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The recovery of noradrenaline in adrenergic nerve terminals of the rat after reserpine treatment*

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Tissue concentrations of endogenous noradrenaline in heart, submaxillary gland, and gastrocnemic muscle have been examined after one large dose of reserpine (10 mg/kg) to rats. After the initial depletion of the amine, the concentration started to rise between 24 and 36 h. For about one week thereafter the amine recovery proceeded comparatively fast, then the rate of the recovery slowed. Between the 4th and the 6th weeks there was a pronounced drop in the noradrenaline concentration in all three tissues, apparently beginning in the 4th week with a maximal decrease of about 20% in the 5th week after reserpine. Thereafter the concentrations increased to approach normal about 6 weeks after reserpine. These results are discussed in relation to the axonal down-transport of newly formed amine storage granules and to the life-span of these granules in the nerve terminals. The different parts of the noradrenaline recovery curve appeared to reflect the axonal down-flow of granules. A theoretical recovery curve was calculated, based on granular transport. This curve was similar to the observed recovery curve. The claim is made that the recovery of adrenergic function and noradrenaline levels after reserpine is due to a down-transport of newly formed, amine storage granules to the nerve terminals. There seems little need for the theory that the storage function reappears in old, reserpine-blocked granules, as a mechanism for noradrenaline recovery after a large dose of reserpine.

Reserpine decreases the tissue levels of 5-hydroxytryptamine (Shore, Silver & Brodie, 1955; Pletscher, Shore & Brodie, 1955) and of catecholamines (Bertler, Carlsson & Rosengren, 1956; Carlsson & Hillarp, 1956). The block in adrenergic transmission after reserpine is most probably a result of the depletion of noradrenaline in the adrenergic neurons (cf. Carlsson, 1965). This depletion is considered to be the result of a long lasting block of the amine storage mechanism in the amine storage granules (see for example: Iversen, 1967; Andén, Carlsson & Häggendal, 1969) while the amine uptake mechanism at the level of the nerve membrane (the "membrane pump") appears to be unaffected (Malmfors, 1965; Hamberger, 1967).

After the initial depletion of the amines by reserpine, the tissue concentrations recover slowly (Carlsson, Rosengren & others, 1957; cf. Carlsson, 1965) and appear to reach normal some weeks after one large dose of the drug (Häggendal & Lindqvist, 1963, 1964; Dahlström & Häggendal, 1966a). Noradrenaline recovery in adrenergic neurons has been observed in the perikarya, long before the amine could be found in the nerve terminals (Dahlström & Fuxe, 1965; Dahlström, Fuxe & Hillarp, 1965;

* The results of this study were presented in part at the Second International Meeting of the International Society of Neurochemistry, Milan, October 1969.

Norberg, 1965; Dahlström, 1967). The amine storage granules are probably formed in the perikarya, and transported rapidly down the axons to the nerve terminals, where they have a life-span of several weeks (Dahlström & Häggendal, 1966b, 1967, 1970). Therefore, it was suggested that the recovery of tissue noradrenaline concentrations after one large dose of reserpine, was due mainly to the down-transport of newly formed amine granules to the nerve terminals (Dahlström & Häggendal, 1966a), rather than to a return of the storage function in the old granules in the nerve terminals (e.g. Alpers & Shore, 1969).

Since reserpine, on account of its amine-depleting effect, is often used as a tool, it appears to be of importance to study in detail the recovery curve for endogenous noradrenaline after reserpine depletion. The question of whether the recovery of transmission, of the capacity of the tissues to take up and retain exogenous noradrenaline, and of the endogenous noradrenaline concentrations, depend upon restitution of the function of the old amine granules in the nerve terminals, or on the transport of newly formed granules to the nerve terminals after reserpine, appears difficult to answer until a detailed picture of the reserpine recovery has been obtained. This paper reports an attempt to do this for endogenous noradrenaline.

MATERIALS AND METHODS

Male albino rats of the Sprague-Dawley strain (200–250 g) were used. The animals were given one single dose of reserpine (Serpasil, 2·5 mg ampoules) (10 mg/kg, i.p.) 12, 18, 24, 36 h, 2, 3, 4, 5, 6, 7, 9, 11, 13 days, 2, 3, 4, 5, and 6 weeks before decapitation. After the reserpine injection the rats were kept at 23–25°, except for the first 6–8 h, during the phase of leakage of amines, when the temperature was 15–17°, which kept mortality to about 1%. The rats were kept 5 in each cage and received food and water freely. Control rats were similarly treated in every way except that they were not given reserpine. At death, heart, submaxillary glands and gastrocnemius muscles were excised, immediately weighed, frozen in dry ice, and kept at − 70° until assay.

The heart, salivary glands and the muscles were homogenized in 10 ml 0·4N perchloric acid (PCA, ice-cooled) with 20 mg of ethylenediamine tetra-acetate (EDTA) and 10 mg of ascorbic acid. After homogenization, using an Ultra-Turrax homogenizer (Janke & Kunkel), and centrifugation, the extracts were purified on columns of strong cation exchange resins (Dowex 50 W-X4). The noradrenaline was estimated by the modified trihydroxyindole fluorometric method (Häggendal, 1963) in an Aminco-Bowman spectrophotofluorometer. In every series of estimations two samples of normal tissues, with known amounts of added noradrenaline (0·1 μg), were included to check recovery.

RESULTS

General observations

After reserpine, body and individual tissue weights were reduced for about one week compared to the controls probably because of diarrhoea, salivation and reduced food and water intake. During the second day after reserpine the rats recovered from the sedation and food and water were taken. Reserpine causes changes in e.g. heart muscle (cf. Zaimis, 1961) which may contribute to the weight reductions observed, although no difference in distribution or amount of nerve terminals has

been observed in reserpine-treated rats compared to normal rats (see e.g. Malmfors, 1965; Hamberger, 1967). Therefore, the figures for noradrenaline concentrations in the different tissues after reserpine were corrected for the weight losses, to avoid falsely high figures, especially during the initial period after the reserpine.

Tissue noradrenaline concentrations

The recovery of noradrenaline in the tissues followed a multiphasic course. As seen from Fig. 1d the concentrations were very low 12–24 h after reserpine in the three tissues (Section I in Fig. 2). Between 24 and 36 h there was a clear increase in all three tissues. After 36 h the concentrations increased rapidly with a smooth gradient, up to approximately day 7 after reserpine (Fig. 1a–c; Section II in Fig. 2).

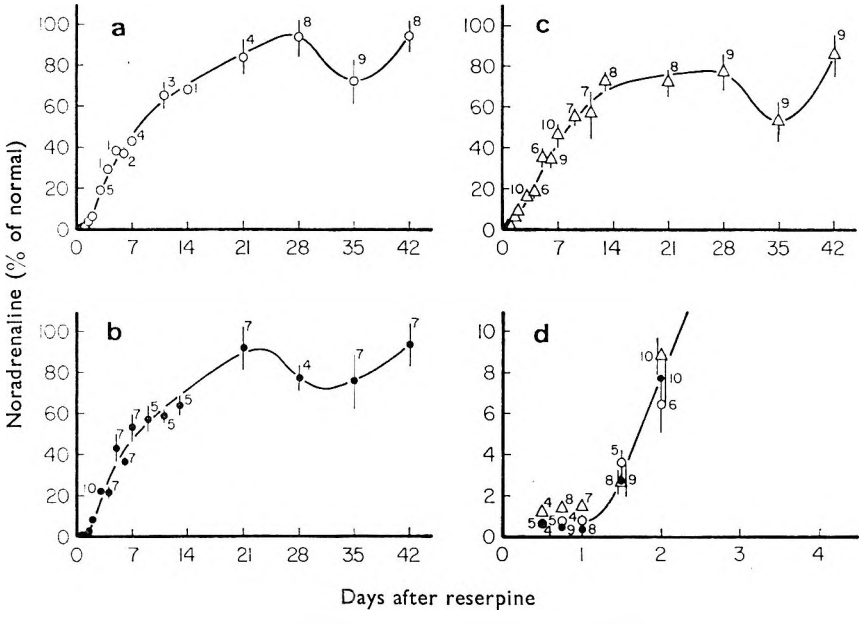

FIG. 1. The course of recovery of endogenous noradrenaline after reserpine treatment (10 mg/kg, i.p.) in different tissues of the rat: (a) gastrocnemius muscle, (b) submaxillary gland, (c) heart. The noradrenaline values are expressed in per cent of the values in control rats, killed and assayed parallelly. Corrections for weight reductions in reserpine-treated rats have been performed. The vertical bars indicate the s.e., and the small figures indicate number of experiments. (d) The noradrenaline concentrations in heart (△), salivary gland (●), and gastrocnemius muscle (○) during the early period after the reserpine injection. In all three tissues the concentrations start to increase between 24 and 36 h after reserpine (details from a–c).

Between day 7 and the third to fourth week the concentrations increased more slowly (Section III in Fig. 2). During the fourth to sixth week there was a drop (Section IV in Fig. 2) in all three tissues (Fig. 1a–c). The maximal decrease was about 20% 5 weeks after reserpine. This decrease was statistically significant when the values from all three tissues were taken together ($P < 0{\cdot}025$). Thereafter the levels slowly increased and appeared to reach normal levels about 6 weeks after the reserpine.

The different sections (I–IV) of the recovery curve will be discussed below and related to possible mechanisms in the adrenergic neuron.

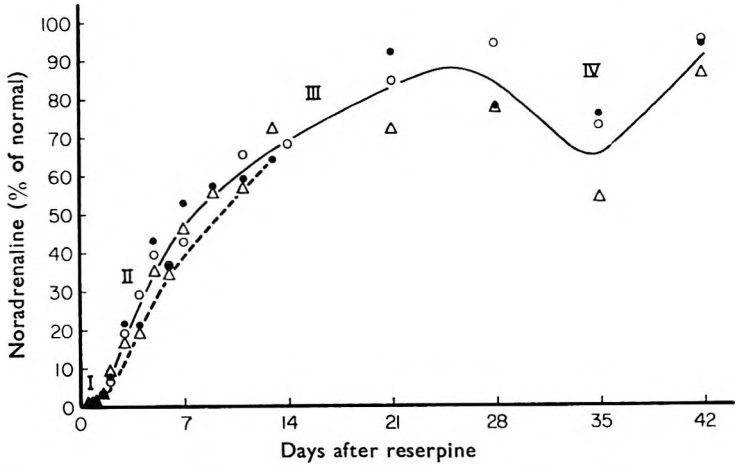

FIG. 2. The noradrenaline recovery in peripheral tissues of the rat after one large dose of reserpine (10 mg/kg, i.p.). The values from Fig. 1a–c have been indicated, with the same symbols and the curve for recovery in the three tissues, taken together, has been drawn. I–IV indicate different parts of the recovery curve, used for convenience in the discussion of the curve. The concentrations at 5 weeks are significantly lower than the values at 4 weeks and 6 weeks, when values from all 3 tissues are taken together ($P < 0{\cdot}025$). The theoretical curve for recovery, calculated on basis of axonal transport of granules, is indicated (- - - -). The values used for the calculations are indicated in Fig. 3. A correction of the values by 25% was made (see discussion).

DISCUSSION

Section I

During this time the endogenous noradrenaline concentrations are very low, resulting from the blockage of the noradrenaline storage mechanism in the amine granules (cf. Carlsson, 1965). Within this time the adrenergic transmission is also blocked, and exogenous noradrenaline (^{3}H-NA) is not retained in the tissues (Muscholl, 1960; Andén, Magnusson & Waldeck, 1964; Iversen, Glowinski & Axelrod, 1965; Häggendal & Dahlström, 1970). All these functions appear to be dependent on intact amine storage granules (e.g. Häggendal & Malmfors, 1969; Jonsson, Hamberger & others, 1969; for review see Andén & others, 1969).

One function so far observed to be undestroyed during this period is the synthesis of noradrenaline in the granules (Kirshner, 1962; Glowinski, Iversen & Axelrod, 1965). Furthermore, a so-called reserpine resistant uptake mechanism has been discussed (see Andén & others, 1969) but for the recovery of endogenous noradrenaline and of nerve function after reserpine *in vivo* this mechanism appears to be of little or no importance.

Section II

Onset of noradrenaline recovery. Fig. 1d shows the onset of increase in tissue noradrenaline concentrations which occurred between 24 and 36 h after reserpine in all three tissues. Previously Andén & others (1964) had not found any increase in rat peripheral organs until 48–72 h after reserpine. This difference may be due to a more sensitive assay and more material were used by us. In the early part of this period the recovery not only of endogenous noradrenaline concentrations, but also of transmission and the capacity to retain small amounts of ^{3}H-NA begins. Thus, between 30 and 48 h after reserpine, nerve function had partially recovered and so

also had the rat heart and femoral muscle capacity to take up and store ^{3}H-NA (Andén & others, 1964). Iversen & others (1965) also observed a partial recovery of ^{3}H-NA uptake capacity in rat heart at 36–48 h. Both ^{3}H-NA retention capacity and endogenous noradrenaline concentrations begin to recover 24–36 h after reserpine in the rat (Häggendal & Dahlström, 1970). As these three functions depend on functioning amine storage granules, these are probably present 24 and 36 h after reserpine in the adrenergic nerve terminals.

Two main possibilities may be discussed for the onset of recovery of these three functions: the first is that the old reserpine-blocked granules may recover their functions, the second is that new functioning granules, formed in the cell bodies *after* the reserpine administration, have been transported to the nerve terminals during this time. The first possibility has been suggested by many authors, lately by Alpers & Shore (1969) based partly on their own results (on the rate of disappearance of [^{3}H]reserpine from peripheral tissues after a small i.v. dose) and partly on the results from Carlsson, Rosengren & others (1957) which indicated that noradrenaline recovery in rabbit heart appears to be completed within 14 days after reserpine. Later, Häggendal & Lindqvist (1964) showed rabbit heart noradrenaline to approach normal levels 5 weeks after reserpine with 2 week values at about 70% of normal (see also Carlsson, 1965). The present findings show that full recovery of noradrenaline concentrations in rat peripheral tissues after reserpine does not occur until after about 6 weeks.

In support of the second possibility, new, functioning, noradrenaline-containing granules are most probably being transported down adrenergic axons in the sciatic nerve, as early as 15—18 h after reserpine (Dahlström, 1967, Dahlström & Häggendal, 1969), and would reach the nerve terminals in the hind limb skeletal muscle around 24 h after reserpine. Thus, there is a good correlation in time between the calculated arrival of new, functioning granules in the nerve terminals and the onset of recovery of transmission, [^{3}H]noradrenaline retention capacity and endogenous noradrenaline levels.

Further support for this second possibility has recently been obtained. Interruption of the axoplasmic flow of material including amine storage granules for 12 h by axotomy caused a delay in the recovery of the tissue capacity to retain small amounts of ^{3}H-NA (Häggendal & Dahlström, 1970) and also the endogenous noradrenaline concentrations after reserpine (Dahlström & Häggendal, unpublished). Since no sign of nerve terminal degeneration was observed and the influence of impulse activity was minimized (by preganglionic denervation), interruption in axoplasmic flow is probably the main reason. A similar delay after axotomy in the onset of increase in the concentration of endogenous noradrenaline in the spinal cord after reserpine has been demonstrated (Dahlström & Häggendal, unpublished). Furthermore, transsection of the spinal cord delayed the recovery of ^{3}H-NA formation and storage after ^{3}H-L-dopa administration in reserpine pretreated rats (Andén & Lundborg, 1970).

Progress of noradrenaline recovery. The increase in endogenous noradrenaline in the nerve terminals during section II of the recovery curve (Fig. 2) was relatively fast, and reached about 50% of control values after 7 days. The accumulation of noradrenaline above a ligation of the sciatic nerve at different intervals after reserpine treatment has been reported (Dahlström & Häggendal, 1969) and Fig. 3 is based on Fig. 1 in that paper. The figures used were obtained by subtraction of the amount

of noradrenaline found in 1 cm of unligated nerve from that found in 1 cm above a ligation, made 6 h before death. Fig. 3 thus shows the amount of noradrenaline transported to the 1 cm nerve above the ligation during 6 h. This would reflect the number of noradrenaline-containing amine granules that, after formation in the cell bodies, are transported along the nerve during the 6 h after ligation. Fig. 3 shows that during the third to sixth day after reserpine the amount of noradrenaline (in granules) transported to the 1 cm of nerve just above the ligation is supranormal, reaching a maximum of about 160% of the control values. After the seventh day, the amounts decrease again, being about 90% of normal during the 9th to 13th (latest period studied) days.

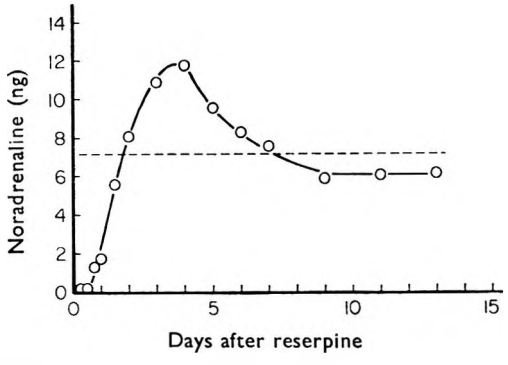

FIG. 3. The noradrenaline amounts transported to the 1 cm part of the rat sciatic nerve just above a 6 h ligation, made at different time intervals after reserpine (10 mg/kg, i.p.). This noradrenaline is probably located within amine granules, transported to this nerve part during 6 h. The curve is based on Fig. 1 in Dahlström & Häggendal (1969) and obtained by subtraction of the amounts of the amine in 1 cm of unligated nerves from those found proximal to a 6 h ligation, at different times after reserpine. The noradrenaline values are given in % of the amounts transported to the 1 cm part of sciatic nerve above a 6 h ligation in normal animals.

Thus, the rise in noradrenaline concentration in the nerve terminals during the first week after reserpine (Section II) not only appears to begin somewhat later than the period after reserpine (15–18 h), when noradrenaline-storing amine granules have reappeared in cell bodies and axons, but also, the recovery appears to be fastest in section II of the curve, when the down-transport of noradrenaline-containing granules has reached normal and supra-normal levels. The close relation between these curves indicates that the noradrenaline recovery in the nerve terminals during this period may mainly depend on axonal transport of new, functioning amine granules to the nerve terminals (see below).

Within Section II, the capacity for ^{3}H-NA retention, and the response to nerve stimulation in the nerve terminals also increases. During the second to fourth day, ^{3}H-NA retention after small intravenous doses approaches normal (Andén & others, 1964; Iversen & others, 1965; Häggendal & Dahlström, 1970). Normal response to nerve stimulation, obviously requiring only a small proportion of normal transmitter stores (Häggendal & Lindqvist, 1963, 1964; Andén & others, 1969), was also observed in this time period (Andén & others, 1964). As with endogenous noradrenaline, the capacity for ^{3}H-NA retention, and the response to nerve stimulation increase markedly in this period, when the down-transport of new amine granules is supranormal, it is possible that all these three parameters are linked to the new granules arriving at the

nerve terminals. Since endogenous noradrenaline concentrations at this time are still low (40–50% of normal), while the capacity for ^{3}H-NA retention and transmission appears to be normal, granules of different ages may have different properties (cf. Dahlström & Häggendal, 1970; Häggendal & Dahlström, 1971).

If the increase in noradrenaline concentrations in section II of the recovery curve is due to the arrival in the nerve terminals of newly formed amine granules, a theoretical calculation of the amines recovery, based on axonal transport of granules, should give a curve similar to that of section II. A calculation was undertaken: (*a*) based on the values from Fig. 3 the amount of adrenaline in granules transported down the axons to the nerve terminals per day was calculated; (*b*) as the amount of the amine normally present in the nerve terminals of the adrenergic axons in the sciatic nerve is known (900 ng, Dahlström & Häggendal, 1966b, probably mainly stored in amine granules cf. Iversen, 1967; Andén & others, 1969), the values obtained can be expressed as a percent increase in noradrenaline per day. The amine granules in the sciatic nerve contain less noradrenaline than they do when approaching the nerve terminals, both in normal and reserpine-pretreated rats (Dahlström, Häggendal, Larsson & Magnusson, unpublished) [this is supported by De Potter, Chubb & De Shaepdryver (1970) who found that nerve trunk granules contained less noradrenaline than nerve terminal granules, compared to their protein content]; it was calculated that the granules in the sciatic nerve contained at least 25% less of the amine than granules in the nerve terminals. For this reason, the figures from Fig. 3 were increased by 25%, to correct for the noradrenaline content in the granules when they had reached the nerve terminals. The theoretical curve for recovery, obtained by these calculations (Fig. 2), together with the curve for the assayed amine recovery are rather similar and suggest that there is little requirement for a restitution of old granules to explain the recovery of noradrenaline after reserpine.

Further support for the "new granule" theory is given by Mueller & Shideman (1968) who found that protein synthesis inhibitors markedly delayed the recovery of endogenous noradrenaline concentrations after reserpine. It appears likely that protein synthesis inhibition affects the formation of new granules in the perikarya more than it blocks the restitution of the storage mechanism in the old granules.

Section III

During this period, from about the second to third week inclusive, the recovery of noradrenaline begins to slow. Fig. 3 shows that the period of overshooting in the amount of produced and down-transported granules, is followed by a period of subnormal production and transport of granules during the second week (7–13 days after reserpine). The decrease in the rate of noradrenaline recovery thus occurred when signs of a decreased down-transport of granules were observed. We therefore suggest that the two phenomena are intimately related.

Section IV

A clear-cut drop in the noradrenaline concentrations was observed in all tissues between the fourth and the sixth weeks. Already during the fourth week the levels start declining. The drop is statistically significant when the values from the three times are taken together, and has regularly been observed in experiments over three years at all seasons. A similar, but earlier drop in noradrenaline concentrations has also been found in the brain (unpublished observation) during the recovery after

reserpine. At present, it is difficult to explain this drop on basis of the theory that noradrenaline recovery is due to an "awakening" of old, blocked granules. With the "new granule" theory, however, this drop may be explained thus: the amine storage granules in the nerve terminals appear to have a life-span of 3–4 weeks, in their capacity to store endogenous noradrenaline (Dahlström & Häggendal, unpublished) taking into consideration the degree of noradrenaline loading of the axonal granules compared to the nerve terminal granules. A supranormal quantity of noradrenaline granules appears to reach the nerve terminals during the first week after reserpine, and these granules have a life-span for storage of 3–4 weeks. Since the amount of granules transported to the nerve terminals per time unit at this time (about 4 weeks) is probably normal or subnormal, the loss of an unusually large number of noradrenaline-containing granules cannot be compensated for immediately. The result will be a drop in the concentration of noradrenaline, followed by gradual return to normal at about 6 weeks after reserpine.

The present study demonstrates that the recovery in nerve terminals of endogenous noradrenaline concentrations after reserpine (10 mg/kg, i.p.) follows a multiphasic course. The levels start to increase after 24 h while new, functioning amine granules are thought to be transported down the rat adrenergic axons somewhat earlier (18 h) (Dahlström, 1967; Dahlström & Häggendal, 1969). Therefore, when experimentally functioning amine granules are not wanted in the nerve terminals (and the tool for this is often reserpine) the reserpine-pretreatment should not be earlier than about 15 h before the start of the experiment, to avoid the possibility that some newly formed functioning amine granules may have reached the nerve terminals by axonal transport.

The effects on concentrations of endogenous noradrenaline in the adrenergic neuron persist up to at least 6 weeks after the reserpine injections. The variations in concentrations after reserpine appear to be well correlated to the down-transport of amine storage granules and the properties of these granules. It is evident that the local synthesis of noradrenaline in the nerve terminals is essential, not only for the economy of the transmitter stores in the normal condition, but also for the amine's recovery after reserpine. However, noradrenaline synthesis, without functioning storage sites in the down-transported amine granules, is unlikely to be in itself responsible for the amine's recovery after reserpine. As proposed earlier (Dahlström & Häggendal, 1966a) after one large dose of reserpine the recovery of the concentrations of endogenous noradrenaline, and also transmission, and the capacity to take up and store small amounts of ^{3}H-NA are now suggested to be mainly accounted for by the axonal transport of new amine granules, formed in the perikarya after the reserpine injection.

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REFERENCES

ALPERS, H. S. & SHORE, P. A. (1969). *Biochem. Pharmac.*, **18**, 1363–1372.

ANDÉN, N.-E., CARLSSON, A. & HÄGGENDAL, J. (1969). *Ann. Rev. Pharmac.*, **9**, 119–134.

ANDÉN, N.-E., MAGNUSSON, T. & WALDECK, B. (1964). *Life Sci.*, **3**, 19–25.

ANDÉN, N.-E., LUNDBORG, P. (1970). *J. Pharm. Pharmac.*, **22**, 233–234.

BERTLER, Å., CARLSSON, A. & ROSENGREN, E. (1956). *Acta physiol. scand.*, **37**, 235–239.

CARLSSON, A. (1965). In: *Handbuch der Exp. Pharmacol.*, XIX, pp. 534–592. Editors: Eichler, O. & Farah, A. Berlin-Heidelberg-New York: Springer.

CARLSSON, A. & HILLARP, N.-Å. (1956). *K. fysiogr. Sällsk. Lund Förh.*, **26**, Nr. 8.

CARLSSON, A., ROSENGREN, E., BERTLER, Å. & NILSSON, J. (1957). In: *Psychotropic Drugs*, pp. 363–372. Editors: Garattini, S. & Ghetti, V. Amsterdam: Elsevier Publ. Co.

DAHLSTRÖM, A. (1967). *Acta physiol. scand.*, **60**, 167–179.

DAHLSTRÖM, A. & FUXE, K. (1965). *Ibid.*, **64**, *Suppl.* 247.

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

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

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

DAHLSTRÖM, A. & HÄGGENDAL, J. (1967). *Ibid.*, **69**, 153–157.

DAHLSTRÖM, A. & HÄGGENDAL, J. (1969). *J. Pharm. Pharmac.*, **21**, 633–638.

DAHLSTRÖM, A. & HÄGGENDAL, J. (1970). In: *Biochemistry of Single Neuronal Models*, Symp. held in Milan, Sept. 1969. pp. 65–93. Editors: Costa, E. & Giacobini, E. New York: Raven Press.

DE POTTER, W. P., CHUBB, W., & DE SHAEPDRYVER, A. F. (1970). *Acta physiol. scand., Suppl.* 357.

