Journal of Pharmacy and Pharmacology

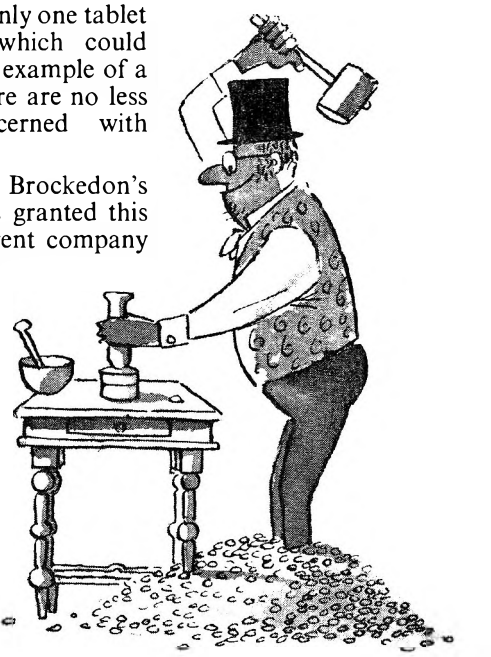
The Pharmaceutical Society of Great Britain

Volume 21 Number 5 May 1969

'You don't take a hammer to a Rotapress'

In 1843 William Brockedon, a North Country Chemist, was granted a patent for the novel process of "Shaping Pills, Lozenge and Black Lead by pressure in Dies." At the time his pre-occupation was the manufacture of pencils and it is thought that he applied the pressure by means of a hammer. No doubt Mr. Brockedon would be not a little startled if he could see the far-reaching consequences both social and scientific that his ingenious idea has unleashed to-day.

In the B.P. of 1898 there was only one tablet formula Tabellae Trinitrini, which could hardly be described as a typical example of a tablet. In the current B.P. there are no less than 227 monographs concerned with Compressed Tablets.



Some 113 years after William Brockedon's application, another patent was granted this time to John Holroyd, the parent company of Manesty Machines. This patent related to the invention of a machine for the production of coated tablets by Compression, an equally novel idea. The machine in question was, in fact, the famous DryCota, which is now accepted the World over for the production not only of coated tablets but more often for complex Pharmaceutical formulations by the Compression method.

MANESTY have been involved in many patents both before and since 1956. It is this ability to harness ingenuity in design to engineering excellence which keeps the Company in the van of World tablet machine manufacturers.

Apart altogether from machines with specialized functions such as the DryCota, Bicota, AutoCota, Layerpress and Accela Cota, MANESTY have a constantly developing line of modern high speed rotaries. The new Mark II Rotapress and the Betapress are its present leaders.

Wherever tablets are made it is an odds-on chance that you will find machines manufactured by MANESTY—in operation and not a hammer in sight!

MANESTY The name behind the greatest names in tablets.
Manesty Machines Ltd, Speke, Liverpool L24 9LQ.
Tel: 051-486 1972

MANESTY

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 5 **May 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**, 273–276 *Received October* 10, 1968

The rate of dissolution of powdered griseofulvin at different stirring rates

P. H. ELWORTHY AND F. J. LIPSCOMB

Department of Pharmaceutical Technology, University of Strathclyde, Glasgow, C.1., Scotland

The non-ionic surfactant, cetomacrogol, increased the rate of solution of powdered griseofulvin in water. At a stirring rate of 200 rev/min, the increase was similar to that found previously for solid discs of this drug. At 60 rev/min, the surfactant had a smaller effect on the rate of solution.

Previously, Elworthy & Lipscomb (1968c) have shown that non-ionic detergents and a polyoxyethylene glycol increased the rate of dissolution of griseofulvin. The results were analysed in terms of a zero order rate constant (k_1) for transfer of the drug from the crystal to the bulk of the solution, and a first order constant (k_2) for the reverse process. The presence of surfactants increased k_1 and decreased k_2, compared with the dissolution in pure water, measurements being made on a disc of griseofulvin. In the present paper the effect of stirring rate and cetomacrogol concentration are extended to a study of dissolution from the powdered drug.

EXPERIMENTAL

Materials

Griseofulvin, crystallized from ethanol, had the same analytical data as reported by Elworthy & Lipscomb (1968a). It was ground in a mortar, passed through a BS44 mesh sieve and had fines removed on a BS350 mesh sieve. Particle size analysis by microscopy gave a mean volume diameter of 104 μm. The same sample of cetomacrogol was used (Elworthy & Lipscomb, 1968b). A sample of fine particle griseofulvin was used as supplied by Glaxo Laboratories Ltd.

Method

Water or solution (1 litre) was placed in a 1 litre conical flask fitted with a 2 bladed stirrer (blade length 3·5 cm), placed 8 cm above the bottom of the flask. The stirrer was driven by a variable speed motor. A magnetic stirrer was not used in case attrition of drug particles took place. Griseofulvin (100 mg) was introduced into the solution, the stirrer started, and 3 ml samples removed through a pipette fitted with a No. 3 sintered glass filter. The samples were analysed spectrophotometrically as before (Elworthy & Lipscomb, 1968a). Incomplete wetting of the sample using pure water caused difficulties. The method adopted was to place 100 mg of the drug in a thin-walled glass bulb, which was then evacuated at 0·01 mm Hg for 10–15 min, sealed under vacuum, and broken beneath the water surface. Fine particle griseofulvin could not be readily wetted, even when washed with solvents, dried, and degassed as above.

Treatment of Results

Each experiment was duplicated, and the extinction-time curves treated as before (Elworthy & Lipscomb, 1968c) to evaluate k_1 and k_2.

Experiments were continued until approximately 2 mg of griseofulvin had dissolved. Calculations show that there is little change of surface area during this time, and it seemed reasonable, as the smallest particles exceeded 45 μm to assume that the number of particles present was constant during a run. Hence to a reasonable approximation the surface area is constant, so that the analysis previously described was applied.

A further difficulty was experienced with experiments in which the stirring rate was between 40–80 rev/min. The griseofulvin settled to the bottom of the flask in a heap. The type of extinction-time graph obtained in these experiments is shown in Fig. 1.

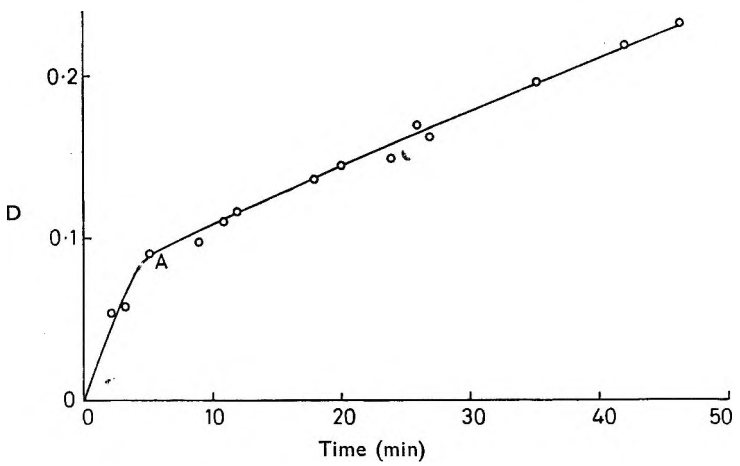


FIG. 1. Extinction in 1 cm cells (D) against time for griseofulvin dissolving in 9·13% w/w cetomacrogol solution at a stirring rate of 60 rev/min.

The initial steep rise in extinction occurs in the time when the particles are sedimenting, and is followed by a gentle curve. The effects would seem to arise from a decrease in surface area exposed to agitated solution as the heap is formed on the bottom of the flask. As we are interested in the dissolution rate when sedimentation is complete, the readings were corrected by subtracting the extinction and time at point A of Fig. 1 from these readings at later times, and evaluating k_1 as before. Alternatively, the curve after point A was extrapolated back to $t = 0$, and the extinction obtained subtracted from subsequent readings. Tests showed that both procedures gave the same value of k_1 ($\pm 1\%$), and the second method was used in evaluating most rate constants.

RESULTS AND DISCUSSION

The zero order rate constant k_1 was measured at different stirring rates (Fig. 2). At low stirring rates, up to 60 rev/min, the heap of griseofulvin is present at the bottom of the vessel, and hence a large proportion of the surface area of the powder is not in direct contact with stirred liquid. k_1 is thus quoted in units of mol litre^{-1} min^{-1} as the effective surface area cannot be measured. At 80 rev/min the heap can be seen to move, but is not in suspension. It is completely suspended above 130 rev/min. The initial increase in k_1 with stirring rate is likely to be due more to increased exposure

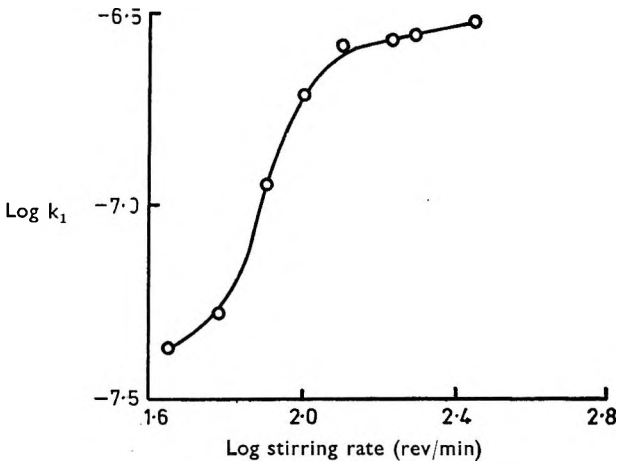


FIG. 2. Effect of stirring rate on zero order rate constant k_1 for 100 mg griseofulvin in 1 litre water.

of powder surface to stirred liquid, than to the effect of stirring itself. The final straight line portion of Fig. 2 has a slope of 0·21, compared with a slope of 0·54 found for dissolution rates from griseofulvin discs. This may be due to stirring being less effective in decreasing the thickness of a diffusion layer when the particles can move with the stirred liquid instead of being fixed as with the disc.

Six repeat experiments at 200 rev/min gave a mean k_1 of $2{\cdot}8 \times 10^{-7}$ mol litre^{-1} min^{-1}. Using the mean particle diameter of 104 μm, and the density of griseofulvin of 1·31 (Matthews & Rhodes, 1968), 100 mg of the drug has a surface area of 22 cm^2, assuming that the particles are spherical. This gives an absolute value of $k_1 = 1{\cdot}3 \times 10^{-8}$ mol litre^{-1} min^{-1} cm^{-2} compared with $1{\cdot}14 \times 10^{-8}$ found from experiments with discs (Elworthy & Lipscomb, 1968c). The agreement is good considering the number of experimental quantities involved.

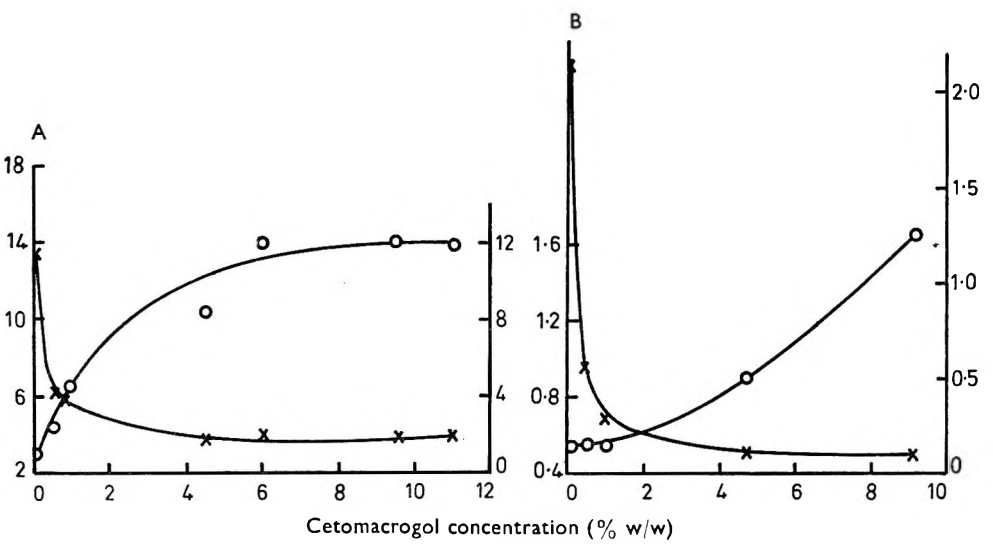


FIG. 3. Effect of cetomacrogol concentration on k_1 (○) and k_2 (×) at a stirring rate of A, 200 rev/min, B, 60 rev/min. Left hand ordinates $10^7 k_1$. Right hand ordinates $10^3 k_2$.

The experimental conditions at low stirring rates are similar in principle to those achieved in the "beaker" method (Levy & Hayes, 1960, Levy & Hollister, 1964). The effect of cetomacrogol was studied at 60 rev/min at which rate the heap of drug was present, and also at 200 rev/min, where suspension was complete.

The rate constants obtained at 200 rev/min are shown in Fig. 3A. The results are similar to those found for griseofulvin discs, in that k_1 increases and k_2 decreases with increase in cetomacrogol concentrations.

The overall effect is an increased dissolution of drug when the surfactant is present. Results of this type were fully discussed earlier (Elworthy & Lipscomb, 1968c), and all that need be said is that the same effects of the surfactant in facilitating the transfer of molecules of griseofulvin from the crystal into the solution apply, while the high viscosity of concentrated surfactant solutions causes the flattening of the plots in Fig. 3A. Linear relations between both $k_1\eta$ and $k_2\eta$ Ve, and surfactant concentration were found as before.

The effect of cetomacrogol on the rate constants determined at a stirring speed of 60 rev/min (Fig. 3B) is different from that on rate constants determined at 200 rev/min. At 200 rev/min the griseofulvin is suspended, at 60 rev/min it is in a pile on the bottom of the vessel and only the higher concentrations of cetomacrogol give an increase of dissolution rate over that in water. The lack of increase of k_1 with cetomacrogol concentration compared with the experiments at 200 rev/min may be due to the amount of surface relatively inaccessible to solvent movement when the drug is in a heap. The viscosity of cetomacrogol solutions only increased markedly when the concentration exceeded 3% w/w (Elworthy & Lipscomb, 1968c). The increased viscosity may allow an increased agitation to be transmitted to the heap, resulting in an increase of surface area exposed to the stirred liquid. This increase in area may be more important than the effects of the higher viscosity solutions on the transport properties of the solute. When the drug is in a heap there are a number of complex factors operating, which cannot be analysed completely.

Hamlin, Nelson & others, 1962, showed that *in vitro* dissolution tests on methylprednisolone correlated with *in vivo* ones only when the rate of stirring was low. Good correlation between *in vitro* dissolution of aspirin and *in vivo* absorption has been found by Levy (1961), using a low rate of agitation. The differences shown here between dissolution at high and low amounts of agitation, make it necessary to choose a stirring rate with care, especially if attempts to correlate *in vivo* and *in vitro* dissolution are to be made.

Acknowledgements

We thank Glaxo Research Limited for a maintenance grant fo F.J.L. and for the gift of griseofulvin.

REFERENCES

ELWORTHY, P. H. & LIPSCOMB, F. J. (1968a). *J. Pharm. Pharmac.*, **20**, 790–792.
ELWORTHY, P. H. & LIPSCOMB, F. J. (1968b). *Ibid.*, **20**, 817–824.
ELWORTHY, P. H. & LIPSCOMB, F. J. (1968c). *Ibid.*, **20**, 923–933.
HAMLIN, W. E., NELSON, E., BOLLARD, B. E., & WAGNER, J. G. (1962). *J. pharm. Sci.*, **51**, 432–435.
LEVY, G. & HAYES, B. A. (1960). *New Engl. J. Med.*, **262**, 1053–1058.
LEVY, G. & HOLLISTER, L. E. (1964). *J. pharm. Sci.*, **53**, 1446–1452.
LEVY, G. (1961). *Ibid.*, **50**, 388–392.
MATTHEWS, B. A. & RHODES, C. T. (1968). *J. Pharm. pharmac.*, **20**, *Suppl.*, 204S–212S.

J. Pharm. Pharmac., 1969, **21**, 277–282 *Received December* 12, 1968

Quantitative determination of physostigmine from aqueous solution

F. W. TEARE AND S. I. BORST

Faculty of Pharmacy, University of Toronto, Toronto 5, *Canada*

A gas chromatographic method is described for the direct determination of physostigmine base from a solution of physostigmine salicylate. The method involves isolation of physostigmine salicylate from an aqueous solution by a freeze drying process, subsequent conversion to a trimethylsilyl (TMS) derivative and gas liquid chromatography. The trimethylsilyl salt dissociates on injection into the flash vapourizer inlet of a gas chromatograph, and the physostigmine–TMS and salicylic acid–TMS derivatives are eluted separately. Due to the specificity of the silylation reaction it is possible to determine by gas chromatography the alkaloid content in the presence of its degradation products. A modification of the method was necessary for the assay of a commercial formulation containing certain additives.

A gas chromatographic method has been developed to estimate physostigmine from aqueous solutions of physostigmine salicylate. Physostigmine from fresh or degraded solutions can be determined with good sensitivity and with greater specificity than in many other procedures, the most useful of which have been listed (Teare & Taylor, 1967; Taylor, 1967). The methods are not entirely satisfactory because of the lack of specificity for physostigmine in the presence of possible decomposition products (Ellis, 1943) or of salicylate.

The proposed method requires the removal of water from aqueous solutions of physostigmine salicylate by a freeze drying process, and subsequent silylation of the salt residue to the corresponding physostigmine trimethylsilyl (TMS) derivative. The physostigmine–TMS derivative obtained is then gas chromatographed directly without prior liberation of the base. By this procedure it is possible to measure quantitatively intact physostigmine obtained from an aqueous solution of its salicylate salt.

Gas chromatography of alkaloidal salts of pharmaceutical interest, including those of physostigmine has been reported by Brochmann-Hanssen & Baerheim Svendson (1962). Few attempts have been reported for the gas chromatography of complex carbamates or corresponding trimethylsilyl derivatives. Some trimethylsilyl pesticidal carbamates have been examined qualitatively by gas chromatography (Fishbein & Zielinski, 1965).

EXPERIMENTAL

Reagents. Analar pyridine distilled and stored over molecular sieves (Linde Air Products Co., type 4A). Bis(trimethylsilyl)acetamide (BSA) (Pierce Chemical Co., Illinois). Physostigmine salicylate B.P. (B.D.H. Canada). Physostigmine base (Mann Research Laboratories, N.Y.). Physostigmine salicylate—0·25% w/v (Esromiotin) (Crookes-Barnes Laboratories, Inc., Wayne, New Jersey).

Apparatus and operating conditions. Samples were freeze dried using a Vertis Unitrap Automatic Freeze-Dryer.

A Beckmann GC-4 gas chromatograph, with a hydrogen flame ionization detector and a Beckmann 10-inch laboratory potentiometric recorder fitted with a disc chart integrator, Model 236 (Disc Instruments Inc.) were used. The chromatographic columns were glass U-tubes (4 ft × 3 mm, i.d.), packed with silicone gum rubber (3·8% SE-30) on Diatoport S, 80–100 mesh (Hewlett-Packard). The columns were operated under the following conditions: oven temperature 145°; injection flash vapourizer inlet 200°; air 350 ml/min, H_2 50 ml/min and N_2 93 ml/min.

Procedure. Samples of either fresh or partially degraded solutions (having intense red colorations) of about 0·5% w/v physostigmine salicylate were used. A 0·2 ml aliquot of either solution was transferred to a micro tube (1 × 7·5 cm) and the sample freeze dried. Dry pyridine (5 μl) and bis(trimethylsilyl)acetamide (10 μl) were added to the residue and the silylating solution was allowed to stand for 1 h before injecting 1·2 μl into the flash vapourizer inlet of the chromatograph.

Calculation of results

The areas under the peaks due to physostigmine–TMS and salicylic acid–TMS derivatives obtained with assay samples are calculated and compared with the areas obtained by gas chromatography of a known (standard) physostigmine salicylate solution similarly prepared. The weight (W_{up}) in grams of physostigmine in the sample is calculated from the following equation:*

$$\frac{W_{up}}{275{\cdot}35} = \frac{1}{R_1} \cdot \frac{A_{us}}{A_{ks}} \cdot \frac{W_{ks}}{138{\cdot}12} \cdot \frac{1}{k}$$

where: *M* for salicylic acid = 138·12; *M* for physostigmine base = 275·35; A_{us} = peak area of salicylic acid–TMS from physostigmine salicylate of *unknown* concentrations; A_{ks} = peak area of salicylic acid–TMS from physostigmine salicylate of *known* concentration; W_{ks} = known weight in grams of salicylic acid present in a known physostigmine salicylate solution; R_1 = ratio of peak areas of salicylic acid–TMS to physostigmine–TMS from the *unknown* physostigmine salicylate solution; k = ratio of peak areas of physostigmine–TMS to salicylic acid–TMS from the *known* physostigmine salicylate solution (freshly prepared).

RESULTS AND DISCUSSION

Preliminary work on the gas chromatography of 0·5% w/v physostigmine base or salt in a solution of acetone revealed 2 or more peaks, depending on the conditions, which reflected decomposition on the column (Brochmann-Hanssen & Baerheim Svendson, 1962). This decomposition was overcome by conversion of the physostigmine moiety of the salt to the trimethylsilyl (TMS) derivative which chromatographs to give a single peak. Conversion of physostigmine base and the salicylate trimethylsilyl derivatives was attempted with conventional silylating reagents. The method of Fishbein & Zielinski (1965) for the silylation of carbamates was tried but the reaction was incomplete. We found bis-(trimethylsilyl)acetamide (Klebe, Finkbeiner & White, 1966) to react rapidly with physostigmine base in the presence of pyridine. Evidence of a more stable derivative was demonstrated by only one peak in the gas chromatogram. Using the same conditions, physostigmine salicylate was silylated. The physostigmine salicylate–TMS apparently dissociates in the flash heater unit at 200°, and the

* Photo copies of the derivation of this formula are available from the Editorial Department, Journal of Pharmacy and Pharmacology, 17 Bloomsbury Square, London, W.C.1.

physostigmine and salicylic acid moieties were eluted as their TMS derivatives (Fig. 1A). No difference in retention time could be observed whether physostigmine–TMS was prepared from the salt or the free base. Similarly the TMS derivatives of the salicylate moiety of this salt and salicylic acid have identical retention volumes. Good resolution for the TMS derivatives of both moieties of the physostigmine salicylate was achieved with a minimum of tailing (Fig. 1A) on the column used.

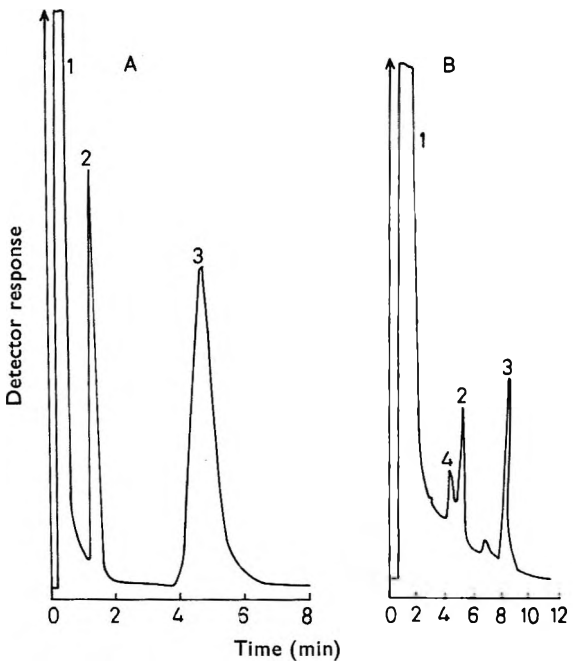


FIG. 1. A. Silylated derivatives of freeze-dried physostigmine salicylate, chromatographed under isothermal conditions. (1) Solvent peak. (2) Salicylic acid–TMS peak (attenuation 2×10^4). (3) Physostigmine–TMS peak (attenuation 2×10^3).

B. Temperature programmed chromatogram of a freeze-dried silylated commercial sample of physostigmine salicylate containing additives.

Conditions: 100° isothermal for 0·6 min, 100° to 150° at 10·3°/min., 154° isothermal for 3·25 min. Other parameters given under earlier isothermal conditions. (1) Solvent peak. (2) Salicylic acid–TMS peak. (3) Physostigmine–TMS peak. (4) Thiomersal peak.

Dissociation of physostigmine salicylate during the silylation reaction would appear not to be possible because pyridine (pK_a 5·12), the solvent for the silylation reaction, will not promote dissociation of the salicylate salt as it is a weaker base than physostigmine (pK_a 6·12 and 12·24). Moreover Brochmann-Hanssen & Baerheim Svendson (1962) reported that dissociation of some alkaloidal salts of organic acids including physostigmine salts always occurs in the flash heater.

In partially degraded aqueous solutions, specificity for physostigmine was satisfactory because the decomposition products, such as rubreserine, eserine blue, eserine brown, will not gas chromatograph, possibly owing to their non-volatility, irreversible adsorption to column material or failure to form TMS derivatives. There was no evidence of the formation of a trimethylsilyl ether of eseroline; this might be expected owing to its rapid oxidation to further decomposition products (Ellis, 1943).

