Journal of Pharmacy and Pharmacology

The Pharmaceutical Society of Great Britain

Volume 21 Number 10 October 1969

Journal of Pharmacy and Pharmacology

Published by THE PHARMACEUTICAL SOCIETY OF GREAT BRITAIN
17 Bloomsbury Square, London, W.C.1. Telephone: 01-405 8967
Volume 21 Number 10 **October 1969**

Notice to Contributors

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

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

Text. Authors should consult a CURRENT issue of the Journal and conform to the typographical conventions, use of headings, lay-out of tables, and citation of references. Texts must be typewritten in double spacing on sheets not larger than 9 × 13 inches (22 × 32 cm) with a 1¼ inch (3 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 to the HARVARD system. In the text the surname of the author(s) and the date of publication are given thus: Lewis & Train (1965) described . . . . or . . . . has been described (Lewis & Train, 1965). The list of references is in alphabetical order of first authors and each reference is arranged as follows: LEWIS, C. J. & TRAIN, D. (1965). *J. Pharm. Pharmac.*, **17**, 33–41. The title of publication is underlined and abbreviated as in *World List of Scientific Periodicals* (4th edn, 1963–1965 and supplements) and is followed by the volume number and first and last page numbers. References to books should be as follows: GOODMAN, L. S. & GILMAN, A. (1965). *The Pharmacological Basis of Therapeutics*, 3rd edn, p. 464, London: Collier-Macmillan.

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

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

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

Copyright

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

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

J. Pharm. Pharmac., 1969, **21**, 633–638 *Received June* 16, 1969

Recovery of noradrenaline in adrenergic axons of rat sciatic nerves after reserpine treatment

ANNICA DAHLSTRÖM AND JAN HÄGGENDAL

The Institute of Neurobiology and the Department of Pharmacology, University of Göteborg, Göteborg, Sweden

The recovery of noradrenaline in adrenergic axons of the rat sciatic nerve after a single dose of reserpine (10 mg/kg i.p.) has been studied in unligated nerves and nerves ligated for 6 h. In unligated nerves the recovery at 24 h after reserpine was about 14% of normal. The noradrenaline content then slowly rose to reach about normal concentrations 6–7 days after reserpine injection. In nerves ligated 6 h before death, about 8·0 ng of noradrenaline accumulated proximal to the ligation in normal animals. At 6 and 12 h after reserpine about 4% of normal amounts of noradrenaline were found. Thereafter the amount of accumulated noradrenaline rapidly increased to about normal levels on day 2 after reserpine. At this time the content in unligated nerves was only about 45% of normal unligated nerve. On days 3–5 after reserpine, supranormal accumulations of noradrenaline were found (statistically highly significant), having a maximum at day 4 of about 145% of normal. At this time the noradrenaline content in unligated nerve was only about 80% of normal. The results may indicate an increased synthesis and increased rate of downtransport of amine storage granules during the early recovery phase after reserpine. This phenomenon may be part of a feed-back mechanism operating after depletion of the transmitter in the nerve terminals.

The accumulation of noradrenaline above a constriction of peripheral adrenergic nerves has been studied both histochemically (Dahlström & Fuxe, 1964; Dahlström, 1965; Kapeller & Mayor, 1966) and quantitatively (Dahlström & Häggendal, 1966a, 1967). The rapidly occurring accumulation has been interpreted to arise from a piling up above the lesion of amine storage granules, synthesized in the nerve cell body and transported proximo-distally in the axon (Dahlström & Häggendal, 1966a, 1967; Dahlström, 1966). The minimal effective rate of this transport and the average life-span of the granules in the terminals have for the rat been calculated to be about 5 mm/h, and several weeks, respectively (Dahlström & Häggendal, 1966a).

The catecholamine-depleting effect of reserpine has been demonstrated to be due to a blockage of the catecholamine-storage mechanism in the amine storage granules of central and peripheral catecholamine-containing neurons (Carlsson, Hillarp & Waldeck, 1963; Carlsson, 1965). This blockage is long-lasting, and after high doses of reserpine it even seems to be irreversible, since recovery to normal levels takes several weeks in peripheral tissues and in the central nervous system (Dahlström & Häggendal, 1966b). With the use of the histochemical fluorescence method of Hillarp, Falck and coworkers (for ref. and description see Corrodi & Jonsson, 1967) it has been demonstrated that the very first reappearance of noradrenaline after

reserpine depletion occurs in the nerve cell body in a zone around the nucleus (Carlsson, Falck & Hillarp, 1962; Dahlström, Fuxe & Hillarp, 1965; Dahlström, 1967). At about 24 h after the reserpine treatment most nerve cell bodies have about normal levels of catecholamine-fluorescence, several, however, having supranormal amounts of catecholamine. This overshooting is more pronounced at 36–48 h and can then be seen in large numbers of cells (Dahlström & others, 1965). In the non-terminal axons noradrenaline-fluorescence has been demonstrated about 3–4 cm away from the perikarya as early as 15–18 h after the injection of the drug. At 30–36 h the recovery has proceeded further, permitting about "normal" amounts of noradrenaline to accumulate above a ligation made 1 h before death (Dahlström, 1967).

Since the histochemical method used for the above experiments is only semi-quantitative (see Corrodi & Jonsson, 1967) the present biochemical study was undertaken to follow more in detail, and quantitatively, the reappearance of noradrenaline in the sciatic nerve of the rat. Of special interest was the question whether the supranormal levels of noradrenaline seen in the nerve cell bodies during the recovery phase was reflected in the axons.

Material and methods

Male albino rats of the Sprague-Dawley strain (200–250 g) were given one single dose of reserpine (Serpasil, 2·5 mg ampoules) intraperitoneally (10 mg/kg) 6, 12, 18, 24, 36 h, 2, 3, 4, 5, 6, 7, 9, 11 and 13 days before death according to Tables 1 and 2. During the time between the reserpine injection and killing the rats, they were kept at a temperature of 23–25°. In half of the animals the sciatic nerve was ligated bilaterally under ether anaesthesia 6 h before death. In the rest of the rats the nerves were used unligated. The rats were killed by a blow in the head, and the sciatic nerves dissected out immediately. In the *ligated group* the proximal 1 cm part of the nerve above the ligation was taken out and assayed in groups of 4 to 8. Nerves from normal rats given no drug, and with bilateral 6 h ligations were also collected and assayed at every experimental series. In the *unligated rats* about 3 cm of the nerve, from the dorsal exit through *foramen infrapiriformis* and distally, was dissected

Table 1. *The noradrenaline content in % (mean ± s.e.) of the normal value/cm unligated sciatic nerve of rat after reserpine treatment* (10 mg/kg). *The normal value* (100%) *corresponds to* 1·91 ± 0·08 ng/cm.

Time after reserpine treatment	Number of experiments	Amount of noradrenaline % of normal ± s.e.
0 h*	24	100 ± 4·3
18 h	1	11
24 h	7	14 ± 2·7
36 h	1	40
2 d	8	44 ± 6·6
3 d	14	72 ± 3·7
4 d	6	83 ± 5·7
5 d	6	72 ± 10·4
6 d	5	85 ± 9·4
7 d	6	103 ± 11·3
9 d	7	108 ± 18·7
11 d	8	92 ± 9·7
13 d	8	92 ± 4·4

* Normal rats not given reserpine.

Table 2. *The noradrenaline content in the proximal 1 cm part of the sciatic nerve of rat after ligation, in normal and reserpine-treated rats. All rats were ligated 6 h before death. The values are expressed in % (mean ± s.e.) of the noradrenaline accumulation estimated in normal, 6 h ligated nerves at every experimental series. The noradrenaline value for 6 h ligated normal nerves (100%) corresponds to 7·99 ± 0·36 ng/nerve.*

Time interval reserpine treatment—death	Number of experiments	Amount of noradrenaline accumulated in % of normal	Difference from normal levels (*P* values)
0 h*	26	100 ± 4·5	
6 h	2	4 ± 1·0	<0·001
12 h	3	4 ± 1·2	<0·001
18 h	8	16 ± 8·6	<0·001
24 h	7	22 ± 7·2	<0·001
36 h	8	69 ± 9·9	<0·001
2 d	9	96 ± 5·7	>0·5
3 d	9	133 ± 10·4	<0·001
4 d	10	145 ± 11·6	<0·001
5 d	11	120 ± 4·9	<0·01
6 d	9	108 ± 11·2	>0·25
7 d	9	105 ± 6·6	>0·5
9 d	10	88 ± 3·6	>0·1†
11 d	11	86 ± 3·9	<0·1†
13 d	10	87 ± 4·5	>0·1†

* Normal rats given no reserpine but ligated 6 h before death.

† When taken together, $P < 0{\cdot}025$.

bilaterally and assayed in groups of 4–8. Unligated nerves of normal rats were collected at every experimental series. At every experimental occasion 1–3 single observations were made in each time group. The noradrenaline values of the single observations were expressed in % of the mean noradrenaline value in the normal control group for each experimental occasion.

The method for bioassay used was the trihydroxyindole method as modified by Häggendal (1963). After the dissection, the nerves were either frozen in dry ice and stored at −70° until bioassay, or immediately put into ice-cooled 0·4 N perchloric acid (7 ml) with 20 mg of ethylenediamine tetra-acetate (EDTA) added. After homogenization, using an Ultra-Turrax (Janke & Kunkel) homogenizer, and centrifugation, the extracts were purified on columns of strong cation exchange resins (Dowex-W 50 X8). The fluorescence was measured in an Aminco-Bowman spectrophotofluorometer. In every experiment at least 2 samples containing 0·5 or 1 cm of normal nerve with an addition of known amounts of noradrenaline (50 or 100 ng) were included in the series to check the recovery through the estimation procedures. The recovery was about 85% in all series, and no correction was made for this.

RESULTS

Unligated nerves

In the normal rat, 1 cm of the sciatic nerve was found to contain somewhat less than 2 ng/cm (1·91 ± 0·08). One day after reserpine treatment the nerve appeared to hold about 4% of the normal value (the fluorescence intensities of the samples, however, being very close to that of the blanks). During the following days the noradrenaline content rose, reaching about normal concentrations on the 6th to 7th

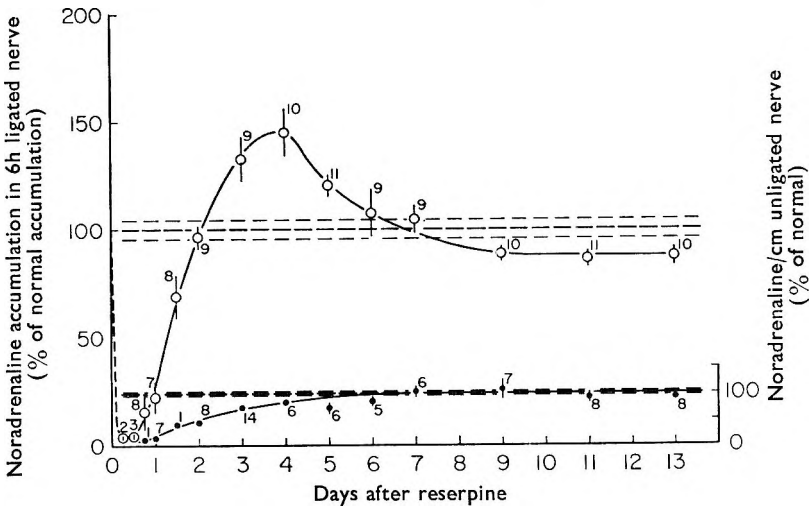


FIG. 1. The noradrenaline content in sciatic nerve of normal and reserpine-treated rats (one single dose of 10 mg/kg i.p.). The upper curve (○———○) indicates the accumulation of noradrenaline in the 1 cm part of nerve above a ligation performed 6 h before death. The values are expressed as % of the mean value for the noradrenaline amount found to accumulate in normal rat nerves above a 6 h ligation at every experimental occasion. 100% corresponds to 7·99 ng ± 0·36 (mean ± s.e., n = 26). The lower curve (●———●) indicates the noradrenaline content in unligated nerves, expressed as % of the content found in normal unligated nerves at every experimental occasion. The amount of noradrenaline/cm normal unligated nerve (100% ordinate to the right) corresponds to 1·91 ng/cm ± 0·08 (mean ± s.e., n = 24). The vertical bars represent the s.e., and the numerals indicate number of experiments.

day. As seen in Fig. 1 the levels remained around normal up to day 13 after the reserpine treatment.

Ligated nerves

In rats without reserpine treatment, about 8 ng of noradrenaline was found above a ligation made 6 h before death (7·99 ± 0·36 ng/cm). After reserpine treatment the amounts were strongly decreased during the first 12 h, but from 18 h and on the accumulated noradrenaline values rapidly reached normal values. A pronounced overshooting was observed between the 3rd and the 5th day (highly significant, $P < 0{\cdot}001$ and $P < 0{\cdot}01$, Table 2), reaching a maximum of about 45% above normal on the 4th day (see Table 2 and Fig. 1). At 9, 11 and 13 days after reserpine, the accumulated noradrenaline was slightly less than normal (86–88%, significant at $P < 0{\cdot}025$ when the 3 groups were taken together).

DISCUSSION

There seems to be little doubt that the recovered noradrenaline, observed in the adrenergic axons of the sciatic nerve, is stored within amine storage granules, since tetrabenazine, a shortlasting blocker of the noradrenaline storage mechanism in the granules (Pletscher, Brossi & Gey, 1962; Häggendal, 1968), or a second injection of reserpine, can disperse the reappeared noradrenaline (Dahlström, 1967).

In earlier histochemical experiments it was found that no noradrenaline accumulated above a 1 h ligation in rats given reserpine (10 mg/kg) 6–12 h previously. At 15 h after reserpine some noradrenaline accumulated, thereafter the recovery proceeded rapidly, and normal fluorescence intensities were found by 30–36 h after

reserpine. In this quantitative study, accumulation of noradrenaline was traced as early as 18 h after reserpine, which is in agreement with the histochemical observations.

In the present experiment the amount of noradrenaline accumulated above a 6 h ligation was found to be about normal at day 2 after reserpine; unligated nerves at the same time held about 45% of normal. Three to 5 days after reserpine, supranormal accumulations of noradrenaline developed, while the noradrenaline content in unligated nerves was still below normal. According to earlier calculations on the rate of transport of granules, made under the presumption that the storage granules do not increase their content of noradrenaline during the time of accumulation (Dahlström & Häggendal, 1966a), these results would indicate an increased rate of transport of granules in the nerve during the 3rd to 5th day after reserpine. It does indeed seem as if the granules do increase to some extent their content of noradrenaline during the time of accumulation. However, this increase appears to be of about the same relative size in normal rats as in rats given reserpine 3 or 4 days beforehand (Dahlström & Häggendal, unpublished observations). Therefore, the supranormal levels of noradrenaline accumulation observed in this work may indicate an increased amount of downtransported granules, and thereby an increased synthesis of granules in the cell bodies, and also, an increased rate of the proximo-distal transport.

It is well-known that the recovery of noradrenaline to normal amounts after reserpine depletion takes a long time (cf. Carlsson, 1965). In a preliminary study, this time was found to be several weeks (Dahlström & Häggendal, 1966b) in the rat, approximately the same time as was calculated to be the average life-span of granules in the nerve terminals (Dahlström & Häggendal, 1966a). The recovery curve after the reserpine injection followed an approximately straight line, suggesting that a continuous, steady downtransport of newly formed granules from the nerve cell bodies is necessary for a complete recovery of noradrenaline levels in the nerve terminals after one high dose of the drug (Dahlström & Häggendal, 1966b). However, it has been observed that within the first week after the injection the recovery curve has a temporary steeper inclination (Häggendal & Dahlström, unpublished observations). The results of the present experiments suggest that this temporary increase in the rate of noradrenaline recovery in the nerve terminals may be due to an increased synthesis and downtransport of amine storage granules within this early time period.

It has been observed that increased nerve activity causes an increased synthesis of proteins in the perikarya (cf. Hydén, 1960). Since reserpine depletes the transmitter stores, transmission is blocked and the functional response to nerve activity abolished. It may be speculated that some feed-back mechanism may cause a compensatory increased impulse activity to the adrenergic neurons in reserpinized animals. This increased nerve activity could possibly induce an increased synthesis of the protein containing storage granules. Even if other mechanisms may explain the results, such an increased formation of granules as observed, may seem biologically adequate in a situation where the granules in the neuron are blocked, as for example exists after reserpine.

Acknowledgements

The present study has been supported by grants from the Swedish Medical Research Council (grants nr B69-14X-2207-03 and B69-14X-166-05 A) and by grants from the

Faculty of Medicine, University of Göteborg, Sweden. For generous supply of Serpasil we are indebted to the Swedish CIBA, Stockholm. The skilful technical assistance of Mr. Pär-Anders Larsson, Miss Ingalill Nordgren, Miss Birgitta Parkner and Miss Agneta Wilén is gratefully acknowledged. For preparation of figure, and statistical treatment of the material we are indebted to Research engineer Tor Magnusson.

REFERENCES

CARLSSON, A. (1965). *Handbuch der Exp. Pharmacol.* Editor: Erspamer, V. Berlin-Göttingen-Heidelberg: Springer.

CARLSSON, A., FALCK, H. B. & HILLARP, N.-Å. (1962). *Acta physiol. scand.*, **56**, Suppl. 196, 1–28.

CARLSSON, A., HILLARP, N.-Å. & WALDECK, B. (1963). *Ibid.*, **59**, Suppl. 215, 1–38.

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

DAHLSTRÖM, A. (1965). *J. Anat.*, **99**, 677–689.

DAHLSTRÖM, A. (1966). *M.D. Thesis.* Stockholm.

DAHLSTRÖM, A. (1967). *Acta physiol. scand.*, **69**, 167–179.

DAHLSTRÖM, A. & FUXE, K. (1964). *Z. Zellforsch.*, **62**, 602–607.

DAHLSTRÖM, A., FUXE, K. & HILLARP, N.-Å. (1965). *Acta pharmac. tox.*, **22**, 277–292.

DAHLSTRÖM, A. & HÄGGENDAL, J. (1966a). *Acta physiol. scand.*, **67**, 278–288.

DAHLSTRÖM, A. & HÄGGENDAL, J. (1966b). *J. Pharm. Pharmac.*, **18**, 750–751.

DAHLSTRÖM, A. & HÄGGENDAL, J. (1967). *Acta physiol. scand.*, **69**, 153–157.

HÄGGENDAL, J. (1963). *Ibid.*, **59**, 242–254.

HÄGGENDAL, J. (1968). *J. Pharm. Pharmac.*, **20**, 364–367.

HYDEN, H. (1960). In "*The Cell*". Editors: Brachet, J. & Mirsky, A., Volume IV, p. 215, New York-London: Academic Press.

KAPELLER, K. & MAYOR, D. (1966). *J. Anat.*, **100**, 439–441.

PLETSCHER, A., BROSSI, A. & GEY, K. F. (1962). *Int. Rev. Neurobiol.*, **4**, 275–306.

J. Pharm. Pharmac., 1969, **21**, 639-647 *Received April* 3, 1969

Histochemical studies on the depletion of noradrenaline by adrenaline in adrenergic nerves of the rat iris

C. G. HAINING, JULIA L. HEYDON AND L. R. MURRAY

From the Pharmacology Section, National Biological Standards Laboratory, Canberra, A.C.T., Australia

The intensity of fluorescence produced in adrenergic nerves by exposure to formaldehyde under controlled conditions and levels of tissue catecholamines were determined in rat irides. Incubation of irides in Krebs solution containing (−)-adrenaline (0·1–10 μg/ml) led to a temperature-dependent reduction of fluorescence intensity, a reduction in tissue noradrenaline and a gain in tissue adrenaline. The changes in fluorescence were reversed by subsequent incubation of the tissue in the presence of (−)-noradrenaline (0·1–1 μg/ml). A reduction of fluorescence intensity and tissue noradrenaline in the rat iris, followed by recovery over 16 h, was also produced by exposing the cornea to a 1 or 5% solution of (−)-adrenaline. The application of these results to studies on noradrenaline uptake in adrenergic nerves is discussed.

The uptake of exogenous adrenaline in tissues is accompanied by a release of noradrenaline (Andén & Magnusson, 1963; Andén, 1964; Angelakos, Bloomquist & King, 1965; Westfall, 1965; Nash, Wolff & Ferguson, 1968) which is thought to result from a competitive exchange reaction (Euler & Lishajko, 1963; Burgen & Iversen, 1965). In recent years it has become possible to follow changes in the level of intraneuronal noradrenaline by histochemical fluorescence methods (Falck, 1962). Such techniques have been used to demonstrate uptake of primary catecholamines at the cellular level in tissues from reserpinized animals (Malmfors, 1965) and displacement of noradrenaline from the sympathetic nerve net-work of the rat iris by adrenaline and other amines (Eränkö & Räisänen, 1966).

The present work was carried out to investigate further the noradrenaline-releasing action of adrenaline and the possibility of employing adrenaline to deplete tissues of noradrenaline in studies on catecholamine uptake into adrenergic nerves. Results of investigations into the suitability of drugs other than reserpine for this purpose have recently been reported by Malmfors & Sachs (1968).

EXPERIMENTAL

Methods

In vitro *experiments.* Wistar rats were killed with ether or carbon dioxide and their eyes removed immediately. Irides with corneas attached were removed from eyes and allotted randomly to treatments. Some irides were treated histochemically immediately after removal from the eye while others were incubated in Krebs solution in each experiment as controls for histochemical treatment and drug effects respectively. Groups consisting of 5 or 10 irides were incubated together in Krebs solution (Ringer-phosphate bicarbonate III; Long, 1961) with or without drug. Equal

numbers were included in control and test groups. Incubations were carried out in 10 ml volumes of solution and gassed with moist preheated 5% carbon dioxide in oxygen at 37° unless stated otherwise.

Dissection of the iris. The method was based largely on that of Malmfors (1965). The eye was placed in a depression in a Perspex plate. Two incisions were made at the same time on opposite sides about 2 mm posterior to the end of the rectus tendon with two razor blades and the anterior segment of the eye was then detached with fine forceps and iris scissors. The lens capsule with adherent vitreous was removed from the anterior segment and the cornea, with attached iris, placed in cold Krebs solution with other similar preparations before being distributed randomly into groups. At the end of the incubation period each preparation was rinsed in cold Krebs solution. The iris and ciliary body were removed from the cornea with fine forceps and a radial incision was made in the iris which was then stretched and allowed to dry on a microscope slide. The ciliary body was separated from the iris with Beaver Mini Blades and the preparation was then placed over phosphorus pentoxide in a desiccator for at least 1 h.

Histochemical treatment of the iris. Condensation of the noradrenaline in adrenergic nerves with formaldehyde gas was as described by Falck (1962). The prepared microscope slides in racks of thirty were placed under vacuum in a desiccator and exposed for 1 h at 40 or 80° to the vapour from paraformaldehyde (6 g/litre) which had been equilibrated at a relative humidity of 70% for one week (Hamberger, Malmfors & Sachs, 1965; Hamberger, 1967). Preparations could be kept without apparent deterioration for 3 or 4 days over phosphorus pentoxide before formaldehyde treatment but after fluorescence had been induced slides were read as soon as possible since some fading appeared to take place within 24 h.

Estimation of fluorescence intensity. The fluorescence intensity of the irides was estimated using either a Zeiss fluorescence microscope fitted with a Zeiss I (BG 12/4) filter for excitation and a combination of Zeiss 53 and Zeiss 44 as barrier filters, or a Reichert fluorescence microscope, fitted with a Reichert E 3(BG 12/6) filter for excitation and a Reichert Sp 3(GG 9/1 + OG 1/1·5) as the barrier filter. In both microscopes the source of illumination was a high pressure mercury lamp, HBO 200 W.

Slides were coded in a random manner so that the treatment received by a preparation could not be identified until all assessments had been made. Scores for fluorescence intensity were always allotted by the same observer on the following basis: 0 = negligible, 1 = indistinct and weak, 2 = distinct and weak and 3 = distinct and strong. The score for each group was expressed as a percentage of that assigned to one of the corresponding control groups of irides included in the same experiment. n = The number of irides examined.

Extraction and estimation of tissue catecholamines. The procedure followed was that of Anton & Sayre (1962).

Source of drugs. (−)-Adrenaline hydrogen tartrate (British Drug Houses Ltd., England). (−)-Noradrenaline (Fluka A.G., Switzerland).

RESULTS

Histochemical evidence for the release of noradrenaline from adrenergic nerves by adrenaline in vitro. Incubation of isolated rat irides for 1 h in Krebs solution at 37°

Table 1. *Loss of fluorescence in adrenergic nerves of the isolated rat iris by incubation with adrenaline.* Groups of rat irides were incubated for 1 h in Krebs solution containing varying concentrations of catecholamine. Fluorescence in adrenergic nerves was induced by treatment with formaldehyde under controlled conditions. The intensity of fluorescence was estimated visually on a 0 to 3 scale and is expressed as a percentage of the value obtained for corresponding control irides incubated in Krebs solution for 1 h. n = The number of irides examined.

Concentration of catecholamine μg/ml	Adrenaline		Noradrenaline	
	n	Intensity of fluorescence %	n	Intensity of fluorescence %
0·01	25	104	20	110
0·1	32	99	30	108
1	70	67	20	109
10	65	34	25	98
100	35	27	20	100

with increasing concentrations of adrenaline led to a graded reduction in the fluorescence intensity of the adrenergic nerve plexus. The threshold concentration of adrenaline necessary to cause a reduction in the intensity of fluorescence was between 0·1 and 1 μg/ml and a near maximum effect was obtained with a concentration of 10 μg/ml (Table 1).

This effect of adrenaline was temperature dependent. When groups of 20 irides were incubated for 2 h with adrenaline (10 μg/ml) at 37, 20 or 0°, little reduction of fluorescence was obtained in the nerve network at the two lower temperatures. Decreases in the fluorescence intensity of adrenaline-treated groups expressed as percentages of values obtained for corresponding control groups were 80% at 37°, 7% at 20 to 21° and 2% at 0°.

Under the conditions employed, fluorescence obtained by exposure to formaldehyde gas was specific and was not produced when stretch preparations of rat irides were heated for 1 h at 40 or 80° in a desiccator in the absence of paraformaldehyde.

No evidence was obtained in any experiments that more prolonged incubation of irides in Krebs solution for periods up to 4 h led to a reduction in the development of fluorescence. Comparison of scores allotted to groups of irides which had been mounted immediately after removal from the eye with those of control preparations incubated in Krebs solution for 1 h did not reveal any difference in the intensity of fluorescence in the two groups.

Incubation of irides with noradrenaline at concentrations from 0·01 to 100 μg/ml did not affect the intensity of fluorescence (Table 1). Irides which had been treated in solutions containing 10 μg/ml or lower concentrations of noradrenaline resembled control preparations which had been incubated in Krebs solution for the same period of time without catecholamine, but incubation with noradrenaline (100 μg/ml) resulted in an overall fluorescence of the tissue. The network of nerves enveloping blood vessels which normally exhibit fluorescence equal to or greater in intensity than other parts of the sympathetic nerve plexus were not clearly discernable, possibly because of the strong background fluorescence.

Since oxygenated Krebs solution containing adrenaline darkened during incubation,

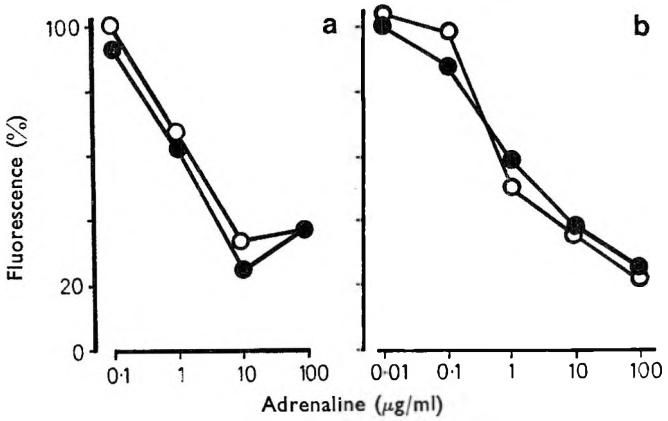


FIG. 1. Failure of ascorbic acid (a) and ascorbic acid plus sodium edetate (b) to modify the effect of incubation for 1 h with adrenaline on the fluorescence intensity of adrenergic nerves in the isolated rat iris. ○, adrenaline; ●, adrenaline + ascorbic acid (10 μg/ml), or ascorbic acid (10 μg/ml), and sodium edetate (20 μg/ml). The effect of each concentration of adrenaline was examined on 15 (a) or 20 (b) irides.

the possibility of protecting catecholamines by the addition of antioxidants was investigated. The presence of ascorbic acid (10 μg/ml) alone or with sodium edetate (EDTA) (20 μg/ml) had a negligible effect on the intensity of fluorescence in irides incubated for 1 h in the absence of catecholamine. The mean fluorescence intensity of irides which had been incubated with ascorbic acid (10 μg/ml) was 90% of maximum (n = 35) compared with 80% for controls (n = 35) whilst corresponding values for those incubated with ascorbic acid (10 μg/ml) + EDTA (20 μg/ml) were 96% (n = 40) and 98% (n = 40) for controls. The presence of ascorbic acid (10 μg/ml) for the same period of incubation alone (Fig 1a) or with EDTA (20 μg/ml) (Fig 1b) failed to modify the effect of adrenaline on the intensity of fluorescence in adrenergic nerves.

Chemical evidence for the release of noradrenaline from adrenergic nerves by adrenaline in vitro. To confirm that a reduction in the intensity of fluorescence indicated

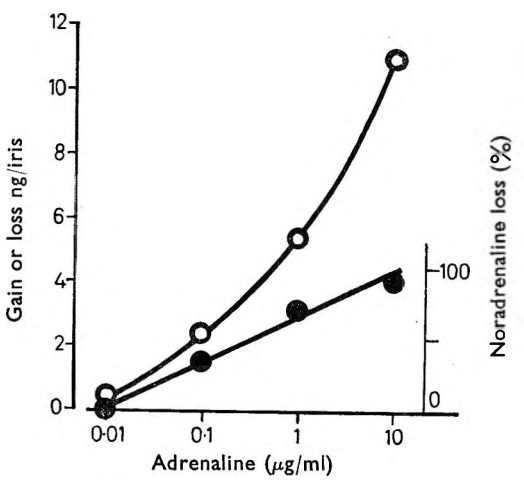


FIG. 2. Changes in noradrenaline and adrenaline content of the isolated rat iris after incubation with adrenaline for 1 h. The effect of each concentration of adrenaline was examined on 25 irides. Catecholamines were determined chemically in groups of 5 or 10 irides. ○, adrenaline gain; ●, noradrenaline loss.

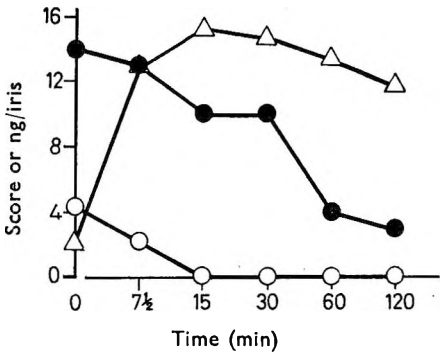


FIG. 3. Time course of noradrenaline depletion from adrenergic nerves of the isolated rat iris incubated with adrenaline (10 μg/ml). ●, Intensity of fluorescence in 5 irides; △, adrenaline and ○, noradrenaline, determined chemically in 5 pooled irides.

loss of noradrenaline, parallel experiments were made in which tissue catecholamine levels were estimated spectrofluorimetrically after extraction. Changes in the adrenaline and noradrenaline content of irides resulting from incubation with varying concentrations of adrenaline for 1 h are shown in Fig 2.

