Journal of Pharmacy and Pharmacology

The Pharmaceutical Society of Great Britain

Volume 23 Number 6 June 1971

SUPPLEMENT 1970 to the British Veterinary Codex 1965

The British Veterinary Codex provides standards of identity and purity for medicines and prophylactic agents used in veterinary practice and gives authoritative information on their actions, uses and dosage. It is produced by the Pharmaceutical Society of Great Britain with the help and advice of the Royal College of Veterinary Surgeons and the British Veterinary Association. *The Veterinary Record* has described it as "an invaluable source of information on agents used in the treatment and prevention of disease in domesticated animals . . . a book which should have a place in every veterinary practice, teaching department and research institute."

This 320-page supplement to the British Veterinary Codex 1965 includes new monographs reflecting recent developments in veterinary drugs, particularly in anthelmintics, antibiotics, coccidiostats and ovulation controllers.

In the formulary section there are new monographs for oral pastes, premixes and pellets, all appearing for the first time in the British Veterinary Codex.

In Part II the standards for antisera, vaccines and related products have been completely revised and Part II of the Supplement replaces Part II of the British Veterinary Codex 1965. Monographs are also included for a number of new vaccines.

The Supplement includes as an appendix a full list of British Veterinary Non-proprietary Names, with graphic formulae, chemical names, trade names, and uses.

SUPPLEMENT 1970 to the British Veterinary Codex 1965
SBN 85369 069 3
320 pages. Price £3.50 post free

British Veterinary Codex 1965
SBN 85369 010 3
880 pages. Price £5.50 post free

Combined price when ordered together
£7.50 post free.

The Pharmaceutical Press, 17 Bloomsbury Square, London WC1A 2NN

Journal of Pharmacy and Pharmacology

Published by THE PHARMACEUTICAL SOCIETY OF GREAT BRITAIN
17 Bloomsbury Square, London, WC1A 2NN Telephone: 01-405 8967

Volume 23 Number 6 **June 1971**

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 (220 × 320 mm) with a minimum margin of 1¼ inch (30 mm). 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. (1970). *The Pharmacological Basis of Therapeutics*, 4th edn, p. 460. 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 a separate sheet of paper and appended to the typescript of the paper. 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) £10, (U.S.A. $28). Single copies £1.25, (U.S.A. $3.50).

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

Member of the Audit Bureau of Circulations.

CONVERT your KYMOGRAPH into a HORIZONTAL RECORDER

411/1323
PALMER KYMOGRAPH FRAME

411/1322
PEN/AMPLIFIER MODULE

411/1325
TIME/EVENT MARKER MODULE

411/1324
STABILIZED POWER SUPPLY

411/1326
ISOTONIC TRANSDUCER

A parallel rod frame has been specially designed to fit Palmer Super 10, Electric 12 and E.P. Kymographs and allow them to be mounted horizontally, thus greatly extending their range of useful applications.
Up to four pen/amplifier modules can be mounted on the frame and may be powered by the special stabilized bench power pack shown, or by any centre-tapped 12V car battery.
An ink-writing time and event marker module is available for operation by any 6V source.

Full technical specifications available on request

C. F. PALMER (LONDON) LTD.

A MEMBER OF THE SEARLE GROUP

LANE END ROAD, HIGH WYCOMBE, BUCKINGHAMSHIRE

Telephone: High Wycombe 21124

J. Pharm. Pharmac., 1971, **23**, 393–398 *Received January* 22, 1971

The displacement of L-tryptophan and dipeptides from bovine albumin *in vitro* and from human plasma *in vivo* by antirheumatic drugs

J. N. MCARTHUR, P. D. DAWKINS AND M. J. H. SMITH

Department of Biochemical Pharmacology, King's College Hospital Medical School, Denmark Hill, London, S.E.5, U.K.

L-Tryptophan occurs in a protein-bound and an unbound form in serum from normal subjects. The amino-acid is displaced from its binding sites *in vitro* by salicylate, phenylbutazone, indomethacin, prednisolone, chloroquine and gold salts and is virtually absent in serum obtained from patients with rheumatoid arthritis receiving therapy with antirheumatic drugs. Some dipeptides bind to bovine albumin *in vitro* and are displaced by salicylate. All the drugs displace L-phenylalanyl-L-phenylalanine from its binding to normal human serum *in vitro*.

There have been many attempts to provide a unifying concept of the mode of action of the drugs commonly used in the treatment of rheumatism. These have included mechanisms based on stimulation of the anterior pituitary and adrenal cortex glands, on an interference with either the formation, release or action of suspected mediators of inflammation, on the abilities of the drugs to uncouple oxidative phosphorylation reactions and on a stabilizing effect of the drugs on lysosomal membranes thus preventing the release of hydrolase enzymes which can degrade joint cartilage. None of these theories adequately explains the clinical anti-inflammatory actions of the salicylates (Smith, 1966) or of the other antirheumatic remedies (Whitehouse, 1965; Domenjoz, 1966; Skidmore & Trnavsky, 1967; Houck & Forscher, 1968; Spector & Willoughby, 1968; Collier, 1969).

It has been shown (McArthur & Dawkins, 1969) that salicylate displaces L-tryptophan from its binding sites to human serum proteins. The present paper describes the effects of the commonly used antirheumatic drugs on the release of the amino-acid and certain dipeptides from their binding sites to bovine serum albumin and to human serum proteins.

MATERIALS AND METHODS

Materials

Pooled human serum was obtained from the National Transfusion Service, Sutton and individual samples of serum were obtained by venepuncture from 3 female and 2 male patients with rheumatoid arthritis under treatment at King's College Hospital. Bovine serum albumin (fraction V), amino-acids and dipeptides were obtained from the Sigma Chemical Company. The sodium salicylate was of British Pharmacopoeial standard, phenylbutazone was obtained from Geigy (U.K.), Ltd., as ampoules each containing 600 mg of the drug plus 30 mg of xylocaine in 3 ml, indomethacin powder

and prednisolone were obtained from Merck Sharp and Dohme Ltd., the latter being in the form of an injection containing 20 mg of prednisolone sodium phosphate, 2·5% (w/v) nicotinamide, 0·01 (w/v) sodium metabisulphite, 0·05% (w/v) sodium edetate and 0·5% (w/v) phenol in a volume of 2 ml. Chloroquine phosphate powder was obtained from ICI Ltd. and the gold salts from May & Baker Ltd. as an injection containing 100 mg of sodium aurothiomalate and 0·002% (w/v) phenylmercuric nitrate in 1 ml.

Measurement of tryptophan in human serum

The concentrations of tryptophan (total and free) were measured in pooled normal serum and in sera from patients with rheumatoid arthritis as described by McArthur & Dawkins (1969).

Tryptophan binding experiments

Samples (10 ml) of either pooled human serum or serum from patients with rheumatoid arthritis were applied to a 36 × 2·8 cm column containing 40 g of Sephadex G-25 medium. The column was washed with a solution containing 0·14M NaCl and 0·01M phosphate buffer, pH 7·4 (buffer A) and the first 65 ml of eluate were discarded. The next 35 ml, containing the proteins but free of amino-acids, was collected, placed in a 65 ml ultrafiltration cell equipped with a Diaflo PM 30 membrane (Amicon N.V., Holland), and the volume reduced to 10 ml. Buffer A (10 ml) was added to the protein solution inside the cell, the volume reduced to 10 ml and this process was repeated three times to ensure that all ultrafilterable ninhydrin-positive substances and as much as possible of the drugs had been removed. To the concentrated protein solution inside the cell was added 10 ml of a 0·1 mM solution of L-tryptophan in buffer A and 10 ml of ultrafiltrate collected. The first 7 ml of the ultrafiltrate were discarded to compensate for the "dead space" in the apparatus and the final 3 ml diluted with an appropriate quantity of buffer A and its content of free tryptophan estimated as described previously (McArthur & Dawkins, 1969). This procedure was repeated successively with 10 ml quantities of 0·3, 1·0 and 3·0 mM tryptophan and with 2·0 mM salicylate.

Binding of dipeptides to bovine albumin and displacement by salicylate

Samples (20 ml) of 3% (w/v) bovine albumin in buffer A were placed in a 65 ml ultrafiltration cell fitted with a Diaflo PM 30 membrane and the volume reduced to 10 ml. This procedure was repeated after the successive addition of 10 ml quantities of the phosphate buffer, and the following concentrations of either amino-acids or the dipeptides, 0·05 mM, 0·1 mM and either 0·5 mM (×2) or 0·3 mM and 1·0 mM, depending on the varying solubilities of the materials, followed by 2 mM salicylate. In each instance the first 7 ml of ultrafiltrate was discarded and the following 3 ml collected and analysed for ninhydrin-positive substances (McArthur & Dawkins, 1969), after appropriate dilution with the phosphate buffer.

Displacement of L-*tryptophan and* L-*phenylalanyl*-L-*phenylalanine from pooled human serum by antirheumatic drugs*

Samples (10 ml) of pooled human serum were cleared of amino-acids as described above except that the Sephadex column was washed with 0·01M phosphate buffer, pH 7·4, and not with buffer A. To the 10 ml of protein solution inside the 65 ml

ultrafiltration cell was added either 10 ml of 0·01M phosphate buffer, pH 7·4, or a 10 ml quantity of the buffer containing either 0·5 mM L-tryptophan or 0·5 mM L-phenylalanyl-L-phenylalanine and the volume reduced to 10 ml. The first 7 ml of ultrafiltrate was discarded and the final 3 ml used for analysis using ninhydrin and the Technicon autoanalyser (McArthur & Dawkins, 1969). This procedure was repeated successively with 10 ml quantities of either buffer alone or buffer containing 100, 200 or 400 μg/ml of each drug.

RESULTS

Tryptophan concentrations in human serum

The free and total tryptophan concentrations (μM/100 ml) in pooled normal serum were: 1·21 $\pm$ 0·22 and 6·36 $\pm$ 0·48 (s.d. n = 6) and in patients with rheumatoid arthritis were 0·33 $\pm$ 0·06 and 1·88 $\pm$ 0·28 (s.d. n = 5) respectively.

The results show that the concentrations of free and total tryptophan in sera obtained from five patients with active rheumatoid arthritis, who were receiving treatment with one or more antirheumatic drugs, were significantly reduced ($P < 0{\cdot}001$) compared to the corresponding concentrations in the normal serum.

Binding of tryptophan to serum from normal subjects and from patients

The patients with rheumatoid arthritis had all received aspirin plus either phenylbutazone, indomethacin or gold salts. The serum samples and pooled normal serum were exhaustively treated by ultrafiltration to remove any amino-acids and drugs present, then exposed to increasing concentrations of L-tryptophan followed by salicylate. Free and total tryptophan concentrations were determined. There was no difference between the results from the pooled normal serum and from the patients' sera for each concentration of the amino-acid but the subsequent displacement of the bound tryptophan by 2 mM salicylate was significantly greater ($P < 0{\cdot}01$) for the normal serum.

Binding of dipeptides to bovine albumin

L-Tryptophan was found to be the only amino-acid to bind to the bovine albumin and none of the following dipeptides; glycylglycine, glycyl-L-serine, L-valyl-L-leucine, glycyl-L-phenylalanine, L-phenylalanylglycine, glycyl-L-tryptophan and L-tryptophanylglycine showed measurable binding to the protein. In contrast, the dipeptides listed in Table 1 resembled tryptophan in their binding characteristics and were all displaced by 2 mM salicylate, a concentration of the drug which is attained and maintained during the treatment of rheumatoid arthritis. These results are too limited to draw any conclusions about possible structural requirements for binding with the obvious reservation that the presence of either two phenylalanyl, two tryptophanyl or one of each amino-acid residues in a peptide may be an important factor in the binding to albumin. L-Phenylalanyl-L-phenylalanine did not displace any of the amino-acid bound to bovine serum albumin.

Displacement of L-tryptophan and L-phenylalanyl-L-phenylalanine from human serum by antirheumatic drugs

Normal human serum, when cleared of amino-acids, binds tryptophan and phenylalanylphenylalanine. When the cleared sera, exposed to 0·5 mM tryptophan or the

Table 1. *Binding of dipeptides to bovine serum albumin.* Each value is given as the % of the total concentration of dipeptide present which is bound to serum albumin and represents the mean of two separate experiments. The results in the presence of salicylate are expressed in the same way except that the total concentrations of dipeptides present are in brackets. The results with L-tryptophan have also been included for comparison.

Dipeptide	Total concentration of dipeptide present (mM) 0·025	0·06	0·20	0·60	In presence of salicylate (2 mM)
L-Phenylalanyl-L-phenylalanine	85	83	75	68	27 (0·48)
L-Phenylalanyl-L-tryptophan ..	76	74	67	63	38 (0·54)
L-Tryptophanyl-L-phenylalanine	89	87	84	77	40 (0·59)
L-Tryptophanyl-L-tryptophan ..	99	95	92	88	71 (0·45)
L-Tryptophan	80	77	64	43	36 (0·47)

dipeptide, are ultrafiltered and additional quantities of buffer added, then the concentrations of unbound tryptophan or dipeptide in the successive ultrafiltrates are progressively reduced. In the presence of either phenylbutazone, indomethacin, prednisolone, chloroquine or gold the amounts of unbound tryptophan or dipeptide in the ultrafiltrates are greater than in the absence of the drugs (Fig. 1). These drugs therefore displace the protein-bound amino-acid and dipeptide, the effect becoming more pronounced as the serum plus bound substance becomes exposed to increasing concentrations of each drug. The results of preliminary experiments also indicated that the effect of gold salts becomes more pronounced if the cleared serum was incubated for 16 h at 4° with the sodium aurothiomalate before exposure to the

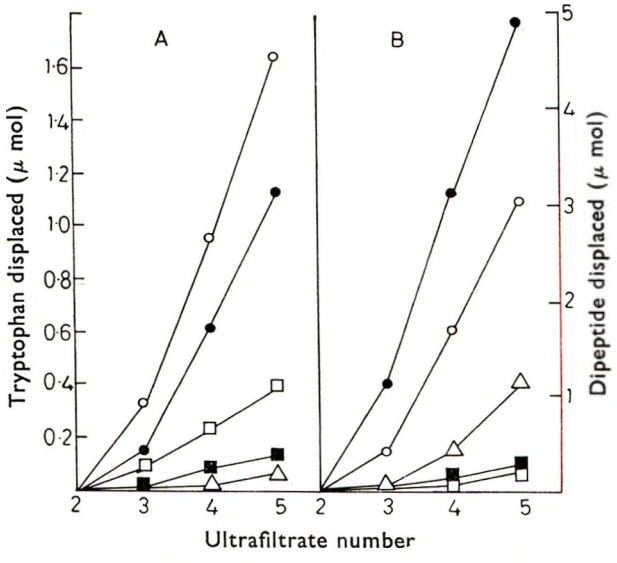

FIG. 1. Displacement of L-tryptophan and L-phenylalanyl-L-phenylalanine from human serum by antirheumatic drugs. The results have been plotted as the cumulative displacement of either the amino-acid or the dipeptide against the ultrafiltrate number. The second ultrafiltrate was obtained after the initial exposure of the cleared serum to 0·5 mM tryptophan or dipeptide, the third, fourth and fifth ultrafiltrates were obtained after the subsequent addition of 100, 200 or 400 μg/ml of the drugs. Each point represents the mean of two separate determinations. A, L-tryptophan; B, L-phenylalanyl-L-phenylalanine; ○, indomethacin; ●, phenylbutazone; □, chloroquine; ■, gold; △, prednisolone.

tryptophan and ultrafiltration. Salicylate has not been included in Fig. 1 since it had already been established (Table 1) that the drug displaced both tryptophan and phenylalanylphenylalanine from albumin.

DISCUSSION

L-Tryptophan is the only amino-acid bound to human serum albumin (McMenamy & Oncley, 1958). It is displaced by salicylates both *in vitro* (McArthur & Dawkins, 1969) and *in vivo* (Smith & Lakatos, 1971). The present work shows that protein-bound and unbound tryptophan may be readily measured in normal human serum but that their concentrations are significantly reduced in the serum of patients with rheumatoid arthritis receiving therapy with one or more antirheumatic drugs (Table 1). However, when the drugs are removed from the patients' sera, tryptophan binds to the proteins but is less easily displaced by salicylate than from normal serum. The other commonly used antirheumatic drugs, phenylbutazone, indomethacin, prednisolone, chloroquine and gold displace the amino-acid from its binding to human serum (Fig. 1).

The results in Table 1 show that certain dipeptides bind to bovine albumin and are displaced by a therapeutic concentration of salicylate. The binding of one of these dipeptides, L-phenylalanyl-L-phenylalanine, is affected by all the antirheumatic drugs (Fig. 1).

Although the slopes of the plots for the individual drugs in Fig. 1 show their relative potencies in displacing either tryptophan or phenylalanyl–phenylalanine from human serum *in vitro* this need not represent their antirheumatic activities since neither the amino-acid nor the dipeptide necessarily mediate clinical anti-inflammatory actions. They can only be interpreted as a qualitative guide that these antirheumatic drugs share a common biochemical action. It remains to be shown if drugs, which bind extensively to circulating proteins but which are devoid of clinical antirheumatic activity, lack the ability to displace either tryptophan or the dipeptide. However, it is possible that many drugs of this type could exert antirheumatic actions in man if they could be administered in sufficient dosage over long enough periods of time. This may apply to long-chain fatty acid anions, such as oleate, which have been shown (McMenamy & Orcley, 1958) to displace tryptophan from its binding sites to human albumin.

All the effective antirheumatic remedies have to be administered in divided doses over long periods of time, amounting to years for rheumatoid arthritis (Bluestone, 1970). The necessity for continued administration coupled with the relapses which occur when the drugs are stopped suggest that they support a natural defensive reaction against chronic inflammatory stimuli. Secondly, all the drugs bind to circulating proteins, particularly albumin. If given in therapeutic amounts only a relatively small fraction of the drugs exist in the unbound form. For example, salicylate is bound up to 90% at therapeutic concentrations. Toxicity, rather than clinical effectiveness, appears to be associated with the accumulation of unbound salicylate (Dawkins & Smith, 1971). Thus the protein-bound rather than the unbound forms of the drugs exert the chronic antirheumatic effects.

When the drugs bind to circulating albumin they displace other biologically active small molecules from their binding sites on serum proteins. The present work shows that this displacement extends to dipeptides which bind to human serum proteins. It is suggested that the unbound forms of some peptides may exert a

protective effect against the actions of mediators of chronic inflammatory insults. The binding of these hypothetical peptides to circulating proteins may be abnormally strong in patients with the rheumatic diseases because in such patients the circulating albumin possesses an abnormal amino acid composition (Denko, Purser & Johnson, 1970). The fraction of the peptides present in the free form would then be insufficient to exert a protective role. It is proposed that the antirheumatic drugs act by increasing the proportion of free peptides in the blood.

Acknowledgements

We should like to thank Dr. E. Hamilton of the Department of Rheumatology, King's College Hospital for permission to study patients under his care. We are grateful for grants received from the Arthritis and Rheumatism Council and the King's College Hospital and Medical School Research Committee.

REFERENCES

BLUESTONE, R. (1970). *Br. med. J.*, **4**, 602–604.

COLLIER, H. O. J. (1969). *Nature, Lond.*, **223**, 35–37.

DAWKINS, P. D. & SMITH, M. J. H. (1971). *J. Pharm. Pharmac.*, **23**. In the press.

DENKO, C. W., PURSER, O. B. & JOHNSON, R. M. (1970). *Arthritis Rheum.*, **13**, 311–312.

DOMENJOZ, R. (1966). *Adv. Pharmac.*, **4**, 143–204.

HOUCK, J. C. & FORSCHER, B. K. (1968). *Chemical Biology of Inflammation*, London: Pergamon Press.

MCARTHUR, J. N. & DAWKINS, P. D. (1969). *J. Pharm. Pharmac.*, **21**, 744–750.

MCMENAMY, R. H. & ONCLEY, J. L. (1958). *J. biol. Chem.*, **233**, 1436–1447.

SKIDMORE, I. F. & TRNAVSKY, K. (1967). *Some Aspects of Anti-Inflammatory Drugs. Acta Rheumatology et Balneologica Pistiniana*, Czechoslovakia.

SPECTOR, W. G. & WILLOUGHBY, D. A. (1968). *The Pharmacology of Inflammation.* London: English Universities Press.

SMITH, M. J. H. (1966). *The Salicylates.* New York: Interscience Publishers.

SMITH, H. G. & LAKATOS, C. (1971). *J. Pharm. Pharmac.*, **23**, 180–189.

WHITEHOUSE, M. W. (1965). *Prog. Drug. Res.*, **8**, 321–429.

J. Pharm. Pharmac., 1971, **23**, 399–406 *Received October* 13, 1970

Salicylate-induced inhibition of collagen and mucopolysaccharide biosynthesis by a chick embryo cell-free system

HIDEO NAKAGAWA* AND J. PETER BENTLEY

Division of Experimental Biology, University of Oregon Medical School, Portland, Oregon, U.S.A.

The ability of a chick embryo cell-free system to synthesize collagen, mucopolysaccharide and non-collagen protein in the presence of sodium salicylate was studied. Added creatine phosphate together with endogenous creatine kinase was the ATP generating system. The incorporation of labelled proline or labelled glucose into collagen or mucopolysaccharide respectively depended on ATP level in the cell-free system used. Salicylate inhibited collagen and mucopolysaccharide synthesis to a greater extent than non-collagen protein synthesis. The ability of the cell-free system to hydroxylate labelled protocollagen was inhibited 50% by storage at −18°. Ferrous iron reversed this inhibition. Salicylate prevented the restoration of the enzyme activity by ferrous iron. Incorporation of radioactivity into hyaluronic acid, when labelled UDP-glucuronic acid and UDP-*N*-acetylglucosamine were supplied, was inhibited 17% by salicylate. Under the same conditions 47% inhibition of incorporation into chondroitin sulphate was seen. This suggests that UDP-*N*-acetylglucosamine-UDP-*N*-acetylgalactosamine epimerase is inhibited by salicylate.

Rokosova-Cmuchalova & Bentley (1968) reported the effect of salicylate on the synthesis of collagen, chondroitin sulphate and non-collagen protein by slices of puppy epiphysial cartilage incubated with appropriate precursors of the above compounds. The biosynthesis of collagen and of chondroitin sulphate was markedly inhibited by a salicylate concentration of 10 mM whilst the synthesis of non-collagen protein was minimally, if at all, affected. The various steps in the biosynthesis of chondroitin sulphate are inhibited by salicylate. No information is available about the inhibition of the final polymerization of UDP-glucuronic acid and UDP-*N*-acetylgalactosamine which leads to the formation of the chondroitin sulphate chain. The present communication describes the biosynthesis of collagen, mucopolysaccharides and non-collagen protein by a chick embryo cell-free, mitochondrial-free preparation, and the effect of salicylate on these processes.

Since salicylate is known to uncouple oxidative phosphorylation (Smith & Smith, 1966) it is entirely possible that some of the effects noted were due to lack of ATP. The use of such a cell-free system permits the concentration of ATP to be controlled more readily since the uncoupling effect can be ignored. It also permits studies of the effect of salicylate on the hydroxylation of proline in labelled protocollagen, and of

* Present address: Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

the incorporation of UDP monosaccharides into mucopolysaccharides. This latter is not possible in tissue slices due to the impermeability of cell membranes to UDP sugars (Telser, Robinson & Dorfman, 1965).

MATERIALS AND METHODS

The chick embryo cell-free system used was a dialysed 15 000 *g* supernatant (S-15) fraction prepared as described by Peterkofsky & Udenfriend (1963).

Incubation procedure

Incubations were carried out in air in rubber-stoppered 50 ml Erlenmeyer flasks at 37° with shaking. The final volume was 10 ml and contained the following components (mM): KCl, 20; $MgCl_2$, 4; sucrose, 250; ATP, 2; glutamine, 1; NAD, 0·5; creatine phosphate, 20; tris-HCl buffer (pH 7·6), 50; dialysed S-15 fraction equivalent to 100 to 120 mg of protein. [^{3}H]L-proline, [^{14}C]glucose and [^{14}C]UDP-glucuronic acid were added as described in the various experiments.

Isolation of imino-acids from collagen and non-collagen protein

After incubation, trichloroacetic acid (TCA) was added to a final concentration of 5%. After centrifugation the precipitate was heated for 60 min at 90° in 5% TCA and the supernatant solution added to that from the previous TCA treatment. This extraction procedure was repeated three additional times. The final insoluble residue was hydrolysed at 105° in 6 N HCl for 16 h. The hydrolysate was treated with nitrous acid followed by ether extraction to remove amino-acids (Myhill & Jackson, 1963) and the imino-acids separated on columns of AG50X8-H^+ (Eastoe, 1961). No hydroxyproline was recovered from the TCA-insoluble residue which is referred to as non-collagen protein. The supernatants from the hot TCA extraction steps were dialysed against 0·5% proline in 0·01 M acetic acid in an attempt to remove adsorbed labelled proline. Dialysis against several changes of 0·01 M acetic acid was continued for a further four days at 4°. Following this step a measured amount of highly purified calf skin collagen was added as carrier* before reduction of volume and hydrolysis. The hydrolysate was treated as before, leading to the isolation of collagen hydroxyproline and collagen proline.

Isolation of mucopolysaccharides

After incubation, TCA was added as before to a final concentration of 5%. The precipitate, after adjustment of pH, was papain-digested as described by Antonopoulous, Gardell & Hamnström (1965), and subjected to a second precipitation step with 5% TCA. The supernatant was added to that from the initial TCA precipitation step and both were dialysed against repeated changes of a 3% glucose solution to remove adsorbed labelled glucose and then against repeated changes of distilled water for four days at 4°. Carrier chondroitin-6-sulphate prepared from shark cartilage was added* followed by cetyl pyridinium chloride (CPC) to a final concentration of 1% in order to precipitate charged mucopolysaccharides and leave much of the uncharged glycopeptide material in solution. The precipitate was dissolved in a

* The specific activity data were not corrected for the addition of carrier collagen or chondroitin sulphate which accounts for the relatively low activity of these fractions when compared with non-collagen protein or with hyaluronic acid.

small amount of 60% propanol, precipitated with ethanol at a concentration of 70% together with traces of sodium acetate, and dried in a vacuum. This mixed mucopolysaccharide precipitate was dissolved in 0·4 M NaCl solution, and a 1% solution of CPC dissolved in 0·4 M NaCl was added to precipitate sulphated polysaccharide [the critical electrolyte concentrations (Scott, 1960) of which exceed this concentration of NaCl]. This is referred to as the "chondroitin sulphate fraction." The supernatant was subjected to ethanol precipitation as before and the precipitate referred as "hyaluronic acid fraction".

Hydroxylation system

[^{3}H]Protocollagen was prepared by incubation of the minced 9-day-old chick embryos (31 g) in a modified Krebs-Ringer buffer containing α,α′-dipyridyl (1 mM) and [^{3}H]proline (2 mCi) for 90 min at 37° (Hutton, Tappel & Udenfriend, 1966). The incubation mixture was then centrifuged at 15 000 *g* at 4° for 15 min and the resulting pellet was extracted with 0·5 M acetic acid. The extract was dialysed against distilled water for 3 days. The precipitate appearing on dialysis was dissolved in 0·1 M acetic acid and NaCl was added to a final concentration of 10%. The resulting precipitate was dialysed against 0·1 M acetic acid for 3 days and centrifuged. Following adjustment to pH 7·6, the supernatant was used as [^{3}H] protocollagen substrate. It contained $3{\cdot}8 \times 10^5$ d/min ml^{-1}, 2·36 mg protein/ml. Incubations and isolation of imino-acids were carried out as above with the addition of 0·3 ml [^{3}H]protocollagen substrate per flask.

Analytical procedures

Proline was determined on the Technicon autoanalyser by a modification of the method of Troll & Lindsley (1955). Hydroxyproline was determined by an automated modification of the Stegemann procedure (Stegemann, 1958). Uronic acid was determined by the procedure of Balazs, Bernsten & others (1965). ATP was determined (Kornberg, 1955) with a Biochemica Test combination (Boehringer, Mannheim). Radioactivity was measured by liquid scintillation spectrometry with adequate correction for quenching.

RESULTS AND DISCUSSION

Under the conditions used, the cell-free system was capable of incorporating labelled proline into collagen hydroxyproline, into non-collagen protein proline and of incorporating labelled glucose into the "chondroitin sulphate fraction". The synthetic activities continued for between 1 and 2 h after which time no further incorporation occurred. This cessation of synthetic activity can in part be accounted for by the rapid utilization and degradation of ATP by the cell-free system. It was found by ATP assay that the half life of ATP in this system was approximately 18 min, and that the conversion of added creatine phosphate into ATP by endogenous creatine kinase had largely ceased after 45 min. It is also probable that most other enzyme systems had become inactive after this time. It has previously been demonstrated that salicylate inhibits the production of ATP from creatine phosphate by creatine kinase (Dawkins, Gould & Smith, 1966).

Fig. 1 shows that the response of collagen synthesis to increasing creatine phosphate concentration is similar to that seen for the two mucopolysaccharide fractions up to

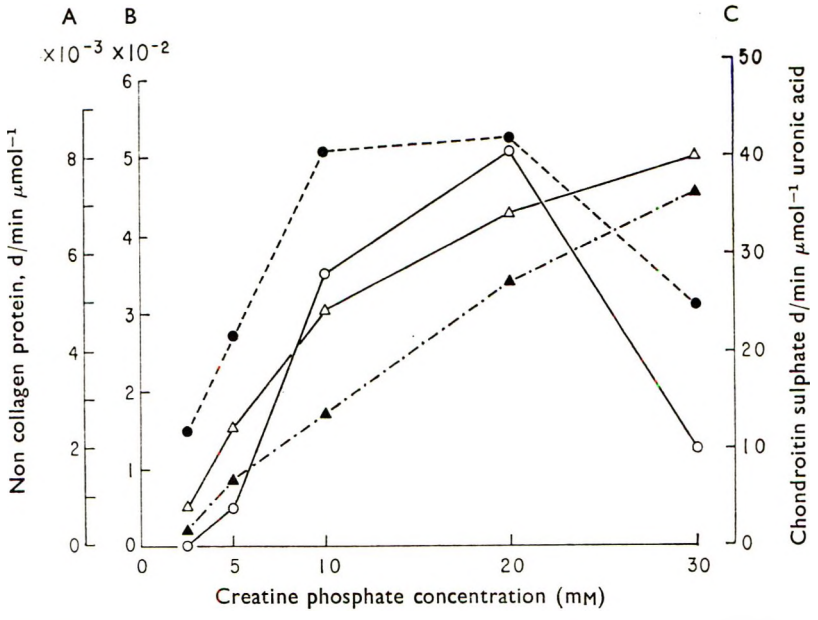

FIG. 1. The effect of creatine phosphate concentration on the incorporation of [^{14}C]glucose into chondroitin sulphate (closed triangles) and into hyaluronic acid (open triangles) and on the incorporation of [^{3}H]proline into collagen hydroxyproline (open circles) and into non-collagen protein proline (closed circles). Ordinate: A = non collagen proline, d/min μmol^{-1}. B = collagen hydroxyproline, d/min μ mol^{-1} and hyaluronate d/min μmol^{-1} uronic acid. C = chondroitin sulphate, d/min μ mol^{-1} uronic acid.

a concentration of 20 mM in that a continuous rise is seen. The specific activity rises by approximately 40% between 10 and 20 mM creatine phosphate. The non-collagen protein curve, however, follows a different course in that a mere 2% stimulation of synthesis is noted between these two creatine phosphate concentrations. Creatine phosphate, 30 mM, further stimulates mucopolysaccharide synthesis whereas it is inhibitory to both collagen and non-collagen protein synthesis. Such differences at very high concentrations of creatine phosphate are not, however, surprising since both the collagen and non-collagen protein are being synthesized on such structures as endoplasmic reticulum and polysomes, whereas the carbohydrate of the mucopolysaccharide is probably being produced by addition of monosaccharide units to preformed protein acceptors. In the range of creatine phosphate concentration between 10 and 20 mM the increased rate of collagen and mucopolysaccharide synthesis noted may be due to the increased availability of ATP, but the increased rate of collagen synthesis may equally be due to the concomitant increase in mucopolysaccharide synthesis.

As shown in Fig. 2, salicylate inhibited the incorporation of labelled proline or labelled glucose into collagen hydroxyproline or chondroitin sulphate respectively to a greater extent than the incorporation of labelled proline into non-collagen protein. The result may be accounted for by the decreased availability of ATP, since salicylate inhibits the production of ATP from creatine phosphate by creatine kinase, and collagen and mucopolysaccharide syntheses, as shown in Fig. 1, depend on the ATP level more than does non-collagen synthesis in the system used.