The unknown concentration of the physostigmine moiety in solutions of physostigmine salicylate could be determined by direct comparison of the peak areas of the

unknown and known physostigmine–TMS derivatives. However, the first method of calculating the physostigmine used in this paper takes advantage of the greater reproducibility of the salicylate–TMS derivative peak areas, which enhances the overall precision of the assay.

It must be emphasized that only in freshly prepared solutions of physostigmine salicylate are the salicylic acid and physostigmine moieties on a 1 : 1 molar basis. Whereas the salicylic acid portion remains constant with time in *all* solutions, in the degraded solutions, some physostigmine decomposition product(s) could be also associated with the salicylic acid moiety. However, of these alkaloidal substances *only* the physostigmine forms a TMS derivative which gives a peak on the chromatogram, giving specificity to the method described here.

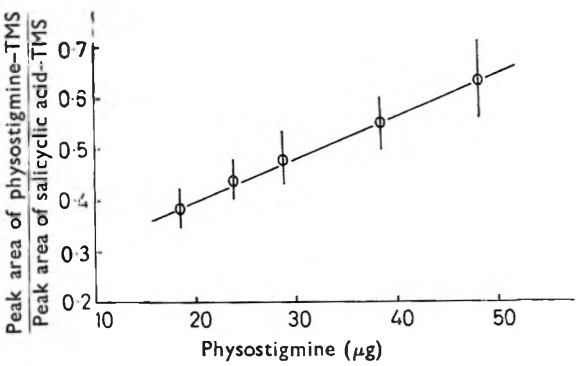


FIG. 2. Linear relation between the ratio of peak areas of physostigmine–TMS and salicylic acid–TMS derivatives and the concentration of physostigmine in known simulated mixtures.

The calibration curve (Fig. 2) is rectilinear for physostigmine–TMS over the range 15–48·4 μg. This calibration curve was constructed by preparing simulated mixtures of salicylic acid and physostigmine base which include those quantities found in the physostigmine salicylate preparations being assayed. The salicylic acid content was kept constant while increasing known amounts of physostigmine base were added to the standards before silylation and subsequent gas chromatography employed for samples of the assay preparation of physostigmine salicylate. The linearity of detector response to increasing amounts of physostigmine salicylate from a freshly prepared solution was demonstrated by the constancy of the ratio of the physostigmine–TMS to salicylic acid–TMS peak areas when increasing volumes of the silylated sample were injected.

Table 1. *Assays of physostigmine solutions I on 5 consecutive days, II on the same day, III on the same day after partial degradation by refluxing for 6 h*

	No. of determinations	Assays made over:	Physostigmine concn (% w/v base ± s.d.) Initial	Found	Found
I	5	5 consecutive days	0·334	0·355 ± 0·041*	0·349 ± 0·045†
II	4	1 day	0·334	0·315 ± 0·031**	0·306 ± 0·038†
III	4	1 day	0·333	0·121 ± 0·009**	0·124 ± 0·008†

* Mean of 6 reference and 6 sample injections.
** Mean of 3 reference and 3 sample injections.
† Results from 1 reference and 1 sample injection.

The results obtained by applying the proposed method to various fresh and degraded solutions are summarized in Table 1. Routine analysis over several days gave a precision of ± 11·5%. Determinations made on the same day show a precision of ± 7 to 10% (Table 1). Assays based on determinations corresponding to single injections of sample and reference show a lower precision. Physostigmine could be detected at about 1·5 μg but quantitative work was not possible in this region. This is most likely due to a combination of factors, irreversible adsorption of the TMS derivative becomes significant at this concentration level, and background noise with baseline drift appears at the high sensitivity settings of the instrument.

Application to stability studies of both salicylic acid and physostigmine present in aqueous solution

Work was initiated to investigate the stability of the salicylic acid moiety of physostigmine salicylate. We hoped to verify the use of the salicylic acid portion of the salt as an internal standard for the assay of the physostigmine moiety in stored solutions of physostigmine salicylate, by showing that the former remained constant throughout the pharmacologically-active life of this salt solution.

The salicylic acid residue of physostigmine salicylate solutions was found by colorimetry and by gas chromatography to remain constant (Table 2) over 5 weeks storage at room temperature. This suggests the salicylic acid portion of the salt could be used as the internal standard for the assay of the physostigmine moiety in the same aqueous solution.

Table 2. *The stability of the salicylic acid moiety of physostigmine salicylate in one solution over the test period*

Physostigmine salicylate stored at 23° (days)	Salicylic acid concentration found in physostigmine salicylate solutions (% w/v) (initial concn 0·169%)		Physostigmine concentration found in physostigmine salicylate solution (% w/v)
	Colorimetric*	GLC†	GLC‡
0	0·170	—	—
2	0·170	0·167	0·326
6	0·172	—	—
9	0·177	0·161	0·333
16	0·178	0·173	0·351
39	0·173	—	—

* Average of 3 determinations from the same sample.
† Average of 6 determinations from the same sample used for the colorimetric assay.
‡ No specific colorimetric or spectrophotometric method is available for the physostigmine moiety in its salts or for the free base.
The GLC method reported for the physostigmine has a precision of about ± 11·5% (see text).

During the 5 week period, aqueous solutions of physostigmine salicylate developed a pink coloration. But such pink solutions do not necessarily reflect significant amounts of decomposition of physostigmine in aqueous solution as estimated by using the salicylic acid moiety as an internal standard. This supports the findings of other workers (Hellberg, 1949).

A modified assay was made on a commercial formulation (Esromiotin-1/4, Crookes-Barnes Laboratories, Inc., Wayne, N.J., U.S.A.) containing physostigmine 0·25% w/v, in boric acid solution with sodium metabisulphite 0·1% and thiomersal 0·01%. Using isothermal conditions, the peak corresponding to the thiomersal overlaps that of

the salicylic acid–TMS derivative. Tailing of the solvent peak is caused by the presence of boric acid which produces significant base line drift and affects the reproducibility of the physostigmine–TMS peak. This base line drift was reduced by temperature programming (Fig. 1B). Using the conditions in Fig. 1B the physostigmine contents of a commercial preparation was successfully determined. It was calculated first by using the equation previously given in which the ratio of the salicylic acid–TMS peak areas is used, and secondly by considering the ratio of the physostigmine–TMS peak areas of the reference and unknown samples only. The partial overlap of the thiomersal and salicylic acid–TMS peaks (Fig. 1B) appears not to interfere in the estimation of physostigmine content (% w/v) when this is calculated using the ratio of the known and unknown salicyclic acid–TMS peak areas (mean 0·160 ± 0·008, n = 5). Calculations based solely on the ratio of the known and unknown peak areas of physostigmine–TMS (mean 0·164 ± 0·012 n = 5) also show close agreement with the labelled strength of this commercial preparation.

Acknowledgement

The authors would like to thank the National Research Council of Canada for partial financial support of this study through on Operating Grant No. A2064.

REFERENCES

Brochmann-Hanssen, E. & Baerheim Svendsen, A. (1962). *J. pharm. Sci.*, **51**, 1095–1098.
Ellis, S. (1943). *J. Pharmac. exp. Ther.*, **79**, 364–372.
Fishbein, L. & Zielinski, W. L. (1965). *J. Chromatog.*, **20**, 9–14.
Hellberg, H. (1949). *Svensk farm. Tidskr.*, **53**, 637–643.
Klebe, J. F., Finkbeiner, H. & White, D. M. (1966). *J. Am. chem. Soc.*, **88**, 3390–3395.
Taylor, K. M. (1967). *J. Pharm. Pharmac.*, **19**, 770–771.
Teare, F. W. & Taylor, K. W. (1967). *Ibid.*, **19**, 257–261.

J. Pharm. Pharmac., 1969, **21**, 283–286 *Received October* 2, 1968

The determination of bromisoval and carbromal in biological material

A. G. RAUWS

National Institute of Public Health, Sterrenbos 1, *Utrecht, the Netherlands*

The bromine determination (van der Meulen) has been adapted to the assay of bromisoval and carbromal in biological material. Interfering bromide is eliminated by pretreatment with silver nitrate. The ashing procedure was simplified by previous precipitation of proteins by acetone. Interference by bromine-containing metabolites of the drugs is absent or minimal. The method is applicable in the range of 0·1 to 1 μmol. Recovery in blood was 95%. The standard deviation of the determination is approximately 5%.

The distribution and biotransformation of the bromocarbamides (bromoureides) were examined early in this century (Takeda, 1911; Kwan, 1912; Impens, 1912; Gensler, 1916). These drugs were largely superseded by the barbiturates, and their place has been only in household medicine in some countries. Some 15 years ago manifestations of bromocarbamide toxicity revived interest, mainly in forensic science laboratories (Schmidt, 1954; Vidic, 1959; Curry, 1963). The methods then developed were qualitative or semiquantitative and not useful for serial work. A method for the determination of bromisoval or carbromal adapted to process larger numbers of samples of biological material in an investigation of bromocarbamide metabolism is now described. After some trial (Rauws, 1968), a procedure was evolved, by which the bromine compounds, after hydrolysis, were determined as bromide. A titration procedure was arrived at which later was recognized as being essentially the same as that developed by Hunter (1953).

Both bromocarbamides are almost wholly degraded in the body with the formation of bromide. It is therefore necessary to separate them from bromide before the determination. Complete extraction of carbromal from blood or plasma with chloroform or ether after denaturation with trichloroacetic acid or wolframate proved to be difficult because of the adsorption of the drug to the precipitated proteins. A complicating factor was its unexpected property of partly volatilizing during evaporation of the extracts. To prevent this, the proteins were precipitated with a water miscible solvent (ethanol, acetone) in which the bromocarbamides are soluble. From the resulting extract, bromide was precipitated with silver nitrate according to Wollheim (1958) and the remaining bromine compounds were determined as bromide after alkaline hydrolysis.

This method has been used for determinations in plasma, blood, brain and liver. For determinations in fat tissue a modified extraction procedure was necessary.

EXPERIMENTAL

Reagents. (Unless otherwise stated the reagents were of analytical grade.) Acetone; 200 mM aqueous solution of silver nitrate; 220 mM aqueous solution of potassium chloride (Merck "bromfrei"); 2 M aqueous solution of sodium hydroxide; 40% w/v aqueous solution of sodium dihydrogen-phosphate monohydrate; 1·0 M sodium

hypochlorite in aqueous 0·1 M sodium hydroxide (BDH, "Low in bromine"); 50% w/v aqueous solution of sodium formate; 6·0 N aqueous solution of sulphuric acid; 10% w/v aqueous solution of potassium iodide, freshly prepared; 0·006 N aqueous solution of sodium thiosulphate, freshly diluted from 0·1 N stock solution; sodium starch glycollate indicator solution, prepared according to Vogel (1951); potassium bromide standard, 1 mM aqueous solution; potassium bromate standard, 1 mM aqueous solution; bromisoval (Ph.Ned.Ed.VI) 10 mM fresh solutions in ethanol or propylene glycol; carbromal (Ph.Ned.Ed.VI) 10 mM fresh solutions in ethanol or propylene glycol.

Separation procedures

Plasma or blood. Plasma or blood (1·0 ml) is added dropwise to acetone (4·0 or 4·5 ml respectively) in a centrifuge tube. After mixing on a vortex mixer, the contents are centrifuged for 10 min in a tabletop centrifuge at 3000 rev/min. The supernantant (4·0 ml) is pipetted into a centrifuge tube and silver nitrate (1·0 ml) added. The contents of the tube are thoroughly mixed and, after 30 s, potassium chloride (1·0 ml) is added. Again the contents are mixed thoroughly and the tube is closed with "Parafilm" to prevent evaporation and centrifuged at 3000 rev/min for 30 min. The silver halide supernatant (5·0 ml) is used for the next steps in the analytical procedure. The precipitation of the silver halides is carried out in semi darkness.

Soft tissues (brain, liver). 1·0 g or less tissue is homogenized in acetone (3–4 ml). The volume of the homogenate is adjusted to 5·0 ml. After centrifugation at 3000 rev/min for 10 min the supernatant (4·0 ml) is treated as for the corresponding blood supernantant.

Fat tissue. Fat tissue (approximately 1 g) is cut in small pieces. It is then extracted three times with chloroform (50 ml). The extract is evaporated from pre-weighed flasks, and the extracted fat is weighed*. The fat is dissolved in light petroleum (b.p. 80–100°) (5 ml) and transferred into a stoppered centrifuge tube. Ethanol (4·0 ml) is added and the contents are mixed. To the homogeneous solution water (1·0 ml) is added. The two phases separate after shaking. An aliquot of the ethanol-water phase (4·0 ml) is processed further.

Analytical procedure

The silver halide supernatant (4·0 or 5·0 ml) is pipetted into a nickel crucible (capacity 25 ml) and 2 drops of sodium hydroxide are added. The mixture is then dried quickly at its boiling point where, for carbromal, hydrolysis competes succesfully with volatilization. The crucible with its dried contents is heated in an electrical oven to dull red for 45 s. The contents are dissolved in 1 ml water and are transferred quantitatively to a 100 ml Erlenmeyer flask. The total volume of the washings (e.g. 3 × 1·5 ml of water) should not exceed 5 ml. Sodium dihydrogenphosphate (5 ml) and sodium hypochlorite (3 ml) are added and the mixture is heated for 20 min in a boiling water bath. After 15 min, sodium formate (1 ml) is added and the mixture stirred. Excess hypochlorite is destroyed and carbon dioxide is evolved. The flask is taken from the bath and cooled to approximately 15°, sulphuric acid (5 ml) and potassium iodide (1 ml) are added. The iodine formed in the reaction is titrated with 0·006 N thiosulphate with a few drops of starch glycollate as an indicator. 1·0 ml thiosulphate is equivalent to 1 μmol of bromine.

* The fat content of the fresh wet tissue of the rats used was 34 ± 5%.

The blank value depends on the varying quality of the hypochlorite reagent. Blood blanks varied between 0·050 and 0·090 ml.

RESULTS

The average recovery from blood, plasma and brain tissue was approximately 95% (Table 1). With fat tissue recovery was less satisfactory. Using olive oil as a model approximately 85% of the added bromocarbamides was recovered (Table 2).

Table 1. *Accuracy and recovery of bromocarbamide determination in blood*

	Titrated (ml)	Concentration determined (μmol/ml)	Bromocarbamide added (μmol/ml)	Recovery (%)
Blank	0·090*	—	—	—
Bromisoval	0·305	0·355		95
	0·300	0·345		92
	0·315	0·370	0·375	99
	0·315	0·370		99
	0·285	0·320		85
mean ± s.d.		0·350 ± 0·020		94 ± 6
Carbromal	0·310	0·365		92
	0·310	0·365		92
	0·315	0·370	0·395	94
	0·325	0·390		99
	0·325	0·390		99
mean ± s.d.		0·375 ± 0·015		95 ± 4

* Volumes and concentrations are rounded off to 0·005 units.

Table 2. *Accuracy and recovery of bromocarbamide determination in olive oil*

	Titrated (ml)	Concentration determined (μmol/ml)	Bromocarbamide added (μmol/ml)	Recovery (%)
Blank	0·045*	—	—	—
Bromisoval	0·380	0·420		84
	0·370	0·405	0·500	81
	0·375	0·410		82
mean		0·410 ± 0·010		82
Carbromal	0·390	0·430		86
	0·385	0·425	0·500	85
	0·380	0·420		84
mean		0·425 ± 0·005		85

* Volumes and concentrations are rounded off to 0·005 units.

The standard deviation for the determination of bromide at 0·5 μ-equiv/ml was 0·010 μ-equiv/ml and at 0·1 μ-equiv/ml it was 0·005 μ-equiv/ml. It thus varied between 2 and 5% of the values to be determined. The determination of covalently bound bromine was, as had been expected, less accurate. In the concentration range of 0·1 to 0·5 μmol/ml the standard deviation varied from 0·010 to 0·020 μmol/ml i.e. maximally 10% of the value to be determined (Table 1). This was also the concentration range found in animal experiments. As the molecular weight of bromisoval is 223 and that of carbromal 237, it corresponds to a range of approximately 2 to 10 mg/100 ml.

DISCUSSION

The method does not discriminate between covalently bound iodine and bromine. Iodine compounds in as far as they are not precipitated with the proteins are determined together with the bromine compounds. Interference however, is not to be

expected, as physiological and pharmacological concentrations of iodine compounds are negligible except perhaps shortly after an intravenous injection of radio-opaque media. Physiological bromine compounds are rare in terrestrial animals. A qualitative analysis will detect external bromine-containing compounds which unexpectedly might be present. In general however an external interference is not to be expected.

What could be called internal interference might be caused by bromine-containing metabolites. Those found are: 2-bromo-2-ethyl-butyric acid (Jindra, Slámová & others, 1964), hydroxycarbromal or 2-bromo-2-ethyl-3-hydroxy-butyryl-carbamide (Butler, 1964) from carbromal, and 2-bromoisovaleric acid (Rauws, 1968) from bromisoval. In acid extracts of blood taken from rats and mini-pigs after administration of bromisoval or carbromal, no hydroxycarbromal or bromoethylbutyric acid have been detected. However, a trace of bromoisovaleric acid has been found. This does not interfere with the quantitative determination since it is precipitated together with the bromide by silver nitrate. Likewise bromoethylbutyric acid will not interfere. But hydroxycarbromal will be determined together with carbromal when present in blood. In mice, appreciable blood levels of this compound have to be reckoned with (Butler, 1964). If a separation of hydroxycarbromal from carbromal is needed, chromatographic separation or extraction will be necessary. The same applies to separation of bromisoval and carbromal after their simultaneous administration. After a tenfold dilution of the acetone supernatant with water, most of the carbromal may be recovered by extracting three times with two volumes of light petroleum. The bromisoval may be extracted next with chloroform (three times the same volume). From the chromatographic properties of hydroxycarbromal compared with those of bromisoval it may be expected that the former could be separated from carbromal by a similar procedure.

The methods described have been applied successfully to a comparative investigation of the distribution and biotransformation of bromisoval and carbromal in which series of 30 to 50 samples at a time were processed. For clinical toxicological purposes the method is time-consuming and gas chromatography would be the method of choice.

This work is part of a doctor's thesis at the University of Leiden. The full text is published in Dutch.

REFERENCES

BUTLER, TH. C. (1964). *J. Pharmac. exp. Ther.*, **143**, 23–29.

CURRY, A. S. (1963). *Poison Detection in Human Organs*, p. 81, Springfield: Thomas.

GENSLER, P. (1916). *Arch. exp. Path. Pharmak.*, **79**, 42–54.

HUNTER, G. (1953). *Biochem. J.*, **54**, 42–45.

IMPENS, E. (1912). *Therapie Gegenw.*, **53**, 158–162.

JINDRA, A., SLÁMOVÁ, L., SIPAL, Z. & KEMÉNYOVÁ, M. (1964). *Pharmazie*, **19**, 244–247.

KWAN, F. P. (1912). *Archs int. Pharmacodyn. Thér.*, **22**, 331–341.

RAUWS, A. G. (1968). Thesis, Leiden, p. 23–29, p. 51–60.

SCHMIDT, G. (1956). *Arch. exp. Path. Pharmak.*, **229**, 67–72.

TAKEDA, S. (1911). *Archs int. Pharmacodyn. Thér.*, **21**, 203–211.

VIDIC, E. (1959). *Arch. Toxikol*, **17**, 373–386.

VOGEL, A. J. (1951). *A Text-book of Quantitative Chemical Analysis*, 2nd edn, pp. 331–2, London: Longmans.

WOLLHEIM, F. (1958). *Acta pharmac. tox.*, **15**, 1–7.

J. Pharm. Pharmac., 1969, **21**, 287–292 *Received October* 23, 1968

Comparison of bromisoval and carbromal in the rat

A. G. RAUWS

National Institute of Public Health, Sterrenbos 1, *Utrecht, the Netherlands*

Distribution experiments *in vitro* and *in vivo* with bromisoval and carbromal have shown carbromal to be more lipophilic and cumulative in brain and fat tissue. The half-life of bromisoval in the rat is approximately 2·5 h, that of carbromal is longer and variable. Stimulation and inhibition experiments revealed that both drugs are metabolized and follow different patterns. Carbromal toxicity is enhanced by bromide treatment, whereas bromisoval toxicity is not. The results show that carbromal is more toxic than bromisoval for the rat.

The hypnotic bromocarbamides (or bromoureides) carbromal and bromisoval, introduced in the beginning of this century, have largely been superseded by the evolution of the barbiturate hypnotics except as household remedies in some countries. Being effective and apparently innocuous when used occasionally they gave little cause for worry. When, however, in the years after the Second World War misuse and abuse of drugs became more widespread, the chronic and acute toxicity of both bromocarbamides became a source for concern (Magnussen, 1947; Anonymous comment, 1947; Kärber, 1952; Copas, Kay & Longman, 1959; Atkinson, 1960; Pihkanen & Harenko, 1962; van Heyst, 1966).

As pharmacological and toxicological knowledge of both drugs was defective, some of their properties which were expected to be related to their toxicity have been investigated. The tissue distribution and relative lipid solubilities were determined. Plasma half-lives were estimated and their dependence on sex and other factors were assessed. As acute intoxication with bromocarbamides often follows a period of prolonged misuse, the influence of bromide intoxication on the toxicity of both drugs was also determined.

EXPERIMENTAL

Materials. Bromisoval (Ph.Ned. Ed. VI) and carbromal (Ph.Ned. Ed.VI) were used and were shown to be pure by thin-layer chromatography. They were dissolved in propylene glycol B.P. for administration.

Animals. Wistar rats of either sex, approximately 150 g were used and were obtained from the TNO animal breeding farm, Zeist, the Netherlands, or from the breeding department of our own institute (RIV).

Partition coefficients. The bromocarbamide (5 mg) dissolved in deionized water (10 ml) was extracted for 4 h with an equal volume of organic solvent (nitrobenzene, chloroform, light petroleum b.p. 80–100°). Samples of both phases were taken, and the bromocarbamide content was determined as described by Rauws (1969), directly in the water phase and, after evaporation of the chloroform or light petroleum phase, in the residue taken up in water. After preceding alkaline hydrolysis of the bromocarbamide, the nitrobenzene phase was removed by steam distillation and bromide was determined in an aliquot of the aqueous residue.

Distribution in vivo. The drugs were administered to the rats by intraperitoneal injection as a solution in propylene glycol. Blood and tissue samples were taken when the animals awoke. It was hoped that this would minimize individual differences in distribution kinetics. Subcutaneous perineal fat was used in the analysis of fat tissue.

Determination of the half-life. Groups of six rats were bled (orbital puncture) to obtain blank values. At zero time 0·7–1·0 mmol/kg of the drug, was injected intraperitoneally. At least three times afterwards the rats were bled again, 0·4 ml of blood from each animal was pooled and the bulked blood of each group was used to determine the bromocarbamide concentration in duplicate. On the assumption that a linear relation exists between the logarithm of the blood level and time, the best fitting straight line was computed (de Jonge, 1962). The apparent half-life was calculated from the parameters of this line. No rate constants are derived from this empirical quantity.

Experimental bromide intoxication. Rats were given 0·1 M sodium chloride and 0·05 M sodium bromide in the drinking water. Control animals received 0·15 M sodium chloride. In three to four weeks blood bromide levels rose to approximately 25 m-equiv/litre. At these bromide levels, which in man are toxic, the rats showed no signs of depression as judged by their spontaneous motility, exploration and feeding habits. Grooming however was distinctly depressed and the otherwise clean fur of the rats became yellow and caked with dried urine.

Analytical procedures in biological material. These were carried out as described by Rauws (1969). As the plasma and blood concentrations in the rats used were essentially the same, determinations were made in whole blood.

Statistical analysis. Wilcoxon's two samples test was used to estimate the significance of some results.

RESULTS

Partition coefficients. The results are shown in Table 1. The two structurally related compounds exhibit large differences in physicochemical properties. The relative difference between the partition coefficients of the two compounds increases with the increase of the ratio for the dielectric constants of water and solvent.

Table 1. *Partition coefficients of bromisoval and carbromal between water and some organic solvents*

	Nitrobenzene: water	Chloroform: water	Light petroleum (80–100°): water
$K_{bromisoval}$	3·58	7·4	0·013
$K_{carbromal}$	26·3	163	0·53
$K_{carbr}/K_{bris o}$	7·35	22·0	40·7
$\epsilon_{water}/\epsilon_{solvent}$	2·27	16·9	42·6

Distribution in vivo. Table 2 shows the results of two distribution experiments. Carbromal accumulates in brain relative to blood and more so in fat tissue, whereas bromisoval concentration in fat tissue is strikingly low.

Comparison of half-lives. Results in Table 3 show a consistent and clear difference in half-life of carbromal and bromisoval in various lines of female rats of the Wistar strain. The same situation prevails in the male rat, as is illustrated in Tables 3 and 5. The unintentioned use of several lines of the Wistar strain in one investigation—a consequence of fluctuations in the supply—is instructive in so far as it shows the large

Table 2. *The distribution of bromisoval and carbromal in blood, brain tissue and fat tissue of the rat* (0·75 mol/kg, *intraperitoneally*). Samples were taken when the animals awoke. Concentration μmol/ml blood or μmol/g wet tissue (mean ± s.d.) Each group consisted of 5 or 6 animals.