In these experiments, estimates of the quantity of adrenaline taken up by tissues exceeded those of noradrenaline displaced, the difference being most marked in irides which had been incubated with adrenaline (10 μg/ml). Ratios of adrenaline gain to noradrenaline loss obtained with increasing concentrations of adrenaline in the medium were 0·1 μg/ml, 1·6; 1 μg/ml, 1·7 and 10 μg/ml, 3·1.

Time course of noradrenaline release from adrenergic nerves by adrenaline in vitro. The time course of noradrenaline release from isolated irides incubated with adrenaline (10 μg/ml) determined by fluorescence microscopy is shown in Table 2. The

Table 2. *Time course of changes in fluorescence intensity in adrenergic nerves of the isolated rat iris incubated with adrenaline.* Groups of rat irides were incubated for varying periods of time in Krebs solution with or without adrenaline (10 μg/ml). Fluorescence in adrenergic nerves was induced by treatment with formaldehyde under controlled conditions. The intensity of fluorescence was estimated visually on a 0 to 3 scale and is expressed as a percentage of the value obtained for corresponding control irides mounted immediately after removal from the eye. *n* = The number of irides examined.

Period of incubation (min)	Krebs solution		Krebs solution + adrenaline 10 μg/ml	
	n	Intensity of fluorescence %	n	Intensity of fluorescence %
0	20	106		
7·5			25	106
15			25	90
30			27	81
60	40	103	33	51
120	35	103	33	34
240	20	121	20	33

results of a single experiment in which both the intensity of fluorescence and catecholamine levels were determined in parallel after incubation with adrenaline (10 μg/ml), for varying periods of time are shown in Fig. 3. Maximum reduction of fluorescence intensity was obtained in tissue only after exposure to adrenaline for 2 h or more (Table 2) but noradrenaline could not be detected chemically after an incubation period of 15 min (Fig. 3).

Restoration of fluorescence in adrenergic nerves after its reduction by incubation with adrenaline in vitro. The fluorescence of adrenergic nerves which had been reduced in intensity by exposure to adrenaline was restored in preparations which had been treated with noradrenaline (Table 3). Incubation of irides with adrenaline (10 μg/ml) for 1 h followed by a further period of 1 h in Krebs solution after a rinse in Krebs

Table 3. *Restoration of fluorescence in adrenergic nerves of the isolated rat iris after its partial reduction by incubation with adrenaline.* Groups of 10 rat irides were incubated for 1 h with or without adrenaline (10 μg/ml) and then for a further 1 h with varying concentrations of noradrenaline. Fluorescence in adrenergic nerves was induced by treatment with formaldehyde under controlled conditions and its intensity estimated visually on a 0 to 3 scale.

Treatment		Intensity of fluorescence		
1st h adrenaline μg/ml	2nd h noradrenaline μg/ml	Expt 1 Score	Expt. 2 Score	%
0	0	29/30	30/30	100
10	0	7/30	6/30	22
10	0·01	7/30	9/30	27
10	0·1	20/30	17/30	63
10	1	29/30	30/30	100
10	10	30/30	30/30	102

solution resulted in a 78% reduction of fluorescence intensity compared with control preparations. However, when noradrenaline at a concentration of 0·1 μg/ml or higher was present during the second incubation period, the intensity of fluorescence increased and was restored to its original level with a concentration of 1 μg/ml.

Noradrenaline release from adrenergic nerves by adrenaline in vivo. Experiments were made to determine whether the displacement of noradrenaline by adrenaline could be demonstrated *in vivo* under conditions similar to those in which adrenaline and other sympathomimetic amines might be employed clinically. Albino rats were allotted to one of two groups and one drop of a 1 or 5% w/v solution of adrenaline as the hydrogen tartrate dissolved in saline and adjusted to pH 3·5 was applied on one eye for 10 s, the other eye acting as control. Two h later the animals were killed with carbon dioxide and stretch preparations prepared from their irides were exposed to formaldehyde. A single application of adrenaline at either concentration reduced the intensity of fluorescence in the iris of the treated eye. Little difference was observed between the response to 1 and 5% solutions. With groups of 25 animals, the reduction in fluorescence intensity of the treated eyes compared with their controls was 31% when a 1% solution of adrenaline was used and 44% with a 5% solution. A more marked reduction of fluorescence was obtained when the adrenaline solution was applied repeatedly at 10 min intervals for 2 h. In groups of 5 animals, the 1% solution produced a 64% reduction of fluorescence and the 5% solution 75%.

The time course of noradrenaline depletion from the rat iris after a single application of adrenaline to the cornea and its subsequent restoration, was followed by estimating catecholamine levels chemically and determining changes in fluorescence intensity. A 5% solution of adrenaline was applied to the cornea of one eye and allowed to remain in contact with it for 10 s. The other eye was untreated and acted as control. Groups of 15 animals were killed with carbon dioxide at suitable time intervals after drug administration. Irides were removed from both eyes of 10 of the rats, rinsed in Krebs solution and dropped into 0·4N perchloric acid before determinations of their adrenaline and noradrenaline content by the trihydroxyindole method. Irides were dissected from the eyes of the remaining five animals and prepared as stretch preparations on microscope slides for histochemical treatment.

The onset of noradrenaline release was rapid. Maximum depletion within 1 h was indicated by histochemical and chemical procedures (Fig. 4). The intensity of fluorescence was reduced by 50% and the level of chemically estimated noradrenaline by 80–100%. Subsequently, levels of catecholamines gradually returned towards pretreatment levels, complete recovery being achieved about 16 h after treatment.

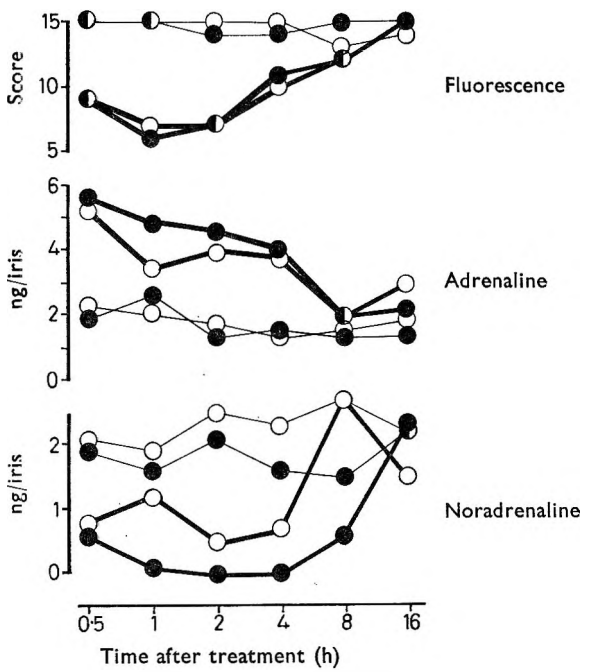


FIG. 4. Time course of noradrenaline depletion and reduction of fluorescence intensity in adrenergic nerves of the rat iris following a single application of adrenaline, 5%, to the cornea. ——, control eye; ——, treated eye. ○, 1st experiment; ●, 2nd experiment. Adrenaline and noradrenaline were determined chemically in 10 pooled irides. Fluorescence intensity was assessed in groups of 5 irides.

DISCUSSION

The histochemical demonstration of noradrenaline is based on the formation of a fluorescent compound by its condensation with formaldehyde under controlled conditions. Since secondary catecholamines require more severe reaction conditions for optimal fluorescence than primary catecholamines (Corrodi & Jonsson, 1967), the reduced intensity of fluorescence obtained in tissues exposed to adrenaline

after treatment with formaldehyde suggests that part of the endogenous noradrenaline has been replaced by adrenaline.

The loss of fluorescence in irides treated with a relatively high concentration of adrenaline and its restoration in preparations incubated with noradrenaline confirms results previously reported by Eränkö & Räisänen (1966). In the present experiments these changes have also been shown to take place with concentrations of catecholamines much lower than those so far reported. The depletion of noradrenaline stores *in vitro* and *in vivo* by adrenaline and their subsequent restoration has in some cases also been followed by chemical determinations of catecholamines. Although qualitatively results obtained by the two methods are compatible, there are disparities between them if the results are considered on a quantitative basis. This is not unexpected since the scoring system used in the histochemical studies was purely arbitrary and the relation between noradrenaline content and fluorescence intensity is not clear since quenching of fluorescence may take place with high concentrations of noradrenaline (Ritzén, 1966). Furthermore, interaction between adrenaline and formaldehyde under the conditions employed may have led to the formation of a fluorescent compound, which, even though of low intensity, could have contributed to the residual fluorescence present in neurons exposed to adrenaline.

In the noradrenaline uptake studies (Table 3) a graded increase in the intensity of fluorescence was obtained with concentrations of noradrenaline from 0·01 to 1 μg/ml (60×10^{-7}M) but no further effect could be demonstrated when the concentration was increased to 10 μg/ml. The results of chemical determinations indicate that although a reduction of fluorescence intensity is associated with depletion of noradrenaline, a large part of the adrenergic transmitter store must be lost before a reduction of fluorescence intensity is detectable. Confirmation of this is to be found in the reports of recent studies by Olson, Hamberger & others (1968) in which fluorescence histochemistry and measurements of [^{3}H]noradrenaline were made in the same tissue. Reasonable correlation was shown by these workers to exist between subjectively estimated fluorescence and the quantity of catecholamine taken up from Krebs-Ringer solution by irides from reserpine-treated rats which had been exposed to noradrenaline over a limited range of concentrations up to about 35×10^{-7}M. When the concentration of catecholamine was raised to 110×10^{-7}M there was no increase in the intensity of fluorescence of the iris although its [^{3}H]noradrenaline content was almost doubled.

The overall fluorescence observed histochemically in tissues which had been incubated with noradrenaline (100 μg/ml) indicates that at high concentrations uptake of the amine was not restricted to adrenergic nerve fibres. A similar effect was not obvious when the incubation was carried out in the presence of adrenaline instead of noradrenaline. However, there was a large increase in the ratio of adrenaline gain to noradrenaline loss determined chemically in irides which had been incubated with 10 μg/ml of adrenaline compared with those exposed to a concentration of 1 μg/ml (Fig. 2) which could be explained by the uptake of adrenaline at extraneuronal sites.

Our results suggest that provided suitable concentrations of catecholamines are employed, tissue which has been depleted of its adrenergic transmitter by previous treatment with adrenaline may be of value in studies on the uptake of noradrenaline into adrenergic neurons. Limitations which are inherent in the histochemical fluorescence technique would however prevent the application of this method in a strictly quantitative manner. These are, the presence of residual fluorescence in

neurons of adrenaline-treated preparations possibly due to an adrenaline formaldehyde condensation product and the fact that exogenous noradrenaline may accumulate intraneuronally beyond the level at which maximum fluorescence intensity is obtained.

Results of *in vivo* studies (Fig. 4) confirm the rapid onset of noradrenaline release from the iris by adrenaline observed *in vitro* (Fig. 3) and indicate that replenishment of noradrenaline by endogenous catecholamine requires only a few hours. Although adrenaline in solution, at concentrations which have been used clinically, reduced the noradrenaline content of the iris after application to the cornea, the implication of this is not clear. Andén & Magnusson (1963) failed to detect any change in adrenergic function in rats and cats after 95% depletion of peripheral noradrenaline by (+)-adrenaline, which had only about 1/10th the agonist activity of (−)-adrenaline.

Acknowledgements

The authors wish to express their appreciation to Miss K. E. McKinstry, Mrs. S.M. Nurmi and Miss E. H. Rowe for skilful technical assistance.

REFERENCES

ANDÉN, N.-E. (1964). *Acta pharmac. tox.*, **21**, 59–75.

ANDÉN, N.-E. & MAGNUSSON, T. (1963). *Biochem. Pharmac.*, **12**, *Suppl.* 66.

ANGELAKOS, E. T., BLOOMQUIST, E. I. & KING, M. P. (1965). *Life Sci.*, **4**, 1515–1520.

ANTON, A. H. & SAYRE, D. F. (1962). *J. Pharmac. exp. Ther.*, **138**, 360–375.

BURGEN, A. S. V. & IVERSEN, L. L. (1965). *Br. J. Pharmac. Chemother.*, **25**, 34–49.

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

ERÄNKÖ, O. & RÄISÄNEN, L. (1966). *Mechanisms of Release of Biogenic Amines*, Editors: Euler, U. S. von, Rosell, S. & Uvnäs, B., pp. 73–78, Oxford: Pergamon.

EULER, U. S. von & LISHAJKO, F. (1963). *Acta physiol. scand.*, **59**, 454–461.

FALCK, B. (1962). *Ibid., Suppl.* 197.

HAMBERGER, B. (1967). *Ibid., Suppl.* 295.

HAMBERGER, B., MALMFORS, T. & SACHS, C. (1965). *J. Histochem. Cytochem.*, **13**, 147.

LONG, C. (1961). *Biochemists' Handbook*, p. 58, London: Spon.

MALMFORS, T. (1965). *Acta physiol. scand., Suppl.* 248.

MALMFORS, T. & SACHS, C. (1968). *Europ. J. Pharmac.*, **2**, 265–275.

NASH, C. W., WOLFF, S. A. & FERGUSON, B. A. (1968). *Can. J. Physiol. Pharmac.*, **46**, 35–42.

OLSON, L., HAMBERGER, B., JONSSON, G. & MALMFORS, T. (1968). *Histochemie*, **15**, 38–45.

RITZÉN, M. (1966). *Expl cell Res.*, **44**, 505–520.

WESTFALL, T. C. (1965). *Acta physiol. scand.*, **63**, 336–342.

J. Pharm. Pharmac., 1969, **21**, 648–655 *Received May* 12, 1969

Some effects of dihydroergocristine and of phentolamine mesylate on renal function in rats

MARY F. LOCKETT, D. L. STUART, ROSALIE WADLEY, A. R. GOSS AND H. H. SIDDIQUI

The Department of Pharmacology, The University of Western Australia, Nedlands, Western Australia

Single injections of dihydroergocristine (DHE) (25, 50 and 100 μg, i.p.) did not depress the systemic arterial pressure but antagonized the pressor effects of (−)-noradrenaline (100 μg, s.c.) in unanaesthetized rats. The effects of these doses of DHE on water diuresis were minimal: slight K retention, a rise in urinary Na : K and a slight but significant fall in the clearance of *p*-aminohippuric acid were noted. Single injections of phentolamine mesylate (10, 20 and 40 μg, i.p.) greatly depressed systemic arterial pressure and reduced the glomerular filtration rate and *p*-aminohippuric acid clearance and the excretion of water and Na proportionately, but markedly. The Na : K in the urine rose. Chemical denervation of the kidneys either with DHE, 32 μg, or phentolamine, 2 mg, thrice daily for 5–7 days decreased, but for 14 days increased, the juxtaglomerular index of the kidneys. Parallel changes were found in the extractable renin. Continued treatment with DHE (32 μg thrice daily for 2 weeks) raised, and hyperduric adrenaline (250 μg twice daily for 1 week) lowered the stores of growth hormone in the adenohypophysis. Continued treatment with DHE, 32 μg thrice daily produced antidiuresis and retention by the third day lasting to the fifth. This condition had reversed by the eighth day.

Recent observations have indicated that the sympathetic nervous system plays some part in the control of the secretion of renin from the juxtaglomerular apparatus (Bunag, Page & McCubbin, 1966; Bozovic & Castenfors, 1967). A rich innervation of the epithelioid cells which lie within the wall of the afferent glomerular arteriole and which constitute the "polkissen" of the juxtaglomerular apparatus has been demonstrated by electronmicroscopy (Barajas, 1964). More recently these fibres have been identified as adrenergic by histochemical fluorescence (Wägermark, Ungerstedt & Ljungquist, 1968). The smooth muscle cells of the afferent arterioles also receive sympathetic innervation.

The object of the present work has been to discover whether the influence of the sympathetic nervous system on the secretion of renin is mediated through α-receptors within the "polkissen" or through changes in tension within the arteriolar wall. Hence, two α-blocking drugs have been selected for use: the first, dihydroergocristine is capable of blocking the vascular action of exogenous noradrenaline without affecting systemic arterial pressure; the second, phentolamine, is not. The urinary changes induced by these drugs have also been examined since renin is secreted into the blood stream and into the interstitial fluid within the kidney (Peart, 1965) and the interstitial fluid contains renin substrate (Thurau, 1964), It is therefore theoretically possible that part of the renal actions of these blocking agents may be due to change in the rate of the intrarenal secretion of renin and hence to change in the rate of the intrarenal formation of angiotensin.

EXPERIMENTAL

Methods

Female Wistar rats, 160–205 g, were housed in a single room at 20–22°, drank water freely and ate a pellet diet supplied by Wesfarmers Ltd. Since the sodium concentration in this diet varied from batch to batch during the period of these experiments it was necessary to stabilize groups of control and experimental animals on each new batch of diet before the start of treatments.

Renin measurements. Rats were anaesthetized with sodium pentobarbitone (6 mg/100 g/weight, i.p.) before the kidneys were removed, weighed separately, and assigned R and L alternately for determination of renin content or of juxtaglomerular index.

Measurement of the renin content of kidneys. Weighed kidneys were chilled and separately homogenized in NaH_2PO_4, 0·05M, pH 7·0 3·5 ml/g tissue, at 8° as described by Katz, Cockett & Moore (1966). Renin was prepared from each kidney homogenate by Skinner's method (1967) modified only in so far that pH 3·0 was substituted for pH 3·3 in the first dialysis at 8° and the subsequent step at 32°. The final renin preparations obtained, free of angiotensinase and endogenous substrate, were diluted 1 in 10 to 1 in 100 with 0·1M NaH_2PO_4 buffer, pH 7·0 for incubation with an excess of ox substrate (Lever, Robertson & Tree, 1964) at 37° and pH 7·0. The incubation mixtures were sampled at 1, 4 and 8 h for immediate assay of the angiotensin formed on the blood pressure of the ganglion blocked rat (Peart, 1955). Aspartyl[1], valyl[5], angiotensin II β amide (Hypertensin, CIBA), similarly incubated, was used as reference standard. All assays were of 2 × 2 Latin Square design and afforded data which were subjected to variance analysis. The renin content of each kidney was expressed as ng angiotensin formed per g kidney per hour.

Measurement of juxtaglomerular index (JG index). Transverse slices of cortex 4 mm thick, were fixed for 18 h in Smith's fluid: $K_2Cr_2O_7$, 5g: 38–40% formaldehyde, 10 ml; glacial acetic acid, 2·5 ml; distilled water 88 ml, washed for 2 h in running tap water and embedded in Paraplast wax using a Shandon Elliot automatic tissue processor. Sections, 7 μm, were stained by Smith's method (1966). The juxtaglomerular granules stained deep purple, the nuclei lavender to pink, the cytoplasm blue, collagen pink and erythrocytes red. The JG index was determined as described by Hartroft & Hartroft (1953): estimates of JG index were invariably found reproducable amongst those experienced in the technique.

Measurement of the concentrations of adenohypophyseal hormones. The pituitary glands were removed from rats decapitated under pentobarbitone anaesthesia; all adenohypophyseal tissue from groups of 4 rats were separately homogenized with 1 ml sample gel, 0·1 ml of which was applied to each column for electrophoresis. Disc electrophoresis was carried out on standard 7·5% acrylamide gel at pH 9·0 as described by Ornstein (1964). Polymerization of the large pore spacer and sample gels was assisted by the addition of tetramethylene diamine (Lewis, 1963). A constant current of 5 mA was applied to each tube for 40 min. All gels were stained for 2 h with Amido Schwartz reagent and were destained electrophoretically. The protein bands were examined by means of a double beam recording and integrating densitometer (Joyce, Loeble & Co. Ltd.) speed 2 mm/s, gain 5.

Measurement of the effects of drugs on mean arterial pressure. Pentobarbitone anaesthesia was used for the insertion of indwelling polythene cannulae, heparin (Evans Medical Ltd.) filled, into carotid arteries 36 h before their use. The cannulae

were exteriorized at the back of the neck. The animals were enclosed in a small dark, well ventilated, fabric space during measurement of mean arterial pressure. The polythene cannulae projected from this space to connect with an E & M transducer coupled to a Heathkit pen recorder.

Experiments made during water diuresis. Every experiment was designed as a series of cross-over tests in which each animal received each treatment and served as its own control. Equal numbers of each treatment were allocated to each day. Tests were made every third day and began with a 2 h period during which rats were deprived of solid food. The oral water load, equivalent to 2·5% body weight, was given at the end of this period immediately before each animal was put into a separate cage for the collection of all urine entering the bladder in the next hour. Gentle suprapubic pressure was applied to empty the animals' bladders at the termination of each collection of urine. All tests constituting a single experiment were performed at a time of day fixed for each experiment. Phentolamine and dihydroergocristine were injected intraperitoneally in 0·1 to 0·2 ml of 0·9% NaCl at the time of water-loading. Creatinine 4·0% and *p*-amino-hippuric acid (PAH) 1% in 0·9% NaCl, neutralized by addition of solid $NaHCO_3$, were injected subcutaneously (0·6 ml per 100g 30 min) before administration of the water load. Concentrations of sodium (Na) and potassium (K) in urine samples were determined by flame photometry. Two channels of an auto analyzer (Technicon Ltd.) were used to estimate urinary concentrations of creatinine and PAH. Predetermined mean plasma values for these substances, found 60 min after their subcutaneous injection were applied to obtain individual clearance values for each animal: mg substance excreted in 1 h divided by mg substance per ml plasma × 60. The data from each experiment was subjected to variance analysis: *t*-tests were applied within groups to determine the significance of drug actions and interactions.

Drugs. Dihydroergocristine (DHE) was received as a gift from Sandoz Basle. Phentolamine mesylate (CIBA), (−)-noradrenaline (Winthrop) and hyperduric (−)-adrenaline chloride (Parke Davis) were obtained commercially. Bovine growth hormone (NIH, GH B9) and thyrotropic hormone (Thyrotropar) were received as gifts.

RESULTS

The influence of dihydroergocristine (DHE) and phentolamine on water diuresis in rats. Nine 4 day cross-over tests (Lees, Lockett & Roberts, 1964) were made on groups of 10 to 12 rats to determine the effect of DHE on water diuresis. The combined results of these experiments are shown on the left of Fig. 1. DHE in doses of 25, 50 and 100 μg/rat was without significant effect on the elimination of a water load and on the excretion of Na. The rate of excretion of K tended to decrease as the dose of DHE increased hence both 50 and 100 μg/rat caused a small but significant increase in the ratio Na : K of the urine. The glomerular filtration rate (GFR) remained unchanged but the clearance of PAH(C_{PAH}) was slightly depressed: this depression reached significance ($P < 0·05$) overall, but not at the individual dose levels in individual experiments. Data obtained from six similarly designed cross-over tests made to determine the effects of phentolamine on water diuresis in rats is summarized on the right of Fig. 1. Phentolamine in doses of 10, 20 and 40 μg/rat caused a small reduction in the urine flow in these water-loaded rats accompanied by retention of Na and K and significant reduction in GFR and C_{PAH}. Since the urinary excretion of K was

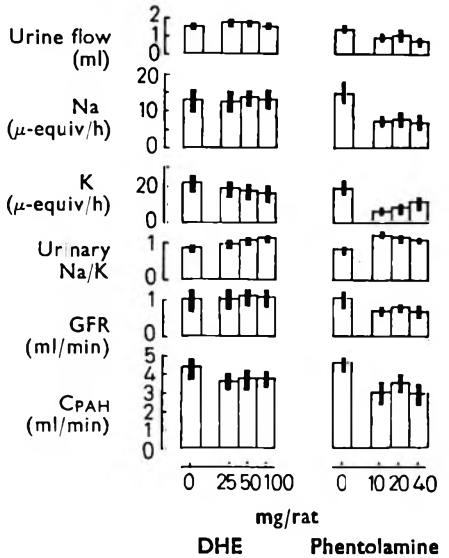


FIG. 1. Contrasting effects of single i.p. doses of dihydroergocristine (DHE) and of phentolamine on water diuresis in rats. All values are expressed per 100g weight. The heights of the columns indicate mean values and the inset columns the standard errors of these means. The columns on the left of each series indicate control values, the other columns represent the effects of treatment with DHE 25, 50 and 100 μg per rat (left) and phentolamine, 10, 20 and 40 μg per rat.

even more markedly reduced than that of Na, the ratio Na:K in the urine rose. The higher the dose of phentolamine the less the effect on this ratio: by contrast, only the higher doses of DHE (25 and 50 μg) produced a rise in the Na:K of the urine.

The influence of DHE *and phentolamine on the systemic arterial pressure of unanaesthetized rats.* The injection of DHE (50 μg, i.p.) into rats equipped with indwelling

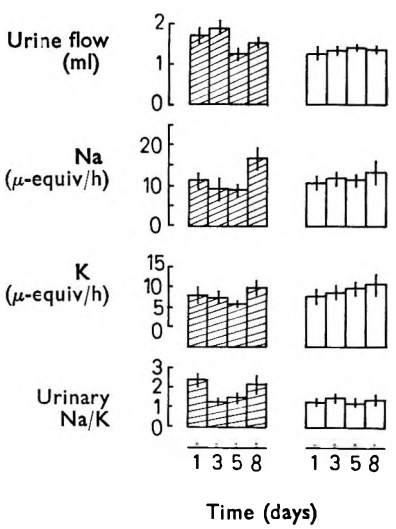


FIG. 2. The effects of continuous treatment with DHE, 32 μg thrice daily per rat, for 8 days, on the excretion of a water load and on urinary electrolytes. Shaded columns are treated and open columns control results. Values per 100g rat per h: on the first, third, fifth and eighth day of the experiment.

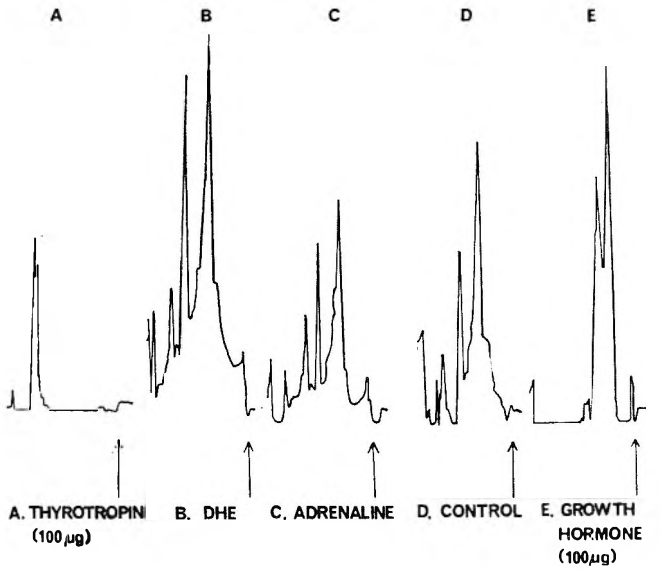


FIG. 3. Typical densitometer tracings of disc electrophoretic patterns obtained by mixing A, 100 μg thyrotropin, E, 100 μg bovine growth hormone, B, C and D each one tenth the homogenate from the adenohypophyses of four rats treated with DHE, 32 μg thrice daily, 2 weeks, hyperduric adrenaline 250 μg, s.c. thrice daily, 1 week and 0·9% NACl thrice daily, 2 weeks, respectively. The arrows indicate the starting line of each tracing.

carotid cannulae caused no significant fall in mean arterial pressure during subsequent periods of observation lasting 1½ to 3 h. By contrast, phentolamine (20 μg) decreased mean arterial pressure by as much as 40 mm Hg within 30 min. Noradrenaline (100 μg, s.c.) produced no sustained elevation of mean arterial pressure in the presence of either 50 μg of DHE or 20 μg of phentolamine.

The effect of continued treatment with DHE 50 μg *thrice daily on water diuresis in rats.* The effect of continuous treatment with DHE (32 μg, i.p.) thrice daily for 8 days is shown in Fig. 3. During this period there were no significant variations in the urinary responses of a group of 24 rats weighing 287 ± 4·3 g to water loading. By contrast, 14 rats (191 ± 4·1 g) receiving DHE significantly reduced their urinary Na by the 5th day of treatment. The K output also tended to fall during this period but to a lesser extent so that the ratio Na : K in the urine was very significantly decreased. This initial effect of continuous treatment with DHE was not maintained: normal ability to excrete Na was restored by the 8th day of treatment.

The influence of renal denervation, adrenal demedullation and of continuous treatment with DHE *or with phentolamine on the renin content and juxtaglomerular index of rat kidneys.* Both 5 and 7 days of treatment with DHE (32 μg, i.p., thrice daily) caused significant decrease in the JG index but phentolamine (2 mg, i.p., thrice daily) did not influence the JG index. Two weeks of continuous treatment with either DHE or phentolamine at the above doses significantly increased both the JG index and the renin content of the kidneys of normal rats (Table 1).

The influence of continuous treatment with DHE (32 μg, *thrice daily*) *on the stored hormones in the adenohypophyses of rats.* Fig. 3 shows typical patterns of the adenohypophyses of control (D), DHE-treated (B) and adrenaline-treated (C) rats. Moving right to left from the starting arrow, the growth hormone peak (E) precedes the albumen

Table 1. *The influence of various treatments on the juxtaglomerular index and the renin content of rat kidneys*

Treatment	Body weight g	Weight of a single kidney mg	Juxtaglomerular index	Renin content ng angiotensin/ g kidney h^{-1}
Normal rats (controls)	183 ± 5·2 (10)	646 ± 14·7	15·5 ± 0·37	371 ± 39·8
Dihydroergocristine 32 μg thrice daily				
for 5 days	141 ± 0·4 (6)	612 ± 8·4	11·6 ± 1·27*	
for 7 days	174 ± 1·6 (4)	642 ± 12·6	12·8 ± 0·63*	
for 14 days	191 ± 3·0 (10)	668 ± 15·2	18·6 ± 0·74	449 ± 41·3*
Phentolamine 2 mg thrice daily				
for 7 days	179 ± 2·1 (6)	632 ± 11·7	16·6 ± 1·08	394 ± 42·4
for 14 days	175 ± 4·8 (10)	660 ± 14·7	19·6 ± 1·82*	456 ± 47·4*

The values shown are means ± their standard errors. The numbers of animals used are shown in parentheses. The significance of differences caused by treatments has been examined by *t*-tests and is indicated by an asterisk if P is $< 0{\cdot}05$.

peak in the normal adenohypophysis (D); thyrotropin (A) and lactogenic hormone (not shown) migrate together more rapidly. Adrenocorticotrophin does not migrate under the conditions of these experiments. Continuous treatment with DHE for two weeks 32 μg, thrice daily) has increased the stores of growth hormone markedly. The albumen content of the glands has also increased. Stores of thyrotropic and lactogenic hormones remain unaffected. Conversely, hyperduric adrenaline (250 μg, twice daily) for 1 week has caused some reduction in the stores of somatotrophin.