The biosynthesis of collagen is distinguished from that of other proteins by the hydroxylation of prolyl and lysyl residues previously incorporated into a large polypeptide chain, protocollagen (Lukens, 1965; Prockop & Juva, 1965; Kivirikko

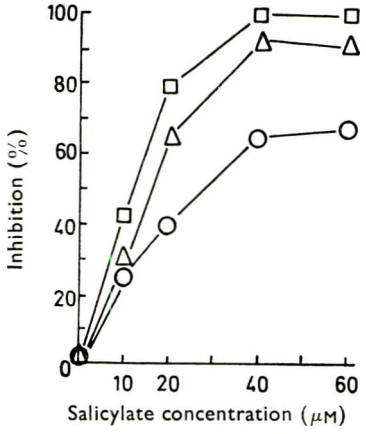

FIG. 2. The effect of salicylate on the incorporation of [^{14}C]glucose into "chondroitin sulphate fraction" (triangles) and on the incorporation of [^{3}H]proline into collagen hydroxyproline (squares) and into non-collagen protein proline (circles).

& Prockop, 1967). The enzyme responsible for the hydroxylation of proline in protocollagen has been referred to as protocollagen proline hydroxylase. Ferrous iron is a required cofactor of the enzyme which can be inhibited by such metal chelators as α,α′-dipyridyl, 1,10-phenanthroline or EDTA (Hurych & Chvapil, 1965; Nordwig, Kobrle & Pfab, 1967; Chvapil, Hurych & others, 1967). Salicylate is capable of acting as a metal chelator (Perrin, 1958), although the stability constant for Fe^{2+}-salicylate complex is markedly less than that of similar complex with α,α′-dipyridyl (Baxendale & George, 1948; Krumholz, 1949). It is thus possible that the results noted above could be due to inhibition of the hydroxylation step. This question was studied by incubating the S-15 fraction with previously prepared chick embryo protocollagen, the proline residues of which were labelled with tritium. The subsequent appearance of [^{3}H]hydroxyproline was taken as evidence of hydroxylation. As shown in Table 1, the S-15 fraction was capable of hydroxylating 17% of the proline residues of the protocollagen, but storage at −18° for 12 days reduced this value by half. Addition of 0·5 or 1·0 mM ferrous sulphate restored the activity of the frozen enzyme system to levels equal to that of the fresh material. The addition of 5 or 10 mM salicylate to the frozen stored enzyme system produced no effect but these concentrations were capable of preventing progressively the restoration of

Table 1. *Effect of ferrous iron and salicylate on the hydroxylation of protocollagen.* Incubation conditions: protocollagen 1·14 × 10^5 d/min; Dialysed S-15 fraction, 80 mg protein; final volume, 8 ml; incubation at 37° for 2 h.

Incubation temp. (°C)	Salicylate concn (mM)	$FeSO_4$, 0 mM d/min Hyp	Pro	Hydroxylation (%)	$FeSO_4$, 0·5 mM d/min Hyp	Pro	Hydroxylation (%)	$FeSO_4$, 1·0 mM d/min Hyp	Pro	Hydroxylation (%)
37°	0α	5 610	26 556	17·4						
1°	0•	401	32 240	1·2						
37°	0•	3 104	29 538	9·5	7 083	29 445	19·4	6930	31 513	18·0
37°	5•	2 978	32 706	8·3	5 207	28 202	15·6	4 179	29 041	12·6
37°	10•	2 731	32 022	7·9	3 665	32 194	10·2	3 285	31 573	9·4

• S-15 fraction stored 12 days at −18°.
α Fresh dialysed S-15 fraction was used.

activity by ferrous iron. It would seem that the enzyme contains more than one iron atom and that these are bound with different degrees of stability. Some of the ferrous iron may be removed by storage or by a chelator such as salicylate with a relatively low affinity for ferrous iron. This removal only partially inactivates the enzyme, though the use of chelators with higher stability constants for ferrous iron complexes leads to complete loss of activity.

Characterization of the mucopolysaccharide fraction

In an attempt to further characterize the polysaccharides produced, samples of "hyaluronic acid fraction" and "chondroitin sulphate fraction" were subjected to the cellulose column fractionation procedure of Antonopoulous & others (1965).The cetyl pyridinium complexes of the polysaccharides were eluted with increasing salt concentrations and the analysis of the material eluted is presented in Table 2. It is

Table 2. *Cellulose column fractionation of cetyl pyridinium (CPC) complexes of the hyaluronic acid (HA) and chondroitin sulphate (CSA) fractions.*

		Eluant			
	1% CPC	0·3 M NaCl	0·3 M $MgCl_2$	0·75 M $MgCl_2$ in acetic acid	0·75 M $MgCl_2$
HA Fraction					
Glucosamine (μmol)	0·12	1·28	—	—	—
Galactosamine (μmol)	0·02	0·04	—	—	—
Glucuronic acid (μmol)	0·25	1·30	0	0	0
Radioactivity (d/min)	—	228	0	0	0
CSA Fraction					
Glucosamine (μmol)	—	—	—	0·02	—
Galactosamine (μmol)	—	—	—	2·20	—
Glucuronic acid (μmol)	0	0	0·2	2·21	0
Radioactivity (d/min)	—	0	25	94	10

Results expressed as μmol/5 ml of 15 000 *g* supernatant.

clear from these results that the "hyaluronic acid fraction" is virtually free of galactosamine and has a glucosamine to glucuronic acid ratio of 1. The analytical figures of the "chondroitin sulphate fraction" agree well with its identity as chondroitin 4 or 6 sulphate. In addition, Table 2 shows that all the radioactivity incorporated into the "hyaluronic acid fraction" can be eluted from the column with 0·3 M NaCl and that most of the activity incorporated into the "chondroitin sulphate fraction" can be eluted with 0·75 M magnesium chloride in acetic acid. These salt concentrations are in agreement with the critical electrolyte concentrations of hyaluronic acid and chondroitin sulphate respectively. Because of the minute amounts available, no attempt was made to characterize further the fractions eluted with 0·3 M $MgCl_2$ and with 0·75 M $MgCl_2$ at neutral pH.

Effect of salicylate on the epimerase and polymerase steps

As previously pointed out (Rokosova-Cmuchalova & Bentley, 1968), salicylate is capable of blocking several reactions in the biosynthesis of mucopolysaccharides. No information is available however regarding the final UDP-*N*-acetylglucosamine

(UDP-GlcNHAc)–UDP-*N*-acetylgalactosamine (UDP-GalNHAc) epimerase reactions or of the UDP-glucuronic acid (UDP-GlcUA)–UDP-GalNHAc or UDP-GlcNHAc polymerase steps. Experiments were made to determine whether salicylate produced different effects on mucopolysaccharide synthesis when labelled glucose only was provided as a precursor and when the direct precursors of hyaluronic acid, UDP-GlcUA and UDP-GlcNHAc were supplied. After incubation, the "hyaluronic acid fraction" and "chondroitin sulphate fraction" were further purified by the cellulose column fractionation procedure described above and their specific radioactivities determined. The results of this experiment are seen in Table 3. Salicylate (20 mM) inhibited the incorporation of [^{14}C]glucose into chondroitin sulphate by 55% and into hyaluronic acid by 43%. A similar degree of inhibition (47%) was noted for the incorporation of [^{14}C]UDP-GlcUA into chondroitin sulphate when UDP-GlcNHAc was supplied, but a much lower degree (17%) of inhibition was seen for the incorporation of [^{14}C]UDP-GlcUA into hyaluronic acid under the same conditions.

Table 3. *Effect of salicylate on mucopolysaccharide synthesis by chick embryo 15 000 g supernatant fraction.*

Labelled precursor	[^{14}C]Glucose (30 μCi)	[^{14}C]Glucose (30 μCi)	[^{14}C]Glucose (30 μCi)	[^{14}C]UDP-GlcUA* (3·3 μCi)	[^{14}C]UDP-GlcUA* (3·3 μCi)	[^{14}C]UDP-GlcUA* (3·3 μCi)
Sodium salicylate	None	None	20 mM	None	None	20 mM
Incubation temp. (°C)	1°	37°	37°	1°	37°	37°
Hyaluronic acid, d/min μ mol^{-1}	5·3	79·8	44·8 (43% inhibition)	7·8	139·7	116·5 (17% inhibition)
Chondroitin sulphate, d/min μ mol^{-1}	2·6	21·6	9·6 (55% inhibition)	1·7	45·4	23·8 (47% inhibition)

Incubation was carried out for 2 h under the conditions as in the text except as follows:
* Contained UDP-glucuronic acid, 0·3 mM; UDP-NHAc glucosamine, 0·3 mM.

It is felt that this 17% inhibition represents the effect of salicylate upon the enzyme system polymerizing UDP-GlcUA and UDP-GlcNHAc. The additional inhibition noted in the chondroitin sulphate synthesis is probably due to the effect of salicylate upon the epimerase responsible for the conversion of UDP-GlcNHAc to-UDP-GalNHAc.

This report serves to point out the complexity of the effect of salicylate on the biosynthesis of connective tissue components.

Acknowledgement

This work was supported in part by U.S.P.H.S. Grants: AM 09530, HE 10647, HE 06336, CA 10016 and by grant from the American Heart Association.

REFERENCES

ANTONOPOULOUS, D. A., GARDELL, S. & HAMNSTRÖM, B. J. (1965). *J. Atheroscler. Res.*, **5**, 9–15.
BALAZS, E. A., BERNSTEN, K. O., KAROSSA, J. & SWANN, D. A. (1965). *Analyt. Biochem.*, **12**, 547–558.
BAXENDALE, J. H. & GEORGE, P. (1948). *Nature, Lond.*, **162**, 777–778.
CHVAPIL, M., HURYCH, J., EHRLICHOVA, E. & TICHY, M. (1967). *Europ. J. Biochem.*, **2**, 229–235.
DAWKINS, P. D., GOULD, B. J. & SMITH, M. J. H. (1966). *Biochem. J.*, **99**, 703–707.

EASTOE, J. (1961). *Ibid.*, 79, 648–652.
HURYCH, J. & CHVAPIL, M. (1965). *Biochim. Biophys. Acta*, **97**, 361–363.
HUTTON, J. J., TAPPEL, A. L. & UDENFRIEND, S. (1966). *Analyt. Biochem.*, **16**, 384–394.
KIVIRIKKO, K. I. & PROCKOP, D. J. (1967). *Proc. natn. Acad. Sci. U.S.A.*, **57**, 782–789.
KORNBERG, A. (1955). *Meth. Enzym.*, **2**, 497–500.
KRUMHOLZ, P. (1949). *Nature, Lond.*, **163**, 724–725.
LUKENS, L. N. (1965). *J. biol. Chem.*, **240**, 1661–1669.
MYHILL, D. & JACKSON, D. S. (1963). *Analyt. Biochem.*, **6**, 193–198.
NORDWIG, A., KOBRLE, V. & PFAB, F. K. (1967). *Biochim. Biophys. Acta*, **147**, 487–496.
PERRIN, D. D. (1958). *Nature, Lond.*, **182**, 741–742.
PETERKOFSKY, B. & UDENFRIEND, S. (1963). *J. biol. Chem.*, **238**, 3966–3977.
PROCKOP, D. J. & JUVA, K. (1965). *Proc. natn. Acad. Sci. U.S.A.*, **53**, 661–668.
ROKOSOVA-CMUCHALOVA, B. & BENTLEY, J. P. (1968). *Biochem. Pharmac., Suppl.*, 315–328.
SCOTT, J. E. (1960). *Meth. biochem. Analysis*, **8**, 145–197.
SMITH, M. J. H. & SMITH, P. K. (1966). *The Salicylates.* London: Interscience.
STEGEMANN, H. (1958). *Z. Physiol. Chem.*, **311**, 41–45.
TELSER, A., ROBINSON, H. C. & DORFMAN, A. (1965). *Proc. natn. Acad. Sci. U.S.A.*, **54**, 912–919.
TROLL W. & LINDSLEY, J. (1955). *J. biol. Chem.*, **215**, 655–660.

J. Pharm. Pharmac., 1971, **23**, 407–411 *Received October* 14, 1970

Evidence that the hypotensive action of methyldopa is mediated by central actions of methylnoradrenaline

M. HENNING AND A. RUBENSON

Department of Pharmacology, University of Göteborg, Göteborg, Sweden

Mean arterial blood pressure was recorded in conscious normotensive rats through indwelling arterial catheters. The effect of L-α-methyldopa (α-MD) (400 mg/kg, i.p.) was studied in animals pretreated with α-methyl-*m*-tyrosine (400 mg/kg i.p.) 27 and 15 h before α-MD, α-methyl-*p*-tyrosine methylester (H 44/68) (250 mg/kg, i.p.) 1 h before α-MD, and DL-α-hydrazino-α-methyl-β-(3,4-dihydroxyphenyl) propionic acid (MK 485, 100 mg/kg, i.p.) 30 min before α-MD. This pretreatment, which resulted in a severe depletion of endogenous catecholamines, did not alter the hypotensive effect of α-MD. The effect of α-MD (200 mg/kg, i.p.) was studied 30 min after pretreatment with the dopamine β-hydroxylase inhibitor, bis (4-methyl-1-homopiperazinyl-thiocarbonyl) disulphide (FLA-63) (25 mg/kg, i.p.). The hypotensive response to α-MD was completely abolished in these experiments. The formation of α-methylnoradrenaline from α-MD was prevented after FLA-63 but there was a significant increase in the amounts of α-methyldopamine formed.

A new possibility for the mechanism of action of hypotensive drugs has recently been suggested by the finding that, after inhibition of peripheral decarboxylase, L-dopa exerts a centrally mediated hypotensive effect (Henning & Rubenson, 1970 a, b). There is evidence that this action is brought about through activation of inhibitory mechanisms, possibly of a noradrenergic nature and it is also exerted by certain analogues of L-dopa, e.g. *m*-tyrosine (Rubenson, 1971).

The structurally related amino-acid, L-α-methyl-3,4-dihydroxyphenylalanine (α-methyldopa, α-MD) lowers arterial pressure in animals and man through mechanisms which are not yet completely understood (for review see Henning, 1969b). Current views on the mode of action of α-MD emphasize the importance of its metabolites, in particular α-methylnoradrenaline (α-MNA), which is thought to act as a pseudotransmitter in sympathetic nerves, thereby producing a functional impairment in these nerves and hence a decrease in blood pressure (for review see e.g. Kopin, 1968). There is no doubt that the hypotensive action of α-MD is related to an enzymatic decarboxylation since potent inhibitors of dopa decarboxylase in the central nervous system and peripheral tissues prevent the lowering of blood pressure (Davis, Drain & others, 1963; Henning, 1968, 1969a). However, it has also been demonstrated that selective inhibition of peripheral decarboxylase leaves the hypotensive effect of α-MD unchanged (Henning, 1969a). Taken together, these findings clearly point to the importance of a decarboxylation of α-MD within the central nervous system for the hypotensive action of the drug (for review see Henning, 1969b).

However, these results do not permit any conclusions about which of the decarboxylation products of α-MD is responsible for the central effect, or if their action is mediated by a false transmitter mechanism in the brain. The recent availability of potent

and relatively specific inhibitors of dopamine-β-hydroxylase (Florvall & Corrodi, 1970) seemed to offer an approach to this question. Therefore, the action of α-MD on blood pressure and tissue monoamine levels was studied before and after pretreatment with bis(4-methyl-1-homopiperazinyl-thiocarbonyl)disulphide (FLA-63), an inhibitor of dopamine β-hydroxylase (Svensson & Waldeck, 1969; Florvall & Corrodi, 1970). It was found that the hypotensive action of α-MD in conscious normotensive rats was abolished after pretreatment with FLA-63.

METHODS

Male Sprague-Dawley rats, 200–250 g, were used. Mean arterial pressure was recorded through indwelling arterial catheters connected to Statham P23 Dc pressure transducers writing on a Grass polygraph (Henning 1969b). Using this method blood pressure varies little with time of day or on subsequent days. Therefore, the basal values were obtained by continuous recording for 15–30 min periods and the average of the last 10 min was taken as the level of blood pressure. Similar recordings were made at intervals after administration of saline (0·9%) or drugs. No animal received the same treatment more than once.

The amine contents of isolated organs were measured. The brains, hearts and spleens from two animals were separately pooled. Dopamine and noradrenaline were determined according to Carlsson & Lindqvist (1962) and Bertler, Carlsson & Rosengren (1958). α-Methyldopamine was determined as described by Carlsson & Lindqvist (1962) and α-methylnoradrenaline as described by Waldeck (1968). To avoid interference of noradrenaline in determinations of α MNA, rats were pretreated with reserpine (10 mg/kg) 16h before the experiments.

RESULTS

Blood pressure

The effect of α-MD (400 mg/kg) on blood pressure was studied in animals pretreated with α-MMT (two doses of 400 mg/kg, 27 and 15 h before α-MD), H 44/68 (250 mg/kg, 60 min before α-MD) and MK 485 (100 mg/kg, 30 min before α-MD). The results are shown in Fig. 1. There was a significant decrease in blood pressure 3 h ($P < 0·025$) and 6 h ($P < 0·001$) after α-MD. In another series of experiments 0·9% saline was given instead of α-MD and produced no significant changes in blood pressure.

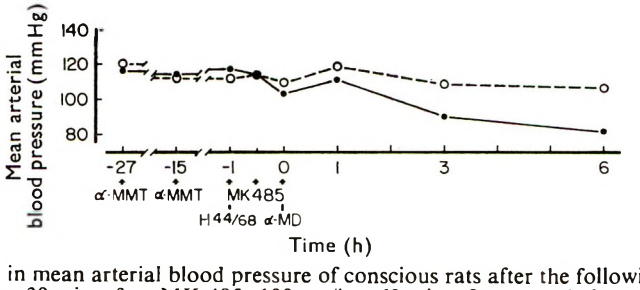

FIG. 1. Changes in mean arterial blood pressure of conscious rats after the following treatments: α-MD, 400 mg/kg, 30 min after MK 485, 100 mg/kg, 60 min after α-methyl-*p*-tyrosine methylester (H 44/68; 250 mg/kg) and 15 h and 27 h after two doses of α-methyl-*m*-tyrosine (α-MMT), 400 mg/kg (solid symbols; error variance 52·3, n = 4); 0·9% saline, 5 ml/kg, 30 min after MK 485, 100 mg/kg, 60 min after H 44/68, 250 mg/kg and 15 h and 27 h after two doses of α-MMT, 400 mg/kg (open symbols; error variance 99·2, n = 4). The values are means in mm Hg; s.e. were calculated by analysis of variance.

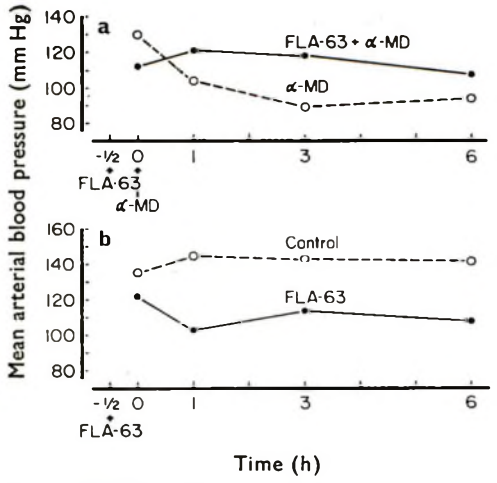

FIG. 2. Changes in mean arterial blood pressure of conscious rats after i.p. injections of drugs as indicated. a. α-MD, 200 mg/kg (open symbols; error variance 100·7, n = 5); α-MD, 200 mg/kg, 30 min after FLA-63, 25 mg/kg (solid symbols; error variance 95·7, n = 5). b. Saline (0·9%) 5 ml/kg, 30 min after FLA-63, 25 mg/kg (solid symbols; s.e. = 3·8, n = 7); two injections of 0·9% saline, 5 ml/kg, 30 min apart (open symbols; s.e. = 4·4, n = 5). The values are means in mm Hg; s.e. were calculated by analysis of variance.

Fig. 2 shows the effect of α-MD alone and after pretreatment with FLA-63. Administration of α-MD (200 mg/kg) lowered mean arterial blood pressure significantly after 1, 3 and 6 h ($P < 0{\cdot}001$). After pretreatment with FLA-63 (25 mg/kg), no significant changes ($P < 0{\cdot}10$) in blood pressure occurred, the hypotensive effect of α-MD (200 mg/kg) being completely abolished. FLA-63 alone (25 mg/kg) caused a slight lowering of blood pressure, particularly 1·5 h after its administration ($P < 0{\cdot}005$) (Fig. 2b). When equivalent volumes of 0·9% saline were injected, no significant changes in blood pressure occurred.

Amine contents of organs

Table 1 shows the levels of dopamine and noradrenaline in brain after pretreatment with αMMT and H 44/68 as described in the blood pressure experiments. There was a marked lowering of both amines. Three h after the injection of α-MD (200 mg/kg) to reserpinized rats, significant amounts of α-methyl-dopamine (α-MDA) and α-MNA had accumulated in all organs examined (Table 2). Pretreatment with FLA-63 (25 mg/kg), significantly prevented the formation of α-MNA

TABLE 1. *Concentrations of dopamine and noradrenaline after administration of two doses of α-methyl-*m-*tyrosine* (α-MMT; 400 mg/kg i.p. 27 *and* 15 h *before death*), *α-methyl-*p-*tyrosine methylester* (H 44/68; 250 mg/kg, i.p. 1 h *before death*) *and* MK 485 (100 mg/kg, i.p. 30 min *before death*). The values are means in μg/g with s.e.

	Brain content of amines (μg/g)			
	No pretreatment		αMMT + H 44/68 + MK 485	
	Dopamine	Noradrenaline	Dopamine	Noradrenaline
Mean	0·640	0·357	0·196	0·028
s.e.	0·011	0·013	0·051	0·005
	(n=2)	(n=2)	(n=3)	(n=3)

Table 2. *Concentration of α-methyldopamine and α-methylnoradrenaline* 3 h *after α-methyldopa* (α-MD) (200 mg/kg, i.p.), *or* FLA-63 (25 mg/kg, i.p.) *plus α-methyldopa* (200 mg/kg, i.p., 30 min *after* FLA-63) in *rats pretreated with reserpine* (10 mg/kg) 16h *previously*. The values are means in μg/g. *P* values are calculated by analysis of variance and *t*-test.

	Brain			Heart			Spleen		
	Control	α-MD	FLA+α-MD	Control	α-MD	FLA+αMD	Control	α-MD	FLA+α-MD
	A	B	C	A_1	B_1	C_1	A^2	B^2	C^2
				α-Methyldopamine					
n =	(1)	(3)	(3)	(1)	(3)	(3)	(1)	(3)	(3)
	0·000	0·252	0·442	0·000	0·045	0·077	0·000	0·066	0·047
ϕ Within group		4			4			4	
Variance within group		0·00567			0·00012			0·00100	
		A–B: $P < 0·001$			A_1–B_1: $P \approx 0·05$			A^2–B^2: $P > 0·1$	
		A–C: $P < 0·001$			A_1–C_1: $P < 0·005$			A^2–C^2: $P > 0·1$	
		B–C: $P < 0·001$			B_1–C_1: $P \approx 0·05$			B^2–C^2: $P > 0·1$	
				α-Methylnoradrenaline					
n =	(4)	(6)	(6)	(3)	(6)	(6)	(4)	(6)	(6)
	0·016	0·175	0·017	0·004	0·176	0·044	0·019	0·200	0·062
		13			12			13	
Variance within group		0·00026			0·00072			0·00443	
		A–B: $P < 0·001$			A_1–B_1: $P < 0·001$			A^2–B^2: $P \approx 0·001$	
		A–C: $P > 0·1$			A_1–C_1: $P > 0·1$			A^2–C^2: $P > 0·1$	
		B–C: $P < 0·001$			B_1–C_1: $P < 0·001$			B^2–C^2: $P < 0·005$	

from α-MD ($P < 0·001$) at the same time. The amounts of α-MDA were significantly increased ($P < 0·001$) in the brain.

DISCUSSION

The results of the present investigation confirm the ability of α-MD to lower mean arterial blood pressure in the conscious normotensive rat (Henning 1967).

Previous work has established that decarboxylation of α-MD in the central nervous system is necessary for its hypotensive action in the rat (Henning, 1968, 1969a). The α-methylated amines could act either directly or indirectly. The present results indicate that an indirect effect would seem less likely since α-MD retained its hypotensive effect after pretreatment with repeated doses of α-MMT in combination with the tyrosine hydroxylase inhibitor H44/68. There is a profound depletion of tissue catecholamines after α-MMT (Hess, Connamacher & others, 1961; Andén, 1964) and the combination used in the present study lowered brain noradrenaline to less than 10% of control values. Further, the lack of effect of α-MMT, in contrast to α-MD, on blood pressure (Henning, 1967) may point to the importance of direct effects of the decarboxylation products of α-MD. There is ample evidence that α-MMT is metabolized to α-methyl-*m*-tyramine and metaraminol in the brain (Carlsson & Lindqvist, 1962; Muscholl, 1966) and it seems likely that these amine products should have indirect actions to a similar extent as those amines formed after α-MD.

The present results show that pretreatment with FLA-63, an inhibitor of dopamine β-hydroxylase (Svensson & Waldeck, 1969; Florvall & Corrodi, 1970), prevented the hypotensive response to α-MD and completely prevented the formation of α-MNA while at the same time there was a significant increase of the amounts of α-MDA found.

Assuming a predominantly direct action of the decarboxylation products of α-MD in mediating the hypotensive action of the drug, the effect of FLA-63 reported here ascribes an important role to α-MNA. The recent observation that FLA-63 may rapidly deplete endogenous noradrenaline stores (Persson & Waldeck, 1970) is

probably less important in the case of α-MD, in view of the results with α-MMT discussed above.

Thus, there is an obvious similarity between the effect of α-MD and that previously observed in similar studies with L-dopa (Henning & Rubenson, 1970b) or *m*-tyrosine (Rubenson, 1971): all three drugs also produce hypotensive effects through central activation of inhibition of sympathetic mechanisms by their decarboxylation products. However, in the case of L-dopa and *m*-tyrosine, indirect mechanisms appear to be of major importance. Although the nature and exact localization of the actions is not known, it is of interest to note that the metabolites of L-dopa (Andén, 1969) as well as α-MD (Andén, Butcher & Engel, 1970) are capable of stimulating noradrenaline receptors in the rat spinal cord, but α-MMT, which lacks hypotensive properties in the rat, had no such effect. Interestingly, the antihypertensive drug clonidine (Catapresan) also stimulates central noradrenaline receptors (Andén, 1969; Andén, Corrodi & others, 1970).

Acknowledgements

The work has been supported by grants from the Faculty of Medicine, University of Göteborg, Göteborg; the Swedish State Medical Research Council (Nos B71-14X-2863-02 and B70-14X-155-06B) and by "Läkemedelsindustriföreningen", Stockholm, Sweden.

For a generous gift of drugs we are obliged to Merck Sharp & Dohme International (α-methyldopa and MK 485), Hoffman-La Roche, Basle, Switzerland (Ro 4–4602), AB Astra, Södertälje, Sweden (FLA-63) and AB Hässle, Göteborg, Sweden (H 44/68). We are greatly indebted to Miss Anna Janson and Miss Ann-Sofie Järnström for skilful technical assistance.

REFERENCES

ANDÉN, N. E. (1964). *Acta pharmac. tox.*, **21**, 260–271.

ANDÉN, N. E. (1969). Paper read at the Laurentian Conference on L-dopa, Val David, Que., Canada, November 9–11, 1969.

ANDÉN, N. E., BUTCHER, S. G. & ENGEL, J. (1970a). *J. Pharm. Pharmac.*, **22**, 548–550.

ANDÉN, N. E., CORRODI, H., FUXE, K., HÖKFELT, T., RYDIN, C. & SVENSSON, T. (1970b). *Life Sci.* **9**, 513–523.

BERTLER, A., CARLSSON, A. & ROSENGREN, E. (1958). *Acta physiol. scand.*, **44**, 273–292.

CARLSSON, A. & LINDQVIST, M. (1962). *Ibid.*, **54**, 87–94.

DAVIS, R. A., DRAIN, D. J., HORLINGTON, M., LAZARE, R. & URBANSKA, A. (1963). *Life Sci.*, **3**, 193–197.

FLORVALL, L. & CORRODI, H. (1970). *Acta pharm.*, **7**, 7–22.

HENNING, M. (1967). *J. Pharm. Pharmac.*, **19**, 775–779.

HENNING, M. (1968). *Br. J. Pharmac.*, **34**, 233 p.

HENNING, M. (1969a). *Acta pharmac. tox.*, **27**, 135–148.

HENNING, M. (1969b). *Acta physiol. scand.*, suppl. 322.

HENNING, M. & RUBENSON, A. (1970a). *J. Pharm. Pharmac.*, **22**, 241–243.

HENNING, M. & RUBENSON, A. (1970b). *Ibid.*, **22**, 553–560.

HESS, S. M., CONNAMACHER, R. H., OZAKI, M. & UDENFRIEND, S. (1961). *J. Pharmac. exp. Ther.*, **134**, 129–138.

KOPIN, I. J. (1968). *A. Rev. Pharmac*, **8**, 377–394.

MUSCHOLL, E. (1966). *Ibid.*, **6**, 107–128.

PERSSON, T., WALDECK, B. (1970). *J. Pharm. Pharmac.*, **22**, 473–478.

RUBENSON, A. (1971). *Ibid.*, **23**, 412–419.

SVENSSON, T. & WALDECK, B. (1969). *Europ. J. Pharmac.*, **7**, 278–282.

WALDECK, B. (1968). *J. Pharm. Pharmac.* **20**, 163–164.

J. Pharm. Pharmac., 1971, 23, 412–419 *Received October* 14, 1970

Analysis of the action of *m*-tyrosine on blood pressure in the conscious rat: evidence for a central hypotensive effect

ALLAN RUBENSON

Department of Pharmacology, University of Göteborg, Göteborg, Sweden

Mean arterial blood pressure was recorded through indwelling arterial catheters in conscious normotensive rats. DL-*m*-Tyrosine, 400 mg/kg, was given intraperitoneally alone and after pretreatment with two inhibitors of dopa decarboxylase [DL-α-hydrazino-α-methyl-β-(3,4-dihydroxyphenyl) propionic acid (MK 485) or N^1-(DL-seryl)-N^2-(2,3,4-trihydroxybenzyl)hydrazine (Ro 4-4602)]. DL-*m*-Tyrosine alone produced a hypertensive response, but after MK 485 it caused a significant lowering of blood pressure after 5–7 min and after Ro 4-4602 (400 + 200 mg/kg) it had no significant influence on blood pressure. The hypotensive response to DL-*m*-tyrosine was not influenced by the central dopamine receptor blocking agent spiroperidol (0·1 mg/kg) or by pretreatment with the tyrosine hydroxylase inhibitor H 44/68 (250 mg/kg). However, the depressor action could be completely inhibited after depletion of central catecholamines by α-methyl-*m*-tyrosine, 400 + 400 + 200 mg/kg, in combination with H 44/68, 250 mg/kg. Further, the depressor action was abolished by the dopamine β-hydroxylase inhibitor bis (4-methyl-1-homopiperazinyl-thiocarbonyl) disulphide (FLA-63) 40 mg/kg. In correlative biochemical experiments the concentrations of the decarboxylation products of *m*-tyrosine were measured in brain and heart. DL-*m*-Tyrosine alone produced an accumulation of *m*-tyramine and *m*-octopamine in these tissues. MK 485 + *m*-tyrosine substantially reduced the levels of *m*-tyramine and *m*-octopamine in the heart, but their accumulation in brain was largely unaltered. The results suggest that when decarboxylation of DL-*m*-tyrosine occurs in both the central and peripheral nervous system, there is a pressor action. When decarboxylation occurs mainly in the central nervous system there is a hypotensive response which is associated with accumulation of decarboxylation products of *m*-tyrosine.

We have recently observed that L-dopa may produce a hypotensive response via its metabolites acting in the central nervous system, possibly through activation of sympatho-inhibitory noradrenergic mechanisms (Henning & Rubenson, 1970 a, b). It is evident from the literature that structural analogues of L-dopa, e.g. *m*-tyrosine, have many pharmacological effects in common with the parent compound. Thus, *o*- and *m*-tyrosine produced sympathomimetic signs as well as central nervous excitatory actions in the rat and these effects were augmented by an inhibitor of monoamine oxidase (Mitoma, Posner & others, 1957). Like L-dopa, *m*-tyrosine increased motor activity of normal mice and had an awakening effect in reserpinized mice; inhibitors of monoamine oxidase augmented these actions (Blaschko & Chruschiel, 1960). Similar findings were reported in rats (Ernst, 1965). *m*-Tyrosine pretreatment largely prevented the appearance of the reserpine syndrome in mice and partially

protected the stores of dopamine and noradrenaline in the brain from the action of reserpine (Carlsson & Lindqvist, 1967).

These similarities between some actions of L-dopa and *m*-tyrosine prompted a study of the effects of *m*-tyrosine on blood pressure and tissue monoamines, using essentially the same approach as that previously employed to study L-dopa (Henning & Rubenson, 1970b). The results show that *m*-tyrosine, like L-dopa, has a central hypotensive effect which is probably of noradrenergic nature and which may be indirect, mediated by displacement of endogenous noradrenaline.