GLOWINSKI, J., IVERSEN, L. L. & AXELROD, J. (1965). *J. Pharm. exp. Ther.*, **151**, 385–399.

HÄGGENDAL, J. (1963). *Acta physiol. scand.*, **59**, 242–254.

HÄGGENDAL, J. & DAHLSTRÖM, A. (1970). *Europ. J. Pharmac.*, **10**, 411–415.

HÄGGENDAL, J. & DAHLSTRÖM, A. (1971). In: *Subcellular Organization and Function in Endocrine Tissues.* Internat. Symp. Bristol, April 1970. In the press.

HÄGGENDAL, J. & LINDQVIST, M. (1963). *Acta physiol. scand.*, **57**, 431–436.

HÄGGENDAL, J. & LINDQVIST, M. (1964). *Ibid.*, **60**, 351–357.

HÄGGENDAL, J. & MALMFORS, T. (1969). *Ibid.*, **75**, 33–38.

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

IVERSEN, L. L. (1967). *The Uptake and Storage of Noradrenaline in Sympathetic Adrenergic Nerves.* London: Cambridge University Press.

IVERSEN, L. L., GLOWINSKI, J. & AXELROD, J. (1965). *J. Pharm. exp. Ther.*, **150**, 173–83.

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

KIRSHNER, N. (1962). *J. biol. Chem.*, **237**, 2311–2317.

MALMFORS, T. (1965). *Acta physiol. scand.*, **64**, *Suppl.* 248.

MUSCHOLL, E. (1960). *Arch. exp. Path. Pharmak.*, **240**, 234–421.

MUELLER, R. A. & SHIDEMAN, F. E. (1968). *Biochem. Pharmac.*, **17**, 451–467.

NORBERG, K.-A. (1965). *Acta physiol. scand.*, **65**, 221–234.

PLETSCHER, A., SHORE, P. A. & BRODIE, B. B. (1955). *Science, N.Y.*, **122**, 374–375.

SHORE, P. A., SILVER, S. L. & BRODIE, B. B. (1955). *Ibid.*, **122**, 284–285.

ZAIMIS, E. (1961). *Nature, Lond.*, **192**, 521–523.

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Differences in the stereospecificity of closely related compounds; a reinvestigation of the enantiomers of procyclidine, benzhexol and their metho- and etho-salts

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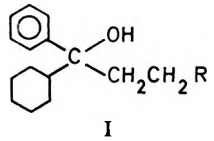
The findings of Duffin & Green (1955) that there are very large differences in the stereospecificity of some closely related phenylcyclohexylhydroxypropyl compounds have been confirmed, and it has been shown that this cannot be ascribed to any errors attached to the methods for assessing biological activity, or to inadequate resolution of some of the compounds. Measurement of the affinity constants of the compounds for the postganglionic acetylcholine receptors of the guinea-pig ileum showed that the (+)-and (−)-isomers of benzhexol differ only 5·5-fold in affinity whereas the (+)- and (−)-isomers of procyclidine differ at least 375-fold. This big variation in stereospecificity indicates that changes in one part of the molecule markedly affect the binding of the rest of the molecule and the effects are different in the different enantiomers. It is not possible to interpret the difference between the affinity of the isomers simply in terms of the fit, or failure to fit, of one group, such as the hydroxyl, attached to the asymmetric centre. In the five pairs of compounds tested, the stereospecificity was greatest in the compounds with lowest affinity, which is the reverse of what would be predicted from Pfeiffer's rule.

The difference between the biological activities of optical isomers has long interested pharmacologists and is one of the most important reasons for believing in the existence of "receptors". Because enantiomers differ only in the arrangement of groups about an asymmetric centre, it is assumed that the difference between their biological activities (the stereospecificity of the pair) yields information about the asymmetry of the receptor. For compounds which are agonists, such as the enantiomeric forms of noradrenaline, adrenaline and isoprenaline, the differences in activity depend upon differences in ability to activate receptors, as well as upon differences in affinity for the receptors; the stereospecificity of such agonists cannot, therefore, be used satisfactorily to analyse receptor structure. With antagonists, however, the stereospecificity is due only to differences in the affinity of the two forms.

Accordingly, the high stereospecificity of the isomers of phenylcyclohexylglycollylcholine as antagonists of furfuryl trimethylammonium on rat intestine (Ellenbroek, Nivard & others, 1965) can be taken to indicate the existence in the "muscarine-sensitive" receptor of groups capable of interacting with cyclohexyl, phenyl and hydroxyl groups as well as with the charged onium group in the antagonists. The discovery by Inch, Ley & Rich (1968) that the more active (−)-isomer has the *R* configuration then makes it possible to indicate how these groups may be arranged in the receptor (see, for instance, van Rossum, 1968).

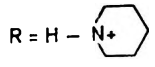
Measurement of the stereospecificity of isomeric antagonists is undoubtedly a valuable method of investigating receptor structure, particularly as there is as yet no means of studying this directly with isolated receptors, but it seems likely that there are limits to the usefulness of results obtained with only one pair of isomers. From measurements of the affinity of many series of antagonists at the postganglionic acetylcholine receptors of the guinea-pig ileum, Abramson, Barlow & others (1969) obtained evidence that the binding of one part of an antagonist at the receptor may be considerably affected by changes in the structure of other parts of the molecule. Changes in chemical structure at points distant from the asymmetric centre may therefore have profound effects on stereospecificity.

The results by Duffin & Green (1955), who measured the atropine-like activity of enantiomeric pairs of phenylcyclohexylhydroxypropyl compounds, I, showed this to

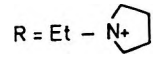


I

be so. The stereospecificity varied, depending on the nature of the group R at the other end of the molecule from the asymmetric centre. The size of the variation was particularly striking. In tests on the guinea-pig ileum the stereospecific index for the piperidino-compounds, with



was 9·8 whereas for the ethylpyrrolidinium compounds, with



it was 290. Because of the important implication of these results it seemed desirable to check them. Drs Duffin and Green very kindly provided samples of the compounds and tests have been made of their biological activity on the guinea-pig isolated ileum and on the optical purity of some of them.

METHODS

Compounds. The substances tested had the basic structure I with R = the groups shown in Table 1.

Biological activity. The affinity constants of the compounds for the "muscarine-sensitive" postganglionic acetylcholine receptors of the guinea-pig ileum at 37° were measured by the method of Abramson & others (1969). Carbachol was the agonist. In most experiments the dose-ratio (Gaddum, Hameed & others, 1955) obtained when the antagonist had come into equilibrium with the tissues was between 20 and 100 but if there was enough material, tests were also made with higher concentrations, which produced dose-ratios between 100 and 1000. The results obtained with high concentrations gave values of the affinity constant similar to those obtained with lower concentrations, which is consistent with competitive antagonism. For example, in an experiment with the (−)-isomer of procyclidine, 364C52, the value of K was $1{\cdot}58 \times 10^8$ for a dose-ratio of 32·6 and $1{\cdot}88 \times 10^8$ for a dose-ratio of 380.

Duffin & Green (1955) measured the atropine-like activity of the compounds on the guinea-pig isolated ileum at 38° by comparing the concentrations of the compounds and of atropine which reduced the size of the response to a standard dose of acetylcholine by one-half (White, Green & Hudson, 1951; Green, 1953).

Optical purity. The rotations of the isomers of procyclidine (363C52 and 364C52), of benzhexol (247C53 and 248C53), and of the (−)-forms of their metho-salts (427C52 and 250C53), were measured with a Bellingham and Stanley Model B Spectropolarimeter. The cell had a 5 cm light-path and readings were taken at 17–20° with solutions in water and in chloroform which were usually 2×10^{-2}M, In some instances it was necessary to use more dilute solutions because of the shortage of material. The wavelengths selected were 546, 320, 300, 290 and 280 nm, but with solutions in chloroform the absorption by the solvent made it impossible to obtain readings below 290 nm and the absorption by the iodide ion similarly limited the readings that could be obtained with the methiodides dissolved in water.

All measurements were made relative to air. Mean values for the rotation at each wavelength were calculated, together with estimates of the standard error, based on the variance of the values for the solutions and the values for the solvent, but it is probable that these are underestimates. The results obtained with 247C53 and 248C53, for instance, indicate that the rotation of the (+)-isomer is significantly less than that of the (−)-isomer ($P < 0{\cdot}05$) at 546 and 320 nm and significantly greater at 290 nm, even though the same solutions were used.

There was a steady increase in the size of rotation with increasing wavelength. Dr. J. C. P. Schwarz and Mr. F. Rutherford kindly measured the rotations of 427C52 and 364C52 with a continuously scanning instrument (Bellingham and Stanley Polarmatic 62) down to 240 nm and observed only small Cotton effects, insufficient to alter the sign of rotation, in the regions corresponding to the absorption maxima (262, 257 and 251 nm). The signs of rotation of the hydrochlorides of the tertiary bases in water, however, were the opposite of those for solutions in chloroform and the size of rotation was smaller. The compound 364C52, designated the (−)-enantiomer of procyclidine because of its rotation in chloroform, was dextrorotatory in water at all the wavelengths studied. The quaternary salts, on the other hand, had the same sign of rotation in water as they had in chloroform, though the size of rotation was again smaller in water.

RESULTS

Table 1 shows the logarithms of the affinity constants for the postganglionic acetylcholine receptors of the guinea-pig ileum at 37°. Mean values are given together with the standard error and the number of estimates. Each estimate was made on a fresh piece of ileum. The three compounds which were not tested in high concentrations to check for competition are indicated. The stereospecific index (difference between the activity of the enantiomers) can be compared with the values obtained by Duffin and Green, which are shown in parentheses. The activity of the compounds relative to atropine has been calculated assuming a value of log K for atropine of 9·0 and are molar; the values obtained by Duffin and Green were calculated according to weight.

Most of the results do not differ from the original estimates by more than a factor of two. The biggest discrepancy is with the pyrrolidino-compounds, 363C52 and 364C52, where the stereospecific index is much higher than that obtained previously.

Table 1. *Logarithms of the affinity constants for postganglionic acetylcholine receptors of the guinea-pig ileum at* 37° *together with the stereospecific index and activity relative to atropine.*

		$\alpha^{20°}_{546}$	log K ± s.e. (*n* results)	Stereospecific index	Activity relative to atropine
Enantiomers of procyclidine					
 HCl	(−) 364C52	− 30	8·266 ± 0·014 (10)	375 (49)	0·18‡ (0·10)
	(+) 363C52	+ 30	5·692 ± 0·043 (6)†		0·00049 (0·002)
	(±)		7·945 ± 0·013 (4)		
Enantiomers of tricyclamol					
MeI	(−) 427C52	− 25	8·702 ± 0·027 (7)	87 (160)	0·50 (1·6)
	(+) 428C52	+ 25	6·732 ± 0·026 (6)		0·0054 (0·01)
EtI	(−) 429C52	− 30	8·684 ± 0·018 (8)	226 (290)	0·48 (1·0)
	(+) 430C52	+ 30	6·330 ± 0·035 (7)†		0·0021 (0·0034)
Enantiomers of benzhexol					
 HCl	(−) 248C53	− 30	8·751 ± 0·015 (7)	5·5 (9·8)	0·56 (0·71)
	(+) 247C53	+ 31	8·008 ± 0·033 (9)		0·10 (0·075)
MeI	(−) 250C53	− 22	9·175 ± 0·050 (20)	86·3 (48)	1·50 (0·86)
	(+) 249C53	+ 23	7·239 ± 0·037 (8)†		0·017 (0·018)
EtI	(−) 375C53	− 18	9·729 ± 0·030 (8)		5·36

* The values of the rotations are those of Duffin & Green (1955). The enantiomer of 375C53 had a rotation of +21 but none was left for testing.
† Not tested in concentrations which produced dose-ratios >100.
‡ Molar and assuming log K for atropine = 9·0; values in parentheses from Duffin & Green are on a weight basis.

The high value, however, is confirmed by tests made with a racemic solution, obtained by mixing equal amounts of equimolar solutions of the two enantiomers. The value of log K for this was less than that for the more active (−)-isomer by 0·321, which is not significantly different from log 2.

The low stereospecificity of the piperidino-compounds, 247C53 and 248C53, and the high stereospecificity of the pyrrolidino-compounds, 363C52 and 364C52, has therefore been confirmed. It is highly unlikely that the logarithms of the stereospecific index are incorrect by as much as 0·1, i.e that the stereospecific index is incorrect by more than a factor of 1·3. It is next necessary to consider whether the low stereospecificity of the piperidino compound might be due to inadequate resolution of the isomers.

Suppose that x mol of the weaker isomer produce the same biological effect as 1 mol of the stronger, when both are absolutely pure. If the weaker isomer is only partly resolved, however, and contains a fraction y, it will also contain a fraction (1 − y) of the stronger isomer, and will be stronger than if it were completely resolved. If z mol of the partly resolved form of the weaker produce the same biological effect

as 1 mol of the pure more active isomer, the observed stereospecificity, z, will be less than the true stereospecificity, x, and

$$\frac{1}{z} = \frac{y}{x} + (1 - y) \text{ so } x = \frac{y}{\frac{1}{z} - (1 - y)} \text{ and } z = \frac{1}{\frac{y}{x} + (1 - y)}$$

For example, if y = 0·5, i.e. for a racemic mixture, z will lie between 1 (when x = 1) and 2 (when x is very large).

For the piperidino-compounds, 247C53 and 248C53, the observed value for z is 5·5, so if y were 0·95, the correct value for the stereospecificity would be 7·2, for y = 0·90 it would be 11 and y = 0·85 it would be 26·6. The limiting value of y, corresponding to one isomer being completely inactive (x = ∞) is 0·818.

The optical rotations are shown in Table 2. The specific rotations for the pyrrolidino-compounds, 363C52 and 364C52, are very close to the values obtained by Duffin

Table 2. *Molar rotations obtained with a 5 cm cell.* For 247C53 in chloroform the concentration was 6·35 × 10^{-3}M; for 248C53 in chloroform it was 10^{-2}M; for all other measurements it was 2 × 10^{-2}M (and the figures indicate the actual angles measured in millidegrees, corrected for the solvent blank). Each figure is the mean of four sets of measurements, except for those marked with an asterisk, which are the means of only three sets. The standard error shown is based on the combined variance of the values for solution and for solvent and may be an underestimate (see text).

		Wavelength, nm				
		546	320	300	290	280
363C52 in $CHCl_3$	..	+103	+421	+527	−607	+740
		±1·8	±5·1	±5·6	±6·3	±4·3
in water	..		−20	−34	−44	
			±1·5	±1·1	±0·9	
364C52 in $CHCl_3$	..	−102	−411	−517	−598	−723
		±1·6	±2·6	±2·3	±2·5	±3·3
in water	..		+15	+29	−37	
			±1·2	±2·4	±0·7	
247C53 in $CHCl_3$	..	+99	+480	+644	−768	+894
		±1·8	±5·2	±6·4	±10·7	±5·7
in water	..		−48*	−66*	−77*	
			±1·5	±2·1	±4·2	
248C53 in $CHCl_3$	..	−126	−505	−642	−738	−908
		±1·7	±3·3	±4·4	±4·8	±4·4
in water	..		+54*	+74*	−84*	
			±0·9	±0·9	±2·2	
427C52 in $CHCl_3$	..	−104	−490	−647		
		±1·4	±3·0	±2·4		
in water	..	−17	−63	−77	−104	
		±1·2	±0·8	±0·9	±1·7	
250C53 in $CHCl_3$	..	−99	−458	−585		
		±1·9	±1·5	±2·8		

The specific rotations in chloroform at 546 nm, with the values obtained by Duffin & Green (1955) shown in parentheses, are:

363C52, +31·8 (+30); 364C52, −31·5 (−30); 247C53, +29·3 (+31); 248C53, −37·3 (−30); 427C52, −24·4 (−25); 250C53, −22·4 (−22).

& Green (1955). The values for the piperidino-compounds are less satisfactory; that for 248C53 is higher than the recorded value and that for 247C53 slightly lower. If the rotations obtained with the biggest measured angles (in chloroform at short wavelengths) are compared, the resolution would appear to have been effective. The rotations are much higher than those for the pyrrolidino-compounds, and these must have been resolved satisfactorily (see below). If the values for the rotations at 546 nm are used to assess the purity, however, the observed rotation, 99°, instead of a rotation of 126°, indicates that the (+)-isomer is a fraction 225/252 = 0·89 pure. If the resolution were only complete to this extent, the true stereospecific index would be 11·9, but this is still a very low figure compared with the value for the pyrrolidino-compounds, 375.

For a true stereospecificity (x) of 100, the observed value, 5·5, would be obtained only when the material was 82·6% pure. The (−)-isomer could, of course, also be impure. This would affect the stereospecificity slightly and if it, too, were only 82·6% pure the difference between the pure isomers would be 100/0·826 = 122; i.e. with both (+)- and (−)-isomers only 82·6% pure, the observed stereospecific index of 5·5 would be obtained when the difference between the pure isomers was 122. In this situation, however, the size of the rotations would be equal for the two isomers; this illustrates the difficulty of using optical rotations to assess purity.

The very high stereospecificity of some of the compounds, however, does indicate their purity. For the pyrrolidino-compounds, 363C52 and 364C52, the value for z is 375 and the limiting value of y, corresponding to one isomer being completely inactive ($x = \infty$), is (1–0·0026), i.e. this material must be at least 99·74% pure. The biological effects of the compounds are produced by antagonizing the actions of acetylcholine and there is little likelihood that the isomers are interacting with each other either chemically or biologically.

If it is argued that the low stereospecificity of the piperidino-compounds 247C53 and 248C53 is due to inadequate resolution, it is possible to obtain an idea of the size of the rotations of the pure compounds. If 247C53, the material with the smallest rotation (M_{546} + 99), were only 82·6% pure, the value for the pure material would be +152 (α_{546} + 44·9). This is much in excess of the values for the pyrrolidino-compounds, 363C52 and 364C52, which must be pure because they have high stereospecificity. With the metho-compounds, 427C53 and 250C53, which also have high stereospecificity, the change from pyrrolidino to piperidino leads to a slight decrease in rotation. It seems, therefore, very unlikely that the rotations of the piperidino compounds 247C53 and 248C53 could be as high as they would need to be if their low stereospecificity were due simply to inadequate resolution.

The results show that the more active isomer is (−)-rotatory in chloroform. The more active isomers of the esters of phenylcyclohexylglycollic acid studied by Ellenbroek & others (1965) were also (−)-rotatory but were tested in solution in methanol; they were all quaternary salts. The absolute configuration of these follows from the synthesis of the phenylcyclohexylglycollic acids and their methyl esters from starting materials of known absolute configuration by Inch & others (1968), who measured the rotations of these compounds both in methanol and in chloroform. As the rotations of the phenylcyclohexylhydroxypropyl compounds tested here did not change sign at shorter wavelengths it seems likely that the more active (−)-isomers have the same absolute configuration (*R*) as the more active (−)-isomers of the esters, even though they contain methylene groups and not a carboxyl group attached

to the asymmetric centre. The configuration cannot be regarded as being certain, however, particularly in view of the effects of solvent on the rotations of this type of compound, which with water have been found even to lead to a change in the sign of rotation.

DISCUSSION

These results confirm the findings of Duffin & Green (1955) that there can be large differences in the stereospecificity of closely related compounds. Any attempt to make deductions about receptor structure must therefore be limited to situations in which many pairs of compounds have been tested. A false impression of the asymmetry of the acetylcholine receptor would be obtained from considering only the piperidino-compounds, for instance.

These particular results also illustrate how variable the effects of a group are on affinity and the need to consider how far the contribution of the group to binding may be offset by rearrangement of the binding of the rest of the molecule. The stereospecificity of the pyrrolidino-compounds, for instance, indicates a difference in the free energy of adsorption ($-\Delta G = RT\ln K$) of 3·65 kcal mol^{-1} (15·3 kJ mol^{-1}), which might be ascribed to hydrogen bonding of the hydroxyl group to the receptor in the (−)-isomer and not in the (+)-isomer, but smaller differences are obtained with the other pairs of compounds. In fact, it is highly unlikely that the difference between the isomers is only due to extra binding by the hydroxyl group in the (−)-compounds (see below). The change from pyrrolidine to piperidine in the (+)-isomers increases log K from 5·69 to 8·01, indicating an increase in the free energy of adsorption of 3·29 kcal mol^{-1} (13·8 kJ mol^{-1}) and it is difficult to see how such a large increase can come simply from binding of the extra methylene group in the piperidine ring. The unexpectedly large effect of the change can be compared with the unexpectedly large effects of introducing the cyclohexyl group into phenylacetyl and phenylglycolloyl esters (Abramson & others, 1969). It could be interpreted by supposing that the binding of the rest of the molecule in the (+)-series of compounds is disturbed much less by the introduction of a piperidino-group than by the introduction of a pyrrolidino-group. Unfortunately, there are not enough results to make it possible to estimate the effects of the change in structure from pyrrolidino- to piperidino-group on the binding of the rest of the molecule (as was possible for the introduction of phenyl and cyclohexyl groups in the compounds referred to above). It seems likely however, that they are large and also that the difference between affinity of optical isomers will be the result of differences in the contributions to binding of all the groups in the molecule, not just of one group (such as the hydroxyl group) in these compounds.

The irregular effects of quite simple changes in structure on affinity can also be seen by considering the replacement of hydrogen by methyl and methyl by ethyl in these compounds. In three instances the replacement of hydrogen by methyl increased affinity (between 2 and 12-fold) but it decreased the affinity of the (+)-isomer of the piperidino-compounds nearly 6-fold. Replacement of methyl by ethyl reduced the affinity of the pyrrolidino-compounds slightly [just over 2-fold in the (+)-isomers and not significantly in the (−)-isomers] but increased the affinity of the (−)-isomer of the piperidino-compounds to the extremely high value of 9·73. The size of these changes is again so large that it is unlikely that they can be ascribed simply to binding of the extra methylene group to the receptors, or its failure to bind. It is necessary

to consider also the effect of the extra methylene group on the binding of the rest of the molecule.

There is no evidence that stereospecificity is greater with the more active compounds as has been suggested by Pfeiffer (1956) or Ariëns, Simonis & van Rossum (1964). With the compounds used in the present work the reverse is true; those with lower affinity have higher stereospecificity. It seems probable that it is not possible to generalize at all because each type of drug and receptor will constitute a separate problem.

Acknowledgements

I wish to record sincere thanks to Drs Duffin and Green for their great kindness in supplying the compounds, Dr. Schwarz and Mr. Rutherford for the results with the Polarmatic 62, Miss Ann Graham for highly competent technical help and the Medical Research Council for the Bellingham and Stanley Model B spectropolarimeter.

REFERENCES

ABRAMSON, F. B., BARLOW, R. B., MUSTAFA, M. G. & STEPHENSON, R. P. (1969). *Br. J. Pharmac.*, **37**, 207–233.

ARIËNS, E. J., SIMONSI, A. M. & ROSSUM, J. M. VAN (1964). *Molec. Pharmac.*, Vol. 1. p. 233. Editor: Ariëns, E. J. New York: Academic Press.

DUFFIN, W. M. & GREEN, A. F. (1955). *Br. J. Pharmac. Chemother.*, **10**, 383–386.

ELLENBROOK, B. W. J., NIVARD, R. J. F., ROSSUM, J. M. VAN & ARIËNS, E. J. (1965). *J. Pharm. Pharmac.*, **17**, 393–404.

GADDUM, J. H., HAMEED, K. A., HATHWAY, D. E. & STEPHENS, F. F. (1955). *Q. Jl. exp. Physiol.*, **40**, 49–74.

GREEN, A. F. (1953). *Br. J. Pharmac. Chemother.*, **8**, 2–9.

INCH, T. D., LEY, R. V. & RICH, P. (1968). *J. chem. Soc. C.*, 1693–1699.

PFEIFFER, C. C. (1956). *Science, N.Y.*, **124**, 29–30.

ROSSUM, J. M. VAN (1968). *Recent Advances in Pharmacology*, 4th edn. Fig. 6, facing page 110. Editor: Robson, J. M. & Stacey, R. S. London: Churchill.

WHITE, A. C., GREEN, A. F. & HUDSON, A. (1951). *Br. J. Pharmac. Chemother.*, **6**, 560–571.

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The action of isoprenaline on the perfused vessels of the rabbit ear

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In rabbit isolated perfused ear preparations isoprenaline in doses of 1 to 5 μg caused a vasodilatation which was specifically blocked by propranolol. In a minority of experiments larger doses of isoprenaline (5 to 10 μg) caused vasoconstriction. The vasodilatations to isoprenaline were increased by raising vascular tone with barium chloride whereas ergotamine and sympathetic stimulation were relatively ineffective. In the isolated ear central artery preparation, isoprenaline caused a vasoconstriction due to α-receptor stimulation. A vasodilator response due to β-receptor stimulation was revealed when perfusion pressure was raised with barium chloride. It is concluded that α- and β-adrenergic receptors are present in both preparations but a higher proportion of β-receptors are present in the whole ear.

The isolated perfused artery preparation from the rabbit ear was first described by de la Lande & Rand (1965) and has since been extensively used for studying the effects of vaso-active drugs (for instance de la Lande & Harvey, 1965; Starr & West, 1966; Day & Owen, 1968).

Gay, Rand & Wilson (1967) investigated the action of isoprenaline on this preparation and found that under a variety of conditions it produced only vasoconstrictor responses which were abolished by α-adrenergic receptor blocking agents. These workers concluded that isoprenaline was acting on α-adrenergic receptors in this preparation and that β-receptors were either absent or stimulation of them did not elicit vasodilator responses.

In view of the widespread use of this preparation for pharmacological studies it was thought worthwhile to re-examine it for the presence of β-adrenergic receptors.

METHODS

Whole ear preparation. A polyethylene cannula was used to cannulate the central artery and the vessels were perfused with Tyrode solution gassed with air and maintained at 37° by means of a constant volume flow inducer. The rate of perfusion varied between 5 and 8 ml/min in different preparations and perfusion pressure was recorded using a mercury manometer.

Isolated artery preparation. This tissue was set up as described by de la Lande & Rand (1965) except that aerated Tyrode solution at 37° was the perfusion fluid and the vessel was suspended in air, not in an organ bath containing the perfusion fluid.