DISCUSSION

It is generally agreed that sympathetic vasoconstrictor tone is small or absent in the normal kidney (Smith, 1939; Thurau, 1964). Therefore, the very marked reduction in GFR and in C_{PAH} caused by single full α-adrenergic blocking doses of phentolamine are attributed to the large decrease in systemic arterial pressure which these doses caused. This decrease in arterial pressure does not appear to have provoked the release of renin since GFR and C_{PAH} were proportionately reduced and so the filtration fraction remained unchanged. Any increase in the plasma concentration of renin would have increased the concentration of circulating angiotensin (Peart, 1965) and a rise in the filtration fraction is characteristic of the renal actions of this peptide (Finnerty, Massaro & others, 1961; Lockett, 1967). Our observations are only superficially in conflict with previous work, for Vander (1965) has demonstrated that electrical stimulation of the renal nerves or infusions of either adrenaline or noradrenaline increase the secretion of renin. Hence the raised rate of renin secretion which results from a fall in systemic mean arterial pressure (Skinner, McCubbin & Page, 1963a, b) is probably a consequence of reflexly increased sympathetic activity. However, the α-receptor blockade induced by phentolamine would be expected to abolish the effects of enhanced sympathetic nervous activity on the release of renin. The reduction in GFR caused by phentolamine suffices to explain both the antidiuresis and reduction in Na excretion caused by single injections of this drug. The doses of DHE used in these single injection experiments were adequate to prevent the pressor effects of (−)-noradrenaline (100 μg, s.c.) per rat, but did not depress mean arterial pressure. These doses of DHE reduced C_{PAH} but not the GFR and therefore caused a rise in the filtration

fraction. This rise in the filtration fraction, observed to result from single doses of DHE is probably attributable to the release of noradrenaline from the terminals of the postganglionic sympathetic nerves supplying the juxtaglomerular apparatus since DHE has been shown to release catecholamine from the adrenal medulla (Lockett & Wadley, 1969). An effect of DHE on the postglomerular renal vasculature cannot however be excluded since DHE is known to possess weak direct constrictor properties (Rothlin & Cerletti, 1949).

The changes induced by the continued administration of these same doses of phentolamine and DHE are more complex. Denervation of a kidney decreases its renin content (Tacquini, Blaquier & Tacquini, 1964) and lowers the JG index, (Tobian, Branden & Maney, 1965). Since 7 days of treatment with full α-adrenergic blocking doses of phentolamine failed to influence the JG index, and the same period of treatment with DHE, in doses sufficient to block the effects of exogenous noradrenaline but insufficient to inhibit vasomotor tone, reduced the JG index, our observations cannot be explained in terms of the blocking action of these drugs on a pure α-receptor mechanism for the release of renin by catecholamine. Moreover, 2 weeks of continuous treatment with these same doses of either drug caused a secondary rise both in the JG index and in the renin content of the kidneys. Possibly a prolonged α-blockade brings about a change in the sensitivity of the juxtaglomerular apparatus either to a feed back system or to an intermediary substance within the apparatus itself or to both.

Rise and fall in the JG index or the renin content of the kidneys, or both, is widely accepted as evidence of a rise or a fall, respectively, in the rate of secretion of renin (Tobian, 1960). The effects of continuous treatment with DHE on the excretion of water and electrolytes by rats do not reflect the changes in renin secretion indicated by the changes in the JG index. A small but significant retention of water and Na was evident from the third to the fifth day of treatment but reversed by the 8th day. Stores of electrophoretically identifiable growth hormone had however accumulated in the adenohypophysis during 2 weeks of treatment with DHE. No conclusion can be drawn until it be determined whether these increased stores of growth hormone reflect change in the rate of synthesis or release or both of these. This hormone has however been shown powerfully Na-retaining in the rat (Lockett & Nail, 1965) and necessary for the normal sensitivity of the nephron to aldosterone (Lees & others, 1964; Lockett & Roberts, 1963).

Acknowledgements

The expenses of this work were defrayed from a Research Grant awarded to M.F.L. by the National Health & Medical Research Council of Australia. D. S. and R. W. gratefully acknowledge the opportunity given to them by N. H. & M. R. C. to join in this research as technologist and graduate assistant respectively.

REFERENCES

BARAJAS, L. (1964). *Lab. Invest.*, **13**, 916–29.

BUNAG, R. D., PAGE, I. H. & MCCUBBIN, J. W. (1966). *Circulation Res.*, **19**, 851–858.

BOZOVIC, L .& CASTENFORS, J. (1967). *Acta physiol. scand.* **70**, 290–292.

FINNERTY, F. A. JR., MASSARO, G. D., CHUPKOVITCH, V. & TUCKMAN, J. (1961). *Circulation Res*, **9**, 256–263.

HARTROFT, P. M. & HARTROFT, W. S. (1953). *J. exp. Med.*, **97**, 415–427.

KATZ, Y. J., COCKETT, A. T. K. & MOORE, R. S. (1966). *Investve Urol.*, **4**, 64–68.

LEES, P., LOCKETT, M. F. & ROBERTS, C. N. (1964). *J. Physiol., Lond.*, **171**, 397–402.

LEWIS, U. V. (1963). *J. biol. Chem.*, **238**, 3330–3335.
LEVER, A. F., ROBERTSON, J. I. S. & TREE, M. (1964). *Biochem. J.*, **91**, 346–352.
LOCKETT, M. F. (1967). *J. Physiol., Lond.*, **193**, 639–647.
LOCKETT, M. F. & NAIL, B. (1965). *Ibid.*, **180**, 147–156.
LOCKETT, M. F. & ROBERTS, C. N. (1963). *Ibid.*, **169**, 879–888.
LOCKETT, M. F. & WADLEY, R. (1969). *Br. J. Pharmac.*, in the press.
ORNSTEIN, L. (1964). *Ann. N.Y. Acad. Sci.*, **121**, 321–349.
PEART, W. S. (1955). *Biochem. J.*, **59**, 300–302.
PEART, W. S. (1965). *Pharmac. Rev.*, **17**, 143–82.
ROTHLIN, E. & CERLETTI, A. (1949). *Verh. dt. Ges. Kreislanfforsch.*, **15**, 158.
SKINNER, S. L. (1967). *Circulation Res.*, **20**, 391–402.
SKINNER, S. L., MCCUBBIN, J. W. & PAGE, I. H. (1963a). *Science, N.Y.*, **141**, 814–816.
SKINNER, S. L., MCCUBBIN, J. W. & PAGE, I. H. (1963b). *Circulation Res.* **13**, 336–345.
SMITH, C. L. (1966). *Stain Tech.*, **41**, 291–294.
SMITH, H. W. (1939–1940). *Harvey Lecture*, **166**, 138.
TACQUINI, A. C., BLAQUIER, P. & TACQUINI, A. C. Jr. (1964). *Can. Med. Assoc. J.*, **90**, 210.
THURAU, K. (1964). *Am. J. Med.*, **36**, 698–719.
TOBIAN, L. (1960). *Physiol. Rev.*, **40**, 280–312.
TOBIAN, L., BRANDEN, M. & MANEY, J. (1965). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **24**, 405.
VANDER, A. J. (1967). *Physiol. Rev.*, **47**, 359–000.
WAGERMARK, J., UNGERSTEDT, U. & LJUNGQUIST, A. (1968). *Circulation Res.*, **22**, 149–153.

J. Pharm. Pharmac., 1969, **21**, 656–661 *Received March* 10, 1969

The effect of SC-15396, atropine and mepyramine on gastrin-, bethanechol- and histamine-stimulated gastric acid secretion in rats and guinea-pigs

MARGITTA ALBINUS AND K.-FR. SEWING

Department of Pharmacology, University of Tübingen, W.-Germany

The effect of SC-15396 ("antigastrin"; 2-phenyl-2-(2-pyridyl)thioacetamide), atropine and mepyramine on gastrin-, bethanechol- and histamine-stimulated gastric acid secretion was studied in rats and guinea-pigs. For all three stimulants parallel dose response curves were obtained except in guinea-pigs where bethanechol even in very high doses displays a poor activity in stimulating gastric acid secretion. The maximal secretory response was found to be 12·7 ± 5·0 μ-equiv HCl/10 min in rats and 53·2 ± 27·9 μ-equiv HCl/10 min in guinea-pigs. All stimulating effects on gastric acid secretion were reduced by SC-15396; atropine abolished the secretory responses to bethanechol. Mepyramine was ineffective. In accordance with these findings the mechanism of action of gastrin and a receptor model on the oxyntic cell are discussed.

Several chemically different compounds are potent gastric acid secretagogues, among those the antral hormone gastrin can be regarded as the physiological stimulus for gastric secretion. It has been postulated by several authors that histamine may be the common final chemostimulator (Code, 1965; Lorenz & Pfleger, 1968). This hypothesis up to now has not been established unequivocally. The present investigation was designed to bring some light to the question as to whether gastrin, bethanechol and histamine act on the oxyntic cell by the same common mechanism. The effects of the alleged specific gastrin antagonist SC-15396 ["antigastrin"; 2-phenyl-2-(2-pyridyl)thioacetamide], the anticholinergic atropine and the antihistamine mepyramine on gastrin-, bethanechol- and histamine-stimulated gastric acid secretion have been examined in anaesthetized rats and guinea-pigs.

EXPERIMENTAL

Methods

The experiments were made on 95 male Wistar rats (180–400 g) and 95 male mongrel guinea-pigs (200–530 g) from which food was withheld for 24 h; there was free access to drinking water. The animals were prepared under urethane anaesthesia (rats 1·25 g/kg, i.p., guinea-pigs 1·5 g/kg i.p.) according to Lai (1964). Gastric acid secretion was collected in 10 min periods and the acid output was estimated titrimetrically with 0·01 N NaOH using bromothymol blue as indicator. The dose response curves were obtained from six different doses of each stimulus. The lowest dose was numbered as 1 and the highest as 6. For convenience, in a single animal three doses (1–3–5, 5–3–1, 2–4–6, 6–4–2) were infused intravenously for 15 min at intervals of 70 min. The infusion volume was 0·935 ml/15 min for rats and 2·35 ml/

15 min for guinea-pigs. Each dose of the agonists was tested in the presence of only one dose of the antagonists. SC-15396 has a rather short duration of action and was therefore injected intravenously 5 min before each infusion. Atropine and mepyramine were administered intravenously as a single dose 20 min before the first infusion. The combination of drugs and groups of drugs (see above) were randomized for each species. Each point of the dose-response curves represents the mean value of 4 single experiments (the highest dose of bethanechol in combination with SC-15396 in rats and the highest dose of gastrin in combination with SC-15396 in guinea-pigs are mean values of only 3 experiments). Differences in the dose-response curves were tested by analysis of variance.

Compounds and dosage

Agonists. Synthetic human gastrin I (American Gastroenterological Association): rats and guinea-pigs 0·2, 0·4, 0·8, 1·6, 3·2, 6·4 μg/kg in 15 min. Bethanechol chloride (Schuchardt, Munich): rats and guinea-pigs 20, 40, 80, 160, 320, 640 μg/kg in 15 min. Histamine dihydrochloride (La Roche, Grenzach): rats 50, 100, 200, 400, 800, 1600 μg/kg in 15 min; guinea-pigs 1·25, 2·5, 5, 10, 20, 40 μg/kg in 15 min.

Antagonists. SC-15396 [2-phenyl-2-(2-pyridyl)thioacetamide (G. D. Searle, Chicago and Dr. Karl Thomae, Biberach]: rats and guinea-pigs 3 × 10 mg/animal. Atropine sulphate (Merck, Darmstadt): rats 0·5 mg/kg; guinea-pigs 5·0 mg/kg. Mepyramine maleate (Bayer, Leverkusen): rats 10 mg/kg; guinea-pigs 1·3 mg/kg.

All compounds were given as weights of the base dissolved in 0·9% saline and were injected or infused into the jugular vein. SC-15396 was dissolved in a mixture of 0·5 ml dimethylsulphoxide and 0·5 ml 0·9% saline. After each injection or infusion the venous cannula was rinsed with 0·2 ml saline.

pA_2 values

The pA_2 values of SC-15396 for histamine and acetylcholine were determined by the method of Schild (1947).

RESULTS

Rats

The basal secretion varied between 2–4 μ-equiv HCl/10 min.

Gastrin (Fig. 1A). The infusion of gastrin resulted immediately in a dose-dependent secretion of HCl. When the infusion was stopped gastric secretion returned to the basal level within 30 min. The maximal response of the dose response curve was not obtained with the doses used. The slightly stronger effect of gastrin in the presence of mepyramine and the weaker in the presence of atropine was not statistically significant. The gastrin effect was significantly reduced by SC-15396.

Bethanechol (Fig. 1B). With bethanechol, 160 μg/kg in 15 min, the maximal response of the dose-response curve was obtained. The bethanechol effect was not influenced by mepyramine, diminished by SC-15396 or abolished by atropine.

Table 1. *pA_2 values of atropine, mepyramine and SC-15396 for acetylcholine and histamine*

	Atropine	Mepyramine	SC-15396
Acetylcholine	8·27+	4·71+	3·90
Histamine	5·73+	8·71+	3·50

+ Values from Schild (1947).

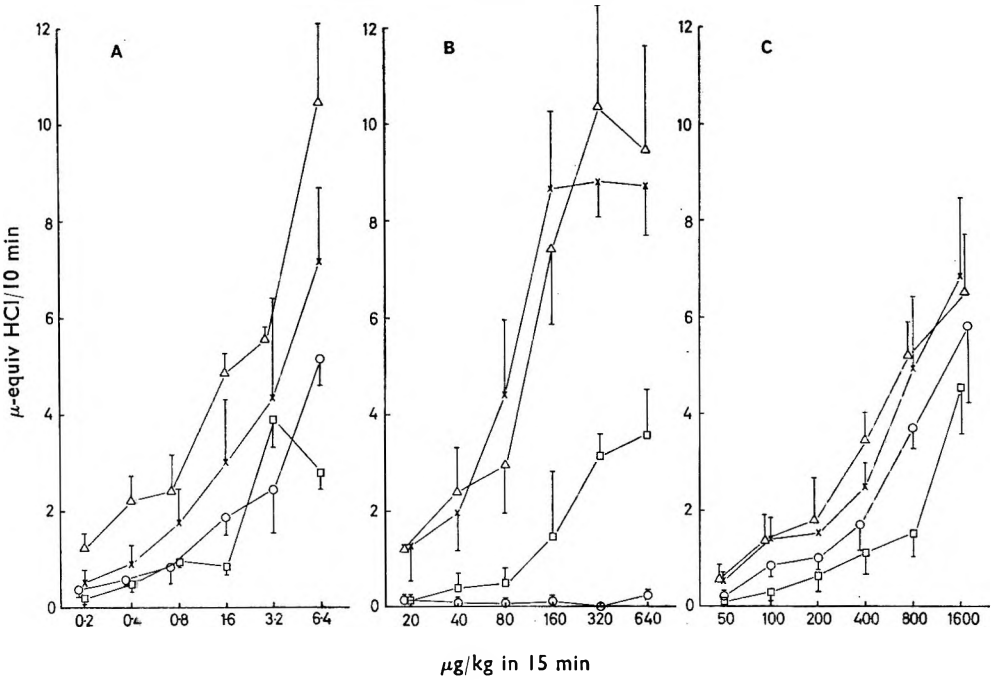


FIG. 1. Dose response curves of gastrin I (A), bethanechol (B) and histamine (C) in rats without pretreatment (×), in the presence of SC-15396 (□), atropine (○) or mepyramine (Δ). Vertical bars = s.e.

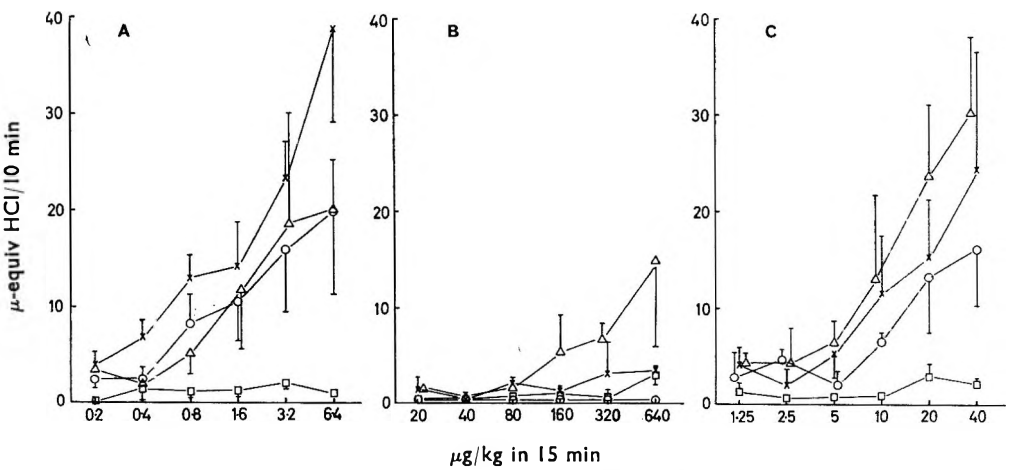


FIG. 2. Dose response curves of gastrin I (A), bethanechol (B) and histamine (C) in guinea-pigs without pretreatment (×), in the presence of SC-15396 (□), atropine (○) or mepyramine (Δ). Vertical bars = s.e.

Histamine (Fig. 1C). The dose-dependent histamine response was not influenced by mepyramine or atropine, but was significantly reduced by SC-15396. The effects of all doses of histamine were submaximal.

In comparing the relative potencies of the three stimulants at a secretory rate of 5 μ-equiv HCl/10 min, it was calculated that on a molar basis gastrin was 4000 times and bethanechol 14 times as potent as histamine.

Guinea-pigs

In guinea-pigs the basal secretion varied over a wide range from 2–15 μ-equiv HCl/10 min.

Gastrin (Fig. 2 A). When the same dose of gastrin used in rats was given to guinea-pigs, much larger responses were obtained. Here also the maximal response was not observed. Atropine and mepyramine had no influence on the gastrin response. A significant inhibition was obtained by SC-15396.

Bethanechol (Fig. 2 B). Doses of bethanechol covering the whole dose-response curve in rats were only weakly potent in guinea-pigs. Even with a dose of 640 μg/kg in 15 min a secretory response of 3·5 μ-equiv HCl/10 min was not exceeded. The augmented secretion rate by mepyramine was insignificant. SC-15396 was ineffective. The bethanechol effect was abolished by atropine. At a constant perfusion rate of the stomach, the outflow from the pyloric cannula was markedly increased by bethanechol. Therefore in separate experiments free (Toepfer's reagent) and total (phenolphthalein) acid was determined. No difference in comparison on titration with bromothymol blue was observed. Furthermore, the protein content of the gastric perfusate was determined (Lowry, Rosebrough & others, 1951). A dose-dependent increase in protein content during the infusion of bethanechol was probably due to the known strong pepsigogue effect of cholinergic stimuli. As a side-effect, a marked salivary secretion was observed.

Histamine (Fig. 2 C). With doses of histamine being about 1/40 of those used in rats a good dose response relation was obtained in guinea-pigs. Doses of 40 μg/kg in 15 min were nearly maximal. The histamine response was almost completely abolished by SC-15396. Neither atropine nor mepyramine had any augmenting or inhibiting effect.

At a secretion level of 10 and 20 μ-equiv HCl/10 min, gastrin was on a molar basis about 270 times as potent as histamine. The relative potency of bethanechol could not be evaluated because of its weak effect.

DISCUSSION

The results demonstrate parallel dose-response curves for all stimulants in rats and for gastrin and histamine in guinea-pigs. The weak bethanechol effect in guinea-pigs may be the consequence of a relative insensitivity of the target organ since salivary and pepsin secretion seemed to be strongly stimulated. In either species gastrin was the most potent secretagogue, underlining the importance of gastrin as the physiological stimulant for gastric acid secretion. The smaller difference in the relative potencies between gastrin and histamine in guinea-pigs than in rats may be due to a less-pronounced sensitivity for histamine in rats.

The maximal secretory response is determined only by the total parietal cell mass and not by the stimulus applied (Makhlouf, McManus & Card, 1966). This would explain the higher secretion rates in guinea-pigs than in rats. Makhlouf's model for describing the dose response relation of gastric acid secretion can be expressed by the following equation:

$$c = \frac{c_{max} \times D}{K + D} \quad .. \quad .. \quad .. \quad .. \quad (1)$$

where c = the observed response, c_{max} = maximal response, D = concentration of the stimulus, K = ED50.

By transformation of equation (1) a linear relation between the reciprocals of both dose and response is obtained:

$$\frac{1}{c} = \frac{1}{c_{max}} + \frac{K}{c_{max}} \cdot \frac{1}{D} \quad .. \quad .. \quad .. \quad .. \quad (2)$$

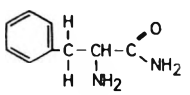
Knowing three parameters of these equations it is possible to determine the unknown. Since in our experiments only the lower ends of the dose response curves were determined (except for bethanechol in rats) it was of interest to get information about the maximal response (c_{max}) in both species and to investigate whether c_{max} was depressed by one or more of the antagonists. Therefore a program was elaborated (Winne, 1969, unpublished) to calculate by a nonlinear regression the parameters by which we could determine c_{max} from the experimental data. From such a calculation c_{max} was found for rats to be $12{\cdot}7 \pm 5{\cdot}0$ μ-equiv HCl/10 min and for guinea-pigs $53{\cdot}2 \pm 27{\cdot}9$ μ-equiv HCl/10 min. Unfortunately we were not able to ascertain whether or not one of the antagonists used depressed c_{max} significantly since in some drug combinations the data available covered too small a range of the total dose response curve to determine c_{max} with a margin of safety.

The inability of antihistamine drugs to antagonize histamine-stimulated gastric acid secretion confirmed earlier observations. In our experiments a stimulating effect of mepyramine on gastric secretion as it has been described for salivary secretion (Lorenz & Pfleger, 1968; Lorenz, Haubensak & others, 1968) was not observed.

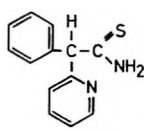
The blockade of cholinergic receptors by atropine was clearly demonstrated in both species when gastric secretion was stimulated with bethanechol. Neither the histamine nor the gastrin response was diminished by atropine.

In both species SC-15396 reduced the effect of all three stimulants. The one exception being the effect of bethanechol in guinea-pigs which was not diminished since the bethanechol effect was already low. The pA_2 values (Table 1) did not give any indication that the effect of SC-15396 was specifically anticholinergic or antihistaminic.

SC-15396 has been reported to be a specific gastrin antagonist in dogs (Bedi, Gillespie & Gillespie, 1967) and rats (Cook & Bianchi, 1967). Connell, Sircus & others (1967) demonstrated an inhibiting effect of SC-15396 on histamine-stimulated dogs. But the opinion that SC-15396 is a specific gastrin antagonist has been changed because it has since been shown to antagonize gastric secretion stimulated by gastrin, histamine and insulin (Connell, Hill & others, 1968).



Phenylalanine amide



SC-15396

SC-15396 chemically resembles the C-terminal phenylalanine amide of the gastrin molecule. This resemblance could be the key to the molecular mechanism of the action of gastrin thus offering the following hypothesis: The active centre of the gastrin molecule covering the entire range of physiological properties is the C-terminal tetrapeptide amide Trp.Met.Asp.Phe-NH_2 (Tracy & Gregory, 1964). The polar end group $-C\begin{matrix}O\\ \diagdown NH_2\end{matrix}$ of the phenylalanine amide is capable of accepting

H^+ ions supplied by intramolecular electron transfer. These H^+ ions may come from the aspartyl residue. The importance of the aspartyl residue and the C-terminal acid amide group is underlined by several observations. Morley (1968) found that modification of the aspartyl group led to either inactive compounds or to compounds whose activity was so weak as to be questionable. The same happened when the amide group was substituted by two methyl groups. On the other hand, the activity of the C-terminal tetrapeptide amide was increased when substituted at the N-terminal end by a β-alanyl residue or a carbamoyl group. Both groups could serve as additional acceptors for H^+ ions supplied by the aspartyl residue. This would explain the increase in activity. These considerations do not allow the conclusion that the activity of the total gastrin molecule *in vivo* is only the action of its C-terminal tetrapeptide amine.

SC-15396, because of its similarity with the phenylalanine amide, is likely to interfere with the receptor for the $-C\begin{matrix}O\\ \diagdown\\ \diagup\\ NH_2\end{matrix}$ group. The large dose necessary to display an antisecretory activity may be due to its poor solubility which limits its use for more detailed molecular studies.

The antagonistic effect of SC-15396 to all three stimulants suggests either a common receptor for gastrin, bethanechol and histamine or one common chemical transmitter for stimulating the secretion of gastric hydrochloric acid. The common chemical transmitter may be gastrin since its active centre shows the closest chemical resemblance to the inhibitor for all three stimulants—SC-15396.

REFERENCES

Bedi, B. S., Gillespie, G. & Gillespie, I. E. (1967). *Lancet*, **1**, 1240–1243.

Code, C. F. (1965). *Fedr. Proc. Fedn Am. Socs exp. Biol.*, **24**, 1311–1321.

Connell, A. M., Hill, R. A., MacLeod, I. B., Sircus, W. & Thomson, C. G. (1968). *Gut*, **9**, 641–654.

Connell, A. M., Sircus, W., Hill, R. A., MacLeod, I. B. & Thomson, C. G. (1967). *Lancet*, **2**, 720.

Cook, D. L. & Bianchi, R. G. (1967). *Life Sci.*, **6**, 1381–1387.

Lai, K. S. (1964). *Gut*, **5**, 327–341.

Lorenz, W., Haubensak, G., Hutzel, M. & Werle, E. (1968). *Arch. Pharmak. exp. Path.* **260**, 416–437.

Lorenz, W. & Pfleger, K. (1968). *Klin. Wschr.*, **46**, 57–71.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.*, **193**, 265–275.

Makhlouf, G. M., McManus, J. P. A. & Card, W. I. (1966). *Gastroenterology*, **51**, 455–465.

Morley, J. S. (1968). *Proc. Roy. Soc. B.*, **170**, 97–111.

Schild, H. O. (1947). *Br. J. Pharmac. Chemother.*, **2**, 189–206.

Tracy, H. J., & Gregory, R. A. (1964). *Nature, Lond.*, **204**, 935–938.

J. Pharm. Pharmac., 1969, **21**, 662–667 *Received May* 15, 1969

The actions of atropine, tropenziline and *N*-butyl hyoscine bromide on the isolated distal colon of the guinea-pig: a comparison of their activities and mechanisms of action

S. LECCHINI, M. DEL TACCA, G. SOLDANI, G. M. FRIGO AND A. CREMA

Department of Pharmacology, University of Pisa, Italy

In the guinea-pig isolated distal colon, the order of anticholinergic activity is as follows: atropine > tropenziline bromide > *N*-butyl hyoscine bromide. The reduction in the responses to pelvic and transmural stimulation produced by tropenziline and *N*-butyl hyoscine bromide is due partly to their ganglion blocking activity. This effect also explains the reduction they cause in acetylcholine output on pelvic nerve and transmural stimulation. Since atropine also reduces acetylcholine release on pelvic nerve stimulation, it is suggested that muscarinic receptors of the parasympathetic ganglia are involved in transmission of pelvic nerve impulses.

A number of investigations have been made on the mammalian colon to analyse quantitatively the blocking effect of anticholinergic drugs on endogenously released and exogenous acetylcholine.

Studies with atropine have been made by Munro (1953), Garry & Gillespie (1955), Lee (1960), Gillespie & MacKenna (1960), Fülgraff & Schmidt (1964), Rand & Ridehalgh (1965) and Campbell (1966). According to Rothlin, Taeschler & others (1954) and Taeschler, Konzett & Cerletti (1960) tropenziline bromide interferes with nervous transmission in the vagal plexus of the intestine and thus exerts a spasmolytic effect, which has also been confirmed clinically by Kewenter, Kock & others (1962) and by Shah, Shet & Shah (1964). The action of *N*-butyl hyoscine bromide on the intestinal tract has been investigated by Wick (1951), who found that it was more active on the intestine than was atropine in blocking the effects produced by vagal stimulation, leading to the conclusion that *N*-butyl hyoscine bromide has strong ganglion-blocking properties. The ganglion blocking activity of *N*-butyl hyoscine bromide has been demonstrated by Holtz & Schümann (1956), Herman, Shaw & Rosenblum (1958), and Pennefather, McCulloch & Rand (1968).

In this paper we deal with comparisons of the activities of atropine sulphate, 6-methoxytropine benzilate-*N*-methylbromide (tropenziline) and *N*-butyl hyoscine bromide on the responses of guinea-pig colon to acetylcholine, and to pelvic and transmural stimulation. Moreover, as there is no direct proof of the existence of a ganglion blocking activity of tropenziline or *N*-butyl hyoscine bromide at the level of the parasympathetic ganglia of the intestinal plexuses, we intend to investigate this problem by examining the effects of these drugs on the amount of acetylcholine released by pelvic nerve stimulation.

EXPERIMENTAL

Guinea-pig isolated colon was prepared similarly to the rabbit preparation described by Garry & Gillespie (1955).

The terminal colon, length 3–4 cm, together with the pelvic nerves was removed from 150 adult female guinea-pigs, 250–300 g. Bipolar electrodes, made with rings of silver wire (2 mm apart), were placed around the nerves. Transmural stimulation was applied by inserting a silver wire into the lumen and by placing the reference electrode into the bath. The preparation was suspended vertically in a 10 ml bath containing an oxygenated Tyrode solution at 35°. The contractions of the longitudinal muscle were recorded with a light frontral isotonic gimbal lever, exerting a tension of 2·5 g. The preparation was left in the bath for about 120 min before beginning the experiment. To stimulate the pelvic and intramural nerves, trains of rectangular pulses with 1 ms pulse duration and a frequency of 10 Hz were applied for 30 s. The pelvic nerve was stimulated supramaximally. The strength of the transmural stimulation (20 V) and the acetylcholine concentration (1×10^{-7} g/ml) were selected to give a response similar to that produced by pelvic nerve stimulation.

For the construction of dose-response curves in each experiment, the activity of an anticholinergic drug was evaluated against one kind of stimulation. Progressively increasing concentrations of drugs were added to the bath at 30 min intervals, each addition being 20 min before stimulation. Since the response to transmural stimulation after atropine became biphasic or triphasic according to the drug concentration used, the height of contraction above the base line was chosen for measurement and was compared with the height of the control contraction (Del Tacca, Lecchini & others, 1968). The heights of contractions produced by acetylcholine, pelvic and transmural stimulation after drug treatment were expressed as percentages of the control contractions to calculate the ED50 values.

For estimating acetylcholine release and the effects of drugs on it, the colon was prepared as described above, and was then incubated for 40 min with physostigmine sulphate (1×10^{-5} g/ml). Concentrations of atropine, tropenziline and *N*-butyl hyoscine bromide causing a reduction of about 60% of the response to pelvic and transmural stimulation were chosen.

The experiments were made as follows: during the first period the preparation was stimulated (pelvic or transmural stimulation) for 10 min, and then was left to rest for 10 min, after which time the Tyrode solution was removed. During the second period, after washing, the preparation was kept at rest for 20 min; then the Tyrode solution was again removed. The first and second periods were taken as a trial cycle, which was repeated 3 times. The first cycle was discarded and the second cycle was used as a control: between the second and the third cycle the drugs were added to the colon. The acetylcholine content in the samples (diluted 1 to 1·2 with distilled water) was assayed against suitable standards on frog rectus abdominis muscle added with 5×10^{-6} g/ml physostigmine sulphate. The amount of acetylcholine released during a 10 min resting period was subtracted from that released during the period of stimulation to obtain the quantity released during the 10 min period of stimulation.

The following drugs were used: atropine sulphate, 6-methoxytropine benzilate-*N*-methylbromide (tropenziline), *N*-butyl hyoscine bromide, acetylcholine chloride, hexamethonium bromide and physostigmine sulphate. The concentrations are expressed in terms of base.

RESULTS

In 13 out of 67 preparations the responses to pelvic and transmural stimulation, but not those to exogenous acetylcholine, were partially resistant to the blocking actions of atropine, tropenziline and *N*-butyl hyoscine bromide, as had also been previously noted for atropine by Del Tacca & others (1968). The resistant preparations were not included in the calculation of the ED50.