METHODS

Male Sprague-Dawley rats, 250–300 g, were used. Mean arterial blood pressure was recorded in conscious unrestrained animals through indwelling arterial catheters connected to Statham P23 Dc pressure transducers writing on a Grass Polygraph (Henning, 1969). The blood pressure values represent averages of recordings for the 10 min periods immediately before the administration of the drugs except those values after *m*-tyrosine which are averages of the pressure for 5–10 min after the injection. For doses and time intervals see results. Tests of significance were conducted by analysis of variance with two independent criteria of classification followed by the *t*-test. For the examination of *m*-tyramine and *m*-octopamine content in heart and brain, organs from 2–4 animals of corresponding body weight were pooled. *m*-Tyramine and *m*-octopamine were purified and separated by cation exchange chromatography and determined spectrophotofluorimetrically after condensation with *o*-phthalaldehyde (Shore & Alpers, 1964). Recovery was checked by adding known amounts of amines to aliquots of the brain extracts. All values in brain were corrected for recovery (see Table 1).

Drugs

The following drugs were used for the purposes stated: DL-α-hydrazino-α-methyl-β-(dihydroxyphenyl)propionic acid (MK 485)—inhibition of peripheral dopa decarboxylase (Porter, Watson & others, 1962); N^1-DL-seryl-N^2-2,3,4-trihydroxybenzylhydrazine hydrochloride (Ro4-4602)—inhibition of both central and peripheral dopa decarboxylase (Burkard, Gey & Pletscher, 1964); bis-(4-methyl-1-homopiperazinylthiocarbonyl)disulphide (FLA-63)—inhibition of dopamine-β-hydroxylase (Florvall & Corrodi, 1970); 8-[3-(4-fluorobenzoyl)-propyl]-4-oxo-1-phenyl-1,3,8-triaza-spiro-[4,5]decane (spiroperidol)—blockade of central dopamine receptors (Andén, Butcher & Engel, 1970); DL-α-methyl-*p*-tyrosine methylester (H 44/68)—inhibition of tyrosine hydroxylase (Corrodi & Hanson, 1966); DL-α-methyl-*m*-tyrosine (α-MMT)—depletion of tissue catecholamines (for review see Muscholl, 1966).

RESULTS

Blood pressure experiments

m-*Tyrosine.* After an injection of DL-*m*-tyrosine (400 mg/kg, i.p.) a rapid increase in mean arterial blood pressure was observed (Fig. 1 a), with a maximal effect after 5–10 min ($P < 0{\cdot}005$). The pressure was still elevated after 20 min ($P < 0{\cdot}01$), but not after 30 min (Fig. 1a).

The animals showed piloerection and exophthalmus during the period of increased blood pressure. In addition, there was an increase in locomotor activity starting

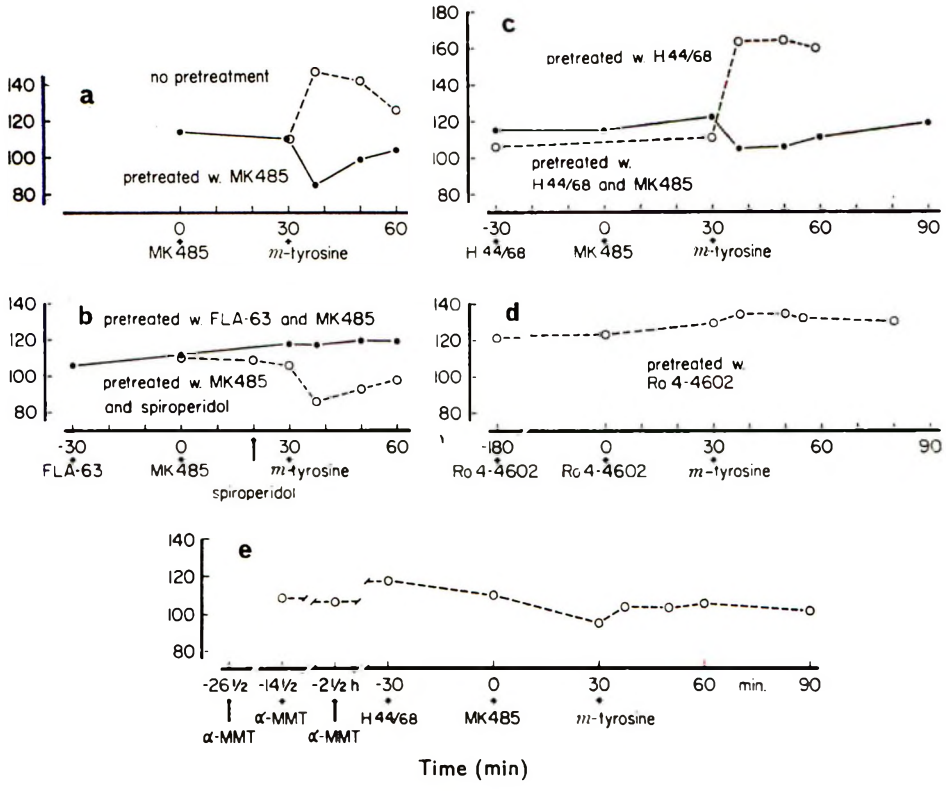

FIG. 1. Changes in mean arterial blood pressure in conscious normotensive rats after i.p. injection of drugs as indicated. The blood pressure values represent averages of recordings during 10 min periods before and after the drugs except the values immediately after *m*-tyrosine which represent the average of the recording 5-7 min after its injection. S.e. were calculated by analysis of variance. a. DL-*m*-Tyrosine, 400 mg/kg (open symbols; s.e. = 7·6; 6 exp.). DL-*m*-Tyrosine, 400 mg/kg, 30 min after MK 485, 100 mg/kg (solid symbols; s.e. = 2·7; 6 exp.). b. DL-*m*-Tyrosine, 400 mg/kg, 30 min after MK 485, 100 mg/kg, and 60 min after FLA-63, 40 mg/kg (solid symbols; s.e. = 2·7; 5 exp.). DL-*m*-Tyrosine, 400 mg/kg, 10 min after spiroperidol, 0.1 mg/kg, and 30 min after MK 485, 100 mg/kg (open symbols; s.e. = 3·4; 5 exp.). c. DL-*m*-Tyrosine, 400 mg/kg, 60 min after α-methyl-*p*-tyrosine methylester (H 44/68) 250 mg/kg (open symbols; s.e. = 2·6; 4 exp.). DL-*m*-Tyrosine, 400 mg/kg, 60 min after H 44/68, 250 mg/kg, and 30 min after MK 485, 100 mg/kg (solid symbols; s.e. = 2·6, n = 7). d. DL-*m*-Tyrosine, 400 mg/kg, 3 h after Ro 4-4602, 400 mg/kg, and 0·5 h after Ro 4-4602, 200 mg/kg (s.e. = 2·5; 5 exp.). e. DL-*m*-Tyrosine, 400 mg/kg, 30 min after MK 485, 100 mg/kg, 60 min after H 44/68, 250 mg/kg, 3 h after α-methyl-*m*-tyrosine (αMMT), 200 mg/kg, 15 h after αMMT, 400 mg/kg, and 27 h after αMMT, 400 mg/kg (s.e. = 5·3, n = 4). Figures on ordinate represent mean arterial blood pressure (mm Hg); figures on abscissa represent time in min.

after 15–20 min. No clear signs of aggressiveness or stereotyped movements were observed.

Dopa decarboxylase inhibition with MK 485 + m-*tyrosine.* Injection of MK 485 (100 mg/kg) did not influence the mean arterial blood pressure in the subsequent 30 min ($P > 0{\cdot}10$). Then *m*-tyrosine (400 mg/kg, i.p.) produced a decrease in blood pressure (Fig. 1 a). The time course of this effect was similar to the hypertensive response to *m*-tyrosine alone, maximum effect being reached after 5–10 min ($P < 0{\cdot}001$). After 20 min there was still a significant lowering of blood pressure ($P < 0{\cdot}001$) when compared to the level before MK 485 ($P < 0{\cdot}01$ when compared to the level before *m*-tyrosine). The values after 30 min were lower than those obtained before MK 485 ($P < 0{\cdot}025$).

In these rats, piloerection and exophthalmus were not apparent but there was an increased spontaneous motility, including "rearing", starting after 15–20 min.

Dopa decarboxylase inhibition with Ro 4-4602 + m-*tyrosine.* After pretreatment with Ro 4-4602, 400 and 200 mg/kg 3 h and 0·5 h before hand respectively, *m*-tyrosine (400 mg/kg) had no significant effect on mean arterial blood pressure (Fig. 1d).

FLA-63 + *MK* 485 + m-*tyrosine.* In this series, rats were pretreated with an inhibitor of dopamine β-hydroxylase, FLA-63 (40 mg/kg), and MK 485 (100 mg/kg) 60 and 30 min, respectively, before injection of *m*-tyrosine (400 mg/kg). The blood pressure level 5–10 min after *m*-tyrosine was not significantly different from the level before *m*-tyrosine ($P > 0{\cdot}1$) or before MK 485 ($P > 0{\cdot}10$) but was significantly higher than the level before FLA 63 ($P < 0{\cdot}01$) (Fig. 1b).

The rats did not differ in gross behaviour from the animals given MK 485 + *m*-tyrosine only.

MK 485 + *spiroperidol* + m-*tyrosine.* Spiroperidol (0·1 mg/kg) was given 20 min after MK 485 (100 mg/kg) and 10 min before *m*-tyrosine (400 mg/kg) (Fig.1 b). *m*-Tyrosine lowered blood pressure to the same extent as in animals pretreated with MK 485 alone. Thus, the level of blood pressure 5–10 min after *m*-tyrosine was significantly lower than the levels before *m*-tyrosine, spiroperidol or MK 485 ($P > 0{\cdot}001$). This was also the case with the level of blood pressure after 20 min ($P < 0{\cdot}025$, $P < 0{\cdot}005$ and $P < 0{\cdot}001$, respectively). With this combination of drugs the increase in locomotor activity was less than in the previous series.

H 44/68 + m-*tyrosine.* Pretreatment with the tyrosine hydroxylase inhibitor, α-methyl-*p*-tyrosine methylester, H 44/68 (250 mg/kg), 60 min before *m*-tyrosine (400 mg/kg), did not influence the hypertensive effect of the latter drug (Fig. 1 c). Thus, there was a highly significant ($P < 0{\cdot}001$) increase in blood pressure at all intervals after *m*-tyrosine. The effects on gross behaviour were the same as rats receiving *m*-tyrosine alone.

H 44/68 + *MK* 485 + *m-tyrosine.* Fig. 1c also shows the effect of tyrosine hydroxylase inhibition on the hypotensive response to *m*-tyrosine after MK 485. H 44/68 (250 mg/kg) was given 30 min before MK 485 (100 mg/kg) and 60 min before *m*-tyrosine (400 mg/kg). In this series, *m*-tyrosine lowered blood pressure significantly after 5–10 min as well as 20 min (*P* values when compared to blood pressure before *m*-tyrosine: $<0{\cdot}001$: before MK 485: $<0{\cdot}025$; before H 44/68: $<0{\cdot}025$).

The behavioural changes were the same as those produced by *m*-tyrosine after MK 485.

α-*MMT* + *H* 44/68 + *MK* 485 + m-*tyrosine.* The effect of *m*-tyrosine was studied in a group of animals pretreated with α-MMT (400, 400 and 200 mg/kg) given 27, 15 and 3 h before *m*-tyrosine, H 44/68 (250 mg/kg) and MK 485 (100 mg/kg) given 60 and 30 min before *m*-tyrosine. There was no significant difference ($P > 0{\cdot}10$) between the initial blood pressure level and the levels after any of the drugs (Fig. 1e).

Heart and brain content of m-*tyramine and* m-*octopamine*

The results of organ analysis are shown in Table 1. All analyses were made at the time of maximal hypotensive effect of *m*-tyrosine after MK 485, i.e. 7 min after the injection of *m*-tyrosine.

Table 1. *Levels of* m-*tyramine and* m-*octopamine in brain and heart of normal rats 7 min after* m-*tyrosine* (400 mg/kg, i.p.) *alone and after pretreatment with MK* 485 (100 mg/kg i.p.) 30 *min previously.* A third group of animals were only given MK 485 (100 mg/kg) 37 min before death. Values of means and s.e. are given.

Treatment: MK 485 100 mg/kg

Brain (μg/g)		Heart (μg/g)	
m-Tyramine	*m*-Octopamine	*m*-Tyramine	*m*-Octopamine
0·19	0·021	0·20	0·029
0·14	0·026	0·15	0·042
—	0·020	0·15	0·033
Mean = 0·17	0·022	0·17	0·035
s.e. = 0·025	0·002	0·017	0·004

Treatment: *m*-tyrosine 400 mg/kg

Brain (μg/g)		Brain recovery (%)		Heart (μg/g)	
m-Tyramine	*m*-Octopamine	*m*-Tyramine	*m*-Octopamine	*m*-Tyramine	*m*-Octopamine
4·00	0·073	129	138	16·25	0·306
6·51	0·060	64	136	17·92	0·247
5·88	0·037	103	107	14·27	0·110
4·90	0·075	71	134	14·42	0·350
Mean = 5·32	0·061			Mean = 15·72	0·253
s.e. = 0·55	0·009			s.e. = 0·86	0·052

Treatment: MK 485 100 mg/kg + *m*-tyrosine 400 mg/kg

Brain (μg/g)		Brain recovery (%)		Heart (μg/g)	
m-Tyramine	*m*-Octopamine	*m*-Tyramine	*m*-Octopamine	*m*-Tyramine	*m*-Octopamine
4·32	0·041	69	136	3·28	0·11
4·22	0·071	73	116	3·01	0·14
5·13	0·049	56	93	3·90	0·078
2·64	0·026	76	113	—	0·042
2·87	0·106	64	91	1·36	0·050
Mean = 3·83	0·059			Mean = 2·88	0·084
s.e. = 0·47	0·014			s.e. = 0·54	0·018

m-Tyrosine alone (400 mg/kg) produced an increase in the *m*-tyramine content of brain and heart. Smaller amounts of *m*-octopamine were also formed. With both amines the concentrations in heart were about three times those in brain.

In animals given MK 485 (100 mg/kg) 30 min before *m*-tyrosine (400 mg/kg) much smaller amounts of *m*-tyramine as well as *m*-octopamine were found in the heart. However, there was also a decreased formation of *m*-tyramine in the brain and the levels of *m*-octopamine were about the same as in the heart. The ratio heart : brain was approximately 1 : 1 for both amines.

In animals treated with MK 485 (100 mg/kg) only and killed after 37 min, the amounts of *m*-tyramine and *m*-octopamine found were always much smaller than after *m*-tyrosine, alone or in combination with MK 485.

DISCUSSION

The results show that injection of *m*-tyrosine, like that of L-dopa, produces sympathomimetic symptoms and an increase in mean arterial blood pressure in conscious rats. The magnitude of the response to *m*-tyrosine was similar to that previously found after a much lower dose of L-dopa (Henning & Rubenson, 1970a,b), except

that the onset was more rapid and duration shorter. Previous work has indicated that *m*-tyrosine may produce sympathomimetic signs in rats (Mitoma & others, 1957).

The pressor effect of *m*-tyrosine was abolished by pretreatment with Ro 4-4602, which inhibits dopa decarboxylase in peripheral tissues and the brain (Pletscher & Gey, 1963; Burkhard, Gey & Pletscher, 1964) and also after inhibition of peripheral dopa decarboxylase alone by MK 485 (Porter, Totaro & Leiby, 1962; Bartholini & Pletscher, 1969). On the other hand, pretreatment with a tyrosine hydroxylase inhibitor, H 44/68, did not influence the increase in blood pressure. Therefore, it may be concluded that the sympathomimetic effects and the hypertensive response to *m*-tyrosine is mediated by its metabolites acting in the periphery. After inhibition of peripheral decarboxylase with MK 485, *m*-tyrosine produced a significant decrease in blood pressure. The time characteristics of this response were similar to those of the hypertensive reaction after *m*-tyrosine alone. A similar effect has been observed with L-dopa after MK 485 (Henning & Rubenson, 1970). Since the hypotensive response to *m*-tyrosine in the present study did not occur after a decarboxylase inhibitor which acts in the central nervous system, Ro 4-4602, the effect may be due to the central actions of *m*-tyrosine metabolites. It is well established that *m*-tyrosine is decarboxylated in the brain to form *m*-tyramine (Mitoma & others, 1957; Carlsson & Lindqvist, 1967; Andén, Butcher & Engel, 1970), and the β-hydroxylated derivative, β-hydroxy-*m*-tyramine has also been demonstrated in brain tissue after treatment with *m*-tyrosine (Carlsson & Lindqvist, 1967).

The response is therefore likely to be mediated via the decarboxylated products of *m*-tyrosine. The amines may exert their effect through different mechanisms: (1) direct stimulation of central nervous receptor mechanisms via (a) *m*-tyramine, (b) *m*-octopamine, or both, or (2) an indirect effect via displacement of endogenous amines by (a) *m*-tyramine, (b) *m*-octopamine, or both.

The present results appear to make a direct stimulation of dopamine and noradrenaline receptors less likely since severe depletion of these amines by pretreatment with α-MMT (Andén, 1964) in combination with the tyrosine hydroxylase inhibitor, H44/68, prevented the hypotensive response to *m*-tyrosine after MK 485.

The hypotensive effect of L-dopa after MK 485 is also prevented following this type of pretreatment (Rubenson, 1971). Since in these experiments the last dose of α-MMT was given only 3 h before *m*-tyrosine or L-dopa, it is possible that the decarboxylation of the latter amino-acids may have been prevented to some extent; α-MMT has a slight inhibitory effect on dopa decarboxylase (Hess, Connamacher & others, 1961; Porter, Watson & others, 1962). However, this appears less likely since identical results were obtained with L-dopa, regardless of whether the last dose of α-MMT was given (Rubenson, 1971).

Study of the flexor reflex activity of spinal rats by Andén & others (1970) has revealed that the metabolites of *m*-tyrosine, in contrast to those of L-dopa, are unable to stimulate central noradrenaline receptors. However, in these experiments the rats were pretreated with reserpine and indirect effects may have been prevented. Pretreatment with an inhibitor of dopamine β-hydroxylase, FLA-63 (Svensson & Waldeck, 1969; Florvall & Corrodi, 1970), blocked the depressor response to *m*-tyrosine after MK 485. Therefore this response may have been produced by the β-hydroxylated product of *m*-tyrosine. Another possibility to be considered is an indirect effect by the *m*-tyramine formed. Recent studies indicate that FLA-63, in

the dose used in the present experiments, may reduce endogenous noradrenaline in the brain (Persson & Waldeck, 1970). The effect of FLA-63 in the present investigation could therefore also be explained by a decreased availability of endogenous noradrenaline for displacement by *m*-tyramine. On the other hand, pretreatment with the tyrosine hydroxylase inhibitor H 44/68, unlike FLA-63, did not influence the hypotensive response to *m*-tyrosine after MK 485. However, the lowering of endogenous noradrenaline after H 44/68 is not as rapid as that after FLA-63 (Persson & Waldeck, 1970).

The *m*-tyrosine injected may be decarboxylated both intra- and extraneuronally in the brain. In this connection, the existence of dopa decarboxylase in the brain capillaries (Bertler, Falck & others, 1966) is of interest. The tendency to a decreased accumulation of *m*-tyramine after MK 485 + *m*-tyrosine compared to that seen after *m*-tyrosine alone might be explained by a decreased extraneuronal accumulation of *m*-tyramine due to inhibition of the capillary decarboxylase by MK 485 (*cf.* Constantinidis, De la Torre & others, 1969). The levels of β-hydroxy-*m*-tyramine (*m*-octopamine) which may be assumed to be formed from *m*-tyramine mainly intraneuronally were not reduced after MK 485 pretreatment and this gives further support for this explanation. In the heart, MK 485 prevented the accumulation of *m*-hydroxylated amines to a large extent.

The biochemical studies thus show a central accumulation of *m*-tyramine and *m*-octopamine at the time of the maximal hypotensive effect.

In conclusion, both the biochemical and functional studies point to a central nervous origin of the hypotensive effect of *m*-tyrosine after peripheral dopa decarboxylase inhibition. The hypotensive effect appears to be elicited by the catabolites of *m*-tyrosine which may act indirectly by displacement of endogenous catecholamines, particularly noradrenaline.

Acknowledgements

The work has been supported by grants from the Faculty of Medicine, University of Göteborg, Göteborg; the Swedish State Medical Research Council (Nos B71-14X-2863-02 and B70-14X-155-06B) and by "Överläkare Albert Wallins fond". For generous gifts of drugs I am obliged to Hoffmann-La Roche & Co., Basle, Switzerland (Ro 4-4602); Merck Sharp & Dohme International (MK 485); AB Astra, Södertälje, Sweden (FLA-63); Janssen Pharmaceutica, Beerse, Belgium (spiroperidol). I am greatly indebted to Miss Anna Janson and Miss Ann-Sofie Järnström for skilful technical assistance.

REFERENCES

ANDÉN, N. E. (1964). *Acta pharmac. tox.*, **21**, 260–271.

ANDÉN, N. E., BUTCHER, S. G. & ENGEL, J. (1970). *J. Pharm. Pharmac.*, **22**, 548–550

BARTHOLINI, G. & PLETSCHER, A. (1969). *Ibid.*, **21**, 323–324.

BERTLER, A., FALCK, B., OWMAN, CH. & ROSENGREN, E. (1966). *Pharmac. Rev.* 18: 1, No. 1, 369–385.

BLASCHKO, H. & CHRUSCHIEL, T. L. (1960). *J. Physiol., Lond.*, **151**, 272–284.

BURKARD, W. P., GEY, K. F. & PLETSCHER, A. (1964). *Arch. Biochem. Biophys.*, **107**, 187–196

CARLSSON, A. & LINDQVIST, M. (1967). *Europ. J. Pharmac.*, **2**, 187–192.

CONSTANTIDINIS, J., DE LA TORRE, J. C., TISSOT, R. & GEISSBUHLER, F. (1969). *Psychopharmacologia*, **15**, 75–87.

CORRODI, H. & HANSON, L. C. F. (1966). *Ibid.*, **10**, 116–125.

ERNST, A. M. (1965). *Ibid.*, **7**, 391–399.

FLORVVLL, L. & CORRODI, H. (1970). *Acta pharm.*, **7**, 7–72.

HENNING, M. (1969). *Acta physiol. scand., Suppl.* 322.

HENNING, M. & RUBENSON, A. (1970a). *J. Pharm. Pharmac.*, **22**, 241–243.

HENNING, M. & RUBENSON, A. (1970b). *Ibid.*, **22**, 553–560.

HESS, S. M., CONNAMACHER, R. H., OZAKI, M. & UDENFRIEND, S. (1961). *J. Pharmac. exp. Ther.*, **134**, 129–138.

MITOMA, C., POSNER, H. S., BOGDANSKI, D. F. & UDENFRIEND, S. (1957). *Ibid.*, **120**, 188–194.

MUSCHOLL, E. (1966). *A. Rev. Pharmac.*, **6**, 107–128.

PERSSON, T. & WALDECK, B. (1970). *J. Pharm. Pharmac.*, **22**, 473–478.

PLETSCHER, A. & GEY, K. F. (1963). *Biochem. Pharmac.*, **12**, 223–228.

PORTER, C. P., TOTARO, J. A. & LEIBY, C. M. (1961). *J. Pharmac. exp. Ther.*, **134**, 139–145.

PORTER, C. C., WATSON, L. S., TITUS, D. C., TOTARO, J. A. & BYER, S. S. (1962). *Biochem. Pharmac.*, **11**, 1067–1077.

RUBENSON, A. (1971). *J. Pharm. Pharmac.*, **23**, 228–230.

SHORE, P. A. & ALPERS, H. S. (1964). *Life Sci.*, **3**, 551–554.

SVENSSON, T. & WALDECK, B. (1969). *Europ. J. Pharmac.*, **7**, 278–282.

J. Pharm. Pharmac., 1971, 23, 420–424 *Received January* 18, 1971

DL-5-Hydroxytryptophan-induced changes in central monoamine neurons after peripheral decarboxylase inhibition*

KJELL FUXE, LARRY L. BUTCHER† AND JÖRGEN ENGEL

Department of Histology, Karolinska Institutet, S-104 01 Stockholm 60 and Department of Pharmacology, University of Göteborg, S-400 Göteborg 33, Sweden

The histochemical effects of 500 and 1000 mg/kg of DL-5-hydroxytryptophan (5-HTP), both alone and in combination with a peripheral decarboxylase inhibitor (seryl-trihydroxy benzyl hydrazine; Ro 4-4602) have been examined on central monoamine neurons of rats by the Falck-Hillarp fluorescence technique that demonstrates monoamines and their precursors. 5-HTP alone or together with Ro 4-4602 caused only weak intraneuronal accumulation of 5-HT in the central 5-HT neurons, in spite of an increased entry of 5-HTP into the brain after Ro 4-4602 treatment, as shown by an increase in the specific neuropil fluorescence and a reduction of 5-HT accumulation in the cells of the capillary walls. Ro 4-4602 markedly potentiated the effects of 5-HTP on the central dopamine neurons, many of which became clearly yellow fluorescent. The mechanism of dopamine depletion by 5-HTP is probably therefore mainly one of displacement. The effects on the noradrenaline neurons were also potentiated by Ro 4-4602 pretreatment, the neurons exhibiting a yellow-green fluorescence. This depletion may therefore also be mainly be due to amine displacement. It is concluded that the ability of the 5-HT neurons to take up and accumulate 5-HT in the presence of 5-HTP is relatively low in spite of large amounts of 5-HTP present in the brain neuropil after extracerebral decarboxylase inhibition.

Recently a detailed histochemical analysis of the effects of L-3,4-dihydroxyphenylalanine (L-dopa) on central monoamine neurons after extracerebral decarboxylase inhibition was presented (Butcher, Engel & Fuxe, 1970). It was reported that in the presence of the decarboxylase inhibitor Ro 4-4602 [N^1-(DL-seryl)-N^2-(2,3,4-trihydroxybenzyl)hydrazine, (Bartholini & Pletscher, 1968)], the intraperitoneal injection of L-dopa to rats resulted in a marked accumulation of dopamine in central dopamine neurons. The intraneuronal dopamine concentrations in dopamine cell bodies, nonterminal axons, and nerve terminals were much increased compared with those obtained when L-dopa was used alone. It was also observed that the 5-hydroxytryptamine (5-HT) neurons contained catecholamines that had probably displaced existing 5-HT stores. This poses the question whether a similar picture might emerge after administration of 5-hydroxytryptophan (5-HTP), the precursor of 5-HT. Using a histochemical fluorescence analysis of monoamines (Falck, Hillarp & others, 1962; Hillarp, Fuxe & other, 1965; Corrodi & Jonsson, 1967), we have assessed the effects of DL-5-HTP on central monoamine neurons after peripheral decarboxylase inhibition by Ro4-4602.

* Portions of these data were presented at the Second International Meeting of the International Society for Neurochemistry; Milan, Italy; September 1–5, 1969, in a Round Table Discussion on the "Correlation between Behaviour and Transmitters in Brain".

† Present Address: Department of Psychology; University of California; Los Angeles, California, 90024, U.S.A.

MATERIAL AND METHODS

Male Sprague-Dawley rats (150–180 g) were injected intraperitoneally either with DL-5-HTP alone (dissolved in 0·9% saline with warming in doses either of 500 or 1000 mg/kg), six rats being in each dosage group; or with 5-HTP preceded by Ro 4-4602 (50 mg/kg in saline) 30 min before the 5-HTP administration; eight rats being in each dosage group. All animals were killed 75 min after precursor administration.

Normal untreated rats were included in each experiment, as were other control animals injected with saline alone in volumes comparable to those used in the experimental groups. Since Butcher & Engel (1969) and Butcher & others (1970) have shown that R o4-4602 (50 mg/kg) injected alone does not affect central monoamine levels, a separate control with this drug treatment was not included.

Immediately after death, brain and spinal cord were taken for histochemical fluorescence analyses of catecholamines and 5-HT (Dahlström & Fuxe, 1965; Fuxe & Jonsson, 1967). Serial transverse sections were made of all regions in each animal. Semi-quantitative estimations of fluorescence intensity were made on coded slides. It is known that a change in fluorescence intensity reflects a change in amine concentration (Olson, Hamberger & others, 1968; Jonsson, 1969).

RESULTS

DL-5-*HTP alone*

5-HT neurons. A slight increase in the fluorescence intensity of the 5-HT cell bodies and nerve terminals in the entire central nervous system, was observed but only at the 1000 mg/kg dose.

Dopamine neurons. The dopamine nerve terminals in the median eminence, which lies outside the blood brain barrier, had a strong yellow fluorescence, probably due to the accumulation of 5-HT and subsequent displacement of dopamine stores. However, the dopamine nerve terminals of the limbic forebrain and in the neostriatum showed no alterations in fluorescence intensity. After the high dose of 5-HTP, however, the fluorescence in these latter two areas changed from green to yellow-green, suggesting an increase in indoleamine. The dopamine cell bodies in the hypothalamus and the mesencephalon showed a small decrease in fluorescence intensity and a shift in colour towards yellow-green.

Noradrenaline neurons. After 1000 mg/kg of 5-HTP there was a clear decrease in the fluorescence intensity of the noradrenaline nerve terminals. In most areas of the brain the fluorescence was green to yellow-green. No changes in fluorescence intensity and colour were observed in the cell bodies.

Cells of the capillary walls (*pericytes and endothelial cells*). After 500 mg/kg of 5-HTP the pericytes and endothelial cells showed a strong yellow fluorescence which was increased with the 1000 mg/kg dose.

Extraneuronal tissue. A small increase, compared with untreated controls, in fluorescence intensity was observed in the neuropil. The fluorescence had a yellowish colour.

5-*HTP in combination with Ro* 4-4602

5-HT neurons. A slight increase in fluorescence intensity was observed in the 5-HT cell bodies and nerve terminals. But whether the increase was greater than that obtained with 5-HTP alone could not be assessed.

Dopamine neurons. In contrast to the results obtained with 5-HTP alone, the yellow fluorescence in the dopamine nerve terminals in the median eminence was no longer observed, probably because the decarboxylase inhibitor could effectively reach this area, which lies outside the blood brain barrier. Therefore, the decarboxylation of 5-HTP could not occur to any great extent. Instead a diffuse yellowish fluorescence was seen throughout the median eminence probably due to the presence in the brain tissue of 5-HTP which itself is converted into a fluorescent β-carboline by the histochemical technique (see Corrodi & Jonsson, 1967). The dopamine nerve terminals in the neostriatum and limbic forebrain showed a shift in fluorescence from green to yellow-green. In the nucleus amygdaloideus centralis and in the dorsolateral part of the nucleus interstitialis striae terminalis, the dopamine nerve terminals were clearly yellow. These effects were obtained with both doses of 5-HTP, a dose-dependent relation being observed.

The dopamine cell bodies of the arcuate nucleus (group A 12 according to Dahlström & Fuxe, 1964), of the substantia nigra (group A 9), and of the ventrolateral part of the mesencephalic reticular formation (group A 8) developed a moderate to strong yellow fluorescence. Normally they exhibit a weak to moderate green intensity. The yellow fluorescence was weaker in the medial part of the substantia nigra although it was more intense in all cell bodies than that formed in the 5-HT cell bodies. The dopamine cell bodies of group A 10 displayed practically no yellow fluorescence but did have a yellow-green colour, of normal intensity. The dopamine cell bodies exhibiting the greatest accumulation of 5-HT were those showing the strongest accumulation of dopamine after dopa treatment (Butcher, Engel & others, 1970). The yellow fluorescence in the dopamine cell bodies was primarily localized in a perinuclear ring as was the endogenous green fluorescence.

Noradrenaline neurons. In contrast to the results with 5-HTP alone, a decrease in fluorescence intensity of the noradrenaline nerve terminals was seen with both less and high doses of 5-HTP. The colour of the fluorescence was yellow-green. The cell bodies were not substantially affected; if anything, a small decrease in intensity was noted.

Cells of the capillary walls. In contrast to the effect after 5-HTP alone, virtually no yellow fluorescence was evident in the pericytes and endothelial cells, even with the high dose of 5-HTP.

Extraneuronal tissue. A strong yellowish fluorescence was observed throughout the neuropil, possibly due to the presence in the brain tissue of 5-HTP itself.

DISCUSSION

Previously, a relative specificity for the 5-HT neurons in the uptake and decarboxylation of 5-HTP, in doses of 20–100 mg/kg, in combination with tryptophan hydroxylase inhibition or monoamine oxidase inhibition has been observed (Fuxe, 1965; Corrodi, Fuxe & Hökfelt, 1967). The presence of 5-HT derived from 5-HTP could be detected only in the 5-HT neurons. The present study has demonstrated that with 5-HTP in doses of 500 or 1000 mg/kg, especially in combination with a peripheral decarboxylase inhibitor, this relative specificity is lost, and changes in the overall fluorescence pattern are also seen in the central dopamine and noradrenaline neurons.