In both preparations the Tyrode solution was filtered through resin filter paper immediately before use. Drugs were injected in volumes not exceeding 0·1 ml into the cannula via a short polyethylene tube fitted with a one way tap system. Each drug injection was given at a constant rate of 0·1 ml/15 s and was flushed in with a further 0·2 ml Tyrode solution injected at the same rate. Infusions were made by addition of the drug to the Tyrode solution in the reservoir.

Sympathetic stimulation was achieved by threading the vessel through a bipolar ring electrode or with the whole ear preparation by using a bipolar hook electrode placed under the upper end of the artery. Stimulation was at supramaximal voltage (10 to 25 V) and at a frequency of 2 to 10 pulses/s.

Drugs

Drugs used were: acetylcholine chloride, isoprenaline sulphate (±) and (−) -noradrenaline hydrochloride, propranolol hydrochloride (Inderal, ICI), phentolamine mesylate (Rogitine, Ciba), ergotamine tartrate (Femergin, Sandoz).

All doses are expressed in terms of the salt.

To avoid loss of potency, solutions of noradrenaline and isoprenaline were made up in 0·01N hydrochloric acid and kept on ice throughout the experiment. This vehicle was without effect on perfusion pressure when injected in volumes similar to those used for drug injections.

RESULTS

Whole ear preparation

In 9 of 13 preparations injections of isoprenaline (1 to 5 μg) caused vasodilatations with a fall in perfusion pressure of 5 to 25 mm Hg. In four of the preparations which responded to low doses of isoprenaline with vasodilations, higher doses caused biphasic responses with a marked vasoconstrictor component with increasing dosage. This is illustrated in Fig. 1A where 1 μg of isoprenaline caused predominantly vasodilatation whereas 10 μg caused a marked vasoconstriction. The vasodilator responses to isoprenaline were specifically abolished by propranolol (25 to 100 ng/ml). This is

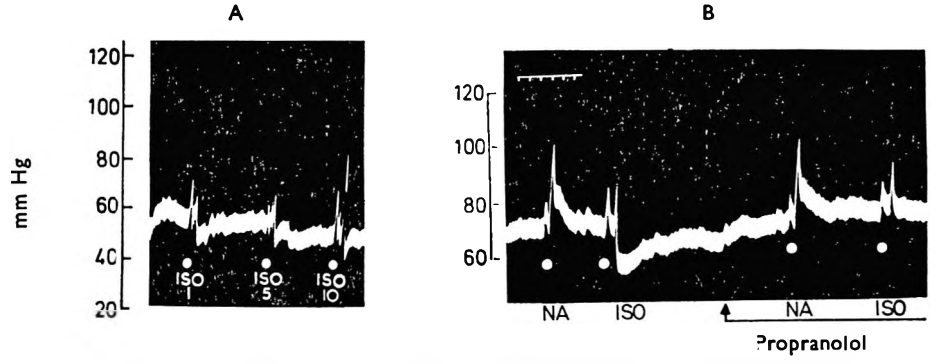

FIG. 1.A. Whole ear preparation: Response to isoprenaline (ISO) 1, 5 and 10 μg showing change from predominantly vasodilator response at 1 μg to predominantly vasoconstrictor response at 10 μg. B. Whole ear preparation: Responses to noradrenaline 50 ng (NA) and isoprenaline 1 μg (ISO) before and in the presence of propranolol 25 ng/ml. Time scale in min.

shown in Fig. 1B where the response to isoprenaline (1 μg) was converted from a dilatation to a constriction in the presence of propranolol whereas the constrictor response to noradrenaline was unaffected. The vasoconstrictions to both isoprenaline and noradrenaline were in all experiments abolished by phentolamine (1 μg/ml). In nine experiments of this series only vasodilatations could be obtained with any dose of isoprenaline in the absence of propranolol.

Effect of isoprenaline administered during raised perfusion pressure

Ergotamine. Ergotamine (2 to 10 ng/ml) raised the perfusion pressure from a resting level of 30 to 60 mm Hg to a level of 80 to 110 mm Hg. However, the increased perfusion pressure was not well maintained and in only two out of five preparations did isoprenaline cause a small fall in perfusion pressure of about 5 mm Hg. No constrictor action of isoprenaline (10 to 100 μg) was obtained in the presence of ergotamine.

Sympathetic stimulation. Arterial spasm was produced by sympathetic stimulation at frequencies of 2 to 10 pulses/s but in only three preparations out of 19 tested was the increased perfusion pressure sufficiently well maintained to enable isoprenaline to be injected during stimulation. In each of these three experiments isoprenaline caused an enhanced vasodilatation compared with the response before stimulation.

Barium chloride. Barium chloride (50 to 500 μg/ml) caused a sustained increase in perfusion pressure and in four out of five experiments the vasodilatation in response to isoprenaline was markedly increased. This is illustrated in Fig. 2 where the vasodilatations to both isoprenaline and acetylcholine are compared before and in the presence of barium chloride. The vasodilatation to isoprenaline was increased more markedly than was that to acetylcholine in the presence of barium ions.

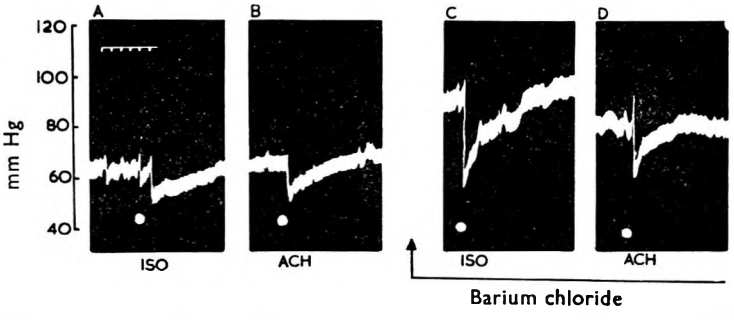

FIG. 2. Whole ear preparation: Responses to isoprenaline 1 μg (ISO) and acetylcholine 1 μg (ACH) before (panels A and B) and in the presence of barium chloride 100 μg/ml (panels C and D). Time scale in min.

The isolated artery preparation

In doses ranging from 1 to 10 μg isoprenaline caused either no change in perfusion pressure or else a small vasoconstriction. In larger doses (10 to 100 μg) it caused a marked vasoconstriction which was abolished by phentolamine (1 μg/ml) confirming the results of Gay & others (1967).

Barium chloride. In the presence of barium chloride (50 to 500 μg/ml) a sustained increase in perfusion pressure occurred and in four out of five preparations a marked vasodilatation occurred in response to injections of isoprenaline (5 to 10 μg). Fig. 3 illustrates an experiment in which vasodilatations in response to isoprenaline (5 and 10 μg doses) were revealed in the presence of barium chloride (100 μg/ml) and were abolished by propranolol (50 ng/ml).

Sympathetic stimulation. Vasodilatations in response to isoprenaline were not obtained in any preparation in which the perfusion pressure was raised by sympathetic stimulation (4 experiments).

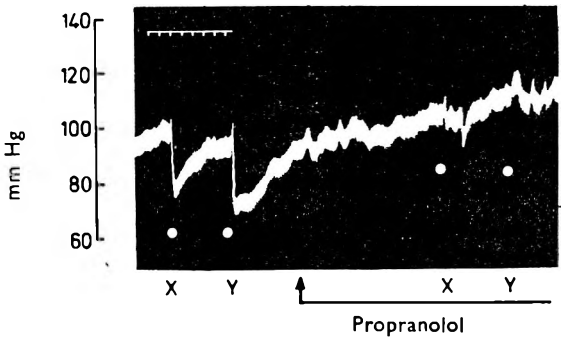

FIG. 3. Isolated central artery preparation: Perfusion pressure raised by the addition of barium chloride (100 μg/ml) present throughout the experiment. The vasodilatations in response to isoprenaline 5 μg (X) and 10 μg (Y) were abolished in the presence of propranolol (50 ng/ml). Time scale in min.

DISCUSSION

The results obtained in this study indicate that isoprenaline has both α and β-adrenergic receptor stimulant properties on the vasculature of the rabbit ear. It was easier to demonstrate a vasodilator response to isoprenaline in the whole ear preparation, even in the absence of raised vascular tone, than in the isolated artery preparation. In addition, it was more difficult to reveal an α-stimulant action of isoprenaline on the whole ear preparation than in the isolated artery. The most likely explanation of these observations is that the whole ear preparation contains a larger proportion of β-receptors, presumably present in the small arterial branches and in the veins, than are present in the arterial preparation. Raising the tone of the whole ear preparation with barium chloride markedly increased the dilatations in response to isoprenaline whereas sympathetic stimulation and ergotamine were relatively ineffective.

The present results confirm the observation of Gay & others (1967) that in the isolated artery preparation the main effect of isoprenaline is vasoconstriction due to α-adrenergic receptor stimulation. We also confirmed their observation that raising the tone by sympathetic nerve stimulation did not reveal a vasodilatation in response to isoprenaline. However, a β-receptor stimulant effect of isoprenaline was readily revealed when the perfusion pressure was raised by adding barium chloride to the perfusion solution. Thus it seems likely that both α- and β-adrenergic receptors are present in the central artery of the rabbit ear. The failure in the present experiments as well as in those of Gay & others (1967) to reveal a β-stimulant action of isoprenaline in the presence of ergotamine may indicate a β-receptor blocking action of this substance.

REFERENCES

DAY, M. D. & OWEN, D. A. A. (1968). *Br. J. Pharmac.*, **34**, 499–507.
DE LA LANDE, I. S. & HARVEY, J. (1965). *J. Pharm. Pharmac.*, **17**, 589–593.
DE LA LANDE, I. S. & RAND, M. J. (1965). *Aust. J. exp. Biol. med. Sci*, **43**, 639–656.
GAY, W. S., RAND, M. J. & WILSON, J. (1967). *J. Pharm. Pharmac.*, **19**, 468–473.
STARR, M. S. & WEST, G. B. (1966). *Ibid.*, **18**, 838–840.

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The selective uptake of mercury by myocardial infarcts in the rat

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Rats with myocardial infarcts, sham operated and control animals were treated with methoxyethyl mercury chloride (MEMC) intraperitoneally or with mercamphamide intravenously or intraperitoneally and the mercury content in the hearts determined spectrophotometrically with dithizone after various time intervals. After MEMC treatment there was no difference between the amounts of mercury found in the hearts of rats with myocardial infarcts and controls. After injection of mercamphamide, mercury was taken up by hearts of rats with myocardial infarcts while those of sham operated and control animals remained free of mercury. Optimal results were yielded by 1 h experiments made within the first 24 postoperative hours. The selective uptake of mercury from organomercurials is much dependent on the organic part of the molecule present in the given individual compound.

Experimentally induced myocardial infarcts have been precisely localized using [^{203}Hg]chlormerodrin by Gorten, Hardy & others (1966). Organically bound mercury accumulated in the infarcted areas but not in undamaged heart tissue. We wished to make a quantitative determination of the mercury accumulated in the heart and as the first step experiments were undertaken to reproduce the findings of Gorten & others in rats, by chemical determination of non-labelled mercury. Rats with myocardial infarcts, sham operated and unoperated control animals were treated with mercamphamide or methoxyethyl mercury chloride and after appropriate time intervals the content of mercury in their hearts was determined.

METHODS

Myocardial infarcts were produced by surgical coronary ligature (Selye, Bajusz & others, 1960) in male albino rats, 155 ± 5 g (n = 315) of an inbred colony maintained on standard semisynthetic pellet diet, with free access to food and drink water throughout the experiment.

The descending branch of the left coronary artery was tied near to its origin. Mortality rates considerably exceeded those reported by Selye & others, and were similar to those of Dušek & Jezdinská (1965).

The hearts of sham-operated animals were lifted from the opened thorax and replaced after a short time and the wound closed.

The organomercurial compounds, methoxyethyl mercury chloride (MEMC; mol wt 295·15; Berk Ltd, London) and mercamphamide (*N*-3-hydroxy-mercury-2-methoxy-propylcamphoramidic sodium; mol. wt 509·95; Novurit inj. Chinoin, Budapest) were used. Mercamphamide was injected in 25 mg/kg intravenous or intraperitoneal doses, diluted with distilled water to give 0·1 ml/100 g weight; MEMC

in 10 mg/kg intraperitoneal doses was suspended with 1% methylcellulose to give 0·1 ml/100 g giving the animals treated with mercamphamide 0·986 mg Hg/100 g and those with MEMC 0·686 mg Hg/100 g.

Organomercurial treatment was given 3 to 5, 18 to 22 or 42 to 46 h after surgery and the animals were killed 5 min, 1, 5 or 16 h after injection by ether. The hearts were excised, opened and the ventricles cleaned from atrial and other adherent tissues. They were thoroughly washed from blood with tap water, then with distilled water, weighed, cut into small pieces with scissors and then homogenized in a Potters flask with 3 volumes of distilled water.

The homogenized hearts were digested and the amount of mercury present in them was spectrophotometrically assessed by the dithizone method. Graded reagents and water distilled twice from glass were used. Carbon tetrachloride was freshly purified according to method recommended for chloroform by (Iwantscheff, 1958a). Dithizone (G. R.; Reanal, Budapest) was dissolved according to Grusz-Harday (1969). The concentrated solution could be stored in a dark glass in a refrigerator for about one month.

For digestion, the method of Grusz-Harday (1969) was adopted for the rat hearts by the following modifications. The homogenate was transferred to a 25 ml Erlenmeyer flask, 0·15 g of potassium chlorate added and the mixture heated on a boiling water bath for 5 min. One ml of concentrated hydrochloric acid was then added and the flask was removed from the bath. After the intensive gas production had ceased, 1 ml of concentrated hydrochloric acid was added again and the flask, covered with glass, was left on the water bath switched off overnight. Distilled water (10 ml) was then added to the mixture which was heated on the bath until the chlorine had evaporated (about 8 to 10 h). Lost water was replaced. The chlorine-free liquid was filtered made up to 80 ml with distilled water to keep the concentration of chloride ions below 0·2N (Iwantscheff, 1958b).

Mercury was determined as mercury dithizonate according to Fischer & Leopoldi (1935). Accidental metallic contamination was removed as described by Iwantscheff (1958c). The absorbance was measured at 485 nm against pure carbon tetrachloride. The blank value of untreated rat hearts, determined in each experimental series and subtracted from the measured absorbance, was $0{\cdot}035 \pm 0{\cdot}003$ ($n = 23$). The mercury content was calculated on the basis of a calibration curve made with pure mercury chloride solution treated according to the above schedule. These modifications increased the sensitivity of the method, originally described for mercury concentrations about 2 μg/ml, by one order of magnitude.

Data in numbers throughout this paper are given as mean values $\pm$ standard error of the mean ($\bar{x} \pm s_{\bar{x}}$). Significance of differences was assessed by the *t* or, if the "F" test revealed significant heteroscedasticity, the "d" test.

RESULTS

Table 1 shows the mercury content of rat hearts after MEMC treatment. The amount of mercury in the hearts of control animals and those with myocardial infarcts increased as a function of time elapsed after MEMC treatment, but although the figures of each group differed significantly from zero there was no significant difference between animals with infarcts and controls.

With mercamphamide (Table 2), the hearts of unoperated control animals contained relatively large amounts of mercury a few minutes after injection, but 1 or more h later

Table 1. *Mercury content in the hearts of rats injected with MEMC intraperitoneally.*

	Time (h): between surgery and MEMC injection	Time (h): after MEMC treatment	Number of animals	Mercury content (μg/heart)	Significance of the difference from: zero *P*	Significance of the difference from: respective controls *P*
Control animals	—	1	18	0·58 ± 0·167	< 0·01	—
		5	27	0·95 ± 0·202	< 0·01	—
		16	9	1·23 ± 0·281	< 0·01	—
Animals with myocardial infarcts	18 to 22	1	23	0·23 ± 0·092	< 0·05	> 0·05
		5	24	1·16 ± 0·154	< 0·01	> 0·20
	3 to 5	16	10	1·53 ± 0·265	< 0·01	> 0·20

the mercury content in the hearts did not significantly differ from zero, with the only exception of the group tested 1 h after the intravenous treatment. Large amounts of mercury were found in the hearts of the rats with myorcardial infarcts at each time. One to five h after the intravenous treatment given within 24 h after surgery there was more than 2 μg of mercury in the hearts. This amount decreased as the time elapsing between surgery and mercamphamide treatment or that between the injection and killing the animals was prolonged to 48 and 16 h, respectively. After intraperitoneal treatment, the mercury content after 1 h was higher, while that after 5 h was much lower than, the respective values found after intravenous mercamphamide. All these values significantly differ from zero and, apart from the

Table 2. *Mercury content in the hearts of rats injected with mercamphamide.*

	Route	Time: between surgery and mercamphamide inj.	Time: after mercamphamide treatment	Number of animals	Mercury content (μg/heart)	Significance of differences from: zero *P*	Significance of differences from: controls *P*	Significance of differences from: sham operated animals *P*
Control animals	i.v.		5 min	5	3·52 ± 0·941	<0·05	—	—
			1 h	14	0·31 ± 0·143	<0·05	—	—
			5 h	12	0·27 ± 0·146	>0·05	—	—
			16 h	8	0·52 ± 0·26	>0·05	—	—
Sham-operated animals		18 to 22 h	1 h	12	0·22 ± 0·123	>0·05	>0·20	—
			5 h	12	0·60 ± 0·185	<0·01	>0·10	—
		3 to 5 h	16 h	6	0·43 ± 0·274	>0·10	>0·20	—
		42 to 46 h	5 h	7	0·37 ± 0·259	>0·10	>0·20	—
Animals with myocardial infarcts		3 to 5 h	1 h	11	2·38 ± 0·293	<0·01	<0·01	—
		18 to 22 h	1 h	13	2·52 ± 0·479	<0·01	<0·01	<0·01
			5 h	14	2·28 ± 0·449	<0·01	<0·01	<0·01
		3 to 5 h	16 h	16	1·18 ± 0·257	<0·01	>0·10	>0·10
		42 to 46 h	5 h	11	1·55 ± 0·610	<0·05	>0·05	>0·10
Control animals	i.p.	—	1 h	9	0·37 ± 0·186	>0·05	—	—
			5 h	13	0·11 ± 0·074	>0·10	—	—
Sham-operated animals		18 to 22 h	1 h	10	0·07 ± 0·070	>0·20	>0·20	—
			5 h	12	0·07 ± 0·067	>0·20	>0·20	—
Animals with myocardial infarcts			1 h	12	3·93 ± 0·215	<0·01	<0·01	<0·01
			5 h	23	0·94 ± 0·209	<0·01	<0·05	<0·01

16 h experiments, also from those of the respective unoperated controls. Thus, after mercamphamide treatment, selective uptake of mercury by rat hearts with myocardial infarcts takes place.

This conclusion is supported by the results of experiments on sham-operated animals. Amounts of mercury, approaching those found in rats with myocardial infarcts, could not be detected.

DISCUSSION

The experiments were made with two different organomercurials. MEMC was chosen because it is a relatively small molecule and its structure is similar to that of chlormerodrin used by Gorten & others (1966). Mercamphamide, also has a structure similar to, and the same biological diuretic action as, chlormerodrin. But the results were totally divergent. Both the dynamics of changes in heart mercury levels and the selectivity of uptake were different. In those rats given MEMC the amount of mercury present in the hearts increased as a function of time elapsed after the organomercurial injection. There was, at the same time, no significant difference between parallel values of groups with myocardial infarcts and unoperated rats. With the animals treated with mercamphamide, after intravenous injection, the mercury content was very high even in the hearts of unoperated control animals in the first 5 min. An hour later, however, it had decreased to near zero and remained there. In those rats with myocardial infarcts the amount of mercury was equally high 1 or 5 h after intravenous injection but decreased by 16 h after the treatment. In those rats with myocardial infarcts that received mercamphamide intraperitoneally, the decrease between the first and fifth h was faster but the conditions were also in this case fundamentally different from those seen after MEMC. This difference can be explained by different resorption rates.

Different resorption rates, however, fail to offer any reasonable explication for the lack of selective uptake of mercury from MEMC. Selective uptake of mercury takes place, in the theory of Gorten & others (1966) because there is increased permeability of capillaries in the ischaemic areas where a large amount of organomercurial passes into the interstitium and is bound by SH groups of cell proteins. After MEMC treatment, the hearts of unoperated animals also contained large amounts of mercury. This may be perhaps explained by the relatively small size of the MEMC molecule, enabling it to pass more readily into the interstitium. It may also be because MEMC is among those organomercurials metabolized to inorganic mercury (Report of an International Committee, 1969). The degradation of MEMC according to Ulfvarson (1962), is a relatively slow process and would not be important in our experiments.

The uptake of mercury according to Gorten & others (1966) is not due to haemorrhages, hyperaemia or inflammation. This view is supported by the fact that in hearts of our sham operated animals, potentially exposed to the action of all these factors, no large amount of mercury could be found.

If mercamphamide was injected 48 h after the coronary ligature, the uptake of mercury was less than in the first 24 postoperative h. Thus, the facility of selective mercury uptake is impaired by progression of the process of tissue necrosis in the heart. Optimum results were yielded by 1 h experiments made within the first 24 postoperative h. In the experiments of Gorten & others (1966) the greatest contrast between healthy and infarcted areas was found 3 to 5 days after surgery. The results were worse and scarcely available on the 6–8 and 9–12 postoperative days,

respectively. Their result agrees with ours. Apparent discrepancies are well explained by differences in the surgical technique and the species used. In the rat, a small animal, biological processes are quicker, and the genesis and cicatrization of myocardial infarcts passes off more rapidly than in the pig.

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REFERENCES

DUŠEK, J. & JEZDINSKÁ, V. (1965). *Cor Vasa*, **7**, 136–142.

FISCHER, H. & LEOPOLDI, G. (1935). *Z. analyt. Chem.*, **103**, 241–257.

GORTEN, R. J., HARDY, L. B., MCCRAW, B. H., STOKES, J. R. & LUMB, G. D. (1966). *Am. Heart J.*, **72**, 71–78.

GRUSZ-HARDAY, E. (1969). *Arch. Kriminol.*, **143**, 33–37.

IWANTSCHEFF, G. (1958a). *Das Dithizon und seine Verwendung in der Mikro- und Spurenanalyse*, p. 58. Weinheim-/Bergstr.: Verlag Chemie Gmbh.

IWANTSCHEFF, G. (1958b). *Ibid.* p. 107.

IWANTSCHEFF, G. (1958c). *Ibid.*, 101.

Report of an International Committee (1969). *Archs envir. Hlth*, **19**, 891–905.

SELYE, H., BAJUSZ, E., GRASSO, S. & MENDELL, P. (1960). *Angiology*, **11**, 398–407.

ULFVARSON, U. (1962). *Int. Arch. Gewerbepath. Gewerbehyg.*, **19**, 412–422.

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Non-competitive spasmolytics as antagonists of Ca^{++}-induced smooth muscle contraction

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The spasmolytic drugs papaverine, phenpropamine, Cxn 2 (1-methylamino-3,3,5-trimethylcyclohexane), khellin, cyclandelate, magnesium chloride, cocaine, 2,4-dinitrophenol and KCN antagonize various types of spasmogens, such as acetylcholine or histamine, non-competitively. They are assumed to act at some level in the excitation-contraction process common to the various spasmogens. Ca^{++} is essential for the induction of a contraction by the spasmogens. In the non-competitive action of some spasmolytics, Ca^{++} is postulated to be involved. To detect possible differences in their mechanism of action the various spasmolytics were tested against Ca^{++} acting as a pseudo-spasmogen on the K^{+}-primed taenia caeci of the guinea-pig in a Ca^{++}-free medium. Although different mechanisms of action for the various spasmolytics are to be expected, they all behaved similarly with respect to Ca^{++} in that they all caused a parallel shift in the log dose-response curve of Ca^{++} to higher concentrations.

Spasmolytics antagonize the contractions induced by spasmogens in smooth muscle tissue. The action of spasmogens involves two main sequences of biochemical events: the excitation in the muscle cell membrane and the contraction related to chemomechanical processes in this cell. In the coupling between excitation and contractile processes, Ca^{++} appears to play an essential role (Durbin & Jenkinson, 1961; Yukisada & Ebashi, 1961; Hurwitz, Battle & Weiss, 1962; Daniel, 1964; Woodbury, Gordon & Conrad, 1965; Bianchi, 1968; Oehme, Bergman & others, 1969; Ebashi, 1970).

Non-competitive spasmolytics (also called non-specific spasmolytics, musculotropic spasmolytics or antispasmodics) do not interact with the receptors of the spasmogens as do the competitive spasmolytics but interfere with the chain of events leading from the receptor occupation by the spasmogen to its effect. The fact that they are called musculotropic spasmolytics indicates that they do not interfere with the neuromuscular transmission but with the contraction process in the muscle fibres. The effect of this type of spasmolytic is insurmountable. This means that in their presence dose-effect curves for spasmogens on smooth muscle tissues are depressed. Papaverine is the prototype of this group of compounds; Mg^{++}, cocaine, cyclandelate, khellin, phenpropamine, Cxn 2 (1-methylamino-3,3,5-trimethylcyclohexane), KCN and 2,4-dinitrophenol have also been found to act as non-competitive spasmolytics (Ariëns, 1970). It is postulated that these non-specific spasmolytics may interfere at any level in the sequence of processes common to all spasmogens, that is, with the excitation-contraction coupling process or with the contraction process. However, the various members of the group may well act at different levels in this sequence. This is likely since the chemical characteristics of the compounds in question are very different.

The action of papaverine does not depend on the type of spasmogen—histamine,

acetylcholine, barium chloride, K^+—involved (Ariëns, 1970). Papaverine has nearly the same activity against the various spasmogens (Ariëns, 1970) but opinions differ on its mechanism of action. Santi, Contessa & Ferrari (1963) postulated an inhibitory action on oxidative phosphorylation, Holtz, Langeneckert & Palm (1968) a β-adrenergic mechanism of action, Kukovetz, Juan & Pöch (1969) suggested an inhibitory action on the phosphodiesterase while Ferrari & Carpenedo (1968a and b) proposed a competitive antagonistic action with respect to Ca^{++}. Interference with Ca^{++} was also assumed to play a role in the spasmolytic action of cocaine (Bianchi, 1968; Hurwitz, Battle & Weiss, 1962; Feinstein & Paimre, 1969) and Mg^{++} (Edman & Schild, 1962). It may be asked whether such an interference is restricted to these compounds and if on this basis a differentiation of the various non-competitive spasmolytics is possible.