Table 1 shows that the potency of blocking activity against both endogenous and exogenous acetylcholine is in the order: atropine > tropenziline > *N*-butyl hyoscine bromide. Atropine and tropenziline were significantly more active in blocking responses to exogenous acetylcholine than in blocking those to either transmural or pelvic stimulation. For *N*-butyl hyoscine bromide, activity against responses to pelvic stimulation, but not that to transmural stimulation, was significantly different from its activity against exogenous acetylcholine. Atropine reduced the response to transmural stimulation at lower concentrations than those necessary to reduce the response to pelvic stimulation. On the other hand, the response to pelvic and transmural stimulation were inhibited by concentrations of tropenziline and *N*-butyl hyoscine bromide which were not significantly different. For all three substances there was a linear relation between log dose and effect.

The amounts of acetylcholine released from the colon vary considerably, not only from one species to another, but also with preparations of colon from a single species.

Table 1. *ED50 and slopes* (b) *of regression lines with 95% fiducial limits of atropine, tropenziline and N-butyl hyoscine bromide on the contraction producted by acetylcholine* (1×10^{-7} g/ml), *transmural* (10 Hz) *and pelvic* (10 Hz) *stimulation in normal guinea-pig colon. The probability values* (*P*) *for the significance of differences between ED50 within groups are given.*

Drugs	No. of expts	Stimulating agents	ED50 with fiducial limits (95%)	b values with fiducial limits (95%)	*P* values
Atropine	8	Acetylcholine (ACh)	$2{\cdot}59 \times 10^{-8}$ M ($3{\cdot}63 \times 10^{-8}$ – $1{\cdot}91 \times 10^{-8}$)	73·13 (69·96 – 76·30)	ACh/P < 0·001
	8	Transmural (T)	$5{\cdot}41 \times 10^{-8}$ M ($8{\cdot}78 \times 10^{-8}$ – $3{\cdot}45 \times 10^{-8}$)	63·21 (56·20 – 70·22)	ACh/T < 0·01
	8	Pelvic (P)	$9{\cdot}95 \times 10^{-9}$ M ($2{\cdot}64 \times 10^{-8}$ – $6{\cdot}50 \times 10^{-9}$)	41·39 (35·85 – 46·93)	PS/T < 0·05
Tropenziline	7	Acetylcholine (ACh)	$1{\cdot}57 \times 10^{-8}$ M ($2{\cdot}56 \times 10^{-8}$ – $1{\cdot}04 \times 10^{-8}$)	57·99 (52·87 – 63·11)	ACh/P < 0·02
	10	Transmural (T)	$2{\cdot}83 \times 10^{-8}$ M ($4{\cdot}49 \times 10^{-8}$ – $1{\cdot}88 \times 10^{-8}$)	54·73 (51·76 – 57·69)	ACh/T < 0·05
	10	Pelvic (P)	$3{\cdot}54 \times 10^{-8}$ M ($6{\cdot}21 \times 10^{-8}$ – $2{\cdot}14 \times 10^{-8}$)	42·68 (40·45 – 44·91)	PS/T = N.S.
N-Butyl hyoscine bromide	6	Acetychloline (ACh)	$8{\cdot}13 \times 10^{-8}$ M ($1{\cdot}40 \times 10^{-7}$ – $4{\cdot}74 \times 10^{-8}$)	73·51 (73·03 – 73·99)	ACh/P < 0·05
	8	Transmural (T)	$1{\cdot}15 \times 10^{-7}$ M ($2{\cdot}16 \times 10^{-7}$ – $5{\cdot}47 \times 10^{-8}$)	54·93 (59·44 – 50·41)	ACh/T = N.S.
	10	Pelvic (P)	$2{\cdot}54 \times 10^{-7}$ M ($6{\cdot}74 \times 10^{-7}$ – $9{\cdot}85 \times 10^{-8}$)	34·25 (33·78 – 34·73)	PS/T × N.S.

Table 2. *Acetylcholine output from the guinea-pig colon. Action of different anticholinergic drugs on transmural stimulation and resting acetylcholine output* (ng/g/10 min ± s.e.).

Drugs	Concn (M)	Stimulation		Resting	
		Control	After drug	Control	After drug
Atropine (10)*	$8{\cdot}60 \times 10^{-9}$	1·089 ± 0·058	1·185 N.S. ± 0·139	0·301 ± 0·025	0·288 N.S. ± 0·027
Tropenziline (9)*	$3{\cdot}09 \times 10^{-8}$	0·948 ± 0·164	0·750 $P < 0{\cdot}02$ ± 0·160	0·327 ± 0·049	0·303 N.S. ± 0·055
N-Butyl hyoscine bromide (9)*	$2{\cdot}27 \times 10^{-7}$	1·083 ± 0·149	0·891 $P < 0{\cdot}05$ ± 0·144	0·312 ± 0·035	0·276 N.S. ± 0·043

* No. of experiments
P = probability values for significance of difference between the mean amounts of acetylcholine released before and after drug addition. N.S. = no significant difference.

Table 3. *Acetylcholine output from the guinea-pig colon. Action of different anticholinergic drugs on resting and pelvic stimulation acetylcholine output* (ng/g/10 min ± s.e.).

Drugs	Concn (M)	Stimulation		Resting	
		Control	After drug	Control	After drug
Atropine (15)*	$1{\cdot}72 \times 10^{-8}$	0·568 ± 0·056	0·424 $P < 0{\cdot}001$ ± 0·039	0·342 ± 0·040	0·297 N.S. ± 0·029
Tropenziline (6)*	$6{\cdot}29 \times 10^{-8}$	0·746 ± 0·051	0·516 $P < 0{\cdot}01$ ± 0·036	0·385 ± 0·035	0·306 N.S. ± 0·014
N-Butyl hyoscine bromide (6)*	$3{\cdot}27 \times 10^{-7}$	0·616 ± 0·065	0·422 $P < 0{\cdot}02$ ± 0·042	0·281 ± 0·030	0·220 N.S. ± 0·024

* No. of experiments
P = probability values for significance of difference between the mean amounts of acetylcholine released before and after drug addition. N.S. = no significant difference.

We have observed this and it is in agreement with the results obtained by Paton & Vizi (1969). Therefore, the effects of the drugs were evaluated by comparing, in each experiment, the amounts of acetylcholine released before and after drug addition. Beani, Bianchi & Crema (1969) observed that a second period of transmural stimulation resulted in the release of a quantity of acetylcholine not significantly different from that of the first period. In 5 experiments, we found, additionally, that with two periods of pelvic stimulation, the second stimulation caused a release of acetylcholine not signicantly different from the first.

Tables 2 and 3 show that atropine, tropenziline, and *N*-butyl hyoscine bromide did not reduce the resting output of acetylcholine, whereas release during pelvic and transmural stimulation was significantly reduced by tropenziline and *N*-butyl hyoscine bromide. Atropine reduced the output only during pelvic stimulation. Expressed in percentages the amounts released by transmural stimulation were reduced by 21% by tropenziline and by 18% by *N*-butyl hyoscine bromide. The acetylcholine released by pelvic stimulation was reduced by 31% by both tropenziline and *N*-butyl hyoscine bromide. The amount released by pelvic stimulation was reduced by 25% by atropine.

In 2 experiments, hexamethonium (1×10^{-4} g/ml) was used to block ganglion transmission and this resulted in a reduction of 39% of the resting release and of 49% of the amount released by pelvic stimulation.

DISCUSSION

In the colon, atropine exhibited a greater anticholinergic activity than either tropenziline or hyoscine *N*-butyl bromide, in agreement with findings in other organs by Wick (1951), Rothlin & others (1954), Holtz & Schümann (1956), Herman & others (1958), and Pennefather & others (1968). All three parasympatholytic agents antagonized the effects of exogenous acetylcholine more readily than those caused by endogenous acetylcholine.

It has been shown (for reviews see Barlow, 1964; Gyermek, 1967) that the quaternization of tropane derivatives increases ganglion blocking activity and decreases the antimuscarinic potency. In the guinea-pig colon the ratio between the ED50 against responses to pelvic stimulation and the ED50 against responses to exogenous acetylcholine is 4·05 for atropine, 2·25 for tropenziline and 3·12 for *N*-butyl hyoscine bromide. These different ratios could indicate that the quaternary derivatives exert a ganglion blocking action also in the colon. The effects of these drugs on the release of acetylcholine confirm this supposition. In fact, concentrations of tropenziline and *N*-butyl hyoscine bromide which reduce the response to pelvic stimulation by 60%, also cause a 31% reduction of acetylcholine output.

It has been shown that hexamethonium reduces the response to submaximal transmural stimulation (Schaumann, 1956; Härtfelder, Kuschinsky & Mosler, 1958; Kern & Lembeck, 1959; Paton & Vane, 1963; Bianchi, Beani & others, 1968) and that it decreases the acetylcholine output associated with transmural stimulation, lending support to the hypothesis that the ganglia of the colon can act as a multiplier of the submaximal transmural stimulus (Bianchi & others, 1968). In our experiments the strength of transmural stimulation was submaximal and, therefore, signs of the ganglion blocking activity of the quaternary compounds could be expected to appear. However, all three drugs, at the concentrations employed, were ineffective on the resting output of acetylcholine, although it was reduced by hexamethonium, confirming previous results (Beani & others, 1969). No further effort was made to investigate whether or not the action of ganglion blocking drugs on the resting output was dependent upon the concentration employed.

Atropine did not modify the acetylcholine release produced by transmural stimulation, which is in agreement with the findings of Schaumann (1956) and Del Tacca & others (1968), but it significantly reduced its release on pelvic stimulation (a similar but not significant effect was noted in a smaller number of experiments by Del Tacca & others, 1968). An explanation for the effect of atropine in reducing the amount of acetylcholine released by pelvic stimulation is that atropine blocks transmission by acting on muscarinic receptors at the level of the ganglia (for reviews see Volle, 1966; Trendelenburg, 1967). If this explanation is correct it implies the existence of muscarinic receptors on parasympathetic ganglion cells, which is at variance with the conclusions of Roszkowski (1961) and Smith (1966). It is possible that physostigmine reveals the presence of muscarinic receptor, as was the case with both the sympathetic and parasympathetic ganglia in which the ganglion blocking action of atropine was unmasked by the presence of anticholinesterase drugs (Flacke & Gillis, 1968), as well as

by the continuous perfusion of acetylcholine (Gebber & Snyder, 1968). Further experiments are needed to investigate the action of atropine on parasympathetic ganglia.

In conclusion, tropenziline and *N*-butyl hyoscine bromide have two sites of action in the guinea-pig colon. In addition to their principal antimuscarinic activity on the smooth muscle, they have a ganglion blocking action exerted through nicotinic receptors and, possibly, also through muscarinic receptors.

REFERENCES

BARLOW, R. B. (1964). *Introduction to Chemical Pharmacology*, London: Methuen & Co. Ltd.

BEANI, L., BIANCHI, C. & CREMA, A. (1969). *Br. J. Pharmac.*, **36**, 1–17.

BIANCHI, C., BEANI, L., FRIGO, G. M. & CREMA, A. (1968). *Europ. J. Pharmac.*, **4**, 51–61.

CAMBPELL, G. (1966). *J. Physiol., Lond.*, **185**, 148–159.

DEL TACCA, M., LECCHINI, S., FRIGO, G. M., CREMA, A. & BENZI, G. (1968). *Europ. J. Pharmac.*, **4**, 188–197.

FLACKE, W. & GILLIS, R. A. (1968). *J. Pharmac. exp. Ther.*, **163**, 266–276.

FÜLGRAFF, G. & SCHMIDT, L. (1964). *Archs int. Pharmacodyn. Thér.*, **149**, 552–559.

GARRY, R. C. & GILLESPIE, J. S. (1955). *J. Physiol., Lond.*, **128**, 557–576.

GEBBER, G. L. & SNYDER, D. W. (1968). *J. Pharmac. exp. Ther.*, **163**, 64–74.

GILLESPIE, J. S. & MCKENNA, B. R. (1960). *J. Physiol., Lond.*, **152**, 191–205.

GYERMEK, L. (1967). *In Drugs affecting the Peripheral Nervous System*, Editor: Burger, A. Vol. 2, pp. 149–326, New York: M. Dekker, Inc.

HÄRTFELDER, G., KUSCHINSKY, G. & MOSLER, K. H. (1958). *Arch. exp. Path. Pharmak.*, **234**, 66–78.

HERMANN, L., SHAW, F. H. & ROSENBLUM, E. I. (1958). *J. Pharm. Pharmac.*, **10**, 348–355.

HOLTZ, P. & SCHÜMANN, H. J. (1956). *Arch. exp. Path. Pharmak.*, **229**, 101–112.

KERN, H. & LEMBECK, F. (1959). *Ibid.*, **236**, 325–330.

KEWENTER, J., KOCK, N. G., POMPEIUS, R. & SCHELLER, S. (1962). *Acta chir. scand.*, **123**, 396–404.

LEE, C. Y. (1960). *J. Physiol., Lond.*, **152**, 405–418.

MUNRO, A. F. (1953). *Ibid.*, **120**, 41–52.

PATON, W. D. M. & VANE, J. R. (1963). *Ibid.*, **165**, 10–46.

PATON, W. D. M. & VIZI, E. S. (1969). *Br. J. Pharmac.*, **35**, 10–28.

PENNEFATHER, J. N., MCCULLOCH, M. W. & RAND, M. J. (1968). *J. Pharm. Pharmac.*, **20**, 867–872.

RAND, M. J. & RIDEHALGH, A. (1965). *Ibid.*, **17**, 144–156.

ROSZKOWSKI, A. P. (1961). *J. Pharmac. exp. Ther.*, **132**, 156–170.

ROTHLIN, E., TAESCHLER, M., KONZETT, H. & CERLETTI, A. (1954). *Experentia*, **10**, 142–145.

SCHAUMANN, W. (1956). *Nature, Lond.*, **178**, 1121.

SHAH, N. R., SHETH, R. D. & SHAH, R. C. (1964). *J. Ass. Phyns India*, **12**, 771–775.

SMITH, J. C. (1966). *J. Pharmac. exp. Ther.*, **153**, 266–275.

TAESCHLER, M., KONZETT, H. & CERLETTI, A. (1960). *Schweiz. Med. Wschr.*, **90**, 136–140.

TRENDELENBURG, U. (1967). *Ergerb. Physiol.*, **59**, 2–85.

VOLLE, R. L. (1966). *Pharmac. Rev.*, **18**, 839–869.

WICK, H. (1951). *Arch. exp. Path. Pharmak.*, **213**, 485–500.

J. Pharm. Pharmac., 1969, **21**, 668–673 *Received April* 8, 1969

Some observations on the toxicology of morphine-*N*-oxide

M. R. FENNESSY AND H. J. FEARN*

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

The intravenous and subcutaneous acute toxicities of morphine-*N*-oxide (MNO) in mice were respectively 3·2 and 8 times less than that of morphine. Amiphenazole or tacrine reduced the acute toxicity of MNO but not that of morphine in mice. The chronic toxicity of MNO was examined in mice and rats. Daily oral doses of 100 mg/kg did not significantly affect growth or condition, or produce gross or microscopic lesions in mice treated for 3 weeks or rats treated for 3 months. No teratogenic effect of MNO or of bromolysergic acid diethylamide was observed in rats.

Woo, Gaff & Fennessy (1968) identified morphine-*N*-oxide (MNO) in the urine of patients treated with morphine in combination with either amiphenazole (2,4-diamino-5-phenylthiazole) or tacrine (1,2,3,4-tetrahydro-9-aminoacridine) but not after morphine alone. They suggested that MNO was an intermediate metabolite in the breakdown of morphine. Earlier reports suggested that MNO was without analgesic activity (Freund & Speyer, 1910; Rosenthäler, 1933; Keil, Schmidt & Günther, 1933; Anton, Theiss & Weissig, 1935; Braenden, Eddy & Halbach, 1955). However, Fennessy (1968) found it to have a weak analgesic activity which was markedly potentiated when it was administered with either amiphenazole or tacrine to rats and mice. The potentiation of the analgesic action of MNO and its presence in urine after morphine was suggested to be due to impairment of metabolism of MNO in the liver by amiphenazole and tacrine.

MNO may be of clinical interest as a metabolite of morphine or as an analgesic in its own right. We have examined its toxicology.

EXPERIMENTAL

Acute toxicity

Swiss mice of either sex, about 25 g were randomly assigned to groups of 10; various doses of morphine, MNO, amiphenazole, tacrine or nalorphine, given subcutaneously, were randomly assigned to these groups and the mice dead in each group after 24 h were recorded. The LD50 values with 95% confidence limits were calculated (Litchfield & Wilcoxon, 1949).

The intravenous LD50 values for morphine and MNO were also determined in normal mice and in mice pretreated 30 min before with amiphenazole, tacrine or nalorphine injected subcutaneously in doses corresponding to one-third and two-thirds of their subcutaneous LD50 doses.

Subacute toxicity

Female Swiss mice, 4 weeks old, about 15 g, were randomly assigned to 3 equal

* Present address: Royal Free Hospital, School of Medicine, London, England.

groups of 10 animals. One group received 100, another 1000 mg/kg of MNO by stomach tube in 0·2 ml of a suspension in 1% gum tragacanth. The third group were given the suspending agent. Food and water were allowed *ad libitum*. Body weights were recorded every 5 days. On the 22nd day, drug treatment was discontinued and body weight was recorded for a further 75 days.

Chronic toxicity

Female Sprague-Dawley rats, mean weight 154 g, were randomly assigned to 3 equal groups of 8 animals. The first group were given a daily dose of 25 mg/kg of morphine, the second group 100 mg/kg of MNO, and the remaining group were untreated. Each rat was given the required dose dissolved in water and mixed with 15 g of diet (Barostoc Growers Pellets). This amount of food was always consumed by the rats. The daily dose of 100 mg/kg of MNO was the maximum the animals would consume, even at the point of starvation. Water was given *ad libitum*. Body weights were recorded for 124 days and mean differences between the groups were tested for significance using the *t*-test. After 124 days the rats were killed and the organs examined histologically.

In another experiment 5 groups, each of 8 female Sprague-Dawley rats, mean weight 255 g, were used. The first group, controls, received no drug treatment; the second received daily a mixture of MNO (50 mg/kg) and amiphenazole (50 mg/kg); the third a mixture of MNO (50 mg/kg) and tacrine (5 mg/kg); the fourth amiphenazole (50 mg/kg), and the fifth group tacrine (5 mg/kg). Each animal received the required dose of drug dissolved in water and mixed with 19 g of food (Barostoc Growers Pellets). Drinking water was allowed *ad libitum*. Weights were recorded weekly for 13 weeks. The animals were then killed, inspected for gross pathological changes and organs were removed for histological examination.

Teratogenic activity

Healthy Sprague-Dawley female rats were mated with healthy males; 33 of 45 becoming pregnant and these were randomly assigned to 3 groups. The rats were individually housed. On the 3rd, 4th and 5th days of gestation, rats in one group received 50 μg/kg of 2-bromo-D-lysergic acid diethylamide (BOL) (an agent reported to have teratogenic activity, Geber, 1967) those in the second group received MNO 50 mg/kg) and those in the third, normal saline. On the 20th day of gestation the rats were killed with chloroform and the uterus and contents removed. Each foetus was examined to determine its viability and development and preserved for study of skeletal structure using the alizarin technique (Mahoney, 1966).

Drugs used were: Morphine sulphate (D.H.A.), Morphine-*N*-oxide, amiphenazole hydrochloride (Nicholas Pty. Ltd.), tacrine (H. W. Woods Pty. Ltd.), nalorphine hydrobromide (Burroughs Wellcome) and 2-bromo-D-lysergic acid diethylamide (Sandoz, Aust.). The concentration of all drugs has been expressed in terms of the base. Morphine-*N*-oxide was prepared according to the method of Freund & Speyer (1910). Solutions for injection were freshly prepared each day by dissolving the MNO in 0·1 N HCl and adjusting to pH 5 with NaOH.

RESULTS

Acute toxicities in mice

The LD50 values and their 95% confidence limits obtained with intravenous and subcutaneous injections of morphine and MNO, and with subcutaneous injections of

Table 1. *Acute toxicities in mice*

Compound	LD50 mg/kg (95% confidence limits) Subcutaneous injection	Intravenous injection
Morphine	675 (527–864)	250 (211–308)
MNO	>5300	820 (773–869)
Nalorphine	731 (594–899)	—
Amiphenazole	260 (226–299)	—
Tacrine	34 (29–39·8)	—

nalorphine, amiphenazole and tacrine are in Table 1. MNO was much less toxic than morphine, the extent depending on the route of administration. The intravenous LD50 of MNO was 3·2 times greater than that of morphine and the subcutaneous LD50 could not be determined as no mice died after 5300 mg/kg, the highest dose given because of the low solubility of MNO.

Changes in the LD50 values for morphine and MNO in mice pretreated with one-third and two-thirds the LD50 doses of nalorphine, amiphenazole and tacrine are shown in Fig. 1. The toxicity of MNO was clearly antagonized by tacrine, but only slightly antagnoized by amiphenazole or nalorphine. Tacrine, however, had no effect on the toxicity of morphine, amiphenazole caused a slight potentiation, and nalorphine produced a slight antagonism.

Subacute toxicity in mice

MNO given orally for 22 days to mice impaired growth compared to controls (Fig. 2).

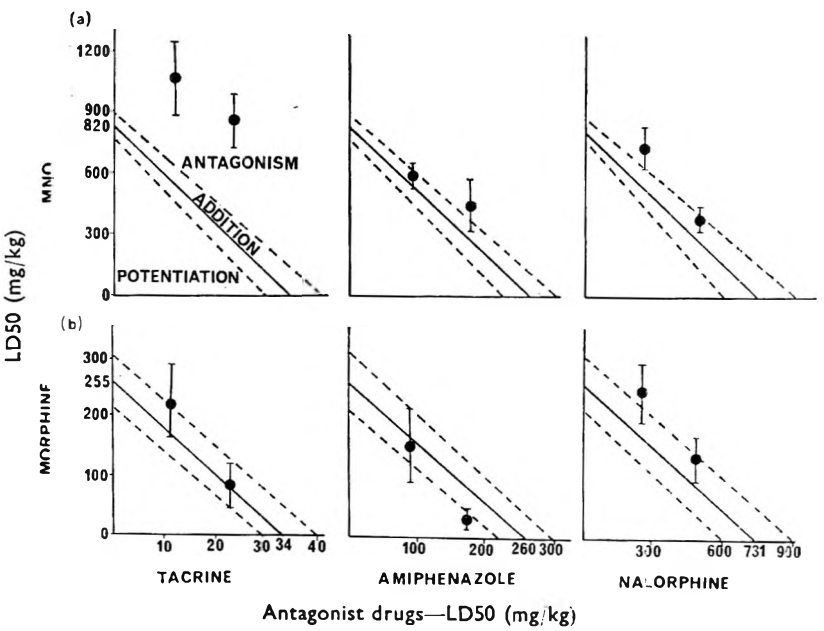


FIG. 1. Interaction between the toxic effects of analgesic drugs and narcotic antagonists in mice. The unbroken lines join the LD50 of (a) MNO or (b) morphine and the LD50 of tacrine, amiphenazole and nalorphine respectively. The broken lines join the 95% confidence limits of the corresponding LD50 of each drug. The vertical lines indicate the confidence limits for the LD50 of (a) MNO or (b) morphine in the presence of one-third or two-thirds LD50 of tacrine, amiphenazole or nalorphine given subcutaneously 30 min before the intravenous administration of either morphine or MNO.

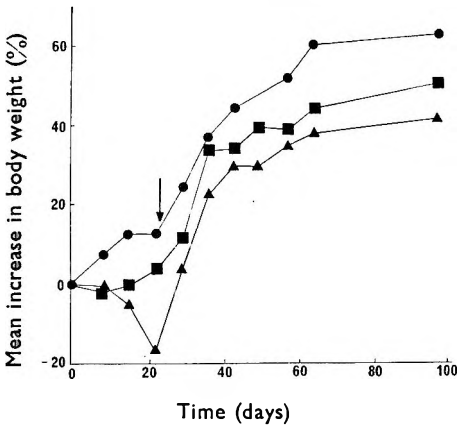


FIG. 2. The effect of MNO on body weight of mice. MNO was administered orally at dose levels of 100 mg/kg (■-■) and 1000 mg/kg (▲-▲). Control mice (●-●) received daily oral administrations of saline. Treatment was discontinued on the 22nd day (arrow). Each point is the mean weight of ten mice.

At 100 mg/kg of MNO daily the mean body weight of mice decreased slightly for the first 10 days then increased, but the weights of mice receiving 1000 mg/kg, were significantly decreased after 22 days compared to the other two groups (*t*-test, $P < 0{\cdot}05$). Discontinuation of the drug was followed by a rapid increase in body weight. After 100 days the mean weight of the mice on the higher dose of MNO was slightly less than the control value, while animals on the lower dose of MNO had a higher mean weight.

Chronic toxicity in rats

The growth rate of rats was slightly increased by MNO (100 mg/kg daily) but was significantly reduced by morphine (25 mg/kg) (Table 2). The general condition of the rats in all three groups remained good. No morphological or histological abnormalities were found in liver, kidneys, brain, bone-marrow, spleen, heart or gastrointestinal tract.

Amiphenazole (50 mg/kg) or tacrine (5 mg/kg) included in the diets of rats receiving MNO (50 mg/kg) daily caused a significant decrease in body weight after 91 days (Table 3), similar to that of rats receiving the same dose of amiphenazole or tacrine alone. No morphological abnormalities were found except that rats receiving

Table 2. *Effect of morphine and* MNO *on body weight in rats.* Morphine (25 mg/kg) or MNO (100 mg/kg) was given orally, mixed with the diet, daily for 124 days. There were 8 rats in each group.

	Mean body weight g ± s.e.		Mean percentage increase in weight ± s.e.
	Before test	After test	
Controls	150·4 ± 8·3	189·7 ± 6·8	26·1 ± 1·3
Morphine	154·1 ± 6·2	162·3 ± 5·9	5·3 ± 2·6*
MNO	158·2 ± 3·1	205·5 ± 4·7	29·9 ± 4·7†

* Significantly different from control (*t*-test, $0{\cdot}01 < P < 0{\cdot}05$).
† Not significantly different from control (*t*-test, $0{\cdot}05 < P < 0{\cdot}1$).

Table 3. *Effects of amiphenazole, tacrine and combinations of amiphenazole + MNO and tacrine + MNO on body weight in rats. Drugs were given orally, mixed with the diet, to each rat for 91 days. There ware 8 rats in each group.*

	Mean body weight in g ± s.e.		Mean percentage change in weight
	Before test	After 91 days	
Control	225·2 ± 13·0	229·0 ± 10·0	+1·7
Amiphenazole (50 mg/kg)	224·6 ± 10·3	212·5 ± 9·2	−5·4
Tacrine (5 mg/kg)	225·5 ± 8·5	192·2 ± 7·4*	−14·7
MNO (50 mg/kg) + amiphenazole (50 mg/kg)	227·0 ± 6·6	210·4 ± 4·2*	− 7·5
MNO (50 mg/kg) + tacrine (5 mg/kg)	222·8 ± 9·2	196·3 ± 8·1*	−11·7

* Significantly different from controls, $P < 0{\cdot}01$.

either tacrine or a mixture of MNO and tacrine had distended stomachs and reduced spleens.

Chronic toxicity studies of MNO given subcutaneously, intraperitoneally or orally were abandoned because the treated rats became autocannibalistic; also, animals receiving MNO subcutaneously developed necrotic lesions at the site of injection within 24 h.

Teratogenic activity

When MNO (50 mg/kg), BOL (50 μg/kg) or saline were injected subcutaneously into pregnant rats on the 3rd, 4th and 5th days of gestation, and foetuses examined on the 20th day, i e., one day before full term, there was no significant difference between the litter sizes or the mean foetal weights of animals in any of the groups (t-test, $P > 0{\cdot}1$). No skeletal abnormalities were observed after fixing and staining the foetuses. Foetal resorptions were observed in all three groups of rats, the highest being in the control group.

DISCUSSION

The acute toxicity of MNO was much lower than that of morphine. It appears probable that *N*-oxide derivatives of opium alkaloids have lower toxicities than the parent compounds, since the LD50 of codeine-*N*-oxide was found by Tagaki & Fukuda (1960) to be 1500 mg/kg when given subcutaneoulsy to mice compared with 356 mg/kg for codeine. The chronic toxicity of MNO was also lower than that of morphine. Thus MNO did not affect growth rate in rats whereas a smaller dose of morphine significantly inhibited growth. However, in mice, large daily concentrations of MNO (1 g/kg) did decrease body weight.

Although both amiphenazole and tacrine potentiate the analgesic action of MNO (Fennessy, 1968), both drugs antagonize the acute toxicity of MNO, tacrine being the better antagonist. However, in rats, tacrine caused greater inhibition of growth than amiphenazole, it also produced definite morphological abnormalities. The evidence indicates that the decreased growth rate produced by combinations of MNO with tacrine and or with amiphenazole is due to the actions of the antagonists and not to MNO.

MNO had no teratogenic activity in rats, but it is difficult to draw conclusions from the negative results obtained.

Acknowledgements

Thanks are due to H. W. Woods Pty. Ltd. for financial support and to Professor M. J. Rand for his criticism of the manuscript.

REFERENCES

ALEXANDER, G. J., MILES, B. E., GOLD, G. M. & ALEXANDER, R. B. (1967). *Science, N.Y.*, **157**, 459–460.

ANTON, G., THEISS, W. & WEISSIG, H. (1935). *Dtsch. Med. Wschr.*, **61**, 1195–1196.

AUERBACH, R. & RUGOWSKI, J. A (1967). *Science, N.Y.*, **157**, 1325–1326.

BRAENDEN, O. J., EDDY, N. B. & HALBACH, H. (1955). *Bull. Wld. Hlth. Org.*, **13**, 937–998.

FENNESSY, M. R. (1968). *Br. J. Pharmac. Chemother.*, **34**, 337–344.

FREUND, M. & SPEYER, E. (1910). *Ber. Dtsch. Chem. Ges.*, **43**, 3310–3314.

GEBER, W. F. (1967). *Science, N.Y.*, **158**, 265–267.

KEIL, W., SCHMIDT, H. & GÜNTHER, A. (1933). *Dtsch. Med. Wschr.*, **59**, 959–960.

LITCHFIELD, J. T. & WILCOXON, F (1949). *J. Pharmac. exp. Ther.*, **96**, 99–113.

MAHONEY, R. (1966). *Laboratory techniques in Zoology.* 1st edn., pp. 339–340, London: Butterworth.

ROSENTHÄLER, L. (1933). *Pharmaz. Zeit.*, **78**, 926–929.

TAGAKI, K. & FUKUDA, H. (1960) *Pharm. Soc. Jap. J.*, **80**, 1501–1506.

WARKANY, J. & TAKACS, E. (1968). *Science, N.Y.*, **159**, 731–732.

WEST, G. B. (1962). *J. Pharm. Pharmac.*, **14**, 828–830.

WOO, J. T. C., GAFF, G. A. & FENNESSY, M. R. (1968). *Ibid.*, **20**, 763–767.