Brain levels of 5-HT are increased approximately 5 times normal after injection of 500 mg/kg of 5-HTP and about 8 times normal after 1000 mg/kg of 5-HTP (Butcher,

(Henning & Rubenson, 1971; Butcher, Engel & Fuxe: unpublished observations). This mechanism would not be at variance with a similar hypothesis advanced to explain the depletion of central 5-HT administration of L-dopa in combination with Ro 4-4602 (Bartholini, DaPrada & others, 1968; Butcher & Engel, 1969).

The reason for the marked accumulation of 5-HT in the dopamine nerve cells may be due to the high capacity of these neurons to take up or to decarboxylate amine precursors, or both. With a high intraneuronal concentration of 5-HTP in dopamine neurons we would expect the 5-HT precursor to compete with normally available dopa for the decarboxylase enzyme, since the same enzyme is thought to catalyse the decarboxylation of both 5-HTP and dopa (Rosengren, 1960). This mechanism would also result in a net decrease of dopamine accompanied by an increase in 5-HT. Furthermore, the injected 5-HTP may compete with normally available L-dopa for entry into the brain.

With both doses of 5-HTP the depletion of noradrenaline by indoleamines is potentiated by Ro 4-4602 pretreatment. After high doses of 5-HTP, noradrenaline concentrations in mouse heart and brain (Andén, 1964) and in rat brain (Butcher, Engel & Fuxe. unpublished data) are reduced. The effect of 5-HTP on the noradrenaline neurons may be attributable to displacement of endogenous noradrenaline stores by the 5-HT formed.

When 5-HTP is given systemically to rats it is probably mainly decarboxylated extracerebrally since the decarboxylase activity is much higher in peripheral organs

like the kidney and the liver than in the brain (Hagen & Cohen, 1966), and, as suggested by the present experiments, the decarboxylase in the cells of the capillary walls may constitute a barrier against the entry of 5-HTP into the brain neuropil. However, by inhibiting the decarboxylase in the peripheral organs and in the cells of the capillary walls, using Ro 4-4602, the entry of 5-HTP into the brain tissue is facilitated, as shown by the marked increase in neuropil fluorescence. In spite of the fact that little intraneuronal 5-HT accumulation was observed, sufficient 5-HT must have been formed to reach the receptor sites since, as assessed by gross observation, the administration of 5-HTP alone, or in combination with Ro 4-4602, results in a syndrome characterized by immobilization, tremors in the head and the forelimbs, extension and abduction of the hindlimbs. Furthermore, the extensor hindlimb reflex activity of the spinal rat, which is dependent on the integrity of 5-HT receptor stimulation (Andén, 1968), is increased by combined Ro 4-4602–5-HTP treatment, but not appreciably by 5-HTP alone (Fuxe, unpublished observations). Finally, the present results illustrate the difficulties attendant on the correlation of histochemical, and particularly biochemical determinations of changes in 5-HT concentrations after 5-HTP with corresponding changes in function.

Acknowledgements

This research was supported by grants (Nos. B71–14X–715–06 and B71–14X–155–07C) from the Swedish Medical Research Council; by grants from the Faculty of Medicine, University of Göteborg; from "Magn. Bergvalls Stiftelse", from "Ollie och Elof Ericssons Stiftelse"; and from "W. och M. Lundgrens Stiftelse". L. L. B. was supported by postdoctoral fellowship 5 F2-AM-33, 747–02 from the National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service. We thank Prof. A. Pletscher (F. Hoffmann-La Roche and Co., Ltd.; Basle, Switzerland) for a generous supply of Ro 4-4602. The skillful technical assistance of Mrs. K. Andreasson, Mrs. U. Axelsson, Mrs. M. Baidins and Mrs. A. Eliasson is gratefully acknowledged.

REFERENCES

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

ANDÉN, N.-E. (1968). *Adv. Pharmac.* pp. 347–349, Editors: Garattini, S. L. & Shore, P. London-New-York: Academic Press Inc.

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

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

BUTCHER, L. L. & ENGEL, J. (1969). *Brain Res.*, **15**, 233–242.

BUTCHER, L. L., ENGEL, J. & FUXE, K. (1970). *J. Pharm. Pharmac.*, **22**, 313–315.

CORRODI, H., FUXE, K. & HÖKFELT, T. (1967). *Ibid.*, **19**, 433–438.

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

DAHLSTROM, A. & FUXE, K. (1964). *Acta physiol. scand.*, **62**, *Suppl.* 232.

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

FUXE, K. (1965). *Z. Zellforsch.*, **65**, 573–596.

FUXE, K. & JONSSON, G. (1967). *Histochemie*, **11**, 161–166.

HAGEN, P. B. & COHEN, L. H. (1966). *Handbook of Experimental Pharmacology*. Editors: Eichler, O. & Farah, A., Vol. XIX, pp. 182–205. Berlin-Heidelberg-New York: Springer.

HENNING, M. & RUBENSON, A. (1971). *Acta pharmac. tox.*

HILLARP, N.-Ä., FUXE, K. & DAHLSTRÖM, A. (1965). *Symposium on "Release of Biogenic Amines"*, pp. 31–57, Proc. Int. Wenner-Gren Center Symp., Stockholm, Editors: von Euler, U.S., Rosell, S. & Uvnas, B., Oxford-London-Edinburgh: Pergamon Press.

JONSSON, G. (1969). *J. Histochem. Cytochem.*, **17**, 714–723.

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

ROSENGREN, E. (1970). *Acta physiol. scand.*, **49**, 364–369.

J. Pharm. Pharmac., 1971, **23**, 425–428 *Received December* 29, 1970

The influence of hexobarbitone on calcium ion movements in isolated heart muscle

H. HAACKE AND P. A. VAN ZWIETEN

Department of Pharmacology, Christian-Albrechts-University, Kiel, W. Germany

In beating atria (frequency 180/min) sodium hexobarbitone ($7{\cdot}8 \times 10^{-4}$ M) inhibited the rate of uptake of $^{45}Ca^{2+}$ from the medium without influencing the total calcium content of the tissues. Concomitantly, the so-called exchangeable calcium fraction was diminished although not at equilbrium. In resting left auricles the barbiturate did not affect $^{45}Ca^{+}$-uptake or the exchangeable calcium fraction. It seems likely that the barbiturate reduces membrane permeability towards Ca^{2+} during excitation without influencing the permeability at rest.

Until now, relatively few studies of the influence of barbiturates on ionic fluxes in heart muscle have been published. Klaus and Lüllmann (1961) demonstrated an impaired K^{+}-efflux as a result of treatment with sodium hexobarbitone. Similarly, the efflux of $^{86}Rb^{+}$ from atrial tissue was also inhibited by hexobarbitone (van Zwieten, 1969). The influence on calcium metabolism has been studied only in brain slices, where Klaus (1967) demonstrated a significant inhibition of the Ca^{2+}-efflux by sodium hexobarbitone. As no report of the influence of barbiturates on cellular calcium metabolism in intact heart muscle tissue appears to have been published, we have examined the influence of sodium hexobarbitone on calcium ion movements in intact atria of the guinea-pig. The effect of the drug was investigated both in beating atria and in resting left auricles.

METHODS

Isolated atria or left auricles were dissected from guinea-pigs of either sex, 350–450 g, according to Hoditz & Lüllmann (1964). The left auricles, being devoid of pacemaker cells did not beat spontaneously. The organs were suspended in Muralt Tyrode solution that was continuously gassed with 5% carbon dioxide in oxygen. The calcium ion content of the medium was 1·8 mmol/litre. The temperature was maintained at 30° throughout. Supramaximal stimulation with rectangular pulses (duration 2 ms) at a frequency of 180/min was with a stimulator from Braun GmbH, Melsungen (W.-Germany). The organs were attached to a strain gauge, connected to an amplifier and Helcoscriptor recorder (type HE 86 t). The volume of the bath was 20 ml. The experiments were limited to a barbiturate concentration of $7{\cdot}8 \times 10^{-4}$ M, since at this concentration the contractile force was reduced by half of its initial value.

Calcium metabolism

Before addition of drug to the medium the organs were equilibrated in Tyrode solution at 30° for 30 min. During equilibration of the isolated atria electrical stimulation was applied. In all experiments, to allow a steady-state reduction of beat in presence of hexobarbitone to be reached, the drug was added to the medium before the uptake of ^{45}Ca was measured. Where the uptake of $^{45}Ca^{2+}$ was studied, the bath

volume was 750 ml. The uptake of $^{45}Ca^{2+}$ by the isolated atria or left auricles was determined according to Hoditz & Lüllmann (1964), Lahrtz, Lüllmann & van Zwieten (1967) and Haacke, Lüllmann & van Zwieten (1970). The total calcium content of the organs was determined spectrofluorimetrically (Zepf, 1966). Sodium hexobarbitone was obtained from Bayer AG, Leverkusen. $^{45}CaCl_2$ (specific activity of the stock solution 5 Ci/mmol) was supplied by the Radiochemical Centre, Amersham, U.K.

RESULTS AND DISCUSSION

Sodium hexobarbitone ($7{\cdot}8 \times 10^{-4}$ M) diminished the contractile force of electrically stimulated atria by 58 $\pm$ 4% of the initial value (mean $\pm$ s.e., n = 6). The initial steep phase of the uptake curves (Fig. 1) probably represents the exchange of Ca^{2+} in the extracellular space against $^{45}Ca^{2+}$ from the medium (Lahrtz & others, 1967). From Fig. 1 it is obvious that the rate of uptake of $^{45}Ca^{2+}$ by beating atria was reduced in the presence of sodium hexobarbitone ($7{\cdot}8 \times 10^{-4}$M). Both in control experiments and in the presence of the drug, equilibrium was achieved after approximately 180 min of incubation. The equilibrium uptake (at t = 180 min) was not affected by the presence of hexobarbitone. Both in control experiments and also in presence of the

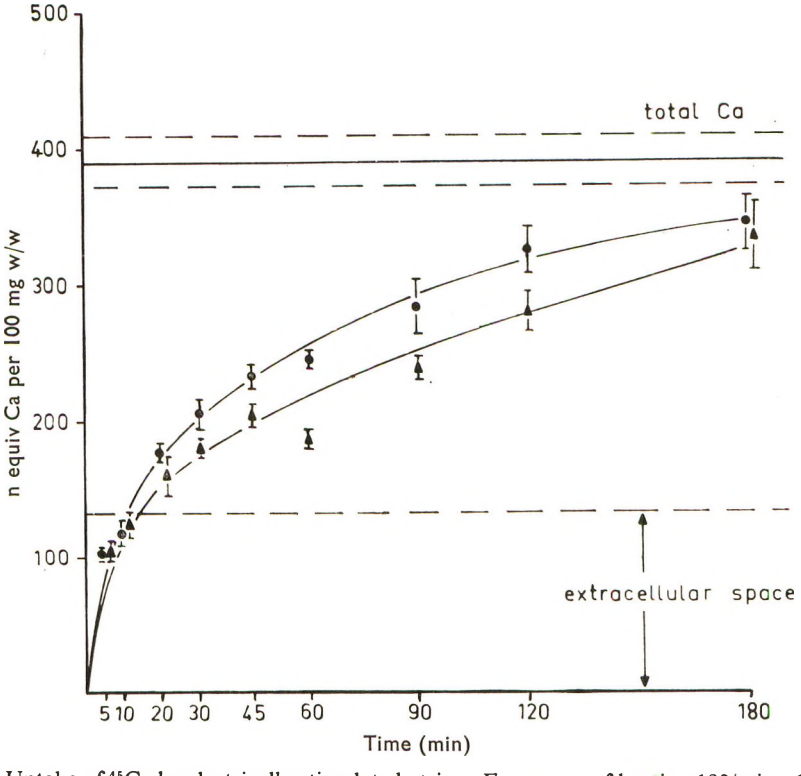

FIG. 1. Uptake of ^{45}Ca by electrically stimulated atria. Frequency of beating 180/min. Calcium content of the Tyrode solution 1·8 mmol/litre. Ordinate: n equiv calcium taken up from the ^{45}Ca-containing medium. The amount of calcium taken up was calculated from the tissue radioactivity. Each point on the curves represents the mean value ($\pm$s.e.) for 8–10 different organs. The size of the extracellular space is also shown (dotted line). The amount of calcium present in the extracellular compartment was calculated, the extracellular space amounting to 0·3 ml/g tissue. ○ - - - ○ ^{45}Ca uptake under control circumstances. △ - - - △ ^{45}Ca uptake in the presence of sodium hexobarbitone ($7{\cdot}8 \times 10^{-4}$ M).

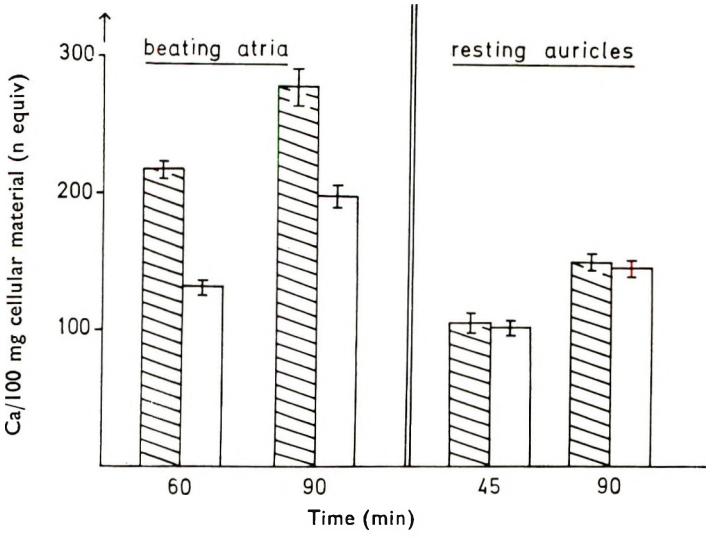

FIG. 2. Cellular exchange of calcium under influence of sodium hexobarbitone, determined by means of ^{45}Ca. For the calculations of the exchange the extracellular space of atrial tissue was assumed to be 0·3 ml per g tissue. The columns represent the mean values (± s.e.) for 8–10 different organs. Hatched columns: controls; open columns: sodium hexobarbitone ($7{\cdot}8 \times 10^{-4}$M).

barbiturate the total calcium concentration of the isolated organs remained unchanged throughout the experiments (control experiments: 383 ± 14 n equiv/100 mg, n = 71; hexobarbitone experiments: 395 ± 21 nl equiv/100 mg, n = 68).

To assess drug effects on calcium exchange, the cellular uptake of $^{45}Ca^{2+}$ was calculated in terms of nequiv Ca/100 mg cellular material. For these calculations an extracellular space of 0·3 ml/g tissue was assumed on the basis of previous measurements of [^{14}C]saccharose (Bauer, Lüllmann & Richter, 1963; Lüllmann & van Zwieten, 1967). Checking experiments using [^{14}C]saccharose confirmed this value and showed that electrical stimulation and the addition of hexobarbitone did not modify the saccharose space.

The results of the calculations are shown in Fig. 2. The differences in ^{45}Ca uptake owing to drug treatment already observed in Fig. 1 were also obvious if the amount of cellular calcium exchanged was taken into account. The determination of the ^{45}Ca-concentration and the total calcium content of the organs allows the calculation of the so-called exchangeable calcium fraction, which is obtained from the relation:

$$\text{exchangeable fraction} = \frac{\text{specific activity of Ca in the tissue}}{\text{specific activity of Ca in the medium}}$$

Since in our studies the total calcium content of the tissues remained virtually unchanged, a certain parallelism will exist between the drug-induced changes in ^{45}Ca uptake and those in the calculated exchangeable fraction (see Table 1). As shown in Fig. 2, the presence of $7{\cdot}8 \times 10^{-4}$M sodium hexobarbitone did not influence the uptake of ^{45}Ca by *resting* left auricles. In comparison with beating atria the rate of uptake of ^{45}Ca by resting organs under control circumstances was much lower (Fig. 2), agreeing with Hoditz & Lüllmann (1964). Both under control circumstances and in a hexobarbitone-containing medium the total calcium content of resting auricles remained unchanged throughout the experiments (controls: 377 ± 14 n equiv/100 mg wet weight, n = 25; hexobarbitone: 391 ± 19 n equiv/100 mg wet weight, n = 22).

Table 1. *Exchangeable calcium fraction in beating atria and in resting auricles.* Influence of sodium hexobarbitone ($7{\cdot}8 \times 10^{-4}$M). The exchangeable fraction was calculated from the data in Figs 1 and 2 (details see text) and was expressed as percentage of total tissue Ca.

	Beating atria exchangeable fraction (%)		Resting auricles exchangeable fraction (%)	
	60 min	90 min	45 min	90 min
Controls	50	69	20	40
Sodium hexobarbitone ($7{\cdot}8 \times 10^{-4}$M)	34	54	20·5	40

In contrast to beating organs the exchange in *resting* left auricles was not affected by sodium hexobarbitone. This observation would suggest that only the additionally occurring membrane permeability for Ca^{2+} during excitation is impaired by sodium hexobarbitone whereas *at rest* the barbiturate does not interfere with the passive calcium movements. Similar observations have been made for K^+ in atrial-tissue (Klaus & Lüllmann, 1971) and for Ca^{2+} and K^+ in brain slices that were studied at rest or when exposed to electrical stimulation (Klaus, 1967).

According to Chimoskey & Gergely (1968), differences in pH of the medium might account for the changes in calcium movements observed in isolated sarcoplasmic reticulum preparations that were exposed to barbiturates (Briggs, Gertz & Hess, 1966). But the pH of the media we used was in the range 7·2–7·3. However, it could be argued that the intracellular pH might be modified and could be of greater significance in affecting cellular calcium disposition than alterations of extracellular pH (cf. Waddell & Bates, 1969).

The present experiments indicate that the amount of calcium entering the cell per single excitation will be reduced in the presence of hexobarbitone. However, it might also be that the number of calcium ions mobilized within the cell is diminished in the presence of hexobarbitone as methods and calculations do not allow any differentiation between these two processes.

Acknowledgement

The skilful technical assistance of Mrs. M. Dorn is gratefully acknowledged. We are largely indebted for the support of this work by the Deutsche Forschungsgemeinschaft.

REFERENCES

Bauer, M., Lüllmann, H. & Richter, M. (1963). *Pflügers Arch. ges. Physiol.*, **277**, 48.

Briggs, F. N., Gertz, E. & Hess, M. L. (1966). *Biochem. Z.*, **345**, 122–131.

Chimoskey, J. E. & Gergely, J. (1968). *Archs int. Pharmacodyn. Thér.*, **176**, 289–297.

Haacke, H., Lüllmann, H. & van Zwieten, P. A. (1970). *Pflügers Arch. ges. Physiol.*, **314**, 113–123.

Hoditz, M. & Lüllmann, H. (1964). *Ibid.*, **280**, 22–29.

Klaus, W. (1967). *Anaesthesiology and Resuscitation*, **11**. Editors: Frey, R., Kern, F. & Mayerhofer, O. Berlin, New York: Springer.

Klaus, W. & Lüllmann, H. (1961). *Arch. exp. Path. Pharmak.*, **241**, 532–533.

Lahrtz, Hg., Lüllmann, H. & van Zwieten, P. A. (1967). *Biochim Biophys. Acta*, **35**, 701–709.

Lüllmann, H. & van Zwieten, P. A. (1967). *Medna Pharmac. exp.*, **16**, 89–94.

Waddell, W. & Bates, R. G. (1969). *Physiol. Rev.*, **49**, 285–329.

Zepf, S. (1966). *Zeiss Mitt. Fortschr. Tech. Opt.*, **4**, 43–57.

van Zwieten, P. A. (1969). *Br. J. Pharmac.*, **35**, 103–111.

J. Pharm. Pharmac., 1971, **23**, 429–433 *Received November* 17, 1970

Spasmolytic action of adenosine on the guinea-pig ileum

S. L. SEHORN AND J. L. BOROWITZ

Department of Pharmacology, Bowman Gray School of Medicine, Winston Salem N.C., U.S.A.

and Department of Pharmacology and Toxicology, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, Indiana, U.S.A.

Adenosine (10^{-5}M) reduces the contractile response of the guinea-pig isolated ileum to 5-hydroxytryptamine or barium more than the responses to acetylcholine or histamine. Adenosine also inhibits the contractile response of the guinea-pig ileum to a calcium-free medium. These results suggest that adenosine effects a blockade of the indirect responses to barium or 5-hydroxytryptamine mediated through intrinsic nerves and has a weak direct inhibitory effect on the smooth muscle cells of this tissue.

Smooth muscle is generally relaxed by adenosine (Bennet & Drury, 1931), although certain smooth muscle structures, such as the kidney vasculature (Buyniski & Rapela, 1969) are contracted. Adenosine produces coronary vasodilatation (Rubio & Berne, 1969), relaxation of bronchioles (Titone, 1914), and decreases spontaneous activity of cat and rabbit intestinal musculature (Drury & Szent-Gyorgyi, 1929).

We have found that, using guinea-pig ileum, indirect-acting agonists are blocked more effectively by adenosine than direct-acting agonists. Thus, in certain smooth muscles, the relaxant effect of adenosine and the antagonism of indirect-acting drugs by adenosine may be due in part to an action on the intrinsic nerve supply.

METHODS

Female guinea-pigs, 400 to 800 g, were killed by cervical dislocation; sections of ileum were removed and Tyrode solution passed through the lumen. Strips of ileum, 4 to 5 cm in length were fixed in a 70 ml bath and attached to a Grass Force Displacement transducer (Ft. 03). The normal bathing fluid was Tyrode solution having a composition (mM): NaCl, 136; KCl, 2·7; $MgCl_2$, 1·4; NaH_3PO_4, 0·04; $CaCl_2$, 1·8; $NaHCO_3$, 11; and glucose, 5·5 (pH = 7·5). Air was vigorously bubbled into the baths which were maintained at 37°. One g of tension was placed on each muscle strip and the change in isometric tension was recorded on a Grass Polygraph.

A 30 min equilibration period was allowed before addition of drugs, which were added directly to the muscle bath in volumes that did not dilute the bath more than 2%. Each intestinal strip was exposed to only one agonist, after each addition of which approximately 20 s was allowed before washing out with Tyrode. The maximum tension reached in the period of contraction was recorded.

Doses of acetylcholine, histamine, 5-hydroxytryptamine (5-HT) and barium required to produce 40% of the maximum response (ED40) were determined from dose response curves (Saferna, Loukomskaya & others, 1966). These doses were repeatedly added to, and washed from, the solution bathing the ileal preparations at 5 min intervals for a total of 105 min. Adenosine was added 20 min after the first

response was obtained, and was removed 45 min later. Control experiments without adenosine, involving at least two muscle strips for each agonist, showed that the responses to the ED40 increased slightly at first, reaching a relatively stable value in 20 to 30 min. No correction was made for the initial increase in response noted in control experiments.

In another series of experiments, dose-response curves were done for each agonist before and after addition of adenosine and after removal of adenosine. Adenosine remained in contact with the ilea for 30 min before addition of agonist. The recovery dose-response curve was obtained 30 min after washing out adenosine. The muscles were washed once after each dose of agonist and at least three times between each dose response curve.

RESULTS

Antagonism of the response to acetylcholine by adenosine. The response of the guinea-pig ileum to the ED40 of acetylcholine was decreased by adenosine, 10^{-5}M, an average of 38% in six experiments. The decrease was significant at the 1% level by comparison of the means and standard deviations of the three control responses before adenosine with the first three responses after addition of adenosine (Fig. 1A). However, the dose response curve to acetylcholine was not significantly affected by 10^{-5}M (Fig. 2A). Reduction in the responses to the ED40 appears to be a more

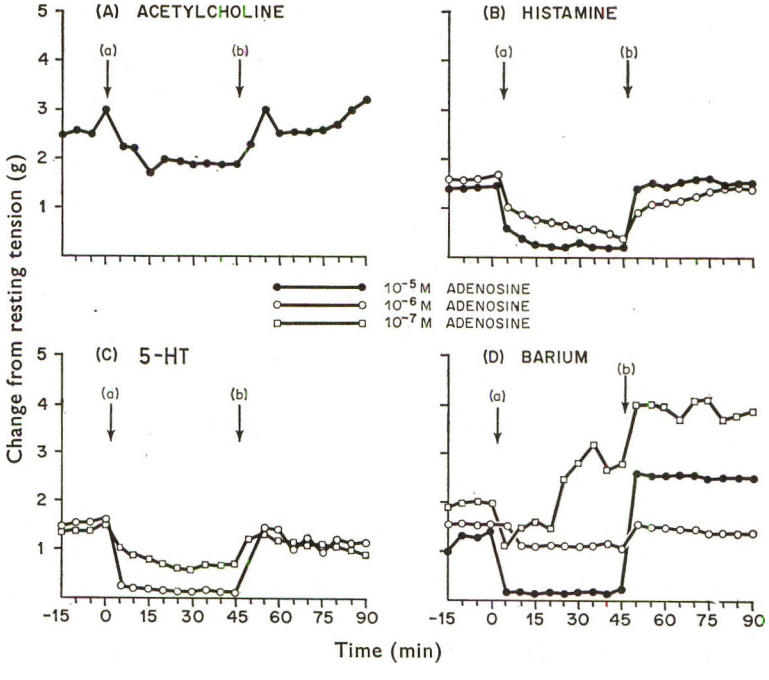

FIG. 1. Antagonism by adenosine of the responses of the guinea-pig ileum to various agonists. A dose of each agonist needed to produce 40% of the maximal response was repeatedly added to, and washed from, the medium bathing the ilea. The maximum response obtained in 20 s was recorded. Adenosine was added at (a) and was washed out at (b). Doses of agonist averaged $7{\cdot}2 \times 10^{-8}$M for acetylcholine, $3{\cdot}6 \times 10^{-7}$M for histamine, $5{\cdot}4 \times 10^{-8}$M for 5-HT, and $8{\cdot}75 \times 10^{-5}$M for barium. Each point is a mean of 5 to 6 experiments, except for barium with 10^{-7}M adenosine where 3 muscles were used.

sensitive measure of the effect of an antagonist. It is concluded that adenosine 10^{-5}M, weakly antagonizes the response of the guinea-pig ileum to acetylcholine.

Antagonism of the response to histamine by adenosine. Fig. 1B shows that the response to the ED40 of histamine is strongly reduced (90%) by 10^{-5}M adenosine ($P < 0{\cdot}01$). Again the effect is not so apparent in the dose-response to the agonist (Fig. 2B), although the responses to lower doses of histamine were significantly antagonized ($P < 0{\cdot}01$, $P < 0{\cdot}01$ and $P < 0{\cdot}02$ respectively for 0·39, 1·3, and 3·9 $\times$ 10^{-7}M histamine). Higher doses of histamine were not significantly affected by adenosine, 10^{-5}M.

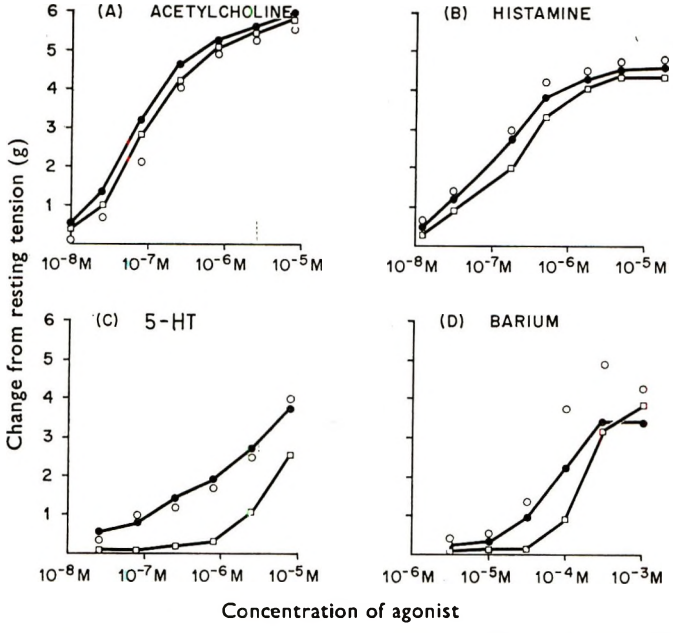

FIG. 2. Dose-response curves to various agonists before (●), during (□), and after (○) exposure to adenosine, 10^{-6}M. Each point is a mean of 8 to 12 experiments.

Antagonism of the response to 5-hydroxytryptamine by adenosine. A nearly complete blockade of the response to the ED40 of 5-HT was seen in the presence of 10^{-6}M adenosine (Fig. 1C). After addition of adenosine the first response of 5-HT was significantly less than the control response before adenosine ($P < 0{\cdot}01$ at 10^{-7}M adenosine and $P < 0{\cdot}001$ at 10^{-6}M adenosine). Also, the dose-response curve to 5-HT was markedly depressed by 10^{-5}M adenosine (Fig. 2C). When the reciprocal of the dose of 5-HT is plotted against the reciprocal of the mean response, straight lines are obtained for the responses in the presence and in the absence of adenosine. The slopes of the lines are significantly different ($P < 0{\cdot}02$) by regression analysis which suggests that this antagonism is competitive.

Antagonism of the response to barium by adenosine. The response to the ED40 of barium was nearly abolished by 10^{-5}M adenosine (Fig. 1D). At lower concentrations of adenosine, antagonism was not marked. The data suggest that adenosine in low concentrations may enhance the response to the ED40 of barium. After exposure for 20 min to 10^{-7}M adenosine the muscles showed an exaggerated response to barium

rather than an antagonism. Also, on removal of adenosine (10^{-7}, 10^{-6}, and 10^{-5}M), the responses to barium were higher on the average than control responses before adenosine and the difference bordered on significance ($P < 0{\cdot}06$). In the dose-response curves to barium, lower doses were markedly antagonized by 10^{-5}M adenosine (Fig. 2D). The nature of the antagonism was obscured by the enhancement above control of the response to barium at the highest dose ($P < 0{\cdot}01$).

Effect of adenosine on the contractile response of guinea-pig ileum to calcium-free medium. Exposure of guinea-pig ileum to a calcium-free medium (containing 5×10^{-6}M Na_2EDTA) after soaking in normal Tyrode solution produces a contraction of 1 to 2 g tension lasting approximately 3 min (30 observations). This type of contraction in guinea-pig ileum is thought to be independent of any intrinsic nerve supply since it is not blocked by procaine or atropine (Irwin & Oliver, 1970). Adensine, 10^{-5}M, inhibits the contractile response to a calcium-free medium when added before removal of calcium (30 observations) and abolishes the contraction when added after the response to a calcium-free medium has been initiated (12 observations).

DISCUSSION

Adenosine strongly antagonized the response of the guinea-pig ileum to 5-HT or barium whereas the antagonism to acetylcholine was only weak. Antagonism of the response to histamine by adenosine was also not marked, especially in the dose response curves. The actions of 5-HT and barium are mediated to a large extent through the intrinsic nerve supply, whereas acetylcholine and histamine are thought to act directly on the smooth muscle of guinea-pig ileum (Gershon, 1967; Paton & Zar, 1968; Henderson, Ariëns & Simonis, 1970). These data suggest that a major part of the inhibitory action of adenosine is exerted on the intrinsic nerve supply of guinea-pig ileum.

These observations are in accord with the report that adenosine inhibits intrinsic intestinal reflexes (Bishop, Frazer & others, 1963). Both the preparatory and emptying phases of the response of the guinea-pig ileum to distention are completely blocked by adenosine. Thus, adenosine blocks reflexes mediated through the intrinsic nerves of guinea-pig ileum, and also blocks the actions of drugs mediated through this system.

Enhancement by adenosine of the response of the guinea-pig ileum to large doses of barium has not been reported previously. The mechanism by which the increased response is produced is not known. Blockade by adenosine of the release of an inhibitory substance by barium is a possible explanation.

A relatively weak blockade by adenosine of the actions of acetylcholine and histamine on guinea-pig ileum was seen. These agonists act directly on this tissue to produce the contractile response. It was also shown that adenosine blocks the contractile response of guinea-pig ileum to a calcium-free solution. This response is also thought to be independent of any intrinsic nerve supply in the isolated ileum (Irwin & Oliver, 1970). Thus, it appears that the relaxant effect of adenosine in the guinea-pig ileum is mediated through the intrinsic nerve supply and to a lesser extent through a direct action on smooth muscle cells.

Cocaine is also a more effective antagonist of the effect of 5-HT on the guinea-pig ileum than of the effects of either acetylcholine or histamine (Roche e Silva, Valle & Picarelli, 1953). This suggests that adenosine may act as a local anaesthetic in

guinea-pig ileum, but intradermal injection of 0·1 ml of 4×10^{-3}M adenosine into guinea-pigs does not alter the response to an electrical stimulus applied to the skin at the injection site making it unlikely that a local anaesthetic action explains the spasmolytic effects of adenosine.