		Experiment I	Experiment II
Bromisoval	Blood	0·200 ± 0·020	0·255 ± 0·055
	Brain	0·210 ± 0·020	0·250 ± 0·060
	Fat tissue	—	0·095 ± 0·025
Carbromal	Blood	0·180 ± 0·080	0·230 ± 0·035
	Brain	0·380 ± 0·050	0·370 ± 0·070
	Fat tissue	—	0·510 ± 0·225
Bromisoval	Brain*/Blood	1·05	1·00
	Fat tissue/Blood	—	0·37
Carbromal	Brain/Blood	2·11	1·60
	Fat tissue/Blood	—	2·23
Significance of difference between bromisoval and carbromal: *P* in %	Brain/Blood	<5	2
	Fat tissue/Blood	—	2

* Concentration ratio's are the mean values of the individual concentration ratio's.

differences that may be a result of slight differences in breeding and housing of animals.

The influence of sex on the half-life of carbromal and its lack of influence on that of bromisoval is shown in Table 3. Other differences between bromisoval and carbromal are the prolongation by SKF 525-A (β-diethylaminoethyldiphenylpropylacetate) of the half-life of bromisoval and the shortening by phenobarbitone pretreatment of the half-life of carbromal (Table 4). The increase in half-life of bromisoval by SKF 525-A

Table 3. *Plasma half-lives* (*h*) *of bromisoval and carbromal in the male and female rat*

Strain and line	Bromisoval		Carbromal	
	Female	Male	Female	Male
Wistar-RIV	3·0		12·5	6·9
Wistar-TNO	2·1		> 15	
	4·5		> 40	
Wistar-RIV inbred	1·2	1·2	6·5	2·8
	1·7		6·9	

Table 4. *Influence of pretreatment with phenobarbitone and SKF 525-A on the plasma half-lives* (*h*) *of bromisoval and carbromal in the rat*

		strain and line	Control	Phenobarbitone	SKF 525-A
Bromisoval	♀	Wistar-TNO	1·7	—	2·9
	♀	Wistar-TNO	4·5	4·9	8·6*
	♀	Wistar-RIV inbred	1·7	1·4	2·9†
	♂	Wistar-RIV inbred	1·2	1·2	3·8
Carbromal	♀	Wistar-TNO	> 15	—	> 15
	♀	Wistar-TNO	> 40	9·5	> 40
	♀	Wistar-RIV inbred	6·9	3·6	5·8
	♂	Wistar-RIV inbred	4·5	1·5	4·0

Pretreatment: phenobarbitone on both days preceding the experiment 50 mg/kg in saline intraperitoneally; SKF 525-A, 45 to 60 min before the experiment 80 mg/kg in saline intraperitoneally.

* 1 animal died (out of 5).

† 3 animals died (out of 6).

was accompanied by some mortality after the administration of doses which were non-lethal in controls. Benziodarone, an inhibitor of mercapturic acid conjugation (Boyland & Grover, 1967) had no effect on either half-life.

Table 5. *Influence of pretreatment with bromide on hypnotic activity and toxicity of bromisoval and carbromal in the rat (Wistar-TNO). Each subgroup consisted of 5 animals*

		Control group (chloride)			Bromide group (blood level 15 m-equiv/litre)		
	Sex	Mortality (24 h) %	Sleeping time (h) mean ± s.d.	Surviving animals arousable after 24 h (%)	Mortality (24 h) %	Sleeping time (h) mean ± s.d.	Surviving animals arousable after 24 h (%)
Bromisoval 1 mmol/kg i.p.	Female	0	4 ± 1	100	0	6 ± 1	100
	Male	0	3 ± 1	100	0	5½ ± ½	100
Carbromal 1 mmol/kg i.p.	Female	0	> 22½ (21, 22, 23, > 24, > 24)	60	60	> 24 (> 24, > 24)	0
	Male	0	9½ ± 1½	100	40	> 23 (21, 23, > 24)	67

Pretreatment (3 weeks): control group, 0·15 M sodium chloride in drinking water; bromide group, 0·10 M sodium chloride plus 0·05 M sodium bromide in drinking water.

In Table 5 the results of one of the experiments comparing the influence of preceding bromide administration on the toxicity of the bromocarbamides are shown. A large increase in the toxicity of carbromal is evident. The prolongation of the sleeping time in the male group is striking. Because of the restricted observation time an effect on the sleeping time in the female group is masked, but the incidence of mortality points to an increased toxicity. On the other hand, the influence of bromide on the sleeping time after administration of bromisoval is slight, but significant at the 5% level. The results of several other analogous experiments, although differing numerically, are consistent with this picture (Rauws, 1969).

DISCUSSION

The results of the partition experiments show a distinct difference between carbromal and bromisoval, although their molecules differ only in the location and length of an alkyl group (2-ethyl versus 3-methyl). Carbromal is more lipophilic. A comparison of the Stuart models of both molecules gives a hint of an explanation. The carbromal molecule is much more compact, the bromine atom being pinched between the two ethyl groups. These groups also limit the free rotation of the carbamide group. In contrast the bromisoval molecule is more flexible. The volatility and smell, and the lower melting point of carbromal are probably also related to this structural compactness. The results also show that the partition coefficient between light petroleum and water is a more sensitive indicator of slight differences in lipophilicity than that between more polar solvents and water. Analogous comparisons are given by Mayer, Maickel & Brodie (1959) and Bickel & Weder (1968).

The distribution pattern *in vivo* is consistent with the results *in vitro*. The samples were taken late in the experiment, to minimize the influence of vascularization on the distribution. When samples are taken shortly after the administration of the drugs, the differences in concentration are much less. Early brain levels are high, reflecting

early blood levels, whereas early levels in tissues with a low degree of vascularization are negligible. Only after redistribution in the tissues are steady state conditions, which reflect physicochemical relations between drug and tissues, approached. From these results, treatment of cases of acute carbromal overdosage would require consideration of the accumulation of the drug in fat depots and treatment with lipid haemodialysis might be well worth trying. In bromisoval poisoning, conventional haemodialysis would be more effective. Pilot experiments revealed no important binding of either bromocarbamide to proteins or cell membranes (Rauws, 1968).

The difference between the half-lives and their respective patterns of stimulation and inhibition suggest that the main biotransformation pathways of both drugs are not identical. A phenobarbitone stimulated dehalogenating, microsomal enzyme system is known (Van Dyke, 1966) and a SFK 525-A-inhibited microsomal amidase has been characterized by Hollunger (1960). These enzyme systems might well represent the main pathways, as 2-ethylbutyrylcarbamide is an important metabolite of carbromal (Butler, 1964) and 2-bromo-isovaleric acid and its mercapturic acid derivative are found as metabolites of bromisoval (Haruna, 1961, Rauws, 1968). Human and rat plasma also contains an enzyme which degrades bromisoval but not carbromal (Rauws, 1968). Assuming a degree of overlap in the biotransformation patterns of bromisoval and carbromal one might, by way of hypothesis, suggest the following scheme (Fig. 1). The difference in pattern could depend on degree of lipophilicity of the compounds, as is suggested in another context by Lien & Hansch (1968). This matter is the subject of further research.

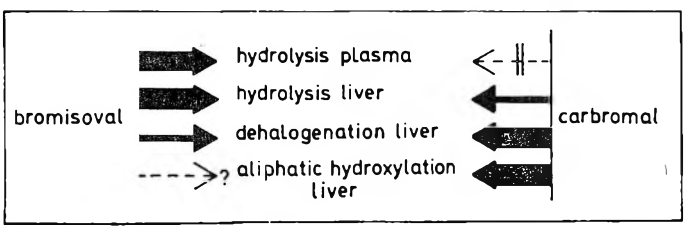


FIG. 1. Schematic representation of probable biotransformation patterns of bromisoval and carbromal.

The long half-life of carbromal in the Wistar-TNO rat is puzzling. It is associated with a greater apparent volume of distribution than in the other Wistar lines. A difference in the composition of the body fat might be the cause. It is tempting to speculate about a possible genetic cause of these differences. To obtain conclusions appreciably more data would be needed. As the TNO-animals grew up in another institute, environmental influences cannot be excluded as a possible cause.

It was to be expected that pretreatment with bromide to a level of approximately 25 m-equiv/litre would intensify the action of both bromocarbamides. Bromide itself has no influence on the biotransformation kinetics (Rauws, 1968) but the intensification of the action is probably not a purely pharmacological phenomenon, at least in the case of carbromal. Early experiments showed a raised brain concentration of carbromal in bromide-treated animals compared to chloride-treated controls. An explanation of this could be that the synergism of bromide with carbromal causes a circulatory depression, which in turn slows down the elimination of carbromal from the brain tissue. That bromisoval toxicity is not augmented by bromide to the same

extent as carbromal toxicity is interesting. Comparative data about the distribution of both compounds within the brain might contribute to an explanation of this contrast. In one respect, however, the two compounds are similar. Prolonged administration of both produces the same, fairly rapid accumulation of bromide in the body (Rauws, 1968).

The investigation, reported here in part, was begun with the expectation that the two compounds would present a closely analogous picture. Many unexpected but mutually consistent differences were found. The present conclusion is that, in the rat, carbromal is more toxic than bromisoval because of several interdependent reasons.

How far this conclusion may be transferred to man is a matter of conjecture. Clinical reports about acute intoxications generally do not contain the data required for an objective comparison.

The possibility of bromide accumulation as a consequence of prolonged administration of both bromocarbamides has already been examined in man by Wollheim (1958). Sufficient data would have now been gathered (Copas & others, 1959; Atkinson, 1960; Pihkanen & Harenko, 1962; Andrews, 1965) to conclude that both bromocarbamides are entirely unsuitable for prolonged use.

This work is part of a doctor's thesis at the University of Leiden. The full text is published in Dutch.

REFERENCES

ANDREWS, S. (1965). *Med. J. Aust.*, **52**, I, 646–652.

ANONYMOUS comment (1947). *J. Am. med. Ass.*, **135**, 659–660.

ATKINSON, I. (1960). *Med. J. Aust.*, **47**, II, 10–13.

BICKEL, M. H. & WEDER, H. J. (1968). *Archs int. Pharmacodyn. Ther*, **173**, 433–463.

BOYLAND, E. & GROVER, L. P. (1967). *Clin. chim. Acta*, **16**, 205–213.

BUTLER, T. C. (1964). *J. Pharmac. exp. Ther.*, **143**, 23–29.

COPAS, D. E., KAY, W. W. & LONGMAN, V. H. (1959). *Lancet*, **1**, 703–705.

HARUNA, K. (1961). *J. Biochem., Tokyo*, **49**, 388–391.

HEYST, A. N. P. VAN (1965). *Pharm. Weekbl.*, **101**, 909–920.

HOLLUNGER, G. (1960). *Acta pharmac. tox.*, **17**, 384–389.

DE JONGE, H. (1962). *Medische Statistiek II*, 2e edn p. 548, Leiden: Nederlands Instituut voor Preventieve Geneeskunde.

KÄRBER, G. (1952). *Arch. Toxikol.*, **14**, 137–139.

LIEN, E. J. & HANSCH, C. (1968). *J. pharm. Sci.*, **57**, 1027–29.

MAGNUSSEN, G. (1947). *Ugeskr. Laeg.*, **109**, 359–363.

MAYER, S., MAICKEL, R. P. & BRODIE, B. B. (1959). *J. Pharmac. exp. Ther.*, **127**, 205–211.

PIHKANEN, T. & HARENKO, A. (1962). *Acta neurol. scand.*, **38**, 209–217.

RAUWS, A. G. (1968). Thesis, Leiden.

RAUWS, A. G. (1969). *J. Pharm. Pharmac.*, **21**, 283–286.

VAN DYKE, R. A. (1966). *J. Pharmac. exp. Ther.*, **154**, 364–369.

WOLLHEIM, F. (1958). *Acta pharmac. tox.*, **15**, 1–7.

J. Pharm. Pharmac., 1969, **21**, 293–296 *Received January* 16, 1969

The binding of salicylate to plasma protein from several animal species

J. L. KUCERA AND F. J. BULLOCK

Arthur D. Little, Inc., Cambridge, Massachusetts 02140, *U.S.A.*

A comparison has been made of the binding of salicylate to plasma from man, green monkey, rabbit, rat, dog, and guinea-pig. Since the total protein content of normal, citrated, pooled plasmas of these species was not identical, comparisons were made with samples adjusted to the same total protein concentration. The % binding to plasmas of 5·3% total protein at 50 μg of drug/ml was found to be: man, 72%; green monkey, 70%; guinea pig, 64%; rabbit, 64%; rat, 47%; dog, 45%. Results for 150 μg and 500 μg of salicylate/ml are also reported. The albumin : globulin ratio as determined by electrophoresis varied widely among the species. Fractionation of the plasmas and comparison of binding, using 3% solutions of the various albumin fractions in buffer, indicated that the low binding of dog and rat plasmas was due primarily to the low binding affinity of the albumins. Adequate characterization of plasma samples is needed when comparative binding studies are made.

The numerous and difficult problems associated with achieving an understanding of species variations in drug response have been the object of several recent summaries (Brodie, Cosmides & Rall, 1965; Proceedings, 1967; Williams, 1967). One aspect of this problem is related to variations in drug binding to plasma protein among species, which may play a role in determining differences in tissue levels of drug (Brodie & Hogben, 1957; Gillette, 1965), in drug toxicity (Loomis, 1968), and in overall drug kinetics, particularly for highly bound drugs (Martin, 1965; Krüger-Thiemer, 1967). A most striking species variation in drug binding was recently reported for salicylate (Sturman & Smith, 1967). The work reported here was undertaken both to confirm these results and, if possible, find the basis for the species variations. Differences in total protein of plasmas among these species are a significant variable and might therefore account in part for many apparent species differences in binding of salicylate. This variable was significant for a number of other drugs, as demonstrated by work in these laboratories as well as by a report of Dayton, Perel & others (1967). Consequently, comparisons reported here were made with samples of identical total protein content.

EXPERIMENTAL

Plasma pools. Fresh pooled citrated plasmas (100–150 ml) were prepared and analysed for protein by the biuret method. The pools were then quantitatively diluted with pH 7·4, 0·075 M phosphate buffer, ionic strength 0·15, to give the same total protein, 5·3%. The pools were divided into 25 ml batches which were frozen for storage. No sample was carried through more than one cycle of freezing and thawing and the results from similar experiments were comparable before and after one cycle. Electrophoresis was done on cellulose acetate strips in pH 8·8, 0·025 M, barbitone buffer. After the protein bands had been stained (Ponceau S) and the strips dried, the

albumin and globulin fractions were cut out and eluted with 3·0 ml of 0·1N NaOH. Eluates were clarified as necessary by gentle centrifugation and the solutions read at 525 nm with a Beckman DU spectrophotometer. Using a standard curve, the ratio of albumin to globulins was calculated. The accuracy of these ratios is estimated as $\pm 0{\cdot}2$.

Plasma fractionations. The globulins were precipitated by repeated dialysis of the plasma sample against 6 litre volumes of 0·001M citrate buffer, pH 5·0 at 5°. The precipitated globulins were removed by centrifugation. The supernatant which was largely albumin was adjusted to pH 7·4 with 0·075 M phosphate buffer and checked for purity by electrophoresis. The major component corresponded to the albumin of the whole plasma and was contaminated with only traces ($<5\%$) of globulins. Each albumin solution after analysis by the biuret method was diluted to 3% with pH 7·4, 0·075 M phosphate buffer.

Methods and materials. Binding studies were made by equilibrium dialysis using 0·25 inch diameter Visking cellulose casing prepared for use as described by Hughes & Klotz (1959). Casings were stored refrigerated in buffer. After addition of the appropriate amount of drug to plasma, 1·0 ml of the drug-plasma mixture was dialysed against 4·0 ml of pH 7·4, 0·075 M phosphate buffer in a screw cap vial. The vial contents were rotated gently at 37 $\pm$ 0·1° until equilibrium (5–6 h). An aliquot of the buffer was then pipetted into Bray's solution (Bray, 1960) for scintillation counting. Sufficient counts were obtained to achieve the 98% confidence limit. The salicylic acid was a [^{14}C]carboxyl-labelled sample obtained from New England Nuclear Corporation, Medford, Mass. It had a specific activity of 2·31 mCi/mg and was chromatographically pure.

RESULTS

Binding studies were made at salicylate levels of 50, 150 and 500 μg/ml. The results, expressed in terms of percent drug bound, are presented in Table 1 along with the ratio of albumin to globulins for our particular plasma pools.

Table 1. *Salicylate bound (%) to various plasmas of constant total protein**

Drug level (μg/ml)	Man	Green monkey	Rabbit	Guinea-pig	Rat	Dog
50	72	70	64	64	47	45
150	60	58	55	53	36	41
500	40	42	37	41	33	37
Albumin/globulin ratio	1·8	1·6	2·0	1·1	0·8	1·2

* Total protein was adjusted to 5·3% with 0·075 M phosphate buffer, pH 7·4, ionic strength 0·15.

The results of a similar binding study using 3% solutions of the various albumin fractions in pH 7·4 buffer are given in Table 2. Percentages in both Tables are rounded to the nearest whole integer and are $\pm 3\%$. Although our albumin fractions contained traces of globulins, these samples were judged suitable for comparative studies. We have confirmed the report of Reynolds & Cluff (1960) that globulins bind salicylate much less than albumin in human plasma. We find that salicylate at 50 μg/ml is 25% bound to a 3% solution of human globulins in pH 7·4, 0·075 M phosphate buffer, ionic strength 0·15. It is 21% bound at 150 μg/ml and 16% bound at 500 μg/ml.

DISCUSSION

Our results for salicylate binding indicate genuine species differences which cannot be accounted for in terms of different protein concentration in the samples. Broadly speaking, our results support some of the conclusions of Sturman & Smith (1967). We agree that the species man, monkey, rabbit and guinea-pig can be considered together as having a higher proportion of salicylate bound than rat and dog which fall into a clearly differentiated group having a lower proportion of salicylate bound at the same total drug concentration. The data of Kurtz & Friemel (1967) for salicylate binding to various plasmas also show rat and dog plasmas to be clearly differentiated from those of man, rabbit and guinea-pig. Some of our absolute values for binding are at large variance with those of Sturman & Smith (1967). Agreement of values for binding to man and monkey plasmas is good and consistent with other reports for human plasma (Gutman, Yü & Sirota, 1955; Moran & Walker, 1968). We do not find rabbit

Table 2. *Salicylate bound (%) to albumin fractions of various plasmas**

Drug level (μg/ml)	Man	Green monkey	Rabbit	Guinea-pig	Rat	Dog
50	73	58	53	57	42	31
150	60	45	46	40	30	30
500	44	32	35	34	31	29

* Albumins were 3% solutions in pH 7·4, 0·075 M phosphate buffer, ionic strength 0·15.

plasma to have an intrinsically higher binding affinity than human at 50 μg/ml as previous work suggests. Our values for dog and rat plasma at this drug level are considerably different from the reported respective values of 14% and 2%. The value for salicylate binding to dog plasma reported by Potter & Guy (1964) also seems low, but the 60% binding value at 2–35 μg/ml (Weiner, Washington & Mudge, 1959) is somewhat more consistent with our data. We cannot explain these differences but note that all workers who have reported very low binding used gel filtration methods. We have observed many acidic drugs, including salicylate, to be reversibly but highly bound to Sephadex. This complicating factor introduces the possibility of competitive phenomena between column support and protein in quantitative studies. McArthur & Smith (1968) have demonstrated that the column length of gel influences the apparent extent of binding of salicylate to bovine serum albumin when it is determined by the gel filtration method. With short columns (90 mm), higher apparent binding was observed than with long columns (240 mm). This is consistent with the possibility of competitive binding of salicylate by the column support.

Kurtz & Friemel (1967) have made the qualitative observation that variations in the binding of salicylate to various animal plasmas are better correlated with the percentage of albumin in the sample than with total protein. Our binding results (Table 2) for the various albumin fractions differentiate rat and dog albumins as having lower binding affinity for salicylate than the other albumins studied. The low binding affinity of this fraction seems the likely cause of the low binding observed with these species.

Acknowledgement

This work was carried out under contract No. PH43–66–1139 from the National Institute of General Medical Sciences, Bethesda, Maryland.

REFERENCES

BRAY, G. A. (1960). *Anclyt Biochem.*, **1**, 279.

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

BRODIE, B. B., COSMIDES, G. J. ,& RALL, D. P. (1965). *Science, N.Y.*, **148**, 1547–1554.

DAYTON, P. G., PEREL, J. M., LANDAU, M. A., BRAND, M. & MARK, L. E. (1967). *Biochem Pharmac.*, **16**, 2321.

GILLETTE, J. R. (1965). Drugs and Enzymes, Editors: Brodie, B. B. & Gillette, J. R., pp. 9–22. New York: Macmillan and Co.

GUTMAN, A. B. YU, T. F. & SIROTA, J. H. (1955). *J. clin. Invest.*, **34**, 711–721.

HUGHES, T. R. & KLOTZ, L. M. (1956). *Methods of Biochemical Analysis*. Editor: Glick, D., p. 265, New York: Interscience.

KRÜGER-THIEMER, D. (1967). *Pharmacologic Techniques in Drug Evaluation*, Editors: Siegler, P. E. & Moyer, J. H., III, pp. 224–230, Chicago: Yearbook Medical Publishers.

KURTZ, H. & FRIEMEL, G. (1967). *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.*, **257**, 35.

LOOMIS, T. R. (1968). *Essentials of Toxicology*, p. 34, Philadelphia: Lea and Febiger.

MARTIN, B. K. (1956). *Nature, Lond.*, **207**. 959–960.

MCARTHUR, J. N. & SMITH, M. J. H. (1968). *FEBS Letters*, **1**, 332.

MORAN, C. J. & WALKER, W. H. C. (1968). *Biochem. Pharmac.*, **17**, 153.

POTTER, G. D. & GUY, J. L. (1964). *Proc. Soc. exp. Biol. Med.*, **116**, 658–660.

Proceedings of an International Symposium on Comparative Pharmacology (1967). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **26**, 964–1262.

REYNOLDS, R. C. & CLUFF, L. E. (1960). *Bull. Johns Hopkins Hosp.*, **107**, 278–290.

STURMAN, J. A. & SMITH, M. J. H. (1967). *J. Pharm. Pharmac.*, **19**, 622.

WAGNER, J. G. (1968). *Ann. Rev. Pharmac.*, 81–82.

WEINER, I. M., WASHINGTON, J. A., II, & MUDGE, G. H. (1959). *Bull. Johns Hopkins Hosp.*, **105**, 284–297.

WILLIAMS, R. T. (1967). *Drug Responses in Man*, Ciba Symposium. Editors: Wolstenholme, G. & Porter, R., pp. 71–82, Boston: Little, Brown and Co.

J. Pharm. Pharmac., 1969, **21**, 297–301 *Received November* 29, 1968

Dopa-induced locomotor stimulation after inhibition of extracerebral decarboxylase

G. BARTHOLINI, J. E. BLUM AND A. PLETSCHER

Research Department, F. Hoffmann-La Roche & Co. Ltd., Basle, Switzerland

In rats, a combination of small doses of the dopa decarboxylase inhibitor Ro 4–4602 [*N*-(DL-seryl)-*N'*-(2,3,4-trihydroxybenzyl)hydrazine] with dopa causes a marked enhancement of the spontaneous locomotor activity which is not seen with dopa alone. If high instead of low doses of Ro 4–4602 are used, locomotor stimulation does not occur. Low doses of Ro 4–4602, owing to selective inhibition of extracerebral decarboxylase, enhance the dopa-induced rise of dopa and catecholamines in the brain, whereas high doses of Ro 4–4602, which also inhibit the cerebral dopa decarboxylase, increase only the level of dopa but not that of the catecholamines. It is concluded that the locomotor activation after small doses of Ro 4–4602 in combination with dopa is due to cerebral accumulation of catecholamines which consist mainly of dopamine.

It has previously been reported that small doses of Ro 4–4602 [*N*-(DL-seryl)-*N'*-(2,3,4-trihydroxybenzyl)hydrazine], an inhibitor of the decarboxylase of aromatic amino-acids, selectively inhibit the decarboxylation of administered L-3,4-dihydroxyphenylalanine (dopa) in extracerebral tissues (e.g. blood and heart). As a consequence, the dopa-induced increase of catecholamines and phenolic carboxylic acids in the blood is diminished, whereas the concentration of dopa is greatly raised, leading to an increased supply of the amino-acid to the brain. Since the cerebral dopa decarboxylase is not significantly affected by Ro 4–4602, an enhanced formation of catecholamines (particularly dopamine) takes place in the brain (Bartholini, Bates & others, 1967; Bartholini & Pletscher, 1968). By fluorescence microscopy it has been demonstrated that the formation of catecholamines occurs in the parenchyma and not in the capillary walls of the brain (Constantinidis, Bartholini & others, 1968).

Since the cerebral catecholamines can be selectively increased by small doses of Ro 4–4602 plus dopa, the effect of this combination on locomotor activity has been investigated and is now reported.

EXPERIMENTAL

Male albino rats, weighing 80–100 g, received Ro 4–4602 (50 or 500 mg/kg, i.p.) alone or followed after ½ h by L-dopa (200 mg/kg i.p.). Controls were given L-dopa only. In some experiments, the animals were treated with reserpine (5 mg/kg, i.p.) 16 h before Ro 4–4602. The locomotor activity of 3 rats per experiment was measured in activity cages (Lehigh-Valley Electronics, Inc., Mod. A 1497), the number of interruptions of light beams during 1 h being recorded for 6 consecutive hours.

In parallel series, rats treated as indicated above were killed by decapitation 2 h after administration of L-[2-^{14}C]dopa (specific activity 2·07 mCi/mmol), and the radioactive metabolites of dopa were isolated from the brain and measured as previously described (Bartholini & Pletscher, 1968).