J. Pharm. Pharmac., 1969, **21**, 674–677 *Received April* 18, 1969

Gas chromatographic identification of thioridazine in plasma, and a method for routine assay of the drug

S. H. CURRY AND G. P. MOULD

*Department of Pharmacology and Therapeutics, London Hospital Medical College, Turner Street, London, E.*1, *England*

Thioridazine had a retention time of approximately 4 min on a 3% OV-17 gas chromatographic column at 260°. Using flame or argon ionization detectors samples as low as 0·01 μg could be detected. The drug could be extracted into n-heptane containing 1·5% isoamyl alcohol or 20% toluene. Recovery of drug added to plasma was 100%. Unchanged thioridazine was identified in the plasma of two experimental samples, on the basis of chromatographic and solvent distribution data.

Studies of thioridazine metabolism in man have included determinations of apparent concentrations of the drug in blood, serum or plasma. Eiduson & Geller (1963), Mellinger (1965) and Mellinger, Mellinger & Smith (1965), have implied but not proved that serum concentrations of the drug vary between zero and 0·9 μg/ml during the 24 h after single doses of 100 mg. However, the techniques used by these authors are not known to be specific for any one compound, and it was not clear whether drug or drug and metabolite concentrations were measured. Indeed, Eiduson, Geller & Wallace (1963) found 97% of excreted radioactivity in man after [^{35}S]thioridazine to be extractable in solvents of the polarity of ether and chloroform even though unchanged phenothiazines are seldom detectable in urine. This suggests that the "apparent thioridazine" is at least in part accounted for by metabolites of the drug.

Solvent extracts of the plasma of patients receiving chlorpromazine can contain large quantities of its metabolites (Curry & Marshall, 1968) but phenothiazines and their metabolites show only slight spectroscopic variations (Beckett & Curry, 1963; Beckett, Curry & Bolt, 1964). As part of a wider investigation of plasma levels of psychoactive phenothiazines, an examination of the gas-liquid chromatographic properties of solvent extracts of the plasma of humans receiving thioridazine has been made. We describe the characterization of the gas-chromatographic response of apparent thioridazine in plasma, and a specific method for the routine assay of the drug in biological fluids.

EXPERIMENTAL

Identification of thioridazine

Two samples of blood from patients receiving chronic thioridazine treatment (oral dose unchanged for one month or more), and receiving no other drugs, were collected into Vacutainer Tubes (B-D Products) containing potassium oxalate, and centrifuged immediately. Aliquots of plasma were examined as below. Details of doses and times of sample collection are given in Fig. 2.

A sample of plasma was mixed with 1 ml of 5% NaOH solution and extracted with 10 ml of n-heptane containing 1·5% isoamyl alcohol. An aliquot of the heptane solution was extracted with 2 ml of 0·01N HCl solution and an aliquot of this aqueous solution was made alkaline with 0·3 ml of NaOH solution and extracted with 50 μl of n-heptane containing 1·5% isoamyl alcohol. Samples (1–10 μl) of the final heptane layer were examined by gas-chromatography.

Gas-chromatographic conditions were as follows: instrument, Barber-Colman Model 5000; inlet temperature, 270°; column packing, 3% OV-17 on Gas-Chrom Q (100–120 mesh) (Applied Science Laboratories); column temperature, 260°; carrier gas, nitrogen flowing at 60 ml/min; detector, 10-mCi ^{90}Sr ionization detector operated at 270° and 750 V.

Signals were assessed by measurement of peak height and comparison with a reference curve obtained from standard quantities of thioridazine. The response was linear over the range 0·1 to 10 μg.

Routine assay of thioridazine

A sample of plasma was mixed with 2·5 ml of 5% NaOH solution and extracted with 5 ml of n-heptane containing 20% toluene. An aliquot of the heptane solution was extracted with 2 ml of 0·1N HCl solution and an aliquot of this aqueous solution was made alkaline with 0·2 ml of N NaOH solution and extracted with 50 μl of the heptane-toluene mixture. Samples (1–2 μl) of the final heptane layer were examined by gas-chromatography.

Gas-chromatographic conditions were as above, except for the substitution of a Pye Series 104 gas-chromatograph with a flame ionization detector (H_2 flow rate, 60 ml/min; O_2 flow rate, 600 ml/min).

The method of Brodie, Udenfriend & Baer (1947) was used for specificity checks by the technique of comparative distribution ratios.

Standard thioridazine solutions

Standard solutions of thioridazine were prepared by dissolving known quantities of the hydrochloride in water, adding 1 ml of N NaOH solution, and extracting the thioridazine into known volumes of either the n-heptane-isoamyl alcohol mixture or the n-heptane-toluene mixture. These extracts contained all of the thioridazine originally dissolved in the water.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatogram obtained from a reference sample of thioridazine, and the chromatograms obtained from extracts of "blank" plasma and from an extract of the same plasma when used as the solvent for thioridazine. Fig. 2 shows chromatograms obtained from the two experimental plasma samples. Table 1 shows solvent distribution data of actual and apparent thioridazine between n-heptane containing 1·5% isoamyl alcohol and aqueous solutions of various pH values. Table 2 shows the actual and found values for thioridazine in plasma for a series of synthetic solutions assayed by the routine method. As can be seen from the data, "blanks" were of minimum significance (the signal from a "blank" extract was comparable with the signal from solvent alone), recovery was virtually complete, and the apparent thioridazine had the same retention time and partition coefficients as actual thioridazine. In view of the fact that the OV-17 column effectively separates

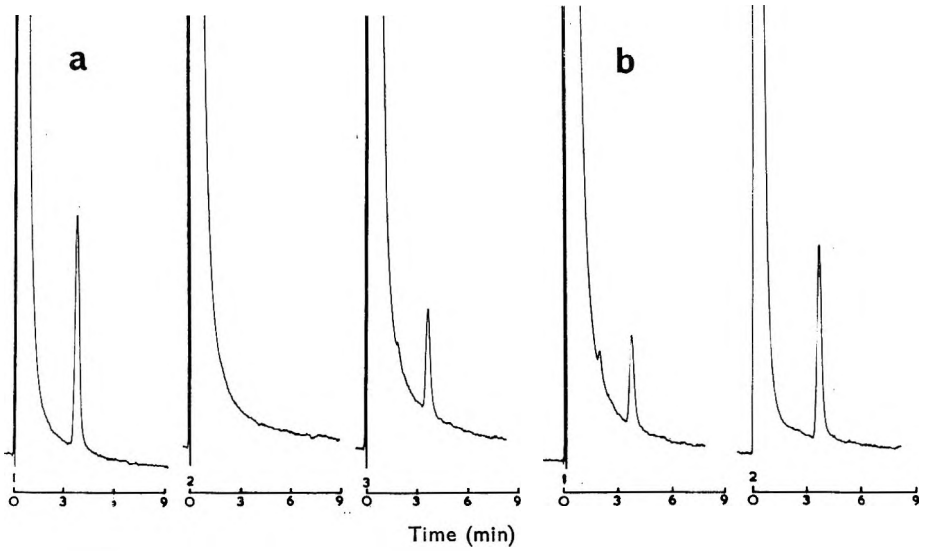


FIG. 1a. Gas-liquid chromatographic traces of: (1) reference thioridazine (0·5 μg) in n-heptane containing isoamyl alcohol; (2) 10 μl of an extract of blank plasma prepared as for "identification of thioridazine"; (3) 10 μl of extract of a second sample of the plasma used in (2), but after addition of 1 μg/ml of thioridazine to the plasma (recovery 89%).

b. Gas-liquid chromatographic traces of experimental extracts from plasma of patients receiving chronic treatment with oral thioridazine: (1) dose 100 mg twice daily; sample 2 h after morning dose; concentration 0·24 μg/ml; (2) dose 300 mg twice daily; sample 2 h after morning dose; concentration 1·80 μg/ml.

the metabolites of the analogous compound chlorpromazine (Curry, 1968), and that solubility of the actual and apparent thioridazine is identical, it is considered that the specificity of the method is proved.

Adsorption of thioridazine on to glass, a major problem in drug extraction (Brodie & others, 1947), could be prevented by the presence in the heptane of either 1·5% isoamyl alcohol or 20% toluene. However, the latter was preferred as the solvent in the later experiments because less emulsion formation occurred in the extractions.

Table 1. *Distribution of thioridazine and apparent thioridazine between water at various pH values and n-heptane containing* 1·5% *isoamyl alcohol*

Aqueous solution or pH value of buffer solution	Fraction of material remaining in organic phases		
	Standard	Recovery solution	Experimental sample
0·05N HCl	0	0	0
4	0·01	0·01	0·02
4·7	0·40	0·41	0·40
5·2	0·89	0·90	—
7	0·96	0·95	0·98
7·3	0·98	0·96	—
10	1·00	1·00	—
0·1N NaOH	1·00	1·00	1·00

Aliquots of the standard solution (1 ml) and of the diluted extracts (50 μl) were shaken with equal volumes of various buffer solutions. The amount of thioridazine remaining in the organic phase after equilibration was determined by GLC and is expressed as a fraction of the amount originally present.

Table 2. *Values of true and found thioridazine in a number of 2·5 ml plasma solutions of the drug. Limits, where given, are ± standard error of the mean*

Thioridazine added to plasma (μg/ml)	No. of Samples	Thioridazine recovered (μg/ml)	Recovery (%)
0·4	4	0·38 ± 0·01	96
1	8	1·05 ± 0·05	105
2	4	1·98 ± 0·04	99
10	6	10·6 ± 0·50	106

Large quantities of either solvent mixture were tolerated by the argon ionization detector, but the response of the flame ionization detector was adversely affected by more than two μl of solvents containing heptane. Evaporation of solvents resulted in high concentrations of undesired contaminants in the extracts, so concentration of extracts to small volumes was achieved by back extractions, using techniques originally described for chlorpromazine (Curry, 1968). The two extraction systems resulted in clean blanks, and adequate extraction of apparently-identical materials. With 5 ml plasma samples, thioridazine could be assayed at concentrations as low as 0·05 μg/ml using the argon ionization detector, and at concentrations as low as 0·2 μg/ml using the hydrogen flame detector.

The analyses of the two plasma samples provide evidence of the adequacy of these techniques for a wider study of thioridazine kinetics and for studies of the relation between kinetics and pharmacological responses. It has recently been shown that plasma concentrations of chlorpromazine vary widely, and that this variation is at least partly responsible for variations in pharmacological effects (Curry & Marshall, 1968).

Acknowledgements

The authors are grateful to Sandoz Pharmaceuticals for the supply of thioridazine; the two experimental plasma samples were kindly supplied by Dr. J. H. L. Marshall of St. Elizabeth's Hospital, Washington, D.C., U.S.A., during an extensive study of kinetics of phenothiazine derivatives in psychiatric patients; preliminary experiments designed to test the suitability of the OV-17 column for reference and extracted thioridazine were made at the Laboratory of Chemical Pharmacology, National Heart Institute, Bethesda, Maryland, U.S.A.

This work was supported (in part) by contract number PH 43-66-1167 with the United States National Institute of General Medical Sciences.

REFERENCES

Beckett, A. H. & Curry, S. H. (1963). *J. Pharm. Pharmac.*, **15**, 246T–252T.
Beckett, A. H., Curry, S. H. & Bolt, A. G. (1964). *Ibid.*, **16**, 500–502.
Brodie, B. B., Udenfriend, S. & Baer, J. E. (1947), *J. biol. Chem.*, **168**, 299–309.
Curry, S. H. (1968). *Analyt. Chem.*, **40**, 1251–1256.
Curry, S. H. & Marshall, J. H. L. (1968). *Life Sci.*, **7**, 9–17.
Eiduson, S. & Geller, E. (1963). *Biochem. Pharmac.*, **12**, 1429–1435.
Eiduson, S., Geller, E. & Wallace, R. D. (1963). *Ibid.*, **12**, 1437–1439.
Mellinger, T. J. (1965). *Am. J. Psychiat.*, **121**, 1119–1122.
Mellinger, T. J., Mellinger, E. M. & Smith, W. T. (1965). *Clin. Pharmac. Ther.*, **6**, 486–491.

J. Pharm. Pharmac., 1969, **21**, 678–680 *Received, May* 5, 1969

Gas chromatographic analysis of acetylsalicylic acid, phenacetin, caffeine, and codeine in APC and codeine tablets

EARL B. DECHENE, L. H. BOOTH AND (MISS) M. J. CAUGHEY

The control laboratories of Merck-Frost Laboratories, Montreal, Quebec, Canada

All active ingredients in APC and codeine tablets are determined by gas chromatographic procedures after separation of acetylsalicylic and phenacetin from caffeine and codeine.

Hoffman & Mitchell (1963) described a gas chromatographic method for determining in a single run the active ingredients in APC tablets. In our hands their method gave satisfactory assays of acetylsalicylic acid and phenacetin, in tablets containing acetylsalicylic acid 0·22 g, phenacetin 0·16 g, caffeine 0·016 g, codeine phosphate 0·008 to 0·032 g. It was not reliable for determining caffeine due to the high ratio of phenacetin to caffeine, and the small difference in their retention times.

Haefelfinger, Schmidli & Ritter (1964) described a gas chromatographic procedure for determining caffeine and phenacetin combined with other active ingredients in tablets. Again we encountered difficulty with this method in obtaining satisfactory assays for caffeine because of interference from phenacetin.

We established in model assays that the chromatographic system described by Haefelfinger could reliably determine caffeine and codeine alone or in combination.

The Hoffman procedure uses chloroform to extract the active ingredients from the tablets whereas the Haefelfinger method uses acetone. Neither of these solvents is suitable for extracting codeine phosphate. We investigated a number of extraction procedures, and developed a procedure that quantitatively extracted caffeine and codeine from tablets while separating them from phenacetin and acetylsalicylic acid. We were able then, to reliably determine caffeine and codeine in combination by the Haefelfinger procedure. We used a higher column temperature to decrease the retention time of codeine. We have not as yet found satisfactory "internal standards" for use in these assay procedures.

EXPERIMENTAL

Apparatus. A linear programmed temperature gas chromatograph, Perkin Elmer Model 880, equipped with a flame ionization detector was used. The detector signal was supplied to a Leeds Northrum Speedomax Model G recorder with chart speed of ½ inch/min, connected to a Perkin Elmer Printing Integrator Model 194B. The chromatograph was operated with dual columns.

Materials. Nitrogen was the carrier gas. The columns were prepared with the materials and by the procedures described by Hoffman & Mitchell (1963), and with the materials and by the procedures described by Haefelfinger & others (1964).

Method for determining aspirin and phenacetin

Two (dual columns) 6 ft × ⅛ inch o.d. stainless steel tubing filled with 2% Dow

Corning Fluid No. 200 absorbed on Haloport F-80 mesh (supplied by F & M Scientific Corp., Avondale, Penn., USA). Gas pressures: air 44 lb/inch², hydrogen, optimized, nitrogen 60–65 lb/inch²; nitrogen flow: 50 ml/min; operating temperature: injection port 300°, detector 200°, column 100°–180°, programmed at 10°/in.

Procedure for sample preparation. Weigh an amount of powdered tablets to yield about 450 mg of acetylsalicylic acid and 325 mg of phenacetin. Place the powder in a 25 ml volumetric flask containing about 20 ml of chloroform AR. Loosely stopper the flask, and shake periodically while maintaining it at 50° for 15 to 20 min. Cool and dilute to volume with chloroform AR. Filter the solution, and discard the first 5 ml of filtrate. Use 2·0 μl of the clear filtrate for each assay. Determine on at least 2 injections the areas of the peaks with retention times corresponding to those shown by the standard preparation.

Procedure for standard preparation. Add 1·80 g of acetylsalicylic acid B.P. and 1·30 g of phenacetin B.P. in a 100 ml volumetric flask. Add 80 ml of chloroform AR, shake until solids dissolve and make to volume with the same solvent. Use 2·0 μl of this solution for each assay. On at least 2 assays, determine the peak areas showing retention times at 4·1 min for acetylsalicylic acid and at 9·6 min for phenacetin.

Method for determining caffeine and codeine phosphate

Two (dual columns) 6 ft × ⅛ inch o.d. stainless steel tubing filled with 10% SE-30 (Chromatographic Specialties, Brookville, Ont.) absorbed on Acid Washed Chromasorb W (F. & M. Scientific Div., Avondale, Penn.). Gas pressures: air 44 lb/inch², hydrogen, optimized, nitrogen 60–65 lb/inch²; nitrogen flow: 50 ml/min; operating temperature: injection port 300°, detector 270°, column 195° for 6 min followed by temperature programming at 48°/min to 260°.

Procedure for sample preparation. Place an amount of powdered tablets to yield about 100 mg of caffeine and 50 mg of codeine phosphate in a 50 ml stoppered centrifuge tube. Add 30 ml of 1% aqueous ammonium hydroxide, stopper the tube, shake for 30 min and centrifuge. Transfer 25·0 ml of the clear supernatant to a beaker, and bring the solution to pH 10 by the addition of concentrated ammonia. Transfer the solution quantitatively to a 125 ml separatory funnel using water to wash, and extract with 4 × 10 ml of chloroform. Combine the chloroform extracts in a small beaker. To the aqueous phase add 3·5 ml of 40% aqueous sodium hydroxide, mix and extract with 2 × 10 ml of chloroform AR. Add these chloroform extracts to those obtained previously, and evaporate the combined chloroform extracts to dryness on a steam bath. Transfer the residue with chloroform AR to a 5 ml volumetric flask, and make to volume with the same solvent. Use 2·0 μl of this solution for each assay, and determine on at least 2 assays the average of the peak areas with retention times corresponding to the standard preparation.

Procedure for standard preparation. Place 162·0 mg of anhydrous caffeine B.P. and 56·0 mg of anhydrous codeine alkaloid B.P.C. in a 10 ml volumetric flask. Dissolve the solids in chloroform AR, and make to volume with the same solvent. Use 2·0 μl of this solution for each assay, and determine on at least 2 assays the average of the peak areas showing retention times of 5 min for caffeine and 10·5 min for codeine.

Table 1. *Results from replicate determinations of standard solutions*

Ingredient	Recovery (%) Aspirin	Phenacetin	Caffeine	Codeine phosphate
High value	102·05	102·62	103·37	106·03
Low value	98·10	97·37	95·40	96·08
Average	100·10	99·99	100·18	100·92
s.d.	±1·46	±1·81	±2·44	±4·05
Number replicates	11	11	18	18

Table 2. *Results of applying the assay procedure to tablets of APC with codeine taken from the same batch*

Ingredient assay	Recovery (%) Aspirin	Phenacetin	Caffeine	Codeine phosphate
Average of 10 assays ..	98·30	100·77	100 00	102·88
High value	103·30	106·20	105·04	109·60
Low value	93·76	95·46	97·92	94·06
s.d.	±2·77	±3·26	±1·16	±4·83
B.P. or B.P.C. limit ..	95–105	95–105	93–107	92·5–107·5
Number of assays outside limits	1	1	0	2

RESULTS AND DISCUSSION

The results obtained in replicate determinations of "standard" solutions are shown in Table 1.

The results obtained by the assay procedures described above in replicate determinations on tablets of APC with codeine taken from the same batch, are shown in Table 2.

The standard deviation values for the replicate assays of the "standard" solutions and of the tablets indicate that no significant increase is contributed by the performance of the assay procedures.

The number of assay values outside the "official limits" for the individual components is considered acceptable relative to the rapidity and accuracy of the assay procedures for routine application.

The gas chromatographic procedures described have been used satisfactorily for the routine analysis of over 200 batches of commercially prepared APC and codeine tablets. An experienced technician can conveniently assay 10 samples of tablets for all active ingredients in 8 h.

REFERENCES

HAEFELFINGER, P. SCHMIDLI, B. & RITTER, M. (1964). *Arch. Pharm., Berl.*, **297**, 641–648.
HOFFMAN, A. J. & MITCHELL, H. I. (1963). *J. pharm. Sci.*, **52**, 305–306.

J. Pharm. Pharmac., 1969, **21**, 681–686 *Received May* 5, 1969

Preservation of fluorescein solutions against contamination with *Pseudomonas aeruginosa*

R. M. E. RICHARDS, P. SUWANPRAKORN, S. NEAWBANIJ AND N. SURASDIKUL

The Armed Forces Pharmaceutical Factory, Soi Kleui Nam Tai, Bangkok, Thailand

A combination of phenylmercuric nitrate and phenylethyl alcohol was more effective in preserving fluorescein solutions against contamination with *P. aeruginosa* than phenylmercuric nitrate alone. *P. aeruginosa* cells, grown in the presence of phenylethyl alcohol and then washed were rendered sensitive to chemical inactivation.

Fluorescein solutions are difficult to preserve effectively against contamination by *Pseudomonas aeruginosa* (Dale, Nook & Barbiers, 1959). Phenylmercuric nitrate (PMN) is generally acknowledged to be the most suitable preservative for these solutions and is recommended by the B.P.C. 1968. Kohn, Gershenfeld & Barr (1963) considered that the rate of kill of aqueous solutions of PMN against *P. aeruginosa* was too slow for PMN to be recommended as a preservative for ophthalmic solutions.

Disodium ethylenediamine tetraacetate (EDTA) enhances the activity of benzalkonium, chlorhexidine and polymyxin against logarithmic phase cells of *P. aeruginosa* (Brown & Richards, 1965). This effect was considered to be the result of EDTA chelating divalent cations from the bacterial cells and thus affecting the permeability properties of the cells to the chemical antibacterials under test. EDTA, however, did not enhance the activity of solutions of PMN against *P. aeruginosa* cells, rather it caused a reduction in the activity of the organic mercurial (Brown, 1968). In this situation it was thought that the EDTA chelated mercury ions and thus rendered the PMN less effective against the *P. aeruginosa* cells.

Phenylethyl alcohol (PEA) has been recommended as a preservative for use in ophthalmic solutions (Brewer, Goldstein & McLaughlin, 1953), but Kohn & others (1963) found PEA, like PMN, to have too slow an action against *P. aeruginosa* cells for use in ophthalmic solutions.

Silver & Wendt (1967) concluded that PEA exerted its antibacterial effect by modifying the permeability properties of the bacterial cell. Therefore PEA would seem to offer similar possibilities of EDTA of being used in combination with other antibacterial agents to enhance their antibacterial activity.

The purpose of this present work was to investigate the effectiveness of combinations of PEA and PMN in the preservation of fluorescein solutions against contamination with *P. aeruginosa*.

EXPERIMENTAL

The test organism used was *P. aeruginosa* strain NCTC 8203, and the growth medium for liquid cultures was Difco nutrient broth, Difco bacto-agar 2% was added to obtain the solid culture medium. Incubation was at 37°. The fluorescein sodium, PMN and EDTA were all BDH laboratory chemicals and the PEA (N.F.) was obtained from S. B. Penick & Company, New York. Cell numbers were estimated by

colony counts. The inactivating broth of Riegelman, Vaughan & Okumoto (1956) without agar, was used for the first dilution and nutrient broth was used for subsequent dilutions. The maintenance of stock cultures has already been described (Brown & Richards, 1964).

Effect of PEA *and* PMN *on survival time*

An end point experiment was made to determine the effect of PEA on the action of PMN in the preservation of fluorescein solutions against contamination with *P. aeruginosa*.

Preparation of inoculum. An overnight culture was used to inoculate nutrient broth at 37° to give a final volume of 100 ml; after incubation for 2·5 h this culture formed the source of inoculum.

Preparation of reaction mixtures. Five formulations (A–E) of fluorescein sodium 2% were prepared. Solutions A–E were preserved with PMN 0·002%; PEA 0·6%; PMN 0·002% with PEA 0·6%; PMN 0·002% with PEA 0·4% and PMN 0·002% with PEA 0·2% respectively. 9·5 ml quantities were filled into glass tubes having plastic screw caps and sterilized at 121° for 20 min. Duplicate tubes of each solution were inoculated with 0·5 ml samples of the prepared culture to give a final bacterial concentration of approximately 3×10^5 cells per ml. These inoculated solutions were designated reaction mixtures and were maintained at 21–22°.

Determination of survival time. Samples of 0·5 ml were taken from each reaction mixture after 10, 20, 30, 60 and 180 min; they were added to 9·5 ml recovery medium and incubated for 7 days. The recovery medium was that of Riegelman & others (1956) without agar. Positive controls to test the efficiency of the recovery medium consisted of 0·5 ml samples of prepared culture separately added to recovery medium

Table 1. *Survival times for* P. aeruginosa *in 2·0% fluorescein solutions*

Preservative	Replicate[a]	Contact time (min) at 21°				
		10	20	30	60	180
PMN 0·002% (A)	1	+	+	+	+	−
	2	+	+	+	+	−
PEA 0·6% (B)	1	+	+	+	+	−
	2	+	+	+	−	−
PMN 0·002% PEA 0·6% (C)	1	+	+	+	−	−
	2	+	−	−	−	−
PMN 0·002% PEA 0·4% (D)	1	+	+	−	−	−
	2	+	+	+	−	−
PMN 0·002% PEA 0·2% (E)	1	+	+	+	−	−
	2	+	+	+	+	−

Controls: Duplicate controls separately containing 0·5 ml of "C" plus 9·5 ml inactivating recovery broth inoculated to a final concentration of approximately 3×10^5 cells/ml log phase *P. aeruginosa*. All gave growth.

a, +, −, visible growth or not after 7 days incubation at 37° in inactivating recovery broth.

N.B. Inocula consisted of 0·5 ml log phase *P. aeruginosa* in nutrient broth to give a final concentration of approximately 3×10^6 cells/ml.

containing 0·5 ml of the fluorescein solution preserved with PMN 0·002% plus PEA 0·6%. The additional control procedure of Brown (1968) was also followed. Samples of all positive tubes were incubated on a milk agar medium consisting of 10% dried defatted milk (Carnation nonfat dry milk) and 2% agar to observe for pigment production and clearing of the casein. The results are in Table 1.

Effect of growth media on survival time

An end point experiment was made to determine whether *P. aeruginosa* cells grown on nutrient agar plus various additives produced cells having different resistance to fluorescein solutions preserved with either PMN 0·002% or the PMN/PEA combination.

Preparation of inoculum. An overnight culture was used to inoculate duplicate 900 ml flat culture bottles containing 200 ml solid media. There were 7 different media consisting of nutrient agar; nutrient agar plus PMN 0·001%; nutrient agar plus

Table 2. *Survival times for* P. aeruginosa *in fluorescein solutions*

Media used to obtain source of inocula[1]	Formulation[2]	Replicate[3]	Contact time (min) at 22°							
			30	60	90	120	150	180	300	24 h
Nutrient agar	A	1	+	+	+	+	+	+	+	−
		2	+	+	+	+	+	+	+	−
	D	1	+	+	+	+	+	+	−	−
		2	+	+	+	−	−	−	−	−
Nutrient agar + PMN 0·001%	A	1	+	+	+	+	+	+	+	−
		2	+	+	+	+	+	+	+	−
	D	1	+	+	+	+	−	−	−	−
		2	+	+	+	+	−	−	−	−
Nutrient agar + EDTA 0·1%	A	1	+	−	−	−	−	−	−	−
		2	+	+	+	−	−	−	−	−
	D	1	+	−	−	−	−	−	−	−
		2	+	−	−	−	−	−	−	−
Nutrient agar + calcium chloride 0·1%	A	1	+	+	+	+	+	+	−	−
		2	+	+	+	+	+	+	+	−
	D	1	+	+	+	+	−	+	−	−
		2	+	+	+	+	−	−	−	−
Nutrient agar + dextrose 1·0%	A	1	+	+	+	+	+	+	+	−
		2	+	+	+	+	+	+	−	−
	D	1	+	+	+	+	+	+	+	−
		2	+	+	+	+	+	+	−	−
Nutrient agar + PEA 0·25%	A	1	+	+	−	−	−	−	−	−
		2	+	+	+	+	−	−	−	−
	D	1	+	−	−	−	−	−	−	−
		2	+	−	−	−	−	−	−	−
Nutrient agar + 0·5% polysorbate 80	A	1	+	+	+	+	+	+	+	−
		2	+	+	+	+	+	+	−	−
	D	1	+	+	+	+	+	−	−	−
		2	+	+	+	+	−	−	−	−

Controls: Duplicate controls separately containing 1·0 ml of each of "A" and "D" plus 9·5 ml inactivating recovery broth were inoculated to a final concentration of approximately 2×10^5 cells/ml of the cells grown on nutrient agar + EDTA 0·1%. All gave growth.

[1] Inoculum gave a final concentration of approximately 2×10^6 cells/ml.

[2] Formulation "A" was fluorescein sodium 2·0% with PMN 0·002%. Formulation "D" was fluorescein sodium 2·0% with PMN 0·002% and PEA 0·4%.

[3] +, −, Visible growth or not after 7 days incubation at 37° in inactivating recovery broth.

EDTA 0·1%; nutrient agar plus anhydrous calcium chloride 0·1%; nutrient agar plus dextrose 1%; nutrient agar plus PEA 0·25% and nutrient agar plus polysorbate 80 0·5%. The inoculated media were then incubated for 48 h after which the surface growth was harvested by gently washing the surface of each culture with 10 ml sterile distilled water. The suspensions thus obtained from the duplicate bottles were bulked. The bulked suspensions were centrifuged, the supernatants removed and the cells resuspended in an equal volume of nutrient broth. A viable count was then made on each of the 7 suspensions. These suspensions formed the source of inocula and were refrigerated at 2–4° until needed.

Preparation of reaction mixtures. 9·5 ml quantities of formulations A and D were prepared as before and duplicate tubes of both solutions were inoculated with 1·0 ml of the prepared suspensions to give a final concentration of approximately 2×10^6 cells per ml. (A further count was made to check the actual inoculum in each case.) These inoculated solutions were designated reaction mixtures and were maintained at 21–22°.

Determination of survival times. Survival times were determined as previously described except that the contact times were 30, 60, 90, 120, 150, 180, 300 min and 24 h. The results are given in Table 2.

Effect of PEA *on growth rate*

Replicate inocula of overnight *P. aeruginosa* cells were used to inoculate 4 × 250 ml flasks of prewarmed nutrient broth (W–Z) to give a final volume of 100 ml. Two of the flasks (W and Y) contained plain nutrient broth and the other 2 flasks (X and Z) contained nutrient broth plus PMN 0·001%. The growth rates of cultures W–Z were followed by means of colony counts. At time 145 min after inoculation, 0·25 ml sterile distilled water prewarmed to 37° was separately added to W and X and 0·25 ml prewarmed PEA was separately added to Y and Z to give a final concentration of 0·26% PEA. The results are shown in Fig 1.

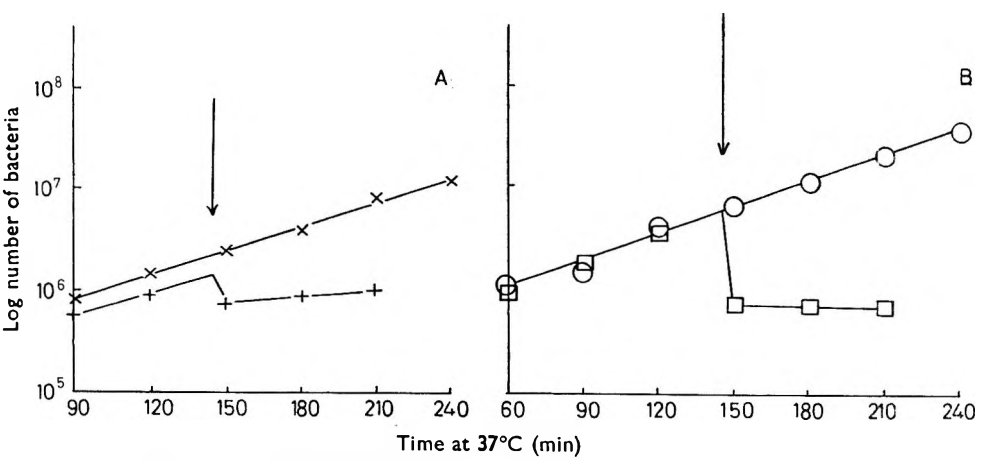


FIG. 1. The effect of PEA 0·26% against growing cultures of *P. aeruginosa* in nutrient broth alone (A) and plus PMN 0·001% (B). × Nutrient broth (W). + Nutrient broth plus PEA 0·26% at 145 min (Y). ○ Nutrient broth plus PMN 0·001% (X). □ Nutrient broth plus PMN 0·001% plus PEA 0·26% at 145 min (Z).