In a Ca^{++}-free medium smooth muscle spasmogens fail to cause a contraction. Addition of Ca^{++} after a spasmogen results in a contraction. On this basis cumulative dose-response curves can be obtained for the contraction of smooth muscle by increasing the Ca^{++} concentration. Ca^{++} then acts as a pseudo-spasmogen. Some spasmolytics have now been tested on such a system as possible antagonists of Ca^{++}.

METHODS

Isolated taenia caeci of young guinea-pigs were used and maintained at 37° in Tyrode through which air was bubbled. On this organ primed with K^+ various spasmolytics were tested against Ca^{++}. Cumulative dose-response curves (van Rossum & van den Brink, 1963; van Rossum, 1963) for Ca^{++} were studied in the absence and in the presence of various concentrations of a spasmolytic. In the period between the experiments the organ was kept in Ca^{++}-free Tyrode solution. Three min before starting a cumulative dose-response curve with Ca^{++}, this solution was replaced by K_2SO_4-Ringer (21·6 g K_2SO_4, 0·2 g $NaHCO_3$ and 1 g glucose to 1 litre of distilled water) and at the start of each curve it was exchanged for KNO_3-Ringer (17·6 g KNO_3, 0·2 g $NaHCO_3$ and 1 g glucose to 1 litre of distilled water). According to Ferrari & Carpenedo (1968a) substitution of the sulphate-Ringer by the nitrate-Ringer has the advantage that precipitation of Ca^{++} is avoided while the contractions are well maintained. The intermediate use of the SO_4^{--}-medium enhances the elimination of Ca^{++}.

RESULTS

In the presence of $MgCl_2$ the log dose-response curves obtained with Ca^{++} on the K^+-primed taenia caeci showed a parallel shift to higher concentrations (Fig. 1A). This suggests a competitive relation between Ca^{++} and Mg^{++} as also reported by Edman & Schild (1962). Similar results were obtained with cocaine as an antagonist (Fig. 1B), which confirms competition with Ca^{++} as mentioned by Hurwitz, Battle & Weiss (1962) and by Feinstein & Paimre (1969). The parallel shift of the Ca^{++}-curve by papaverine as reported by Ferrari & Carpenedo (1968a,b) was confirmed, even over a large dose range (Fig. 1C). Other papaverine-like amines such as phenpropamine and Cxn 2 behaved in the same way. The question arises whether a correlation exists for these papaverine-related amines between their Ca^{++}-antagonizing potency and their non-competitive spasmolytic potency against spasmogens such as cholinergics and histamine in the classical test on the guinea-pig isolated ileum. Such a correlation was indeed found. The relative antagonistic potencies against both Ca^{++} and the

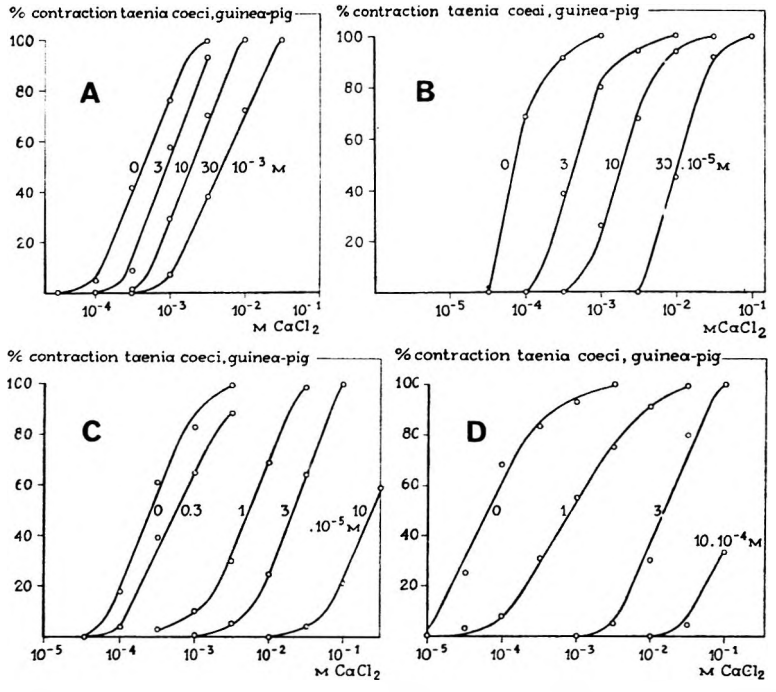

FIG. 1. Cumulative log dose-response curves for $CaCl_2$ as a pseudo-spasmogen on the K^+-primed isolated taenia caeci muscle of the guinea-pig in a Ca^{++}-free KNO_3-Ringer and the influence thereon of various non-competitive spasmolytics: (A) $MgCl_2$, (B) cocaine, (C) papaverine, (D) khellin. Note that all spasmolytics cause a parallel shift of the $CaCl_2$-curve to higher Ca^{++}-concentrations.

classical spasmogens for papaverine, phenpropamine and Cxn 2 were about 100, 1000 and 5 respectively. This indicates that the parallel shift observed with respect to Ca^{++} is probably related to the non-competitive papaverine-like activity of these drugs. Structurally unrelated non-competitive "papaverine-like" spasmolytics such as the non-ionizable nitrogen-free spasmolytics khellin (Fig. 1D) and the ester-type cyclandelate also displace the log dose-response curve obtained with Ca^{++} to the right of the control and in a parallel manner.

Remarkably, however, a metabolic blocker such as 2,4-dinitrophenol—a compound which acts as an uncoupling agent—also causes a parallel shift in the log dose-response curve obtained with Ca^{++}. Ferrari & Carpenedo (1968a) reported a similar phenomenon for KCN, also using the K^+-primed depolarized taenia caeci smooth muscle of the guinea-pig.

The non-competitive spasmolytics cyclandelate, papaverine, phenpropamine, Cxn 2, magnesium chloride, cocaine, khellin, 2,4-dinitrophenol and KCN all show a similar behaviour in their antagonism of Ca^{++} acting as a pseudo-spasmogen on the potassium-primed taenia caeci muscle of the guinea-pig, despite the fact that these spasmolytics very probably have different mechanisms of action. The desired differentiation of the various non-competitive spasmolytics was not obtained. The undifferentiated behaviour of the chemically heterogeneous and biochemically probably differently acting non-competitive spasmolytics in their action against Ca^{++} awaits further elucidation.

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REFERENCES

ARIËNS, E. J. (1970). In: *Proc. IV Int. Congress Pharmac.*, Basel, 1969, III, 16. Basel: Schwabe.

BIANCHI, C. P. (1968). *Cell calcium.* In: *Molecular biology and medicine series.* Editor: Bittar, E. E. London: Butterworth.

DANIËL, E. E. (1964). *Ann. Rev. Pharmac.*, **4**, 189–222.

DURBIN, R. P. & JENKINSON, D. H. (1961). *J. Physiol., Lond.*, **157**, 90–96.

EBASHI, S. (1970). *Proc. IV Int. Congress Pharmac.*, Basel, 1969, I, 32. Basel: Schwabe.

EDMAN, K. A. P. & SCHILD, H. O. (1962). *J. Physiol., Lond.*, **161**, 424–441.

FEINSTEIN, M. B. & PAIMRE, M. (1969). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **28**, 1643–1648.

FERRARI, M. & CARPENEDO, F. (1968a). *J. Pharm. Pharmac.*, **20**, 317–318.

FERRARI, M. & CARPENEDO, F. (1968b). *Archs int. pharmacodyn. Thér.*, **174**, 223–232.

HOLTZ, P., LANGENECKERT, W. & PALM, D. (1968). *Arch. exp. Path. Pharmak.*, **259**, 290–306.

HURWITZ, L., BATTLE, F. & WEISS, G. B. (1962). *J. gen. Physiol.*, **46**, 315–332.

KUKOVETZ, W. R., JUAN, H. & PÖCH, G. (1969). *Arch. exp. Path. Pharmak.*, **264**, 262.

OEHME, P., BERGMANN, J., RUDEL, M., EICHSTADT, M. & JUNG, F. (1969). *Archs int. Pharmacodyn. Thér.*, **179**, 6–22.

ROSSUM, J. M. VAN (1963). *Ibid.*, **143**, 299–330.

ROSSUM, J. M. VAN & VAN DEN BRINK, F. G. (1963). *Ibid.*, **143**, 240–246.

SANTI, R., CONTESSA, A. R. & FERRARI, M. (1963). *Biochem. Biophys. Res. Commun.*, **11**, 156–159.

WOODBURY, J. W., GORDON, A. M. & CONRAD, J. T. (1965). *Muscle*, Ch. 5. In: *Physiology and Biophysics.* Editors: Ruch, T. C. & Patton, H. D. Philadelphia: Saunders.

YUKISADA, N. & EBASHI, F. (1961). *Jap. J. Pharmac.*, **11**, 46–53.

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The gas-liquid chromatographic estimation of phenacetin and paracetamol in plasma and urine

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Methods are described for the gas-liquid chromatographic estimation of phenacetin and paracetamol in plasma and free and conjugated paracetamol in urine. *p*-Chloracetanilide and *p*-bromacetanilide were used as internal standards. The drugs were extracted with ethyl acetate and before chromatography were converted to trimethylsilyl derivatives with *N*,*O*-bis(trimethylsilyl)acetamide (simultaneous assay of phenacetin and free paracetamol in plasma), or *N*-trimethylsilylimidazole (assay of paracetamol alone). Phenacetin was extracted with chloroform and chromatographed directly. Paracetamol glucuronide and sulphate were hydrolysed enzymatically to the parent compound before extraction. Recovery of added phenacetin and paracetamol in plasma at concentrations of 1–100 μg/ml was complete, and the limit of detection of the drugs in plasma was 0·05 μg/ml.

Existing methods for the estimation of phenacetin and its metabolite paracetamol in plasma and urine are non-specific, time consuming or lack sensitivity (Brodie & Axelrod, 1948, 1949; Welch & Conney, 1965; Büch, Pfleger & Rüdiger, 1967; Cummings, King & Martin, 1967; Routh, Shane & others, 1968). A gas-liquid chromatographic method providing a more sensitive and specific technique for the assay of these drugs either separately or together in biological fluids is now described. The gas-liquid chromatography of paracetamol has been reported by Klutch & Bordun (1968), but in the present studies the drug could not be chromatographed directly in low concentrations without peak tailing and absorption losses. It was therefore converted to trimethylsilyl (TMS) derivatives using *N*-trimethylsilylimidazole (TMSI) or *N*, *O*-bis(trimethylsilyl)acetamide (BSA). TMSI is a powerful silylating reagent which is selective for -OH groups (Pierce, 1968) and paracetamol is converted to the TMS ether derivative. BSA reacts with both -NH- and -OH groups (Klebe, Finkbeiner & White, 1966; Pierce, 1968) and mono- and di- TMS derivatives are formed with phenacetin and paracetamol respectively. *p*-Chloracetanilide and *p*-bromacetanilide were used as internal standards. The gas-liquid chromatographic properties of some phenacetin metabolites and related compounds were also investigated.

MATERIALS AND METHODS

Hewlett Packard Model 5755B and Pye Series 104 Model 14 gas chromatographs with flame ionization detectors were used in conjunction with Moseley Model 7128A and Leeds & Northrup Speedomax "W" recorders respectively. Argon or nitrogen was used as the carrier gas at a flow rate of 50 ml/min. Hydrogen and air flow rates were approximately 40 and 400 ml/min respectively. The colmns were glass, $\frac{1}{4}$ inch diameter and 5 or 6 ft in length. Pyridine, TMSI and BSA (Pierce) were obtained

from Phase Separations Ltd., Deeside Industrial Estate, Queensferry, Flintshire, and prepared column packings (Applied Science) from Field Instruments Ltd., Orchard Road, Richmond, Surrey.

Preparation of TMS derivatives

TMSI, BSA and anhydrous pyridine were stored separately under nitrogen in capped vials and standard precautions were taken to avoid hydrolysis of the TMS derivatives (Pierce, 1968). All transfers were made with clean dry microsyringes.

The reactions were carried out in glass vials, 36 × 14 mm with capillary end-bulbs of about 30 μl capacity (Glass Appliances Ltd., Holburn Street, Aberdeen). The vials were flushed with nitrogen, sealed with silicone rubber caps and 15 μl of a pyridine solution of the compound or extract under investigation injected through the cap into the end-bulb. An equal volume of the silylating reagent was added and the solutions mixed thoroughly with the syringe. The tubes were left at room temperature (20°) for 15–30 min and 2–3 μl aliquots were injected directly into the chromatograph.

Assay of free paracetamol in plasma and urine

Plasma or urine samples (2·0 ml) containing up to 40 μg of paracetamol and 1M phosphate buffer pH 8·0 (1·0 ml) were extracted in stoppered glass tubes with redistilled ethyl acetate (5·0 ml) containing 3·0 μg/ml of *p*-chloracetanilide. The tubes were shaken mechanically for 20 min and then centrifuged (vigorous shaking causes emulsions). The organic phase only was transferred to 15 ml tapered glass centrifuge tubes and evaporated to dryness on a rotary vacuum evaporator with the tubes immersed in water at 26°. The residue in the tips of the tubes was dissolved in 15 μl of anhydrous pyridine with the aid of a vortex mixer, transferred to reaction vials and 15 μl of TMSI added as described above. Samples containing 20–200 μg/ml of free paracetamol were extracted with ethyl acetate containing 30 μg/ml of *p*-chloracetanilide. Appropriate dilutions of more concentrated samples were made before analysis. The columns were packed with 10% OV17 on 80/100 mesh Gaschrom Q and run at 200°.

Assay of total free and conjugated paracetamol in urine

Urine samples (0·5 ml), 0·2M sodium acetate buffer pH 5·0 (0·5 ml) and glusulase* (0·1 ml) (Endo Laboratories, Garden City, N.Y., USA) were mixed in stoppered glass tubes and incubated at 37° for 16 h. Phosphate, 0·2M, pH 9·9 (2·0 ml) was then added and the mixture extracted with ethyl acetate (5·0 ml) containing 30 μg/ml of *p*-chloracetanilide and assayed as described for free paracetamol. Preliminary dilutions were made if the urine contained more than 1000 μg/ml of total paracetamol. Hydrolysis of paracetamol conjugates in urine with glusulase has been shown to give a significantly higher recovery of free paracetamol than hydrolysis with hydrochloric acid (Prescott, 1969).

Simultaneous assay of phenacetin and free paracetamol in plasma

The assay was the same as that for free paracetamol except that *p*-bromacetanilide was used as the internal standard. BSA was used as the silylating reagent, and all three compounds were converted to TMS derivatives. The column was packed with either 5% OV1 or 5% UC W98 on 80/100 mesh Gaschrom Q, and run at 160°.

* Glusulase contains 100 000 units of β-glucuronidase and 50 000 units of aryl sulphatase per ml.

Assay of phenacetin alone in plasma

Phenacetin was extracted as for free paracetamol in plasma using chloroform containing 3 μg/ml of *p*-bromacetanilide. The extract was evaporated to dryness and the residue redissolved in 20 μl of chloroform. Aliquots were injected directly into the gas chromatograph. The column was packed with 3% XE 60 on 80–100 mesh Gaschrom Q and run at 160°. Other suitable liquid phases were: 3% QF1, diethyleneglycol succinate, HI-EFF 8BP or Carbowax 20M, 10% OV17 and 10% SE 52 plus 1% HI-EFF 8BP.

Calibration

Aqueous solutions containing known amounts of the drugs were run through the entire procedures and the ratios of the peak heights of the drugs (or their TMS derivatives) to those of the internal standards were plotted against the concentrations of phenacetin and paracetamol. Paracetamol was used for the standardization of the assay for total free and conjugated paracetamol as the glucuronide and sulphate were not available. Linear working curves relating the peak height ratios and drug concentrations were obtained over the range 1–100 μg/ml. The concentration of the drugs in plasma or urine can be determined from previously constructed calibration graphs, but a more accurate result may be calculated from the peak height ratio of a standard solution of the drug run with the samples since there were minor day-to-day variations in peak height ratios.

RESULTS

Aniline, acetanilide, *p*-phenetidine, *p*-aminophenol and phenacetin were all chromatographed directly without difficulty on Gaschrom Q coated with 3% XE 60, 3% HI-EFF 8 BP, 5% Carbowax 20M, 10% OV17 or mixed phases consisting of 10% SE 52 plus 1% HI-EFF 8BP and 0·5% SE 30 plus 0·5% Carbowax 20M. *N*-Methylacetanilide, acet-*p*-toluidide, *p*-chloracetanilde and *p*-bromacetanilide can be used as internal standards. With the exception of *N*-methylacetanilide, all of these compounds were converted to TMS derivatives by treatment with BSA and could then be chromatographed on "non-polar" liquid phases such as OV1 and UC W98. With TMSI, selective silylation of the -OH groups of paracetamol, 2-hydroxyphenacetin and *p*-aminophenol occurred, although with the latter compound derivative formation was apparently incomplete. Paracetamol could not be chromatographed directly on any of the columns without significant peak tailing. The retention times of the different compounds and their TMS derivatives relative to *p*-aminophenol on four different liquid phases are listed in Table 1. In general, normal human plasma and urine samples run through the procedures did not show interfering peaks at the attenuation used for the most sensitive assays. A large extraneous peak eluting after paracetamol-TMS was sometimes encountered with plasma, thus delaying the injection of subsequent samples. Spurious (but non-interfering) peaks were also traced to impurities in some batches of TMSI and ethyl acetate. Chromatograms of extracts of plasma and urine from individuals taking therapeutic doses of paracetamol are shown in Fig. 1 (a, b and c).

Recovery studies

The results of 10–20 analyses of samples of plasma and urine containing known amounts of added phenacetin and paracetamol are shown in Table 2. Except for the

Table 1. *Relative retention of some aromatic amine derivatives (p-aminophenol = 1·00).*

Temperature	3% XE 60 160°C	3% HI-EFF 8BP 180°C	10% OV17 200°C	10% OV17 200°C	5% OV1 155°C
	Without derivative formation			As TMS derivatives*	
Aniline	0·10	—	0·26	—	0·22
N-Methylacetanilide	0·36	0·20	1·01	—	0·33
p-Phenetidine	0·33	0·27	1·00	—	0·79
Acetanilide	1·49	0·91	1·60	—	0·33
p-Aminophenol	1·00	1·00	1·00	1·00†	1·00
Acet-*p*-toluidide	2·10	1·28	2·38	—	0·51
p-Chloracetanilide	4·67	2·90	3·75	—	0·73
p-Bromacetanilide	6·41	3·84	5·85	—	1·08
Phenacetin	7·64	4·95	5·80	—	1·29
Paracetamol	—	—	—	4·40	1·56
2-Hydroxyphenacetin	—	—	—	5·52	3·46
Approximate retention time of *p*-aminophenol (min)	2	5	3	4	10

* Compounds run on OV17 and OV1 were treated with TMSI and BSA respectively.
† Incomplete derivative formation.

assays of paracetamol alone, the recoveries were calculated using previously constructed calibration equations derived from the assay of aqueous solutions of the drugs in the same concentration ranges. The values presented for paracetamol alone were obtained using an aqueous standard run with the samples.

The recovery of both drugs from plasma was essentially complete, and the reproducibility of the assays good, down to concentrations of 1 μg/ml. The limits of detection in plasma are about 0·05 μg/ml. Phenacetin and paracetamol are likely to be taken in combination with acetylsalicylic acid, caffeine, codeine and barbiturates. Caffeine and codeine may be extracted from biological fluids under the present conditions, but salicylates and barbiturates will remain largely in the aqueous phase. These compounds all form TMS derivatives with BSA but do not interfere with the present methods.

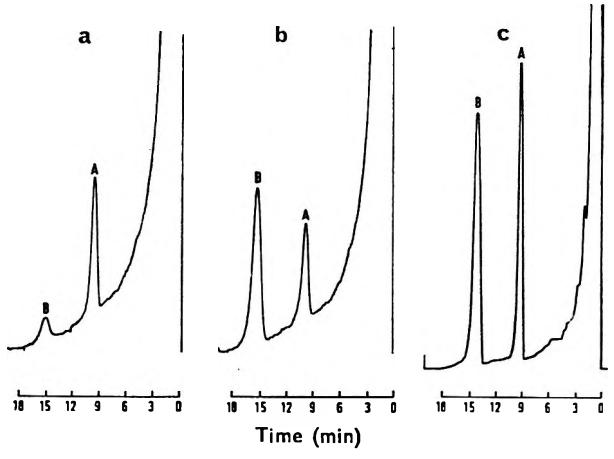

FIG. 1. Chromatograms of extracts of plasma obtained from a patient (a) 30 and (b) 90 min after administration of 1·5 g of paracetamol. The samples were assayed for free paracetamol and the measured concentrations were 1·6 μg/ml (a) and 16·5 μg/ml (b). (c) Chromatogram of an extract of hydrolysed urine obtained from a healthy subject 12–24 h after taking 1·5 g of paracetamol. The urine was assayed for total free and conjugated paracetamol and the measured concentration was 482 μg/ml. The internal standard in all three chromatograms was *p*-chloracetanilide (peak A) and paracetamol was chromatographed as the mono-TMS derivative (peak B).

Table 2. *Recovery of phenacetin and paracetamol added to normal human plasma or urine in replicate assays.*

Assay	Concentration or concentration range (μg/ml)	Mean recovery ± s.d. (%)
Phenacetin in plasma	20–100	101·5 ± 4·1
	1– 10	96·6 ± 10·4
Phenacetin and paracetamol together in plasma		
phenacetin	25–100	100·8 ± 3·0
paracetamol	25–100	102·6 ± 4·0
phenacetin	1– 10	99·7 ± 6·7
paracetamol	1– 10	99·6 ± 9·0
Paracetamol in plasma	10	100·1 ± 1·8
Paracetamol in urine	500	100·9 ± 2·8

DISCUSSION

The gas-liquid chromatographic assay of phenacetin and paracetamol compares favourably with other techniques in respect of sensitivity and reproducibility. Spectrophotometric methods are liable to interference by other aromatic amines or extractable drugs with ultraviolet absorption, and sensitivity is compromised by high blank values if preliminary thin-layer chromatographic separation is used. The inherent specificity of gas-liquid chromatographic analysis is further increased by the formation of TMS derivatives. Paracetamol can be identified with certainty by differential silylation and chromatography on columns of differing polarity.

A disadvantage is that the method can be more time consuming than other techniques, but on the other hand it is particularly useful to be able to estimate phenacetin and paracetamol together in a single analysis. A further drawback is that analytical precision depends on quantitative conversion to TMS derivatives which are readily hydrolysed by moisture, even in the presence of excess reagent. The N-TMS derivatives are more susceptible to hydrolysis than O-TMS derivatives and TMSI is therefore the preferred silylating reagent for the analysis of paracetamol alone. However, the solvent front elutes rather slowly after injection of TMSI, and this limits sensitivity. Minor decomposition of N-TMS derivatives can occur on ageing columns, but this seems to affect the drugs and the internal standards to a comparable degree.

REFERENCES

BRODIE, B. B. & AXELROD, J. (1948). *J. Pharmac. exp. Ther.*, **94**, 22–28.

BRODIE, B. B. & AXELROD, J. (1949). *Ibid.*, **97**, 58–67.

BÜCH, H., PFLEGER, K. & RÜDIGER, W. (1967). *Z. klin. Chem.*, **5**, 110–114.

CUMMINGS, A. J., KING, M. L. & MARTIN, B. K. (1967). *Br. J. Pharmac. Chemother.*, **29**, 150–157.

KLEBE, J. F., FINKBEINER, H. & WHITE, D. M. (1966). *J. Am. chem. Soc.*, **88**, 3390–3395.

KLUTCH, A. & BORDUN, M. (1968). *J. pharm. Sci.*, **57**, 524–526.

PIERCE, A. E. (1968). *Silylation of organic compounds.* Pp. 7 and 22. Rockford, Illinois: Pierce Chemical Co.

PRESCOTT, L. F. (1969). *Clin. Pharmac. Ther.*, **10**, 383–394.

ROUTH, J. I., SHANE, N. A., ARREDONDO, E. G. & PAUL, W. D. (1968). *Clin Chem.*, **14**, 882–889.

WELCH, R. M. & CONNEY, A. H. (1965). *Ibid.*, **11**, 1064–1067.

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The sizing of some powder and liquid sprays from pressurized packs by air sedimentation

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The construction and testing of an apparatus suitable for the size analysis of certain pressurized sprays by air sedimentation is described. Talc spray size was related to the size of the talc used to prepare the pressurized pack. For other powders (UFI and neomycin sulphate) the sprays were much larger than the powder used to prepare the pack. The use of propellents of higher vapour pressures, higher pack temperatures and the addition of 0·5% sorbitan monolaurate resulted in smaller talc sprays. Talc spray size was increased by the addition of 0·5% isopropyl myristate. Increasing the viscosity of the liquid product and increasing the concentration of propellent gave a reduction in liquid spray size; for both liquid and talc sprays, smaller sprays resulted from the use of a valve housing with a vapour tap.

The size analysis of sprays from pressurized packs by microscopy (C.S.M.A., Aerosol Guide, 1966; Lefebvre & Tregan, 1964) is tedious and time-consuming and the results may be unreliable because of the necessity for calibration, the likelihood of size changes taking place between collection and sizing, and because the sampling is often biased. The use of the Cascade Impactor can shorten the analysis (Polli, Grim & others, 1969) but an initial calibration of the apparatus by microscopy is still required and drops with diameters greater than 50 μm settle out of the spray before being impacted on the first slide.

A sedimentation column of height 6 m and diameter 0·74 m with an automatically recording balance at the bottom of the column was used to size non-volatile liquid and solidified liquid fuel drops of 5–250 μm diameter (Tanasawa & Hiroyasu, 1962). Such a tall column was necessary for adequate separation of the different sized drops. Further, the time taken for a drop to reach its terminal sedimentation velocity was small compared to its overall fall time so that it could be assumed that each drop travelled down the whole of the column at its terminal velocity.