RESULTS

L-Dopa (200 mg/kg) does not markedly affect either the locomotor activity (Fig. 1) or the brain catecholamine concentration. Ro 4–4602 (50 and 500 mg/kg) also has no major influence. However, the combination of 50 mg/kg of the inhibitor with 200 mg/kg of dopa strongly increases the number of movements. A maximum is

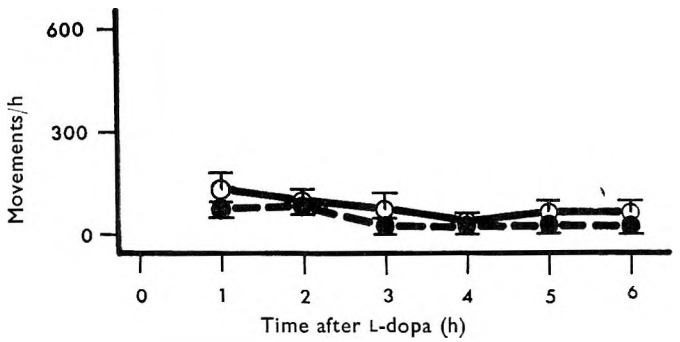


FIG. 1. Effect of L-dopa on locomotor activity of rats. In each experiment, the locomotor activity of 3 rats was measured for 6 h after injection of 200 mg/kg L-dopa i.p. Untreated rats were used as controls. ——— controls; - - - - - - L-dopa. Each value represents an average with s.e. of 3 experiments.

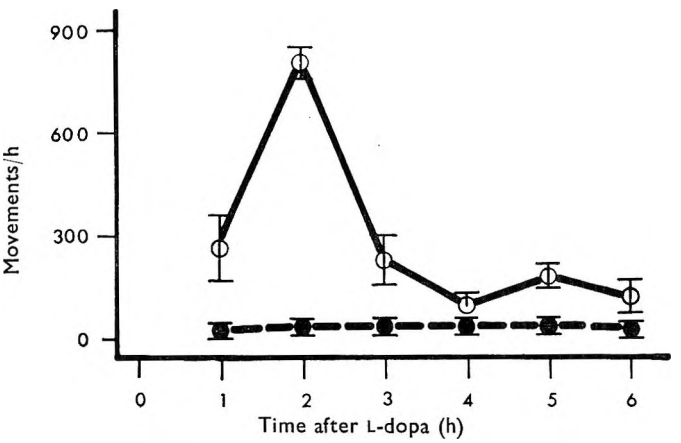


FIG. 2. Effect of two different doses of Ro 4–4602 combined with 200 mg/kg L-dopa on the locomotor activity of rats. Ro 4–4602 (50 or 500 mg/kg) was administered i.p. 30 min before L-dopa i.p. In each experiment, the locomotor activity of 3 rats was measured for 6 h. ——— Ro 4–4602 (50 mg/kg) + dopa; - - - - - - Ro 4–4602 (500 mg/kg) + dopa. Each value represents an average with s.e. of 3 experiments.

reached 2 h after dopa administration (Fig. 2). At the same time, a large rise of catecholamines, dopa and *O*-methyldopa occurs in the brain of animals treated with Ro 4–4602 plus dopa compared with rats injected with dopa alone. The dopa-induced increase of phenolic carboxylic acids is also enhanced by Ro 4–4602, but less markedly than the rise of amino-acids and catecholamines (Table 1).

The administration of 500 mg/kg of Ro 4–4602 followed by 200 mg/kg of dopa does not change the locomotor behaviour of the animals (Fig. 2). Compared to rats treated with dopa alone, only the amino-acid fraction strongly increases after this combination, whereas the catecholamine content does not significantly change

Table 1. *Effect of* L-*dopa and its combination with different doses of Ro* 4–4602 *on the concentration of catechol derivatives in rat brain*

Dopa metabolites	Dopa	Ro 4–4602 (50 mg/kg) + dopa	Ro 4–4602 (500 mg/kg) + dopa
Dopa	0·18 ± 0·03	22·54 ± 1·97	36·13 ± 7·98
O-Methyldopa	2·03 ± 0·13	17·60 ± 0·17	7·65 ± 1·27
Catecholamines	0·59 ± 0·06	3·35 ± 0·25	0·74 ± 0·08
Phenolic carboxylic acids ..	8·12 ± 0·88	13·47 ± 1·15	1·21 ± 0·02

L-[2-^{14}C]Dopa (200 mg/kg, i.p.) was injected alone or 30 min after 50 or 500 mg/kg of Ro 4–4602, i.p., and the animals were killed 2 h later.

The values are expressed in % of the radioactivity injected per gram of body weight and represent averages with s.e. of 3 experiments.

Table 2. *Effect of dopa and Ro* 4–4602 + *dopa on the catechol concentration in brain of reserpinized rats*

Dopa metabolites	Dopa	Ro 4–4602 (50 mg/kg) + dopa
Dopa	0·17 ± 0·01	17·65 ± 1·25
O-Methyldopa	2·30 ± 0·20	26·70 ± 0·50
Catecholamines	0·02 ± 0·00	2·26 ± 0·04
Phenolic carboxylic acids ..	6·60 ± 0·20	17·30 ± 0·50

Reserpine pretreated animals (5 mg/kg i.p. 16½ h before administration of [L-2-^{14}C]dopa) received 50 mg/kg Ro 4–4602 followed after 30 min by 200 mg/kg [L-2-^{14}C]dopa or were given [L-2-^{14}C]dopa only. The animals were killed 2 h after [L-2-^{14}C]dopa.

The values are expressed in percent of the radioactivity injected per gram of body weight and represent averages with s.e of 3 experiments.

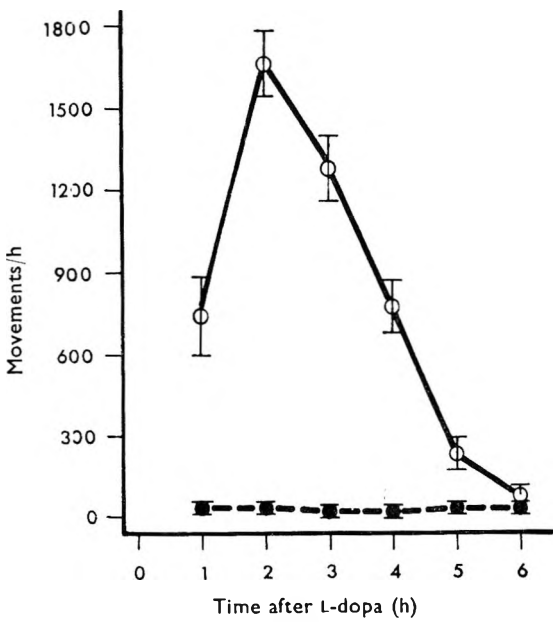


FIG. 3. Effect of dopa and Ro 4–4602 plus dopa on the locomotor activity of reserpinized rats. Reserpine-pretreated animals (5 mg/kg i.p. 16½ h before administration of dopa) received 50 mg/kg Ro 4–4602 followed after 30 min by 200 mg/kg L-dopa, or were given L-dopa only. In each experiment, the locomotor activity of 3 rats was measured for 6 h. ——— Reserpine + Ro 4–4602 + dopa; - - - - - - reserpine + dopa. Each value represents an average with s.e. of 3 experiments.

($P > 0{\cdot}05$), and the phenolic carboxylic acid fraction considerably decreases (Table 1). The ratio of dopa to 3-*O*-methyldopa is higher than in the experiments in which 50 mg/kg of Ro 4–4602 plus dopa were used (Table 1).

The well-known reserpine-induced depression of the locomotor activity is not modified by 50 mg/kg of Ro 4–4602 or by 200 mg/kg of dopa, whereas the combination of the two substances induces a marked hyperactivity for at least 2 h. This increase in locomotor activity is greater and longer-lasting than that induced by the combination of Ro 4–4602 plus dopa without reserpine pretreatment (compare Figs 2 and 3). In the brain, the amino-acids, catecholamines and phenolic carboxylic acids are much increased compared to their levels in reserpinized rats administered dopa alone (Table 2).

DISCUSSION

The present experiments confirm previous findings that 50 mg/kg of Ro 4–4602, intraperitoneally, strongly enhances the dopa-induced rise of amino-acids in the brain as well as of the catecholamines and their breakdown products, the phenolic carboxylic acids (Bartholini & others, 1967; Bartholini & Pletscher, 1968). Furthermore, they show that administration of 500 mg/kg of Ro 4–4602 plus dopa causes only the amino-acid fraction to increase. The relatively marked rise of dopa, compared to 3-*O*-methyldopa, possibly indicates a partial inhibition of catechol-*O*-methyltransferase by the high doses of Ro 4–4602.

As mentioned above, the action of small doses of Ro 4–4602 (50 mg/kg) on the metabolism of dopa is probably due to a selective inhibition of extracerebral dopa decarboxylase in consequence of which dopa, 3-*O*-methyldopa, catecholamines and phenolic carboxylic acids accumulate in the brain. High doses of Ro 4–4602 (500 mg/kg) also inhibit the cerebral dopa decarboxylase enhancing the dopa-induced accumulation of amino-acids, especially dopa, without major formation of catecholamines and phenolic carboxylic acids in the brain (Bartholini, Tissot & Pletscher, 1968).

The marked increase of locomotor activity induced by dopa after pretreatment with 50 mg/kg Ro 4–4602 is in agreement with previous findings according to which low doses of Ro 4–4602 + 200 mg/kg of dopa markedly increased lever pressing by rats in a continuous avoidance experiment (Scheckel, Boff & Pazery, 1965). The locomotor activation seems to be caused by the cerebral accumulation of catecholamines rather than of dopa. Thus, after high doses (500 mg/kg) of Ro 4–4602 plus dopa, the level of dopa in the brain is at least as high as after the combination with the low dose of Ro 4–4602, but no increase of locomotor activity occurs nor are the cerebral catecholamines elevated. A peripheral effect of catecholamines can be excluded since it has been previously shown (Bartholini & Pletscher, 1968) that the rise of the catecholamines as well as of the phenolic carboxylic acids in the blood is markedly less pronounced after the combination than after dopa alone. The latter, in the doses used in the present experiments, does not enhance locomotor activity.

The more marked and prolonged hyperactivity induced by 50 mg/kg Ro 4–4602 plus dopa in reserpinized, compared to non-reserpinized, rats is not due to a higher increase of the total catecholamines in the brain (compare Table 1 and 2). It may, however be the consequence of an enhanced catecholamine concentration at the receptor sites after reserpine pretreatment. Thus, reserpine probably inhibits the uptake of newly formed catecholamines into the storage granules where the amines are protected from

monoamine oxidase (Carlsson, 1966). The significant increase of phenolic carboxylic acids after reserpine pretreatment compared to non-reserpinized controls (compare Table 1 to Table 2) is compatible with this view since this finding indicates an increased exposure of catecholamines to monoamine oxidase.

It has previously been shown that low doses of Ro 4–4602 plus dopa increase the cerebral dopamine much more than the noradrenaline (Bartholini & Pletscher, 1968) Preliminary experiments show that, in the present experiments with non-reserpinized animals, the dopamine and noradrenaline rose by a factor 7 and 1·5 respectively with the combination Ro 4–4602 (50 mg/kg) plus dopa compared to dopa alone. It may therefore be assumed that the increase of cerebral dopamine is a major causative factor in the enhancement of locomotor activity although a possible role of noradrenaline cannot be excluded. The enhanced locomotor activity might also be connected with a liberation of endogeneous cerebral monoamines, e.g. by the dopamine formed from the exogenous dopa. Thus, Ro 4–4602 (50 mg/kg) plus dopa (25–200 mg/kg) decrease the 5-hydroxytryptamine (Bartholini, Da Prada & Pletscher, 1968) and according to preliminary results also the noradrenaline content of the brain. A major involvement of endogenous monoamines is, however, unlikely since locomotor stimulation also occurs in animals whose endogenous monoamine depots have been depleted by previous reserpinization.

REFERENCES

BARTHOLINI, G., BATES, H. M., BURKARD, W. P. & PLETSCHER, A. (1967). *Nature, Lond.*, **215**. 852–853.

BARTHOLINI, G. & PLETSCHER, A. (1968). *J. Pharmac. exp. Ther.*, **161**, 14–20.

BARTHOLINI, G., TISSOT, R. & PLETSCHER, A. (1968). *Proc. 3rd Symp. on Parkinson's Disease*, Edinburgh, May 20–22, in the press.

BARTHOLINI, G., DA PRADA, M. & PLETSCHER, A. (1968). *J. Pharm. Pharmac.*, **20**, 228–229.

CARLSSON, A. (1966). *Handbook of experimental Pharmacology*, vol. XIX: 5-Hydroxytryptamine and related compounds, pp. 529–592. Berlin: Springer.

CONSTANDINIDIS, J., BARTHOLINI, G., TISSOT, R. & PLETSCHER, A. (1968). *Experientia*, **24**, 130–131.

SCHECKEL, C. L., BOFF, E. & PAZERY, L. M. (1965). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **24**, 195.

J. Pharm. Pharmac., 1969, **21**, 302–308 *Received November* 22, 1968

The influence of ATP in brain extracts on the estimation of acetylcholine assayed on the frog rectus abdominis muscle

I. H. STOCKLEY

Pharmacy Department, University of Nottingham, Nottingham, England

Comparative studies of the sensitizing properties of creatine phosphate, ATP and a trichloroacetic acid brain extract on the acetylcholine-induced contractures of the frog rectus muscle indicate that alkaline boiling increases the sensitizations due to these substances threefold. This increase is reflected in a 25% reduction in the values for acetylcholine in brain extracts when assayed on the rectus muscle using Feldberg's method. Since tissue extracts made with acid-alcohol, formic acid-acetone, eserine-saline and sucrose-saline also contain sensitizing substances, the acetylcholine values obtained by this assay method may be in error.

Because substances present in brain extracts increase the contracture of the frog rectus muscle produced by acetylcholine, Feldberg (1945) introduced a modification of the assay of acetylcholine to allow for their presence. This involved boiling part of the brain extract briefly in alkali to destroy the acetylcholine present, and after neutralization, adding a known amount of acetylcholine. The untreated extract was then assayed against the boiled extract on the rectus muscle using a bracketing dose technique. The substances responsible for the increase in contracture appear to be adenosine triphosphate and creatine phosphate (Babskii, Koronevskaya & Minaev, 1945; Feldberg & Hebb, 1947; Babskii & Minaev, 1946a, b, c; 1947 a, b; Minaev, 1947; Golubtsova & Minaev, 1947).

In recent experiments made in this department, results have been obtained which suggest that Feldberg's modification introduces an error into the assay which is as big as that it was designed to obviate. The experimental evidence on which this observation is based forms the content of this report.

EXPERIMENTAL

Preparation of tissue extracts

Mice, guinea-pigs, rats and rabbits were killed either by submersion in liquid oxygen (or nitrogen), or by decapitation under sodium pentobarbitone anaesthesia. The anaesthetic was given by the intraperitoneal route in doses sufficient to cause deep anaesthesia as evidenced by the loss of all somatic reflexes. The brain tissue was removed immediately at death, weighed and extracted with either trichloroacetic acid, acid-alcohol, or formic acid-acetone, according to the methods of Crossland, Pappius & Elliott (1955), Stone (1955) and Toru & Aprison (1966) respectively.

Frozen brain tissue from animals killed by submersion in liquid oxygen was subjected to similar extraction procedures after first being powdered in a cooled steel anvil and homogenized with the extraction medium in a cooled glass mortar. The dissection was made quickly to minimize tissue autolysis. All extracts were

adjusted to pH 7 before assay, those being examined solely for brain tissue sensitizer were boiled for 3 min at pH 11 to destroy the acetylcholine present. Solutions (0·1N) of NaOH and HCl were used in conjunction with BDH Universal indicator paper for pH adjustment.

Unless otherwise stated, the expression "brain tissue sensitizer" refers to trichloroacetic acid extracts of brain which had been subjected to the alkaline boiling procedure.

The assay of ATP and creatine phosphate

The assay of ATP and creatine phosphate in brain extracts was according to Lepage (1957) and is based on a comparison of the extinctions at 680 nm of test and control solutions which had been treated with ammonium molybdate solution and Fiske and Subarrows reagent.

Bioassay of brain tissue sensitizer

The frog rectus muscle was suspended in a 3 ml bath in amphibian Ringer-Locke solution (NaCl 6·5, $CaCl_2$ 0·12, NaH_2PO_4 0·01, KCl 0·14 g/litre of water) containing neostigmine sulphate in a concentration of 1 : 1,000,000. Muscle contractures were recorded on a smoked drum using a gimbal lever with a tenfold magnification. Only muscles showing a similar sensitivity were employed for assay, namely those responding to 0·03 μg/ml acetylcholine with magnified contractions of approximately 1–2 cm. The per cent sensitization remains the same for a constant dose of sensitizer when estimated on acetylcholine-induced contractures which are less than 50% of maximal. Under these same conditions the dose-response relation of sensitizer is linear. The same dose of acetylcholine was administered to the muscle on a regular cycle (within the range 4–10 min depending on the relaxation time of the muscle) and kept in the bath for 1 min. Bioassay was by administering the acetylcholine and sensitizer simultaneously, the per cent sensitization being calculated by comparing the size of the sensitized contracture with the mean of the previous and subsequent unsensitized contractures. When for example a contraction of 2 cm is increased to 2·4 cm by the sensitizer, it is said to be a 20% sensitization. Aliquots of 1 ml of diluted extract containing the equivalent of approximately 100 mg of fresh tissue were used when assaying both for acetylcholine and for the substances causing sensitization.

Bioassay of acetylcholine

Bioassays of acetylcholine were made on the isolated frog rectus muscle sensitized with neostigmine sulphate using a bracketing dose technique, the method of Feldberg (1945) being used to allow for the presence of sensitizing substances.

RESULTS

A comparative examination of ATP, creatine phosphate and brain tissue sensitizer

Brain tissue sensitizer potentiated (or sensitized) the contracture of the frog rectus abdominis muscle produced by acetylcholine but had no direct stimulant action of its own. The sensitization was immediate and maximal on the first acetylcholine-induced contracture (Fig. 1). This sensitization appears to be of a different type from that caused by cerebrospinal fluid, according to the reports of Bhattacharya, Feldberg & Vogt (1957) and Ramwell (1964). This latter sensitization was only

evident after a 60–90s latent period and became maximal on the second or third contraction.

Solutions of ATP sensitized the acetylcholine-induced contracture of the frog rectus muscle, thus confirming the observations of Babskii & others (1945). Similar experiments failed to confirm the reports of Torda & Wolff (1945, 1946) that creatine phosphate has a slight but definite sensitizing effect.

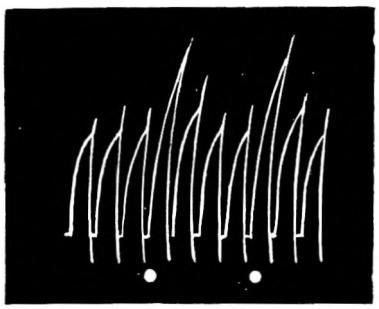


FIG. 1. Contractions of a frog rectus muscle to 0·03 μg/ml acetylcholine in the presence of neostigmine sulphate 1 : 1,000,000. The sensitizing effect of brain tissue sensitizer, the equivalent of 100 mg of fresh brain is shown at the dots.

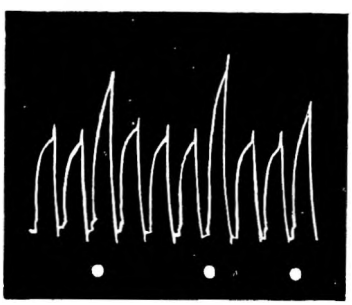
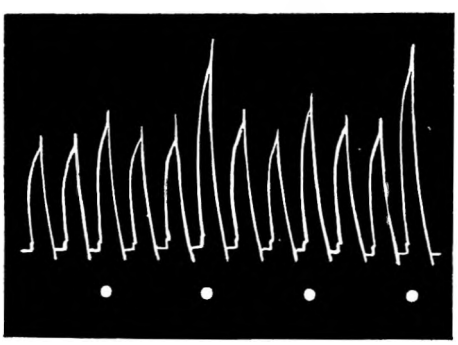


FIG. 2(a). The effects of 90 μg ATP (A), 90 μg ATP + 250 μg creatine phosphate (B), and 250 μg creatine phosphate (C) on the acetylcholine-induced frog rectus muscle contracture. All three solutions had been boiled in alkali for 3 min and reneutralized.



(b). The effects of 100 μg ATP (D) and 100 μg ATP which had been boiled for 3 min at pH 11 and reneutralized (E) on acetylcholine-induced frog rectus muscle contracture. The muscles were responding to 0·06 μg/ml acetylcholine in the presence of neostigmine sulphate 1 : 1,000,000.

Since the preparation of brain tissue sensitizer involves boiling the extract at pH 11 for 3 min to destroy the acetylcholine which is extracted by the trichloroacetic acid, further experiments were made on solutions of ATP and of creatine phosphate that had been submitted to the alkaline boiling procedure. Biological assay showed that creatine phosphate now caused slight sensitization, and the effect of ATP was much increased by alkaline boiling. Frog-Ringer solution subjected to the alkaline boiling procedure was found not to sensitize the contracture of the rectus. There was a marked similarity between the character of the sensitizations induced on the one hand by brain tissue sensitizer and on the other hand by ATP with creatine phosphate. The sensitizations were immediate with their maximal effect on the first acetylcholine-induced contraction, with diminishing effects on the subsequent contractions (Figs 2a, b). A small difference was that the sensitizing effects of the brain extract persisted longer than those of the ATP solutions.

Pooled rat brain tissue was assayed for ATP and creatine phosphate using the method of Lepage (1957). Based on the figures obtained, solutions were made containing equivalent amounts of ATP and creatine phosphate, and both these and the brain tissue extract were assayed for sensitization on the frog rectus muscle. The mean sensitization induced by a brain extract containing 100 μg ATP and 62 μg creatine phosphate was 60%, compared with a sensitization of 66% induced by a solution containing equivalent amounts of ATP and creatine phosphate.

The effect of alkaline boiling on the sensitization caused by ATP and brain tissue sensitizer

Solutions of authentic ATP were assayed for sensitizing activity both before and after brief alkaline boiling. Concurrent assays were also made using a brain tissue

Table 1. *Sensitizations of the frog rectus muscle contracture in the presence of either* 100 μg *ATP or an extract from* 100 *mg of brain tissue containing sensitizer*

Sensitizations due to ATP

Muscle No.	% sensitization: Before alkaline boiling	% sensitization: After alkaline boiling	Increase in sensitization
I	36	91	2·5
II	23	75	3·3
II	26	63	2·4
III	26	73	2·8
III	20	68	3·4
Mean values	26	74	2·8

Sensitizations due to brain tissue sensitizer

Muscle No.	% sensitization: Before alkaline boiling	% sensitization: After alkaline boiling	Increase in sensitization
I	33	90	2·7
I	31	107	3·4
II	38	93	2·4
II	28	89	3·1
III	25	65	2·6
Mean values	31	89	2·9

extract which contained too little acetylcholine to cause contraction of the rectus muscle when the equivalent of 100 mg brain was added to the organ bath. The results of the assays are in Table 1 from which it can be seen that alkaline boiling increased the sensitization in both cases by a factor of almost 3. This increase in sensitization is shown in Fig. 2b.

Feldberg (1945) does not state precisely the conditions for alkaline boiling but it was found that raising the temperature to 100° only momentarily was sufficient to cause a marked increase in sensitization.

The effect of the increase in sensitization due to the alkaline boiling on the assay of acetylcholine in brain tissue extracts

The results of the previous experiments indicated that the three-fold increase in the sensitizing activity of brain tissue extracts when boiling briefly in alkali would be reflected in a reduction in the values for acetylcholine obtained when using Feldberg's acetylcholine assay modification (1945). To confirm this experimentally, assays were made on a series of brain extracts (see Table 2) using Feldberg's method.

Table 2. *The results of acetylcholine assays made on the frog rectus muscle using the Feldberg* (1945) *method. The amounts of acetylcholine added to each extract and the amounts recovered are placed alongside one another*

Animals and method of killing	(i) Acetylcholine content of extract (μg/g brain)	(ii) Acetylcholine added to extract (μg/g brain)	(iii) Acetylcholine recovered (μg/g brain)	(iv) % recovery of added (col. ii) acetylcholine
Liquid nitrogen Rats 1 and 2 ..	0·75	1·68	1·16	69
Liquid nitrogen Rats 3 and 4 ..	1·23	1·11	0·95	86
Decapitation Rats 5 and 6 ..	1·80	0·77	0·57	74
Decapitation Rats 7 and 8 ..	1·38	1·45	1·02	70
				Mean: 75

Known amounts of acetylcholine were then added to the extracts and each was re-assayed by the same method. The amounts of added acetylcholine were not revealed to the assayist until the assays had been made. The mean percentage recovery of the added acetylcholine was 75%. This is in agreement with a theoretical expectation of an approximately 70% recovery which may be derived from a consideration of the probable effect of a threefold increase in sensitization due to alkaline boiling. For example, it is usual when assaying extracts for acetylcholine to add the equivalent of about 100 mg of fresh brain to the rectus muscle in a 3 ml organ bath. This amount of extract would contain about 0·05–0·2 μg of acetylcholine, and about 100 μg of ATP, sufficient to cause a 20–25% sensitization. The alkaline boiling would increase this to 60–70% approximately. Under these circumstances, 0·1 μg (say) of acetylcholine with unboiled sensitizer would cause the same sized rectus muscle contracture as would be obtained with approximately 0·07 μg of acetylcholine in the presence of the same amount of boiled sensitizer.