Since adenosine occurs physiologically (Douglas, 1966; Rubio & Berne, 1969), it is possible that variation in its endogenous levels may give rise to variation in the action of certain drugs on the gastrointestinal tract.

Acknowledgement

This work was supported by a grant from the National Science Foundation.

REFERENCES

Bennet, D. W. & Drury, A. N. (1931). *J. Physiol., Lond.*, **72**, 288–320.

Bishop, H., Frazer, A. C., Robinson, G. B. & Schneider, R. (1963). *Br. J. Pharmac. Chemother.*, **21**, 238–243.

Buyniski, J. P. & Rapela, C. E. (1969). *Am. J. Physiol.*, **217**, 1660–1664.

Daniel, E. E. & Irwin, J. (1965). *Can. J. Physiol. Pharmac.*, **43**, 89–109.

Douglas, W. W. (1966). *Pharmac. Rev.*, **18**, 471–480.

Drury, A. N. & Szent-Gyorgyi, A. (1929). *J. Physiol., Lond.*, **68**, 213–237.

Gershon, M. D. (1967). *Br. J. Pharmac. Chemother.*, **29**, 259–279.

Henderson, P. T., Ariëns, E. J. & Simonis, A. M. (1968). *Europ. J. Pharmac.*, **4**, 62–70.

Irwin, R. L. & Oliver, K. L. (1970). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **29**, 249, Abstract.

Paton, W. D. M. & Zar, M. A. (1968). *J. Physiol., Lond.*, **194**, 13–33.

Roche e Silva, M., Valle, J. R. & Picarelli, Z. P. (1953). *Br. J. Pharmac. Chemother*, **8**, 378–388.

Rubio, R. & Berne, R. M. (1969). *Circ. Res.*, **25**, 407–415.

Saferna, I., Loukomskaya, N., Kadlec, O. & Janku, I. (1966). *J. Pharm. Pharmac.*, **18**, 501–506.

Titone, F. P. (1914). *Pflügers Arch. ges Physiol.*, **155**, 77–81.

J. Pharm. Pharmac., 1971, **23**, 434–437 *Received January 7, 1971*

The effect of the histidine decarboxylase inhibitor brocresine (NSD-1055) on gastric acid secretion in rats*

M. BECKER AND K.-FR. SEWING

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

The effect of the histidine decarboxylase inhibitor brocresine (NSD-1055) given by mouth, intraperitoneally or intravenously on tetragastrin-, histamine- and bethanechol-stimulated gastric acid secretion was examined in rats. Intravenous injection of brocresine slightly reduced the tetragastrin-stimulated secretion. Histamine-stimulated secretion was markedly increased by both intraperitoneal and intravenous injection of brocresine but it had no effect on the bethanechol-stimulated secretion. It was concluded that either histidine decarboxylase is not effectively inhibited by brocresine or any inhibition induced does not affect gastric acid secretion. The enhanced histamine-stimulated secretion points towards an inhibition of diamine oxidase by brocresine.

Histamine has been proposed to be the common final chemostimulator for all physiological (gastrin, vagal stimulation) and pharmacological (i.e. reserpine) gastric acid stimulants (Code, 1965; Levine, 1965; Lorenz & Pfleger, 1968). This assumption is based on the following facts. Large amounts of histamine are present in the gastric mucosa and are stored there in the mast cells and in the so called enterochromaffin-like cells, which are distributed throughout the glandular stomach, the latter histamine stores can be released by cholinergic stimuli and gastrin (Stubrin, Dyce & others, 1965) and have a high rate of turnover. Changes in the histamine metabolism are followed by changes in gastric acid secretion: gastric acid secretion is inhibited by injection of diamine oxidase, the main histamine-metabolizing enzyme (Haverback, Stubrin & Dyce, 1965), whereas the diamine oxidase inhibitor aminoguanidine enhances the secretory response of gastrin and histamine (Amure & Ginsburg, 1964); gastrin is a necessary link for the stimulation of the gastric histidine decarboxylase (Aures, Johnson & Way, 1970).

We describe the effect of the histidine decarboxylase inhibitor brocresine (NSD-1055, 4-bromo-3-hydroxybenzyloxyamine dihydrogenphosphate) on the tetragastrin-, bethanechol- and histamine-stimulated gastric acid secretion in rats.

METHODS

The experiments were made in a randomized order on male rats (FW-49 Biberach, 300–400 g) anaesthetized with urethane (1·25 g/kg, i.p.). Animals were kept in single cages and had free access to drinking water but food was withheld for 24 h. The experiments were divided into three groups: in the first group gastric acid secretion was stimulated by intravenous infusion of tetragastrin (14·4 μg/kg in 15 min), in the

* Supported by a grant from the Deutsche Forschungsgemeinschaft.

second with bethanechol (56 μg/kg in 15 min) and in the third with histamine (1280 μg/kg in 15 min). The doses were submaximal and nearly equi-effective. In each group six rats were treated (a) orally, (b) six intraperitoneally and (c) six intravenously with brocresine (100 mg/kg) which was given to sub-groups (a) and (b) 4 h before the experiment was started, and to sub-group (c) between two periods of stimulation (see below). Groups of six animals receiving saline in a corresponding amount and route served as controls. No particular controls were necessary for the (b) sub-group since the effects of the gastric acid stimulants before and after the brocresine were compared.

The effect of an intraperitoneal injection of brocresine (100 mg/kg) given 1 h before the experiments were started on tetragastrin-stimulated acid secretion was compared with a saline-treated group of rats in separate experiments. In addition, brocresine (100 mg/kg, i.v. in 15 min) was infused into three animals to study its effect on basal secretion.

Gastric acid secretion was estimated according to Lai's (1964) method with the modification that bromothymol blue instead of phenolphthalein was used as a titration indicator and that the total acid output less the basal secretion, instead of an average secretion rate per 10 min, was evaluated. For investigating the effects of oral and intraperitoneal administration of brocresine the experiments were performed as follows: After the preparation of the animal, basal secretion was determined for 30 min, then two infusions of the stimulant were given at an interval of 70 min. To study the effect of an intravenous injection of brocresine, 60 min after the second infusion of the stimulant, brocresine (100 mg/kg) in a concentration of 50 mg/ml was slowly injected, 30–40 min later, depending on the time taken to return to base-line secretion, the third infusion was started.

Since the first infusion occasionally gave irregular results in the orally and intraperitoneally pretreated animals, only the effect of the second infusion of the stimulus was compared with the second infusion of the control animals and statistically analysed by the *t*-test for groups. In the intravenous experiments the second infusion of the stimulant was compared with the third and statistically analysed by the *t*-test for pairs.

Compounds. Brocresine (NSD-1055, 4-bromo-3-hydroxybenzyloxyamine dihydrogenphosphate), American Cyanamid Company, Pearl River, N.Y. and Smith & Nephew Research Ltd., Gilston; tetragastrin (Trp.Met.Asp.Phe-NH_2), Dr. Karl Thomae, Biberach; bethanecholcholride, Schuchardt, Munich; histamine dihydrol chloride, La Roche, Grenzach. All weights are given as the base of the compounds. Brocresine was freshly dissolved before each experiment.

RESULTS

The effect of the different routes of administration of brocresine on tetragastrin-, bethanechol- and histamine-stimulated gastric acid secretion and the statistical analyses are summarized in Table 1. The results demonstrate that the tetragastrin-stimulated acid secretion was significantly reduced by 22·4% by an intravenous injection of brocresine. However, the histamine-stimulated secretion was significantly enhanced by intraperitoneal (137·5%) and intravenous (60·3%) injection of brocresine. The stimulating effect of an oral administration of brocresine on tetragastrin- and histamine-stimulated secretion was statistically non-significant. None

Table 1. *The effect of different routes of administration of brocresine* (100 mg/kg) *on tetragastrin-, histamine- and bethanechol-stimulated gastric acid secretion in rats.*

Stimulus	Route for brocresine	Time between pretreatment and experiment	Total acid output (μequiv means ± s.e.) saline	brocresine	change (%)	*P*
Tetragastrin ..	oral	4 h	14·3 ± 4·1	19·3 ± 3·2	+ 34·9	0·3 < p < 0·4
	i.p.	4 h	16·5 ± 3·0	23·5 ± 5·9	+ 42·4	0·3 < p < 0·4
	i.p.	1 h	12·2 ± 1·7	14·7 ± 2·3	+ 20·5	0·3 < p < 0·4
	i.v.	30–40 min	18·3 ± 2·8	14·2 ± 2·5	− 22·4	< 0·01
Histamine	oral	4 h	41·5 ± 7·7	56·6 ± 4·5	+ 36·4	0·1 < p < 0·2
	i.p.	4 h	25·6 ± 5·3	60·8 ± 9·1	+137·5	< 0·01
	i.v.	30–40 min	21·1 ± 3·0	33·8 ± 5·0	+ 60·3	<0·025
Bethanechol ..	oral	4 h	21·4 ± 3·1	20·2 ± 3·2	− 5·6	0·7 < p < 0·8
	i.p.	4 h	22·3 ± 4·0	19·6 ± 5·6	− 12·1	0·6 < p < 0·7
	i.v.	30–40 min	32·1 ± 5·0	34·1 ± 4·4	+ 6·2	0·2 < p < 0·3

of the routes had any significant effect on the bethanechol-stimulated gastric acid secretion. The basal secretion was slightly elevated for 20–40 min by a continuous intravenous infusion of brocresine (100 mg/kg) for 15 min.

DISCUSSION

The results demonstrate that brocresine does not effectively inhibit stimulated or unstimulated gastric acid secretion in rats. The tetragastrin-stimulated secretion was slightly depressed by an intravenous injection of brocresine. In contrast the histamine-stimulated secretion was markedly increased by both intraperitoneal and intravenous injection of brocresine.

The results are not in agreement with those of Levine (1965) and of Thayer & Martin (1967) who described a strong inhibitory effect of brocresine (150 mg/kg) on the gastric acid response of the Shay rat to gastrin, pentagastrin, bethanechol, insulin and reserpine. In contrast, Fletcher, Pitts & others (1969) could not detect any inhibition of gastric acid secretion in Pavlov and Heidenhain pouch dogs stimulated with histamine, pentagastrin, gastrin and feeding, after a long term treatment with daily brocresine (75–160 mg/kg) for several weeks. The lack of inhibition in our experiments may be because of the following reasons.

(i) The time elapsed between the pretreatment of the animals and the stimulation of gastric secretion—particularly when brocresine was given intraperitoneally or orally—was too long. However, Levine, Sato & Sjoerdsma (1965) demonstrated a maximal effect 3–6 h after intraperitoneal administration. On the other hand, Wustrack & Levine (1969) found a rapid decline of the histidine decarboxylase-inhibiting activity within 60–90 min after the administration of brocresine. (ii) It is possible that an intact histidine decarboxylase system is not a necessary intermediate step in the stimulating process of gastric acid secretion. Up to now there is no convincing evidence that the decarboxylation of histidine is an essential link in the chemostimulation of gastric acid secretion. In contrast, Johnson & Aures (1970) claimed that histamine is not the mediator for gastric secretion in the rat.

The enhancement of the histamine-stimulated secretion by intraperitoneal and intravenous brocresine suggests an inhibition of the histamine catabolism in the body. Maudsley & Kobayashi (1969) reported a 60% inhibition of the diamine oxidase, the main histamine-metabolizing enzyme, 30 min after the application of brocresine. If such an inhibition is the reason for our findings with histamine, then it is likely that

not brocresine itself but a metabolite is the active principle, since the histidine decarboxylase-inhibiting activity disappears from the plasma at the same time (Wustrack & Levine, 1969) as the optical characteristics of brocresine extracted from rat blood into ethyl ether (Sewing, unpublished). The failure of the orally administered brocresine to provoke the same strong effect on the histamine-stimulated secretion may be the result of metabolism during gastrointestinal absorption. There is no published information on the metabolism of brocresine.

From this study it is concluded that the failure of an inhibition of gastric acid secretion in rats by brocresine leaves doubt about the contribution of the histamine synthesis to the stimulation of gastric acid secretion. Furthermore, the results suggest that the diamine oxidase is inhibited by brocresine.

REFERENCES

AMURE, B. O. & GINSBURG, M. (1964). *Br. J. Pharmac. Chemother.*, **23**, 476–485.

AURES, D. JOHNSON, L. R. & WAY, L. W. (1970). *Am. J. Physiol.*, **219**, 214–216.

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

FLETCHER, T. L., PITTS, C. L., EVERETT, M. T. & GRIFFITH, C. A. (1969). *Proc. Soc. exp. Biol. Med.*, **132**, 205–211.

HAVERBACK, B. J., STUBRIN, M. J. & DYCE, B. J. (1965). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **24**, 1326–1330.

JOHNSON, L. R. & AURES, D. (1970). *Proc. Soc. exp. Biol. Med.*, **134**, 880–884.

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

LEVINE, R. J. (1965). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **24**, 1331–1333.

LEVINE, R. J., SATO, T. L. & SJOERDSMA, A. (1965). *Biochem. Pharmac.*, **14**, 139–149.

LORENZ, W. & PFLEGER, K. (1968). *Klin. Wschr.*, **46**, 57–71.

MAUDSLEY, D. V. & KOBAYASHI, Y. (1969). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **28**, 353.

STUBRIN, M. J., DYCE, B., BREM, T., TECIMER, L. B. & HAVERBACK, B. J. (1965). *Am. J. dig. Dis.*, **10**, 901–908.

THAYER, W. R. & MARTIN, H. F. (1967). *Ibid.*, **12**, 1050–1061.

WUSTRACK, K. O. & LEVINE, R. J. (1969). *Biochem. Pharmac.*, **18**, 2465–2471.

J. Pharm. Pharmac., 1971, **23**, 438–443 *Received November* 11, 1970

The influence of test conditions on the disintegration time of gelatin capsules

B. E. JONES AND W. V. J. COLE

Laboratories of Elanco Division, Eli Lilly and Company Limited, Basingstoke, Hampshire, U.K.

The British Pharmacopoeia test for capsule disintegration is basically the same test as that used to measure tablet disintegration and as such it has some shortcomings, particularly in the determination of the end point. The results were significantly affected by the capsule size, the nature of the test solution, the temperature, and whether single capsules were tested instead of groups of five. The effect of temperature on capsule solubility was demonstrated. A modified test was applied to both hard and soft capsule products selected from the British Pharmacopoeia. Recommendations are made for new specifications for an official pharmacopoeial test.

The British Pharmacopoeia 1968 uses for the "Disintegration test for capsules" the same test and apparatus as that used for tablets except that a repeat test using a guided disc is not permitted. One of the main problems with the capsule disintegration test is the determination of a finite end point. The official apparatus was designed for tablets (Hoyle, 1946; Prance, Stephenson & Taylor, 1946) and when applied to capsules, with their different physical properties, it has given rise to a number of problems. Capsules disintegrate by first opening at the weakest point, the radius or seal, releasing the contents and leaving a partly dissolved empty shell (Czetsch-Lindenwald, 1962). The end point as defined by the British Pharmacopoeia is "the capsules are disintegrated when no particle of solid remains above the gauze which would not readily pass through it". The exact determination of this point is difficult because as the capsule disintegrates it releases its contents and leaves an adhesive gelatin mass. A more exact end point would be when all the capsule and its contents have passed through the mesh, and on this basis experiments have been made to examine the effect of altering some of the test variables on the disintegration time of hard gelatin capsules.

METHODS

Effect of temperature on capsule solubility

The solubility of capsules was measured at different temperatures using the method of Boymond, Sfiris & Amaker (1966). This consists of placing a ball-bearing inside the capsule, suspending the capsule body in the test solution and measuring the time for it to fall from the capsule.

Five capsules were placed in holders in a strip of stainless steel, the diameter of each hole being such that the body and not the cap could pass through. The holder was suspended over a crystallizing dish 12 cm in diameter so that the bodies were immersed in 500 ml of test solvent, which was stirred at 70 rev/min with a small blade (4·5 cm diameter) polythene stirrer, the position of which in relation to the sample holder was kept constant. The beaker was placed in a water bath and the

temperature of the test liquid maintained within ±0·2° of the nominal figure. The test was performed over a range of temperatures from 33° to 41° at 2° intervals. Opaque white capsules (Elanco) were used, size 0 and size 4, containing ball bearings of weight 1·046 g ± 1 mg and 0·258 g ± 1 mg and diameter 6·35 mm and 4·76 mm respectively. Two test solvents were compared, water and 0·6% hydrochloric acid solution. The end point was taken when the ball bearing hit the base of the beaker. Three determinations on groups of five capsules were made for each set of conditions.

Effect of alteration of test conditions on filled capsules

A Manesty Tablet Disintegration Apparatus Mark II was used. This complies with the specifications of the British Pharmacopoeia 1968. The tests were made at 37° ± 0·2° which was achieved by placing the centre tube and rack assembly of the apparatus in a Techne Tempette water bath. The end point was taken as the time at which no further particles of capsule shell or contents remained above the mesh. Opaque white capsules were used throughout to aid in the observation of this end point which was made directly from above the tubes.

Five factors were varied and each tested at two levels: the test solution (W) and its container (B), the capsule size (S) and contents (C) and the treatment of the surface of the apparatus (T). The British Pharmacopoeia specifies water (W_1) as the test solvent. This was compared with 0·6% hydrochloric acid solution (W_2) which is used in the monograph for Erythromycin Estolate capsules and for enteric coated products. The quantity of water is defined as "having a depth of not less than 15 cm" but it does not indicate the volume or size of the container. Two sizes of beakers were compared; one 4·5 cm diameter (Manesty Apparatus) (B_1) containing 250 ml at 15 cm depth; and one 9·0 cm diameter (1 litre tall-form) (B_2) containing 950 ml at 15 cm depth. Two sizes of hard gelatin capsules were compared, a large capsule size 0 (S_1) and a small capsule size 4 (S_2). Two common pharmaceutical diluents were compared, lactose B.P. (C_1), which is soluble, and starch B.P. (C_2), which is insoluble. These were filled into capsules by hand to ensure well-packed capsules having fill weights within ±5%. As gelatin becomes adhesive when it melts, an attempt to prevent the capsule adhering to the sides of the tube and the mesh was made by treating the apparatus with an aerosol silicone release agent (T_1); comparative tests were made with an untreated apparatus (T_2).

Two series of experiments were performed in a 2^3 factorial experiment in a full factorial design (Davies, 1960). Series I experiments compared medium, capsule size and capsule contents, Series II experiments compared container size, capsule size and untreated and siliconed apparatus.

In Series I experiments the apparatus was treated with an aerosol silicone release agent. In Series II experiments the test solution was 0·6% hydrochloric acid solution and the capsules were filled with equal parts of starch and lactose. Each series consisted of two sets of experiments, one using a sample of five capsules as specified in the British Pharmacopoeia and the other using single capsule samples. Six replicates were made for each set of conditions.

Effect of modified test on B.P. capsule products

Experiments were made with hard and soft capsule products, randomly selected from those included in the 1968 B.P. and the addendum 1969 to the B.P., to assess the effect of using 0·6% hydrochloric acid instead of water and of using single capsules

instead of samples of five. For the single capsule test, six determinations were made for each product. The temperature was maintained at 37° ± 0·2°. The test solution container was a beaker 4·5 cm in diameter. The end point was taken as the time at which no further particles of capsule remained above the mesh. For single capsules the disintegration time of each capsule was recorded and that for the slowest sample was the one used.

RESULTS AND DISCUSSION

The end point in the disintegration test as defined in the British Pharmacopoeia involves a decision being taken on what would readily pass through the mesh in the test apparatus. The course of capsule disintegration shows that the last particle to remain is usually a piece of adhesive shell which sticks to the mesh and the end point is taken at the time when all the capsule and its contents has dissolved or disintegrated. The behaviour of the last fragments of capsule shell appear to be the rate controlling step in most of the experiments, except for capsules filled with non-wetting insoluble materials where the contents remain and are the final particles to pass through the mesh.

The analysis of variance showed that two factors have a significant effect on disintegration time; the nature of the test solution and the capsule size. There is a significant difference between the results obtained with single capsules and samples of five; five capsules always took longer to disintegrate (see Table 1).

Table 1. *Effect of altering test variables on the disintegration time (s) of capsules.* Mean figures of six replicates.

		Disintegration time (s) for capsules of sizes:				
		$0(s_1)$	$4(s_2)$	$0(s_1)$	$4(s_2)$	
		Series I experiments, variables:				
Sample size	Contents	Water (W_1)		Acid (W_2)		
5	Lactose (C_1)	544	538	493	387	mean
5	Starch (C_2)	619	447	498	372	488
1	Lactose (C_1)	495	377	353	330	mean
1	Starch (C_2)	463	368	282	273	368
		Series II experiments, variables:				
	Treatment	(B_1) (4·5 cm)		(B_2) (9·0 cm)		
5	Untreated (T_1)	689	496	636	517	mean
5	Siliconed (T_2)	533	506	558	462	550
1	Untreated (T_1)	488	362	555	392	mean
1	Siliconed (T_2)	452	383	558	377	433

The instruction to take five capsules in the official test presumably has the intention of evaluating intra-batch variation in disintegration, the end point being the breakdown of the slowest capsule to disintegrate. In practice this appears not to occur because after the contents have emptied from the shells these collapse and if they come into contact with each other they form an adhesive mass, the thickness of which may be several times that of a normal capsule. The larger the size of the capsules the greater the probability of agglomeration. This we believe to be the reason why the size factor had a greater effect in our experiments with samples of five capsules than in individual trials. In all our experiments, the size of capsule

was a significant factor because it affected the numbers of wires in the mesh that became coated with gelatin as the shell collapsed, the greater the number of wires coated the longer the shell took to dissolve. A measure of intra-batch variation can best be obtained, therefore, by measuring individual capsules in replicate. For tablet testing, the U.S.P. XVIII has adopted the use of six replicates.

In the single capsule experiments there was a significant interaction between beaker and capsule size effects (see Table 3). The size of the beaker affects only the results on the larger size capsules; capsules take longer to disintegrate in the larger beaker. A separate parts analysis showed that beaker size and capsule size were both significant as main effects except at the size 4 (S_2) levels. This we believe to be due to the fact that as the beaker diameter decreases, the amount of turbulence caused by the reciprocal motion of the tube increases, which is significant only with the larger size capsules and reflects the retarded dissolution of the final pieces of shell.

Treatment of the apparatus with a silicone release agent did not produce a significant effect on the results (see Table 3). The adhesion of the final particles of capsule shell to the mesh was not prevented, but it was apparent during the tests that the capsule contents did not coat the surface of the test tube or mesh.

The effect of changing the test medium from water to an acid solution significantly shortened the disintegration time as shown in Tables 1 and 2. An acid solution more closely approximates to the *in vivo* situation. The Czechoslovakian (3rd edition, 1970) the German (B.R.D. VIII, 1968) and the Japanese (1961) Pharmacopoeias have all adopted an acid solution for this test.

The change in rate of solution of empty capsules over the range 35°–39° of the B.P. test was shown to be about 30% (see Fig. 1). There was no significant difference between acid and water but there was a difference between capsule sizes. These variations were probably caused by the mechanical conditions of the test and the way in which the ball bearing left the capsule. Some fell straight through the bottom of the capsule whereas others were held suspended by strands of gelatin after the shell

Table 2. *Effect of altering test variables on the disintegration time of capsules. Magnitude of effects.* All results subtracted from means (x) of each experiment in Table 1.

Sample size	Contents	*Disintegration time (s) for capsules of sizes:* 0(s_1)	4(s_2)	0(s_1)	4(s_2)
		Series I experiments, variables:			
		Water (W_1)		Acid (W_2)	
5	Lactose (C_1)	+56	+50	+5	−101
5	Starch (C_2)	+131	−41	+10	−116
1	Lactose (C_1)	+127	+9	−15	−38
1	Starch (C_2)	+95	0	−86	−95
	$\bar{x}$ for sample of 5 capsules = 488 s	for sample of 1 capsule = 368 s			
	Treatment	*Series II experiments, variables:* (B_1) (4·5 cm)		(B_2) (9·0 cm)	
5	Untreated (T_1)	+149	−54	+86	−33
5	Siliconed (T_2)	−17	−46	+8	−88
1	Untreated (T_1)	+55	−71	+122	−41
1	Siliconed (T_2)	+19	−50	+125	−56
	$\bar{x}$ for sample of 5 capsules = 550 s	for sample of 1 capsule = 433 s			

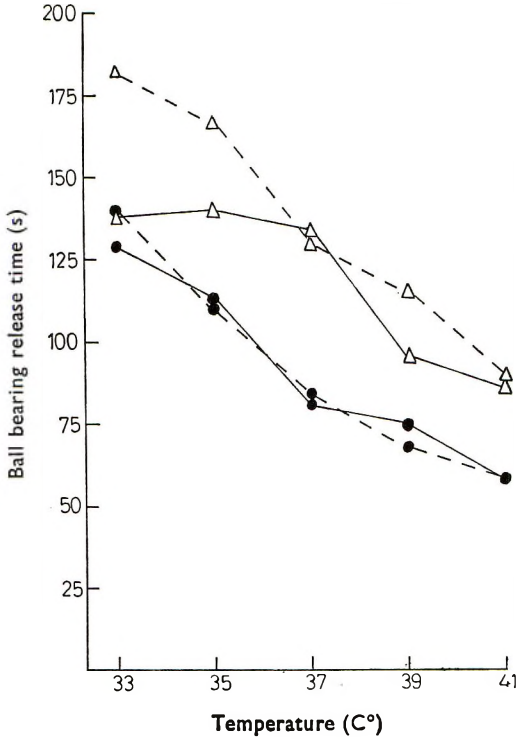

FIG. 1. The variation of capsule solubility with temperature. Each point represents the mean of fifteen determinations using opaque white capsules. △ = size 0 capsules; ● = size 4 capsules; —— = in 0·6% hydrochloric acid solution; — — = in water.

Table 3. *Statistical analysis of changes in the test variables on the disintegration time of hard gelatin capsules.* The figures in this table represent the variance ratios (F).

Effect	Source	Sample 5	Sample 1	Source	Sample 5	Sample 1
Main factors ..	Medium (W)	16·97**	64·72**	Beaker (B)	0·12	(14·40) +
	Size (S)	17·83**	34·15**	Size (S)	7·99**	(111·82)+
	Contents (C)	0·08	1·66	Treatment (T)	3·28	(0·10)+
Interaction between pairs	W × S	0·31	2·28	B × S	0·00	8·56**
	W × C	0·01	0·02	B × T	0·06	0·08
	S × C	3·81	0·38	S × T	1·47	0·01
Interaction of all factors	W × S × C	2·42	0·03	B × S × T	0·82	2·58

+ Approximate figures, invalidated by interaction B × S.

** Highly significant.

At $P = 0{\cdot}05$, F = 4·08 and at $P = 0{\cdot}01$, F = 7·31 (Fisher & Yates, 1963).

had collapsed. The other experiments with filled capsules indicated that the last piece of capsule to remain above the mesh was a piece of shell. Therefore any change in gelatin solubility caused by faulty temperature control could materially alter the results. A reduction in the temperature range would be expected to give more reproducible results.

The results obtained in the modified test on B.P. capsule products were analysed statistically using a non parametric significance test, the Wilcoxon matched-pairs signed-ranks T test (Beyer, 1968). For samples of 5 capsules ($n = 29$) $T = 299$ at $P = 0{\cdot}05$ $T = 141$ and at $P = 0{\cdot}01$ $T = 111$; and for single capsules ($n = 28$) $T = 165$ at $P = 0{\cdot}05$ $T = 130$ and at $P = 0{\cdot}01$ $T = 102$. These showed that the results taken in acid solution were significantly different from those in water. The approximate mean times for the experiment were: samples of 5 capsules in acid = 550 s and in water = 800 s; and samples of single capsules in acid = 480 s and in water 570 s. The disintegration times were shorter in acid solution than in water and shorter when single capsules were tested rather than samples of five. Not all products are soluble in acid solutions and, because of this, cloxacillin and phenoxymethylpenicillin capsules produced a fine adhesive powder that coated the mesh.

The experiments showed that if a finite end point is taken "the time for all the capsule to pass through the mesh" certain test conditions need to be redefined. To obtain a measure of intra-batch variation and to obtain a more distinct end point, the test sample should be a single capsule and six replicates should be performed simultaneously. If this is done the diameter of the test beaker needs to be defined to control the volume of the test solution and to overcome some of the effects of capsule size on the results. The use of 0·6% hydrochloric acid solution, although it shortens disintegration in most cases, cannot be applied universally and consideration needs to be given to the nature of the capsule contents. To improve the reproducibility of results the temperature range over which the test is performed needs to be narrowed.

Acknowledgements

The author would like to thank Dr. J. M. Newton for helpful discussion, Mr. J-F. V. Tornblum for statistical advice and Messrs. I. Barker and V. Winstanley for technical assistance.

This work was carried out at the request of the British Pharmacopoeia Sub Committee on capsule and tablet standards.

REFERENCES

BEYER, W. R. (1968). *Handbook of tables for probability and statistics*, 2nd edn, p. 399. Cleveland, U.S.A.: The Chemical Rubber Company.

BOYMOND, P., SFIRIS, J. & AMACKER, P. (1966). *Pharm. Ind., Berl.*, **28**, 836–42.

CZETSCH-LINDENWALD, H. (1962). *Ibid.*, **24**, 110–4.

DAVIES, O. L. (1960). *Design and analysis of industrial experiments*, 2nd edn, p. 258. London: Oliver and Boyd.

FISHER, R. A. & YATES, F. (1963). *Statistical tables, for biological, agricultural and medical research*, 6th edn, p. 53. London: Oliver & Boyd.

HOYLE, H. (1946). *Q. J. Pharm. Pharmac.*, **19**, 279–86.

PRANCE, H. P., STEPHENSON, D. & TAYLOR, A. (1946). *Ibid.*, **19**, 286–92.

J. Pharm. Pharmac., 1971, **23**, 444–447 *Received August* 19, 1970

Application of orthogonal functions to spectrophotometric analysis of weakly absorbing compounds in tablets

H. ABDINE, A. M. WAHBI AND M. A. KORANY

Pharmaceutical Analytical Chemistry Department, Faculty of Pharmacy, University of Alexandria, Alexandria, U.A.R.

Glenn's method of orthogonal functions has been applied to correct for irrelevant absorption during the analysis of tablets of chlorpheniramine maleate, phenyltoloxamine dihydrogen citrate, diphenhydramine hydrochloride and ephedrine hydrochloride. The results suggest that the method can be used for routine analysis.

The need to correct for irrelevant absorption has occasioned many formulae ranging from the simple (Banes & Eby, 1946) to the highly sophisticated (Tunnicliff, Rasmussen & Morse, 1949), depending upon the shape of the irrelevant absorption spectrum. The method developed by Ashton & Tootill (1956) for the assay of griseofulvin in fermentation samples depends upon the use of orthogonal polynomials. In 1963, Glenn outlined general procedures for the use of orthogonal functions to correct for irrevelant absorption in spectrophotometric analysis. The method is based upon the fact that a gross absorption curve $f(\lambda)$ can be expanded in terms of orthogonal functions as follows:

$$f(\lambda) = p_0P_0 + p_1P_1 + p_2P_2 + \ldots + p_nP_n \quad \ldots \quad (1)$$

where $f(\lambda)$ denotes the absorption of the sample at $(n + 1)$ wavelengths, P_j are the orthogonal polynomials given in standard works on numerical analysis (Milne, 1949; Fisher & Yates, 1953) and p_j are their respective coefficients. These coefficients are proportional to concentration (Glenn, 1963). Thus, $p_j = \alpha_j c_a$ where α_j is the coefficient of P_j for the A (1%, 1 cm) of the pure compound, a, c_a is the concentration. In the presence of irrelevant absorption, each observed coefficient is the sum of two terms; thus,

$$p_j = \alpha_j c_a + p_j\,(z) \quad \ldots \quad \ldots \quad \ldots \quad \ldots \quad (2)$$

where z denotes "contribution from irrelevant absorption". Equation (2) therefore contains two unknowns c_a and p_j (z) and can only be used to evaluate c_a from p_j when there are good grounds for supposing p_j (z) to be negligible relative to $\alpha_j c_a$. To minimize p_j (z) to a negligible value, great care must be taken in choosing the polynomial and range, the number of wavelengths and the mean wavelength, all these choices being made with reference to the irrelevant absorption curve.

Glenn's method of orthogonal functions has been successfully applied to the assay of vitamin A in cod liver oil without saponification and atropine sulphate injections (Wahbi, 1967, 1970).

The present work represents an application of the method to the determination of a single substance in the presence of irrelevant absorption. The choice of polynomial, number of points, wavelength range and intervals is illustrated by the analysis of tablets containing a single, weakly-absorbing active constituent. These are: chlorpheniramine maletate (4 mg), phenyltoloxamine dihydrogen citrate (15 mg), diphenhydramine hydrochloride (25 mg) and ephedrine hydrochloride (30 mg) per tablet.