RESULTS AND DISCUSSION

Effect of PEA *and* PMN *on survival time*

Formations C and D both achieved sterility within 60 min of being contaminated with *P. aeruginosa* cells to a final concentration of approximately 3×10^5 cells per ml (Table 1). This was a faster sterilization time than was obtained with the official B.P.C. formulation A. The formulations containing PEA 0·6% both showed a marked precipitate after autoclaving.

Effect of growth media on survival time

From Table 2 it is seen that formulation D has a shorter sterilization time than formulation A against 6 of the 7 cell suspensions. In the seventh case, further time intervals between 3 and 24 h would be needed to determine which formulation has the more rapid action against the cells of this suspension.

These results again indicate that the formulation containing the combination of PMN 0·002% with PEA 0·4% would seem to be an improvement on the existing B.P.C. formulation which contains PMN 0·002% alone.

The cells grown on nutrient agar in the presence of EDTA 0·1% and the cells grown on nutrient agar in the presence of PEA 0·25% were both more sensitive to the action of PMN and the PMN/PEA combination than the cells grown on nutrient agar alone. EDTA is known to affect the permeability of *P. aeruginosa* cells making them more sensitive to several chemical agents (Brown & Richards, 1965). These results show that PEA also has an effect on the cell that enhances the activity of the chemical agents tested.

The lowered resistance of the cells grown on the EDTA and the PEA media is considered to result from these agents having an action on the cells during the growth period. It is not considered to be caused by chemical carried over in the inocula. The washing of the bacterial suspensions and their subsequent thousand-fold dilution, when used as inocula, was thought to ensure that any agent carried over would be present in such high dilution as to be unable to influence the results. Both EDTA and PEA have to be present in fairly high concentration to influence antibacterial action. Furthermore, Brown (1968) showed that PMN in the presence of EDTA was less effective against *P. aeruginosa* cells than PMN alone. Our own results with EDTA and PMN confirmed those of Brown.

Cells grown in the presence of polysorbate 80 or dextrose appeared to have a similar resistance to the cells grown on plain nutrient agar. Excess calcium did not seem to affect the resistance of the cells. It is concluded that the significant concentration of calcium is that concentration that is sufficient for normal cell synthesis. Concentrations higher than this do not appear to affect the resistance of the pseudomonas cells. Growth in the presence of PMN did not seem to affect the resistance of the cells to subsequent chemical inactivation.

Effect of PEA *on growth rate*

From Fig. 1A it can be seen that PEA 0·26% effected a 45% kill of *P. aeruginosa* cells growing in nutrient broth at 37°. The same concentration of PEA, however, caused an 80% kill of cells growing in nutrient broth plus PMN 0·001% (Fig 1B). It is considered that the PEA enabled the PMN, which at this concentration is not effective against the organism, to kill a significant number of cells. We believe the PEA to be

acting on the permeability of the *P. aeruginosa* cells thus enabling concentrations of PMN which could not on their own effect penetration into the cell to pass into the cell and exert an antibacterial effect. Therefore, under the conditions of this experiment, the PMN and PEA have a synergistic action against growing cells of *P. aeruginosa*. PEA therefore might be used with advantage in combination with PMN and possibly in combination with other antibacterial agents in the preservation of pharmaceuticals against contamination with *P. aeruginosa*.

It is suggested that the effectiveness of PEA, in combination with another chemical antibacterial agent, against *P. aeruginosa* may be related to the permeability of the pseudomonas cell to the agent. If the antipseudomonal activity of the agent is limited because it cannot penetrate into the pseudomonas cell then it may be expected that combining it with PEA will result in increased antipseudomonal activity. If the agent, however, is already able to penetrate the pseudomonas cell, then a combination with PEA is unlikely to increase its activity.

Acknowledgement

This work was supported in part by grants from the Thai Military Defence and Thai Military Surgeons Association.

REFERENCES

BREWER, J. H., GOLDSTEIN, S. W. & MCLAUGHLIN, C. B. (1953). *J. Am. pharm. Ass. (Sci. Edn)*, **42**, 584–585.

BROWN, M. R. W. (1968), *J. pharm. Sci.*, **57**, 389–392.

BROWN, M. R. W. & RICHARDS, R. M. E. (1964). *J. Pharm. Pharmac.*, **16**, *Suppl.* 41*T*–45*T*.

BROWN, M. R. W. & RICHARDS, R. M. E. (1965). *Nature, Lond.*, **207**, 1391–1393.

DALE, J. K. NOOK, M. A. & BARBIER, A. R. (1959). *J. Am. pharm. Ass. Pract. Pharm. Edn*, **20**, 32–35.

KOHN, S. R , GERSHENFELD, L. & BARR, M. (1963). *J. pharm. Sci.*, **52**, 967–974.

RIEGELMAN, S., VAUGHAN, D. G. & OKUMOTO, M. (1956). *J. Am. pharm. Ass.* (*Sci. Edn*), **45**, 93–98.

SILVER, S. & WENDT, L. (1967). *J. Bact.*, **93**, 560–566.

J. Pharm. Pharmac., 1969, **21**, 687

LETTERS TO THE EDITOR

The subcellular distribution of (+)-amphetamine and (±)-*p*-chloroamphetamine in the rat brain as influenced by reserpine

Amphetamine and its *p*-chloro derivative, *p*-chloroamphetamine have some common pharmacological actions. Both produce hypermotility in mice (Pfeifer, György & Fodor, 1968); *p*-chloroamphetamine is somewhat less effective than amphetamine. Amphetamine increases the basal metabolism of rats and mice and this effect has a central origin (Issekutz & Gyermek, 1949; Pfeifer, Vizi & others, 1964). *p*-Chloroamphetamine also increases metabolism in rats and mice (Pfeifer, unpublished). Amphetamine hypermotility occurs in the presence of reserpine, while that of *p*-chloroamphetamine is inhibited by reserpine (Pfeifer & others, 1968). Amphetamine slightly decreases brain noradrenaline (McLean & McCartney, 1961; Sanan & Vogt, 1962) and does not influence the brain level of 5-hydroxytryptamine (5-HT) whereas *p*-chloroamphetamine decreases brain 5-HT levels considerably without influencing the noradrenaline content of the brain (Pletscher, Bartholini & others, 1964; Fuller, Hines & Mills, 1965). It is supposed that amphetamine may act by releasing extra-granular catecholamines (Carlsson, Lindqvist & others, 1966) and in large doses it has a direct releasing effect on the amine storing granules (Lundborg, 1969).

The aim of the present work was to see if studies on subcellular distribution of amphetamine and its *p*-chloro-derivative in the brain could further elucidate their mode of action.

C.F.E. albino rats (120–150 g) were given 5 mg/kg of (+)-amphetamine sulphate together with 10 nCi[^{3}H]-(+)-amphetamine or 15 mg/kg of (±)-*p*-chloroamphetamine intraperitoneally 30 or 60 min, respectively before decapitation. Reserpine was administered $4\frac{1}{2}$ h before. Amphetamine was determined by the liquid scintillation method with a four channel Packard instrument after extraction according to Axelrod (1954). *p*-Chloroamphetamine was determined spectrophotometrically (Axelrod, 1954).

The subcellular fractions were prepared (Kataoka & De Robertis, 1967) with a MSE ultracentrifuge. Nuclear (N), crude mitochondrial (M), microsomal (Ms) and soluble (S) fractions were prepared. From the crude mitochondrial fraction three subfractions were isolated after hyposmotic shock: mitochondrial (Mi), vesicular (Ves) and the soluble exoplasm (A). The identification of the fractions was made by electron microscopy. The protein content was determined as N_2 by a Coleman N_2-analyser after precipitation with trichloroacetic acid.

The subcellular distribution of amphetamine and *p*-chloroamphetamine is very different (Fig. 1). While the greatest amount of amphetamine is in the soluble fraction of the primary fractions and in sub-mitochondrial fractions, *p*-chloroamphetamine is bound chiefly to the particulate fraction both in the primary and in the submitochondrial fractions. The *p*-chloroamphetamine content is 1·87 μg/g and the amphetamine content is 0·356 μg/g, in the vesicular fraction, while the physiological noradrenaline content is 0·034 μg/g according to De Robertis (1966). Reserpine does not influence the absolute content of amphetamine in the whole brain or in the subcellular fractions and has no effect on the subcellular distribution either. On the other hand, *p*-chloroamphetamine, given after reserpine, accumulates to a lesser degree in the whole brain, and significantly lower drug levels are found in the

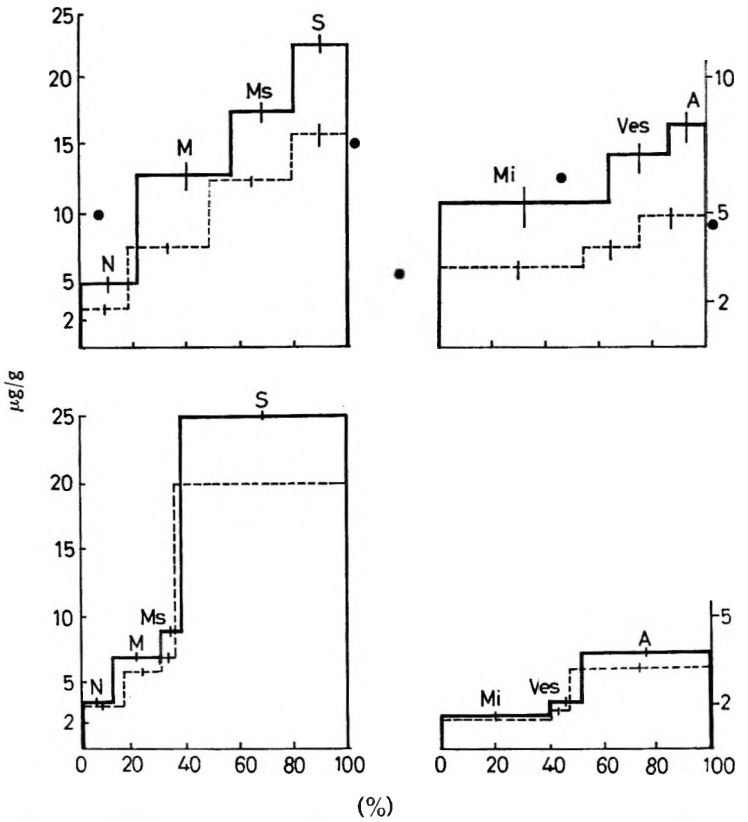


FIG. 1. Diagram showing the concentration in μg/g wet tissue and % distribution of (−)-amphetamine and (±)-*p*-chloroamphetamine in nuclear (N), crude mitochondrial (M), microsomal (Ms) and soluble (S) fractions of the primary fractions and in the submitochondrial fractions: mitochondrial (Mi), vesicular (Ves) and soluble axoplasm (A). Upper: ——— amphetamine - - - - - reserpine + amphetamine. Lower: ——— *p*-chloroamphetamine, - - - - - reserpine + *p*-chloroamphetamine. ● $P < 0{\cdot}05$.

crude mitochondrial fraction, in the mitochondrial, vesicular and soluble axoplasm fractions after hyposmotic shock. There are also alterations in the percentage distribution (Fig. 1).

Table 1 shows the relative specific concentrations (RSC) of amphetamine and *p*-chloroamphetamine in the presence and absence of reserpine treatment in the primary subfractions and in the submitochondrial fractions after hyposmotic shock. For amphetamine the RSC is low in the particulate fractions and very high in the supernatant both in the primary and in the submitochondrial fractions. Reserpine has no influence on the RSC of amphetamine. On the other hand, *p*-chloroamphetamine has a high RSC in the particulate fractions. Reserpine increases the RSC in the microsomal fraction and in the soluble axoplasm.

The results presented suggest that *p*-chloroamphetamine and to a lesser degree amphetamine can be taken up into the catecholamine or 5-HT storage sites. As the absolute quantity of the drug detected in these fractions is several times more than the amount of the endogenous catecholamine or 5-HT, it may be assumed that the capacity of storage sites is not entirely utilized in normal conditions. This idea is supported by the work of Richards & Tranzer (1969) who demonstrated by electron microscopy that the pineal gland of rat can take up so much 5-hydroxydopamine that practically all the vesicles became electron dense. Furthermore, it is also possible

Table 1. *Subcellular distribution of* (+)*-amphetamine and* (±)-p-*chloroamphetamine in rat brain. Effect of reserpine.* Relative specific concentrations = % of drug in the fraction/% of protein in the fraction

Fractions	Protein %	Relative specific concentrations			
		Amphetamine		*p*-Cl-amphetamine	
		Control	After reserpine	Control	After reserpine
Nuclear	34·8	0·41	0·48	0·58	0·52
Crude mitochondrial	35·0	0·45	0·39	1·04	0·85
Microsomal	14·6	0·50	0·40	1·50	2·17
Soluble	15·6	4·22	4·09	1·40	1·34
Submitochondrial fractions:					
Mitochondrial	79·6	0·51	0·50	0·79	0·68
Synaptic vesicle	12·6	0·85	0·60	1·83	1·65
Soluble (axoplasm)	7·7	6·31	6·81	1·91	3·27

that the excess catecholamine or 5-HT stores can take up substances without loosing their endogenous content. Perhaps that is why amphetamine depletes noradrenaline only in large (nearly toxic) doses. On the other hand, the 5-HT depleting effect of *p*-chloroamphetamine can be explained by its greater ability to be bound to the vesicular fractions.

Whether or not the difference in subcellular distribution of amphetamine and *p*-chloroamphetamine and the lack of influence of reserpine on the amphetamine and its definite influence on *p*-chloroamphetamine distribution is casual remains to be solved.

Institute for Experimental Medicine of the Hungarian Academy of Sciences, Budapest 9, *P.O.B.* 67, *Hungary*.

A. K. PFEIFER
L. CSÁKI
M. FODOR
L. GYÖRGY
I. ÖKRÖS

May 31, 1969

REFERENCES

AXELROD, J. (1954). *J. Pharmac. exp. Ther.*, **110**, 315–326.

CARLSSON, A., LINDQVIST, M., FUXE, K. & HAMBERGER, B. (1966). *J. Pharm. Pharmac.*, **18**, 128–130.

DE ROBERTIS, E. (1966). *Pharmac. Rev.*, **18**, 413–424.

FULLER, R. W., HINES, C. W. & MILLS, J. (1965). *Biochem. Pharmac.*, **14**, 483–488.

ISSEKUTZ sen., B. & GYERMEK, L. (1949). *Archs int. Pharmacodyn. Thér.*, **78**, 174–196.

KATAOKA, K. & DE ROBERTIS, E. (1967). *J. Pharmac. exp. Ther.*, **156**, 114–125.

LUNDBORG, P. (1969). *J. Pharm. Pharmac.*, **21**, 266–268.

MCLEAN, J. R. & MCCARTNEY, M. (1961). *Proc. Soc. exp. Biol. Med.*, **107**, 77–79.

PFEIFER, A. K., VIZI, E. S., SÁTORY, E. & GALAMBOS, E. (1964). *Archs int. Pharmacodyn. Thér.*, **149**, 126–135.

PFEIFER, A. K., GYÖRGY, L. & FODOR, M. (1968). *Acta med. hung.*, **25**, 441–450.

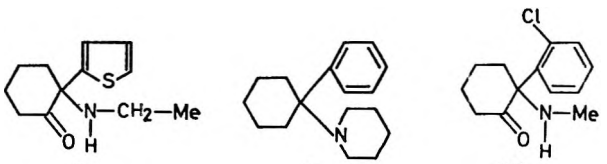
PLETSCHER, A., BARTHOLINI, G., BRUDERER, H., BURKARD, W. P. & GEY, K. F. (1964). *J. Pharmac. exp. Ther.*, **145**, 344–350.

RICHARDS, J. G. & TRANZER, J. P. (1969). *Experientia*, **25**, 53–54.

SANAN, S. & VOGT, M. (1962). *Br. J. Pharmac. Chemother.*, **18**, 109–127.

The effects of a phencyclidine derivative, CI-634, on blood pressure in rats

2-(Ethylamino)-2-(2-thienyl) cyclohexanone (CI-634, I) is a derivative of phencyclidine which has been found useful as an intravenous anaesthetic in cats (Chen & Ensor, 1968) and as an immobilizing agent in rabbits (Chen & Bohner, 1968). Phencyclidine (II) is sympathomimetic by an action on the peripheral nervous system, and a rise in blood pressure can be elicited in spinal cats and pithed rats (Ilett, Jarrott & others, 1966). Ketamine (CI-581, III) probably has no peripheral component in its action on the cardiovascular system (Dowdy & Kaga, 1968; Stanley, Hunt & others, 1968). The sympathomimetic effects of CI-634 in rats are now described. To avoid the problems associated with changes in depth of anaesthesia which occur when a drug, known itself to be an anaesthetic, is administered to an anaesthetized animal, CI-634 was injected into acute preparations of unanaesthetized rats. Experiments on urethane-anaesthetized and also in pithed rats were made to assess further the site of action of CI-634.



I CI-634 II Phencyclidine III Ketamine CI-581

Male albino rats (180–200 g) were anaesthetized with ether. The carotid artery was cannulated with a length of nylon tubing and exteriorized. The incision area was filled with gelatin sponge, to reduce bleeding, before closing the opening. The femoral vein was cannulated and the animal wrapped in a black cloth and allowed to recover in a restraining cage. Heparin (1,000 iu/kg) was given intravenously to minimize clotting and blood pressure recorded using a Statham P23AC transducer. Adrenalectomies were made in animals under ether. Rats (250–300 g) were anaesthetized with urethane (0·6 ml/100 g of 20% solution, intraperitoneally) or were pithed by passing a long steel probe through the orbit and down the spinal cord.

In conscious rats, the intravenous injection of CI-634 hydrochloride (1 mg/kg) produced a rise in blood pressure of from 22–35 mm Hg lasting from 10 to 30 min (Fig. 1). There was no tachyphylaxis of this pressor response at 45 min intervals and no potentiation of adrenaline or noradrenaline pressor responses. The pressor response to CI-634 was abolished by pretreatment with 3 mg/kg reserpine given intraperitoneally 24 h previously (Fig. 1). It was not abolished by adrenalectomy. CI-634 (10 mg/kg) produced slight anaesthesia and a depression of blood pressure followed by a slow increase in pressure. CI-634 (20 mg/kg) produced anaesthesia and depression of blood pressure.

In lightly anaesthetized rats, low doses of CI-634 (0·125 and 0·25 mg/kg) caused a biphasic pressor response. If these doses were repeated rapidly, a depressor phase appeared. This triphasic response was also characteristic of the initial response to higher doses (0·5 and 1·0 mg/kg) of CI-634. On repeated dosage with 1 mg/kg the depression of blood pressure became predominant and obscured any pressor response. All the doses of CI-634 examined had no effect on the blood pressure of pithed rats. Responses to CI-634 in anaesthetized rats were abolished by intravenous pentolinium tartrate (3 mg/kg) or hexamethonium bromide (5 mg/kg) or by reserpine-pretreatment.

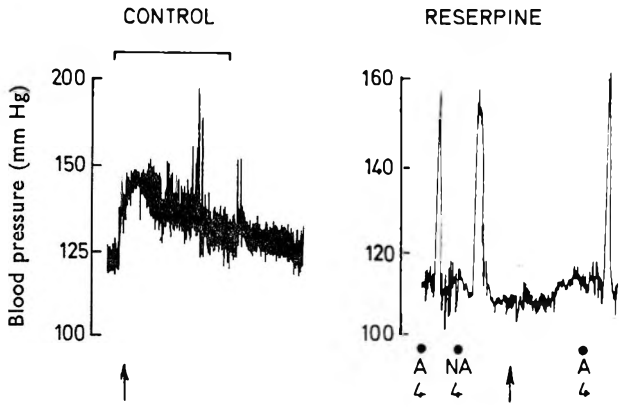


FIG. 1. Blood pressure recordings (mm Hg) from the carotid artery of two conscious rats. CI-634 (1 mg/kg) was administered intravenously at ↑ and 4μg of adrenaline at A and of noradrenaline at NA. The pressor response to CI-634 observed in the control animal was absent in the reserpine-pretreated animal. Time bracket: 10 min.

The rise in blood pressure produced by lower doses of CI-634 is not a peripheral sympathomimetic response since no response to the drug could be elicited in pithed rats. However, the observed block of the pressor response to CI-634 by ganglion blockade and reserpine would suggest involvement of the sympathetic nervous system. Depression of blood pressure produced by anaesthetic doses of CI-634 in conscious rats, and also observed as part of the response in anaesthetized rats, appears to be linked with the anaesthetic properties of the drug. At higher doses of CI-634 or after repeated administration of lower doses, the anaesthetic properties of CI-634 might be expected to summate with urethane to depress blood pressure and also mask pressor responses resulting from other mechanisms. Thus CI-634 requires an intact central nervous system to elicit effects on the blood pressure of rats and in this respect it resembles ketamine more than phencyclidine. Ketamine has been suggested to act either to depress baroreceptor reflex activity (Dowdy & Kaga, 1968) or on central cardiovascular regulatory mechanisms (Stanley, Hunt & others, 1968).

We thank Dr. B. G. Lucas, Parke-Davis and Co. Ltd., Australia, for supplying CI-634. This work was supported in part by a grant from the National Health and Medical Research Council of Australia.

Department of Physiology,
University of Queensland,
St. Lucia, 4067,
Queensland, Australia.

STELLA R. O'DONNELL
SANDRA L. WHYTE

May 24, 1969

REFERENCES

CHEN, G. & BOHNER, B. (1968). *Am. J. Vet. Res.*, **29**, 869–875.
CHEN, G. & ENSOR, C. R. (1968). *Ibid.*, **29**, 863–867.
DOWDY, E. G. & KAGA, K. (1968). *Anaesthesiology*, **29**, 931–943.
ILETT, K. F., JARROTT, B., O'DONNELL, S. R. & WANSTALL, J. C. (1966). *Br. J. Pharmac. Chemother.*, **28**, 73–83.
STANLEY, V., HUNT, T., WILLIS, K. W. & STEPHEN, C. R. (1968). *Anaesth. Analg.*, **47**, 760–768.

Treatment of adjuvant arthritis in rats with the histidine decarboxylase inhibitor hypostamine

Many reports suggest a significant role for histamine in inflammatory and allergic diseases. Again, factors such as the so-called adjuvants, can enhance hypersensitivity states and inflammatory changes in some species of animals; such a factor was also found to increase histidine decarboxylase activity (Schayer & Ganley, 1961).

The widely used model of animal pathology, resembling in some respects human rheumatoid disease, is adjuvant arthritis in rats. This is characterized by disseminated inflammatory lesions of joints and skin of the animals.

The aim of the present work was to investigate the influence of the histidine decarboxylase inhibitor, hypostamine (Trioqualine) (Parrot & Laborde, 1959) on the course of adjuvant arthritis in rats. Additional experiments were also made to test inhibitory activity of hypostamine on histidine decarboxylase in rats.

The experiments were made on 3-month old rats of either sex, of inbred August strain and of randomly bred Wistar rats. The animals were injected with lyophillized tubercle bacilli of $H_{37}Rv$ strain suspended in liquid paraffin into the plantar surface of hind paw in amount of 0·5 mg bacilli per animal. Hypostamine, in daily doses of 250 mg/kg weight, was administered, orally, beginning 8 days after adjuvant injection to the 30th day of the experiment. The animals of control groups were treated with the suspensoid, i.e. 1·25% methylcellulose. Each group of animals consisted of 8 rats.

Immediately after the injection of adjuvant, transitory swelling of the paw was observed; the secondary symptoms of polyarthritis and skin lesions appeared after a latent period of 14–25 days in control Wistar rats and after 12–20 days in both control and hypostamine-treated August rats. The intensity of the disease was then evaluated by scoring according to a conventional 18 point pattern (Giełdanowski, Pelczarska & others, 1969).

The results of 36 days observation of Wistar adjuvant rats are shown in Fig. 1.

The information obtained from experiments on August rats is not reproduced graphically because the results were almost identical in both control and hypostamine-treated animals.

To test inhibitory properties of hypostamine on histidine decarboxylase activity in rats, examinations with liver as a source of the enzyme, were made (Telford &

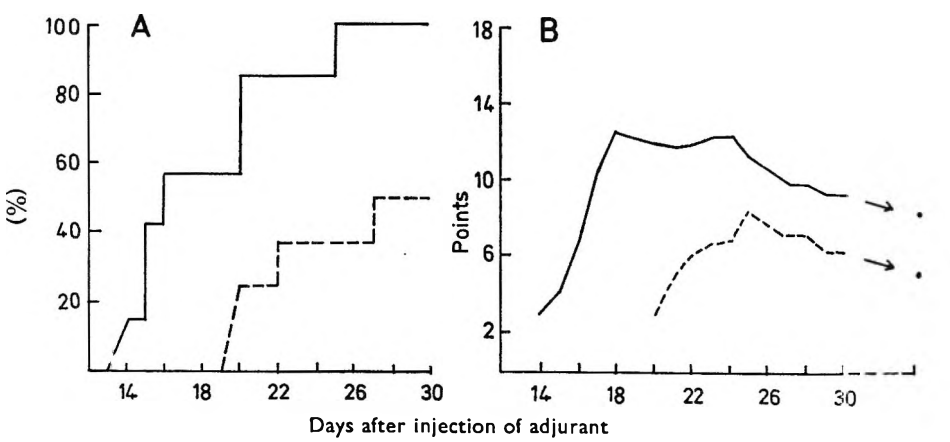


FIG. 1. A. Morbidity rate (%) in Wistar rats.

B. Intensity of symptoms. Average count of points (Giełdanowski & others, 1969) in animals affected with polyarthritis. ——— Control - - - - - Hypostamine-treated rats.

West, 1961). Food was withheld from male and female 3 month-old rats of the previously mentioned strains for 12 h. Next day one group of August rats and one group of Wistar rats were treated orally with a single dose of 250 mg/kg weight of hypostamine. The other two groups of animals were the controls. The animals were killed 3 h later and the livers were excised, cleaned, weighed, cut into small pieces and ground in a mortar with sand and Tyrode solution. The L-histidine zwitterion was prepared from commercial L-histidine monohydrochloride (Fluka) (Mackay & Shepherd, 1960).

Mixtures of supernatant of liver homogenate (800 mg/4 ml), L-histidine zwitterion (15 mg/1 ml) and phosphate buffer pH 8 (5·0 ml) with 1 drop of benzene were incubated for 3 h at 37°. The reaction was then stopped by reducing the pH of the solution to 4·0 with N HCl, and boiling for 3 min. After neutralizing the mixtures with N NaOH, their histamine content was measured on the guinea-pig ileum. Control mixtures containing no homogenate or no histidine were similarly treated and assayed.

The results are in Table 1.

As it has been shown in Fig. 1 hypostamine when administered in Wistar rats had a favourable effect upon the syndromes of adjuvant arthritis. On the contrary, in August rats no therapeutic effect of the drug was observed. These observations were supported by findings that hypostamine inhibited liver histidine decarboxylase in Wistar rats whereas this was not so with August rats.

It seems that genetic differences condition different mechanisms of action of the drug in the two strains of animal.

Table 1. *Influence of hypostamine on histidine decarboxylase activity*

Sex	Wistar					August	
	Control	Hypostamine	Inhibition (%)	t*	P*	Control	Hypostamine
Female ..	3·25 ±0·50	1·75 ±0·54	46·2	5·03	0·001	4·50 ±0·71	4·50 ±0·82
Male ..	3·25 ±0·50	2·12 ±0·25	34·8	5·65	<0·001	3·06 ±0·52	3·12 ±0·43

The histidine decarboxylase activity expressed as μg histamine formed per g tissue per 3 h.

* Data of statistical analysis according to Student's t test.

This evidence also draws attention to the question whether genetically defined, inbred strains of animals are a better choice for pharmacological investigations.

In my opinion, since inbred strains are not common under natural conditions, they are less useful for pharmacodynamic studies than randomly bred, or intentionally "not inbred" animals.

Department of Immunopharmacology,
Institute of Immunology and Experimental Therapy,
Polish Academy of Sciences,
Wroclaw, Poland.

A. PELCZARSKA

June 6, 1969

REFERENCES

GIEŁDANOWSKI, J., PELCZARSKA, A., PATKOWSKI, J., MICHALSKI, Z. & SZAGA, B. (1969). *Arch. Immunol. Ther. Exper.*, **17**, 380–392.

MACKAY, D. & SHEPHERD, D. M. (1960). *Br. J. Pharmac. Chemother.*, **15**, 552–556.

PARROT, J. L. & LABORDE, C. (1959). *J. Physiol., (Paris)*, **51**, 546–547.

SCHAYER, R. W. & GANLEY, O. (1961). *J. Allergy*, **32**, 204.

TELFORD, M. J. & WEST, G. B. (1961). *J. Pharm. Pharmac.*, **13**, 74–82.

Effect of amphetamine and amphetamine-like drugs on homovanillic acid concentration in the brain

It is generally assumed that amphetamine excitatory behaviour (hypermotility and stereotyped activity) and hyperthermia are related to an interaction with the brain catecholamines. Monoamine oxidase inhibition, release of intra- or extra-granular stored catecholamines (Hanson, 1967; Stein & Wise, 1967), blockade of the amine pump at the cell membrane (Carlsson, Lindqvist & others, 1965; Carlsson, Fuxe & others, 1966), modifications of noradrenaline and dopamine turnover (Javoy, Thierry & others, 1968) have been alternatively implicated.

We report here the effect of amphetamine on homovanillic acid (HVA) concentration in the neostriatum. In an attempt to correlate the modifications of HVA with the hyperthermic response we have compared amphetamine with two amphetamine-like drugs lacking hyperthermic activity, fenfluramine (Le Douarec, Schmitt & Laubie, 1966; Jespersen, Bonaccorsi & Garattini, 1969) and S 992 compound [trifluoromethylphenyl(benzoyloxy)ethylamino-2-propane; Laboratoires Servier, Paris, France]. As all the agents show anorexic properties, the anorexigenic activity was also measured in relation to the reported biochemical modification.

Sprague-Dawley female rats (150 $\pm$ 10 g) and female Swiss mice (20–22 g) were used. HVA was measured on pooled neostriata of 4 animals for each sample (Anden, Roos & Werdinius, 1963).

Increase in striatum HVA after (+)-amphetamine sulphate administration appears in mice and rats at relatively high dosages. These results confirm the evidence obtained by Laverty & Sharman (1965) in cats and by Pletscher (1969) in rats and are compatible with the reported increase in synthesis (Javoy & others, 1968) and turnover (Javoy & others, 1968; Costa & Groppetti, 1969) of dopamine into striatum after amphetamine, but they would not support the possibility that high doses of amphetamine may significantly block the monoamine oxidase *in vivo* (Carlsson, 1969; Glowinski, 1969).

Fig. 1 shows a correlation of neostriatum HVA concentration and hyperthermia in rats. But as it appears in Table 1, this correlation is only casual, because amphetamine elicits the usual effect on neostriatum HVA also in particular experimental conditions such as the isolation of animals and the lowering of room temperature when the hyperthermic effect was reduced. Furthermore fenfluramine and S 992 increased neostriatum HVA without affecting body temperature.