Preliminary investigations were made on the presence of ATP in extracts of brain tissue made with media other than trichloroacetic acid. Extracts of equivalent amounts of brain tissue made with acid-alcohol (Stone, 1955) and formic acid-acetone (Toru & Aprison, 1966) were found to induce sensitizations of the same order as those induced by TCA extracts.

DISCUSSION

The experiments described in this paper add experimental evidence to the suggestion made by Feldberg & Hebb (1947) that the sensitizing substances in brain extracts might be ATP and creatine phosphate. In addition they support the results of experiments of Minaev, Babskii, Koronevskaya and Golubtsova (Babskii & others, 1945; Babskii & Minaev, 1946a, b, c; 1947a, b; Minaev, 1947; Golubtsova & Minaev 1947).

The large increase in the sensitizing activities of brain tissue extracts and solutions of ATP after brief alkaline boiling seems to cast some doubt on the accuracy of those acetylcholine assays made on the frog rectus muscle using Feldberg's method (1945). This method appears to have been accepted uncritically and used widely even by the Russian workers who observed that '. . . exposure (of the rectus muscle) to acetylcholine with unboiled, but especially with boiled (brain tissue) emulsion caused greater contraction of the muscle than with acetylcholine alone'. (Golubtsova & Minaev, 1947). This is all the more surprising since Babskii & Minaev (1946) had at that time published a method for the assay of acetylcholine on the rectus muscle which allowed for the presence of sensitizing substances and which appears to be virtually identical with Feldberg's method (1945).

In view of the experimental evidence already described, it is probable that all the acetylcholine assays which have been made using the rectus muscle on aqueous extracts of brain (and other tissues which contain significant amounts of ATP) may be considerably in error. Aqueous extraction media known to extract sensitizing substances from tissues include trichloroacetic acid, acid-alcohol, formic acid-acetone, eserine-saline (Slater, 1966) and eserine-sucrose (Slater, 1966). The sensitizing activity of creatine phosphate on the other hand is small compared with ATP and it would seem unlikely to cause an error of any magnitude.

ATP is relatively stable to alkaline but not to acid boiling. Hock & Huber (1956) reported that when ATP is boiled for 1 h at pH 10·5, only some 30% is destroyed. The breakdown products of alkaline hydrolysis are ADP, AMP and orthophosphate, none of which together or individually induce a sensitization greater than ATP. Acid hydrolysis is much more rapid and liberates 2 of the 3 phosphates as inorganic phosphate with the production of adenine and ribose-5-phosphate (Tsujimoto & Yamaba, 1956; Michelson, 1963) none of which induce sensitization (Torda & Wolff, 1946). With the experimental evidence at present available it is only possible to make the most tentative suggestions about the cause of the increased activity of ATP when boiled in alkali. For example, it might result from an osmotic effect due to the HCl and NaOH added to the extract, or an ionic imbalance which increases the uptake of ATP by the muscle. These and other possibilities are the basis of further investigations into the phenomenon of sensitization by ATP of the rectus muscle contracture.

Acknowledgement

My thanks are due to Dr. J. Crossland for his advice and help in this investigation.

REFERENCES

BABSKII, E. B., KORONEVSKAYA, O. G. & MINAEV, P. F. (1945). *Bull. biol. Med. exp. U.R.S.S.*, **20**, 60–62.

BABSKII, E. B. & MINAEV, P. F. (1946a). *Nature, Lond.*, **158**, 238.

BABSKII, E. B. & MINAEV, P. F. (1946b). *Ibid.*, **158**, 268.

BABSKII, E. B. & MINAEV, P. F. (1946c). *Bull. biol. Med. exp. U.R.S.S.*, **22**, 5–8.

BABSKII, E. B. & MINAEV, P. F. (1947a). *Biokhimiya*, **12**, 231–240.

BABSKII, E. B. & MINAEV, P. F. (1947b). *Bull. biol. Med. exp. U.R.S.S.*, **23**, 98–101.

BHATTACHARYA, B. K., FELDBERG, W. & VOGT, W. (1957). *J. Physiol. Lond.*, **137**, 460–472.

CROSSLAND, J., PAPPIUS, H. M. & ELLIOT, K. A. C. (1955). *Am. J. Physiol.*, **183**, 27–31.

FELDBERG, W. (1945). *J. Physiol. Lond.*, **103**, 367–401.

FELDBERG, W. & HEBB, C. (1947). *Ibid.*, **106**, 8–17.

GOLUBTSOVA, A. V. & MINAEV, P. F. (1947). *Bull. biol. Med. exp. U.R.S.S.*, **23**, 169–172.

HOCK, A. & HUBER, G. (1956). *Biochem. Z.*, **328**, 44–55.

LEPAGE, G. A. (1957). *Manometric Techniques and Tissue Metabolism*, 2nd edn p. 268–281. Editors: Umbreit, W. W., Burris, R. H. & Stauffer, J. J. Minneapolis: Burgess.

MICHELSON, A. M. (1963). *The Chemistry of Nucleosides and Nucleotides*. 1st edn p. 154. New York: Academic Press.

MINAEV, P. F. (1947). *Bull. biol. Med. exp. U.R.S.S.*, **24**, 290–292.

RAMWELL, P. W. (1964). *J. Physiol. Lond.*, **170**, 21–38.

SLATER, P. (1966). *Ph.D. thesis, Nottingham University*, p. 38.

STONE, W. E. (1955). *Archs Biochem. Biophys.*, **59**, 181–192.

TORDA, C. & WOLFF, H. G. (1945). *Proc. Soc. exp. Biol. Med.*, **58**, 29–31.

TORDA, C. & WOLFF, H. G. (1946). *Am. J. Physiol.*, **145**, 419–429.

TORU, M. & APRISON, M. H. (1966). *J. Neurochem.*, **13**, 1533–1544.

TSUJIMOTO, T. & YAMABA, T. (1956). *Wakayama. Med. rept.*, **3**, 77–83.

J. Pharm. Pharmac., 1969, **21**, 309–313 *Received November* 4, 1968

The bronchodilator action of analeptics in the guinea-pig

A. W. LESSIN AND R. L. KRAMER

Research Department, Roche Products Ltd., Welwyn Garden City, Herts., England

Picrotoxin, leptazol, nikethamide and bemegride were found to antagonize the bronchoconstrictor effects of acetyl-β-methylcholine in the guinea-pig anaesthetized with urethane. The analeptics were also active in pithed animals but not in animals that had been adrenalectomized or pretreated with propranolol. Catecholamine release from the adrenals can account for the effects observed.

A given total respiratory ventilation can be achieved by a wide variety of combinations of tidal volume and frequency. The optimal breathing frequency for a given ventilation may be defined as the one that leads to the least energy cost. That this frequency is the one chosen has been confirmed in man and various animals (Milic-Emili & Petit, 1959; Agnostoni, Thimm & Fenn, 1959; Crosfill & Widdicombe, 1961). The equations used in calculations of optimal frequency involve a number of factors including the flow-resistive properties of the system (Otis, Fenn & Rahn, 1950).

It might therefore be predicted that a change in the flow-resistive properties of the system would accompany changes in the ventilation pattern, namely alterations in tidal volume and frequency. That such alterations do occur after bronchodilator or bronchoconstrictor agents has often been reported (Stroud, Lambertsen & others 1955; Dautrebande, Philippot & others, 1942a, b). Conversely one might postulate that analeptic agents, which have a well-defined effect upon respiratory rate and frequency (Dautrebande & Stolport, 1948; Marshall, Walzl & Le Mesurier, 1937), might also have effects upon the diameter of the bronchioles; but the action of none of these drugs has ever been reported, except in the guinea-pig isolated perfused lung (Warnant, 1930).

In preliminary plethysmographic experiments using conscious rabbits we have consistently observed bronchodilator effects with picrotoxin. This agent, together with other analeptics, have consequently been examined to assess their direct bronchodilator effects uncomplicated by alterations of frequency and tidal volume, which always characterize the actions of these agents in the intact animal. For this purpose the Konzett-Rössler preparation of the guinea-pig was used.

EXPERIMENTAL

Method

The method was based on that of Konzett & Rössler (1940) and modified as suggested by Collier, Holgate & others (1960). Electrical recording was used and the piston recorder replaced by a pneumotachograph.

Animals of 300–350 g were anaesthetized with urethane (1·5–2·5 g/kg, i.p.), further doses being administered when necessary to abolish spontaneous respiratory movements.

The trachea was cannulated and inflated by means of a pump delivering about 5 ml stroke volume at 72 strokes/min. A side arm from the cannula allowed about 2 ml of air to escape through a water valve offering a resistance of 10 cm water, the flow being measured by means of the pneumotachograph connected to a sensitive differential pressure transducer. Increases of overflow volume are therefore represented as an increased excurison on the recorder. Bilateral vagotomy was carried out since this seemed to make the response of the preparation more consistent. In some experiments animals were also adrenalectomized or pithed.

Solutions of drugs in saline were injected into the external jugular vein at regular intervals. In all experiments acetyl-β-methylcholine (methacholine) (3–8 μg/kg, i.v.) was used to produce an increase in resistance to inflation. Isoprenaline and papaverine were also used as reference drugs. Doses are of the bases.

RESULTS

Picrotoxin (1·0–3·0 mg/kg, i.v.) consistently reduced the increased resistance to inflation produced by methacholine (Fig. 1). This effect of picrotoxin lasted up to 2 h when the larger doses were used. Isoprenaline (4 μg/kg) and papaverine (2 mg/kg) were also effective though their effects only lasted 10–15 min.

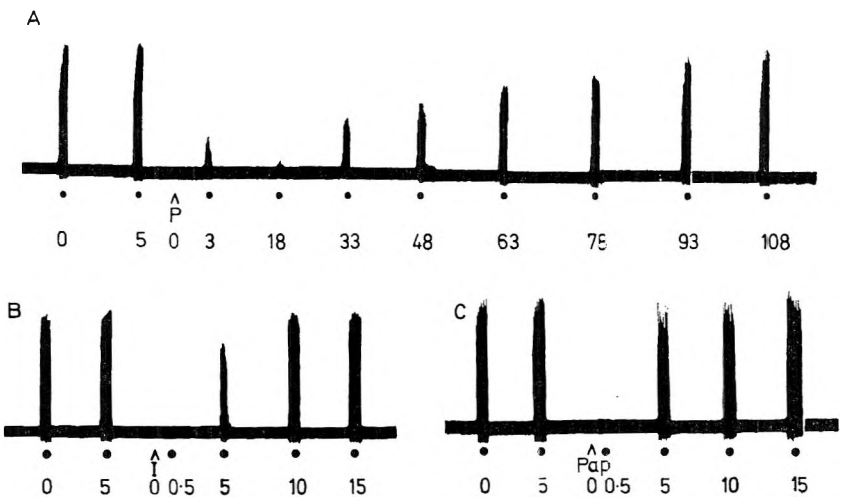


FIG. 1. Vagotomized guinea-pig. Increased resistance to inflation caused by methacholine and its reduction by picrotoxin (P), isoprenaline (I) and papaverine HCl (Pap). Doses were administered intravenously at the intervals shown (min).

In pithed animals the effects of picrotoxin were still apparent (Fig. 2), in fact there was a tendency for the effects of this agent to be more pronounced and of longer duration in these preparations. Also effective in the pithed animal were isoprenaline and papaverine.

However, in the adrenalectomized animal, picrotoxin was ineffective in reducing the increase in resistance produced by methacholine (Fig. 3) though isoprenaline and papaverine were still able to do so.

As may be seen in Fig. 4, pretreating the animals with a β-sympathetic blocker (propranolol, 1·5 mg/kg, i.v.) nullified the effects of both picrotoxin and isoprenaline. The effects of papaverine were perhaps slightly reduced.

Leptazol (10 mg/kg, i.v.), bemegride (10 mg/kg, i.v.) and nikethamide (100 mg/kg, i.v.) were examined in the guinea-pig preparation and found to abolish the responses to methacholine (Fig. 5) but, in the animals given propranolol (1·5 mg/kg, i.v.) these three analeptic agents agents were no longer effective (Fig. 6).

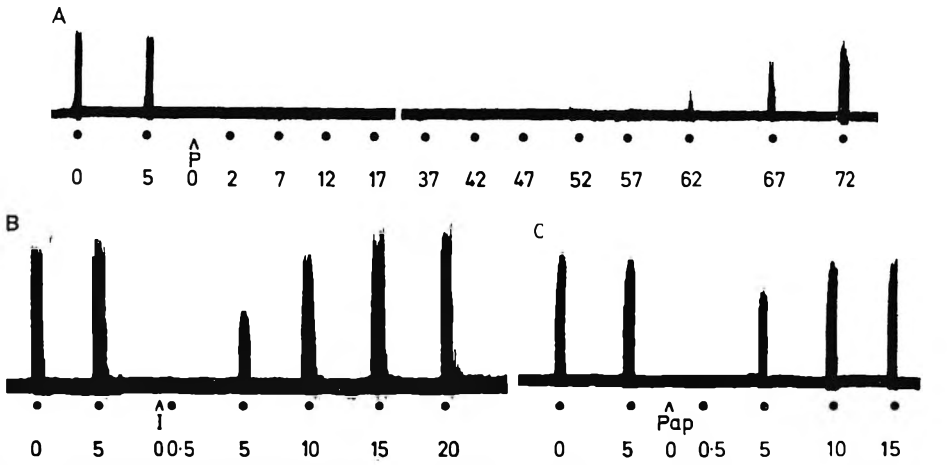


FIG. 2. Vagotomized, pithed guinea-pig. Increased resistance to inflation caused by methacholine and its reduction by picrotoxin (P), isoprenaline (I) and papaverine (Pap). Doses were administered intravenously at the intervals shown (min).

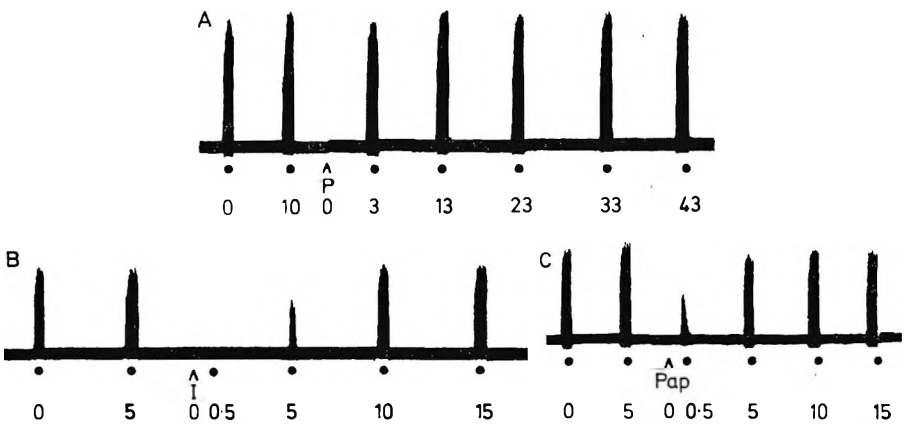


FIG. 3. Vagotomized and adrenalectomized guinea-pig. Increased resistance to inflation caused by methylcholine and to modification by picrotoxin (P), isoprenaline (I) and papaverine (Pap). Doses were administered intravenously at intervals shown (min).

FIG. 4. Vagotomized guinea-pigs. Increased resistance to inflation caused by methylcholine and its modification by picrotoxin (P), isoprenaline (I) and papaverine (Pap) in animals given propranolol (Pro). Doses were administered intravenously at the intervals shown (min).

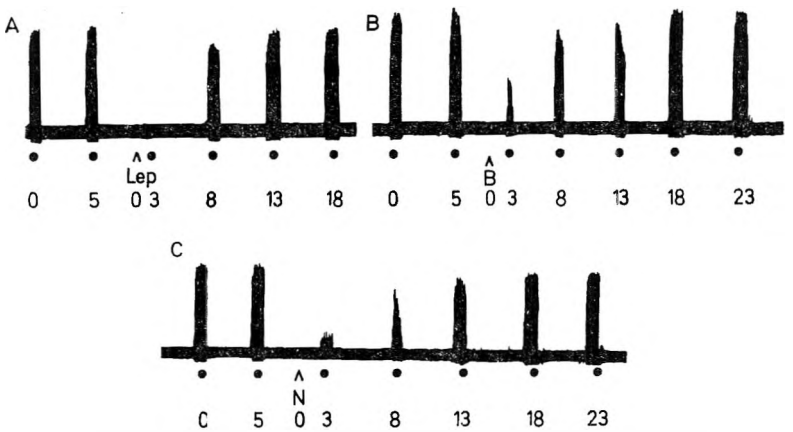


FIG. 5. Vagotomized guinea-pig. Increased resistance to inflation caused by methylcholine and its reduction by leptazol (Lep), bemegride (B) and niketha:nide (N). Doses were administered intravenously at the intervals shown (min).

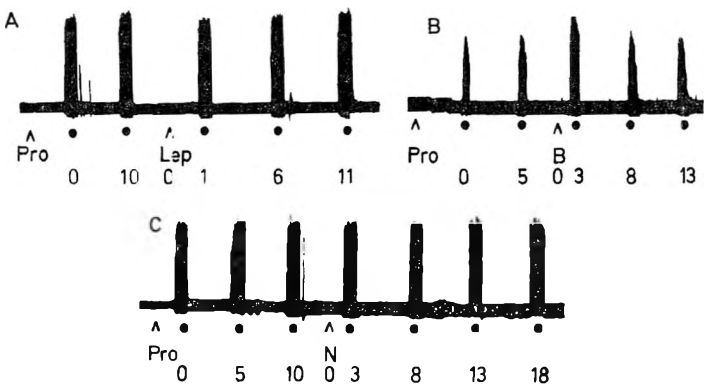


FIG. 6. Vagotomized guinea-pigs. Increased resistance to inflation caused by methylcholine and its persistence after leptazol (Lep), bemegride (B) and niketha:nide (N) in animals given propranolol (Pro). Doses were administered intravenously at the intervals shown (min).

DISCUSSION

The experiments indicate that picrotoxin, leptazol, bemegride and nikethamide can exert a "bronchodilator" effect, as measured in the Konzett-Rössler preparation, and that, moreover, their potency ratios in this test situation are similar to their activities as convulsive agents (Hahn, 1941) which might suggest that they are exerting a central action. However, since these agents are still active in the pithed animal, central properties cannot be invoked to explain these results, and some peripheral mechanism must therefore be sought.

The absence of activity when using these analeptics in adrenalectomized or propranolol-pretreated animals suggests that the analeptic drugs tested exert their effects on the lungs by releasing catecholamines from the adrenals. Such a concept is in accord with the findings of Vogt (1954) who has already shown that picrotoxin in convulsive doses depletes the adrenal medulla of its adrenaline, though she did not observe the same depleting effect using leptazol. Nevertheless, the failure of leptazol to deplete does not necessarily imply that this drug does not release adrenaline, since depletion might only occur after large repeated doses which might not have been

realized in her experiments. Certainly the pressor effects of leptazol in dogs can be reduced by adrenalectomy and Cicardo (1954) concluded that release of catecholamines was involved.

As far as the experiments on guinea-pigs are concerned, we may conclude that, picrotoxin, leptazol, bemegride and nikethamide all have one property in common, in that they exert an apparent bronchodilator effect by the release of catecholamines from the adrenals.

REFERENCES

AGNOSTINI, E. THIMM, F. F. & FENN, W. O. (1959). *J. appl. Physiol.*, **14**, 679–683.

COLLIER, H. O. J. HOLGATE, J. A., SCHACHTER, M. & SHORLEY, P. G. (1960). *Br. J. Pharmac. Chemother.*, **15**, 290–297.

CICARDO, V. H. (1954). *C.r. Séanc. Soc. Biol.*, **148**, 1639–40.

CROSFILL, M. L. & WIDDICOMBE, I. G. (1961). *J. Physiol., Lond.*, **158**, 1–14.

DAUTREBANDE, L., PHILIPPOT, E., CHARLIER, R., DUMOULIN, E., & NOGARÈDE, F. (1942a). *Archs int. Pharmacodyn. Thér.*, **68**, 117–210.

DAUTREBANDE, L., PHILIPPOT, E., CHARLIER, R., DUMOULIN, E., & NOGARÈDE, F. (1942b). *Ibid.*, **68**, 451–469.

DAUTREBANDE, L. & STALPORT, J. (1948). *Ibid.*, **76**, 213–227.

HAHN, F. (1941). *Arch. exp. Path. Pharmak.*, **198**, 491–508.

KONZETT, H. & RÖSSLER, R. (1940). *Ibid.*, **195**, 71–74.

MARSHALL, E. K. JR., WALZL, E. M. & LE MESURIER, D. D. (1937). *J. Pharmac. exp. Ther.*, **60**, 472–486.

MILIC-EMILI, G. & PETIT, J. M. (1959). *Archs Sci. Biol.*, **43**, 326–330.

OTIS, A. B. FENN, W. O. & RAHN, H. (1950). *J. appl. Physiol.*, **2**, 592–607.

STROUD, M. W., LAMBERTSEN, C. J., EWIG, J. H., KOUGH, R. H., GOULD, R. A. & SCHMIDT, C. F. (1955). *J. Pharmac. exp. Ther.*, **114**, 461–469.

VOGT, M. (1954). *J. Physiol., Lond.*, **123**, 451–481.

WARNANT, H. (1930). *Archs int. Pharmacodyn. Thér.*, **37**, 61–86.

J. Pharm. Pharmac., 1969, **21**, 314–316 *Received October* 1, 1968

Possible oestrogenic and anti-androgenic effects in rats treated with the oral hypoglycaemic agents tolbutamide and acetohexamide

SAMIR FAHMY SAAD

Department of Pharmacology, Faculty of Pharmacy, Cairo University, Cairo, U.A.R.

Tolbutamide has an oestrogenic activity; when administered in four oral daily doses of 125, 250 and 500 mg/kg to ovariectomized young rats, it significantly increased their uterine weight. Tolbutamide, when administered in five oral daily doses to castrated young male rats, decreased the weight of the prostatic tissue; the effect was significant with a dose of 250 mg/kg. Acetohexamide in oral doses of 125, 250 and 500 mg/kg, had no significant effect either on the prostatic or the uterine weight.

Since the discovery of the oral hypoglycaemic agents, much work has been done to investigate their effects on different endocrine systems especially those concerned with the regulation of carbohydrate metabolism. No marked attention has been paid to their effects on sex hormones.

The effect of tolbutamide and acetohexamide on the activities of the gonadal hormones has been examined.

EXPERIMENTAL AND RESULTS

Determination of oestrogenic activity

The activity was determined as described by Bülbring & Burn (1935) using the increase in the uterine weights of ovariectomized rats as a criterion.

Thirty-five young female rats, each weighing about 40 g, were ovariectomized. Two days later, the animals were divided into 7 equal groups. One group, used as control, received no drug. Tolbutamide and acetohexamide were given orally in doses of 125, 250 and 500 mg/kg to respective groups, each dose being administered daily for 4 successive days, the animals were then left for a day, and killed on the following day. Thus if the day of the operation was the first day, drugs were given on the third, fourth, fifth and sixth days, and the animals were killed on the eighth day. The dissected uteri were fixed separately in modified Bouin's solution for 24 h, dried between pieces of filter paper and weighed. The weight of uterus per 100 g of rat was calculated. The results are in Table 1.

Determination of anti-androgenic activity

The activity was determined as described by Burn, Finney & Goodwin (1950). The changes in weight of the prostate and seminal vesicles of castrate rats were taken as criteria. Young male rats were used because, according to Sollmann (1957), they are more sensitive to the effect of oestrogens.

Table 1. *The effect of four oral daily doses of tolbutamide and acetohexamide on uterine weights of ovariectomized young rats compared with controls*

Sample number and statistical data	Weight of uterus (mg/100 g)						
	Controls	Tolbutamide (mg/kg)			Acetohexamide (mg/kg)		
		125	250	500	125	250	500
1	40·0	45·3	66·7	50·2	36·8	38·0	40·6
2	42·4	60·2	70·0	60·0	39·0	40·2	43·7
3	44·0	71·0	78·4	78·9	48·6	45·4	48·9
4	57·0	80·3	90·0	82·5	57·5	60·2	58·5
5	58·2	82·5	92·0	108·7	60·0	65·3	68·9
$\bar{x}$	48·32	67·86	79·42	76·06	48·38	49·82	52·12
± s.e.	3·8459	6·8797	5·1077	10·1050	4·6925	5·4745	5·1798
*P**		s.	s.	s.	n.s.	n.s.	n.s.

* The difference between the mean weight of uteri after treatment, and the mean of the control weight is considered significant if $P = <0{\cdot}05$.