MATERIALS AND METHODS

Apparatus

A sedimentation column (Fig. 1), 27·5 feet high and 27 inches in diameter, was made from 3/16 inch "Darvic" PVC sheeting. An earthed coating of powdered graphite applied to the inner surface of the column kept the PVC uncharged. The column was erected against the outside wall of the building and the top and bottom entrance boxes projected into the first floor and basement of the building respectively.

Thermistors (S.T.C., type F.S.) were inserted through the column wall at several points for the measurement of air temperatures.

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An automatic continuous recording balance (Fig. 1) recorded the weight of sedimented particles, the position of a sliding weight on the balance pointer being set to give a linear balance response over the range 0–150 mg. The balance scale pan with a diameter just less than the internal diameter of the column, was made of "Propafilm O" gauge 50/515 supported by a light-weight frame. Movements of the balance beam were recorded by reflection of a light beam from a mirror on the balance beam onto photographic recording paper on the kymograph. A time marker was obtained on the kymograph recording by interrupting, at 1 min intervals, the current to the light source. Balance oscillations were damped by the small vane at the end of the balance pointer moving in a dash-pot containing liquid paraffin B.P.

Even after the column had been sealed for several hours there were air currents in the region of the balance. Insulating or heating the whole or part of the column, or both, did not reduce these movements. The use of more efficient damping and the attachment of springs to the balance beam reduced balance movements but also greatly reduced the sensitivity of the balance. The air movements were reduced by

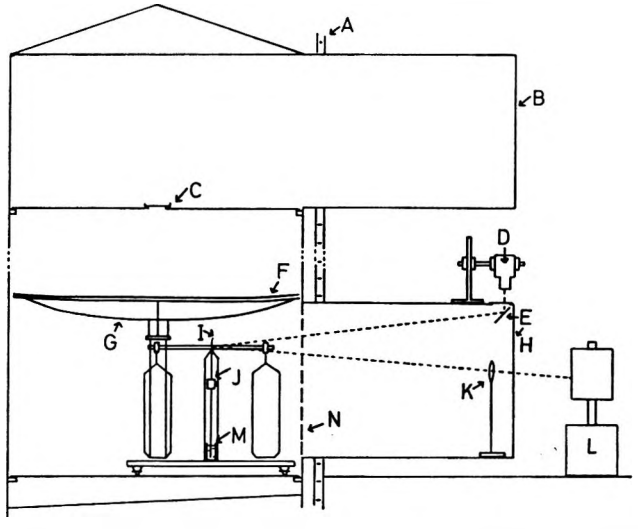

FIG. 1. Sedimentation column and balance. A, wall of building; B, top entrance box; C, shutterplate; D, lamp; E, front-silvered plane mirror; F, scale pan; G, scale pan supports; H, bottom entrance box; I, front-silvered concave mirror; J, sliding weight; K, convex lens; L, kymograph; M, dash-pot containing damping liquid; N, position of baffle.

introducing carbon dioxide through the bottom of the column at flow rates up to 1·8 litre/min; the performance of the balance was unaffected. Higher flow rates increased air movements. The introduction of carbon dioxide at 1·8 litre/min for 60 min abolished balance movements and even after the gas flow was stopped they remained at this low level for several hours. Determination of the carbon dioxide content of the air in the column using a CO_2/O_2 electrode (Norman, Ledsome & Linden, 1965) showed that the carbon dioxide concentration fell off rapidly with height above the floor of the column. The results are shown in Table 1. Because of the difficulty of maintaining stability for very long settling times particles having diameters less than 5 μm were not sized.

The introduction of a sample for analysis was controlled by a shutterplate mounted over the sampling hole in the centre of the platform at the top of the column. So

Table 1. *Percentage of carbon dioxide in the gas removed from the column before, during and after the introduction of carbon dioxide gas at the rate of* 1·8 *litre/min.*

Time (min) after the start of input of carbon dioxide	Position up the column		
	Between floor of column and thermistor 1	Level with thermistor 1	Level with thermistor 2
0	0·2	0·1	0·1
30	5·6	0·3	0·2
60	17·0	0·9	0·3
90	26·6	3·4	0·4
After 90 min carbon dioxide input stopped.			
120	21·9	3·3	
150	16·2	3·3	
180	12·9	2·8	
210	11·5	2·3	

Thermistor 1 was level with the balance scale pan; thermistor 2 was 5·5 ft higher up the column.

that the spluttering of the valve on opening and closing was not included in the sample analysed, the shutterplate movements were controlled electrically to give the following sequence: depression of the actuator button, opening of the shutterplate and sample introduction, closing of the shutterplate, release of actuator button. Any spray released before opening and after closing of the shutterplate was collected on the shutterplate. The sampling time, 1–2 s for the introduction of 100 mg of spray, was marked on the kymograph recording.

Each pressurized pack was kept in a temperature-controlled water bath for a standard time before operation and sampling. During sampling the pack was held horizontally with the orifice of the actuator button above the centre of the shutterplate, the open end of the dip tube being covered by the liquid in the pack. In accordance with the manufacturer's instructions the urea formic iodide (UFI) packs were held at an angle of 45°, with the orifice of the actuator button 12·5 inches from the centre of the shutterplate. The talc, neomycin sulphate and liquid product packs were held with their actuator buttons 3·5 inches above the shutterplate. Evaporation of propellent appeared to be complete from the spray thus introduced into the column. As the spray sample had to remain unchanged during its sedimentation and collection, the investigation was confined to powders and non-volatile liquid products.

Preparation of pressurized packs

With the exception of the UFI packs all packs were assembled from components in the Precision Valve Kit (Metal Box Company).* Except for the actuator button, which was shaved down so that the orifice no longer lay in a recess, all were used exactly as supplied. The UFI packs used were either the standard commercially available pack or a modification specially prepared by the manufacturer. The other packs were prepared in the laboratory using an ambient temperature technique.

The talc was talc B.P. passed through a 200 mesh sieve to remove particles that might clog the valve (Herzka & Pickthall, 1966).

* Components: Actuator button, 0·018 inch standard button; valve stem, 0·018 inch nylon stem, natural; valve housing, 0·025 inch L.D. housing, red; spring, stainless steel; gasket, standard buna gasket; dip tube, standard dip tube.

Preliminary sizing of powder samples

Talc samples were sized by Andreasen's method (B.S. 3406, 1963) using 80% ethanol as sedimentation liquid and 0·1% Lissapol as wetting agent.

Size data provided by the suppliers of the neomycin sulphate powder showed that 90% of the particles were below 10 μm, 99% below 20 μm and only single particles up to 60 μm.

No size data were available for the UFI powder but the powder was 'micronized' as a slurry in liquid propellent before pack assembly.

Calculation of particle sizes

The coefficient of drag, C_d, of a particle sedimenting in a fluid is a function of the shape of the particle and the Reynolds number, Re, of the system. For systems where Stokes' Law holds, i.e. where the Reynolds number is in the range 10^{-4} to 0·2, $C_d = 24/Re$ and the diameter of the particle may be calculated using the equation:

$$d_{st} = \sqrt{\frac{18\,\eta v}{(\rho - \rho_0)g}} \qquad (1)$$

(The symbols have their usual meanings.)

When the Reynolds number is in the range 0·2 to 1000 there is no constant relation between C_d and Re. The relation derived by Schiller & Naumann (1933), $C_d = 24(1 + 0{\cdot}15Re^{0{\cdot}687})/Re$, is reliable for Re values up to 800 (Coulson & Richardson, 1968). From this equation it may be shown that the particle diameter,

$$d^2 = d_{st}^2 (1 + 0{\cdot}15Re^{0{\cdot}687}) \qquad (2)$$

A process of successive approximations was used to obtain d from equation (2). A value for the Reynolds number was obtained using the Stokes' diameter of the particle and this value was substituted together with the Stokes' diameter into equation (2) to give d. From d a second value for R_e was found. From equation (2) a second value of d was thence obtained. This was repeated until successive values of d differed by less than 0·1 μm.

Heywood's tables (1948) of log Re against log $\frac{1}{2}(C_d/Re)$ also may be used to calculate the Reynolds number and hence the diameter of a particle having a known terminal velocity and a Re value in the range 0·2 to 1000.

Both the Schiller-Naumann equation and Heywood's tables were used to calculate the diameter of particles having Reynolds numbers greater than 0·2. The diameter of particles having Reynolds number of 0·2 or less were calculated from equation (1).

Using the calculated particle sizes a cumulative weight oversize curve was derived from the kymograph recording.

The viscosity of air at the temperature of the analysis was taken from International Critical Tables (1929).

Verification of the applicability of the sedimentation column to the size analysis of particles dispersed in the top of the column

A 100 mg sample of glass beads having a nominal size range of 10–100 μm was sized by sedimentation in the column, the sample being blown into the column from a piece of metal foil using a sphygmometer pump. Two analyses were made. The beads were also sized microscopically.

Both powder and liquid pressurized sprays were sized. The powder sprays examined were talc (10%), neomycin sulphate (10%) and UFI (200 g in 300 g of propellent). n-Tetradecane was used as the test liquid for liquid spray packs because of its low volatility, low viscosity, miscibility with halogenated hydrocarbon propellents and compatibility with the valve components.

All spray size determinations were in triplicate.

The rate of hydration of the dried neomycin sulphate was so rapid that all powder entering the column was hydrated. The density of hydrated neomycin sulphate was thus used in the calculation of particle sizes. The neomycin sulphate was dried over phosphorus pentoxide in a vacuum desiccator at 60°.

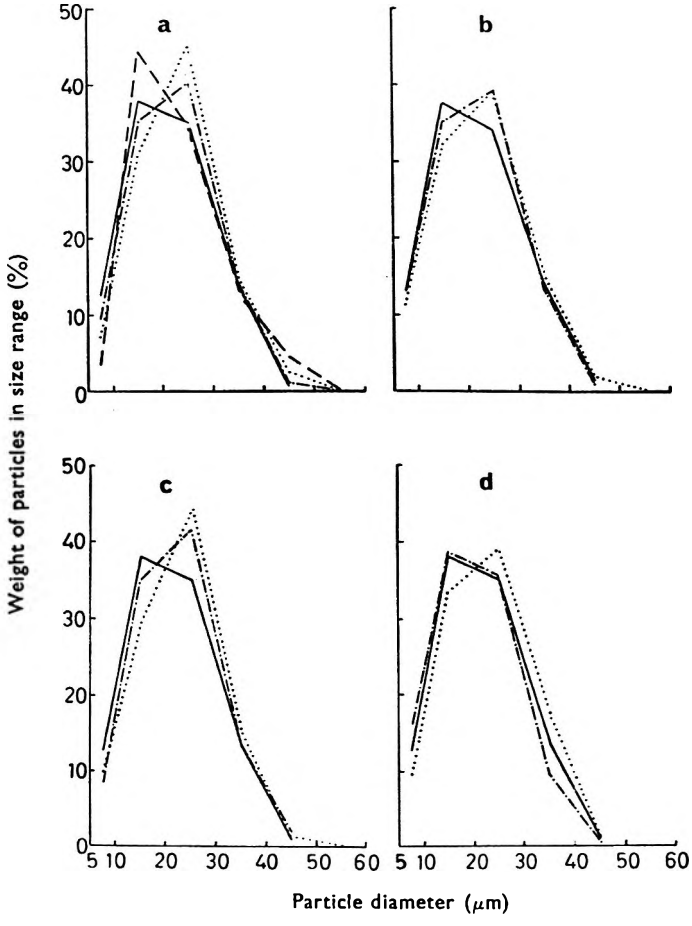

FIG. 2. Weight frequency polygons for sample I talc and sprays from packs prepared with sample I talc obtained at 20° unless otherwise stated.

a – – – Andreasen's Analysis of sample I talc. —— 10% talc I in Arcton 12. –·–·– 10% talc I in Arcton 12 and Arcton 114 in the ratio 80:20, by weight. · · · · 10% talc I in Arcton 12 and Arcton 114 in the ratio 60:40, by weight.

b —— 10% talc I in Arcton 12. –·–·– 10% talc I in Arcton 12 and Arcton 114 in the ratio 80:20, and · · · · 60:40, by weight. All with vapour tap in valve housing.

c —— 10% talc I in Arcton 12, 20°; –·–·– at 15°; · · · · at 10°.

d —— 10% talc I in Arcton 12, 20°. –·–·– 10% talc I and 0·5% Arlacel 20 in Arcton 12 at 20°. · · · · 10% talc I and 0·5% isopropyl myristate in Arcton 12 at 20°.

RESULTS

Agreement between the two sets of results for the sizing of the glass beads was satisfactory (% weight of particles with diameters less than 20 μm 0·1%, 0%; <40 μm 3·7%, 4·2%; <60 μm 72·9%, 68·5%; <80 μm 99·6%, 98·6%; <100 μm 100%; 100% measured by microscopy and air sedimentation respectively) and it was decided to use the column to size particles and drops from pressurized packs.

Comparison of the results from Andreasen's analysis of the four talc samples and from the size analysis of the talc sprays (Fig. 2a and Table 2) shows that spray size was related to the size of the talc sample used to prepare the pack. Reduction in the proportion of Arcton 12 in the propellent and lowering of pack temperature both resulted in larger talc sprays (Fig. 2a and c, Table 2).

Surfactants are sometimes added to pressurized products. Addition of 0·5% w/w of sorbitan monolaurate (Arlacel 20) to a pack containing sample I talc and Arcton 12 slightly reduced the talc spray size whereas incorporation of 0·5% w/w of isopropyl myristate resulted in an increase in spray size (Fig. 2d). Isopropyl myristate is non-volatile and is used as a lubricant to aid the passage of powder through the valve and as a suspending agent for the powder (Herzka & Pickthall, 1966).

Replacement of the valve housing by a valve housing with a 0·025 inch vapour tap in its side led to a small reduction in talc spray size (Fig. 2b).

Storage of the packs containing sample III talc led to a progressive reduction in spray size with increasing time of storage (Table 2).

Table 2. *The effect on talc spray size of propellent composition, particle size of talc sample, and storage.*

Pressurized formulation/talc sample	% Weight of particles undersize at particle diameters (μm) of: 10	20	30	40
Talc II; Andreasen's Analysis	5·5	33·5	76·0	91·7
10% talc II in Arcton 12 at 20°	8·9	37·7	81·4	97·8
10% talc II in Arcton 12 and Arcton 114 in the ratio 80:20, by weight, at 20°	8·8	37·4	81·0	97·4
10% talc II in Arcton 12 and Arcton 114 in the ratio 60:40, by weight, at 20°	6·7	34·4	76·8	96·9
Unsieved talc; Andreasen's Analysis	2·4	34·0	77·7	93·2
10% unsieved talc in Arcton 12 at 20°	9·1	41·3	82·9	97·5
Talc III: Andreasen's Analysis	7·2	70·5	99·9	
10% talc III in Arcton 12 at 20°. Before storage	20·9	81·1	98·5	99·9
10% talc III in Arcton 12 and Arcton 114 in the ratio 80:20, by weight, at 20°. Before storage	17·7	73·3	98·7	99·9
10% talc III in Arcton 12 and Arcton 114 in the ratio 60:40, by weight, at 20°. Before storage	12·3	65·2	98·2	99·8
10% talc III in Arcton 12 at 20°. After 3 months storage	25·7	79·9	99·3	100·0
10% talc III in Arcton 12 and Arcton 114 in the ratio 80:20, by weight, at 20°. After 3 months storage	24·7	77·2	98·5	100·0
10% talc III in Arcton 12 and Arcton 114 in the ratio 60:40, by weight, at 20°. After 3 months storage	22·2	74·0	98·8	100·0
10% talc III in Arcton 12 at 20°. After 6 months storage	23·5	88·0	99·6	100·0
10% talc III in Arcton 12 and Arcton 114 in the ratio 80:20, by weight, at 20°. After 6 months storage	19·2	83·1	99·5	100·0
10% talc III in Arcton 12 and Arcton 114 in the ratio 60:40, by weight, at 20°. After 6 months storage	17·1	80·8	98·3	99·9

Vapour pressure (p.s.i.g.) of Arcton 12 and Arcton 114 mixtures:
100% Arcton 12 at 20°, 67·9; 15°, 56·3; 10°, 47·0.
80% Arcton 12 + 20% Arcton 114 at 20°, 59·0.
60% Arcton 12 + 40% Arcton 114 at 20°, 49·0.

The neomycin sulphate sprays were much larger than the powder used in their preparation; drying the powder before pack assembly resulted in a small increase in spray size (Table 3). The UFI powder was micronized before packaging so it seems likely that the spray particles were agglomerates of single particles. The greater the proportion of the high vapour pressure component in the propellent the smaller the UFI spray (see Table 3).

The size of the n-tetradecane sprays (Table 4) decreased as propellent concentration was increased. The use of a valve housing having a vapour tap of 0·025 inch in its side gave a marked reduction in spray size.

The behaviour of a mixture of 6·5% n-tetradecane and 93·5% light liquid paraffin

Table 3. *The effect of propellent composition on the size of UFI sprays and of drying the neomycin sulphate powder on the size of neomycin sulphate sprays.*

Pressurized formulation	% Weight of particles undersize at particle diameter (μm) of: 10	20	30	40	50	60
Commercial UFI pack at 20°	19·1	52·4	81·6	95·4	98·5	99·4
UFI pack containing a higher proportion of the high vapour pressure propellent component than the commercial pack; 20°	32·7	61·2	83·5	95·9	99·6	100·0
10% undried neomycin sulphate in Arcton 12 at 20° C	21·2	60·1	88·0	98·4	99·9	
10% dried neomycin sulphate in Arcton 12 at 20° C	18·1	57·0	85·2	97·9	100·0	

Table 4. *The effect on liquid spray size of propellent composition, valve structure and product viscosity.*

Pressurized formulation	% Weight of particles undersize at particle diameters (μm) of: 20	40	60	80	100	120	140	160	180	200	220	240	260	280
n-Tetradecane and Arcton 12 in the ratio 20:80, by weight, at 20°	20·1	80·9	97·8	99·5	99·7									
n-Tetradecane and Arcton 12 in the ratio 40:60, by weight, at 20°	1·6	28·9	70·8	90·8	97·7	99·5	99·9	100·0						
n-Tetradecane and Arcton 12 in the ratio 60:40, by weight, at 20°. Schiller-Naumann equation used to calculate the size of particles having Re>0·2		0·7	5·1	13·2	25·2	41·9	58·2	70·5	81·0	88·7	93·4	97·0	99·5	100·0
n-Tetradecane and Arcton 12 in the ratio 60:40, by weight, at 20°. Heywood's tables used to calculate the size of particles having Re>0·2		0·7	5·1	12·8	24·9	40·7	56·3	69·1	79·2	87·1	93·1	97·6	99·8	99·9
n-Tetradecane and Arcton 12 in the ratio 40:60, by weight, at 20°. Vapour tap in valve housing ..	15·4	55·5	84·7	96·1	99·3	99·9								
A mixture of 93·5% light liquid paraffin and 6·5% n-tetradecane with Arcton 12 in the ratio 40:60, by weight, at 20°	6·7	37·6	70·8	88·0	95·1	98·6	99·8	99·9						
Light liquid paraffin and Arcton 12 in the ratio 40:60, by weight, at 20°	14·6	43·3	73·5	90·6	97·7	99·6	100·0							

The mixture of 93·5% light liquid paraffin and 6·5% n-tetradecane has a viscosity of 20 cP (20×10^{-3} N s m^{-2}) at 20°. Light liquid paraffin has a viscosity of 27 cP (27×10^{-3} N s m^{-2}) at 20° and n-tetradecane a viscosity of 2 cP (2×10^{-3} N s m^{-2}) at 20°

B.P. was compared with that of light liquid paraffin and that of n-tetradecane to find the effect of liquid product viscosity on spray size. The packs contained 40% liquid product and 60% Arcton 12. Increase in product viscosity led to a decrease in spray size (Table 4).

When the valves of the talc packs were taken apart it was found that powder had accumulated in the valve housings. Comparison of photomicrographs of this talc and of the talc used to prepare the packs showed that the talc from the valve housing contained a larger proportion of particles of 50 μm or more diameter.

DISCUSSION

A reduction in the internal pressure of a pack either by cooling the pack or by changing the composition of the propellent resulted in an increase in spray size. At lower pack pressures, particles suspended in the liquid propellent pass more slowly through the valve where they are subjected to less turbulence and smaller shearing forces. Propellents of lower vapour pressure volatilize more slowly and gently on leaving the valve and thus disperse the powder agglomerates less efficiently. Root (cited by Johnsen, 1961) and Lefebvre & Tregan (1964) reported similar findings for pressurized packs containing a mixed propellent and *liquid* product.

Addition of surfactant to a powder pack may reduce the interfacial tension between the powder particles and the liquid propellent and lead to a better dispersion of powder in the liquid propellent. Propellent surface tension lowering was not investigated; a pronounced effect does not seem likely as the surface tension of organic liquids is small and but little affected by surfactants. Polli & others (1969) found that addition of 0·2% sorbitan trioleate resulted in a decrease in the size of steroid aerosol particles.

Inclusion of 0·5% isopropyl myristate in a talc pack gave larger spray particles. Whilst the isopropyl myristate may marginally lower the vapour pressure of the propellent it probably exerts its action by retarding propellent vaporization and hence powder dispersion (Root, 1961).

The spray from the pack containing unsieved talc had fewer particles with diameters greater than 40 μm than did the unsieved talc used to prepare the pack. The photomicrographs of the talc from the valve housing indicated some retention of large particles. This effect could also explain the reduction in talc spray size following storage.

For packs containing liquid propellent and a miscible liquid product the higher the concentration of propellent the smaller was the liquid spray. The higher the concentration of propellent the greater the work of dispersion per unit volume of product and therefore the finer the spray produced. The propellent vapour pressure will be progressively lowered by increasing concentration of dissolved product. Hence for higher concentrations of liquid product the vaporization energy will be less and the dispersion of the product less vigorous. Lefebvre & Tregan (1964) found that for a kerosene spray having a propellent of Arcton 11 and Arcton 12 the spray became finer and more uniform with increasing propellent concentration up to 78% propellent.

By using a valve housing with a vapour tap smaller powder and liquid sprays were produced. The vapour tap enables propellent vapour to be drawn into the valve by liquid passing through the valve. Turbulence inside the valve is thus increased and spray size reduced.

No wholly satisfactory explanation can be given for the reduction in spray size associated with increased product viscosity. The viscosity of the product-propellent mixture must be related, though not necessarily directly, to the viscosity of the product. There was little difference between the surface tension values for the three liquids, determined by the Du Noüy Tensiometer, and the spray size differences cannot be explained on this basis. The three liquids may not lower the vapour pressure of the propellent the same amount but this was not investigated. Searls & Synder (1936), using an oil atomizer, showed that increasing the viscosity of the oil reduced the size of the spray drops. The amount of oil sprayed by their atomizer during any time interval decreased as product viscosity increased, i.e. as product viscosity was increased a given volume of air dispersed a smaller volume of liquid. In each of our liquid spray packs the same volume of liquid was dispersed by the same volume of propellent. Further, our observed effects of viscosity on spray size do not agree with the suggestion of Wiener (1958) and Root (1961).

Acknowledgements

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REFERENCES

British Standard No. 3406 (1963). Methods for Determination of the Particle Size of Powders, Part 2: Liquid Sedimentation Methods.

Chemical Specialties Manufacturers Association (C.S.M.A.) (1966). Aerosol Guide, 5th edn, New York.

COULSON, J. M. & RICHARDSON, J. F. (1968). *Chemical Engineering*, Vol. 2, London: Pergamon.

HERZKA, A. & PICKTHALL, J. (1966). *Pressurized Packaging (Aerosols)*, 2nd edn, London: Butterworth.

HEYWOOD, H. (1948). *J.I.C. Chem. Engng Soc.*, **4**, 17–27.

International Critical Tables (1929). International Critical Tables of Data, Physics, Chemistry and Technology, Vol. 5, London: McGraw-Hill.

JOHNSEN, M. A. (1961). *Aerosols: Science and Technology*, Chapter 7. Editor: Shepherd, H. R. New York: Interscience.

LEFEBVRE, M. & TREGAN, R. (1964). *Parfums Cosmét. Savons*, **7**, 276–292.

NORMAN, J., LEDSOME, J. R. & LINDEN, R. J. (1965). *Br. J. Anaesth.*, **37**, 466–479.

POLLI, G. P., GRIM, W. M., BACHER, F. A. & YUNKER, M. H. (1969). *J. pharm. Sci.*, **58**, 484–486.

ROOT, M. J. (1961). *Aerosols: Science and Technology*, Chapter 8. Editor: Shepherd, H. R. New York: Interscience.

SCHILLER, L. & NAUMANN, A. (1933). *Ver. deut. Ing.* **77**, 318.

SEARLS, E. M. & SYNDER, F. M. (1936). *J. econ. Ent.*, **29**, 1167–1170.

TANASAWA, Y. & HIROYASU, H. (1962). *Tech. Rep. Tohoku Univ.*, **27**, 67–89.

WIENER, M. A. (1958). *J. Soc. cosm. Chem.*, **9**, 289–297.

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Dissolution rate studies using the B.P. disintegration apparatus

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The application of the B.P. disintegration apparatus in dissolution rate studies has been examined. The dissolution profiles of some brands of chloramphenicol capsules and tolbutamide tablets have been examined at various tube speeds with and without the guided disc. For both drugs a tube speed of 20 strokes/min gave the optimum difference in dissolution rates. The effect of the guided disc depended on the disintegration time of the product tested. For poorly disintegrating products the use of the guided disc resulted in about twofold increase in dissolution whilst insignificant effect was found for products with fast dissolution rate.

It is well recognized that the dissolution of solid drugs can be the rate limiting step in the absorption process. Various types of dissolution apparatus have been suggested (Hersey, 1969) in an attempt to introduce a dissolution test for routine work. The widespread availability of the disintegration apparatus makes it ideally suited for providing constant and reproducible agitation necessary for a dissolution test. Many workers have used the U.S.P. disintegration apparatus in dissolution studies (Schroeter, Tingstad & others, 1962; Middleton, Davies & Morrison, 1964; Withey & Mainville, 1969). No data were reported on the optimum conditions of the test.

This paper examines the effects of tube speed and the guided disc on the dissolution rates of some brands of chloramphenicol capsules and tolbutamide tablets. These drugs were selected because of the reported variations in absorption and dissolution rates (see Aguiar, Wheeler & others, 1968, for choramphenicol capsules and Levy, 1964, and Varley, 1968, for tolbutamide tablets).