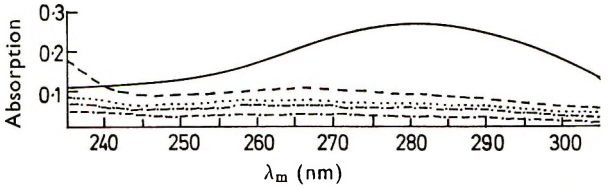

FIG. 1. Irrelevant absorption curves due to: —— lactose, – – – gelatin, ... stearic acid, –·–·– magnesium stearate, – – · – – · starch.

Shapes of irrelevant absorption curves in tablets

Irrelevant absorption in spectra from tablets originates from the diluents, e.g., lactose, starch, sucrose, the moistening agents, e.g., acacia mucilage, gelatin, liquid glucose and the lubricants, e.g., talc, stearic acid and magnesium stearate. Different grades of these ingredients from different sources were separately investigated. Of the ingredients, lactose was found to be the main source of interference (Fig. 1). Furthermore, grade to grade differences were negligible.

Choice of assay polynomial

Following the general rules collated by Wahbi (1967) for the choice of an assay polynomial, the results presented in Table 1 were obtained from the general shapes of the spectra of the compounds to be assayed (Figs 2 and 3).

Number of wavelengths

Eight-point orthogonal polynomials have been preferred for two reasons. Firstly, the irrelevant absorption curves were not too complex to require more than that

Table 1. *Choice of assay polynomial.*

Compound	Segment	Polynomial
Chlorpheniramine maleate	a b c	P_2
Phenyltoloxamine dihydrogen citrate ..	d e f	P_2
Diphenhydramine hydrochloride	g h i j	P_3
Ephedrine hydrochloride	k l m n	P_4
	o p q r s	P_5

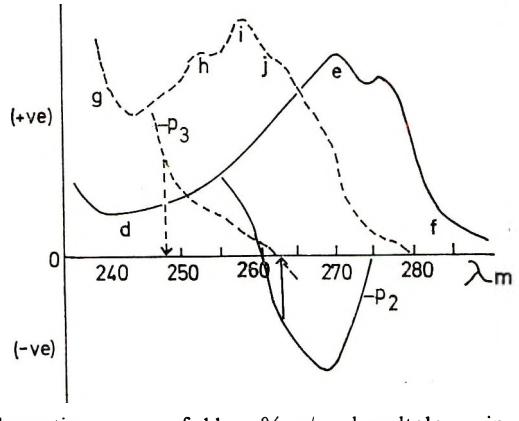

FIG. 2. ———— Absorption curve of 11 mg% w/v phenyltoloxamine dihydrogen citrate in 0·1N sulphuric acid and its p_2-convoluted curve. – – – – – – Absorption curve of 37 mg% w/v diphenhydramine hydrochloride in 0·1N sulphuric acid and its p_3-convoluted curve.

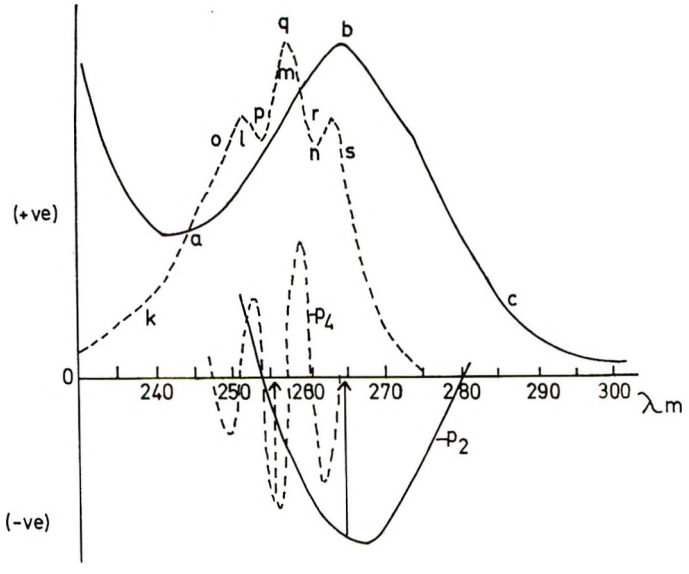

FIG. 3. ——— Absorption curve of 4 mg% w/v chlorpheniramine maleate in 0·1N sulphuric acid and its p_2-convoluted curve. – – – – – Absorption curve of 97 mg% w/v ephedrine hydrochloride in 0·1N sulphuric acid and its p_4-convoluted curve.

number of points for their correction. Secondly, the calculation of any of the coefficients required no more than 5 min using a desk calculator. However, where the irrelevant absorption possessed high frequency components or its shape was completely unknown, more points needed to be used.

Choice of mean wavelength λ_m

λ_m, the mean of the set of wavelengths, was obtained by plotting convoluted absorption curves (Agwu & Glenn, 1967), i.e., p_j calculated at different intervals versus λ_m for both pure substance (Figs 2 and 3) and irrelevant absorption. The optimum wavelength range and intervals were finally selected to maximize p_j and minimize p_j (z) to a negligible value (eqn 2).

Magnitude of coefficients

According to a theory contributed by Dr. A. L. Glenn (see Wahbi, 1967) the comparative coefficients, $|q_j|$ ($q_j = p_j.N_j^{1/2}$; N_j is the normalizing factor) must exceed 140×10^{-3} if the coefficient of variation of p_j calculated at the optimum set of wavelengths is to be less than 1. This requirement was achieved by increasing the intervals of the set of points so that a greater coefficient was obtained. With the exception of ephedrine hydrochloride, observed values of $|q_j|$ were above the stated figure (Table 2).

Table 2. *Comparative coefficients calculated at the optimum set of wavelengths*

Solution	A_{max}	j	$p_j \times 10^3$	$\lvert q_j \rvert \times 10^3$
Chlorpheniramine maleate	0·840	2	−42·530	551·2
Phenyltoloxamine dihydrogen citrate	0·583	2	−28·208	365·6
Diphenhydramine hydrochloride	0·663	3	+17·992	292·3
Ephedrine hydrochloride	0·578	4	−2·125	52·7
		5	−2·056	96·1

Table 3. *Assay results*

Tablets*	P_j	λ_m (nm)	Intervals (nm)	Mean % recovery ($P = 0.05$)
Chlorpheniramine maleate (5)	P_2	264·5	6	99·2 ± 1·5
Phenyltoloxamine dihydrogen citrate (10) ..	P_2	263	6	98·5 ± 1·35
Diphenhydramine hydrochloride (10)	P_3	247·5	3	99·2 ± 1·8
Ephedrine hydrochloride (10)	P_4	249·5	3	99·8 ± 1·7
	P_5	256	2	101·0 ± 0·7

* Prepared separately using five different grades of lactose. Figures in parentheses indicate number of experiments (separate weighings).

For the reason that the three peaks of ephedrine hydrochloride occur within a narrow range of wavelengths (Fig. 3), it was found that by widening the wavelength intervals (more than 3 nm for p_4 and 2 nm for p_5), the magnitude of the coefficients and accordingly $|q_j|$ decreased. Nevertheless, the assay of ephedrine hydrochloride tablets was made at the selected wavelength range and intervals given in Table 3 taking in consideration that the coefficient of variation (p_j) may exceed 1·0.

METHODS

Tablets. These were prepared to contain the previously specified doses per 0·30 g tablet powder. Five different grades of lactose were separately used for their preparation.

Assay. An accurately weighed quantity of the powdered tablets (0·2–0·5 g) was extracted with 0·1N sulphuric acid, filtered and suitably diluted for measurement by a Unicam SP 500 photoelectric spectrophotometer.

RESULTS AND DISCUSSION

Sources of error in the above results are due to (i) the non-zero coefficient which may have been contributed by the irrelevant absorption to the "tablet coefficient", (ii) wavelength setting errors which affect absorbances measured on steep slopes in the absorption curves and (iii) overall shifts in the spectrophotometer's wavelength calibration. The latter source of error affects mainly the coefficients sited on slopes in their respective convoluted absorption curves (Figs 2, 3) (Agwu & Glenn, 1967).

Glenn's method of orthogonal functions proved to be powerful in discounting irrelevant absorption contribution. Thus, the method can be applied for the routine analysis of tablets without separating the active constituent from the tablet fillers. Great care must be taken in the choice of the assay parameters.

REFERENCES

Ashton, G. C. & Tootill, J. P. R. (1956). *Analyst*, **81**, 225–227, 232–238.

Agwu, I. & Glenn, A. L. (1967). *J. Pharm. Pharmac.*, **19**, *Suppl.*, 76S–87S.

Banes, F. W. & Eby, L. T. (1946). *Analyt. Chem.*, **18**, 535–538.

Fisher, R. A. & Yates, F. (1953). *Statistical Tables for Biological, Agricultural and Medical Research*, 4th edn, p. 80 *et seq.* Edinburgh: Oliver & Boyd.

Glenn, A. L. (1963). *J. Pharm. Pharmac.*, **15**, *Suppl.* 123T–130T.

Milne, W. E. (1949). *Numerical Calculus*, 1st edn, p. 265 *et seq.* and p. 375 *et seq.* Princeton: Princeton University Press.

Tunnicliff, D. D., Rasmussen, R. S. & Morse, M. L. (1949). *Analyt. Chem.*, **21**, 895–900.

Wahbi, A-A. E-B. M. (1967). *Ph.D. Thesis*, University of London.

Wahbi, A. M. (1970). *Die Pharmazie.* In the press.

J. Pharm. Pharmac., 1971, **23**, 448–450 *Received January* 4, 1971

Constituents of two varieties of Indian dill

C. S. SHAH, J. S. QADRY AND (MRS.) M. G. CHAUHAN*

Department of Pharmacognosy, L.M. College of Pharmacy, Ahmedabad-9, India

Two varieties of Indian dill, *Anethum sowa*, are available. They differ morphologically and have a different oil composition. They have a low dillapiole content (12–15%) and have a high content of carvone-dihydrocarvone (50–64%).

Anethum graveolens and *A. sowa* represent two separate species, yielding the so called European and Indian dill respectively. The former is official in many pharmacopoeias while the latter is not considered a suitable substitute, as it is reported to contain 40 to 50% of a toxic constituent, dillapiole (Adhikari, 1965; Betts, 1969). However, sowa-dill is used extensively in India and is also exported in large amounts (Wallis, 1965).

We have found that in Gujarat two kinds of sowa-dill are available and these have different external characters and oil composition. Our findings for the oil composition differ from earlier reports (Malaviya & Dutt, 1940: Guenther, 1953; Chakravarty & Bhattacharyya, 1954 & Verma, 1960) as well as from the recent findings of Adhikari (1965) and Betts (1969). Except for Chakravarty & Bhattacharyya's (1954) findings of 19% dillapiole, almost all the workers have reported up to 50% dillapiole in Indian dill oil. Of the two kinds examined, one consists mostly of cremocarps and is considered to be superior; it is referred to as *Variyali sowa* (Fennal sowa). It seems to be that described by Wallis (1965) as Indian dill. The other variety, like *A. graveolens*, consists only of separate mericarps and is considered inferior. It is used in veterinary practice and is thus called *Ghoda sowa* (Horse sowa).

MATERIALS AND METHODS

Materials. Samples of sowa-dill were obtained from the local as well as other drug markets of the states of Gujarat, Delhi, Punjab, Maharashtra, Andhra Pradesh, Mysore and Kerala. Except Gujarat state, the samples received were those of Ghoda sowa.

Methods

Extraction of the oil. The apparatus was that described earlier (Schratz & Qadry, 1966) and the extraction time was 5 h.

Thin-layer chromatography. This was used for testing the purity of the separated fractions of the oil and reference substances. Plates were coated with Silica gel G (E. Merck) and activated at 110° for 1 h. Solvent systems used were: benzene–chloroform (50 : 50); light petroleum (b.p. 40–60°)–ethyl acetate (90 : 10) and benzene–methylene chloride (50 : 50). Vanilin–sulphuric acid and anisaldehyde reagents were used as detecting reagents and the spots were viewed in daylight and under ultraviolet light.

* Work carried out by M. G. C. in partial fulfilment for Ph.D. degree of Gujarat University.

Table 1. *Constituents of the two varieties of Indian dill*

Variety of sowa-dill	Carvone % g.l.c.*	Dihydro-carvone % g.l.c.*	Total carbonyl compounds % titrimetry	Dillapiole % Spectrophoto-metrically	g.l.c.*	Limonene % g.l.c.*
Variyali sowa	21	43	66	15	13	20
Ghoda sowa	35	15	54	12	12	34

* Uncorrected for differences in detector response.

Dillapiole was present in all the samples and its spot was about $\frac{1}{4}$ of the area of carvone. Carvone and dihydrocarvone had almost the same R_F values and they were subjected to g.l.c. separation.

Ultraviolet spectrophotometric evaluation of dillapiole. The absorbance of dillapiole was measured at 288 in a Beckman DU spectrophotometer. The reference solution of dillapiole for the standard curve as well as the oil samples were prepared in methanol (Analar, BDH). The percentage of dillapiole in both the samples found by this method and by quantitative g.l.c. was in fair agreement (Table 1).

Assay for carvone (carbonyl compounds). Carvone and dihydrocarvone present in the oils were estimated as carvone by the method described in the Indian Pharmacopoeia (1966). The results are in Table 1.

Gas-liquid chromatography. A Packard gas chromatograph fitted with argon ionization detector (500 V) was used. The column consisted of 20% Reoplex 400 on polypropylene glycol adipate; temperature was increased from 50 → 200° at 3°/min; carrier gas, argon, flow rate of 77 ml/min; chart speed 1 inch/5 min. Samples of the oil were diluted in pentane and aliquots of 2 μl were injected. In all, 8 peaks were detected. Only 4 main constituents namely dillapiole, carvone, dihydrocarvone and limonene were identified and estimated. The other components were in traces and together formed about 3–4% of oil. Thymol was found only in Ghoda sowa.

RESULTS AND DISCUSSION

The volatile oil of sowa-dill has been reported to have a carvone-dihydrocarvone content of 20–40% and a dillapiole content of 40–50%. Betts (1969) mentions that in the dillapiole-containing forms of dill that he examined, the carvone content was about half the amount of dillapiole. Our findings were different. In the two varieties we examined dillapiole was present to the extent of 12 to 15%, while the carvone-dihydrocarvone total in one variety was 50% (35% and 15% respectively) and in the other the carvone-dihydrocarvone was 64% (21 and 43% respectively). The lower specific gravity (0·9475 to 0·9523) of the volatile oils also gave indirect evidence of a lower percentage of dillapiole. According to Adhikari (1965), the specific gravity of the sowa-dill oils that he examined was always >1.

Findings that official *Anethum graveolens* also contains dillapiole (Chaudhry, Singh & Handa, 1956; Khafagy & Mnajed, 1968; Baslas & Baslas, 1969) suggest that the samples containing a low percentage of dillapiole and high percentage of carvone-dihydrocarvone may be substituted for *Anethum graveolens.*

Acknowledgements

The authors acknowledge the collaboration of the Institute fuer Pharmazeutische-Arzneimittellehre, Munich and thanks Mrs. V. Dunker for technical assistance in gas chromatography. Authentic European dill samples and reference substances were kindly supplied by Dr. Willmar Schabe and Co., Karlsruhe, West Germany.

REFERENCES

ADHIKARI, V. M. (1965). D.Sc. *Thesis (Pharmacognosy)*. Saarland University, Saarbrucken, West Germany.

BASLAS, R. K. & BASLAS, K. K. (1969). *Proc. 56th Indian Sci. Cong.* Part III (Abstracts), Bombay.

BETTS, T. J. (1969). *J. Pharm. Pharmac.*, **21**, 259–262.

CHAKRAVARTY, K. K. & BHATTACHARYA, S. C. (1954). *Indian Pharmst*, **9**, 218.

CHAUDHRY, S. S., SINGH, H. & HANDA, K. L. (1957). *Indian J. Pharm.*, **19**, 76–77.

GUENTHER, E. (1953). *The Essential Oils*, 4th Vol. Sec. Printing, p. 634; New York.

Indian Pharmacopoeia (1966), 2nd Edn, p. 491, India: The Government of India Press.

KHAFAGY, S. M. & MNAJED, H. K. (1968). *Acta pharm. suec.*, **5**, 155–162.

MALAVIYA, B. K. & DUTT, S. (1940). *Proc. Indian Acad. Sci.*, **12**, A, 251–265.

SCHRATZ, E. & QADRY, J. S. (1966). *Planta med.*, **14**, 310.

WALLIS, T. E. (1967). *Text book of Pharmacognosy*, 5th edn, p. 243, London: J. A. Churchill Ltd.

J. Pharm. Pharmac., 1971, **23**, 451

LETTERS TO THE EDITOR

The relation between clinical anti-inflammatory activity and the displacement of L-tryptophan and a dipeptide from human serum *in vitro*

It has been reported (McArthur, Dawkins & Smith, 1971) that salicylate, phenylbutazone, indomethacin, prednisolone, chloroquine and gold salts share a common action in displacing L-tryptophan, and several dipeptides, particularly L-phenylalanyl-L-phenylalanine, from their binding sites to bovine albumin and to human serum proteins *in vitro*. We have observed that this action is shared by two other potent anti-inflammatory substances, mefenamic and flufenamic acids, but not by other drugs that resemble the commonly used antirheumatic remedies in being administered over long periods of time.

We have found that phenobarbitone, penicillin V, ampicillin, cloxacillin, ascorbic acid or paracetamol, when studied in a range of concentrations at least twice those encountered in the circulation during therapy, did not displace either L-tryptophan or L-phenylalanyl-L-phenylalanine, from human serum when this was investigated by the techniques described by McArthur, Dawkins & Smith (1971). The failure of paracetamol to show this effect is of particular interest since the drug does not possess clinical anti-inflammatory activity (Fremont-Smith & Bayles, 1965; Boardman & Hart, 1967) but is frequently administered as an analgesic in rheumatoid arthritis.

It has been proposed (McArthur, Dawkins & others, 1971) that antirheumatic drugs act by displacing certain peptides from their binding sites to circulating proteins and that the free fractions of these peptides protect susceptible tissues against the effect of chronic inflammatory reactions. In patients with rheumatoid arthritis and and similar disorders the peptides are bound to an abnormal extent to the serum proteins and the drugs act by restoring the equilibrium to normal. The behaviour of L-tryptophan and L-phenylalanyl-L-phenylalanine mimics that of the hypothetical protective peptides. The present report provides additional evidence in favour of this hypothesis since it shows that a displacing action is common to antirheumatic drugs but is not given by other drugs that bind to human serum proteins and are administered in divided doses over similar periods of time.

Department of Biochemical Pharmacology,
King's College Hospital Medical School,
Denmark Hill,
London, S.E.5, U.K.

M. J. H. SMITH
P. D. DAWKINS
J. N. MCARTHUR

April 8, 1971

REFERENCES

BOARDMAN, P. L. & HART, F. D. (1967). *Br. med. J.*, **4**, 264–268.
FREMONT-SMITH, K. & BAYLES, T. B. (1965). *J. Am. med. Ass.*, **192**, 1133–1136.
MCARTHUR, J. N., DAWKINS, P. D. & SMITH, M. J. H. (1971). *J. Pharm. Pharmac.*, **23**, 393–398.
MCARTHUR, J. N., DAWKINS, P. D., SMITH, M. J. H. & HAMILTON, E. B. D. (1971). *Br. med. J.* In the press.

The effect of additives on the release of drug from hard gelatin capsules

The release of drugs from hard gelatin capsules has been shown to be affected by the presence of additives (Paikoff & Drumm, 1965; Withey & Mainville, 1969, Samyn & Jung, 1970). In these studies a single factor was varied at any one instance. To study the effect on drug release produced by combining a drug (ethinamate) with a diluent (0, 10 and 50% lactose), a lubricant (0, 1 and 5% magnesium sterate) and a wetting agent (0, 1 and 10% sodium lauryl sulphate) a preliminary experiment has been undertaken. The design used is set out in Table 1. The drug release from capsules has also been shown to be dependent on the capsule fill weight and the particle size of the drug (Newton & Rowley, 1970). The design in Table 1 was carried out with a 76–105 and a 251–420 μm size fraction at a low and high capsule fill weight. The drug availability was assessed by the dissolution test described by Newton & Rowley (1970), employing eight capsules from each combination of drug, diluent, lubricant and wetting agent.

The results for the percentage of drug released from the capsule into solution were treated by an analysis of variance. The values of the variance ratios are given in Table 2. An important feature of the results is the presence of significant interaction between diluent, lubricant and wetting agent. The restricted design of the experiment is such that when interactions are present, it is only possible to obtain indications of how additives influence drug release. To assess the effect of each factor, the average value for each level of the factor is compared with the overall mean for one series of experiments (i.e. one particle size, one capsule density, one time interval).

Table 1. *Combinations of additives tested.*

D_0	L_0	W_0	D_{10}	L_0	W_1	D_{50}	L_0	W_{10}
D_0	L_1	W_1	D_{10}	L_1	W_{10}	D_{50}	L_1	W_0
D_0	L_5	W_{10}	D_{10}	L_5	W_0	D_{50}	L_5	W_1

Where D represents diluent, L lubricant and W wetting agent. The subscript represents the % of additive present.

Table 2. *Results of analysis of variance of the effect of additives on the release of ethinamate from capsules.*

Size fraction of Drug	Time (mins)	Variance ratio							
		Low capsule denstiy				High capsule density			
		F_D	F_L	F_W	F_{DLWI}	F_D	F_L	F_W	F_{DLWI}
251–420 μm	5	0·60	8·43	47·82	18·32	1·77	17·94	42·00	5·54
"	10	14·90	21·35	93·23	44·42	2·70	3·76	31·04	3·71
"	20	8·31	25·91	75·73	26·32	1·21	3·34	9·52	0·32
"	30	1·05	3·11	10·69	0·82	3·57	0·81	1·28	2·63
"	40	5·91	5·55	15·15	0·02	4·53	0·51	0·39	3·28
76–105	5	16·19	10·59	33·71	11·39	7·43	25·28	28·08	0·04
"	10	61·09	27·30	91·76	26·62	28·82	28·34	73·63	7·48
"	20	80·76	28·16	96·71	19·62	70·73	34·76	188·05	5·88
"	30	69·06	22·93	68·08	11·81	78·53	33·69	206·68	1·93
"	40	72·54	26·96	68·14	8·81	81·43	33·23	206·00	0·53

Variance ratio—this is obtained from the mean square calculated as follows:

$$F_D = \frac{S^2_D}{S^2_O},\ F_L = \frac{S^2_L}{S^2_O},\ F_W = \frac{S^2_W}{S^2_O} \text{ and } F_{DLWI} = \frac{S^2_{DLWI}}{S^2_O}$$

where the subscript gives the source of variation: D, diluent; L, lubricant; W, wetting agent; DLWI interaction and O, residual error.

The tabulated values for the variance ratio for 2 and 60 degrees of freedom at the 5, 1 and 0·1% probability levels are 3·15, 4·98 and 7·76 respectively.

Table 3. *Magnitude and significance of the effects of diluent, lubricant and wetting agent on the release of ethinamate from capsules.*

Time (min)	Average % drug released for all factors	Diluent content 0	Diluent content 10%	Diluent content 50%	Lubricant content 0	Lubricant content 1%	Lubricant content 5%	Wetting agent content 0	Wetting agent content 1%	Wetting agent content 10%	
5	5·75	+0·41*	−0·02*	−0·39*	+1·36	+0·17	−1·18	−2·20	+0·68	+1·53	
10	12·69	+0·69*	+0·33*	−1·01*	+1·22	−0·70	−0·51	−3·42	+1·07	+2·36	
20	22·27	+1·04*	−1·05*	+0·01*	+0·67	−1·99	+1·30	−3·35	+1·16	+2·29	A1
30	29·83	−0·31	−2·44	+2·73	−0·11*	−1·19*	+1·29*	−1·57*	+0·09*	+1·46*	
40	35·53	−0·61	−3·10	+3·72	+0·33*	−1·28*	+0·95*	−0·97*	+0·08*	+1·05*	
5	5·31	+0·25*	+0·01*	−0·23*	+0·97	−0·12	−0·96	−2·38	+0·55	+1·83	
10	12·89	+1·84	−0·10	−1·75	+2·20	−2·09	−0·12	−4·45	−0·07	+4·52	
20	21·45	+2·27	−0·40	−1·88	+3·83	−3·59	−0·24	−5·51	−1·44	+6·94	A2
30	28·68	+1·02*	−1·91*	+0·90*	+3·29	−1·39	−1·89	−2·29	−3·76	+6·07	
40	34·82	+0·03	−3·51	+3·48	+3·62	+0·18	−3·80	−0·16	−6·06	+6·22	
5	12·86	−0·81	−2·55	+3·35	+6·22	−4·62	−1·61	−6·55	+1·64	+4·89	
10	17·95	+0·28	−7·13	+6·86	+7·38	−6·40	−1·09	−11·97	+1·78	+10·20	
20	24·88	+1·00	−9·89	+8·83	+6·06	−6·99	−0·92	−16·62	+3·31	+13·48	B1
30	29·34	+1·76	−11·05	+9·29	+5·84	−7·37	+1·54	−18·01	+3·11	+14·91	
40	33·40	+2·05	−11·84	+9·79	+6·29	−7·54	+1·26	−18·66	+2·78	+15·88	
5	12·59	+0·47	−4·43	+3·95	+3·85	−1·25	−2·61	−6·55	+1·09	+5·65	
10	17·83	−0·88	−8·33	+9·22	+6·07	−5·69	−0·38	−12·10	+3·49	+8·63	
20	24·31	−1·47	−10·47	+11·93	+6·05	−7·13	+1·07	−14·02	+4·81	+9·20	B2
30	28·81	−0·91	−11·42	+12·35	+6·64	−7·06	+0·43	−13·35	+4·21	+9·15	
40	32·96	−1·28	−11·81	+13·10	+7·80	−7·44	−0·35	−13·00	+4·19	+9·48	

The figures in the Table represent the difference between the mean of the percentage of drug released, under the influence of each level of each factor and the grand mean, at each time interval. All the results were found to be significant at the 5% level except those marked *.

A = 251–410 μm size fraction. B = 76–105 μm size fraction.
1 = high capsule fill weight. 2 = low capsule fill weight.

The results in Table 3 provide the following indications.

(i) All the additives have a greater effect on drug release when the finer particle size fractions are used. This can be related to the less permeable structure of powder beds formed by small particle size fractions (Newton & Rowley, 1970).

(ii) The addition of 50% of diluent is required to increase the drug release. The lower level of diluent is presumably insufficient to change the hydrophobic nature of the powder bed.

(iii) The presence of the lubricant at a 1% concentration reduces drug release, but there is no further decrease when the content is increased to 5%. The lubricant will enhance the hydrophobic character of the powder mass, however, it appears that there is a limit to this effect.

(iv) The presence of wetting agent increases drug release, the higher level producing a greater increase. The mechanism is no doubt associated with the wetting of the hydrophobic drug.

(v) The range of capsule fill weights used does not appear to greatly influence the effect which additives have on drug release. The results show the complex way in which combining additives influences the release of drugs from capsules. Further studies are required in which it is possible to evaluate the contribution of interactions.

Lilly Research Centre Limited,
Erl Wood Manor,
Windlesham, Surrey, U.K.

J. M. NEWTON
G. ROWLEY
J-F. V. TÖRNBLOM

February 23, 1971

REFERENCES

NEWTON, J. M. & ROWLEY, G. (1970). *J. Pharm. Pharmac., Suppl.* 163S–168S.
PAIKOFF, M. & DRUMM, G. (1965). *J. pharm. Sci.*, **54**, 1693–1694.
SAMYN, J. C. & JUNG, W. Y. (1970). *Ibid.*, **69**, 169–175.
WITHEY, R. J. & MAINVILLE, C. A. (1969). *Ibid.*, **58**, 1120–1126.

Interactions between prostaglandin E_1 and noradrenaline in anaesthetized rats

The effector response to nerve stimulation in the vas deferens, oviduct and spleen is inhibited by prostaglandins (PG) of the E series (Brundin, 1968; Euler & Hedqvist, 1969; Hedqvist & Brundin, 1969). Hedqvist & Brundin (1969) have shown that the inhibitory effect *in vitro* of PGs on the splenic response to nerve stimulation is accompanied by a reduction in the noradrenaline outflow, suggesting that PG might exert an inhibitory action on the process of noradrenaline release. These results prompted us to study the interactions between PGs and noradrenaline in anaesthetized rats with an intact nervous system.

Male Wistar rats, 200 ± 10 g, anaesthetized with sodium ethylmethylbutyl barbiturate (Mebubarbital, 30 mg/kg, i.p.) were infused intravenously with PGE_1, dissolved in isotonic glucose, at the subdepressive dose of 1·25 μg/kg min^{-1} (25 μl/min). Blood pressure was recorded throughout the experiment via an indwelling arterial catheter connected to a strain-gauge manometer. (±)-[^{3}H]Noradrenaline hydrochloride* (^{3}H-NA) (25 μCi; specific activity 7·7 Ci/mmol), dissolved in 0·5 ml of isotonic saline was injected 25 min after the PG infusion was begun. Rats were killed 5, 100 or 300 min later, the PG infusion having continued until death. Control experiments were run simultaneously on anaesthetized rats given ^{3}H-NA and infused with isotonic glucose at rate of 25 μl/min. The action of PGE_1 on tyramine-induced noradrenaline release was investigated in another series of rats receiving infusions of both PGE_1 and tyramine hydrochloride (50 μg/min; 25 μl/min) throughout the experimental period; the animals were killed 100 min after the injection of ^{3}H-NA. Controls received the ^{3}H-NA and a tyramine infusion.

^{3}H-NA was estimated in tissue homogenates, prepared according to Robinson & Watts (1965), by alumina column chromatography (Anton & Sayre, 1962). Radioactivity was determined by liquid scintillation on effluents from the alumina column. Endogenous noradrenaline was estimated fluorimetrically (Euler & Lishajko, 1961).

The results (Table 1) indicate that PGE_1 infusion did not alter the endogenous noradrenaline concentration except in the kidney and in the heart. Endogenous noradrenaline was increased in kidney and decreased in heart respectively 5 and 300 min after the injection of ^{3}H-NA. Five min after ^{3}H-NA injection in PGE_1-infused rats, the ^{3}H-NA concentration (Table 2) was significantly increased in kidney, and significantly reduced in adrenals, but after 100 and 300 min it was significantly higher in adrenals. The ^{3}H-NA concentration measured after the simultaneous infusion of tyramine and PGE_1 was significantly higher in heart than that measured during infusion of tyramine alone (Table 3).

These results do not give a clear idea of a possible interaction of PGE_1 with the sympathetic nervous system. In the kidney, PGE_1 was found to increase noradrenaline uptake, a result which may be related to the vasodilator effect of PGE_1 in this tissue (Lee, 1967). In the adrenals, PGE_1 reduced ^{3}H-NA uptake; the increased ^{3}H-NA concentrations observed 100 and 300 min after the ^{3}H-NA injection in PGE_1-infused rats may therefore be secondary to a decrease in noradrenaline release. In the heart, the uptake of the amine was not modified by PGE_1 although the level of endogenous amine was reduced by 300 min. The association of PGE_1 with tyramine markedly reduced the release of noradrenaline by tyramine (Burn & Rand, 1958) in the heart since the ^{3}H-NA concentration was higher than after tyramine alone. The lack of effect on noradrenaline release in the spleen disagrees with the releasing effect demonstrated *in vitro*.

* 2-Amino-1-(3,4-dihydroxyphenyl)-[1-^{3}H]ethanol from the Radiochemical Centre, Amersham.

Table 1. *Effect of PGE_1 on endogenous noradrenaline content.*

		Heart	Spleen	Vas deferens	Kidney	Adrenals
5 min	Control	606 ± 53	233 ± 28	6959 ± 387	106 ± 3	570447 ± 54730
(n = 12)	PGE_1	660 ± 62	283 ± 54	6806 ± 636	132 ± 8*	572177 ± 62012
100 min	Control	571 ± 58	342 ± 60	6241 ± 516	116 ± 8	560837 ± 40909
(n = 26)	PGE_1	495 ± 46	281 ± 30	7187 ± 616	112 ± 6	577179 ± 50103
300 min	Control	408 ± 31	252 ± 42	5482 ± 335	111 ± 17	462038 ± 26699
(n = 26)	PGE_1	210 ± 15*	279 ± 63	4959 ± 332	93 ± 14	459483 ± 37353

Results are expressed in ng/g. Mean ± s.e. $*P < 0.05$.