Thirty-five young male rats, each weighing from 30 to 40 g, were castrated, left for 30 days before the experiment, and then divided into 7 equal groups. One group, used as control, received no drug. Tolbutamide and acetohexamide were given orally in doses of 125, 250 and 500 mg/kg, each dose being administered daily for 5 days. The animals were killed on the sixth day, and the seminal vesicles and prostatic tissue dissected. The tissue consists of the cranial lobes that are attached to the seminal vesicles and more specifically the dorso-lateral lobes around the base of the seminal vesicles and almost encircling the urethra. Particular care was taken not to remove the ventral lobes. The organs of each rat were individually fixed overnight in modified Bouin's solution after removing fat. The tissues were dried between pieces of filter paper and weighed. The weight of each prostatic tissue was calculated in mg/100 g of rat. The results are in Table 2.

Table 2. *The effect of five oral daily doses of tolbutamide and acetohexamide on the weight of prostatic tissue of* 30 *day castrated young rats compared with the controls*

Sample number and statistical data	Weight of prostatic tissue (mg/100 g)						
	Controls	Tolbutamide (mg/kg)			Acetohexamide (mg/kg)		
		125	250	500	125	250	500
1	17·5	14·1	13·6	16·3	15·0	15·1	18·8
2	19·5	15·2	15·5	16·4	17·9	20·6	19·9
3	22·1	18·5	17·5	17·1	18·8	24·0	21·2
4	22·4	19·5	20·2	20·4	20·2	24·0	21·8
5	27·8	23·9	21·3	22·6	21·8	25·0	22·9
$\bar{x}$	21·86	18·24	17·62	18·56	18·70	21·74	20·92
± s.e.	1·7352	1·7330	1·4290	1·2574	1·1367	1·8198	0·7179
*P**		n.s.	s.	n.s.	n.s.	n.s.	n.s.

* The difference between the mean weight of prostatic tissue after a certain treatment, and the mean of the control weight is considered significant if $P = <0{\cdot}05$.

DISCUSSION

Because of the wide and prolonged use of tolbutamide and acetohexamide in the treatment of certain types of diabetes, it is of importance to determine whether or not they possess oestrogenic and anti-androgenic effects.

Tolbutamide was given to ovariectomized and castrated young rats in oral doses of 125, 250 and 500 mg/kg. These doses have an effective hypoglycaemic action in rats, and were non-toxic. Mukherjee & De (1958) found that oral administration of tolbutamide in doses of 250, 500 and 1000 mg/kg to rats lowered their blood sugar content. The maximal hypoglycaemic effect was produced by a dose of 500 mg/kg body weight. The oral LD50 of tolbutamide in rats was determined by Scholz & Bänder (1956) to be 4 g/kg, and by Penhos (1957) to be 4–5 g/kg body weight. The oral LD100 of tolbutamide in rats was determined by Penhos (1957) to be 6 g/kg body weight.

During tolbutamide and acetohexamide administrations and at the end of the experiments, the rats were normal in behaviour, not excited or depressed. Their body weight during the period of drug administration did not vary more than $\pm$ 3 g. The prostatic tissue weight and uterine weight were expressed as proportions of body weight to compensate for any difference in animal's body weight.

The results of Table 1 show that tolbutamide when administered orally in doses of 125, 250 and 500 mg/kg body weight to ovariectomized young rats for 4 successive days produced a significant increase in the mean uterine weight compared with the control. This suggests an oestrogenic activity for tolbutamide.

The results in Table 2 show that tolbutamide when administered orally for 5 successive days in doses of 125, 250 and 500 mg/kg to 30 day castrated young rats, reduced the weight of prostatic tissue compared with the control. The effect was significant with a daily oral dose of 250 mg/kg body weight. This points to a possible anti-androgenic effect for tolbutamide, although the decrease in the mean weight of prostatic tissue of castrated young rats may reflect the oestrogenic activity of tolbutamide. According to Sollmann (1957), continued administration of oestrogens produces atrophy of the accessory sex organs including the seminal vesicles in immature animals.

Acetohexamide, as compared with tolbutamide, when similarly tested in ovariectomized and castrated young rats had neither an oestrogenic nor an anti-androgenic activity. This suggests that the actions of tolbutamide on uterine and prostatic tissue is not due to its hypoglycaemic action, but instead reflects a specific secondary pharmacological action.

These hormonal effects of tolbutamide would seem to need clinical evaluation.

REFERENCES

BÜLBRING, E. & BURN, J. H. (1935). *J. Physiol., Lond.*, **85**, 320–333.

BURN, J. H., FINNEY, D. J. & GOODWIN, L. G. (1950). *Biological Standardization*, 2nd edn, p. 262, Oxford: University Press.

MUKHERJEE, S. K. & DE U. N. (1958). *Indian J. med. res.*, **46**, 223–233.

PENHOS, J. C. (1957). *Rev. Soc. Arg. biol.*, **33**, 44–50.

SCHOLZ, J. & BÄNDER, A. B. (1956). *Dt. med. Wschr.*, **81**, 825–825.

SOLLMANN, T. (1957). *A Mcnnual of Pharmacology and its Application to Therapeutics and Toxicology*, 8th edn, p. 615. Philadelphia: Saunders.

J. Pharm. Pharmac., 1969, **21**, 317–322 *Received February* 17, 1968

Interaction between histamine and monoamine oxidase inhibitors

G. S. ALLEN AND M. J. RAND

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

The monoamine oxidase inhibitors tranylcypromine, nialamide and iproniazid potentiated the actions of histamine on the blood pressure of the cat and on the tracheo-bronchial muscle of the guinea-pig. These findings may have relevance to side-effects of treatment with monoamine oxidase inhibitors.

It is well known that interactions occur between monoamine oxidase inhibitors and other drugs or pharmacologically active substances in foodstuffs. Much of the literature on this topic is concerned with the presence of tyramine in foodstuffs and with other sympathomimetic drugs (Blackwell & Marley, 1964). The most common clinical effects resulting from interaction of these substances with monoamine oxidase inhibitors are hypertension and headache. However, several cases have been reported where headache without concurrent hypertension has occurred in patients on monoamine oxidase inhibitors after eating certain foodstuffs (Horwitz, Lovenberg & others, 1964; Rondell, 1967). It has been suggested, on the basis of animal experiments and clinical observations, that potentiation of the actions of histamine by monoamine oxidase inhibitors may explain the occurrence of headache and other symptoms that could be caused by histamine (Blackwell, Morley & Ryle, 1964; Blackwell, Marley & Mabbitt, 1965; Keele, 1966; Cooper, 1967). We have investigated the effects of some monoamine oxidase inhibitors on cardiovascular responses to histamine in anaesthetized cats, and bronchoconstrictor responses to histamine in anaesthetized guinea-pigs.

EXPERIMENTAL

Cats of either sex and of 2–4 kg were anaesthetized with chloralose (100 mg/kg) dissolved in normal saline and injected peritoneally. Blood pressure was measured from the femoral artery with an ether pressure transducer and recorded on a Gilson Polygraph. Heart rate was also recorded using a cardiotachometer coupler connected to electrocardiogram electrodes. Drugs were injected into a femoral vein.

Guinea-pigs, approximately 700 g, were anaesthetized with chloralose (20 mg/kg) in 25% urethane; 2 ml/kg was given intraperitoneally. The effects of drugs on tracheo-bronchial smooth muscle were observed using the methods described by McCulloch, Proctor & Rand (1967).

Nialamide (50–100 mg/kg) and iproniazid (50–100 mg/kg) were used as represenatatives of the hydrazine group of monoamine oxidase inhibitors, and tranylcypromine (20 mg/kg) as a representative of the non-hydrazine group. These doses produced marked potentiation of cardiovascular responses to tyramine and amphetamine (Rand & Trinker, 1968).

RESULTS

In experiments on cats, the responses to a range of doses of histamine were obtained. Then, one of the monoamine oxidase inhibitors was given in a single intravenous injection. In some experiments, half-hourly injections of a single dose of histamine were given to follow the rate of change of the response. The first sign of potentiation of the depressor action of histamine was seen after about 1 h and maximal potentiation occurred about 4 h after injection of monoamine oxidase inhibitors. In all experiments, the responses to doses of histamine were determined again 4 h after the monoamine oxidase inhibitors.

A marked pressor response was observed on intravenous administration of tranylcypromine. The sympathomimetic activity of this drug on the cardiovascular system has been described previously (McCulloch, Trinker & others, 1967). However, this effect wore off and the blood pressure had returned to its control level before 4 h had elapsed.

A marked potentiation of the depressor action of histamine was observed in ten out of the 14 cats treated with a monoamine oxidase inhibitor. The potentiation was greatest after tranylcypromine and least after nialamide. Blackwell, Marley & Taylor (1965) reported that some of the actions of histamine in the cat were potentiated by the monoamine oxidase inhibitor mebanazine, but they gave no details.

The potentiation of depressor responses to histamine in doses ranging from 0·001 to 1·0 μg/kg by tranylcypromine (20 mg/kg) is shown in Fig. 1. Both the extent and the duration of the depressor response to each dose was increased. The increase in potency of histamine caused by tranylcypromine in this experiment, as estimated from the dose-response curves, was approximately 300-fold.

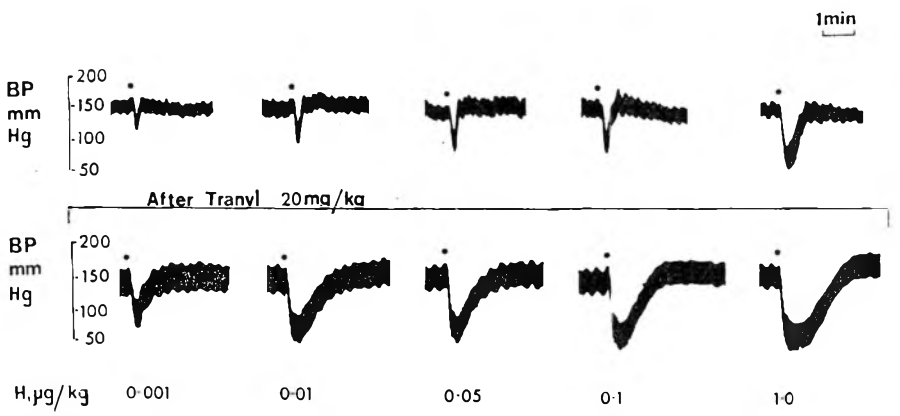


FIG. 1. Records of blood pressure in an anaesthetized cat, 2·4 kg. Histamine was injected intravenously at the points indicated by the dot above each record. The doses are indicated below the records. The upper records were obtained before tranylcypromine. The lower records, obtained 4 h after tranylcypromine, show marked potentiation of the depressor responses to histamine.

The effect of histamine on blood pressure is not always a simple decrease: a biphasic effect consisting of a fall followed by a rise may be seen (for example, in Fig. 3). The secondary pressor response is due to the release of catecholamines, largely from the adrenal medulla, by histamine (Trendelenburg, 1954). In some experiments tranylcypromine not only potentiated the depressor action of histamine,

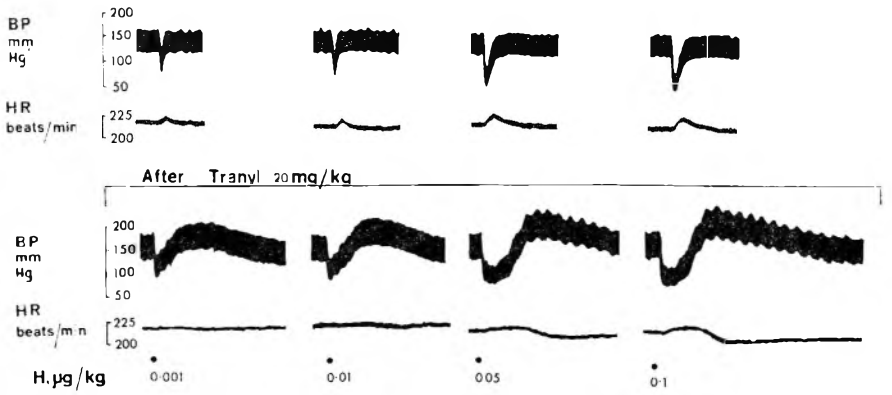


FIG. 2. Records of blood pressure and heart rate in an anaesthetized cat weighing 4·4 kg. 4 h after tranylcypromine, the depressor effect of histamine was enhanced and a secondary pressor phase appeared in the response. Changes in heart rate are in the opposite direction to changes in blood pressure, suggesting they were reflexly induced. Time scale as in Fig. 3.

but also caused the appearance of a secondary pressor effect (see Fig. 2). Tranylcypromine does not potentiate the effects of catecholamines on the cardiovascular system; in fact, it reduces them slightly (Trinker, Fearn & others, 1967; Rand & Trinker, 1968). The appearance of the secondary pressor effect of histamine after tranylcypromine, as shown in Fig. 2, indicates therefore that there was a much increased output of catecholamines.

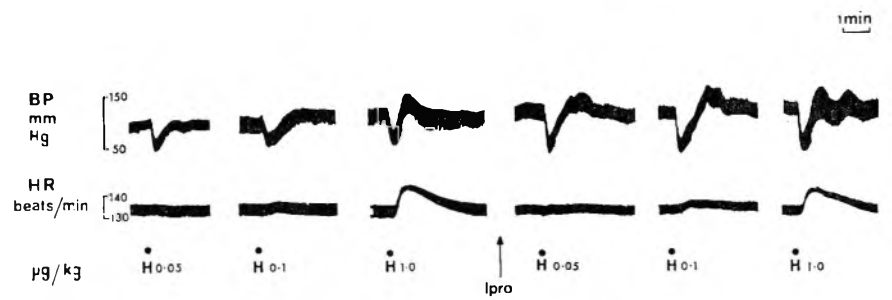


FIG. 3. Records of blood pressure and heart rate in an anaesthetized cat, 2·3 kg. At Ipro, iproniazid (50 mg/kg) was injected intravenously and 4 h later the effects of histamine on the blood pressure were enhanced.

In an experiment with iproniazid (Fig. 3), the control responses to the higher doses of histamine are biphasic. Iproniazid caused increases both in the depressor and pressor phases of the responses.

Histamine produced only slight changes in heart rate and these were not greatly affected by monoamine oxidase inhibitors. The results in Figs 2 and 3 are compatible with the suggestion that changes in heart rate were reflexly induced by the changes in blood pressure.

On account of the complex nature of the action of histamine on the cat blood pressure, and the qualitative change in the response sometimes seen after monoamine oxidase inhibitors, quantitative expression of the results in terms of dose-response is difficult and involves oversimplification of the findings. The results from one experiment in which there was a purely depressor response to histamine both before

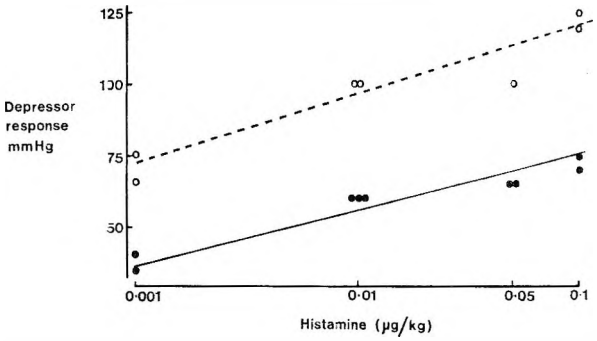


FIG. 4. Log dose-response lines for histamine before and 4 h after 20 mg/kg tranylcypromine in a 2·4 kg cat. The response was the maximal fall in blood pressure from immediately before each injection of histamine. The lines are the calculated regressions. Before tranylcypromine, ● and ——; after tranylcypromine, ○ and ----. Analysis of variance showed that each regression line is significant and they do not differ significantly from parallel. In this experiment there was a 76-fold increase in sensitivity to histamine.

and after tranylcypromine are expressed graphically in Fig. 4, and these data were analysed statistically (see legend).

Bronchospasm produced by histamine in guinea-pigs was enhanced by iproniazid (Fig. 5) or tranylcypromine. The depressor response to histamine in the guinea-pig was not increased by iproniazid (Fig. 5). In contrast, monoamine oxidase inhibitors reduced histamine-induced bronchospasm in the cat although the effects of histamine on blood pressure were markedly enhanced (Fig. 6).

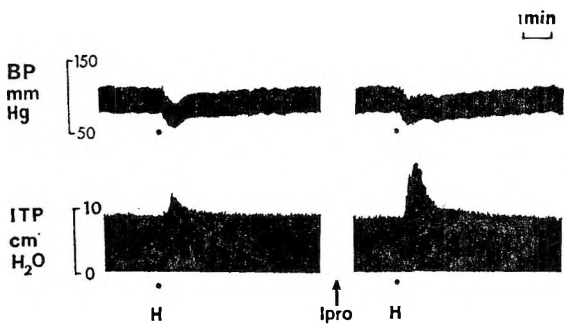


FIG. 5. Records of blood pressure (upper record) and intratracheal pressure (lower record) in an anaesthetized guinea-pig 700 g. At H, histamine was injected intravenously in a dose of 1 mg. At Ipro, iproniazid (50 mg/kg) was injected intravenously, and 4 h later the effect of histamine on intratracheal pressure, but not on blood pressure, was enhanced.

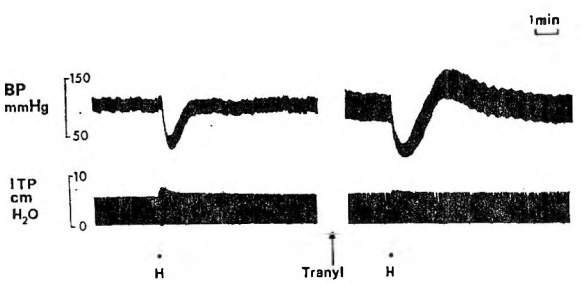


FIG. 6. Records as in Fig. 4, but in an anaesthetized cat, 2·3 kg. At H, histamine (2·5 μg) was injected. At Tranyl, tranylcypromine (20 mg/kg) was injected. 4 h later, the effect of histamine on blood pressure was enhanced, but the effect on intratracheal pressure was reduced.

DISCUSSION

The potentiation by monoamine oxidase inhibitors of the actions of histamine is probably due to impairment of metabolic inactivation. Monoamine oxidase can act on histamine itself (Zeller, Stern & Blacksma, 1956), or methylhistamine which is formed in an alternative metabolic pathway of histamine (Schayer & Cooper, 1956). Monoamine oxidase inhibitors affect many other enzymes; those concerned in histamine metabolism include diamine oxidase (Burkard, Gey & Pletscher, 1960; Gey, Pletscher & Burkard, 1963; Shore & Cohn, 1960), and the histamine methylating enzymes (Schayer, 1953; Schayer & Karjala, 1956).

There are species differences in the metabolism of histamine (Schayer, 1956; Tabor, 1956; Zeller, 1956) and these may account for the observed effects of monoamine oxidase inhibitors on responses to histamine in cats and guinea-pigs. That the effects of histamine on blood pressure were enhanced in the cat but not in the guinea-pig, whereas bronchoconstrictor responses were enhanced in the guinea-pig but not in the cat demands a more sophisticated explanation. Possibly, there is a difference in tissue distribution of histamine-metabolizing enzymes in the two species; we have no information about this. Another explanation might be found in the relative sensitivity of the two effector symptoms in the two species. In the cat, histamine is much the more active on the blood pressure, whereas in the guinea-pig it is the more active on bronchial smooth muscle; McCulloch & others (1967) also observed this. Monoamine oxidase inhibitors potentiated the more active component of histamine action in each species. The lack of potentiation of the less active component may be because secondary counteracting mechanisms are enhanced. Thus, in the cat, histamine releases adrenaline as well as having a depressor action, and this is sometimes manifested as a secondary pressor effect, which is enhanced by monoamine oxidase inhibitors. It could also result in bronchodilatation counteracting the bronchoconstrictor action of histamine; Fig. 6 shows such a reduction in bronchospasm together with a marked increase in the secondary pressor response to histamine. The failure of monoamine oxidase inhibitors to enhance the depressor action of histamine in the guinea-pig may be because the dose-response relation for this effect is shallow, as has alo been noticed by our colleagues L. Q. Pun and J. Atkinson (unpublished observations).

Impairment of histamine metabolism by monoamine oxidase inhibitors may allow the accumulation of histamine in the tissues from endogenous sources or from the intake in the diet. The presence of histamine in foodstuffs, along with other amines, has been demonstrated by Blackwell & others (1965) and by Marks (1965). This exogenous histamine is normally metabolized by enzymes present in the gastrointestinal mucosa and thereby detoxified. But, after monoamine oxidase inhibitors, histamine may be absorbed from the intestine and not detoxified. Blackwell & others (1965) found that yeast extracts, including Marmite, contained histamine as well as tyramine. The pharmacological effects of this histamine were greatly enhanced after intraduodenal administration of yeast extract in a cat pretreated with the monoamine oxidase inhibitor mebanazine.

There is no clear evidence that any of the unwanted symptoms occurring with monoamine oxidase inhibitors are due to an increased sensitivity to histamine. Thus, Blackwell & others (1965) noted that asthma, hay fever, allergies and peptic ulcer were not aggravated by monoamine oxidase inhibitors in psychiatric patients. The incidence of headache was high, but it was not effected by dietary items. However,

it is conceivable that potentiation of the action of endogenous histamine may be concerned in the aetiology of these headaches.

Another possible side-effect of monoamine oxidase inhibitors in which histamine may be implicated is hypotension and hypotensive collapse. This has generally attributed to impairment of sympathetic vasoconstrictor tone, but it could be due to histamine-induced vasodilation.

Acknowledgements

This work was supported by a grant from the National Heart Foundation of Australia. We are grateful for gifts of drugs from the Australian Medical Directors of the following companies: Smith, Kline & French Laboratories for tranylcypromine; Pfizer Laboratories for nialamide; and Roche Products for iproniazid.

REFERENCES

BLACKWELL, B. & MARLEY, E. (1964). *Lancet*, **1**, 530–531.

BLACKWELL, B., MARLEY, E. & MABBITT, L. A. (1965). *Ibid.*, **1**, 940–943.

BLACKWELL, B., MARLEY, E. & TAYLOR, D. (1965). *Ibid.*, **1**, 1166.

BLACKWELL, B., MORLEY, H. & RYLE, A. (1964). *Ibid.*, **1**, 722–723.

BURKARD, W. P., GEY, K. F. & PLETSCHER, A. (1960). *Biochem. Pharmac.*, **3**, 249–255.

COOPER, A. J. (1967). *Br. med. J.*, **4**, 420.

GEY, K. F., PLETSCHER, A. & BURKARD, W. (1963). *Ann. N.Y. Acad. Sci.*, **107**, 1147–1151.

HORWITZ, D., LOVENBERG, W., ENGELMAN, K. & SJOERDSMA, A. (1964). *J. Am. med. Ass.*, **188**, 1108–1110.

KEELE, C. A. (1966). *Background to Migraine*, pp. 126–133. London: Heinemann.

MARKS, J. (1965). *Sci. Basis of Drug Therapy in Psychiat.*, 191, 201. Oxford.

MCCULLOCH, M. W., TRINKER, F. R., CAIRNCROSS, K. D. & STORY, D. F. (1967). *Aust. J. exp. Biol. med. Sci.*, **45**, p. 8.

MCCULLOCH, M. W., PROCTOR, C. & RAND, M. J. (1967). *Europ. J. Pharmac.*, **2**, 214–223.

RAND, M. J. & TRINKER, F. R. (1968). *Br. J. Pharmac.*, **33**, 878–895.

RONDEL, R. K. (1967). *Br. med. J.*, **4**, 357.

SCHAYER, R. W. (1953). *J. biol. Chem.*, **203**, 787–793.

SCHAYER, R. W. (1956). *Histamine. Ciba Foundation Symposium*, pp. 183–188. London: Churchill.

SCHAYER, R. W. & COOPER, J. A. D. (1956). *J. appl. Physiol.*, **4**, 481–483.

SCHAYER, R. W. & KARJALA, S. A. (1956). *J. biol. Chem.*, **221**, 307–313.

SHORE, P. A. & COHN, Jr, V. R. (1960). *Biochem. Pharmac.*, **5**, 91–95.

TABOR, H. (1956). *Histamine* Ciba Foundation Symposium, pp. 318–338. London: Churchill.

TRENDELENBURG, U. (1954). *Br. J. Pharmac. Chemother.*, **9**, 481–487.

TRINKER, F. R., FEARN, H. J., MCCULLOCH, M. W. & RAND, M. J. (1967). *Aust. Dental J.*, **12**, 297–303.

ZELLER, E. A. (1956). *Histamine.* Ciba Foundation Symposium, pp. 339–355. London: Churchill.

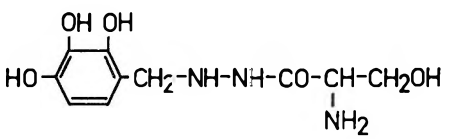
ZELLER, E. A., STERN, P. & BLACKSMA, L. A. (1956). *Naturwissenschaften*, **43**, 157.

J. Pharm. Pharmac., 1969, **21**, 323

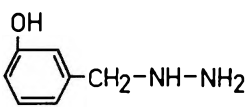
LETTERS TO THE EDITOR

Effect of various decarboxylase inhibitors on the cerebral metabolism of dihydroxyphenylalanine

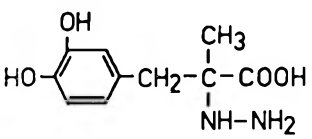
Small doses of Ro 4–4602 [*N*-(DL-seryl)-*N'*-(2,3,4-trihydroxybenzyl)hydrazine], an inhibitor of decarboxylase (DC), enhance the dopa-induced increase of catecholamines in the brain of rats. This was attributed to a poor penetration of the drug through the blood-brain barrier leading to a preferential inhibition of DC in extracerebral tissues like liver, heart and kidney. As a consequence, the concentration of administered dopa in plasma rose and the supply to the brain of this amino-acid was enhanced. This was followed by an increased formation of cerebral catecholamines and their metabolites, the phenolic carboxylic acids (Bartholini, Bates & others, 1967; Bartholini & Pletscher, 1968; Bartholini, Tissot & Pletscher, 1968; Constantinidis, Bartholini & others, 1968). We now report the effect of some other known DC inhibitors: MK485 [β-(3,4-dihydroxyphenyl)-α-hydrazino-α-methyl propionic acid]; NSD 1015 (*m*-hydroxybenzylhydrazine) and α-methyldopa.