MATERIALS AND METHODS

Materials

Four commercial brands of chloramphenicol capsules and three brands of tolbutamide tablets were purchased. These were designated as products A, B, C, D for the capsules and I, II, III for the tablets.

Apparatus

The apparatus used was a standard unit of the B.P. distintegration apparatus equipped with a variable speed motor to control the tube speed.*

The dissolution medium (800 ml) was placed in 1 litre beaker (18 cm high and 10·5 cm i.d.). The tube, at its highest position, just touched the liquid and at the lower position it was 5 cm from the bottom of the beaker. During one complete stroke, the tube travelled 7 cm.

* M. R. Suppliers Ltd., London: rev/min 20, B.S.S. 170 + rheostat. The motor needed running about 30 min to give controlled speeds of 45 ± 2, 30 ± 2, 20 ± 2, 15 ± 1, 10 ± 1 rev/min (as checked every 10 min).

Dissolution media

Hydrochloric acid (0·1N) and a 0·05M phosphate buffer (pH 6·8 ± 0·02) were used for chloramphenicol and tolbutamide respectively. The buffer was used on account of the poor solubility of tolbutamide in 0·1N hydrochloric acid.

All experiments were made at 37° ± 0·1°.

Procedure

At zero time one capsule (or tablet) was placed in the tube and the apparatus operated. At various time intervals, a 5 ml aliquot was sampled using a pipette fitted with a glasswool filter. No solid particles were observed in the aliquots when examined microscopically. Fresh volumes of either 0·1N hydrochloric acid or the phosphate buffer at 37° ± 0·1° were added to compensate for the aliquots withdrawn. Adjustments were made in the calculation for removal and replacement. Determinations of chloramphenicol and tolbutamide were made spectrophotometrically at 278 and 227 nm respectively. The 100% dissolution was obtained by stirring the content in the beaker at the end of the experiment. Four replicate experiments were made and averaged.

RESULTS

Fig. 1 shows representative plots showing the dissolution rates of the various brands of chloramphenicol capsules and tolbutamide tablets examined at a tube speed of 30 strokes/min. No correlation was found between the disintegration time of a brand and its dissolution rate. This is shown in Table 1, where both tablets I and II have approximately the same disintegration times but differed widely in their dissolution rates (Fig. 1b). However, delayed disintegration was often coupled with

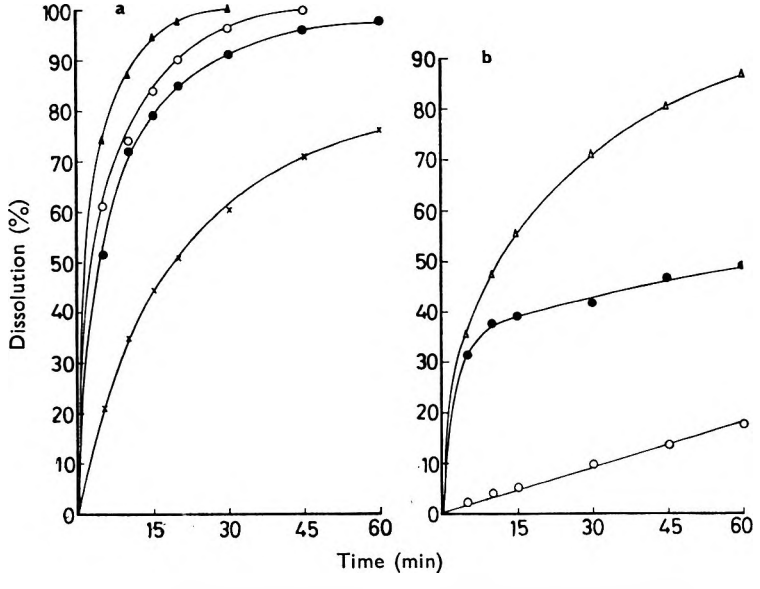

FIG. 1. a. Dissolution rate of chloramphenicol capsules in 0·1 N HCl at 37° ± 0·1°. Speed: 30 strokes/min (no guided disc). ▲—▲ capsule A; ○—○ capsule B; ●—● capsule C, ×—× capsule D. b. Dissolution rate of tolbutamide tablets in a 0·05M phosphate buffer (pH 6·8) at 37° ± 0·1. Speed: 30 strokes/min (no guided disc). △—△ tablet I; ●—● tablet II; ○—○ tablet III.

Table 1. *Disintegration times (in min) at various tube speeds with and without the guided disc*

Tube speed*	Capsules A	B	C	D†	I	Tablets II	III‡
10	12	—	—	>60	2·5	—	>60
15	9	—	—	>60	2·5	—	>60
20	7	5	14	>60	2·5	2	>60
30 without disc	5	5	11	35	2	·5	>60
30 with disc ..	3	2·5	6	5	1	1	>60
45	5	4	7	35	1·5	1	>60

* No. of complete strokes/min.
† This brand gave large aggregates on disintegration at all speeds.
‡ This brand remained as a non-disintegrating disc at all speeds.

poor dissolution as with capsule D and tablet III. Capsule D yielded on disintegration few large aggregates which settled at the bottom of the beaker whilst tablet III failed to disintegrate over 1 h. For tablet III the dissolution curve was linear (Fig. 1b) similar to the data reported for the dissolution of non-disintegrating discs.

Effect of tube speed

The effect of tube speed on dissolution was studied on capsules A and D (Fig. 2a) and tablets I and III (Fig. 2b). An increase in tube speed resulted in a faster dissolution and consequently shorter times for a particular percentage of dissolution. Table 2 shows, at various tube speeds, the calculated values for t90%, t50% and t10% (times for the respective percentages of dissolution). The increase in the tube speed also affected the initial dissolution. Fig. 2a shows that, for capsule A, about 30%

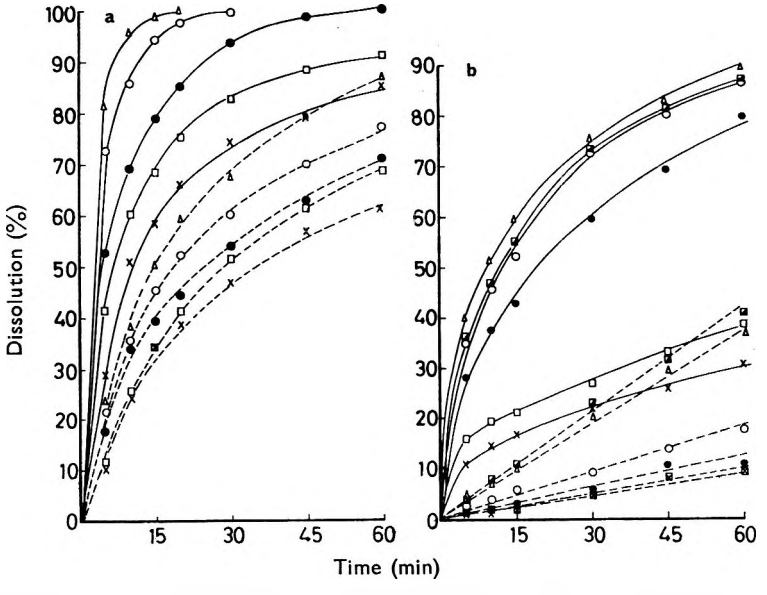

FIG. 2. a. Effect of tube speed on the dissolution rate of chloramphenicol capsules A (——) and D (– – –). Tube speed in strokes/min. (×) 10; (□) 15; (●) 20; (○) 30; (△) 45.
b. Effect of tube speed and the guided disc on the dissolution rate of tolbutamide tablets I (——) and III (– – –). Tube speeds in strokes/min. (×) 10; (□) 15; (●) 20; (○) 30; (△) 45; (◪) 30 with disc.

Table 2. *Calculated values of the times (in min) for 90%, 50% and 10% dissolution at various tube speeds*

Tube speed*	t 90% Capsule A	t 50% Capsule D	t 50% Tablet I	t 10% Tablet III
10	>60 min	35	>60	57
15	50	28	>60	~57
20	25	25	21	48
30 without disc	12	19	12	31
30 with disc ..	7	11	11	14
45	7	15	9	15

* No. of complete strokes/min.

increase in dissolution occurred after 5 min when the tube speed was increased from 20 to 45 strokes/min. The poorly disintegrating capsule D, on the other hand, showed only about 7% increase. It is probable that the increase in tube speed has aided the dispersion of the fine particles produced from capsule A but did not affect significantly the dispersion of the quickly sedimenting aggregates of capsule D. The dissolution of the non-disintegrating tablet III followed the same pattern at various tube speeds, where linear plots were obtained (Fig. 2b). This suggests that variations in the tube speed did not influence the mechanism of dissolution of this brand. The increase in dissolution at higher tube speeds may be attributed to the rapid erosion of the tablet surface.

Effect of the guided disc

The effect of the guided disc on dissolution rate was studied on tablets I and III (Fig. 2b) and capsule A, B and D (Fig. 3). The tube speed selected was 30 strokes/min as specified in the B.P. test for disintegration. As shown in Fig. 2b, the use of the guided disc resulted in an insignificant effect on the dissolution of the fast dissolving tablet I. For capsules A and B only a slight increase in dissolution occurred (Fig. 3). The effect of the disc, however, was well pronounced in the dissolution of the non-

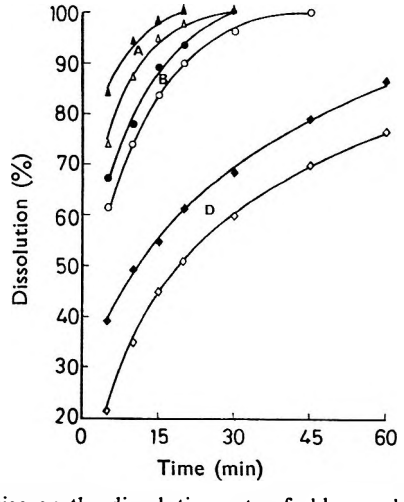

FIG. 3. Effect of guided disc on the dissolution rate of chloramphenicol capsules A, B and D. Speed: 30 strokes/min. △, ○, ◇, without the disc; ▲, ●, ◆, with the disc.

disintegrating tablet III and the poorly disintegrating capsule D. For tablet III about twofold increase in dissolution was observed. The mechanical effect of the guided disc may be responsible for this increase. It was observed that although the use of the disc did not affect the disintegration time of this brand III (Table 1), yet fragments of the tablet were formed and passed into the dissolution medium.

Area under dissolution curve at various tube speeds with and without the guided disc

The areas under the dissolution curves were calculated for the fastest and slowest dissolving products (capsules A and D and tablets I and III). Area measurement was made on graph paper by counting squares. Fig. 4 shows at various tube speeds, the ratios of area for capsule A to that of capsule D and for those of tablet I to tablet III.

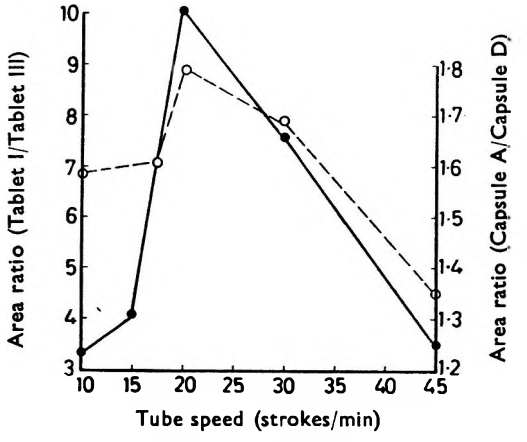

FIG. 4. Effect of tube speed on the area ratio of tablet I/tablet III (●—●) and capsule A/capsule D (○ - - ○).

A tube speed of 20 strokes/min gave the highest ratios for both drugs and therefore it was regarded as the best discriminating speed. When the guided disc was used at tube speed 30 strokes/min the ratio: area for tablet I to area for tablet III was decreased from 7·51 (Fig. 4) to 3·10. This is due to the pronounced increase in the dissolution of tablet III compared to tablet I (Fig. 2b), in the presence of the disc. The ratio for capsules A/D fell from 1·68 (Fig. 1) to 1·46 with the disc.

DISCUSSION

The suitability of the B.P. disintegration apparatus in discriminating the dissolution rates of the various brands tested is seen from Fig. 1. Although the U.S.P. disintegration apparatus has been used by many workers, no critical evaluation of the speed of agitation was reported. A tube speed of 30 strokes/min, as specified in the disintegration test, has been currently used without justification. In the study of Marcus (1969) the speed produced by the U.S.P. apparatus could not differentiate between the dissolution rates of four tablet formulations of different absorption characteristics. When a milder agitation technique was used, variations in the dissolution rates were observed. In the present work a tube speed of 20 strokes/min gave the best discrimination between the dissolution rates of the brands tested. This speed, therefore, is regarded as the optimum speed. Speeds higher than twenty pro-

duced marked agitation of the particles in the beaker. This enhanced dissolution of both fast and slow dissolving brands and subsequently the area ratios were low (Fig. 4). At speeds lower than twenty the particles resting at the bottom of the beaker were not subjected to visible agitation and remained unstirred. This suppressed dissolution particularly of the brands with good dissolution characteristics. Brands with poorer dissolution, on the other hand, were less affected and therefore lower area ratios were obtained (Fig. 4).

Although the B.P. permits the repeat test using the guided disc in the disintegration test for tablets, its use is not permitted for the capsules (B.P. 1968). In the dissolution test, it appears that the use of the guided disc increases the dissolution of both tablets and capsules through its mechanical effect. This effect was more pronounced in the poorly disintegrating products as tablet III (Fig. 2b). The use of the guided disc may, therefore, lead to poorer discrimination between fast and slow dissolving products. The ratios of the areas under dissolution curves produced, at 30 strokes/min, with (1·46 for capsules A/D 3·10 for tablets I/III) and without (1·68 for capsules A/D and 7·51 for tablets I/III) the guided disc show this effect. Campagna, Cureton & others (1963) have also found that omission of the disc of the U.S.P. apparatus gave dissolution data for prednisone tablets which agreed with *in vivo* results.

Beside the tube speed and the guided disc, other factors may affect the dissolution test using disintegration apparatus. Of these factors mention may be made to the mesh number of the wire of the tube and the distance between the bottom of the tube and the beaker on the downward stroke. This latter factor seems to markedly influence the stirring action imparted to the particles which have passed through the mesh to the beaker.

REFERENCES

AGUIAR, A. J., WHEELER, L. M., FUSARI, S. & ZELMER, J. E. (1968). *J. pharm. Sci.*, **57**, 1844–1850.

BRITISH PHARMACOPOEIA (1968). p. 1368, London: The Pharmaceutical Press.

CAMPAGNA, F. A., CURETON, G., MIRIGIAN, R. A. & NELSON, E. (1963). *J. pharm. Sci.*, **52**, 605–606.

HERSEY, J. A. (1969). *Mfg Chem.*, **40**, 32–45.

LEVY, G. (1964). *Canad. med. Ass. J.*, **90**, 978–979.

MARCUS, A. D. (1969). *Am. J. pharm.*, **141**, 28–38.

MIDDLETON, E. J., DAVIES, J. M. & MORRISON, A. B. (1964). *J. pharm. Sci.*, **53**, 1378–1380.

SCHROETER, L. C., TINGSTAD, J. E., KNOECHEL, E. L. & WAGNER, J. G. (1962). *Ibid.*, **51**, 865–874.

VARLEY, A. B. (1968). *J. Am. med. Ass.*, **206**, 1745–1748.

WITHEY, R. J. & MAINVILLE, C. A. (1969). *J. pharm. Sci.*, **58**, 1120–1126.

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LETTERS TO THE EDITOR

NOR_2Chlorpromazine sulphoxide, a "pink spot" produced *in vivo* and *in vitro* from chlorpromazine

Friedhoff & Van Winkle (1962) examining the urine of patients with schizophrenia, reported a substance characterized by its R_F values in different chromatographic systems and by a positive reaction with ninhydrin and thereafter a pink colour with Ehrlich reagent ("pink spot"). The authors claimed this substance to be 3,4-dimethoxyphenylethylamine (DMPE). Closs, Wad & Ose (1967) thought that the "pink spot" was not DMPE but rather the main metabolite of chlorpromazine (CP) excreted in human urine, 2-chloro-1,5-oxide-10-(3-aminopropyl)phenothiazine (NOR_2CPSO) (Fishman & Goldenberg, 1960).

In this report we present the final analysis of "pink spot" and also an *in vitro* experiment with rabbit liver which transforms CP to "pink spot".

"Pink spot" was isolated (Friedhoff & Van Winkle, 1962) from the urine of a patient who received chlorpromazine daily. The substance was fractionated on thin layers of silica gel using acetone–1-butanol–ammonia (25% aqueous) (70 : 5 : 1), extracted with alkali (NaOH at pH 10) and this solution was then further extracted with chloroform. The extract was evaporated to dryness and the residue purified chromatographically on thin-layer silica gel plates using chloroform–2-propanol–acetic acid–water (45 : 35 : 20 : 5). Fifteen μg of the isolated substance gave a distinct ninhydrin-positive spot with the same R_F value as DMPE on paper in 1-butanol–acetic acid–water (4 : 1 : 1). But it did not give any fluorescence after treatment with the highly specific fluorescence method of Bell & Somerville (1966) in which as little as 0·03 μg DMPE can be detected, and, unlike DMPE, it became pink with Ehrlich reagent without previous treatment with ninhydrin.

Comparison of the infrared spectra of CP and "pink spot" suggests that the "pink spot" is a sulphoxide and a primary amine derivative of CP. That it is a sulphoxide derivative is also supported by the ultraviolet spectra, the curves for "pink spot" and of CP oxidized with H_2O_2 (Kofoed, Korczak-Fabierkiewicz & Lucas, 1966) being almost identical. There was no similarity between the ultraviolet spectrum of "pink spot" and that of CP. "Pink spot" behaved like a sulphoxide derivative of a phenothiazine when tested with the reagent described by Forrest & Forrest (1960).

No hydroxyl groups could be detected by the characteristic colour reaction described by Huang (1967), and Beckett & Hewick (1967) for hydroxylated derivatives of CP.

The presence of a non-substituted amino-group was confirmed using dansyl chloride, which reacts with hydroxy- and amino-groups (Gray & Hartly, 1963); this gave a fluorescent substance with "pink spot".

Since the reaction with ninhydrin is not specific for primary amino-groups the substance was treated with nitrite, after which the positive ninhydrin reaction disappeared; this is characteristic for primary amines. "Pink spot" cannot be a secondary amine since there was no reaction with the nitroprusside-acetaldehyde reagent of Sweeley & Horning (1957). Finally, the presence of a chlorine atom in the molecule was demonstrated by Beilstein's test. It therefore seems likely that the "pink spot" is identical with NOR_2CPSO.

We confirmed that incubation of CP with a suitable fortified rat liver homogenate did not result in NOR_2CPSO formation (Robinson, 1966; Beckett & Hewick, 1967). According to Gaudette (1956) rabbit liver has the most suitable demethylation enzymes among animal species.

Liver (6 g) from a male rabbit was homogenized in 30 ml of 0·25M sucrose, centrifuged at 25 000 *g* for 15 min at 2° and the supernatant withdrawn and centrifuged at 110 000 *g* for 60 min at 2°. The supernatant was decanted and the sediment dissolved in 6 ml 0·1M phosphate buffer pH 7·4. The reaction medium was prepared according to Hook & Smith (1967) replacing tris buffer with 0·1M phosphate buffer, pH = 7·4. One ml portions of microsome suspension, total volume 3·5 ml, were mixed in 50 ml beakers with 1 mg of CP or CPSO. The solutions were incubated at 35° for 1·5 h in a shaking incubator. The incubation mixture was deproteinized with 1 ml M trichloroacetic acid and centrifuged. The supernatant was made alkaline (pH 10) and extracted with chloroform. The extract was evaporated to dryness, the residue was dissolved in ethanol and applied on thin-layer silica gel plates with NOR_2CPSO, CP and CPSO as standards. A solvent system of acetone–1-butanol–ammonia (25% aqueous) (70:5:1) was used. The chromatogram was visualized first with 0·2% ninhydrin in acetone. Both substrates, CP and CPSO, were transformed to a small extent to NOR_2CPSO. The purple-coloured spots had the same R_F (0·53) as standard NOR_2CPSO. NOR_2CPSO was produced in greater yield when CPSO was used as substrate. The purple colour changed to pink when sprayed with 50% H_2SO_4, which also produced colours with the non-primary amine metabolites of CP formed *in vitro*.

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REFERENCES

BECKETT, A. H. & HEWICK, D. S. (1967). *J. Pharm. Pharmac.*, **19**, 134–136.
BELL, C. E. & SOMERVILLE, A. R. (1966). *Biochem. J.*, **98**, 1c.
CLOSS, K., WAD, N. & OSE, E. (1967). *Nature, Lond.*, **214**, 483.
FISHMAN, V. & GOLDENBERG, H. (1960). *Proc. Soc. exp. Biol. Med.*, **104**, 99–103.
FORREST, I. S. & FORREST, F. M. (1960). *Clin. Chem.*, **6**, 11–15.
FRIEDHOFF, A. J. & VAN WINKLE, E. (1962). *Nature, Lond.*, **194**, 897–898.
GAUDETTE, L. E. (1956). Ph.D. Thesis, Georgetown Univ., Washington, D.C. cit: WILLIAMS, R. T. 1959. *Detoxication Mechanisms*, 2nd edn., p. 723. London: Chapman & Hall Ltd.
GRAY, W. R. & HARTLY, B. S. (1963). *Biochem. J.*, **89**, 59.
HOOK, G. E. R. & SMITH, J. N. (1967). *Ibid.*, **102**, 504.
HUANG, C. L. (1967). *Int. J. Neuropharmac.*, **6**, 1–13.
KOFOED, J., KORCZAK-FABIERKIEWIEZ, C. & LUCAS, G. H. (1966). *Nature, Lond.*, **211**, 147–150.
ROBINSON, A. E. (1966). *J. Pharm. Pharmac.*, **18**, 19–32.
SWEELEY, C. O. & HORNING, E. C. (1957). *J. Am. chem. Soc.*, **79**, 2620.

Pharmacological studies of fluphenazine and nortriptyline in combination in man

Combinations of fluphenazine and nortriptyline are being assessed in the management of patients with anxiety states complicated by depression according to Drs N. G. Lambert and P. V. Pigott (personal communication). Although fluphenazine in single doses of 1 and 2 mg has been shown to have no significant effect on critical flicker frequency (Turner, 1966; Lind & Turner, 1968), it was considered desirable to study the effects of these combinations on central nervous function in man, after single and multi-dose administration, and to compare them with those of an alternative form of treatment, such as diazepam.

Eight healthy volunteers of either sex aged 18–30 years received no other oral or parenteral medication during the week before entering the study or during the experimental period. Pregnant women were excluded. Subjects were randomly allocated, on the basis of two latin square designs, to the following treatments: (1) fluphenazine 0·5 mg + nortriptyline 10 mg, 1 capsule three times daily for 4 days; (2) fluphenazine 0·5 mg + nortriptyline 20 mg, 1 capsule three times daily for 4 days; (3) diazepam 2 mg, three times daily for 4 days; (4) matching placebo capsules, 1 three times daily for 4 days. All subjects received all treatments with 10 days elapsing between treatments.

Immediately before starting each treatment, 3 h after the first dose and 3 h after the last dose the following tests were made: (i) critical flicker frequency (Turner, 1968); (ii) disc dotting (Hedges, Hills & others, 1971); (iii) serial subtraction (Hedges & others, 1971) and (iv) reaction time, using electronic recording apparatus. Salivary volume was measured by the method of Herxheimer & Haefeli (1966) 3 h after the last dose.

The results were subjected to an analysis of variance, the treatment sum of the squares being proportioned to provide independent tests of (*a*) placebo x active drugs, (*b*) diazepam x both combination treatments (*c*) fluphenazine plus nortriptyline 10 mg x fluphenazine plus nortriptyline 20 mg.

The salivary volume showed a significant difference between treatments ($P < 0{\cdot}05$), and this was due to the difference of both combination forms from placebo ($P < 0{\cdot}01$). Although the mean sputum volumes after each combination were not significantly different, the greater fall in volume when compared with placebo was seen, not unexpectedly, with that combination containing nortriptyline 20 mg. The only other statistically significant treatment differences were at the 5% level between the combination containing nortriptyline 10 mg and that containing nortriptyline 20 mg in disc dotting (day 1) and reaction time (day 5). Neither differed from placebo, however, and it is unlikely that these were important, as there was no significant difference in the changes from initial control values to those after treatment on day 1 or day 5.

It would appear, therefore, that while both treatments containing the anticholinergic compound nortriptyline produced a significant reduction in salivary volume, none of the treatments significantly impaired central nervous function as measured by critical flicker frequency, disc dotting or reaction time, either after single-dose administration or after treatment for 4 days. The critical flicker frequency in particular is a sensitive test of central function, permitting discrimination of many centrally acting drugs in single normal therapeutic doses from placebo preparations (Turner, 1968).

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REFERENCES

HEDGES, A., HILLS, M., MACLAY, W. P., NEWMAN-TAYLOR, A. J. & TURNER, P. (1971). *J. clin. Pharm.*, in the press.
HERXHEIMER, A. & HAEFELI, L. (1966). *Lancet*, **2**, 418–421.
LIND, A. N. & TURNER, P. (1968). *J. Pharm. Pharmac.*, **20**, 804.
TURNER, P. (1968). *Br. J. Ophthal.*, **52**, 245–250.

Promethazine on hand-eye co-ordination and visual function

Although it is widely recognized that compounds with central depressant and sedative effects may seriously impair driving ability and other skills, there is a lack of screening tests that will demonstrate such impairment in small numbers of subjects. Measurement of critical flicker frequency (c.f.f.) may show significant changes in visual discrimination induced by single therapeutic doses of many centrally acting drugs (Turner, 1968).

Molson, Mackey & others (1966) described a test that demonstrated significant impairment of hand-eye co-ordination after promethazine hydrochloride (50 mg) in four subjects. The apparatus then used has been adapted slightly to study the effect of promethazine hydrochloride (25 mg) in this test, and other aspects of visual function have also been examined.