However, in the case of fenfluramine this lack of correlation of pharmacological and biochemical responses may be attributed to a possible central adrenolytic property of the compound (Gomulka & Bonaccorsi, unpublished data) masking the typical hyperthermic activity of amphetamine-like drugs. It is interesting in this respect that fenfluramine is able to elicit, in given experimental conditions, an anti-amphetamine activity (Bizzi, Bonaccorsi & others, 1969).

Other adrenolytic drugs such as chlorpromazine (Da Prada & Pletscher, 1966a,b) or haloperidol (Pletscher & Da Prada, 1967), showing hypothermic activity, also induce an important increase in HVA brain concentration following an increased turnover of dopamine. This effect was suggested to be related to a feed-back mechanism that enhances the dopamine synthesis as a result of a block of dopaminergic receptors evoked by chlorpromazine (Nybäck, Sedvall & Kopin, 1967; Gey & Pletscher, 1968; Da Prada & Pletscher, 1966a,b). A similar effect might explain the increase in neostriatum HVA induced by fenfluramine. However, an effect of fenfluramine on the removal of HVA from brain cannot be excluded.

As far as correlations of anorexic activity and HVA are concerned, the observation that the anorexic effect of amphetamine and fenfluramine occurs at doses which do

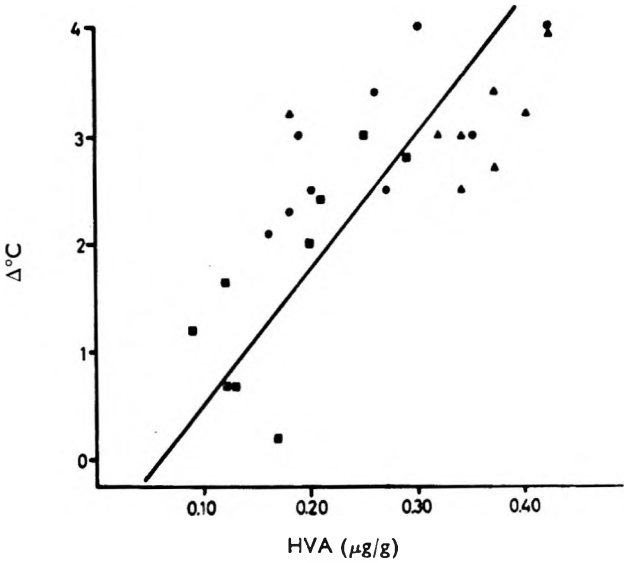


FIG. 1. Correlation between increase of body temperature and neostriatum HVA 1 h after 7·5 (■), 15 (●) or 30 (▲) mg/kg, i.p. of (+)-amphetamine sulphate to rats. Each point represents a pool of 4 animals.

not elicit an increase in neostriatum HVA is relevant (Bizzi, Bonaccorsi & others, 1969). Accordingly data reported in Table 2 indicate that after repeated treatments with fenfluramine, tolerance to anorexia develops without a corresponding modification of the effect on HVA in the neostriatum. On the contrary, repeated treatments with amphetamine, do not modify the effect on food intake, at least in the 2 h after the last injection, but completely remove the effect on HVA.

Table 1. *Effect of (+)-amphetamine sulphate and amphetamine-like drugs on body temperature and brain HVA concentration in rats and mice*

Determinations	Species	Treatment (mg/kg, i.p.)		Body temperature (° C ± s.e.)	Striatum HVA (μg/g ± s.e.)
11	Mouse	Saline		37·0 ± 0·2	0·13 ± 0·01
14	,,	Amphetamine	7·5	38·9 ± 0·4*	0·26 ± 0·01*
6	,,	Fenfluramine	15	36·7 ± 0·2	0·26 ± 0·04*
5	,,	S 992	15	37·0 ± 0·2	0·20 ± 0·03*
13	Rat	Saline		36·7 ± 0·1	0·14 ± 0·01
9	,,	Amphetamine	7·5	38·1 ± 0·3*	0·18 ± 0·02
9	,,	,,	15	39·6 ± 0·3*	0·26 ± 0·03*
8	,,	,,	30	41·0 ± 0·2*	0·36 ± 0·01*
4	,,	,, †	15	38·1 ± 0·6	0·26 ± 0·01*
4	,,	,, ‡	15	37·2 ± 0·5	0·30 ± 0·04*
7	,,	Fenfluramine	15	38·0 ± 0·1	0·28 ± 0·01*
6	,,	,,	30	37·2 ± 0·5	0·34 ± 0·03*
9	,,	S 992	15	36·9 ± 0·1	0·28 ± 0·01*
5	,,	,,	30	36·7 ± 0·3	0·36 ± 0·03*

Drugs were given 1 h before determinations.
Experiments were usually made at a room temperature of 22°.
Animals were grouped (4 rats and 6 mice per cage).
Each determination is the mean of 4 animals.
* $P < 0{\cdot}01$ versus saline.
† Animals were isolated.
‡ Room temperature 18°.

Table 2. *Effect of (+)-amphetamine sulphate and (±)-fenfluramine chloride on food intake and neostriatum HVA concentration*

Determinations	Treatment (mg/kg i.p.)		Food intake g/2 h per rat ± s.e.	Neostriatum HVA (μg/g ± s.e.)
7	Saline		16·8 ± 0·9	0·15 ± 0·01
2	Amphetamine	15	0·5	0·37
2	Fenfluramine	15	1	0·32
7	*Amphetamine	15	0	0·09 ± 0·02‡
4	*Fenfluramine	15	10·2 ± 1·4†	0·38 ± 0·02†

* Rats receiving (±)-fenfluramine chloride or (+)-amphetamine sulphate (5 mg/kg, i.p.) daily for 4 days.

24 h after the last treatment, animals were injected with 15 mg/kg of drugs.

Experiments were made with 40 h fasted rats.

Food intake and HVA concentration were measured 2 h after the last treatment.

Each determination is the mean of 4 animals.

† $P < 0.01$ versus saline.

‡ $P < 0.05$ versus saline.

In conclusion these experiments suggest that hyperthermic and anorexic activity of amphetamine as well as the anorexigenic effect of fenfluramine are not related to the alterations of dopamine metabolism in neostriatum or, at least, that HVA neostriatum concentration is not a suitable parameter to evaluate central biochemical mechanisms responsible for these pharmacological actions.

The technical assistance of Mr. Luciano Guarnieri was particularly appreciated.

The useful advice of Drs. Bartholini and Da Prada to carry out the method for the dosage of HVA is gratefully acknowledged.

Istituto di Richerche Farmacologiche "Mario Negri," A. JORI
Via Eritrea, 62, 20157 *Milan, Italy.* D. BERNARDI

June 19, 1969

REFERENCES

ANDEN, N.-E., ROOS, B. E. & WERDINIUS, B. (1963). *Life Sci.*, **2**, 448–458.

BIZZI, A., BONACCORSI, A., JESPERSEN, S., JORI, A. & GARATTINI, S. (1969). *Amphetamine and related compounds*, Proc. International Symposium, Milan, 1969, in press. New York: Raven Press.

CARLSSON, A. (1969). In *Amphetamine and related compounds*, Proc. International Symposium, Milan, 1969, in press. New York: Raven Press.

CARLSSON, A., FUXE, K., HAMBERGER, B. & LINDQVIST, M. (1966). *Acta physiol. scand.*, **67**, 481–497.

CARLSSON, A., LINDQVIST, M., DAHLSTRÖM, A., FUXE, K., MASUOKA, D. (1965). *J. Pharm. Pharmac.*, **17**, 521–524.

COSTA, E. & GROPPETTI, A. (1969). In *Amphetamine and related compounds*, Proc. International Symposium, Milan, 1969, in press. New York: Raven Press.

DA PRADA, M. & PLETSCHER, A. (1966a). *Experientia*, **22**, 465–466.

DA PRADA, M. & PLETSCHER, A. (1966b). *J. Pharm. Pharmac.*, **18**, 628–630.

GEY, K. F. & PLETSCHER, A. (1968). *Experientia*, **24**, 335–336.

GLOWINSKI, J. (1969). In *Amphetamine and related compounds*, Proc. International Symposium, Milan, 1969, in press. New York: Raven Press.

HANSON, L. C. F. (1967). *Psychopharmacologia*, **10**, 289–297.

JAVOY, F., THIERRY, A. M., KETY, S. S. & GLOWINSKI, J. (1968). *Communs. Behav. Biol.*, Part A, **1**, 43–48.

JESPERSEN, S., BONACCORSI, A. & GARATTINI, S. (1969). *J. Pharm. Pharmac.*, in press.

LAVERTY, R. & SHARMAN, D. F. (1965). *Br. J. Pharmac. Chemother.*, **24**, 759–772.

LE DOUAREC, J. C., SCHMITT, H. & LAUBIE, M. (1966). *Archs int. Pharmacodyn. Thér.*, **161**, 206–232.

NYBÄCK, H., SEDVALL, G. & KOPIN, I. J. (1967). *Life Sci.*, **6**, 2307–2312.

PLETSCHER, A. (1969). In *Amphetamine and related compounds*, Proc. International Symposium, Milan, 1968, in press. New York: Raven Press.

PLETSCHER, A. & DA PRADA, M. (1967). In *Neuropsycho-pharmacology*, Proc. Vth International C.I.N.P. Congress, pp. 304–311. Amsterdam: Excerpta Medica Foundation.

STEIN, L. & WISE, G. D. (1967). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **26**, 651.

The effects of α- and β-sympathicomimetics on rumen motility and heart rate frequency in conscious goats

In the ruminal smooth muscle preparation *in vitro* there exist α-stimulatory and β-inhibitory adrenergic receptors. The effect of adrenaline, either contraction or relaxation, is the result of interactions with both types of receptors (van Miert & Huisman, 1968). It is of interest to note that Titchen & Newhook (1968), who made their experiments with anaesthetized vagotomized sheep and lambs or with vagotomized decerebrate preparations of lambs, reported similar adrenergic mechanisms near the reticulo-omasal orifice.

Adrenaline is known to cause a single slow contraction of reticulum, rumen and abomasum in unanaesthetized vagotomized sheep (Habel, 1956). This is also the case in the anaesthetized goat with intact vagi. However, normal cyclical movements of the reticulo-rumen cease after the vagus nerves have been cut or after induction of anaesthesia. An intravenous injection of adrenaline in the conscious ruminant always gives an inhibition of the regular contractions of the rumen, although it is not known whether α- or β-adrenergic receptors, or both, are involved in this phenomenon. I now report the effects of sympathicomimetics activating α- or β-adrenergic receptors.

Materials and methods. An open-ended water filled polyethylene tube was passed into the rumen intra-nasally, and the other end connected to a pressure transducer. An electrically driven slow-infusion pump was also connected to prevent occlusion of the tube with food particles. The volume of fluid administered was 1 ml/min. Pressure records made in this way show the well-known regular contractions of the rumen occurring with a frequency of about 1/min. The frequency and the amplitude

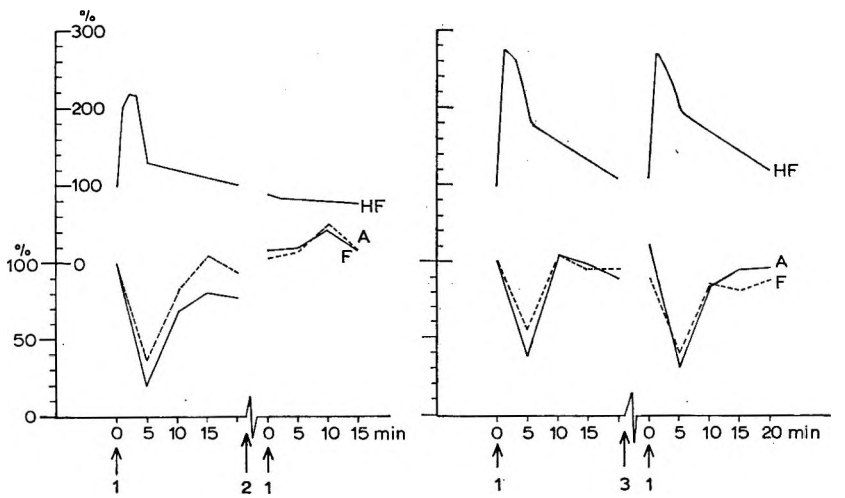


FIG. 1. Mean change in rumen motility for 3 goats to isoprenaline 2·5 μg/kg i.v. (= 1) before and after propranolol 0·5 mg/kg i.v. (= 2) or dibenamine 2·5 mg/kg i.v. (= 3) respectively. A—Summation during 5 min intervals of amplitude, expressed as percentage of the initial value. F—Frequency/5 min expressed as percentage of the initial value. HF—Change in heart rate.

of the contractions were measured every 5 min, and expressed as percent of the initial value. Heart rate was taken from an electrocardiogram using a Elema-Schönander Mingograph. The drugs were dissolved in saline and given as an intravenous injection or by a continuous slow infusion.

For specific α-receptor stimulation phenylephrine 2·5 μg/kg min^{-1} and oxymetazoline 0·2 μg/kg min^{-1} were used; for specific β-stimulation isoprenaline 0·1 μg/kg min^{-1} or 2·5 μg/kg, adrenaline 1 μg/kg min^{-1} or 7·5, 10 or 20 μg/kg, dibenamine 15 μg/kg min^{-1} or 2·5 mg/kg, propranolol 0·5 and 1 mg/kg or 10 μg/kg min^{-1}, Du 21445 [1-isopropyl-amino-3-(2-methylthiophenoxy)-propan-2-ol], also a strong β-blocking agent 10 μg/kg min^{-1} or 1 mg/kg, were also used.

Results and discussion. After an intravenous injection or infusion, during 15 min of isoprenaline, the heart rate is suddenly increased, while rumen motility is depressed. Both effects were completely blocked by propranolol or Du 21445 but not by dibenamine (Fig. 1). Therefore the inhibitory response to isoprenaline must involve activation of β-receptors.

Infusions of oxymetazoline or phenylephrine were accompanied by bradycardia and a reduction of the amplitude and frequency of rumen contractions. After pretreatment with dibenamine—an injection or infusion during 90 min—the bradycardia caused by these α-sympathicomimetics is less and the inhibition of rumen

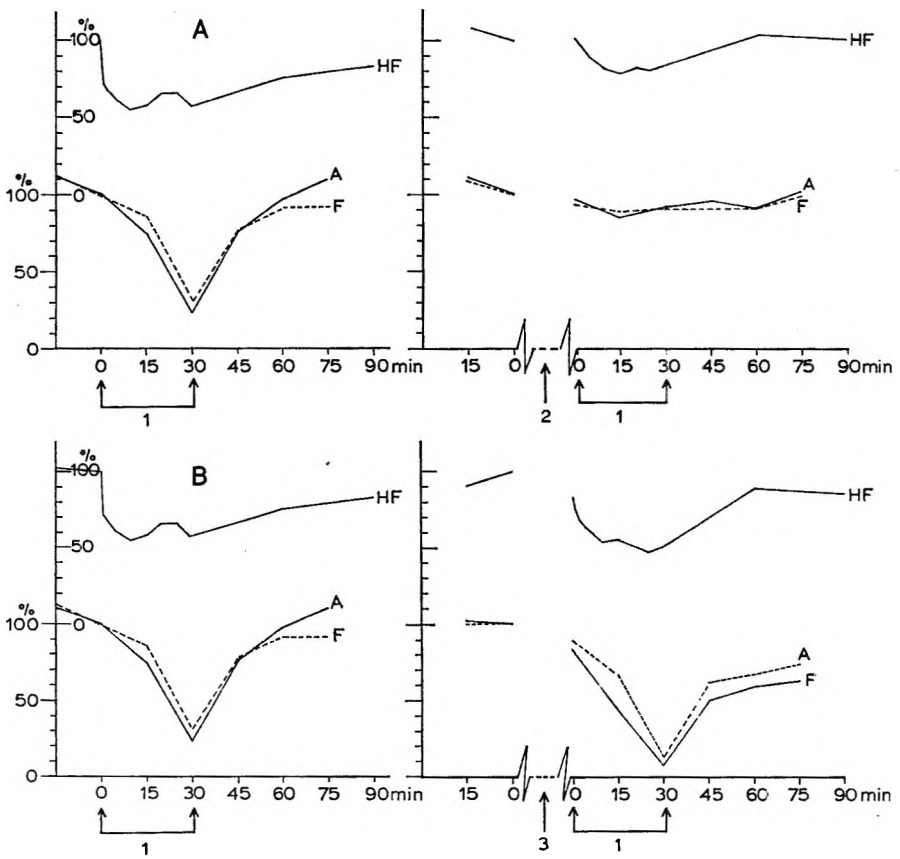


FIG. 2. Mean changes in rumen motility and heart frequency for 3 goats to phenylephrine 2·5 μg/kg min^{-1} (= 1). A. Before and after an infusion of dibenamine 15 μg/kg min^{-1} during 90 min (= 2). B. Before and after an infusion of propranolol 10 μg/kg/min during 90 min (= 3).

contractions is blocked. On the other hand, propranolol has no influence on the inhibitory response to phenylephrine (Fig. 2). Therefore this inhibitory response to phenylephrine must involve activation of α-receptors. The bradycardia caused by phenylephrine could also be prevented by a prior intravenous injection of atropine 0·15 mg/kg. Therefore it seems to be probable that the bradycardia induced by phenylephrine or oxymetazoline is reflex in origin, due to the pressor response of these α-sympathicomimetics, which causes stimulation of the baroreceptors in the carotid sinus and aortic arch with consequent vagal slowing of the heart.

Before propranolol or Du 21445, an injection of adrenaline, 20 μg/kg, caused tachycardia, but after β-blockade the heart rate was depressed, probably attributable to a greater vasoconstrictor response (α-receptor stimulation). There was however no change of the inhibitory response of rumen motility to adrenaline. A low dose of adrenaline as injection, 7·5 μg/kg, or infusion caused bradycardia; β-blocking agents had no influence on this effect. After α-receptor blockade by dibenamine, as an injection or infusion during 120 min, the inhibition of the rumen contractions by adrenaline, 10 or 20 μg/kg, is less, while tachycardia is antagonized. The antagonistic effect of a combination of dibenamine and propranolol in relation to the effect of adrenaline on rumen motility is no greater than with dibenamine alone. An analysis of the cardiac responses and the effects on rumen motility to phenylephrine and isoprenaline after pretreatment with dibenamine or propranolol, indicates that the dosage of both blocking agents was sufficient to produce significant blockade of α- and β-receptors in the cardiovascular system and reticulo-ruminal wall. Adrenaline activates both α- and β-receptors in the ruminal smooth muscle strip. However, *in situ* only dibenamine was able to antagonize the adrenaline effect; this was not so with β-blockers, although the doses of both blocking agents were adequate. On the basis of *in vitro* observations it has been concluded that in the ruminal wall both α-stimulatory and β-inhibitory adrenergic receptors are present (van Miert & Huisman, 1968). *In situ*, α- and β-sympathicomimetics both interfere with the normal rhythmic reticulo-ruminal contractions. The effect of adrenaline seems to be primarily affected by interaction with α-adrenergic receptors. What exactly happens *in situ*, when α- or β-adrenergic receptors in the ruminal wall are stimulated, is not known. Increased tonic activity of the reticulo rumen after stimulation of α-adrenergic receptors, followed by reflex inhibition of the normal cyclical movements, is only one possibility. However, with the method I have used it is not possible to check this hypothesis.

I am grateful to ICI Ltd., N.V. Phillips Duphar and Merck Darmstadt A.G. for gifts of propranolol, Du 21445 and oxymetazoline respectively.

I wish to thank Miss C. Th. M. van Duin for technical assistance.

Institute of Veterinary Pharmacology and Toxicology, University of Utrecht, Utrecht, The Netherlands.

A. S. J. P. A. M. VAN MIERT

June 23, 1969

REFERENCES

HABEL, R. E. (1956). *Cornell Vet.*, **46**, 555–628.

MIERT, A. S. J. P. A. M. van & HUISMAN, E. A. (1968). *J. Pharm. Pharmac.*, **20**, 495–496.

TITCHEN, D. A. & NEWHOOK, J. C. (1968). *Ibid.*, **20**, 947–948.

A smooth muscle-stimulating substance in bovine plasma

We have investigated the properties of smooth muscle-stimulating substances present in plasma of man, guinea-pig, chicken, pig, ox and rat. Plasma (3 ml) obtained by centrifuging heparinized blood at 4,000 *g* for 15 min at 0° was transferred to a Sephadex G 25 column (diameter 2 cm, length 100 cm) which was then eluted with a solution of sodium chloride (0·1M, pH 6·7, temperature 4°) passing at a rate of 30 ml/h. The eluate was collected in 5 ml samples and tested on the rat isolated uterus (30°, de Jalon fluid) and the isolated guinea-pig ileum (37°, Tyrode solution) using bradykinin and histamine respectively as standard spasmogens. Only those samples which contained protein (detected by gel electrophoresis) were active on both preparations and showed dose-response relations. Therefore, the spasmogens are probably not compounds of low molecular weight like kinins. Furthermore, their activities were not modified by antihistamine or anti-5-hydroxytryptamine drugs or by atropine, and incubation with chymotrypsin also did not reduce activity.

The plasma proteins of ox were then fractionated by Cohn's method and freeze-dried. Fraction IV-1 with more than 50% α_2-globulin contained nearly all the active material and this fraction potentiated both the action of bradykinin on the uterus and that of histamine in the ileum. Fraction IV-1 was then transferred to a Sephadex G 200 column (diameter 2 cm, length 30 cm) which was eluted with Tris-buffer (pH 8·05) passing at a rate of 18 ml/h. Samples of eluate (3 ml) were collected and tested for protein by measuring light absorption at 280 nm and by studying gel electrophoresis. Three peaks of absorbancy were identified but only the first (contained in samples 6–14 and therefore in 18–42 ml of eluate) possessed the smooth muscle-stimulating activity on both the uterus and the ileum. This contained most of the α_2-globulin (over 85%) and had a carbohydrate moiety of about 8% and a molecular weight about 800,000. The second peak (samples 15–18 or 45–54 ml of eluate) consisted mostly of β-globulin and γ-globulin whilst the third (samples 19–24 or 57–72 ml of eluate) was predominantly albumin (see Fig. 1).

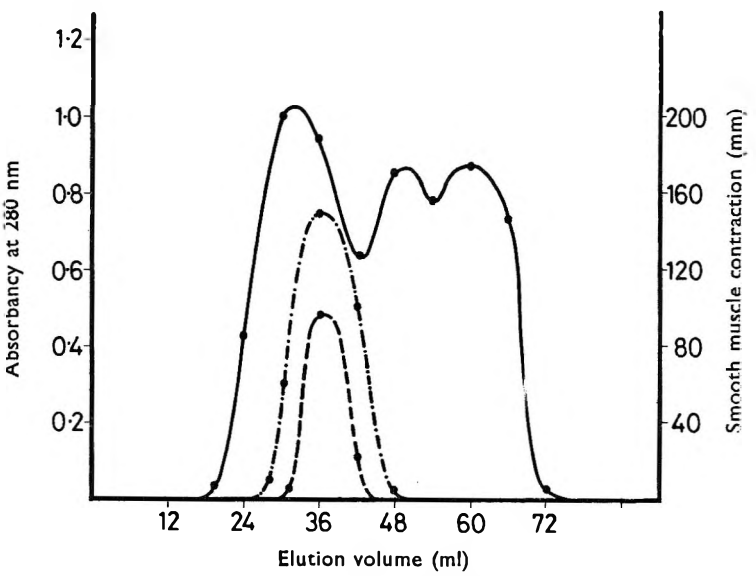


FIG. 1. Protein content (absorbancy at 280 nm) and biological activity of bovine plasma Cohn fraction IV-1 after elution from Sephadex G 200. ●——● protein content; ●— · —● activity on rat uterus; ● — — ● activity on guinea-pig ileum. Note that there are 3 peaks of protein but only the first possesses activity on both preparations.

Since the plasma level of α_2-globulin in man is often raised in conditions of inflammation or stress, this finding may be of pathological importance.

Institute of Pathophysiology and Department of Hygiene, University of Szeged School of Medicine, Szeged, Hungary.

A. GECSE
S. KARADY
A. LOZSA
E. ZSILINSZKY

British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, England.

G. B. WEST

June 18, 1969

Gastrointestinal absorption of two polymorphic forms of aspirin

Two forms of aspirin have been prepared and characterized (Tawashi, 1968). Form II dissolved half as fast again as form I from the planar surface of compressed tablets. Methods based on solubility-temperature dependence (Nogami, Nagai & others, 1969) failed to establish a thermodynamic difference between the two forms, apparently because of the thermodynamic instability of form II and its rapid reversion to form I in solution. Reversion to form I takes place within minutes with ultrasonic energy.

The thermodynamic relation between the two forms was studied by differential thermal analysis (DTA) and thermal gravimetric analysis (TGA). The analyses were made on 10 mg samples, in a dynamic flow of Argon, at 4°/min with alumina as a reference material in a Mettler recording vacuum thermoanalyser. Conditions of particle size, packing of the sample and rate of heating were examined. Fig. 1A shows the temperature curve, DTA and TGA diagrams for forms I and II. The differences in thermal behaviour and mass effects were clearly observed in both forms. From the area of the endothermic peak (of the DTA curves) the heat of fusion was measured, after calibrating the instrument with a material of a known heat of fusion (Barshad, 1952; Garn, 1965). Comparing the endothermic peak areas of both forms, with that of benzoic acid (10 mg), analysed under the same conditions, form I gave a heat of fusion of 29·1 cal/g and form II gave 36·9 cal/g.

Therefore, it was of interest to determine the rate of gastrointestinal absorption of the two different forms in normal human subjects, by measuring the serum salicylate concentration after the oral ingestion of 600 mg of aspirin. After an overnight fast, each subject was given form II crystals dispersed in 50 ml water (room temperature) followed by another 50 ml of water used to wash the containing vessel. The time between the addition of the aspirin to water and the administration was 3 min. Blood samples were taken 10, 20, 30, 45 and 60 min after the oral ingestion, allowed to clot, and the serum separated by centrifugation. The total salicylate was determined by the method of Trinder (1962). Form I was given after 1 week to the same subjects under the same conditions, and with both forms of about the same particle size. Each point on the salicylate concentration-time curve (Fig. 1B) represents the average of 6 determinations within $\pm$4·1 as standard error. The data obtained are in agreement with the previous dissolution rate studies of the two forms, and with differences obtained in the thermal analysis.

In this study Form II increased the salicylate concentration 70% above the value obtained by Form I for the same period of time.

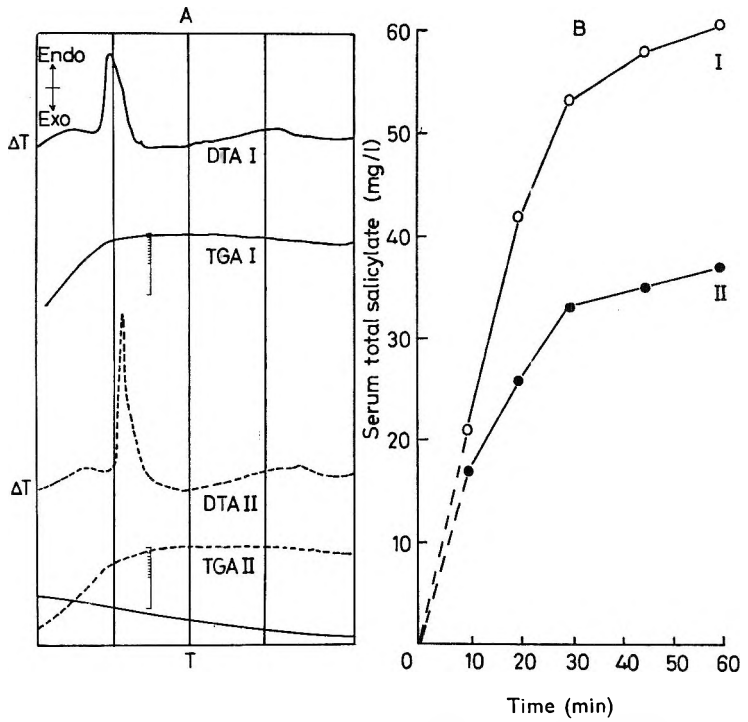


FIG. 1. A. Simultaneous differential thermal analysis and thermal gravimetric analysis of aspirin: forms I and II.

B. Serum total salicylate concentration after the oral ingestion of aspirin forms I and II.

The blood levels given by Form I are similar to that after aspirin (640 mg) tablet reported by Leonard (1963). Form II gave a blood level value higher than other investigated aspirin preparations including a solution of sodium acetylsalicylate.

Faculty of Pharmacy,
University of Montreal,
Quebec, Canada.

R. TAWASHI

May 23, 1969

REFERENCES

BARSHAD, I. (1952). *Am. Mineral.*, **37**, 667–694.

GARN, P. D. (1965). *Thermoanalytical methods of investigation.* New York: Academic Press.

LEONARD, J. R. (1963). *Clin. Pharmac. exp. Ther.*, **4**, 478.

NOGAMI, H., NAGAI, T., FUKUOKA, E., & YOTSUYANAGI, T. (1969). *Chem. Pharm. Bull. (Japan)*, **17**, 23–24.

TAWASHI, R. (1968). *Science, N.Y.*, **76**, 160.

TRINDER, P. (1962). *Biochem. J.*, **57**, 301–303.

Hypoxia and methionine sulphoximine seizures in mice

Acute exposure to hypoxia imparts protection against drug-induced convulsions (Baumel, Schatz & others, 1969a,b). If this is due to the elevated brain γ-aminobutyric acid observed in hypoxic animals (Wood, Watson & Ducker, 1968), drugs which cause convulsions by impairing γ-aminobutyric acid synthesis should become less toxic under hypoxia. Methionine sulphoximine inhibits synthesis of glutamine (Lamar & Sellinger, 1965), a precursor of brain γ-aminobutyric acid (Roberts & Frankel, 1951), and causes severe convulsions (Johnson, Goldring & O'Leary, 1965; Proler & Kellaway, 1962). We now report that acute hypobaric hypoxia antagonizes seizures produced by methionine sulphoximine.

Swiss albino, random-bred male mice (Charles River Farms), 30–35 g were housed at 21–23° with room lights alternating on a 12 h light-dark cycle. The hypobaric chambers (Baumel, Robinson & Blatt, 1967) were plexiglass desiccators (internal diameter 10 in, height 14 in) connected, in parallel, to a manifold which exhausted room air.

Drug solutions were freshly prepared immediately before intraperitoneal injection. The animals were injected and immediately placed, in pairs, in the 4 chambers which were then decompressed in 10 min to 364 mm Hg (10% O_2) or 12 min to 280 mm Hg (7·5% O_2).

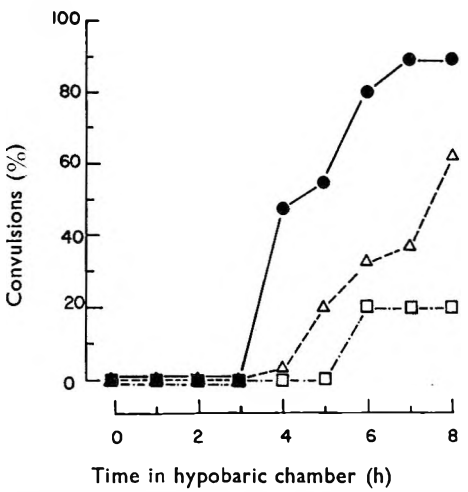


FIG. 1. Effect of hypobaric hypoxia on methionine sulphoximine (200 mg/kg intraperitoneally) convulsions. (—) Sea level, (- - -) hypobaric, ● 760 mm Hg, ▲ 364 mm Hg, ■ 280 mm Hg. Open symbols denote significant difference (P <0·005) from sea level.