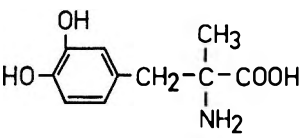
Ro 4–4602



NSD 1015



MK 485



α-methyldopa

Albino rats, 80–100 g, fasted for 16 h, were given α-methyldopa and DC inhibitors of the hydrazine type, i.e. Ro 4–4602, MK 485, NSD 1015, 30 min before 3 mg/kg of [^{14}C]dopa (specific activity 2·07 mCi/mmol), orally. The animals were decapitated 60 min after [^{14}C]dopa. Rats treated with [^{14}C]dopa alone served as controls. Three [^{14}C]catechol fractions containing the [^{14}C]amino-acids—mainly *O*-methyldopa and dopa, the [^{14}C]catecholamines—mainly dopamine and noradrenaline, and the [^{14}C]phenolic carboxylic acids—mainly homovanillic and acid dihydroxyphenylacetic acid, were isolated from two pooled brains in each experiment and measured (Bartholini & Pletscher, 1968).

All four inhibitors act similarly. They cause an enhancement of the dopa-induced rise of amino-acids, phenolic carboxylic acids and catecholamines in the brain and this is dose-dependent at lower doses. Ro 4–4602, NSD 1015 and MK 485 have a stronger effect than α-methyldopa.

The hydrazine type inhibitors also differ among themselves. Lower doses of NSD 1015 and Ro 4–4602 have a more marked effect on the catecholamine concentration than equimolar doses of MK 485. On the other hand, at higher doses, Ro 4–4602—and more so NSD 1015—contrary to MK 485, cause a reduction of the

increase of catecholamines and phenolic carboxylic acids, but not of amino-acids in the brain. This points to some penetration of NSD 1015 and Ro 4–4602 into the brain with consequent inhibition of cerebral DC. The decrease of the amino-acids after high doses of Ro 4–4602 may be explained by an inhibition of *O*-methyl transferase leading to a reduction in the content of *O*-methyldopa (Bartholini, Blum & Pletscher, 1969). This metabolite is an important component of the amino-acid fraction and, in contrast to dopa, accumulates in the brain (Bartholini & Pletscher, 1968).

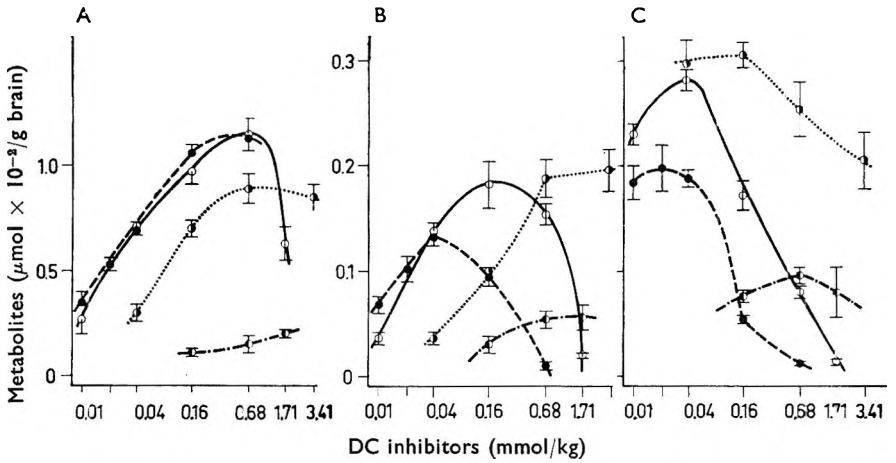


FIG. 1. Effect of the DC inhibitors (Ro 4-4602; - - - NSD 1015; . . . . MK 485; - · - α-methyldopa) on the [^{14}C]dopa-induced rise of [^{14}C]catechol metabolites in the brain of rats. A. [^{14}C]Amino-acids. B. [^{14}C]Catecholamines. C. Phenolic carboxylic acids. The inhibitors were given 30 min before 3 mg/kg [^{14}C]dopa, orally; rats were killed 60 min after [^{14}C]dopa. The points indicate averages with s.e. of 2–9 experiments. The values obtained 60 min after [^{14}C]dopa alone were (in μmol $\times$ 10^{-2}/g brain): [^{14}C]Amino-acids: 0·034 $\pm$ 0·003. [^{14}C]Catecholamines: 0·0011 $\pm$ 0·0003. [^{14}C]Phenolic carboxylic acids: 0·027 $\pm$ 0·002.

In conclusion, the four DC inhibitors may be tentatively characterized: NSD 1015 causes an inhibition of extracerebral DC, but—owing to penetration into the brain—also inhibition of the brain enzyme in low doses. Ro 4–4602 inhibits the extracerebral DC. It interferes only in high doses with the cerebral DC. MK 485 is less potent than NSD 1015 and Ro 4–4602 in inhibiting extracerebral DC, but even in high doses little appears to penetrate the brain. α-Methyldopa has only a slight effect on the extracerebral and no demonstrable effect on cerebral DC.

Research Department,
F. Hoffmann-La Roche & Co. Ltd.,
Basle, Switzerland.

G. BARTHOLINI
A. PLETSCHER

February 19, 1969

REFERENCES

BARTHOLINI, G., BATES, H. M., BURKARD, W. P. & PLETSCHER, A. (1967). *Nature, Lond.*, **215**, 852–853.

BARTHOLINI, G. & PLETSCHER, A. (1968). *J. Pharmac. exp. Ther.*, **161**, 14–20.

BARTHOLINI, G., BLUM, J. E. & PLETSCHER, A. (1969). *J. Pharm. Pharmac.*, **21**, 297–301.

BARTHOLINI, G., TISSOT, R. & PLETSCHER, A. (1968). *Proc. 3rd Symp. on Parkinson's Disease*, Edinburgh, May 20–22, in the press.

CONSTANTINIDIS, J., BARTHOLINI, G., TISSOT, R. & PLETSCHER, A. (1968). *Experientia*, **24**, 529–592.

Influence of pretreatment with testosterone and nandrolone on the reactivity of guinea-pig isolated seminal vesicles to angiotensin and tyramine

It was previously reported (Gascon & Walaszek, 1969) that neither angiotensin nor tyramine stimulates the guinea-pig isolated seminal vesicle. When tested after the addition of adrenaline, however, both compounds induce in this smooth muscle a contraction which is followed by rapid tachyphylaxis.

We now report the influence of chronic treatment with testosterone propionate and nandrolone propionate on the reactivity of guinea-pig isolated seminal vesicles to angiotensin and tyramine.

Male guinea-pigs, 200–225 g, were divided into four groups. Animals in the first and third groups were injected intraperitoneally daily with testosterone (8 mg/kg) and nandrolone (2 mg/kg), respectively, while those in the second and fourth groups served as controls. The influence of testosterone and nandrolone was evaluated on individual groups of 3 animals each day for 7 days of treatment. 24 h after the last injection, the seminal vesicles were suspended at 37° in a 10 ml bath containing Krebs-Henseleit solution gassed with 5% carbon dioxide in oxygen and the reactivity of the organs from treated animals to each agonist was compared with that of the controls.

No difference was observed between the reactivity of the seminal vesicles of the treated animals and those of the controls after the first 4 days of pretreatment with testosterone. In such preparations, angiotensin and tyramine whether alone or in combination, failed to stimulate the isolated smooth muscle. However, after pretreatment with testosterone for 6 days, the agonists induced contraction of the seminal vesicles (Fig. 1).

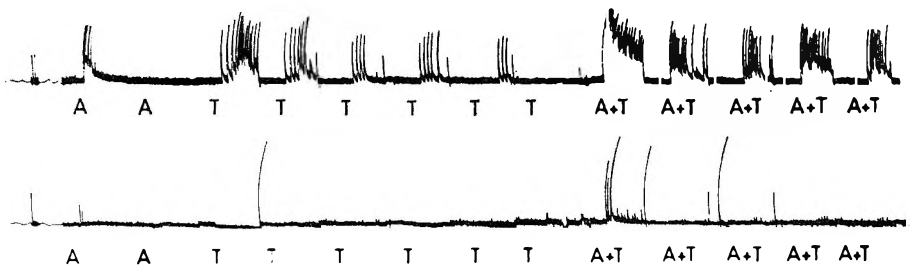


FIG. 1. Influence of pretreatment with testosterone on the reactivity of the guinea-pig isolated seminal vesicles to angiotensin and tyramine. A, Angiotensin 10 μg/ml; T, Tyramine 20 μg/ml. Upper tracing: seminal vesicle of a pretreated animal (6 days). Lower tracing: seminal vesicle of a control animal.

Similar results were observed after pretreatment with nandrolone, the only difference being that modification of the reactivity of the seminal vesicles of animals receiving nandrolone became evident after 4 days pretreatment. The contractions induced by either angiotensin or tyramine in preparations taken from pretreated animals showed tachyphylaxis and were abolished by phenoxybenzamine. On the other hand, the effect induced by the combination of angiotensin and tyramine was antagonized by desipramine (2×10^{-5}M) (Fig. 2).

The mechanism by which testosterone and nandrolone produce this modification of reactivity is not clearly understood. It was recently reported that the contraction induced in normal seminal vesicles by angiotensin and tyramine after the addition of adrenaline is entirely mediated by a release of catecholamines from extraneuronal stores (Gascon & Walaszek, 1969). In the present study, both agonists were able to stimulate the seminal vesicles of the treated animals even when adrenaline was not

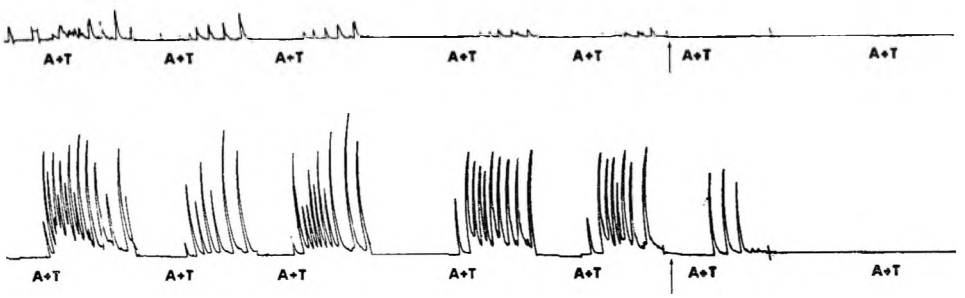


FIG. 2. Effect of pretreatment with nandrolone on the reactivity of the guinea-pig seminal vesicle to the combination of angiotensin and tyramine: influence of desipramine. A, Angiotensin 10 μg/ml. T, Tyramine 20 μg/ml. At ↑, desipramine (2×10^{-5}M) was added to the perfusion mixture. Upper tracing: seminal vesicle of a control animal. Lower tracing: seminal vesicle of a pretreated animal (5 days).

previously added. This observation, and the fact that the stimulant activity of both agonists was abolished either by phenoxybenzamine or by desipramine, indicates that we may be dealing with a release of endogenous catecholamines. If this is so, the site of action is probably located at the nerve endings, since Sjöstrand (1962, 1965) showed that in the seminal vesicles most of the catecholamines are stored in adrenergic nerves as noradrenaline. From these results, it may be concluded that pretreatment with testosterone and nandrolone induces some modification in the noradrenaline content of the seminal vesicles or in the distribution of the biological amine, making it more available for release by angiotensin and tyramine. This type of action has been observed in the uterus and vagina of rats after pretreatment with oestrogens (Sjöberg, 1967; Spratto & Miller, 1968a,b).

This work was supported by the Medical Research Council of Canada, grant MA-3130.

Department of Pharmacology,
Faculty of Medicine,
University of Montreal,
Montreal, Canada.

A. L. GASCON*
J. BRODEUR
M. VAILLANCOURT†

February 12, 1969

* Scholar of the Medical Research Council of Canada, † Fellow of the Medical Research Council of Canada.

REFERENCES

GASCON, A. L. & WALASZEK, E. J. (1969). *Archs int. Pharmacodyn. Thér.*, **175**, 265–272.
SJÖBERG, N. O. (1967). *Acta physiol. scand., Suppl.* 305.
SJÖSTRAND, N. O. (1962). *Ibid.*, **56**, 376–380.
SJÖSTRAND, N. O. (1965). *Ibid.*, **65**, Suppl. 257.
SPRATTO, G. R. & MILLER, J. W. (1968a). *J. Pharmac. exp. Ther.*, **161**, 1–6.
SPRATTO, G. R. & MILLER, J. W. (1968b). *Ibid.*, **161**, 7–13.

Stabilization of erythrocyte membrane by non-steroid anti-inflammatory drugs

An intensive anti-denaturing (stabilizing) effect of anti-inflammatory drugs at therapeutic concentrations on serum proteins has previously been reported by Mizushima & Kobayashi (1968) and Mizushima (1968). Silvestrini & Catanese (1968) obtained a similar effect. Brown, Mackey & Riggilo (1967) and Inglot & Wolna (1968) reported that non-steroid anti-inflammatory drugs such as phenylbutazone and indomethacin stabilized canine and human erythrocytes to heat-induced and hypotonic haemolysis. These findings suggest that stabilization of the erythrocyte membrane by anti-inflammatory drugs may arise from a stabilizing effect of the drugs on some proteins in the cell membrane.

Skidmore & Whitehouse (1965) found that the heat denaturation (coagulation) of serum albumin treated with 2,4,6-trinitrobenzene sulphonic acid (TNBS) was no longer inhibited by anti-inflammatory drugs. Since TNBS reacts with the lysyl ϵ-amino-group of albumin, Skidmore & Whitehouse believed that anti-inflammatory drugs became bound to the ϵ-amino-group and thus stabilized albumin. We have now found canine erythrocytes treated with TNBS to be no longer stabilized to heat-induced haemolysis by anti-inflammatory drugs like phenylbutazone.

After the method of Brown & others (1967), blood was removed from mongrel dogs by a heparinized syringe. The plasma was discarded and the erythrocytes washed twice with saline. Four ml of 40 mM TNBS in saline was added to 36 ml of 5% suspension of the erythrocytes in 0·2 M bicarbonate buffer at pH 8·1. After incubation for 90 min at room temperature (20°), the suspension was centrifuged. The treated erythrocytes were washed with saline and resuspended in 36 ml of 0·15 M phosphate buffer at pH 7·4. To 2·7 ml of the TNBS-treated erythrocyte suspension, or non-treated 5% erythrocyte suspension in the phosphate buffer, were added 0·3 ml of 5 mM anti-inflammatory drugs in saline (pH 7), or saline alone. After standing at room temperature for 15 min, the mixtures were heated in a water bath at 50–51° for 20 min. Samples were cooled with water and promptly centrifuged for 20 min at 14,000 *g*. The haemoglobin content of clear supernatants was measured with a spectrophotometer at 540 nm. The heated control tubes both of non-treated and TNBS groups showed a reading of the spectrophotometer at –log transmission 0·7–1·0 in the method used. Mean haemoglobin content of the heated control tubes, from which the haemoglobin content of the corresponding unheated control tubes had been subtracted, was expressed as 100, as a relative amount of haemolysis. The relative amount of haemolysis of each tube tested was calculated. Erythrocytes treated with 4 mM TNBS were no longer or only minimally stabilized by anti-inflammatory drugs against heat-induced haemolysis, while non-treated erythrocytes were strongly stabilized by them (Table 1).

Table 1. *Effect of TNBS on stabilization of erythrocytes by anti-inflammatory drugs against heat-induced haemolysis*

	Relative amount of haemolysis and standard error	
	non-treated erthrocytes	TNBS-treated erythrocytes
None (control)	100 ± 3·2 (7*, 19**)	100 ± 1·5 (7, 24)
Phenylbutazone	25 ± 2·8 (7, 16)	91 ± 3·3 (7, 18)
Flufenamic acid	34 ± 3·1 (5, 10)	109 ± 4·0 (3, 11)
Indomethacin	7 ± 3·2 (4, 9)	103 ± 4·4 (4, 11)

4·5% of canine erythrocytes treated with and without 4 mM 2, 4, 6-trinitrobenzene sulphonic acid (TNBS) were heated for 20 min at 50–51°C with and without 0·5 mM anti-inflammatory drugs.
* No. of animals used.
** No. of samples tested.

It seems, therefore, that stabilization of the erythrocyte membrane by anti-inflammatory drugs is probably due to a stabilizing effect of the drugs on some proteins in the cell membrane in a manner similar to that observed by us in the experiments on protein denaturation (Mizushima, 1968).

We are grateful to Kowa Ltd., Nagoya for financial support.

Department of Physical Therapy and Medicine,
University of Tokyo,
Tokyo, Japan.
Y. MIZUSHIMA
S. SAKAI

January 25, 1969

REFERENCES

BROWN, J. H., MACKEY, H. K. & RIGGILO, D. A. (1967). *Proc. Soc. exp. Biol. Med.*, **125**, 837–842.
INGLOT, A. D. & WOLNA, E. (1968). *Biochem. Pharmac.*, **17**, 269–279.
MIZUSHIMA, Y. (1968). In *Inflammation*, p. 37–44, ICS 163, Amsterdam: Excerpta Medica.
MIZUSHIMA, Y. & KOBAYASHI, M. (1968). *J. Pharm. Pharmac.*, **20**, 169–173.
SILVESTRINI, B. & CATANESE, B. (1968). *Arzneimittel-Forsch.*, **18**, 425–426.
SKIDMORE, I. F. & WHITEHOUSE, M. W. (1965). *J. Pharm. Pharmac.*, **17**, 671–673.

Inhibition of the noradrenaline uptake in guinea-pig vas deferens by continuous nerve stimulation

The release and the uptake of noradrenaline by nerve terminals can be modulated by different regulatory mechanisms. Gillis (1963) observed that short-lasting stimulation of sympathetic nerves increased the uptake of noradrenaline by the cat perfused heart. Blakeley & Brown (1964) found that nerve stimulation produced an inhibition of the uptake of noradrenaline infused into the cat spleen. Chang & Chiueh (1968) demonstrated that intermittent nerve stimulation of the branches of the facial nerve increased the uptake of tritiated noradrenaline (^{3}H-NA) in rat submaxillary gland. We now report how continuous nerve stimulation with different frequencies can affect the noradrenaline uptake. The hypogastric nerve-vas deferens preparation was chosen because of the abundance of adrenergic synapses in this organ and the opportunity it offers for pre- and postganglionic stimulation (Birmingham & Wilson, 1963).

Male guinea-pigs of about 400 g provided the vasa deferentia and hypogastric nerve preparations which were incubated in a 50 ml bath in modified Krebs solution (Huković, 1961) at 37° for 30 min. The vas deferens from one side was stimulated while that on the other side served as control. The electrical stimulation of the hypogastric nerve was performed as described by Huković (1961), and transmural stimulation as described by Birmingham & Wilson (1963). Monophasic square stimuli of supramaximal strength and 1 ms duration were applied continuously for 30 min. The contractions of the vas deferens were recorded on smoked paper. For the transmural stimulation the vas deferens was dissected and cleaned of peritoneum and surrounding fat tissue.

^{3}H-NA (specific activity 190 mCi/mg) was added to the bath (final concentration, 5 ng/ml of medium). According to Avakian & Gillespie (1968) this concentration is low enough to ensure the uptake of noradrenaline exclusively by the nerve terminals. Oxidation of ^{3}H-NA was prevented by the addition of EDTA (10 mg/litre) and ascorbic acid (20 mg/litre). The pH of the medium was 7·4. After incubation, the preparations were washed twice in 5 ml of saline for 30 s and blotted dry on filter paper. The tissue was homogenized in 0·4N perchloric acid. ^{3}H-NA was separated on alumina columns (Whitby, Axelrod & Weil-Malherbe, 1961). The [^{3}H]radioactivity was measured by a Packard liquid scintillation counter. The amount of ^{3}H-NA metabolites was calculated by subtracting the specific ^{3}H-NA radioactivity from the total [^{3}H]radioactivity.

Low frequency (1 and 6/s) pre- and post-ganglionic stimulation produced 34–44% inhibition of the ^{3}H-NA uptake (Table 1). High frequency (50/s) reduced the uptake by 90–95%. The amount of ^{3}H-NA metabolites varied, also, with the frequency of stimulation; the highest level being observed after 6/s postganglionic stimulation. After 1 and 50/s, the level of ^{3}H-NA metabolites was somewhat lower or did not differ from control value.

Table 1. *Effect of continuous nerve stimulation on the ^{3}H-NA uptake. The results are expressed in* nCi/g *tissue and* P *values show the degrees of significance between control and stimulated preparations. Mean of* 4–6 *experiments*

	^{3}H-NA		^{3}H-NA-metabolites	
	Preganglionic	Postganglionic	Preganglionic	Postganglionic
Control	319·89 ± 15·92	303·50 ± 8·52	20·42 ± 5·36	27·41 ± 15·06
1/s	198·94 ± 19·91 $P < 0.005$	—	15·21 ± 1·75 n.s.	—
6/s	181·00 ± 13·59 $P < 0.001$	201·53 ± 18·75 $P < 0.001$	67·83 ± 11·08 $P < 0.005$	92·72 ± 17·41 $P < 0.01$
50/s	34·02 ± 3·25 $P < 0.001$	17·52 ± 10·11 $P < 0.001$	52·86 ± 16·10 $P < 0.05$	28·73 ± 8·13 n.s.

Our results show that the uptake of ^{3}H-NA is impaired by continuous preganglionic and postganglionic stimulation. This effect was frequency-dependent. One possible explanation for the observed effect is to assume that the depolarization of the neuronal membrane produced by nerve stimulation inhibits the re-uptake and favours the release of noradrenaline. Therefore, at high frequency the inhibiting effect on the uptake process becomes more evident because the depolarization lasts longer. If this were true, then the release and the re-uptake are not simultaneous but sequential processes for a certain area of the synaptic membrane. The alternative explanation could be the existence of a concentration barrier in the synaptic cleft in which a high concentration of endogenous noradrenaline released by high frequency stimulation exceeds the capacity of the re-uptake mechanism and blocks the access of ^{3}H-NA to the synaptic membrane. Recently, Malmfors (personal communication), using a histochemical fluorescence technique, observed a similar effect of nerve stimulation on the uptake of noradrenaline. In the light of these results a reconsideration of the role of the re-uptake mechanism in adrenergic neurotransmission cannot be avoided. It appears that this mechanism has a limited capacity and does not occur simultaneously with the release process at certain areas of the synaptic membrane.

Department of Pharmacology,
Faculty of Medicine,
University of Montreal,
Montreal, Canada.

DJURO PALAIC
JEAN CLAUDE PANISSET

February 11, 1969

REFERENCES

AVAKIAN, O. V. & GILLESPIE, J. S. (1968). *Br. J. Pharmac. Chemother.*, **32**, 168–184.
BIRMINGHAM, A. T. & WILSON, A. B. (1963). *Ibid.*, **21**, 569–580.
BLAKELEY, A. G. H. & BROWN, G. L. (1964). *J. Physiol., Lond.*, **172**, 19P–21P.
CHANG, C. C. & CHIUEH, C. C. (1968). *J. Pharm. Pharmac.*, **20**, 157–159.
GILLIS, C. N. (1963). *Biochem. Pharmac.*, **12**, 593–595.
HUKOVIĆ, S. (1961). *Br. J. Pharmac. Chemother.*, **16**, 188–194.
WHITBY, L. J., AXELROD, J. & WEIL-MALHERBE, H. (1961). *J. Pharmac. exp. Ther.*, **132**, 193–204.

Metabolic effect of *p*-aminosalicylic acid on human erythrocytes

p-Aminosalicylic acid (PAS) induces haemolysis in glucose-6-phosphate dehydrogenase (G-6-PD)-deficient subjects (Szeinberg, Sheba & others, 1957).

We have found that, after *in vitro* incubation with PAS under experimental conditions similar to those of Beutler's glutathione stability test (Beutler, 1957), erythrocytes from G-6-PD-deficient subjects showed no decline of reduced glutathione (G-SH) concentration. Erythrocytes with normal G-6-PD activity washed and suspended in buffered saline also showed no decline of G-SH content, although both PAS concentration and incubation time were increased two-fold (Table 1). No methaemoglobin was formed in erythrocytes after incubation with PAS although methaemoglobin has been claimed to be an obligate intermediary in the biochemical sequence of drug-induced haemolysis (Allen & Jandl, 1961). There was also no Heinz body formation in erythrocytes after incubation with PAS, as happens with acetylphenylhydrazine (APH). Similar experiments with haemolysate prepared from erythrocytes with normal G-6-PD activity also showed no decline of G-SH content or the formation of methaemoglobin (Table 1). Hence, permeability is not the limiting factor.