Table 2. *Effect of PGE_1 on [^3H]noradrenaline content.*

		Heart	Spleen	Vas deferens	Kidney	Adrenals
5 min	Control	270·4 ± 9·0	26·7 ± 2·4	59·3 ± 3·1	50·1 ± 3·0	75·6 ± 6·6
(n = 31)	PGE_1	279·0 ± 17·4	21·5 ± 1·6	61·5 ± 4·5	72·2 ± 4·8*	66·2 ± 3·4*
100 min	Control	208·8 ± 12·3	15·7 ± 1·8	42·7 ± 5·1	15·2 ± 1·3	46·2 ± 3·8
(n = 26)	PGE_1	205·2 ± 14·7	13·9 ± 1·2	41·9 ± 3·3	13·6 ± 0·8	68·2 ± 10·0*
300 min	Control	161·0 ± 9·3	12·5 ± 1·4	31·4 ± 2·7	7·6 ± 0·3	53·9 ± 4·8
(n = 26)	PGE_1	171·0 ± 10·8	14·5 ± 1·5	31·6 ± 2·2	8·0 ± 0·9	63·6 ± 5·8*

Results are expressed in counts/mg tissue; mean ± s.e. $*P < 0.05$.

Table 3. *Effect of PGE_1 on [^3H]noradrenaline content in rats infused with tyramine.*

	Heart	Spleen	Vas deferens	Kidney
Control (tyramine)	74·4 ± 6·3	17·9 ± 1·7	33·1 ± 3·9	9·7 ± 1·2
PGE_1 + tyramine (n = 18) ..	101·2 ± 10·3*	16·5 ± 1·3	38·6 ± 2·8	11·5 ± 1·3

Results are expressed in counts/mg tissue; mean ± s.e. $*P < 0.05$.

It is concluded that PGE_1 at subdepressive doses may reduce *in vivo* the release of noradrenaline in adrenals and heart. That the results were more obvious in previous studies performed *in vitro* may be the consequence of the large inactivation of PGE_1 occurring in the pulmonary circulation (Ferreira & Vane, 1967).

The authors wish to thank Drs J. R. Week and J. R. Pike of the Upjohn Company, Kalamazoo, Mich. who kindly provided PGE_1.

Hypertension Laboratory,
Hospital Broussais,
96, *rue Didot,*
75-*Paris* 14, *France.*

NICOLAS PAPANICOLAOU
PHILIPPE MEYER
PAUL MILLIEZ

March 3, 1971

REFERENCES

ANTON, A. H. & SAYRE, D. F. (1962). *J. Pharmac. exp. Ther.*, **138**, 360–375.
BRUNDIN, J. (1968). *Acta physiol. scand.*, **73**, 54–57.
BURN, J. H. & RAND, M. J. (1958). *J. Physiol., Lond.*, **144**, 314–336.
EULER, U. S. VON & HEDQVIST, P. (1969). *Acta physiol. scand.*, **77**, 510–512.
EULER, U. S. VON & LISHAJKO, F. (1961). *Ibid.*, **51**, 348–385.
FERREIRA, S. H. & VANE, J. R. (1967). *Nature, Lond.*, **216**, 868–873.
HEDQVIST, P. & BRUNDIN, J. (1969). *Life Sci.*, **8**, 389–395.
LEE, J. B. (1967). *New Eng. J. Med.*, **20**, 1073–1079.
ROBINSON, R. L. & WATTS, D. T. (1965). *Clin. Chem.*, **11**, 986–997.

The release of prostaglandin from the oesophagus and the stomach of the frog (*Rana temporaria*)

Vogt & Distellkötter (1966) first reported the spontaneous release of a prostaglandin into fluid bathing the frog intestine. They also showed an increase of release of prostaglandin when the intestine was placed in distilled water. Bartles, Vogt & Willie (1968) showed that acetylcholine (10^{-5} g/ml) increased the concentration of free prostaglandin in frog intestine. The release of prostaglandin from the oesophagus and the stomach of *Rana temporaria* has now been investigated under conditions of rest and of electrical stimulation.

The tissue strips were stimulated electrically between two parallel electrodes (Birmingham & Wilson, 1963), using a frequency of 5 Hz at 0·2 m/s pulse width for oesophagus and 0·1 m/s width for stomach at maximal voltage (150v), in a bath containing 5 ml "frog" Krebs solution* at room temperature bubbled with 5% carbon dioxide in oxygen. Parallel experiments were made in which the tissues from one frog were set up for measurement of the spontaneous release of prostaglandin and the tissues from another frog were stimulated electrically. At hourly intervals for the whole day (5 h per day) the bath contents were removed for assay and replaced by fresh "frog" Krebs solution.

The five samples were pooled and acidified with *N* hydrochloric acid to a pH 2·0–2·5, then shaken up with an equal volume of diethyl ether for 2 min. The ether was then removed in a separator funnel. This process was repeated three times. The volume of ether collected in this way was evaporated to dryness in a stream of nitrogen and the residue was dissolved in Krebs solution (1 ml) and assayed on the rat fundus strip (Vane, 1957). Standard solutions of prostaglandin E_1 (1 mg/25 ml) were prepared and taken through the procedure just described.

The results (Table 1) are the outcome of five experiments on both stomach and oesophagus. The spontaneous release of prostaglandin was the same from stomach and oesophagus. Both tissues showed increased release on electric stimulation. On electric stimulation the release was greater from the oesophagus than from the stomach. The recovery of a known amount of prostaglandin E_1 by the method was 70–80%.

The released prostaglandin was identified according to Green & Samuelson (1964) and to Fleshler & Bennett (1969). These methods can only distinguish between E and F types of prostaglandins, but they do not differentiate between E_1 and E_2 or $F_{1\alpha}$ and $F_{2\alpha}$. The prostaglandin released from the stomach and the oesophagus in these experiments was predominantly E type.

The results presented here show that there was a small spontaneous release in the resting state from the frog oesophagus and stomach which could be increased by stimulation of the intramural nerves. This release was not as high as that reported by Vogt & Distellkötter (1966). The prostaglandin released by stimulation

Table 1. *Release of prostaglandin from the oesophagus and the stomach of* R. temporaria *in resting and electrically stimulated states. Means ± s.e. of means.*

State	Oesophagus	Stomach
Resting (Spontaneous)	1·33 μg/g (n = 5) s.e. ± 0·07	1·17 μg/g (n = 5) s.e. ± 0·26
Electrically stimulated	27·0 μg/g (n = 5) s.e. ± 1·78	6·16 μg/g (n = 5) s.e. ± 0·14

* 1 litre of "frog" Krebs solution = 700 ml of Krebs solution + 300 ml of distilled water; pH after gassing, 7·3.

may have come from the stores in the muscle itself or from the nerve fibres but the experiments do not allow a firm conclusion to be drawn about the origin of the prostaglandin E.

Department of Pharmacology,
King's College,
Strand, London, W.C.2, U.K.
February 19, 1971

SHAHID RASHID*

* Present address: Department of Pharmacology, Wyeth Institute of Medical Research, Taplow Bucks., U.K.

REFERENCES

BARTLES, J., VOGT, W. & WILLIE, G. (1967–68). *Arch. Pharmak. exp. Path.*, **259–260**, 153–154.
BIRMINGHAM, A. T. & WILSON, A. B. (1963). *Br. J. Pharmac. Chemother.*, **21**, 569–580.
FLESHLER, B. & BENNETT, A. (1969). *J. Lab. clin. Med.*, **74**, 872–873.
GREEN, K. G. & SAMUELSON, B. (1964). *J. Lipid Res.*, **5**, 117–120.
VOGT, W. & DISTELLKÖTTER, B. (1966). *II Nobel Symposium on Prostaglandin* 1967, pp. 237–240.
VANE, J R. (1957). *Br. J. Pharmac. Chemother.*, **12**, 344–349.

Cannabis-induced vocalization in the rat

A disadvantage of previous methods of assaying the potency of cannabis preparations (Dixon, 1899; Gayer, 1928; Valle, 1967a,b) is that they cannot be used for determining effect of cannabis in rats. One possible approach is to use the degree of ataxia or catalepsy induced by the drug, another is to use vocalization as reported by Carlini & Kramer (1965). They found that, under the influence of cannabis, rats vocalize when they are touched.

We have set out to find if vocalization is a relevant indicator of cannabis effect. As reported by Boyd, Hutchington & others (1963) we also found that even low doses of cannabis produce a decrease in fixed ratio responding for food in rats. We have therefore compared the minimal doses necessary to affect vocalization with bar-pressing behaviour for food in a fixed ratio (FR) program.

Adult male albino rats of the Sprague-Dawley strain, weighing 300–350 g, 10 animals to each dose, were tested with cannabis extract (75% tetrahydrocannabinol, 7% cannabinol, 11% cannabidiol) or synthetic Δ^9- and Δ^8-tetrahydrocannabinol (THC) intraperitonally or orally, to assess the drugs' ability to dispose the animals to vocalization. The drugs were dissolved either in propylene glycol or olive oil. In an inhalation experiment, raw material (3·2% tetrahydrocannabinol, 1·2% cannabinol, 5·1% cannabidiol), as smoke, was tested, the rats being confined in a closed acrylic cage (22 × 15 × 15 cm) for 10–15 min after it had been filled with pure cannabis- or tobacco-smoke. The amount used varied between 0·60–0·80 g/rat.

Animals were tested in their individual cages. After the administration of the drugs, the rats were gently pressed with thumb and forefinger by the experimenter 2–4 times bilaterally behind their forelimbs on the ventral aspect of the frontal costal region every 5 min, to find the onset and duration of the vocalization behaviour and to see if habituation occurred.

In the fixed ratio experiment, six animals, trained at a FR30 schedule of reinforcement performing in daily sessions of 15 min, were used. The apparatus was standard operant conditioning equipment.

Vocalization could be produced in animals given: extract, in propylene glycol, in doses of 5 mg/kg, i.p. or more (one of ten animals did not vocalize at 10 mg/kg); extract, in propylene glycol or olive oil, orally in doses of 50 mg/kg or more; raw

material, inhaled as smoke; Δ^9-THC, in propylene glycol, in doses of 1·5 mg/kg, i.p. or more (two of ten animals did not vocalize at 1·5 mg/kg); Δ^8-THC, in propylene glycol, in doses of 2 mg/kg, i.p. or more (one of ten animals did not vocalize at 5 mg/kg).

Vocalization did not occur when the animals were given: extract in olive oil, in doses of 70 mg/kg, i.p., or less, or in doses of 35 mg/kg, orally, or less (four out of ten animals vocalized at 25–35 mg); solvents only, given i.p. or orally; tobacco, inhaled as smoke; Δ^9-THC, in propylene glycol, 0·80 mg/kg, i.p.

The vocalization behaviour generally appeared within 15 min of the drug being given, except after the oral administration of extract in oil, where the onset appeared after 1 h. In some animals it occurred after 5 min. No habituation to touching was noted even after 5–6 h.

To see if there was any sensitization to pressing, one experimental and one control group (n = 20/group) were given tetrahydrocannabinols daily for six days and then tested with solvent only. No vocalization occurred at the day of testing. The lack of effect with olive oil as solvent is probably because the drug absorption was prolonged. In a comparison of the minimal doses that affected vocalization with those affecting fixed-ratio behaviour it was found that when vocalization was present there was also a dose-dependent reduction in responding. Δ^9- and Δ^8-THC, 1·5–5·0 and 2·0–10·0 mg/kg, produced decrements in bar-pressing ranging from 20–100% and 8–100% respectively.

Not on every occasion did a usually effective dose of the drugs affect the operant-behaviour but in these cases there were neither vocalization, nor any other overt behavioral sign. This might be the result of faulty administration of drug or of individual variations of the animals in reacting to the drugs. We did observe that every time the behaviour was affected the rats always vocalized before and after the test. We conclude that vocalization may be used as an indicator of the existence of cannabis effects in rats and is useful when very low doses are involved.

This work was in part supported by a research grant from the Swedish Medical Research Council, Stockholm, Sweden (B71-27P-2904-02).

The authors wish to thank Dr. I. Dureman and Dr. U. Söderberg for their assistance and advice and Dr. T. Petrzilka, University of Zürich, for kind supply of the tetrahydrocannabinols and Dr. S. Agurell, University of Uppsala, for kind supply of extract and raw material.

Department of Psychology, University of Uppsala, Uppsala, Sweden.
Neurophysiological Laboratory, Psychiatric Research Centre, Ulleråker Hospital, University of Uppsala, Uppsala, Sweden.

BENGT G HENRIKSSON
TORBJÖRN JÄRBE

February 10, 1971

REFERENCES

BOYD, E. S., HUTCHINGTON, E. D., GARDNER, L. C. & MERRITT, D. A. (1963). *Archs int. Pharmacodyn. Thér.*, **144**, 533–544.

CARLINI, E. A. & KRAMER, C. (1965). *Psychopharmacologia*, **7**, 175–181.

DIXON, W. E. (1899). *Br. med. J.*, **2**, 1354–1357.

GAYER, H. (1928). *Arch. exp. Path. Pharmak.*, **129**, 312–318.

VALLE, J. R., SOUZA, J. A. & HIPPOLITO, N. (1967a). *Il Farmaco (Scient. Ed)*, **22**, 27–36.

VALLE, J. R., BARATELLA, J. R. S., TANGARY, M. R. & SILVA, N. (1967b). *Anais. Acad. bras. Cienc.*, **39**, 445–452.

Propranolol interferes with inhibitory behaviour in rats

Although there have been clinical reports that propranolol is associated with psychotic depression in patients receiving the drug for cardiac conditions (Waal, 1967) and that it is as effective as chlordiazepoxide in reducing anxiety in psychiatric out-patients (Wheatley, 1969), Laverty & Taylor (1968) did not find behavioural effects of propranolol in rats and no subsequent reports of behavioural effects of the drug in animals have appeared. However, we have found that propranolol disrupts the performance of rats on a DRL-20 operant conditioning schedule, a task requiring the inhibition for 20 s of a previously learned response in order to receive reinforcement.

Twenty male hooded rats, maintained at 80% of their free feeding body weight by food deprivation, were trained to press the bar in a Skinner Box for food reinforcement. Seven daily 45 min sessions in which each bar press was reinforced, were followed by 15 daily 45 min sessions of DRL-20 on which only responses at least 20 s apart were reinforced. Five min before each DRL-20 session, 5 rats were given a 5 mg/kg intraperitoneal injection of propranolol dissolved in 0·9% saline, 5 rats received 12·5 mg/kg of the drug, 5 rats had saline, and 5 rats had no injection.

A two way analysis of variance of the per cent reinforced responses on the DRL sessions showed a significant difference between the control groups and the two drug groups [$F(2,17) = 3{\cdot}99$; P 0·05]. Fig. 1 shows that less than 10% of the responses of the two drug groups were reinforced on most of the 15 days of DRL, while the two control groups rapidly improved to 30% reinforced responses.

Pellegrino (1968) found that ablation of the baso-lateral amygdala of rats disrupted the performance of a DRL-20 task in much the same way as propranolol did in the present study. Horovitz (1966) suggested that the amygdala is the site of action of antidepressant drugs and therefore that the amygdala is implicated in depression psychoses. Schallek & Kuehn (1965) concluded that the amygdala may be involved in the production of anxiety. Thus the effects of propranolol reported by Waal (1967) on depression, by Wheatley (1969) on anxiety, and by our experiments on the performance of a task requiring inhibition of a previously learned response, all parallel suggested functions of the amygdala.

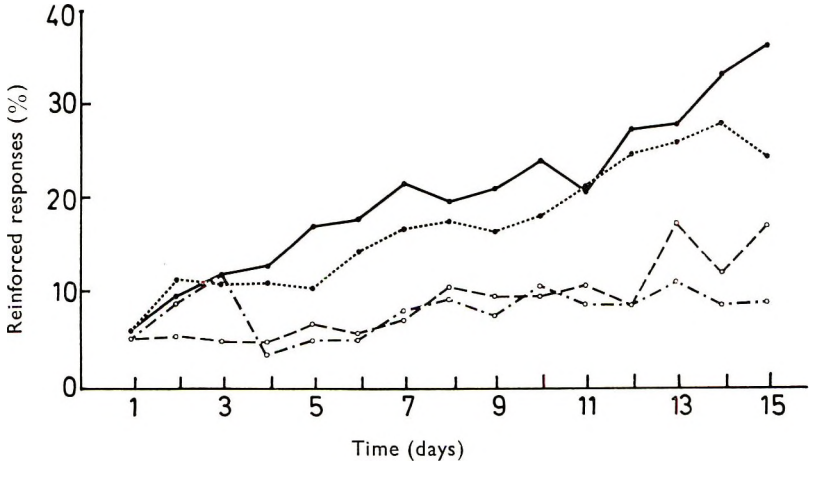

FIG. 1. Mean number of correctly inhibited responses expressed as % of total responses for each group of 5 rats on each of the 15 daily 45 min DRL-20 sessions. ●—● Normal. ● . . . ● Saline. ○— —○ Propranolol 5 mg/kg. ○—·—○ Propranolol 12·5 mg/kg.

This work was supported in part by U.S.A.F.O.S.R. grant F 44620-69-C-0001 to Dr. R. E. Musty, investigator. We are grateful to Dr. A. Sahagian-Edwards, Ayerst Laboratories, New York, for supplies of propranolol.

Neuropsychology Laboratory,
Department of Psychology,
University of Vermont,
Burlington, Vermont, U.S.A.

J. STEVEN RICHARDSON
P. DAVID STACEY
PETER W. CERAUSKIS
RICHARD E. MUSTY

January 25, 1971

REFERENCES

HOROVITZ, Z. P. (1966). *Recent Adv. Biol. Psychiat.*, **8**, 21–31.
LAVERTY, R., & TAYLOR, K. M. (1968). *J. Pharm. Pharmac.*, **20**, 605–609.
PELLEGRINO, L. (1968). *J. comp. physiol. Psychol.*, **65**, 483–491.
SCHALLEK, W. & KUEHN, A. (1965). *Medna. Pharmac. exp.*, **12**, 204–208.
WAAL, H. J. (1967). *Br. Med. J.*, **2**, 50.
WHEATLEY, D. (1969). *Br. J. Psychiat.*, **115**, 1411–1412.

Influences of cholinergic mechanisms on the function and turnover of brain dopamine

Anticholinergic drugs have long been used in the treatment of both spontaneous and drug-induced parkinsonism. The dopamine receptor stimulating drug apomorphine has also been reported to have a beneficial effect on Parkinson's disease, though weaker than L-dopa (Cotzias, Papavasiliou & others, 1970). Furthermore, hallucinations can be evoked both by blockade of central acetylcholine receptors and by stimulation of central catecholamine receptors. In the present investigation we have compared the effects of anticholinergics and of apomorphine on the function and turnover of dopamine in the rat corpus striatum both after and without treatment with the neuroleptic drug haloperidol.

The following drugs were used: DL-α-methyltyrosine methylester HCl (H 44/68; *Hässle, Mölndal), haloperidol (*Leo, Hälsingborg), *N*-ethyl-2-pyrrolidylmethyl-cyclopentyl-phenyl-glycolate HCl plus *N*-ethyl-3-piperidyl-cyclopentyl-phenyl-glycolate HCl (70 + 30% = Ditran; *Lakeside, Milwaukee), trihexyphenidyl HCl (*Kabi, Stockholm), (±)-hyoscyamine sulphate (atropine; Sigma, St. Louis), (−)-hyoscine hydrobromide (scopolamine; Merck, Darmstadt), *N*-methylscopolamine nitrate (Pharmacia, Uppsala), apomorphine HCl (Sandoz, Basle). The doses given refer to the salts.

The corpus striatum of adult hooded rats (150–300 g) was removed on one side by suction during diethylether anaesthesia. All brains were examined after the experiment and only animals with correct lesions were considered. The drugs tested were given 2–5 h after the operation. As previously described (Andén, Dahlström & others, 1966), haloperidol (1 mg/kg, i.p.) produced a longlasting and marked turning of the head and the tail to the unoperated side. When the haloperidol-induced asymmetry was well established after about 2 h, hyoscine (20 mg/kg, i.p.) was administered to 16 rats. It caused a clearcut and long lasting change in the position in 13 of these rats: their position became almost symmetrical in 5 min and they could turn to the operated side. A larger dose of hyoscine did not modify this response. In the remaining 3 rats, no obvious change was observed. Hyoscine (20–100 mg/kg, i.p.) given alone did not cause any observable asymmetry in unilaterally treated rats. In contrast, apomorphine (1 mg/kg, i.p.) did not change the haloperidol-induced asymmetry but evoked by itself a strong turning of the head and the tail to the operated side for about 1½ h (Andén, Rubenson & others, 1967).

The rats were examined for catalepsy immediately before death by placing one foreleg on supports of different heights and by measuring the period during which the imposed posture was maintained (Morpurgo, 1962). After treatment with haloperidol (1 mg/kg, i.p., $4\frac{1}{4}$ h) plus H 44/68 (250 mg/kg, i.p., 4 h), all the rats showed a catalepsy of the highest degree. In agreement with Morpurgo (1962), atropine (100 mg/kg, i.p., $4\frac{1}{2}$ h) and hyoscine (100 mg/kg, i.p., $4\frac{1}{2}$ h) completely abolished this catalepsy. Ditran (10 mg/kg, i.p., $4\frac{1}{2}$ h) and trihexyphenidyl (50 mg/kg, i.p., $4\frac{1}{2}$ h) partly suppressed it whereas methylscopolamine (100 mg/kg, i.p., $4\frac{1}{2}$ h) and apomorphine (1·25 mg/kg, s.c. for $4\frac{1}{4}$ h) were without effect.

The dopamine and noradrenaline were in each experiment determined in the pooled brains from two male Sprague-Dawley rats, 150–250 g, by spectrofluorometry after cation exchange chromatography of the amines and their oxidation (Bertler, Carlsson & Rosengren, 1958; Carlsson & Waldeck, 1958; Carlsson & Lindqvist, 1962). The results are in Tables 1 and 2. Inhibition of the enzyme tyrosine hydroxylase by H 44/68 (250 mg/kg, i.p.) caused in 4 h a decrease in brain dopamine and noradrenaline by about 70 and 50%, respectively (*cf.* Corrodi & Hanson, 1966). Pretreatment with

Table 1. *Influence of anticholinergics and apomorphine on the disappearance of dopamine in the rat brain induced by H* 44/68 (250 mg/kg, i.p., 4 h) *alone or together with haloperidol* (1 mg/kg, i.p., $4\frac{1}{4}$ h).

	Dopamine concentration*				Difference ± s.e.†		
Drug in *C* and *D*	*A* H 44/68	*B* Haloperidol + H 44/68	*C* Drug + haloperidol + H 44/68	*D* Drug + H 44/68	*B–A*	*C–B*	*D–A*
Ditran (10 mg/kg i.p.)	31·9 (5)	20·1 (5)	26·6 (5)	39·0 (5)	−11·8 ± 1·45 ($P < 0·005$)	6·4 ± 1·37 ($P < 0·01$)	7·1 ± 1·45 ($P < 0·01$)
Trihexyphenidyl (50 mg/kg i.p.)	30·9 (5)	21·2 (5)	25·8 (5)	37·7 (5)	−9·7 ± 3·48 ($P \approx 0·05$)	4·6 ± 1·49 ($P < 0·05$)	6·8 ± 2·40 ($P < 0·05$)
Atropine (100 mg/kg i.p.)	27·6 (5)	16·6 (5)	24·2 (5)	35·5 (5)	−11·0 ± 1·74 ($P < 0·005$)	7·6 ± 1·67 ($P < 0·02$)	7·8 ± 1·90 ($P < 0·02$)
Scopolamine (100 mg/kg i.p.)	27·6 (5)	16·6 (5)	25·1 (5)	30·3 (5)	−11·0 ± 1·74 ($P < 0·005$)	8·5 ± 2·64 ($P < 0·05$)	2·7 ± 1·66 ($P > 0·05$)
Methylscopolamine (100 mg/kg i.p.)	31·7 (5)	19·8 (5)	20·9 (5)	35·8 (5)	−11·9 ± 3·99 ($P < 0·05$)	1·1 ± 0·95 ($P > 0·05$)	4·1 ± 4·38 ($P > 0·05$)
Apomorphine (0·5+0·25× 3 mg/kg s.c.)	30·1 (6)	17·1 (6)	19·0 (6)	55·4 (6)	−13·1 ± 6·45 ($P < 0·001$)	1·9 ± 0·85 ($P > 0·05$)	25·2 ± 2·18 ($P < 0·001$)

* Mean values in per cent of untreated controls (0·72 μg/g = 100%). Number of experiments in parentheses.
† Statistical significance by Student's *t*-test after pairing of samples.

Table 2. *Influence of anticholinergics and apomorphine on the disappearance of noradrenaline in the rat brain induced by H* 44/68 (250 mg/kg, i.p., 4 h) *alone or together with haloperidol* (1 mg/kg, i.p., $4\frac{1}{4}$ h).

	Noradrenaline concentration*				Difference ± s.e.†		
Drug in *C* and *D*	*A* H 44/68	*B* Haloperidol + H 44/68	*C* Drug + haloperidol + H 44/68	*D* Drug + H 44/68	*B–A*	*C–B*	*D–A*
Ditran (10 mg/kg, i.p.)	60·1 (5)	47·0 (5)	40·2 (5)	51·1 (5)	−13·0 ± 2·77 ($P < 0·01$)	−6·8 ± 2·26 ($P < 0·05$)	−8·9 ± 3·16 ($P < 0·05$)
Trihexyphenidyl (50 mg/kg, i.p.)	53·4 (5)	45·8 (5)	39·7 (5)	46·3 (5)	−7·6 ± 2·12 ($P < 0·02$)	−6·0 ± 3·26 ($P > 0·05$)	−7·1 ± 3·02 ($P > 0·05$)
Atropine (100 mg/kg, i.p.)	53·7 (5)	42·6 (5)	36·3 (5)	40·2 (5)	−11·1 ± 4·22 ($P \approx 0·05$)	−6·4 ± 5·18 ($P > 0·05$)	−13·5 ± 3·37 ($P < 0·02$)
Hyoscine (100 mg/kg, i.p.)	53·7 (5)	42·6 (5)	41·7 (5)	40·4 (5)	−11·1 ± 4·22 ($P \approx 0·05$)	−0·9 ± 3·19 ($P > 0·05$)	−13·3 ± 3·16 ($P < 0·02$)
Methylscopolamine (100 mg/kg, i.p.)	52·5 (5)	40·7 (5)	39·3 (5)	44·3 (5)	−11·7 ± 3·20 ($P \approx 0·05$)	−1·4 ± 2·74 ($P > 0·05$)	−8·3 ± 4·89 ($P > 0·05$)
Apomorphine (0·5+0·25× 3mg/kg s.c.)	49·1 (6)	42·0 (6)	45·2 (6)	52·3 (6)	−7·1 ± 0·85 ($P < 0·001$)	3·1 ± 1·97 ($P > 0·05$)	3·3 ± 5·07 ($P > 0·05$)

* Mean values in per cent of untreated controls (0·36 μg/g = 100%). Number of experiments in parentheses.
† Statistical significance by Student's *t*-test after pairing of samples.

haloperidol (1 mg/kg, i.p. 15 min before H 44/68) significantly accelerated the disappearance of brain dopamine and noradrenaline induced by H 44/68.

If one of the centrally-active anticholinergics Ditran, trihexyphenidyl, atropine or hyoscine was given 15 min before haloperidol, the haloperidol-induced acceleration of the dopamine loss was significantly reduced. On the other hand, the effect of haloperidol on the noradrenaline turnover was, if anything, potentiated by these anticholinergics. Methylscopolamine did not change the acceleration of the brain dopamine or noradrenaline turnover observed after haloperidol, which agrees with the quaternary's difficulty in entering the brain. The ineffectiveness of methylscopolamine and the opposite effects on brain dopamine and noradrenaline of the centrally-active anticholinergics favour a specific action. Apomorphine did not influence the haloperidol-induced acceleration of the dopamine or noradrenaline turnover.

The anticholinergic drugs by themselves seemed to slightly reduce the H 44/68-induced rate of disappearance of brain dopamine in contrast to the slight acceleration observed for noradrenaline. Atropine has also been reported to slightly lower the level of homovanillic acid in the mouse corpus striatum and to reduce the increase in this dopamine metabolite after neuroleptics (O'Keeffe, Sharman & Vogt, 1970). In contrast to the anticholinergics, apomorphine caused a marked deceleration of the H 44/68-induced disappearance of brain dopamine whereas that of noradrenaline was largely unaffected. Apomorphine and the anticholinergics did not by themselves change the endogenous dopamine and noradrenaline levels significantly (data not shown).

The present study provides evidence for influence on the striatal function and the turnover of the striatal dopamine both of centrally active anticholinergics and of apomorphine. The effects are not the same, however. The actions of the anticholinergics were clearly observed only after pretreatment with haloperidol. On the other hand, the clearcut changes induced by apomorphine alone were virtually completely inhibited by haloperidol. Assuming that haloperidol and apomorphine act on the dopamine receptors (see Andén, Carlsson & Häggendal, 1969), the anticholinergics probably exert an effect on the striatal function beyond the dopamine receptors.

This work was supported by the Swedish Medical Research Council (B71-40X-2457-04). P.B. is a recipient of a fellowship from the Medical Research Council of Canada. For generous supplies of drugs we thank the companies indicated by* above. Skilful technical assistance was given by Mrs. Inger Oscarsson.

Department of Pharmacology,
University of Göteborg,
Sweden.

NILS-ERIK ANDÉN
PAUL BÉDARD

January 29, 1971

REFERENCES

ANDÉN, N.-E., CARLSSON, A. & HÄGGENDAL, J. (1969). *Ann. Rev. Pharmac.*, **9**, 119–134.
ANDÉN, N.-E., DAHLSTRÖM, A., FUXE, K. & LARSSON, K. (1966). *Acta pharmac. tox.*, **24**, 263–274.
ANDÉN, N.-E., RUBENSON, A., FUXE, K. & HÖKFELT, T. (1967). *J. Pharm. Pharmac.*, **19**, 627–629.
BERTLER, Å., CARLSSON, A. & ROSENGREN, E. (1958). *Acta physiol. scand.*, **44**, 273–292.
CARLSSON, A. & LINDQVIST, M. (1962). *Ibid.*, **54**, 87–94.
CARLSSON, A. & WALDECK, B. (1958). *Ibid.*, **44**, 293–298.
CORRODI, H. & HANSON, L. C. F. (1966). *Psychopharmacologia*, **10**, 116–125.
COTZIAS, G. C., PAPAVASILIOU, P. S., FEHLING, C., KAUFMAN, B. & MENA, I. (1970). *New Engl. J. Med.*, **282**, 31–33.
O'KEEFFE, R., SHARMAN, D. F. & VOGT, M. (1970). *Br. J. Pharmac.*, **38**, 287–305.
MORPURGO, C. (1962). *Archs int. Pharmacodyn. Thér.*, **137**, 84–90.

Biliary excretion of penicillins in the rat

Excretion of penicillins occurs in the bile of animals and man (see for example Stewart & Harrison, 1961; Ayliffe & Davies, 1965 for ampicillin and Acred, Brown and others, 1961; Henegar, Silverman & others, 1961 for methicillin), and there seems to be differences in the amounts of the various penicillins excreted. I now report variations in the biliary excretion of different penicillins in the rat when a standardized technique was used to measure the amounts of penicillin eliminated into the bile and to relate these to the logarithm of partition coefficients and chromatographic R_M-values of the penicillins.

The penicillins used were obtained from the Research Laboratories, Astra Läkemedel AB, Södertälje, Sweden. Rats of the Sprague-Dawley strain, with bile fistulae, 350 g, and under pentobarbitone anaesthesia with the temperature at 38 ± 1°, had the compounds injected into the femoral vein (dose 15 mg/kg) and bile collected for 4 h after administration. Antibiotic concentrations were determined by the cylinder-plate biological assay method (Grove & Randall, 1955), using pseudomonas of the Ellsworth strain for carboxybenzyl penicillin and *Sarcea lutea* ATCC 9341 for the other penicillins. The dilutions for the standard curve were prepared in pooled rat bile and the bile samples were diluted in the same medium to give a concentration within the range of the standard curve. Free acids of the penicillins were extracted with n-octanol from acetate buffer solutions (0·01M, pH 4·70 ± 0·02) and measured (Fujita, Iwasa & others, 1964). The concentrations of penicillins were measured by a hydroxylamine method (Boxer & Everett, 1949). The pK_a values of the various penicillins (for calculation of partition coefficients) were obtained from the literature (Rapson & Bird, 1963; Hon & Poole, 1969). The chromatographic R_M-values $\left[R_M = \log\left(\frac{1}{R_F} - 1\right)\right]$ were according to Biagi, Barbaro & others (1969).

Variations in the bile concentrations of biologically active penicillins were noted (Table 1). The concentration range for benzylpenicillin, ampicillin, methicillin and carboxybenzyl penicillin was 696–838 μg/g bile during the 0–1 h time interval. The concentration range for the other penicillins was 330–398 μg/g bile over 0–1 h. The bile concentration of penicillin gradually diminished during the 1–4 h. The cumulative excretions of penicillins into the bile are in Table 2. The penicillins showing the highest concentrations were also excreted to the greatest extent since the bile flow was of about the same rate in all experiments. Four h after administration, more than 30% of the doses of methicillin, ampicillin or carboxybenzyl penicillin had been eliminated as biologically active penicillin. Benzylpenicillin was also eliminated in

Table 1. *Concentration of biologically active penicillin in the bile after intravenous administration of various penicillins to rats* (15 mg/kg).