Hypobaric hypoxia protected against methionine sulphoximine convulsions throughout the exposure period (Fig. 1). The anticonvulsant effect at 280 mm Hg was greater than at 364 mm Hg (8h convulsions, P <0·02). This resembles the dependence of brain γ-aminobutyric acid elevation under reduced oxygen tension on the degree of hypoxia employed (Wood & others, 1968).

Our data, along with previous findings with semicarbazide (Baumel, Schatz & others, 1969a, b), suggest that acute hypoxia prevents convulsions which are caused by the impairment of γ-aminobutyric acid synthesis.

This work was supported by the training grant PHS-1TO1ES-00104 from the Division of Environmental Health Sciences at the Institute of Environmental Biology.

Department of Pharmacology,
University of Rhode Island,
Kingston, Rhode Island 02881, *U.S.A.*
June 10, 1969

IRWIN BAUMEL
ROBERT SCHATZ
JOHN J. DEFEO
HARBANS LAL

REFERENCES

BAUMEL, I. P., ROBINSON, S. M. & BLATT, W. F. (1967). *J. pharm. Sci.*, **56**, 918–919.
BAUMEL, I. P., SCHATZ, R., DEFEO, J. J. & LAL, H. (1969a). *J. Pharm. Pharmac.*, **21**, 119–120.
BAUMEL, I., SCHATZ, R., DEFEO, J. J. & LAL, H. (1969b). *Ibid.*, **21**, 475–476.
JOHNSON, W. L., GOLDRING, S. & O'LEARY, J. L. (1965). *Clin. Neurophysiol.*, **18**, 229–238.
LAMAR, C., JR. & SELLINGER, O. Z. (1965). *Biochem. Pharmac.*, **14**, 489–506.
PROLER, M. L. & KELLAWAY, P. (1962). *Epilepsia*, **3**, 117–130.
ROBERTS, E. & FRANKEL, S. (1951). *J. biol. Chem.*, **190**, 505–512.
WOOD, J. D., WATSON, W. J. & DUCKER, A. J. (1968). *J. Neurochem.*, **15**, 603–608.

The nature of the products from the reaction between Mayer's reagent and tertiary amines

Mayer's reagent (an aqueous solution of potassium tetra-iodomercurate) gives precipitates with alkaloids and synthetic tertiary amines. This reaction forms the basis of a qualitative test for such compounds. Szász (1965; 1966) has reported, from a study of seventeen tertiary amines, that each amine is capable of forming two compounds with potassium iodomercurate, the nature of the compound formed being dependent upon the concentration ratio of the reactants. An excess of potassium tetra-iodomercurate yields coloured (yellow or brown) products, whereas when the amine is in excess the resultant compound is white or only faintly coloured.

Because the "amine-Mayer's reagent" products are formed only in acidic medium, it is reasonable to assume that they are simple salts with a protonated amine cation and either a tri-iodomercurate or tetra-iodomercurate anion. Microanalytical data support the stoichiometry of this proposal (Szász & Buda, 1969). The reactions may, therefore, be expressed by the following equations:

$$(BH)^+ + (HgI_4)^{--} \rightarrow (BH)^+(HgI_3)^- + I^-$$

and

$$2(BH)^+ + (HgI_4)^{--} \rightarrow (BH)_2{}^{++}(HgI_4)^{--}$$

To confirm the salt-like character of the "amine-Mayer's reagent" products, a proton magnetic resonance (pmr) investigation of two representative tertiary amines, codeine and *NN*-dimethylaniline and their iodomercurates was undertaken (Table 1).

The pmr spectrum of dimethylaniline in acetone-D_6 shows a normal $-N-(CH_3)_2$ singlet at 7·20 τ and a complex AA'BB'C aromatic pattern centred on 3·08 τ. This spectrum differs only marginally from that recorded in deuterochloroform (Ma & Warnoff, 1965; Anderson & Silverstein, 1965; Thompson, Warren & others, 1966). Protonation of the nitrogen, in the hydrochloride salt, results in the expected downfield shift of the *N*-methyl absorption to 6·59 τ and of the aromatic multiplet to 2·05 τ. The overall appearance of the aromatic multiplet is little changed. Dimethylaniline tri-iodomercurate and dimethylaniline tetra-iodomercurate exhibit their *N*-methyl signals at 6·36 and 6·42 τ respectively and both aromatic multiplets centre on 2·24 τ. Although these values correspond well with those of the simple hydrochloride and hydroiodide salts, the appearance of the aromatic multiplet is much changed in the iodomercurates.

Table 1. *Pmr data on dimethylaniline, codeine and their derivatives.* Pmr spectra (60 MHz) were determined in the solvent indicated with chemical shifts reported in ppm (τ) downfield from tetramethylsilane as an internal reference (Perkin-Elmer Model R-12).

Compound	>N—CH_3			—O—CH_3			Aromatic *H*		
	Acetone-D_6	DMSO-D_6	$CDCl_3$	Acetone-D_6	DMSO-D_6	$CDCl_3$	Acetone-D_6	DMSO-D_6	$CDCl_3$
Dimethylaniline ..	7·20s	—	7·18s	—	—	—	3·08m	—	3·02m
Dimethylaniline hydrochloride*	6·59s	—	—	—	—	—	2·05m	—	—
Dimethylaniline hydroiodide ..	6·67s	—	—	—	—	—	2·10m	—	—
Dimethylaniline triiodomercurate	6·36s	—	—	—	—	—	2·24m	—	—
Dimethylaniline tetraiodo-mercurate ..	6·42s	—	—	—	—	—	2·24m	—	—
Codeine	7·60s	7·72s	7·54s	6·17s	6·30s	6·12s	3·37q	3·47q	3·17q
Codeine hydro-chloride* ..	6·82s	7·05s	—	6·12s	6·20s	—	3·17q	3·28q	—
Codeine triiodo-mercurate ..	6·61s	—	—	6·11s	—	—	3·16q	—	—
Codeine tetraiodo-mercurate† ..	—	7·01s	—	—	6·22s	—	—	3·29q	—

s = singlet; q = quartet; m = multiplet.

* Hydrochloride salts were prepared *in situ* by passing dry hydrogen chloride through a solution of the base.

† Codeine tetra-iodomercurate is only sparingly soluble in acetone.

Similarly, codeine tri-iodomercurate and codeine tetra-iodomercurate exhibit *N*-methyl signals at values close to that of codeine hydrochloride. No significant variation in chemical shift or spectrum appearance was observed in either the aromatic AB quartet or the aromatic *O*-methyl signal of codeine and its derivatives.

These observations clearly indicate that the precipitates formed from the interaction of tertiary amines and potassium tetra-iodomercurate are tri- and tetra-iodomercurate salts. The change in the form of the aromatic pattern with the dimethylaniline iodomercurates may be attributed to the close proximity of the aromatic protons to the bulky mercury-complex anion.

G.Sz. wishes to thank Professor J. B. Stenlake for the use of laboratories and facilities of the Department of Pharmaceutical Chemistry, University of Strathclyde.

Part of this work was done during a WHO fellowship.

Institute of Pharmaceutical Chemistry, G. SZÁSZ
Medical University of Budapest,
Budapest, Hungary.

Department of Pharmaceutical Chemistry, R. T. PARFITT
University of Strathclyde,
*Glasgow, C.*1, *Scotland.*

June 19, 1969

REFERENCES

ANDERSON, W. R. & SILVERSTEIN, R. M. (1965). *Analyt. Chem.*, **37**, 1417–1418.
MA, J. C. N. & WARNOFF, E. W. (1965). *Can. J. Chem.*, **43**, 1849–1869.
SZÁSZ, G. (1965). *Acta pharm. hung.*, **35**, 256–258.
SZÁSZ, G. (1966). *Ibid.*, **36**, 120–125.
SZÁSZ, G. & BUDA, L. (1969). *Ibid.*, in the press.
THOMPSON, W. E., WARREN, R. J., ZAREMBO, J. E. & EISDORFER, I. B. (1966). *J. pharm. Sci.*, **55**, 110–111.

Comparative spasmolytic potencies of atropine sulphate and *N*-butyl hyoscine bromide following intravenous injection, injection into a mesenteric vein, and intraduodenal instillation

It is generally accepted that drugs can be absorbed from the gastrointestinal tract only if they are unionized and sufficiently lipid-soluble (Travell, 1938, 1940; Schanker & Hogben, 1957; Schanker, Shore & others, 1957; Travell, 1960; Schanker, 1962). If this were true for all drugs, it would be anticipated that atropine sulphate would not be absorbed because it is completely ionized up to a pH of 7·5 and is poorly lipid-soluble (only 0·25% w/v in chloroform). These considerations should also apply to *N*-butyl hyoscine bromide, which, being a quaternary base, is also completely ionized, although it is somewhat more lipid-soluble (approx. 10% w/v in chloroform). However, with atropine sulphate, it is well known that dryness of the mouth occurs after small doses given orally, the oral effective dose being only 5 times higher than the corresponding subcutaneous dose (Unna, Glaser & others, 1950).

These discrepancies between prediction and clinical observation prompted us to investigate the spasmolytic potency of atropine sulphate after intravenous and intraduodenal administration and, to exclude problems of absorption and to evaluate the role played by the liver, also after injection into a mesenteric vein. The spasmolytic activity of *N*-butyl hyoscine bromide was evaluated under identical conditions.

Female mongrel dogs (32) were premedicated with 1 mg/kg morphine sulphate subcutaneously and anaesthetized with chloralose-urethane (80 mg/kg chloralose: 400 mg/kg urethane) intravenously. Pressure in the urinary bladder was measured with a Statham pressure transducer by means of a catheter in the urethra. The nerves in the ligamentae vesicae urinariae were stimulated with a square wave of duration 1ms and of pulse rate 50/s using a Grass stimulator. This stimulation was repeated every 5 min. Contractions of the urinary bladder, blood pressure, and heart rate were recorded on a Grass Polygraph. Atropine sulphate and *N*-butyl hyoscine bromide were given (*a*) intravenously, (*b*) intravenously into a mesenteric vein, and (*c*) intraduodenally.

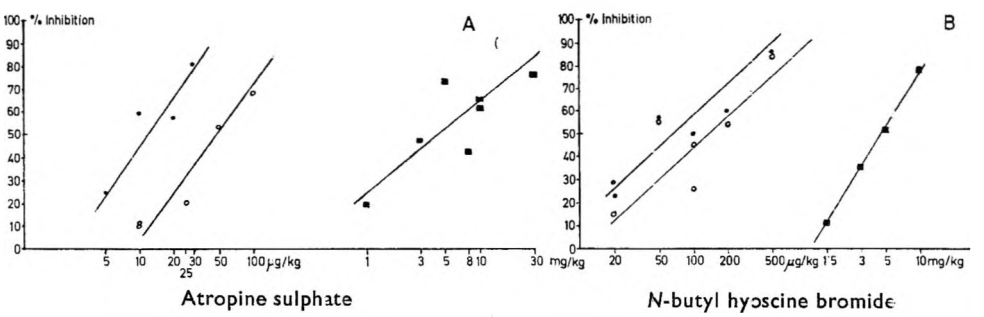


FIG. 1.A. Inhibitory effect of atropine sulphate on electrically stimulated urinary bladder contractions in dogs. Only the effect of the first dose of atropine sulphate given in each experiment is included in this diagram. B. Inhibitory effect of *N*-butyl hyoscine bromide on electrically stimulated urinary bladder contractions in dogs. Only the effect of the first dose of *N*-butyl hyoscine bromide given in each experiment is included in this diagram. —●—●— intravenous injection v. femoralis; —○—○— intravenous injection into a mesenteric vein; —■—■— intraduodenal instillation.

The results are presented in two graphs plotted on a semilogarithmic scale (Fig. 1A and B). Each point of the dose-response curves represents one experiment with a dog, and only the first dose given is included in the diagrams. By interpolation, the doses of atropine sulphate and of *N*-butyl hyoscine bromide which diminished the contractions by 50% were determined, the results are summarized in Table 1.

Table 1. *Inhibitory effect of atropine sulphate and* N-*butyl hyoscine bromide on electrically stimulated urinary bladder contractions in dogs*

Compound	ED 50 intravenously	ED 50 intravenously into a mesenteric vein	ED 50 intraduodenally	Ratio of equi-effective doses
Atropine sulphate ..	0·01 mg/kg	0·05 mg/kg	4·5 mg/kg	1:5:450
N-Butyl hyoscine bromide	0·07 mg/kg	0·14 mg/kg	4·7 mg/kg	1:2:67

With both drugs, a large difference was found between the doses required to produce an equivalent spasmolytic effect after intravenous and intraduodenal administration. One explanation for this quantitative difference could be that in the case of atropine sulphate only 0·25% is absorbed from the gastrointestinal tract and with *N*-butyl hyoscine bromide, only 1·5% of the oral dose is absorbed. Alternatively, both drugs may be well absorbed after intraduodenal administration and the difference in potency could be due to inactivation by the liver, either by storage, metabolism or excretion into the bile.

Comparison of the doses required for an equivalent spasmolytic effect after intravenous administration into the femoral or mesenteric veins has distinguished between these two possibilities. Atropine sulphate retained 20% and *N*-butyl hyoscine bromide retained 50% of its activity after circulation through the liver, indicating that low activity after intraduodenal administration is due to poor enteric absorption of the two drugs. However, this conclusion is surprising in view of the known therapeutic efficacy of oral atropine sulphate and the recently reported efficacy of *N*-butyl hyoscine bromide after oral administration (Schmid, Bleichert & others, 1968).

One could be tempted to conclude that there exists a species difference between man and dog in enteral absorption, the dog absorbing the two drugs much less than the man. On the other hand, the ratio of equi-effective doses after intravenous and oral administration varies according to the test organ chosen for comparison. *N*-Butyl hyoscine bromide is more effective in dogs after intrajejunal instillation when the spontaneous contractions of the duodenum are used as a parameter for efficacy; in these experiments the ratio of the intravenous dose to the dose administered into the jejunum is in the order of 1 to 20 (Bauer, Gross & others, 1968) compared with 1 to 70 reported here. It is concluded that additional pharmacological data are required before precise conclusions can be drawn about the enteral absorption rates of the two drugs.

Our thanks are due to Mr. H. Arslan for his skilful technical assistance.

Department of Pharmacology,
C. H. Boehringer Sohn,
6507 *Ingelheim/Rhine*,
Germany.

R. BAUER
H. WICK

May 30, 1969

REFERENCES

BAUER, R., GROSS, E., SCARSELLI, V., STOCKHAUS, K. & WICK, H. (1968). *Arzneimit.-Forsch.*, **18**, 1132–1137.

SCHANKER, L. S. & HOGBEN, C. M. A. (1957). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **16**, 334.

SCHANKER, L. S., SHORE, P. A., BRODIE, B. B. & HOGBEN, C. M. A. (1957). *J. Pharmac. exp. Ther.*, **120**, 528–539.

SCHANKER, L. S. (1962). *Pharmac. Rev.*, **14**, 501–530.

SCHMID, E., BLEICHERT, A., ÜBERLA, K., RITTER, U. & FEHLHABER, E. (1968). *Arzneimit.-Forsch.*, **18**, 1449–1453.

TRAVELL, J. (1938). *J. Pharmac. exp. Ther.*, **63**, 37.

TRAVELL, J. (1940). *Ibid.*, **69**, 21–33.

TRAVELL, J. (1960). *Ann. N.Y. Acad. Sci.* **90**, 13–30.

UNNA, K. R., GLASER, K., LIPTON, E. & PATTERSON, P. R. (1950). *Pediatrics*, **6**, 197–207.

Some effects of resibufogenin: an aglycone of animal origin

Current textbooks of pharmacology still carry references to the importance of a hydroxyl group at C-14 in cardioactive glycosides such as digitalis (Goodman & Gilman, 1965; Bowman, Rand & West, 1968). This may be because the glycosides and aglycones which have received most attention have been those of plant origin.

The aglycones of animal origin are less thoroughly documented in Western pharmacology although they figure prominently in traditional Eastern medicine. One such biologically active compound, resibufogenin, can be obtained from the skin gland venom (Ch'an Su) of the Chinese toad, and has been widely used in clinical medicine in Japan (Kawashima & Furuhashi, 1963; Iwatsuki, Yusa & others, 1965). In structure, it resembles digitoxigenin but has an epoxide link between C-14 and C-15 with an unsaturated 6-membered lactone at C-17 (Meyer, 1952; Theissen, 1958). Unlike digitalis, resibufogenin has a respiratory stimulant action (Drs. M. F. Tyrell & J. M. Leigh, personal communication). We now report cardiotonic activity of resibufogenin in hypotensive dogs made hypotensive (50–70 mm Hg) in two ways.

Anaesthesia was induced with sodium thiopentone, 15 mg/kg, followed by intubation of the trachea. Maintenance was by nitrous oxide - oxygen (2 : 1) with supplementation by halothane (0·5–1·5%). Mean arterial blood pressure was measured intravascularly with a strain gauge transducer; pulse rate was counted from an electrocardiograph trace; and the cardiac output was determined by the dye dilution technique using indo-cyanine green.

Two groups of 7 dogs were used. The 7 normotensive dogs of Group A were given resibufogenin, allowed to recover and then became Group B animals. Group B were rendered hypotensive by bleeding 20–30% of estimated blood volume and Group C were made hypotensive by pentolinium tartrate. Resibufogenin in 50% propylene glycol was given intravenously at a rate of 0·017 mg/kg min^{-1}, the total dose being between 0·5 and 6·0 mg/animal.

In Group A animals, resibufogenin, like digitalis, had no apparent cardiac action, but unlike digitalis, it did have a respiratory stimulating action. Both hypotensive groups, i.e. Group B with reflexes intact and Group C with autonomic ganglionic traffic modified, showed significant rises in mean systemic arterial pressure following the administration of resibufogenin due to increases in cardiac output without a significant change in pulse rate, i.e. a largely inotropic action. The peak rise in arterial pressure was seen at 1 min and lasted between 3 and 5 min. The respiratory effects considerably out-lasted the cardiovascular effects.

It is of interest that cardiotonic activity has been demonstrated despite the absence of the C-14 hydroxyl group. It may be that the stereo-configuration of the C and D ring is of more importance to cardiotonic activity than possession of the hydroxyl

group at C-14 although epimerization of this latter group was previously considered to destroy such activity.

*Departments of Anaesthetics and Clinical Measurement, Westminster Medical School, Westminster Hospital, London, S.W.*1, *England.* J. M. LEIGH

Pharmax Ltd., Thames Road, Crayford, Kent, England. A. D. S. CALDWELL

July 15, 1969

REFERENCES

BOWMAN, W. C., RAND, M. J. & WEST, G. B. (1968). *Textbook of Pharmacology*, p. 798. Oxford: Blackwell.

GOODMAN, L. S. & GILMAN, A. (1965). *The Pharmacological Basis of Therapeutics.* 3rd Edn, p. 668. New York: Macmillan.

IWATSUKI, J., YUSA, T., KATOAKA, Y. & SATO, K. (1965). *Tohoku J. exp. med.*, **86**, 93–97.

KAWASHIMA, K. & FURUHASHI, M. (1963). *Jap. J. Anaesth.*, **12**, 472–473.

MEYER, K. (1952). *Helv. chim. Acta*, **35**, 2444.

THEISSEN, W. F. (1958). *Chemy Ind.*, 440–441.

Cobalt ion action on the vascular permeability and mast cells of the rat

The action of cobalt ion has been reported to be similar to that of histamine (Niebroj, 1958). Recent evidence suggested that only the second phase of cobalt-induced diphasic change in the vascular permeability of the guinea-pig skin was accompanied by local necrosis (Steele & Wilhelm, 1967). The present report supplies additional evidence on the mechanism of action of the metal on the vascular permeability and on the mast cells.

Male Wistar rats anaesthetized with urethane (600 mg/kg intraperitoneally) were used. $CoCl_2.6H_2O$ (AR) and histamine phosphate solutions were made up with saline (0·85%). The water used for solutions was previously deionized. Intradermal injections of 0·005, 0·01 and 0·02 M of cobalt chloride (pH 6·0) and 4·0 and 8·0 μg of histamine were made and control intradermal injections with 0·1 ml saline. The conventional technique for study of the vascular permeability using a 1% azovan (Merck) blue solution was used (Rocha e Silva & Dragstedt, 1941; Miles & Miles, 1952). In some of the animals promethazine hydrochloride (1 mg/kg) was previously injected intravenously. The cobalt ion effect on the morphology of the mast cells of the mesentery was assessed using a technique described to us by I. Mota (unpublished). This procedure allows microscopic observation of the mast cells of the mesentery *in situ* fixed and stained. 1 ml of a 0·02 M cobalt chloride solution was intraperitoneally injected. After 20, 40, 60 and 80 min, and under light ether anaesthesia, 10 ml of a 0·5% acetic acid, 10% formalin and 0·5% toluidine blue solution was intraperitoneally injected. After 2 h, small fragments of the mesentery were collected, washed in distilled water, attached to a slide and dried at ambiental temperature.

At the concentrations used, cobalt ion induced an immediate increase in the vascular permeability of the rat skin similar to that provoked by histamine. The strongest colour intensity provoked by the metal was always weaker than that elicited by histamine. Promethazine hydrochloride completely prevented the effect of histamine but not that of the cation. The microscopic examination of the mesentery showed that the morphological aspects of the mast cells were not altered by the cation.

Three alternative hypotheses may be formulated to explain the cobalt effect on the vascular permeability: the first, that the effect arose from the hydrogen ion concentration of the unbuffered solution of the salt; the second, an indirect mode of action mediated through the local liberation of histamine; and the third, a direct action of the metal. The first hypothesis seems unlikely since it was demonstrated that pH influences vascular permeability only when it is markedly acid or alkaline (Opie, 1963). Again, the effect does not seem to be mediated through a local histamine liberation, since the action was not inhibited when an antihistamine agent was previously injected and there was demonstrably a lack of action of the metal on the mast cells of the mesentery. The third, and as yet unexplained effect of cobalt in increasing vascular permeability by a direct action of the metal, must now be elucidated.

Department of Pathology
and Department of Pharmacology,
Faculty of Veterinary Medicine,
University of São Paulo,
São Paulo, Brazil.

M. MARIANO
B. MARIA DE LOURDES
S. DE MORAES
J. PALERMO NETO

June 3, 1969

REFERENCES

OPIE, E. L. (1963). *J. exp. Med.*, **117**, 425–449.
MILES, A. A. & MILES, E. M. (1952). *J. Physiol., Lond.*, **118**, 228–257.
NIEBROJ, T. (1958). *Nature, Lond.*, **181**, 991.
ROCHA E SILVA, M. & DRAGSTEDT, C. A. (1941). *J. Pharmac. exp. Ther.*, **73**, 405–411.
STEELE, R. H. & WILHELM, D. L. (1967). *Br. J. exp. Path.*, **48**, 592–607.

Dissolution characteristics of reserpine-polyvinylpyrrolidone co-precipitates

This is a report of a preliminary investigation made to ascertain the dissolution characteristics of a relatively water-insoluble drug reserpine in the form of a co-precipitate or solid dispersion with polyvinylpyrrolidone (PVP). The use of solid dispersions or co-precipitates to facilitate dissolution has been previously reported (Sekiguchi & Obi, 1961; Gibaldi; Feldman & Bates, 1968 & workers there cited; Decato, Malone & others, 1969; Stoll, Bates Nieforth & Swarbrick, to be published).

Reserpine–PVP solid dispersions, in ratios of 1:3 and 1:6, were prepared by dissolving both components in reagent grade chloroform and subsequently removing the solvent by vacuum evaporation. The co-precipitates were dried *in vacuo* to constant weight, screened through standard mesh screens and the 40 to 50-mesh (297–420 μm) fraction collected for use in the dissolution rate studies. Pure reserpine (6–30μm crystals) and a 1:3 reserpine–PVP physical mixture (6–30 μm crystals used) were also subjected to dissolution rate testing.

The dissolution apparatus consisted of a 500 ml three neck round bottom flask containing 350 ml of a 0·005M acetic acid solution (pH 3·65) maintained at 37° ± 0·1°. The solution was agitated at 60 rev/min by a Teflon stir blade of 70mm diam. connected to a Servodyne-constant torque motor assembly. At time zero, a quantity of reserpine equivalent to 10 mg was introduced into the medium. Periodically 5 ml samples were removed from the flask, subjected to Millipore filtration (0·45 μm pore size) and assayed for drug content using a Beckman DB-G recording spectrophotometer. Reserpine in acetic acid obeys Beer's law at a wavelength of 268 nm. Following the removal of each sample, a 5 ml quantity of fresh dissolution medium was pipetted

into the dissolution flask. The amount of drug in solution at any time during the dissolution run was corrected for this dilution effect (Bates, Gibaldi & Kanig, 1966). PVP in the concentrations present in the assay solutions did not interfere with the determination of reserpine.

A weak acetic acid solution was selected as the dissolution medium because of the extremely low aqueous solubility of reserpine.

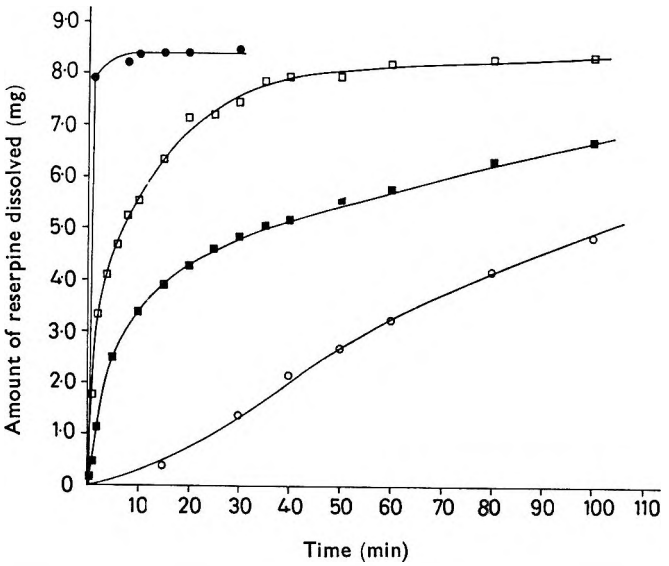


FIG. 1. Dissolution rates of reserpine and reserpine : PVP co-precipitates at 37°. ○ pure reserpine. ■ 1 : 3 physical mixture. □ 1 : 3 co-ppt. ● 1 : 6 co-ppt.

The dissolution behaviour of the four test samples at 37° is shown in Fig. 1. Each curve is drawn through points which represent an average of at least three dissolution runs. It is apparent from an examination of this figure and the dissolution half-lives (reserpine 106 min, 1 : 3 reserpine–PVP mix 35 min, 1 : 3 co-precipitate 7 min, 1 : 6 co-precipitate 0·5 min) that the dissolution rates of the samples decrease in the following order: 1 : 6 co-ppt > 1 : 3 co-ppt > 1 : 3 physical mixture > 1 : 0 pure reserpine. The dissolution half-life data indicate an approximately 15-fold increase in the dissolution rate of reserpine from the 1 : 3 reserpine–PVP co-precipitate and a 200-fold increase for the 1 : 6 co-precipitate over that for the pure reserpine sample. These differences would have been greater had the dissolution rates of the co-precipitates been compared with that for 297–420 μm reserpine particles. However, the 6–30 μm particles were used due to the extremely slow dissolution rate of the former. The marked enhancement in the dissolution characteristics of the co-precipitates most probably reflects a significant reduction in the particle size of reserpine during the preparation of the samples. The reduced particle size and the concomitant increase in the surface area of reserpine exposed to the dissolution medium appears to be the major factor responsible for the observed potentiation. That the dissolution rate of reserpine is particle size dependent is illustrated in Fig. 2 for 6–30 and 297–420 μm reserpine particles. It can be seen from these curves that the finer particles display a significantly greater rate of solution than do the coarser reserpine particles.

Although PVP possesses the ability to form water-soluble complexes with a variety of drugs (Kuramoto & Higuchi, 1954), only about a 20% increase in the equilibrium

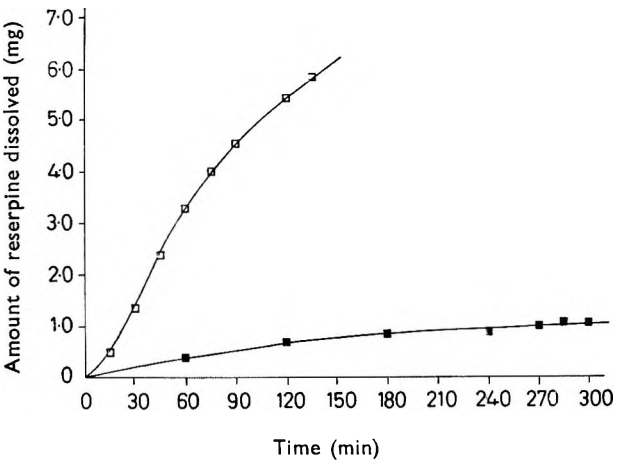


FIG. 2. Effect of particle size on the dissolution rate of reserpine at 37°. □ 6–30 μm particles. ■ 297–420 μm particles.

solubility of reserpine was noted at 37° over a PVP concentration range of 0–1%. This observation, coupled with the fact that the amount of PVP used would yield a maximum concentration of 0·014%, precludes it from functioning to increase the bulk solubility of reserpine and thereby potentiating the dissolution properties of this pharmaceutical. There is, however, a possibility that some complexation could occur in the micro-environment (diffusion layer) immediately surrounding the dissolving solid particles. This mechanism would help to explain the three-fold increase in the dissolution rate of the 1 : 3 reserpine–PVP physical mixture over that for pure reserpine, even though the particle size of the drug in both preparations is comparable (6–30 μm). Nevertheless, it appears that only a change in the physical state of reserpine (i.e., *via* particle size reduction) could account for the five fold increase in the dissolution characteristics of the 1 : 3 co-precipitate compared with the physical mixture of similar composition.

The ability of the water-soluble polymer, PVP, to enhance the *in vitro* dissolution properties of reserpine when the two substances are intimately combined in the form of a co-precipitate or solid dispersion is of importance as it relates to the gastrointestinal absorption of this hydrophobic drug.

The author would like to thank Mr. Richard Kahrimanis and Mr. Thomas Lysz for their expert technical assistance and the CIBA Pharmaceutical Company for their generous supply of reserpine.

Division of Pharmaceutics,
School of Pharmacy,
University of Connecticut,
Storrs, Connecticut 06268, *U.S.A.*

THEODORE R. BATES

June 3, 1969

REFERENCES

BATES, T. R., GIBALDI, M. & KANIG, J. L. (1966). *Nature, Lond.*, **210**, 1331–1333.
DECATO, L., MALONE, M., STOLL, R. & NIEFORTH, K. (1969). *J. Pharm. Sci.*, **58**, in the press.
GIBALDI, M., FELDMAN, S. & BATES, T. R. (1968). *Ibid.*, **57**, 708–709 and refs cited.
KURAMOTO, R. & HIGUCHI, T. (1954). *J. Am. Pharm. Ass., Sci. Edn*, **43**, 393–397.
SEKIGUCHI, K. & OBI, N. (1961) *Chem. pharm. Bull., Tokyo*, **9**, 866–872.

Journal of Pharmacy and Pharmacology

Volume 21 Number 10 October 1969

Original Papers

Letters to the Editor