Table 1. *G-SH and Methaemoglobin levels after incubation with PAS Na and APH.* Twice washed normal human erythrocytes were suspended in isotonic buffered saline (pH 7·4) to give a haematocrit of 50%. A haemolysate was prepared from washed normal erythrocytes by freezing and thawing. Erythrocyte suspension or haemolysate were incubated in the presence of various substances at 37° with continuous shaking for 4 h. G-SH was measured by the nitroprusside method (Beutler, 1957) and methaemoglobin spectrophotometrically (Brewer, Tarlov & Alving, 1960)

Additions to erythrocyte suspension or haemolysate (mM)	G-SH (μmol/ml erythrocytes)	Methaemoglobin (% of total Hb)
Erythrocyte suspension		
None	1·80	0
APH, 30	0·03	31
APH, 30 and glucose, 25	1·95	29
PAS Na, 30	1·90	0
PAS Na, 60	1·90	0
Haemolysate		
None	2·18	2·3
APH, 30	0	51
PAS Na, 60	2·19	2·3

The failure of PAS to oxidize intracellular G-SH and haemoglobin places the drug apart from other known haemolytic drugs such as APH, primaquine, nitrofurantoin, α- and β-napthoquinone and certain vitamin K derivatives which have been shown to oxidize G-SH of G-6PD-deficient erythrocytes (Beutler, 1959). Only, napthalene, like PAS, has no oxidative action (Beutler, 1959).

Department of Haematology,
Burma Medical Research Institute,
5 Zafar Shah Road, Rangoon, Burma.

U HLA-PE
AUNG-THAN-BATU

January 30, 1969

REFERENCES

ALLEN, D. W. & JANDL, J. H. (1961). *J. clin. Invest.*, **40**, 454–475.
BEUTLER, E. (1957). *J. Lab. clin. Med.*, **49**, 84–95.
BEUTLER, E. (1959). *Blood*, **14**, 103–139.
BREWER, C. J., TARLOV, A. R. & ALVING, A. S. (1960). *Bull. Wld Hlth Org.*, **22**, 633–640.
SZEINBERG, A., SHEBA, C., HIRSHORN, N. & BODONYI, E. (1957). *Blood*, **12**, 603–613.

Changes of thirst threshold produced by chlorpromazine

The effect of chlorpromazine on the activity of the hypothalamohypophyseal antidiuretic system has been described by Kovacs, Kovacs & others (1957), Moses (1964) and Boris & Stevenson (1967). As this system is morphologically and functionally close to the hypothalamic thirst centre it could be supposed that chlorpromazine might also effect the mechanism of thirst.

We have investigated the effect of chlorpromazine on the osmotic reactivity of thirst mechanism. The experiments were made with 10 mongrel dogs, 13–22 kg, fasted for 18 h but with free access to water. The osmotic reactivity of the thirst mechanism was examined under control conditions and after intravenous infusion of chlorpromazine, and was measured as the thirst threshold in relation to osmotic stimuli (Wolf, 1950). A solution of saline (5%) was infused (6·4 ml/min) into the saphenous vein of a dog having free access to water and with freedom of movement. When the dog began to drink, the infusion was stopped, and it was assumed that the osmotic load induced by the infusion had reached the thirst threshold. Measurements of the volume of water ingested, the volume of urine produced, and quantity of sodium excreted during the infusion were taken.

The level of thirst threshold was expressed by the magnitude of the sodium load (i.e. the number of m-equiv of Na^+ in the infusion less the number of m-equiv of Na^+ excreted in urine) necessary to induce the drinking response. The cellular dehydration produced at the point of thirst threshold by infusion of hypertonic NaCl was also calculated. The total body water and the extracellular water were measured in each dog. The plasma Na^+ concentration was also measured and the total amount of Na^+ calculated. The amount of Na^+ and water in the infusion are known, hence the shift of water caused by hypertonic infusion and cellular dehydration inducing the drinking reaction could be calculated (threshold cellular dehydration). In each dog, control measurements of thirst threshold were checked 4–6 times.

Chlorpromazine, (Specia-Largactil) 0·22 mg/kg, dissolved in 1·4 ml of 0·9% saline, was given by intravenous infusion 1 h before the thirst threshold measurement. Total body water was measured using tritiated water (Chwalinski, Mikulski & Kossakowska, 1965), and extracellular water by using sodium thiocyanate. The sodium concentration was measured by the Zeiss III flame photometer.

In 9 dogs, chlorpromazine, 0·22 mg/kg, lowered the osmotic reactivity of the thirst mechanism. One dog treated with 0·44 mg/kg showed a higher thirst threshold. The difference between cellular threshold dehydration (%) in controls ($4 \pm 0{\cdot}5$) and after chlorpromazine infusion ($6{\cdot}7 + 0{\cdot}7$) was statistically significant ($P < 0{\cdot}1$).

The volume of water drunk under the threshold stimulus did not differ significantly from the controls. Diuresis increased in 8 dogs during chlorpromazine infusion in a range from 14–204% with an average of 10%. It remained unchanged in one dog and in another decreased by 13%.

The lowering of osmotic reactivity of the thirst mechanism by chlorpromazine may be connected with its direct action upon the central nervous system. A secondary effect of this drug on the thirst mechanism, which might well be due to

its peripheral action in inhibiting vasomotor reflexes, would be expected to produce a rise not a fall of osmotic reactivity of thirst mechanism.

Department of Pharmacology,
Medical Academy of Warsaw,
Poland.

I. GAWECKA
E. SZCZEPANSKA

December 17, 1968

REFERENCES

BORIS, A. & STEVENSON, R. H. (1967). *Archs int. Pharmacodyn. Thér.*, **166**, 486–498.

CHWALINSKI, S., MIKULSKI, A. & KOSSAKOWSKA, M. (1965). *Acta physiol. polon.*, **16**, 141–149.

KOVACS, K., KOVACS, G. S., KOVACS, B. M. & PETRI, C. (1957). *Archs int. Pharmacodyn. Thér.*, **109**, 1–7.

MOSES, A. M. (1964). *Endocrinology*, **74**, 889–893.

WOLF, A. V. (1950). *Am. J. Physiol.*, **161**, 75–86.

The effect of chloral hydrate on the metabolism of ethanol in mice

The enhancement of the hypnotic effect of ethanol by chloral hydrate is well documented (Maynert, 1965). Bardodej (1965) described an disulfiram-like reaction in man after this drug combination and suggested increased acetaldehyde as the cause. However, Kaplan, Forney & others (1967) were unable to show any alteration in ethanol metabolism in man after chloral hydrate and attributed the additive effect to the formation of trichloroethanol. Gessner & Cabana (1967) showed significant differences in the rate of disappearance of chloral hydrate and of the formation of trichloroethanol and trichloroacetic acid in mice when chloral hydrate was given with ethanol. We have now measured ethanol and acetaldehyde in the blood after intravenous injection of ethanol into mice pretreated with chloral hydrate.

Adult male DBA/2 mice, 25 g, received chloral hydrate 200, 400, or 600 mg/kg intraperitoneally. Thirty min later they were injected with ethanol 1·33 g/kg as an 8·33% solution in isotonic saline into the tail vein. The duration of the injection was 1 min and blood samples were withdrawn at 5, 20, 35 and 50 min after the injection was complete. Samples were taken from the retro-orbital sinus directly into a 50 μl disposable micropipette and the acetaldehyde and ethanol concentrations determined by the method previously described (Roach & Creaven, 1968).

With 200 mg/kg of chloral hydrate, increases in blood acetaldehyde of 131% ($P < 0{\cdot}005$), 60% ($P = 0{\cdot}01$), 42% ($P = 0{\cdot}05$), and 27% (N.S.) are seen at 5, 20, 35, and 50 min after ethanol injection. With 400 mg/kg the increases are 318, 341 and 121% ($P < 0{\cdot}001$ for each) and 55% ($P = 0{\cdot}01$) at the same four time intervals; with 600 mg/kg, they are 270, 342, 171 and 93% ($P < 0{\cdot}001$ for each). With 200 mg/kg of chloral hydrate, but twice the dose of ethanol (2·67 mg/kg), acetaldehyde is significantly greater than control values only at 5 min (207%, $P < 0{\cdot}005$). At all four times the values are lower than those found with the same dose of chloral hydrate and the smaller dose of ethanol, and at 35 and 50 min they are significantly lower ($P = 0{\cdot}02$). (For treated groups n = 6; for the control group n = 13.)

The effect on blood ethanol levels are less dramatic. In control animals the decline in the level of blood ethanol is nearly linear with time, in agreement with previous findings in this laboratory for the dose of ethanol (1·33 g/kg) used. Chloral hydrate, 200 mg/kg, causes a 10% increase in the blood ethanol level at 5 min but the rate of decrease is the same as for the controls (Fig. 1). At doses of 400 and 600 mg/kg of chloral hydrate, the 5 min blood ethanol levels are somewhat higher than the control values (16 and 22% respectively; n = 6 for each experiment) but the rate of decrease is no longer constant, being greatest in the first 15 min and

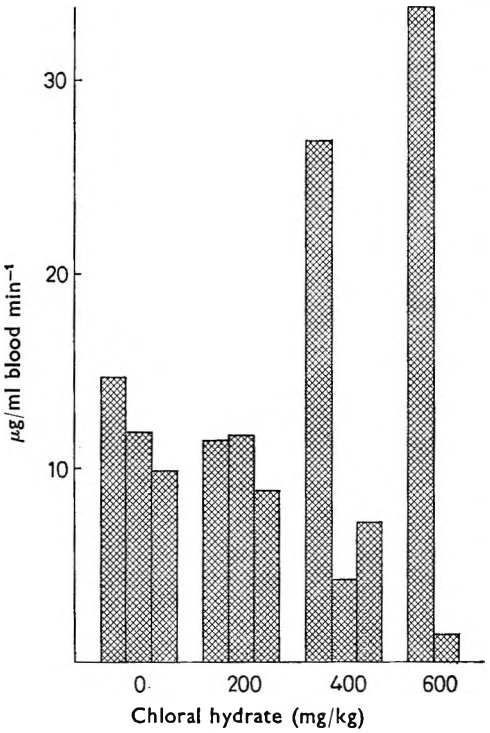


FIG. 1. Rate of decline in blood ethanol levels in each of the three 15 min periods after ethanol (1·33 g/kg), preceded by chloral hydrate in the doses shown. For treated groups n = 6; for the control group n = 13. The first column at each dose represents the 15 min period 5–20 min, the second column 20–35 min, the third 35–50 min (this is on the base line at the 600 mg/kg dose). At 200 mg/kg there are no significant differences. At 400 mg/kg, columns 1 and 2 differ significantly from the control, $P = 0{\cdot}01$. At 600 mg/kg, columns 1 and 3 show a highly significant, difference, $P = 0{\cdot}001$ and column 2 a significant difference, $P = 0{\cdot}01$, from the control.

declining thereafter. This is the pattern of ethanol metabolism previously shown to occur after a dose of ethanol (2·67 g/kg) twice as large as that used here (Roach & Creaven, 1969).

It is clear from these findings that chloral hydrate alters both ethanol and acetaldehyde metabolism and that this can explain at least in part the synergistic effect of the two drugs.

This work was supported by grant MH 144340–1, U.S. Public Health Service.

Section of Biochemistry,
Texas Research Institute of Mental Sciences,
Houston, Texas 77025, *U.S.A.*
February 18, 1969

P. J. CREAVEN
MARY K. ROACH

REFERENCES

BARDODEJ, Z. (1965). *Československ. farm.*, **14**, 478–481.

GESSNER, P. K. & CABANA, B. E. (1967). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **26**, 2, 568.

KAPLAN, H. L., FORNEY, R. B., HUGHES, F. W. & JAIN, N. C. (1967). *J. forensic Sci.*, **12**, 295–304.

MAYNERT, E. W. (1965). *Drills' Pharmacology in Medicine*, 3rd edn, p. 171. Editor: DiPalma, J. R. New York: McGraw-Hill, Inc.

ROACH, M. K. & CREAVEN, P. J. (1968). *Clin. Chim. Acta*, **21**, 275–278.

ROACH, M. K. & CREAVEN, P. J. (1969). *Experientia*, in the press.

Simple chromatography of cannabis constituents

For some years thin-layer chromatography of cannabis has relied upon reversed-phase systems for effective resolution of tetrahydrocannabinol (THC) from other natural constituents (Korte & Sieper, 1964; Betts & Holloway, 1967; Caddy & Fish, 1967). Two alternatives involve multi-component solvent systems (Aramaki, Tomiyasu & others, 1968; Parker, Wright & others, 1969). The advantages of a mono-solvent system are self-evident and such a system has been discovered which resolves 5 main components of cannabis in a running time of under 10 min.

Any of the 3 common aromatic hydrocarbons, toluene, xylene or benzene, resolve cannabidiol (CBD), cannabinol (CBN) and THC on Eastman Chromagram Sheet 6061 (silica gel) as 3 distinct spots in a running time of 20–30 min, and as a triple banded streak in 5–10 min (Table 1). Toluene and xylene give similar results, and with their low volatility, complete equilibration of the tank is of little consequence. A completely open tank has been frequently found more convenient, and provided it is at least twice as high as its width, and reasonably narrow, any vessel may be used; a straight-sided-tall-form drinking tumbler was the easiest "tank" to obtain. Benzene is not so suitable because of its high volatility, and variable Rf values were obtained across the same sheet. The high volatility of many solvents in common use may be a cause of the lack of resolution on silica gel.

The sample was prepared as a light petroleum (boiling range 40–60°) solution (1 vol resin or herb to 1 vol solvent), and spotted-on in a maximum volume of 0·5μl, keeping the spot diameter below 2 mm. Failure to comply with these conditions may result in some loss of resolution. The solvent runs approximately 5 cm in 10 min, allowing 5 cm^2 pieces of sheet to be used for quick monitoring, or 8–9 cm in 30 min for complete identification and determination of the relative proportions of 5 main cannabinoid components. For precise work, xylene in an equilibrated tank runs 16–17 cm in 1½ h, and gives 0·5 cm gaps between the 3 main spots. The spots were visualized by spraying with a fresh 0·1% aqueous methanol solution (1 :3) of Fast Blue B salt, and allowed to dry naturally.

The Chromagram sheets were not activated before use and it is obvious that the grade of silica gel used by the manufacturers is of some importance. Some silica gel plates prepared conventionally may not resolve all the components, and Whatman silica gel-loaded paper yields only 4 spots; THC and CBN run together. Aramaki, with his benzene system on silica gel plates, found that CBN ran ahead of THC, and it was not until a basic component was added that he obtained the same sequence as I have obtained. Parker and his co-workers do not mention CBD, and in view of the similarity of their solvent systems to that of Aramaki, they may have the same

Table 1. *Typical Rf values for aromatic solvents on Chromogram* 6061 *sheets*

Colour		Unknown 1 orange	Unknown 2 violet	CBN violet	Δ^9-THC scarlet	CBD orange	
Benzene	A	56	59	70	74	81	
	B	79	83	91	96	98 (tight bands)	
	C	46	50	57	64	69	
Toluene	A	50	52	62	70	80	
	B	53	60	69	78	86	
	C	42	46	53	61	67	
Xylene	A	40	43	52	58	68	
	B	44	47	57	67	74	
	C	28	39	46	54	64	$\Delta^9 = 57$

Rf values are measured from the leading edge as a more reliable parameter.
System A—open tank, 10 min solvent run. B—open tank, 30 min solvent run. C—equilibrated tank, 16·5 cm solvent run. Benzene time 105 min; toluene 95 min; xylene 90 min.

experience in which CBD runs with one of the other two main components. On Chromagram sheets, CBD runs with THC in Parker's solvent I (n-hexane–1,4-dioxan, 9:1), and this can be clearly illustrated using this system as the second solvent in 2-dimensional chromatography, with xylene as first solvent.

Chromagram sheets may not be the only material available giving such good resolution, but their practical convenience of use for small samples and subsequent ease of storage, have proved overriding considerations against other systems examined.

I wish to thank Dr. A. Scaplehorn of the Home Office Central Research Establishment, Aldermaston, for the gift of authentic CBD, CBN, and Δ^9-THC.

Ministry of Technology, M. J. DE FAUBERT MAUNDER
Laboratory of the Government Chemist,
Cornwall House, Stamford Street,
London, S.E.1, England.
March 25, 1969

REFERENCES

ARAMAKI, H., TOMIYASU, N., YOSHIMURA, H. & TSUKAMOTO, S. (1968). *Chem. Pharm. Bull. (Tokyo)*, **16**, 5, 822–826.

BETTS, T. J. & HOLLOWAY, P. J. (1967). *J. Pharm. Pharmac.*, **19**, *Suppl.*, 97S–102S.

CADDY, B. & FISH, F. (1967). *J. Chromatog.*, **31**, 584–587.

KORTE, F. & SIEPER, H. (1964). *Ibid.*, **13**, 90–98.

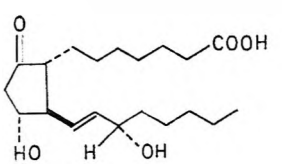
PARKER, K. D., WRIGHT, J. A., HALPERN, A. F. & HINE, C. H. (1968). *Bull. Narcot.*, **20**, (4), 9–14.

Inhibition of gastric acid secretion in the rat by synthetic prostaglandins

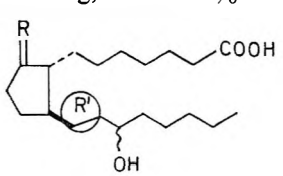
The prostaglandins (PGs) are a group of naturally occurring, long-chain, unsaturated oxygenated fatty acids with potent pharmacological activities (Bergström, Carlson & Weeks, 1968). I have examined the effects of four synthetic PGs (AY–20,524, AY–16,809, AY–21,669, AY–21,670) on gastric acid secretion in the rat.

Basal gastric acid secretory activity was measured (Shay, Sun & Gruenstein, 1954) in Charles River female albino rats (Canadian Breeding Laboratories; 150–170 g) caged individually and from which food had been withheld 48 h before pyloric ligation and drug administration. After the first 24 h of food deprivation the animals were given access to 8% sucrose in 0·2% sodium chloride for 8 h. Water was permitted *ad libitum* except during the 8 h access to sucrose. Four h after pyloric ligation the animals were killed with ether and the amount of acid in the stomach determined (6–9 animals for each treatment) by titration against 0·1N NaOH in a direct reading pH meter to pH 7·0.

The PGs were dissolved as follows: for each mg, 0·1 ml 95% ethanol was added



PGE$_1$



	R	R'		R	R'
AY-20,524	O	—CH=CH—	AY-21,669	H, OH	—CH_2—CH_2—
AY-16,809	H, OH	—CH=CH—	AY-21,670	OH, H	—CH_2—CH_2—

Table 1. *Inhibition of gastric acid secretion in the rat by synthetic prostaglandins*

Compound	Dose mg/kg, s.c.	Gastric acid secretion m-equiv/4 h ± s.e. Exp 1	Exp 2	Exp 3	Exp 4	% of control
None		0·59 ± 0·05	0·52 ± 0·04	0·47 ± 0·05	0·47 ± 0·05	
PGE_1	0·8	0·09 ± 0·02 $P < 0·001$	0·18 ± 0·04 $P < 0·001$			15, 35
	0·4		0·27 ± 0·03 $P < 0·001$			52
AY–20,524	8·0	0·16 ± 0·02 $P < 0·001$				28
	4·0			0·24 ± 0·03 $P < 0·001$		51
	2·0			0·45 ± 0·05 $P < 0·9$		95
AY–16,809	32			0·31 ± 0·03 $P < 0·01$	0·26 ± 0·03 $P < 0·01$	65, 57
	16			0·49 ± 0·05 $P < 0·9$		104
AY–21,669	32				0·31 ± 0·03 $P < 0·05$	67
AY–21,670	32				0·30 ± 0·03 $P < 0·02$	63
	16				0·53 ± 0·05 $P < 0·5$	114

(to a maximum of 1·4 ml which was sufficient to dissolve the greatest amounts of compounds) and solution achieved; this was followed by 0·18 mg Na_2CO_3 and water to the desired volume. The control animals were injected with an aliquot of this vehicle at the appropriate times. The PGs were administered subcutaneously (in 0·2 ml), half the amount stated in Table 1 being injected immediately after pyloric ligation and the other half 2 h later. Student's *t*-test was used to evaluate results.

PGE_1 inhibited gastric acid secretion (Table 1) and the level of inhibitory activity was similar to that obtained by others (Robert, Nezamis & Phillips, 1968). The related synthetic PGE analogue, AY–20,524, also inhibited the gastric acid secretion exhibiting an activity about one-tenth that of PGE_1. The synthetic PGF analogues, AY–16,809, AY–21,669 and AY–21,670, were about one-eighth as active as AY–20,524.

The synthetic PGE was a more potent antisecretory agent than the corresponding synthetic PGF (AY–20,524 *vs* AY–16,809). In a different type of assay, a natural PGE inhibited gastric acid secretion at a level at which the corresponding PGF was not effective (Robert, Nezamis & Phillips, 1967).

PGE_1 is optically pure whereas the synthetic PGs are all racemates with 4 possible optical isomers and thus it is possible that the appropriate synthetic PGs are even more potent than the racemates.

The author acknowledges the technical assistance of Mrs. Susan Schaal.

The compounds were synthesized by Drs J. F. Bagli, T. Bogri and R. Deghenghi of Ayerst Laboratories.

Biogenic Amines Laboratory,
Ayerst Laboratories,
Montreal, Quebec, Canada.

W. LIPPMANN

January 10, 1969

REFERENCES

BERGSTRÖM, S., CARLSON, L. A. & WEEKS, J. R. (1968). *Pharmac. Rev.*, **20**, 1–48.
ROBERT, A., NEZAMIS, J. E. & PHILLIPS, J. P. (1967). *Am. J. dig. Dis.*, 1073–1076.
ROBERT, A., NEZAMIS, J. E. & PHILLIPS, J. P. (1968). *Gastroenterology*, **55**, 481–487.
SHAY, H., SUN, D. C. H. & GRUENSTEIN, M. (1954). *Ibid.*, **26**, 906–913.

UNIVERSITY OF CAPE TOWN/PROVINCIAL ADMINISTRATION OF THE CAPE OF GOOD HOPE LECTURESHIP IN PHARMACOLOGY

Applications are invited from medical graduates for the post of Lecturer in the Department of Pharmacology. The duties include systematic teaching of medical students (lectures, tutorials and experimental work). Lectures are also given to Sister-tutors for the Nursing Diploma course. The time-table allows ample time for research. The duties in the department are full-time.

The Lectureship is on the Specialist salary scale, viz R6000 × 300 – R6600 p.a.

Applicants should state age, qualifications, teaching experience, publications, and research interests and should indicate the date when they could assume duty. The names and addresses of two referees whom the University may consult should be given.

Two copies of the application should reach the Secretary-General, Association of Commonwealth Universities (Appts), 36 Gordon Square, London, W.C.1 (from whom memoranda giving the conditions of service, including transport expenses on appointment, and information on the work of the Department should be obtained) not later than 15 *May*, 1969. A third copy of the application with a recent photograph and medical certificate should be sent direct by airmail to the Registrar, University of Cape Town, Private Bag, Rondebosch, Cape Town, South Africa, by the same date.

The University reserves the right to appoint a person other than one of the applicants, or to make no appointment.

UNIVERSITY OF LEIDEN
THE NETHERLANDS

The FACULTY of SCIENCE invites applications or suggestions for the position of

PROFESSOR OF PHARMACEUTICAL CHEMISTRY

The position involves teaching and research in the chemistry of therapeutic agents, especially with regard to the correlation of the physical and chemical properties of such compounds with their pharmacological effect.

The Department of Pharmacy comprises sections of (1) pharmaceutical analysis, (2) pharmaceutical chemistry, (3) pharmaceutical technology and (4) pharmacognosy.

Salary H.Fl. 52.000 ($14.300)

Applications, including curriculum vitae and references, or suggestions, should be sent as soon as possible to: The Faculty of Science, University of Leiden, Rapenburg 46, Leiden, The Netherlands.

The Pharmaceutical Press

OVERSEAS DISTRIBUTORS

Australia: The Australasian Pharmaceutical Publishing Co. Ltd., 18/22 Saint Francis Street, Melbourne, Vic 3000.

Canada: McAinsh & Co. Ltd., 863 Bay Street, Toronto 5, Ontario.

Japan: Maruzen Co. Ltd., 6 Tori-Nichome Nihonbashi, Tokyo 103.

U.S.A.: Rittenhouse Book Store Inc., 1706 Rittenhouse Square, Philadelphia, Pennsylvania 19103.

Publications of THE PHARMACEUTICAL PRESS may be ordered through medical and scientific bookshops anywhere in the world. In case of difficulty please write direct to The Pharmaceutical Press, 17 Bloomsbury Square, London, W.C.1, England.

UNIVERSITY OF HONG KONG
CHAIR OF PHARMACOLOGY

Applications are invited for the Chair of Pharmacology which will fall vacant on January 1, 1970, on the retirement of Professor R. C. Y. Lin.

Annual salary (superannuable) is HK$ 83,200 fixed (or approximately £5,718 according to present sterling equivalent).

Further particulars and application forms may be obtained from the Secretary-General, Association of Commonwealth Universities (Appts), 36, Gordon Square, London, W.C.1, or the Registrar, University of Hong Kong.

Closing date for applications 25 May, 1969.

Printed by W Heffer & Sons Ltd Cambridge England

Journal of Pharmacy and Pharmacology

Volume 21 Number 5 May 1969

Original Papers

Letters to the Editor