Compound	Concentration in bile (μg/g)* 0–1 h	1–2 h	2–4 h
Dicloxacillin	378 ± 76	14 ± 9	2 ± 1
Cloxacillin	330 ± 31	11 ± 2	1 ± 0
Oxacillin	368 ± 41	30 ± 3	1 ± 0
Azidocillin	357 ± 28	99 ± 71	4 ± 2
Pheneticillin	339 ± 45	18 ± 7	1 ± 0
Phenoxymethylpenicillin	398 ± 20	19 ± 1	9 ± 7
Benzylpenicillin	696 ± 110	37 ± 12	1 ± 0
Carboxybenzylpenicillin	838 ± 70	195 ± 50	21 ± 7
Methicillin	836 ± 84	299 ± 90	68 ± 38
Ampicillin	780 ± 146	352 ± 53	57 ± 19

* Each value represents the mean of 3–7 experiments ± s.e.

Table 2. *Cumulative excretion of biologically active penicillin in the bile after intravenous administration of various penicillins* (15 mg/kg) *to rats.* Results expressed as % of the administered dose are given together with the logarithm of the partition coefficients (log P) and chromatographic *RM*-values of the penicillins.

Compound	Amount excreted (%)* 0–1 h	0–2 h	0–4h	log P	R_M
Dicloxacillin	12·3 ± 2·0	12·7 ± 2·0	12·7 ± 2·0	3·24	1·62
Cloxacillin	10·8 ± 1·8	11·1 ± 1·8	11·2 ± 1·9	2·49	1·34
Oxacillin	17·4 ± 1·7	18·8 ± 2·6	18·9 ± 2·6	2·38	1·05
Azidocillin	10·8 ± 1·5	12·7 ± 1·8	12·9 ± 1·9	2·29	—
Pheneticillin	11·8 ± 1·5	12·3 ± 1·7	12·3 ± 1·7	2·20	1·03
Phenoxymethylpenicillin	14·1 ± 0·3	14·7 ± 1·8	15·1 ± 1·8	2·03	0·89
Benzylpenicillin	23·1 ± 3·2	24·0 ± 3·4	24·1 ± 3·4	1·72	0·55
Carboxybenzylpenicillin	27·3 ± 1·7	32·5 ± 2·5	33·2 ± 2·6	1·13	0·46
Methicillin	28·4 ± 4·0	36·3 ± 4·7	38·4 ± 5·1	1·06	0·47
Ampicillin	22·5 ± 5·5	30·6 ± 5·0	33·2 ± 4·8	—	0·07

* Each value represents the mean of 3–7 experiments ± s.e.

appreciable amounts since about 25% of the dose was in the bile. The other penicillins showed a more moderate excretion with a range from 11–19%. Table 2 also shows the logarithm of the partition coefficients and R_M-values of the compounds. When relating the amount of biologically active penicillin excreted in the bile during the 0–4 h period to the logarithm of the partition coefficients or R_M-values of the penicillins, correlation coefficients of −0·87 or −0·84 were found, respectively. This indicates that, with the penicillins used, a relation exists between increasing polarity in the side-chain of the penicillin molecule and biliary excretion. The variation in polarity may express differences in for example inactivation, affinity for transport systems or protein binding of the penicillins.

The author wishes to thank Dr. L. Magni, Research Laboratories, Astra Läkemedel AB, for performing the microbiological analyses.

Toxicology Laboratories,
AB Astra,
Södertälje,
Sweden.

ÅKE RYRFELDT

March 4, 1971

REFERENCES

ACRED, P., BROWN, D. M., TURNER, D. H. & WRIGHT, D. (1961). *Br. J. Pharmac. Chemother.*, **17**, 70–81.

AYLIFFE, G. A. J. & DAVIS, A. (1965). *Ibid.*, **24**, 189–193.

BIAGI, G. L., BARBARO, A. M., GAMBA, M. F. & GUERRA, M. C. (1969). *J. Chromat.*, **41**, 371–379.

BOXER, G. E. & EVERETT, P. M. (1949). *Analyt. Chem.*, **21**, 670–673.

FUJITA, T., IWASA, J. & HANSCH, C. (1964). *J. Am. chem. Soc.*, **86**, 5175–5180.

GROVE, D. C. & RANDALL, W. A. (1955). In: *Assay methods of antibiotics: a laboratory manual.* New York: Medical Encyclopedia.

HENEGAR, G. C., SILVERMAN, M., GARDNER, R. J., KUKRAL, J. C. & PRESTON, F. W. (1961). *Antimicrob. Ag. Chemother.*, 348–351.

HON, J. P. & POOLE, J. W. (1969). *J. pharm. Sci.*, **58**, 1510–1515.

RAPSON, H. D. C. & BIRD, A. E. (1963). *J. Pharm. Pharmac.*, **15**, 222T–231T.

STEWART, G. T. & HARRISON, P. M. (1961). *Br. J. Pharmac Chemother.*, **17**, 414–419.

Pressor responses in sheep to angiotensin II, noradrenaline and tyramine

Previous reports have dealt with a possible relation between the actions of angiotensin II, noradrenaline and tyramine and their effects on the autonomic nervous system (McCubbin & Page, 1963a,b; Louis & Doyle, 1966; Scroop & Walsh, 1968; Day & Owen, 1969). We have examined the pressor effects of the three compounds after intravenous injections in sheep. Fourteen Kerry Hill and Welsh Mountain ewes and wethers of average weight 36 kg (s.d. = ±4 kg) were used. The general preparation and recording of blood pressure were according to Osborn, Hughes & others (1969). The angiotensin II was asparaginyl[1]-valyl[5]-angiotensin II (*Hypertensin*, Ciba), the noradrenaline was noradrenaline acid tartrate (*Levophed*, Bayer Products) and tyramine hydrochloride was supplied by British Drug Houses. Injections were made into the jugular vein as 5 ml solutions followed by a wash with 2 ml of saline over 2 s.

Doses of angiotensin II (10 μg) and of noradrenaline (20 μg) which, by themselves, raised the blood pressure by 25–30 mm Hg, or mixtures of these doses, were injected in the sequence angiotensin II, noradrenaline, angiotensin II *plus* noradrenaline. Three series of injections were made in each of the twelve experiments, the injections being made at 6 min intervals. The average maximum rise of 28 mm Hg with either hormone alone was increased to 45 mm Hg when the two were mixed while doubling the dose of angiotensin II from 10 to 20 μg and of noradrenaline from 20 to 40 μg only raised the blood pressure to 32 mm Hg.

The effect of sequential injection of angiotensin and noradrenaline was investigated by injecting 20 μg of noradrenaline 4 and 10 min after 10 μg of angiotensin II, and also in the reverse order by giving 10 μg of angiotensin II 2 and 8 min after 20 μg of noradrenaline. Three series of injections were made in each of two animals; there was no evidence of potentiation of either hormone by the other.

The possible potentiation of the effects of angiotensin II and noradrenaline by tyramine was studied in eight experiments. Tyramine (2–4 mg) alone raised the blood pressure, on average, by 26 mm Hg and its effect was observable for up to 4 min. Angiotensin II (10 μg) and noradrenaline (20 μg) produced rises of 25 and 27 mm Hg respectively. When tyramine was combined with 10 μg of angiotensin II the average maximum pressor response was 49 mm Hg and when combined with 20 μg of noradrenaline the rise in blood pressure was 51 mm Hg.

Sequential injections of tyramine and the hormones showed that if tyramine preceded the injection of the angiotensin and noradrenaline by 4 min their pressor effects were enhanced (angiotensin II, on average, by 22% and noradrenaline by 11%).

Our findings indicate that mixtures of angiotensin II and noradrenaline are much more effective in raising the blood pressure of sheep than either hormone itself; however, this does not necessarily indicate that one potentiates the other; their mode of action on the small blood vessels differs (Peart, 1965) and this may explain the present results. Injections of mixtures of tyramine and angiotensin II, and of tyramine and noradrenaline, were also more effective than any of these compounds alone.

Sensitization to the pressor effects of angiotensin II by tyramine may be related to the noradrenaline-releasing action of tyramine and the observed potency of mixtures of angiotensin II and noradrenaline are in keeping with this suggestion.

These studies were made possible by the expert technical assistance of Mr. J. Wilson and Mr. O. F. Mason.

*Departments of Medicine and Metabolic Medicine,** E. C. OSBORN
Welsh National School of Medicine, G. TILDESLEY
Cardiff Royal Infirmary, L. P. O'GORMAN
Cardiff, U.K. R. F. MAHLER

February 13, 1971

REFERENCES

DAY, M. D. & OWEN, D. A. A. (1968). *Br. J. Pharmac.*, **34**, 499–507.
LOUIS, W. J. & DOYLE, A. E. (1966). *Clin. Sci.*, **31**, 247–254.
MCCUBBIN, J. W. & PAGE, I. H. (1963a). *Science, N.Y.*, **139**, 210–211.
MCCUBBIN, J. W. & PAGE, I. H. (1963b). *Circulation Res.*, **12**, 553–559.
OSBORN, E. C., HUGHES, S. G., PIRIE, A. T., WILLICOMBE, P. R., & MAHLER, R. F. (1969). *Cardiovasc. Res.*, **3**, 134–146.
PEART, W. S. (1965). *Pharmac. Rev.*, **17**, 143–182.
SCROOP, G. C. & WALSH, J. A. (1968). *Aust. J. exp. Biol. med. Sci.*, **46**, 573–580.

* Correspondence to Dr. E. C. Osborn at this address.

The effect of angiotensin I and II on hind-limb blood flow in sheep

It was shown by Ng & Vane (1967; 1968) that angiotensin I was substantially removed during a single passage through the hind-limb of the dog but without decreasing blood flow through the limb, and these authors suggested that it required conversion to angiotensin II in the pulmonary circulation before it acquired biological activity. However, earlier studies (Carlini, Picarelli & Prado, 1958; Halvorsen, Fasciolo & Calvo, 1959; Gross & Turrian, 1960; Barac, 1962) using angiotensin I of biological origin had shown that angiotensin I itself reduced the blood flow after perfusion of the hindlimb or hindquarters of the dog and of other species. In this study we report the effect of a synthetic angiotensin I, identical with human angiotensin I, on the hind-limb blood flow of the sheep.

The angiotensin was supplied by Schwarz BioResearch, Orangeburg, New York. It was aspartyl^{1}-isoleucyl^{5}-angiotensin I and was synthesized by the solid-phase technique pioneered by Merrifield (1963). Gel chromatography was used to purify the material and thin-layer chromatography and amino-acid analysis (Spackman, Stein & Moore, 1958) to establish its nature. These procedures indicated that less than 5% of impurities were present (Schwarz BioResearch, 1970; Dr. W. C. Roberts, personal communication). This angiotensin I preparation was tested for the presence of angiotensin II by comparing its action on the rat isolated colon with that of angiotensin II (asparaginyl^{1}-valyl^{5}-angiotensin II, Hypertensin, Ciba, Basle). This assay preparation responds weakly, or not at all, to angiotensin I (Osborn, Tildesley & others; unpublished observations). The results showed that the angiotensin I contained less than 1% of angiotensin II.

The animals we used were Kerry Hill and Welsh Mountain rams, wethers and ewes of average weight 28 kg (s.d. = $\pm$4 kg). Anaesthesia and monitoring of blood pressure were as described previously (Osborn, Hughes & others, 1969). Hind-limb blood flow was determined by a method involving direct collection of femoral vein blood.*

The effects of both hormones were studied in ten experiments. The animals were initially given several injections of saline and of 2 μg of angiotensin II into the femoral artery to accustom them to the procedure. When good reproducibility had been

* Full details on request.

achieved (i.e. values usually within $\pm 10\%$ of the means), angiotensin I (12 μg) was compared with various doses (0·5, 1·0, 2·0, 5·0 and 10 μg) of angiotensin II.

The procedure was as follows: each dose of angiotensin was alternated with one of saline at least 5 min after the injection of the hormone; longer periods were allowed for the larger doses. Two injections of angiotensin I were made initially. These were followed by two injections of each of any three of the five doses of angiotensin II. These were followed by a further two injections of angiotensin I after which the remaining two doses of angiotensin II were employed, each dose being injected twice. A further two injections of angiotensin I were than made to conclude the experiment.

The hind-limb blood flow in the control periods averaged 1·1 ml/s (s.d. = $\pm$0·2 ml/s). Both angiotensin I and angiotensin II caused an immediate fall in hind-limb blood flow; the effects of the injections of the hormones are shown in Table 1. The percentage reduction in blood flow has been calculated relative to the flow during the control period preceding and after the injection of each hormone. On average, angiotensin I (12 μg) had the same effect as a dose of 1–2 μg of angiotensin II.

The replication of the procedure was tested with 1·0 and 0·3 μg of angiotensin II in ten other animals of about the same size. These doses were chosen to give reductions in blood flow about equal to, and appreciably less, than those given by 12 μg of angiotensin I. Injections were made in the sequence saline, 0·3 μg of angiotensin II, saline and 1·0 μg of angiotensin II. Ten series of injections were made in each experiment and the effect of the angiotensin injection was calculated with respect to the two control values before and after it. The results (Table 2) were analysed in terms of five pairs of injections for each dose. The average control blood flow in these studies was 1·0 ml/s (s.d. = $\pm$0·3 ml/s). The results indicate that the method has acceptable reproducibility over the range of blood flow reductions reported in Table 1.

Table 1. *The reduction in hind-limb blood flow in ten sheep after injections of angiotensin I and angiotensin II into the femoral artery.* The s.d. is given in brackets.

Hormone	Dose (μg)	Reduction in blood flow (%)
Angiotensin I	12	24 ($\pm$7)
	0·5	19 ($\pm$5)
	1·0	23 ($\pm$5)
Angiotensin II	2·0	27 ($\pm$6)
	5·0	30 ($\pm$5)
	10	30 ($\pm$6)

Table 2. *The reductions in hind-limb blood flow after injections of* 0·3 *and* 1·0 μg *of angiotensin II into the femoral artery in ten sheep.* The s.d., for the five paired estimations with each dose, is given in brackets. The experiments have been arranged from left to right order of increasing sensitivity to the smaller dose of the hormone. Analysis of variance (Fisher & Yates, 1948; Moroney, 1951) showed that the method could distinguish ($P < 0{\cdot}05$ or $\simeq 0{\cdot}05$) the effects of the two doses in seven of the experiments.

Dose of angiotensin II (μg)	Reduction in blood flow (%)										Mean and s.d.
0·3	8 ($\pm$2)	12 ($\pm$3)	13 ($\pm$3)	14 ($\pm$3)	15 ($\pm$4)	18 ($\pm$4)	18 ($\pm$4)	18 ($\pm$3)	26 ($\pm$4)	31 ($\pm$6)	17 ($\pm$7)
1·0	11 ($\pm$2)	19 ($\pm$4)	17 ($\pm$3)	22 ($\pm$3)	21 ($\pm$3)	21 ($\pm$2)	29 ($\pm$2)	31 ($\pm$2)	31 ($\pm$4)	38 ($\pm$5)	24 ($\pm$8)

The present investigations show that the injection of either hormone into the hind-limb has an immediate effect on blood flow but that angiotensin I is much less effective than angiotensin II. We have found in other investigations that both hormones used in the present studies are removed equally by the hind-limb of the sheep. A possible explanation for the present findings is that much of the angiotensin I is inhibited or destroyed rather than converted to angiotensin II when it is removed from the circulation. Our findings in the sheep differ from those of Ng & Vane (1968) in the dog and they are in agreement with those reported in several species by the earlier workers. These studies were made in the Dr. Leonard West Research Laboratory of Sully Hospital, Sully, Glamorgan, and we gratefully acknowledge the expert technical assistance of Mr. J. Wilson, Mr. O. F. Mason and Mr. P. Stock.

*Departments of Medicine and Metabolic Medicine,** E. C. OSBORN
Welsh National School of Medicine, G. TILDESLEY
Cardiff Royal Infirmary, K. G. LEACH
Cardiff. R. F. MAHLER
Sully Hospital,
Sully,
Glamorgan, U.K.

February 13, 1971

* Correspondence to Dr. E. C. Osborn at this address.

REFERENCES

BARAC, G. (1962). *C.r. Séanc. Soc. Biol., Paris*, **156**, 1722–1725.

CARLINI, E. A., PICARELLI, Z. P. & PRADO, J. L. (1958). *Bull. Soc. chim. Biol.*, **40**, 1825–1834.

FISHER, R. A. & YATES, F. (1948). *Statistical Tables for Biological, Agricultural and Medical Research.* Ch. 5. London and Edinburgh: Oliver and Boyd.

GROSS, F. & TURRIAN, H. (1960). *Polypeptides which Affect Smooth Muscles and Blood Vessels*, pp. 137–151. Editor: Schachter, M. Oxford, London, New York, Paris: Pergamon Press.

HALVORSEN, K. A., FASCIOLO, J. C. & CALVO, R. (1959). *C.r. Séanc. Soc. Biol., Paris*, **153**, 489–490.

MERRIFIELD, R. B. (1963). *J. Am. chem. Soc.*, **85**, 2149–2154.

MORONEY, N. J. (1951). *Facts from Figures.* Ch. 19. London, Reading and Fakenham: Cox and Wyman.

NG, K. K. F. & VANE, J. R. (1967). *Nature, Lond.*, **216**, 762–766.

NG, K. K. F. & VANE, J. R. (1968). *Ibid.*, **218**, 144–150.

OSBORN, E. C., HUGHES, S. G., PIRIE, A. T., WILLICOMBE, P. R. & MAHLER, R. F. (1969). *Cardiovasc. Res.*, **3**, 134–146.

SCHWARZ BIORESEARCH (1970). *Schwarz BioResearch Peptide and Peptide Synthesis Catalogue*, p. 13.

SPACKMAN, D. H., STEIN, W. H. & MOORE, S. (1958). *Analyt. Chem.*, **30**, 1190–1206.

Is 5-hydroxytryptamine involved in the mechanism of action of fenfluramine?

Jespersen & Scheel-Krüger (1970) recently reported that methysergide blocked the hypothermic effect of fenfluramine in dogs and concluded that 5-hydroxytryptamine (5-HT) played an important role in the mechanism of action of fenfluramine, an anorectic drug that does not produce central stimulation in most animals (Le Douarec, Schmitt & Laubie, 1966). On the other hand, Opitz (1967) found that fenfluramine inhibited the appetite of rats in which brain 5-HT had been depleted by *p*-chlorophenylalanine, an experiment that strongly suggested that 5-HT was not required for the action of fenfluramine.

We have confirmed the results obtained by Opitz (1967) in rats trained to eat their day's food in 2 h (Hollifield & Parson, 1962). A single intraperitoneal injection of 300 mg/kg of *p*-chlorophenylalanine lowered food intake by 30% in a group of 15 rats on the third day; an effect which may arise from irritation of the gut since, in other experiments we have found marked intestinal damage after intraperitoneal administration of the drug. On the fourth day, food consumption was inhibited about 80% after the administration of 8 mg/kg of (±)-fenfluramine hydrochloride. This is about the same inhibition as that previously found after the use of fenfluramine alone (Fig. 1). Chemical analyses of the brains of 15 other rats given 300 mg/kg of *p*-chlorophenylalanine showed that the 5-HT content was markedly decreased on the fourth day.

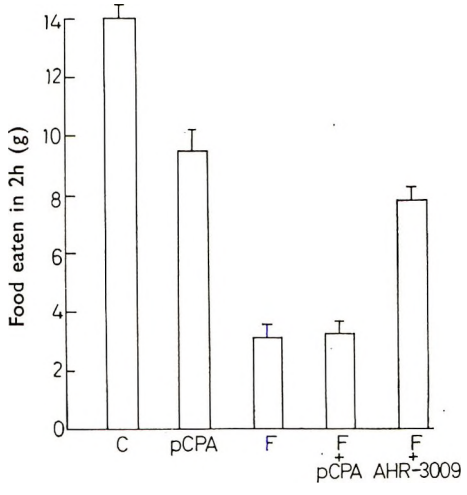

FIG. 1. Effect of drugs on food consumption of rats (n = 15) trained to eat their daily diet in 2 h. Control animals (C) received 1 ml of saline intraperitoneally. *p*-Chlorophenylalanine (pCPA), at 300 mg/kg, lowered food consumption by 30% after 3 days (thought to be due to gastrointestinal irritation; see text). Fenfluramine (F) markedly inhibited food intake when administered alone and to rats pretreated with pCPA 4 days earlier. 8β-Carbobenzyloxy-aminomethyl-1-methyl-10α-ergoline (AHR-3009) antagonized the action of fenfluramine when these drugs were given together.

In a further 15 trained rats, appetite depression usually produced by 8 mg/kg of fenfluramine intraperitoneally was partially blocked by 1 mg/kg by the same route of AHR-3009 (8β-carbobenzyloxy-aminomethyl-1-methyl-10α-ergoline), a potent inhibitor of 5-HT (Beretta, Glässer & others 1965). This is consistent with the finding of Jespersen & Scheel-Krüger (1970) who also found evidence that a 5-HT antagonist blocked the appetite depressant action of fenfluramine. Attempts to antagonize the anorectic action of (+)-amphetamine or chlorphentermine with AHR-3009 have been unsuccessful.

Since the appetite-depressant action of fenfluramine in rats can be blocked by 5-HT antagonist at least in part and it continues to act at a time when the brain content is much decreased, fenfluramine may act by stimulating tryptaminergic neurons directly.

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

WILLIAM H. FUNDERBURK
JEAN C. HAZELWOOD
ROBERT T. RUCKART
JOHN W. WARD

January 28, 1971

REFERENCES

BERETTA, C., GLÄSSER, A. H., NOBILI, M. B. & SILVESTRI, R. (1965). *J. Pharm. Pharmac.*, **17**, 423–428.

HOLLIFIELD, G. & PARSON, W. (1962). *J. clin. Invest.*, **41**, 250–253.

JESPERSEN, S. & SCHEEL-KRÜGER, J. (1970). *J. Pharm. Pharmac.*, **22**, 637–638.

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

OPITZ, K. (1967). *Arch. Pharmak. exp. Path.*, **259**, 56–65.

Acetylcholine and "auto-inhibition"

The evidence that the release of noradrenaline from sympathetic fibres is due to the acetylcholine released by the nerve impulse, has now been strengthened by the work of Eränkö, Rechardt & others (1970), and by that of Malik (1970). The former have stained the pineal gland of the rat by the thiocholine method, and have shown that adrenergic terminals seen in electron micrographs are closely invested with acetylcholinesterase. The pineal gland is innervated entirely by fibres from the superior cervical ganglion, and when the ganglia of both sides are removed, both the acetylcholinesterase and the small granular vesicles containing noradrenaline disappear.

Malik perfused the superior mesenteric artery and its branches in the rat, and recorded the constrictor response in the arteries to postganglionic stimulation. He found that the response to stimulation of frequencies from 1 to 6 s was increased when anticholinesterases were added to the perfusion fluid, and in about 100 experiments showed that the increase was greatest at the lowest frequency, diminishing as the frequency rose until at 6 s the increase was imperceptible. Since the investigations of Eränkö & others (1970) and of Malik (1970) provide very clear evidence, the recent work of Löffelholz (1971) requires consideration.

Löffelholz has carried out experiments on the isolated heart of the rabbit, in which the sympathetic postganglionic nerves were stimulated, and the noradrenaline appearing in the effluent was measured. In the course of these experiments either acetylcholine (plus atropine) was added to the perfusion fluid for a short period, or nicotine, or DMPP, was added, and the noradrenaline released by these substances was measured.

The concentration of acetylcholine infused was large, $2{\cdot}1 \times 10^{-4}$M, and this caused a release of a large amount of noradrenaline. However this release was very brief, continuing for 5 to 10 s only, although the infusion of acetylcholine was maintained for 9 min.

The author considered that the cessation of noradrenaline release after 5 to 10 s was due to "auto-inhibition", the receptors for acetylcholine being blocked by the infusion. The important point was that he found that during this "auto-inhibition" the response to sympathetic stimulation was unchanged. He said "when the nicotinic block was established, the noradrenaline released by electrical stimulation was not inhibited", and his implication was that the receptors on which acetylcholine acted to release noradrenaline were not involved in the release of noradrenaline by sympathetic stimulation.

Since the evidence from anticholinesterases shows that sympathetic stimulation involves receptors for acetylcholine, it follows that the "auto-inhibition" must occur at some other point. The same problem was raised by the experiments of Daly & Scott (1961) who found that acetylcholine, injected into the splenic artery, released noradrenaline, but that this release was blocked by hexamethonium, whereas the response to stimulation of the splenic nerves was not. It seemed possible that the

block of the injected acetylcholine by hexamethonium was a block of the access of acetylcholine to the receptors, and not a block of the receptors themselves. Burn & Gibbons (1964) decided to test this, and did so by choosing bretylium which is chemically similar to acetylcholine. They used the Finkleman (1930) preparation of the rabbit ileum to discover whether hexamethonium would block the action of bretylium in abolishing the response to sympathetic stimulation. They found that it did, from which it followed that hexamethonium was blocking the access of bretylium to the receptors, and thus there was reason to think that hexamethonium also blocked the access of acetylcholine to the receptors.

In the course of these experiments, observations were made to find out if the tertiary compound pempidine, which is a ganglion-blocking agent having a chemical resemblance to nicotine, had any effect in blocking sympathetic nerve endings. When pempidine was added to the bath containing the Finkleman preparation of the rabbit ileum, the following result was consistently obtained. In a concentration of 5×10^{-5} g/ml, pempidine in the first 10 min acted like bretylium in causing a gradually increasing block of sympathetic stimulation. Then the blocking action stopped although not more than 50% complete, and during the next hour the block became less. In the next 2 h the block became complete. The observations appeared to indicate that at first pempidine reached the nicotinic receptors on which the sympathetic impulse acts and began to block them. Then further access of pempidine to these receptors was prevented by pempidine itself, due to "auto-inhibition".

To return to the experiments of Löffelholz, he found in some of his experiments on the perfused heart that the amount of noradrenaline released by sympathetic stimulation was very much increased during the infusion of acetylcholine. This occurred when the concentration of acetylcholine infused was $5{\cdot}5 \times 10^{-5}$M, less than the concentration used previously, and when it had been infused for 1 min only. He could not offer a satisfactory explanation for this, but I think it likely that the infused acetylcholine added its effect to that released by stimulation, to release, in turn, more noradrenaline. We know that acetylcholine is concerned in the release of noradrenaline in the isolated rabbit heart from the work of Huković (1966).

University of Oxford, J. H. BURN
Oxford, U.K.
April 13, 1971

REFERENCES

BURN, J. H. & GIBBONS, W. R. (1964). *Br. J. Pharmacol. Chemother.*, **22**, 549–557.
DALY, M. DE B. & SCOTT, M. J. (1961). *J. Physiol., Lond.*, **156**, 246–259.
ERÄNKÖ, O., RECHARDT, L., ERÄNKÖ, L. & CUNNINGHAM, A. (1970). *Histochem. J.*, **2**, 479–489.
FINKLEMAN, B. (1930). *J. Physiol., Lond.*, **70**, 145–157.
HUKOVIĆ, S. (1966). *Br. J. Pharmac. Chemother.*, **28**, 273–281.
LÖFFELHOLZ, K. (1970). *Arch. Pharmak.*, **267**, 64–73.
MALIK, K. U. (1970). *Circulation Res.*, **27**, 647–655.

The spectrophotometric determination of ampicillin in body fluids

Smith, De Grey & Patel (1967) described a specific spectrophotometric method for the determination of ampicillin in pharmaceutical preparations, based on the copper facilitated formation of the stable acid degradation products, for which the presence of the intact antibiotic molecule is essential.

We have now adapted the method to the assay of ampicillin in chicken blood, bile

Table 1. *Detection of concentrates of ampicillin in plasma and bile*

Ampicillin added (μg)	Concentration (μg/ml) found in plasma Spectrophotometric assay	Microbiological assay	Ampicillin added (μg)	Concentration (μg/ml) Spectrometric assay found in bile
31	30 (90%)	34 (103%)	250	232 (93%)
62	60 (97%)		500	488 (98%)
125	121 (97%)		1000	975 (97%)
125	125 (100%)		2000	2010 (101%)
250	239 (95%)	298 (118%)	3000	3135 (104%)
250	242 (97%)	298 (118%)		
250	230 (92%)	241 (96%)		
250	230 (92%)	270 (108%)		
250	234 (94%)	221 (88%)		
500	468 (94%)			
500	476 (95%)			

and urine; this enabled us to know the antibiotic concentrations in body fluids during the movement of ampicillin through the body.

Plasma assay. Plasma (0·2 ml) from heparinized blood is taken in a 1·5 ml centrifuge tube containing absolute ethanol (0·4 ml). After thorough mixing and centrifuging for 5 min at 4500 rev/min, clear supernatant (0·2 ml) is taken in a 5 ml tube with citrate buffer solution (0·8 ml; pH 5·2) containing copper (15 μg/ml) [98·5 parts of a buffer prepared by mixing citric acid (46·4 ml of 0·1M) and disodium hydrogen phosphate (53·6 ml of 0·2M), and copper sulphate pentahydrate (1·5 parts of a 0·393% solution)]. The tube, agitated at 120 strokes/min, is incubated at 75° for 30 min and then cooled in ice. The sample is taken in a 1·5 ml cuvette and read at 320 nm against the blank given by the residual non-incubated fraction of the supernatant.

Bile and urine assay. Bile or urine (0·1 ml) is taken in a 10 ml centrifuge tube containing trichloroacetic acid solution (0·1 ml; 20%), immediately mixed and then citrate buffer (4·8 ml; pH 5·8) containing copper (15 μg/ml) is added [98·5 parts of a buffer prepared by mixing citric acid (39·5 ml of a 0·1M) and disodium hydrogen phosphate (60·5 ml of 0·2M), and copper sulphate pentahydrate (1·5 parts of a 0·393% solution)]; the final pH is 5·2.

After thorough mixing and centrifugation at 4500 rev/min for 5 min, two similar portions of the supernatant are poured into two tubes, one of which is incubated as above. The procedure is then as described for plasma.

The data in Table 1 show that the spectrophotometric assay can detect with reliability plasma concentrations of ampicillin down to 30 μg/ml. The results are reproducible and are in good agreement with those recorded with microbiological assay (plate diffusion test; *Sarcina lutea*).

The main advantages of the method are the comparatively short time (about 1 h), and the small volume of plasma needed to make the assay.

This work was supported by grants from the Consiglio Nazionale delle Ricerche Rome.

Istituto di Farmacologia,
Università di Camerino,
Camerino, Italy.

L. ANGELUCCI

Istituto di Farmacologia,
Università di Roma,
Rome, Italy.

M. BALDIERI

December 11, 1970

REFERENCE

SMITH, J. W. G., DE GREY, G. E. & PATEL, V. J. (1967). *Analyst*, **92**, 247–252.

ISOLATION and IDENTIFICATION of DRUGS in pharmaceuticals body fluids and post-mortem material

A practical manual and data book for forensic scientists, toxicoloists, analytical chemists, pharmacists, biochemists, pathologists and police surgeons

Edited by

E. G. C. CLARKE M.A., Ph.D., D.Sc., F.R.I.C.

Professor of Chemical Toxicology in the University of London at the Royal Veterinary College

Part 1: Isolation and Identification Techniques: Screening tests for common drugs; extraction methods in toxicology; paper chromatography; thin-layer chromatography; gas chromatography; ultraviolet absorption spectrophotometry; infra-red absorption; spectrophotometry colour tests; microcrystal tests; metabolism of drugs.

Part II: Individual monographs on over 1000 drugs: (a) Nomenclature, formulae, physical data, related salts; (b) analytical data and specific tests; (c) data on metabolism and toxicology; (d) references and abstracts.

Part III: Analytical data tabulated: Data from the monographs in Part II tabulated in sequential order as an aid to identification.

Infra-red spectra: A unique collection of nearly 450 infra-red spectra for drugs and similar substances with major peaks clearly labelled. Many of the spectra have been specially prepared for this book.

EXTRACTS FROM REVIEWS

A real working manual containing a prodigious amount of collected data . . . a book planned to do a job and will prove of inestimable value to the user.—*The Analyst.*

A monumental work of great value.—*British Medical Bulletin.*

Such an inclusive volume should be within easy reach of every practising analytical drug chemist.—*Journal of the Association of Official Analytical Chemists (U.S.A.)*

One of the most useful books on analytical methods and data that has been produced in the last decade.—*Australasian Journal of Pharmacy.*

Will become an essential reference book for all those who are concerned with the extraction and identification of drugs.—*Manufacturing Chemist.*

896 pages (22½ × 15 cm). **Price £14 post free. Detailed prospectus on request**

THE PHARMACEUTICAL PRESS 17 Bloomsbury Square, London, WC1A 2NN

Journal of Pharmacy and Pharmacology

Volume 23 Number 6 June 1971

Original Papers

Letters to the Editor