times 10^{-3}$–$59{\cdot}2 \times 10^{-3}$)	28·5	$\geqslant 160 \times 10^{-3}$	<7·7*	$\geqslant 160 \times 10^{-3}$
Etorphine s.c.	$2{\cdot}52 \times 10^{-3}$ ($1{\cdot}85 \times 10^{-3}$–$3{\cdot}42 \times 10^{-3}$)	52·0	$8{\cdot}65 \times 10^{-3}$ ($6{\cdot}0 \times 10^{-3}$–$16{\cdot}0 \times 10^{-3}$)	16·7	$12{\cdot}0 \times 10^{-3}$
Morphine s.c.	3·36 (2·57 × 4·40)	11·5	1·65 (0·80 — 3·80)	27·8	>200

* Not significantly different from controls.

avoid spillage from the buccal cavity. Trial experiments showed that analgesia was maximal within 15 min of sublingual etorphine administration and began to wear off after 45 min.

Maternal respiratory rates were measured before treatment and again 30 min later, before the mothers were killed by dislocation of the neck. The young were delivered immediately by Caesarian section and incubated individually in the chambers of a respirometer (Blane, 1967).

It was evident that sublingual etorphine (Table 1) caused less depression in maternal respiratory rate than did equi-analgesic doses by the subcutaneous route ($P = <0{\cdot}01$), although it was still greater than morphine. Of greater interest was the absence of effect of even large maternal doses of sublingual etorphine on the new-born. There was no significant depression of oxygen consumption at twice the ED80 for maternal analgesia. When the young were allowed to remain *in utero* for periods longer than 30 min after maternal administration of the drug (up to 2 hr) the oxygen consumption on delivery still fell within the control range. There was also no neonatal mortality at the highest maternal doses of etorphine used so far (160 μg/kg).

It is not yet possible to put forward a completely adequate explanation for the apparent absence of neonatal respiratory depression seen in the young rat delivered from mothers receiving analgesic doses of etorphine sublingually. For the present it is suggested that this route avoids the rapid uptake from the site of administration which results in the occurrence of a sharply defined peak in the maternal blood-concentration curve almost immediately after parenteral etorphine (Blane & Dobbs, 1967). It is possible that the levels of etorphine in neonatal brain which cause respiratory depression and sometimes deaths after parenteral administration are consequent to the development of this short-lived peak blood level on the maternal side of the placental barrier and are avoided when the blood-concentration curve is smoothed by slower absorption after sublingual administration to the mothers.

Department of Pharmacology,
Research and Development Laboratories,
Reckitt & Sons Ltd.,
Hull.
September 1, 1967

G. F. BLANE

References

Blane, G. F. (1967). *Br. J. Pharmac. Chemother.*, **30**, 478–487.
Blane, G. F., Boura, A. L. A., Fitzgerald, A. E. & Lister, R. E. (1967). *Ibid.*, **30**, 11–22.
Blane, G. F. & Dobbs, H. E. (1967). *Ibid.*, **30**, 166–172.
Campbell, D., Lister, R. E. & McNicol, G. W. (1964). *Clin. Pharmac. Ther.*, **5**, 193–200.

Selective protection of 5-hydroxytryptamine stores against the action of reserpine by treatment with 5-hydroxytryptophan

SIR,—Recently we have observed by means of repeated injections of *m*-tyrosine, given shortly before and after reserpine, that it is possible partially to protect the catecholamine stores of the mouse brain against the action of this alkaloid (Carlsson & Lindqvist, 1967). This protection was evident 24 hr after the injection of reserpine, long after the acute effects of *m*-tyrosine had worn off. At the same time the animals were protected against the gross behavioural actions of reserpine. Since *m*-tyrosine was unable to prevent 5-hydroxytryptamine (5-HT) depletion by reserpine, these results underline the importance of catecholamine depletion for the reserpine syndrome.

We have now observed that repeated injections of 5-hydroxytryptophan (5-HTP), given to mice shortly before and after reserpine, will protect 5-HT stores against the action of this alkaloid, while the catecholamine stores are left unprotected (Table 1). Thus, 24 hr after the injection of reserpine (3 mg/kg, i.p.) the brains of the 5-HTP-treated animals still contained about 60% of the normal 5-HT level, compared to 20% in the controls treated with reserpine alone. The catecholamine levels were about 10% of normal irrespective of 5-HTP treatment. It could be proved that here we are dealing with a true protection of 5-HT stores; if animals protected with 5-HTP as described above received a second injection of reserpine after 24 hr, the 5-HT level was rapidly reduced from about 60 to 20%. Further, it was found that animals given 5-HTP in the same dosage but no reserpine, had normal brain 5-HT levels after 24 hr.

TABLE 1. EFFECT OF REPEATED INJECTIONS OF 5-HYDROXYTRYPTOPHAN (5-HTP) ON THE RESERPINE-INDUCED MONOAMINE DEPLETION IN THE BRAIN Female white mice received 400 mg/kg DL-5-HTP subcutaneously. After 30 min reserpine (3 mg/kg) was injected intraperitoneally. Two additional injections of 5-HTP (200 mg/kg) were given after another 1½ and 3½ hr. Controls received the same dose of reserpine only. The animals were killed 24–28 hr after the injection of reserpine. (In two of the experiments a slightly different dosage schedule for 5-HTP was used.) In two experiments indicated in the Table the same dose of reserpine was given 24 hr after the first dose, and the animals were killed after another 4 hr. Data (μg/g) are single values, obtained from 3 to 9 pooled organs.

	5-hydroxytryptamine (μg/g)	Noradrenaline (μg/g)	Dopamine (μg/g)
5-HTP + reserpine	0·29, 0·28 0·25, 0·34 0·29	0·04, 0·04	0·09, 0·08
Reserpine	0·07, 0·06, 0·13	0·04, 0·05	0·07, 0·09
5-HTP + reserpine + reserpine (after 24 hr)	0·10, 0·06	—	—

In spite of the efficient protection of 5-HT stores, the animals treated with 5-HTP and reserpine when examined 24 hr after the injection of the alkaloid, i.e. long after the acute 5-HTP effects had worn off, could not be distinguished from their controls treated with reserpine alone. Hypokinesia, inhibition of exploratory behaviour, hunched back posture and blepharospasm, were thus equally pronounced in both groups.

These experiments, as well as those of Carlsson & Lindqvist (to be published) indicate that the gross reserpine syndrome in the mouse is largely due to catecholamine depletion, whereas 5-HT depletion plays a minor role, if any. Needless to say the possibility remains that 5-HT depletion plays a role for less conspicuous effects of reserpine.

Acknowledgements. This research has been sponsored in part by the Air Force Office of Scientific Research through the European Office of Aerospace Research OAR, United States Air Force under Grant AF EOAR 67–36 and by the Swedish State Medical Research Council (B68–14X-155-04B). For valuable technical assistance we are indebted to Mrs Ingrid Bergh and Miss Birgitta Schultz.

Department of Pharmacology,
University of Göteborg,
Göteborg, Sweden.

ARVID CARLSSON

September 15, 1967

Journal of Pharmacy and Pharmacology

NOVEMBER 1967 VOL. 19 No. 11

Contents

Letters to the Editor