The apparatus consists of a rotating metal drum 16 cm long and 14 cm diameter covered with an insulating material. Punched into this material are 224 holes, each 5 mm in diameter, in the form of an irregular spiral. The drum is turned at a constant speed of 8 rev/min by an electric motor. A metal pointer with a graphite top can be moved across the drum by means of a small steering wheel 16 cm in diameter and a shaft mechanism. When the pointer is accurately controlled along the course of the spiral track, an electrical current is completed each time the pointer strikes a hole in the insulating material. The number of such contacts is recorded electrically by a digital counter.

Retinal sensitivity, colour vision, oculomotor balance, pupil diameter and amplitude of accommodation were measured by conventional methods (Bedwell, 1967; Austen, Gilmartin & Turner, 1971).

Six male students, aged 20–22 years, in good health, with visual acuities of 6/4·5 or better in both eyes, and who were receiving no other medication, were given promethazine, 25 mg, orally or a placebo in random order under double-blind conditions. At least one week elapsed between each treatment. Each subject was fully familiarized with the techniques to minimize learning effects, and abstained from stimulants, alcohol and nicotine during the experiments. Measurements were made before and at $1\frac{1}{2}$ and 3 h after administration of the treatment. The tests were made between 12 noon and 3 p.m.

Promethazine produced a significant reduction in the hand-eye co-ordination test score when compared with placebo, which was most marked at 3 h ($d = 16{\cdot}8$, s.e. $= 4{\cdot}23$, $t = 3{\cdot}906$, $P < 0{\cdot}02$). No significant differences were observed on retinal sensitivity, colour vision, oculomotor balance, pupil diameter and amplitude of accommodation.

Turner (1968) found that promethazine (25 mg) produced significant reduction in c.f.f. and the present study has also shown that the same dose impairs hand-eye co-ordination. The absence of effects on the peripheral components of visual function that were measured would suggest that these effects of promethazine are predominantly, if not wholly, central. No subjective effects of sedation were reported during the 3 h of the experiment when changes in co-ordination were observed, although sedation about 6 h after taking promethazine was noted by all the subjects. This suggests that the hand-eye co-ordination test described, like c.f.f., is able to demonstrate central effects of drugs at a time, or at a dose, when subjective evidence of sedation is not noted. For this reason, and because of its simplicity and the few subjects required, it may prove a useful test in the screening of new drugs for effects on the central nervous system.

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REFERENCES

AUSTEN, D. P., GILMAR, B. A. & TURNER, P. (1971). *Br. J. Physiol. Opt.*, in the press.
BEDWELL, C. N. (1967). *Ophthalmic Optician*, 7, 566–568, 616–618, 613.
MOLSON, G. R., MACKEY, J. A., SMART, J. V. & TURNER, P. (1966). *Nature, Lond.*, **209**, 516.
TURNER, P. (1968). *Br. J. Ophthal.*, **52**, 245–250.

Brain acetylcholine and monoamines during experimental catatonia

Disorders of the extrapyramidal motor system occur in man after large doses of chlorpromazine and reserpine. When given to animals in correspondingly large doses, these drugs bring about a state of catatonic immobility. Bulbocapnine, a drug classically associated with experimental catatonia (De Jong, 1945) also evokes extrapyramidal signs in man (Henner, 1928). It is known that chlorpromazine and reserpine affect the function of monoamine-containing neurons in the brain. We have, therefore, measured the concentrations of 5-hydroxytryptamine (5-HT), noradrenaline and dopamine in the brains of rats made catatonic with bulbocapnine, and also in rats subjected to sound-induced seizures, a procedure which is followed immediately by a period of catatonia (Stainbrook & De Jong, 1943). There is also some evidence that the catatonic state in animals might be associated with an excess of free acetylcholine in the brain, since intracerebral injection of acetylcholine or cholinesterase-inhibiting drugs produces catatonia (Feldberg & Sherwood, 1954; Wada, 1962; Kassil, Latash & Ruthman, 1963). Furthermore, remission from catatonic stupor has been obtained both in animals (Sherwood, Ridley & McCullock, 1952) and in man (Sherwood, 1952) by the intraventricular administration of cholinesterase. We have examined the effect of drug-induced and post-seizure catatonia on the concentration of "free" and "bound" acetylcholine and also the total concentration of acetylcholine in rat brain. No assertions have been made concerning the identity and significance of the "free" and "bound" fractions of brain acetylcholine, but a number of drugs have been shown to affect them differently

(Crossland & Slater, 1968). "Bound" acetylcholine probably represents the stores of transmitter, some of which will be contained in synaptic vesicles, whereas the "free" fraction might be thought of as a mixture of three components comprising newly synthesized acetylcholine in cell cytoplasm, acetylcholine that has been released from neurons and some "bound" acetylcholine which must inevitably be released during the homogenization.

Female Wistar rats, 100–120 g, had catatonia produced by intraperitoneal injection of bulbocapnine, chlorpromazine or reserpine phosphate. Control animals received intraperitoneal injection of 0·9% saline solution. The rats were positioned with their front paws resting on a horizontal rod placed 12 cm above the bench surface, and scored as catatonic if they maintained this unnatural position for at least 1 min. The rats observed in the experiments involving post-convulsive catatonia were all of one particular strain of which approximately 60% were susceptible to sound-induced seizures. A brief exposure to the sound of an electric bell produced convulsions, invariably followed by a period of catatonia. For the estimation of total brain acetylcholine, rats were killed by immersion in liquid air. The frozen brains were crushed and the total acetylcholine was extracted with acid-alcohol. "Free" and "bound" acetylcholine was extracted from the non-frozen brains of rats killed by decapitation. These brains were first homogenized in saline solution to extract "free" acetylcholine and then extracted with acid-alcohol to obtain the "bound" fraction (Crossland & Slater, 1968). The acetylcholine in the final extracts was assayed biologically using the neostigminized frog rectus abdominis muscle. Noradrenaline (Anton & Sayre, 1962), dopamine (Anton & Sayre, 1964) and 5-hydroxytryptamine (Mead & Finger, 1961) were assayed in the brains of decapitated rats. Rats injected with bulbocapnine had the 5-HT extracted from the brain by the butanol-heptane extraction procedure and this interfered with the fluorimetric assay. The bulbocapnine was removed, therefore, by shaking the final brain extract for 1 min with two 5 ml volumes of chloroform.

Rats that had been injected with bulbocapnine (50 mg/mg) or chlorpromazine (10 mg/kg) were killed 30 min after injection when the catatonia was most pronounced. After treatment with reserpine (2·5 mg/mg), catatonia was most marked at 12 h. A

Table 1. *Free and bound acetylcholine content of rat brain during catatonia induced by bulbocapnine, chlorpromazine, reserpine and audiogenic seizures.* The rats were killed 30 min after bulbocapnine or chlorpromazine and 12 h after reserpine. The number of animals used are shown in parentheses. Significance of difference from control values:—* $P < 0.05$, ** $P < 0.01$.

Treatment	Brain acetylcholine (μg/g ± s.e.) "Free"	"Bound"	Total
Control	0·21 ± 0·02 (5)	2·92 ± 0·21 (5)	3·13 ± 0·21 (5)
Bulbocapnine (50 mg/kg)	0·24 ± 0·02 (5)	2·86 ± 0·10 (5)	3·10 ± 0·11 (5)
Control	0·22 ± 0·02 (5)	2·91 ± 0·15 (5)	3·12 ± 0·14 (5)
Chlorpromazine (10 mg/kg)	0·19 ± 0·03 (3)	2·56 ± 0·18 (3)	2·75 ± 0·22 (3)
Chlorpromazine (40 mg/kg)	0·14 ± 0·01 (5)**	2·51 ± 0·13 (5)	2·65 ± 0·14 (5)*
Control	0·31 ± 0·07 (5)	2·44 ± 0·15 (5)	2·75 ± 0·17 (5)
Reserpine (2·5 mg/kg)	0·39 ± 0·07 (5)	2·70 ± 0·11 (5)	3·08 ± 0·11 (5)
Control	0·22 ± 0·01 (5)	2·96 ± 0·15 (6)	3·17 ± 0·19 (5)
Audio-seizure (10 min of post-seizure catatonia)	0·19 ± 0·03 (4)	2·39 ± 0·07 (5)	2·52 ± 0·05 (4)

larger dose of chlorpromazine (40 mg/kg) was also used, although this dose produced a flaccid paralysis rather than catatonia. The mean concentration of total acetylcholine showed no statistically significant change in animals rendered catatonic by drugs or by audiogenic convulsions. Bulbocapnine, chlorpromazine and reserpine, in doses sufficient to produce catatonia, were found not to affect either the "free" or "bound" fractions of brain acetylcholine (Table 1). The only significant drug-induced change in brain acetylcholine was that seen after a large dose of chlorpromazine which caused prostration and, at the same time, reduced the "free" acetylcholine and also the total amount (the sum of "free" and "bound"). This is in contrast to the findings of Zeleny, Lindaur & Kosak (1957) who observed a small increase in the total acetylcholine content of rat brain after chlorpromazine. The administration of reserpine has been reported to raise the concentrations of acetylcholine in whole brain (Haas & Wulzinger, 1960; Giarman & Pepeu, 1962) and to cause varying changes in the acetylcholine concentrations of different brain areas (Malhotra & Pundlik, 1959; Beani, Ledda & others, 1966; Malpica, Jurupe & Campos, 1970). We observed only non-significant increases. Convulsive activity induced by various other physical and chemical agents has been consistently shown to result in a fall in cerebral acetylcholine levels (Richter & Crossland, 1949; Torda, 1953; Giarman & Pepeu, 1962). It seems likely, therefore, that in our experiments, the decrease in acetylcholine was a consequence of the audiogenic convulsions rather than of the catatonia. No significant changes were found in the concentrations of cerebral noradrenaline, dopamine or 5-HT during the catatonia induced by bulbocapnine or during post-convulsive catatonia.

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REFERENCES

ANTON, A. H. & SAYRE, D. F. (1962). *J. Pharmac. exp. Ther.*, **138**, 360–375.
ANTON, A. H. & SAYRE, D. F. (1964). *Ibid.*, **145**, 326–336.
BEANI, L., LEDDA, F., BIANCHI, C. & BALDI, V. (1966). *Biochem. Pharmac.*, **15**, 779–784.
CROSSLAND, J. & SLATER, P. (1968). *Br. J. Pharmac.*, **33**, 42–47.
FELDBERG, W. & SHERWOOD, S. L. (1954). *J. Physiol., Lond.*, **125**, 488–500.
GIARMAN, N. J. & PEPEU, G. (1962). *Br. J. Pharmac. Chemother.*, **19**, 226–234.
HAAS, H. & WULZINGER, H. (1960). *Archs int. Pharmacodyn. Thér.*, **128**, 239–252.
HENNER, K. (1928). *J. Neurol. Psychopath.*, **9**, 176–177.
DE JONG, H. H. (1945). *Experimental Catatonia.* Baltimore: Williams & Wilkins.
KASSIL, G. N., LATASH, L. P. & RUTHMAN, E. M. (1963). *Doklady. Biol. Sciences*, **149**, 464–467.
MALHOTRA, C. L. & PUNDLIK, P. G. (1959). *Br. J. Pharmac. Chemother.*, **14**, 46–47.
MALPICA, J. F., JURUPE, J. & CAMPOS, H. A. (1970). *Archs int. Pharmacodyn. Thér.*, **185**, 13–19.
MEAD, J. A. R. & FINGER, K. F. (1961). *Biochem. Pharmac.*, **6**, 52–53.
RICHTER, D. & CROSSLAND, J. (1949). *Am. J. Physiol.*, **159**, 247–255.
SHERWOOD, S. L., RIDLEY, E. & MCCULLOCK, W. S. (1952). *Nature, Lond.*, **169**, 157.
SHERWOOD, S. L. (1952). *Brain*, **75**, 68–75.
STAINBROOK, E. J. & DE JONG, H. H. (1943). *J. comp. Psychol.*, **36**, 75–78.
TORDA, C. (1953). *Am. J. Physiol.*, **173**, 179–183.
WADA, J. A. (1962). *Ann. N. Y. Acad. Sci.*, **96**, 227–250.
ZELENY, A., LINDAUR, V. & KOZAK, J. (1957). *Arch. exp. Path. Pharmak.*, **231**, 593–595.

Effect of cholinergic drugs on the somatosensory evoked potentials

Cortical application of acetylcholine or eserine to the exposed cerebral cortex increases the amplitude of the repetitive after-discharges of the contralateral somatosensory evoked potentials of the rat anaesthetized with pentobarbitone (Bhargava, 1969). These drugs do not produce any effect on the primary complex (short latency positive-negative waves) of the somatosensory evoked potentials. It was suggested that the afferent pathways responsible for the primary complex of the cortical evoked potentials are not cholinergic, whereas those responsible for the repetitive after-discharges following an afferent volley, are cholinergic. The present paper reports the effect of acetylcholine on the somatosensory evoked potentials on prior eserinization of the cortex, to further strengthen this hypothesis.

Male albino rats of CFE strain (Sprague Dawley from Carworth, Europe), 250–300 g, were anaesthetized with pentobarbitone (50 mg/kg initially, then 12 mg/kg half hourly) intraperitoneally.

Computer derived averages of thirty-two consecutive somatosensory evoked potentials from both cortices in response to the ipsilateral and the contralateral stimulation of the forepaws were recorded (Bhargava & Meldrum, 1969). Drugs dissolved in artificial cerebrospinal fluid (CSF) (Bradbury & Davson, 1964) were applied on the somatosensory area through the specially designed cortical cups mounted on the exposed cortices. Manually, the drugs were applied to one cortex while the other cortex was bathed with normal CSF and served as control during the experimental period.

In each experiment 10^{-5} or 10^{-3}M eserine was applied to one cortex for 30 min, and thereafter 10^{-3}M acetylcholine was applied on the same cortex (eserinized) for 15 min.

Cortical application of acetylcholine after prior treatment of the cortex with eserine did not produce any significant effect on the amplitude of the positive and the negative waves of the primary evoked potentials. However, in all experiments, application of 10^{-5}M eserine for 30 min produced an increase in the amplitude of the repetitive after-discharges, which were further augmented when the eserinised cortex was treated with acetylcholine (10^{-3}M). On average, the amplitude of the repetitive after-discharges after 10^{-3}M acetylcholine was 172 ± 82 μV following eserinization of the cortex ($n = 10$), and 75 ± 7 μV in non-eserinized cortex ($n = 15$). In some experiments the augmentation of the repetitive after-discharges was more marked after 5–10 min of washing off the drug, this was often seen when 10^{-3}M eserine and 10^{-3}M acetylcholine was used. The effect of these concentrations of eserine and acetylcholine on the amplitude of the repetitive after-discharges was also less marked.

Fig. 1 shows a typical experiment of this series. 10^{-5}M eserine was applied to one cortex for 30 min, followed by 10^{-3}M acetylcholine for 15 min on the same cortex. Eserine on its own increased the amplitude of the repetitive after-discharges in 20–30 min, which were further augmented 8–12 min after application of 10^{-3}M acetylcholine. The effect on the primary evoked potentials was not significant. Both positive and negative waves were slightly increased after eserine, but were not affected after application of acetylcholine, whereas the amplitudes of the repetitive after-discharges were augmented.

In the control (non-drug treated) hemisphere, cortical potentials following ipsilateral and contralateral stimulation of the forepaws were not affected when eserine and acetylcholine were applied on the other cortex.

Cortical evoked potentials following stimulation of the ipsilateral forepaw also remained constant on both cortices in all experiments.

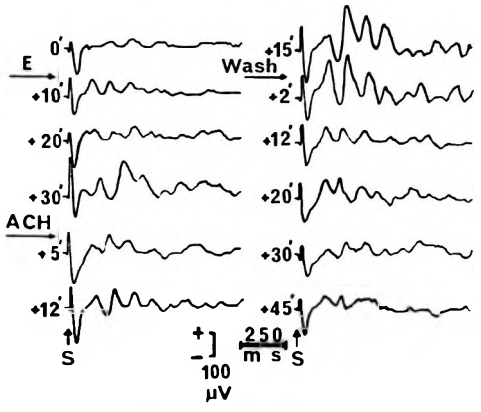

FIG. 1. Each trace is the average of thirty-two consecutive responses recorded during a 1 s epoch following stimulation of the contralateral forepaw. Positivity at the surface of the primary receiving area is shown upwards. Eserine (10^{-6}M) was applied to the right cortex for 30 min followed by acetylcholine (10^{-3}M) to the same cortex for 15 min. Note: that eserine (10^{-5}M) itself increased the amplitude of the repetitive after-discharges, which were further augmented after application of acetylcholine to the same cortex. Calibration 100 μV. Time base 250 ms.

The accumulated evidence presented above is consistent with the existence of functionally significant cholinergic synapses in the cerebral cortex. The somatosensory evoked potentials following an afferent volley from a peripheral nerve consist of an initial positivity and a late negativity of the primary receiving area (Adrian, 1941; Marshall, 1941; Amassian, 1952). The primary evoked response represents the sequence of events in cortex following an afferent volley, the positive and negative waves represent depolarization of the pyramidal cells and the apical dendrites respectively (Bhargava & Meldrum, 1969). The primary evoked potential is followed by a series of positive–negative waves called repetitive after-discharges. These waves occur at a frequency of 6–10/s and arise as a result of successive post synaptic excitatory and inhibitory potentials. The present study supports the view (Bhargava, 1969) that afferent pathways responsible for the primary evoked potentials are not cholinergic, whereas those responsible for the repetitive after-discharges are cholinergic. The repetitive after-discharges following an afferent volley are augmented after cortical application of cholinomimetic drugs, while the primary complex of the somatosensory evoked potentials remains unaffected after such treatment. This effect was best obtained when smaller concentrations of eserine (10^{-5}M) and acetylcholine (10^{-3}M) were employed. A lesser effect was seen with higher concentrations of eserine. This may be due to accumulation of an excessive amount of acetylcholine, resulting in continuous, random firing of cholinoceptive units which may preclude the generation of evoked potentials, or as a result of persistent depolarization of cholinoceptive units due to excessive acetylcholine.

The present study thus also provides electrophysiological evidence for the presence of cholinergic thalamo-cortical pathways, responsible for the augmenting and repetitive after-discharges, as shown by Collier & Mitchell (1966–67), on the basis of their studies on the spontaneous and the evoked release of acetylcholine from the surface of the brain.

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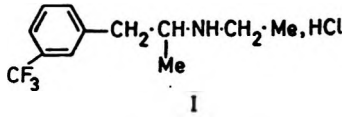
REFERENCES

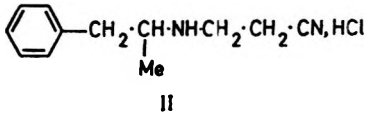
ADRIAN, E. D. (1941). *J. Physiol., Lond.*, **100**, 159–191.
AMMASSIAN, V. E. (1952). *Res. Publs Ass. Res. nerv. ment. Dis.*, **30**, 371–402.
BHARGAVA, V. K. (1969). Ph.D. Thesis Council for National Academic Awards, London.
BHARGAVA, V. K. & MELDRUM, B. S. (1969). *Br. J. Pharmac.*, **37**, 112–122.
BRADBURY, M. W. B. & DAVSON, H. (1964). *J. Physiol., Lond.*, **170**, 195–211.
COLLIER, B. & MITCHELL, J. F. (1966). *Ibid.*, **185**, 22–23*P*.
COLLIER, B. & MITCHELL, J. F. (1967). *Ibid.*, **188**, 83–98.
MARSHALL, W. H. (1941). *J. Neurophysiol.*, **4**, 25–43.

Inhibition by appetite suppressants of the pressor response to (+)-amphetamine in anaesthetized cats

Recently Jespersen & Bonaccorsi (1969a) reported an anti-amphetamine activity of fenfluramine (I) in the isolated tail artery of the rat. These authors observed that the constrictor response to tetrabenazine in the presence of amphetamine was inhibited by fenfluramine in this preparation. At the time of publication we were investigating the effects of another appetite suppressant, fenproporex (II), on the cardiovascular system of the anaesthetized cat where we found that after intravenous administration of fenproporex, (+)-amphetamine failed to produce a substantial pressor response. As a result of this observation we made further experiments which included studies of the effects of both fenproporex and fenfluramine on the pressor responses to tyramine as well as (+)-amphetamine. Cats anaesthetized with sodium pentobarbitone were used and all agents were injected intravenously.



I



II

Fenproporex was shown to have no pressor activity and relatively large doses (3–10 mg/kg) caused marked but transient reductions in blood pressure. When (+)-amphetamine, 0·01–0·1 mg/kg, was administered after a 10 mg/kg dose of fenproporex little or no rise in blood pressure resulted. These doses of (+)-amphetamine produced pressor responses in cats that received no fenproporex, or when given before fenproporex (Fig. 1A). Increasing the dose of (+)-amphetamine did not overcome the inhibitory effect of fenproporex, in fact larger doses (1–10 mg/kg) in the presence of fenproporex caused dose-dependent reductions in blood pressure (see also Fig. 1A). In further experiments, a dose of (+)-amphetamine to produce a marked pressor response (usually 0·03–0·1 mg/kg) was administered before and after various doses of fenproporex. We observed that whereas a 1 mg/kg dose of fenproporex caused only a slight reduction of the (+)-amphetamine pressor response, a 3 mg/kg dose usually caused complete inhibition. At this stage, larger doses of (+)-amphetamine again produced depressor responses.

When the above experiments were repeated using tyramine instead of (+)-amphetamine, no inhibition by fenproporex of the pressor responses to tyramine was observed (Fig. 1B) and in some instances the tyramine response was potentiated. Subsequent doses of (+)-amphetamine (0·1–5 mg/kg) failed to elicit pressor responses, the blood pressure being either unaffected or reduced according to the dose.

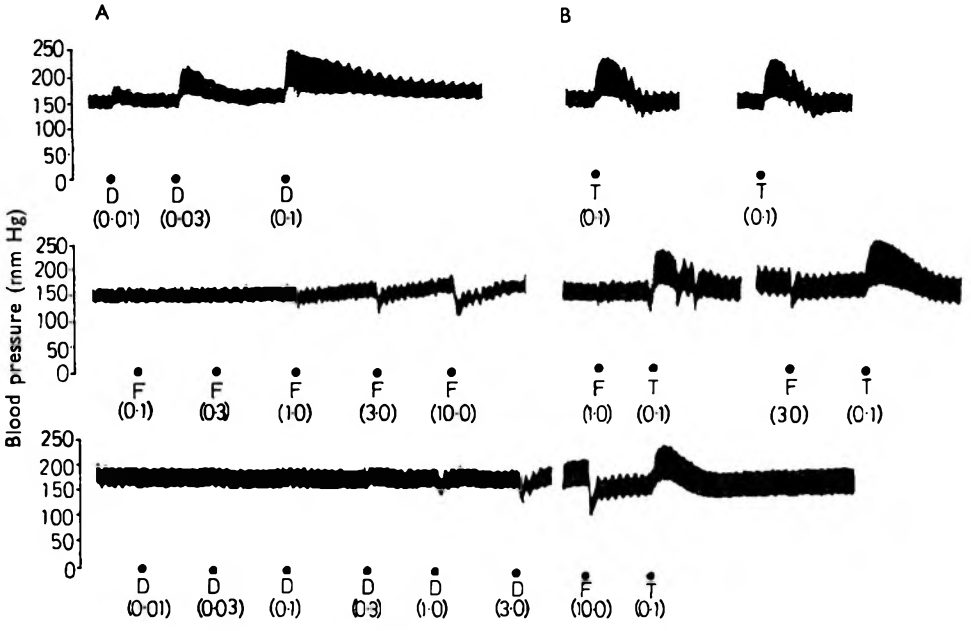


FIG. 1.A. Blood pressure responses to (+)-amphetamine (D) in an anaesthetized cat before (top trace) and after (bottom trace) repeated administration of fenproporex (F, middle trace). Both drugs were administered intravenously. Doses in mg/kg.

B. Blood pressure responses to tyramine (T) in an anaesthetized cat before (top trace) and after (middle and bottom traces) increasing doses of fenproporex (F). Both drugs were administered intravenously. Doses in mg/kg.

Unlike fenproporex, fenfluramine had some pressor activity and doses of 0·3–3·0 mg/kg caused marked elevations in blood pressure after an initial short-lasting fall. Pressor responses to (+)-amphetamine (0·01–0·3 mg/kg) were reduced by fenfluramine; subsequently, higher doses of (+)-amphetamine (1, 2, 3 and 5 mg/kg) produced a prolonged and dose-related fall in blood pressure. Conversely, the pressor response to fenfluramine was reduced by previous administration of (+)-amphetamine. The tyramine response was unaltered or potentiated by fenfluramine.

Both fenproporex and fenfluramine had little effect on the pressor response to noradrenaline at times when the pressor response to (+)-amphetamine had been modified.

Tachyphylaxis to the pressor effects of certain indirectly-acting sympathomimetic amines such as mephentermine or phenylethylamine follows the repeated administration of these agents (Day, 1967); concomitantly, the pressor responses to (+)-amphetamine but not those to tyramine are reduced. Day (1967) suggested that indirectly-acting sympathomimetic amines could be divided into two classes depending on whether their administration reduced the responses to (+)-amphetamine or tyramine.

Fenfluramine is a pressor amine and tachyphylaxis to its pressor response has been reported (Franko, Honkomp & Ward, 1965). However, in the present studies tachyphylaxis to fenfluramine was not a prerequisite to the observation of reduced responses to (+)-amphetamine. Under similar conditions, the pressor response to tyramine was unaltered or potentiated.

The results with fenproporex are interesting as this drug does not raise the blood pressure and yet is very effective in its selective inhibition of the (+)-amphetamine pressor response.

It appears that fenproporex and fenfluramine can interact with and inhibit the mechanism by which (+)-amphetamine but not tyramine releases noradrenaline. Our results lend support to the suggestion by Day (1967) that indirectly-acting sympathomimetic amines may produce their effects by at least two distinct mechanisms.

We are not aware of any previous reports of a dose-dependent reduction in blood pressure to ascending doses of (+)-amphetamine; this phenomenon occurs after pretreatment with either fenproporex or fenfluramine but the underlying mechanism has yet to be elucidated.

In further work it will be interesting to observe if fenproporex and fenfluramine antagonize the stimulant action of (+)-amphetamine on the central nervous system. Jespersen & Bonaccorsi (1969b) have shown fenfluramine to decrease the toxicity of (+)-amphetamine in grouped mice.

We are grateful to Bottu Laboratories of Paris for supplying us with a sample of fenproporex for pharmacological studies.

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REFERENCES

DAY, M. D. (1967). *Br. J. Pharmac. Chemother.*, **30**, 631–643.
FRANKO, B. V., HONKOMP, L. J. & WARD, J. W. (1965). *J. Pharm. Pharmac.*, **17**, 222–226.
JESPERSEN, J. & BONACCORSI, A. (1969a). *Ibid.*, **21**, 776–777.
JESPERSEN, J. & BONACCORSI, A. (1969b). *Europ. J. Pharmac.*, **8**, 364–368.

Modification by a tricyclic series of compounds of the noradrenaline effect on the cat nictitating membrane

There is evidence that the noradrenaline-potentiating action of imipramine and amitriptyline and their desmethyl derivatives is due to their ability to prevent the uptake of noradrenaline by sympathetic nerve endings (Hertting, Axelrod & Whitby, 1961; Iversen, 1965). Another characteristic property of this class of antidepressants is their sympathetic α-receptor blocking action (Sigg, 1959). In an attempt to separate these two actions and determine the importance of the position of the methyl group, the six tricyclic compounds listed in Table 1 have been investigated for their ability to modify the response of the cat nictitating membrane to doses of noradrenaline (2–20 μg/cat).

Cats were anaesthetized with a mixture of chloralose and pentobarbitone. Blood pressure was recorded from the right carotid artery. Changes in the response of the left nictitating membrane to noradrenaline, injected into the left lingual artery retrogradely, by intravenous doses of the tricyclic compounds were measured. In some experiments, the nictitating membrane was chronically denervated by removal of the superior cervical ganglion 21 days previously, a procedure that eliminates uptake of injected noradrenaline into sympathetic fibres (Hertting, Axelrod & others, 1961).

All the compounds modified the noradrenaline action on the intact nictitating membrane; potentiation at low doses, possibly by reducing the uptake of noradrenaline into sympathetic nerve endings, was followed by inhibition at high doses which is indicative of the sympathetic α-receptor blockade. In all the denervated preparations, potentiation of the response was absent and only inhibition was found.

Table 1. *Maximum percentage increase of the cat nictitating membrane to intra-arterial noradrenaline (2–20 μg) produced by intravenous doses of the tricylic series of compounds.* Control responses are taken as 100%. Figures in brackets refer to the number of determinations.

Compound	Dose (mg/kg) base	Increase (% ± s.e.)
Imipramine	0·9	220 ± 46 (7)
Desipramine	0·09	260 ± 29 (7)
Trimipramine	0·02	160 ± 30 (6)
Desmethyltrimipramine ..	0·002	125 ± 7 (5)
Amitriptyline	0·2	160 ± 21 (4)
Nortriptyline	0·5	300 ± 37 (5)

Maximum potentiation for the iminodibenzyl compounds covered a ten-fold range in dosage between the desmethyl derivatives and the parent compounds, imipramine and trimipramine (Table 1). Furthermore, both the β-methyl substituted iminodibenzyl compounds (trimipramine and desmethyltrimipramine) were 45 times more potent than their respective unsubstituted compounds. On the other hand, doses of amitriptyline and nortriptyline producing the maximum potentiation were of a similar order although the degree of potentiation was different. In fact, the potentiation produced by nortriptyline was the highest found with the six tricyclic compounds, probably because it possesses only a weak sympathetic α-receptor blocking action. Besides uptake and blockade of the injected noradrenaline, there may be a local alteration by the tricyclic compounds in the blood flow to the nictitating membrane, although there was no significant alteration in the arterial blood pressure of the cat.

The present results show that the most potent of the tricyclic compounds that potentiated the noradrenaline effect was desmethyltrimipramine, in which the sole methyl group is substituted on the β-carbon atom.

I am grateful to Dr. D. R. Maxwell of May & Baker Ltd., for his encouragement and interest in this work.

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REFERENCES

HERTTING, G., AXELROD, J., KOPIN, I. J. & WHITBY, L. G. (1961). *Nature, Lond.*, **189**, 66.
HERTTING, G., AXELROD, J. & WHITBY, L. G. (1961). *J. Pharmac. exp. Ther.*, **134**, 146–153.
IVERSEN, L. L. (1965). *Adv. Drug. Res.*, **2**, 5–46.
SIGG, E. B. (1959). *Fedn Proc. Fedn Am. Socs exp. Biol.*, **18**, 144.

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A modified competitive protein binding method for measuring plasma progesterone levels

Neill, Johansson & others (1967) described a technique for measuring progesterone in plasma based on the competitive protein binding analysis of Murphy (1964). Tritiated corticosterone is bound to corticosteroid binding globulin (CBG) of dog plasma and subsequently displaced by the addition of progesterone extracted from plasma and separated by thin-layer chromatography. The corticosterone remaining bound to the CBG is counted and the reaction quantitated by comparing these counts with those on a standard curve produced by adding known amounts of progesterone to the CBG-tritiated corticosterone system. The values obtained were corrected for procedural losses by measuring the recovery of tritiated progesterone from plasma subjected to identical extraction and chromatographic procedures—a "parallel standard".

Neill & others (1967) obtained a mean recovery of 75·3%. However, we have found that in some instances there is variation in recovery, even between aliquots of the same plasma sample run on one chromatoplate. An internal standard of tritiated progesterone has therefore been incorporated in each plasma sample and the procedural losses subsequently measured for each sample rather than using a mean value for all the samples on one chromatoplate. The amount of tritiated progesterone added is insufficient to interfere significantly with the subsequent counts of the tritiated corticosterone.

Aliquots (0·1 ml) of a solution containing 2000 d/min tritiated progesterone in ethanol is added to each of 1·5 × 15 cm test tubes and evaporated to dryness under nitrogen on a water bath at 45°. Similar aliquots are added to each of two counting bottles to measure the counts added. Plasma is added to the tubes and after 10 min at room temperature, the progesterone is extracted and isolated as described by Neill & others (1967). Progesterone is eluted from the chromatoplate with methanol and the volume made up to 3·0 ml. One ml is transferred to a counting bottle and hence the recovery of progesterone after extraction and chromatography is measured. The remaining 2 ml is evaporated to dryness and thereafter the procedure of Neill & others (1967) followed.

Table 1 shows plasma progesterone levels, measured in duplicate, in 6 goats at various stages of pregnancy. On one chromatoplate the recoveries are fairly uniform,

Table 1. *Plasma progesterone levels (duplicate samples) before and after correction for procedural losses.*

	Sample		Recovery %	Progesterone (ng) Before correction	After correction
Chromatoplate 1	G	A	70·9	0·6	0·8
		B	78·1	0·8	1·0
	F	A	78·2	3·3	4·2
		B	83·6	3·2	3·8
	M	A	79·1	7·3	9·2
		B	73·7	6·9	9·4
Chromatoplate 2	138	A	69·5	2·4	3·5
		B	21·6	0·7	3·2
	114	A	64·8	1·1	1·7
		B	65·1	1·3	2·0
	940	A	39·9	2·4	6·0
		B	73·1	5·7	5·2

which is the assumption made in the original method. In the second case however, the recoveries are variable, but the use of individual internal standards to correct for procedural losses brings the corrected duplicate values closer.

Progesterone was added to male goat plasma, devoid of any measurable endogenous activity, to give concentrations of 5·0 ng/0·5 ml and 2·4 ng/0·5 ml. When these were assayed the results obtained were 5·3 ± 0·44 (s.d.) with a coefficient of variation of 7·8% (n = 25). In the second case the mean was 2·4 ± 0·18 (s.d.) with a coefficient of variation of 7·6% (n = 15).

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REFERENCES

MURPHY, B. E. P. (1964). *Nature, Lond.*, **201**, 679–682.

NEILL, J. D., JOHANSSON, E. D. B., DATTA, J. K. & KNOBIL, E. (1967). *J. clin. Endocr.*, **27**, 1167–1173.

The effect of anti-inflammatory drugs on the protein-binding of [1,2-^{3}H] cortisol in human plasma *in vitro*

We have shown that protein-binding of endogenous 11-hydroxysteroids measured by a fluorimetric technique in the plasma of rheumatoid arthritic patients is unaffected by the administration of aspirin, indomethacin and phenylbutazone (Stenlake, Davidson & others, 1968). Similar results were obtained in *in vitro* studies for indomethacin, ibufenac and phenylbutazone at plasma concentrations four times the therapeutic level and with supra-normal levels of 11-hydroxysteroids. Under the same conditions, however, aspirin increased and oxyphenbutazone decreased the concentration of unbound 11-hydroxysteroids (Stenlake, Williams & others, 1969). Recent work, however, has shown that in a group of normal subjects non-specific fluorogens consisting of free and esterified cholesterol average 3·4 μg of apparent cortistol/100 ml, equivalent to 22·4% of the total fluorogen present (Stenlake, Davidson & others, 1970). In order, therefore, to confirm our earlier findings, we have studied the effect of anti-inflammatory drugs on the protein-binding of [1,2-^{3}H]cortisol in human plasma.

Plasma pooled from groups of three normal or three rheumatoid arthritic patients, untreated with anti-inflammatory drugs for at least seven days, was added to the dried residue from radiocortisol solutions (1 ml) containing purified [1,2-^{3}H]cortisol (9·7 ng/ml; 2×10^6 d/min ml^{-1}) and non-radioactive cortisol (0·0 or 20 μg/ml), so that the concentration of added cortisol was equivalent to 0 or 50 μg/100 ml of plasma. The mixtures were incubated at 37° (30 min), and aspirin (1·25 or 5·0 mg), ibufenac (0·1 or 0·4 mg), indomethacin (25 or 100 μg), oxyphenbutazone (0·25 or 1·0 mg) or phenylbutazone (0·25 or 1·0 mg) representing therapeutic or four times therapeutic plasma concentrations, was dissolved in separate aliquots (5 ml) of the incubated plasma. The solutions and plasma controls without added drugs were ultra-filtered at 37° for 30 min (Stenlake & others, 1968), and the percentage of unbound radiocortisol determined in each experiment as the mean of three duplicate results.

Table 1. *The effect of anti-inflammatory drugs at therapeutic and supra-therapeutic concentrations on the protein-binding of* [1,2-^{3}H]*cortisol in the plasma of rheumatoid arthritic and normal subjects with endogenous and raised cortisol levels.*

			Unbound [1,2-^{3}H]cortisol[4] %					
Subject[1]	Plasma cortisol[2]	Drug concentration in plasma[3]	Control	Aspirin	Ibufenac	Indomethacin	Oxyphenbutazone	Phenylbutazone
RA	E	T	10·9	11·3	10·8	10·8	10·9	11·0
N	E	T	11·9	12·4	12·1	12·0	12·0	11·8
RA	E	4T	12·2	12·5	11·8	12·3	12·5	12·3
N	E	4T	10·9	11·2	11·0	11·1	10·7	11·0
RA	E + 50	4T	24·2	30·6	23·9	23·9	20·0	23·7
N	E + 50	4T	24·3	31·0	24·1	24·1	18·9	24·3

[1] Normal (N); rheumatoid arthritic (RA).
[2] Endogenous (E); endogenous + 50 μg/100 ml (E + 50).
[3] Therapeutic (T); four times therapeutic (4T).
[4] Mean of three duplicate results in each experiment.

The results (Table 1) show that neither therapeutic nor four times therapeutic drug concentrations have a discernible effect on the protein-binding of [1,2-^{3}H]cortisol at normal endogenous levels of 11-hydroxysteroids. However, at cortisol concentrations raised 50 μg/100 ml above endogenous levels, aspirin at four times therapeutic concentrations increased the unbound radiocortisol by 6·4 and 6·8% in rheumatoid arthritic and normal plasma samples respectively. In contrast, oxyphenbutazone at four times therapeutic concentrations caused decreases of 4·2 and 5·3%, whilst the remaining drugs had no effect. These results agree with those obtained by our previous fluorimetric method.

A second similar series of experiments in which endogenous steroids were removed by the method of Heyns, van Baelen & de Moor (1967) and the drugs dissolved in the plasma before the addition of radiocortisol, was designed to test the postulate (Miller, 1965) that anti-inflammatory drugs do not displace cortisol, but instead occupy vacant cortisol-binding sites and so render them unavailable to newly-available cortisol. The results were essentially the same as those in Table 1, showing that protein-bound anti-inflammatory drugs do not interfere with protein-binding of subsequently added [1,2-^{3}H]cortisol to any greater extent than when they are added to plasma already containing [1,2-^{3}H]cortisol.

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REFERENCES

Heyns, W., van Baelen, H. & de Moor, P. (1967). *Clinica. Chim. Acta*, **18**, 361–370.

Miller, F. P. (1965). M.S. Thesis, George Washington University, p. 37.

Stenlake, J. B., Davidson, A. G., Jasani, M. K. & Williams, W. D. (1963). *J. Pharm. Pharmac.*, **20**, *Suppl.*, 248s–253s.

Stenlake, J. B., Williams, W. D., Davidson, A. G., Downie, W. W. & Whaley, K. (1969). *Ibid.*, **21**, 451–459.

Stenlake, J. B., Davidson, A. G., Williams, W. D. & Downie, W. W. (1970). *J. Endocr.*, **46**, 209–220.

A quick method for testing antituberculosis drugs

Gaugas & Rees (1968) showed that progress of tuberculosis can be very rapid if the immunosuppressive antilymphocytic serum (ALS) is used in thymectomized mice. Here I propose a quick method for screening antituberculosis drugs using the above techniques.

Albino mice about 6 weeks old were thymectomized by sucking out the gland under anaesthesia and used after 14 days. Antilymphatic serum was raised against mouse thymocytes in rabbits (Greaves, Tursi & others, 1969) and assayed by using an agglutination test.

Mice, in groups of 10 were given ALS, 0·2 ml intramuscularly, every alternate day. The infective dose of 1 mg of H37Rv in 0·4 ml was given intravenously, and the drugs were administered on the 4th day. All the drugs were fed orally, except streptomycin which was given intramuscularly. The mortality of the animals was checked till 80% of the animals died.

From preliminary work it was found that more uniform and quicker results were achieved if the mortality of the animals was recorded at 80%, in one week than the customary method of recording 100% mortality. The drugs were administered only once on the 4th day, at which time the growth of the bacilli would be logarithmic. The results (Table 1) are taken to be significant if the survival period was increased from 7 in control to 9 or more in the drug-treated group. Conzelman & Jones (1956) found that the half life of cycloserine in mice was too short to have any effect. This was confirmed.

Table 1. *Time in days to kill* 8 *of ten infected mice.*

Drug	Dose/kg	Days required for 80% mortality
Saline	0·4 ml	7
Isoniazid	20 mg	11
PAS	400 mg	11
Streptomycin	25 mg	12
Cycloserine	100 mg	7
Ethionamide	40 mg	10
Pyrazinamide	20 mg	9
Thiacetazone	40 mg	11

According to Youmans & Youmans (1964) a screening test for *in vivo* bacteristasis should be conducted using animals in which only acute progressive tuberculous disease is manifest, thus eliminating the factor of acquired immunity. So the present method of using immunosuppressed animals would provide an ideal model. The only precaution required is that the animals do not die of other infections.

I thank Dr. N. K. Dutta, Director, Haffkine Institute for the constant interest and encouragement. I also thank Themis laboratories and Unichem Laboratories, India for their supply of antitubercular drugs. The technical assistance of Miss Kushe and Miss Dhairyawan is greatly appreciated.

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REFERENCES

CONZELMAN, G. M. JR. & JONES, R. K. (1956). *Am. Rev. Tuberc. pulm. Dis.*, **74**, 802–806.

GAUGAS, J. M. & REES, R. J. W. (1968). *Nature, Lond.*, **219**, 408–409.

GREAVES, M. F., TURSI, A., PLAYFAIR, J. H. L., GORRIGIANI, G., ZAMIR, R. & ROITT, I. M. (1969). *Lancet*, **1**, 68.

YOUMANS, G. P. & YOUMANS, A. S. (1964). *Experimental Chemotherapy*, Vol. II, pp. 393–499. Editors: Schnitzer, R. J. & Hawking, F. London: Academic Press.

Interactions in phenol–sodium dodecyl sulphate–water systems

Changes in environment resulting from micelle formation and solubilization can be followed by observing chemical shifts in the nuclear-magnetic-resonance spectra of the components. We have used nmr spectroscopy to study interactions between phenol, water and the surfactant sodium dodecyl sulphate.

Spectra were obtained in $D_2O:H_2O$ (60:40) at 35° using a Perkin-Elmer R-12 high resolution spectrometer. Chemical shifts were determined by locking to tetramethylsilane as an external standard and expanding the field to 50 or 100 Hz per chart width; the accuracy of the shifts is within about ±0·2 Hz. Corrections for diamagnetic susceptibilities were made where necessary.

A change of medium from a polarizing to a more inert environment (such as the hydrocarbon environment of a micelle of the surfactant) may cause a considerable high-field shift (Eriksson & Gillberg, 1966). Fig. 1a shows the high-field shift for the phenol ring protons with increasing surfactant concentration. As this concentration increases, the ratio of micellar phenol to free phenol increases. Since the observed chemical shift is the weighted average of the free and solubilized peak positions this gives rise to a high-field shift.

Extrapolation of the phenolic proton shifts to zero chemical shift gives an intercept

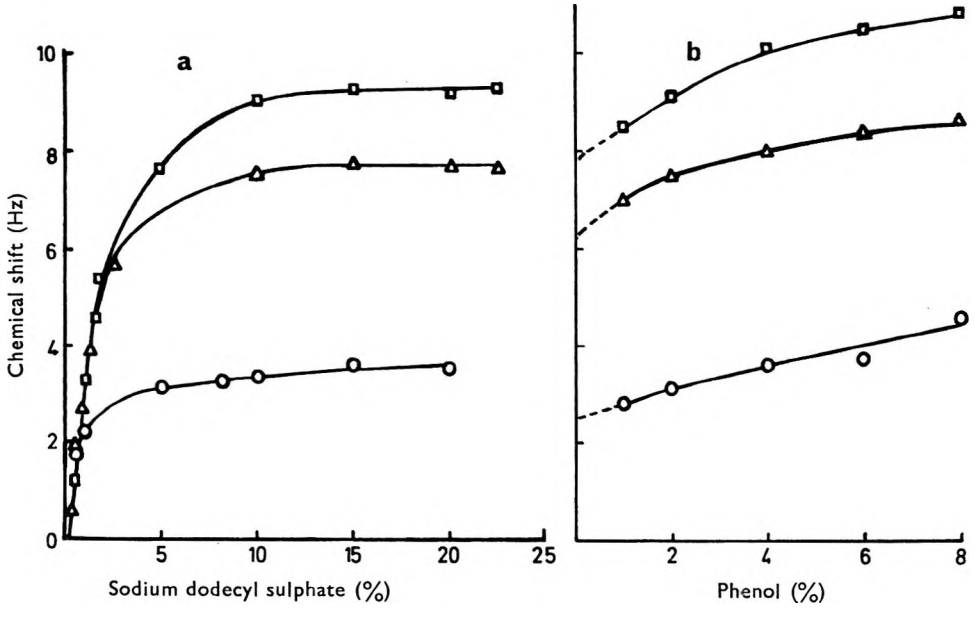

FIG. 1. Chemical shifts of ring protons of phenol: (a) 2% phenol in the presence of varying concentrations of sodium dodecyl sulphate (measured with respect to the peak positions of 2% phenol in water), (b) varying concentrations of phenol in the presence of 10% sodium dodecyl sulphate (measured with respect to the corresponding positions in water). □ *meta* protons; △ *para* protons; ○ *ortho* protons.

at about 0·15% which represents the critical micelle concentration for the surfactant in the presence of 2% phenol at 35°.

The greater high-field shift of the *meta* and *para* protons with respect to the *ortho* protons indicates a greater change in the environment of the former. This could be explained in terms of palisade solubilization of the phenol, with the hydroxyl group directed towards the outside of the micelle.

Fig. 1b shows the high-field shift of the phenol ring protons with increasing phenol concentration in the presence of 10% of the surfactant. Extrapolating to infinite dilution (zero phenol concentration) gives the total shift in terms of shift due to the diamagnetic anisotropic shielding of the aromatic rings and the shift due to the change from an aqueous to a hydrocarbon medium. The high-field shift due to the diamagnetic anisotropy of the aromatic rings indicates that the planes of the rings are randomly arranged and are not stacked or layered on top of one another.

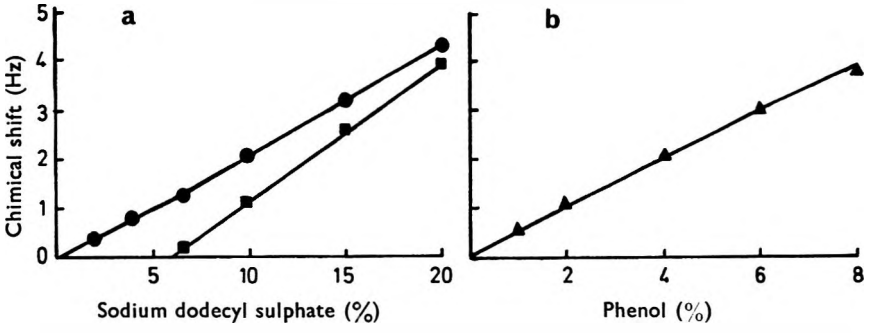

FIG. 2. Chemical shifts of water protons in the presence of (a) ● varying concentrations of sodium dodecyl sulphate (measured with respect to water). ■ 2% phenol and varying concentrations of sodium dodecyl sulphate (measured with respect to water containing 2% of phenol). (b) ▲ Varying concentrations of phenol (corrected for shift contributions by the phenolic hydroxyl protons and susceptibility changes).

It is interesting to note the high-field shift of the water protons in the presence of surfactant (Fig. 2a). This effect was first observed by Clifford & Pethica (1964) and is attributed to disturbance of the water structure by the surfactant. Hydration of the ions tends to oppose this shift and the observed effect is the net result of the two opposing shifts (Pople, Schneider & Bernstein, 1959). The presence of phenol reduces this shift and none is observed until the surfactant concentration reaches about 6%. It is suggested that phenol also disrupts water structure, so that no high-field shift is observed until sufficient surfactant is present to remove most of the phenol to the micellar pseudophase. This postulate is supported by (*a*) the marked high-field shift of water protons in the presence of increasing concentrations of phenol (Fig. 2b), only about half of which can be attributed to the diamagnetic anisotropic shielding of the aromatic rings, and (*b*) by the decrease in the water-peak shift (from 1·6 to 1·1 to 0·5) with increase in phenol concentration (from 1 to 4 to 8% w/v) at constant (10%) surfactant concentration.

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REFERENCES

CLIFFORD, J. & PETHICA, B. A. (1964). *Trans. Faraday Soc.*, **60**, 1483–1490.
ERIKSONN, J. C. & GILLBERG, G. (1966). *Acta chem. scand.*, **20**, 2019–2027.
POPLE, J. A., SCHNEIDER, W. G. & BERNSTEIN, H. J. (1959). *High-Resolution Nuclear Magnetic Resonance*, pp. 449–451, New York: McGraw-Hill.

Absorption of some organic compounds from the biliary system of the rat

The bile is an important pathway of excretion for many drugs and their metabolites (Smith, 1966). However, it is not clear what factors determine their elimination in the bile rather than in the urine. It has been suggested that bile, during its passage down the biliary tract, may be altered in composition by reabsorption and secretion of water and solutes (Andrews, 1955; Brauer, 1959; Sperber, 1959; Goldfarb, Singer & Popper, 1963; Wheeler, 1968). If reabsorption occurs then it could have a decisive bearing on the extent to which a drug appears in bile. It might be postulated, therefore, that substances poorly eliminated in the bile are those that after being cleared by the liver into the primary bile are then reabsorbed as the bile passes along the biliary tract. Conversely, compounds found in large amounts in bile may be relatively poorly reabsorbed from the primary bile.

We have therefore investigated the absorption from the biliary tree of the rat of two groups of organic compounds given by retrograde biliary infusion and selected on the basis of either low or high biliary excretion in this species. Firstly, we examined hippuric acid, 3-aminophenylsulphate, 2-aminophenylglucuronide and succinylsulphanilamide, which are relatively poorly excreted by the rat in the bile, and then stilboestrol glucuronide, phenolphthalein disulphate, phenolphthalein glucuronide and indocyanine green, which are extensively excreted in the bile.

The compounds used were either available in this laboratory or were purchased commercially. They were estimated in bile and urine by procedures previously described thus, [^{14}C]hippuric acid, 3-aminophenylsulphate and 2-aminophenylglucuronide according to Abou-El-Makarem, Millburn & others (1967); succinylsulphanilamide, Millburn, Smith & Williams (1967b), [^{14}C]stilboestrol glucuronide, phenolphthalein disulphate and phenolphthalein glucuronide, Millburn, Smith & Williams (1967a) and indocyanine green, Levine, Millburn & others (1970). Biliary fistulae were established in female Wistar albino rats (210–250 g wt) as described by Abou-El-Makarem & others (1967). In some experiments the renal pedicles were ligated to prevent urinary excretion. The method of retrograde biliary infusion is described in Table 1.

The extent of biliary excretion of the two groups of compounds following their intravenous administration to rats with ligated renal pedicles is shown in Table 1. Those compounds in Group I are poorly excreted in bile in comparison with those of Group II. Thus, the biliary excretion of the compounds in the first group accounted for about 10% or less of the dose in 3 h. By contrast, more than 75% of the dose of the four compounds in Group II appeared in the bile within 3 h. Paper chromatography of bile samples (for details of solvent systems and detection methods see references to analytical methods above) indicated that all the compounds appeared in bile essentially unchanged.

Table 1 also showed that when the four compounds in Group I are given by retrograde biliary infusion less than 20% of the dose is recovered in the bile in 30 min, in fact the values for 3-aminophenylsulphate and succinylsulphanilamide are less than 10%. These compounds appear to be rapidly absorbed from the biliary tract and excreted by the kidneys since following the retrograde biliary infusion of [^{14}C]hippuric acid and succinylsulphanilamide, 47 and 59% of the dose respectively is found in the urine removed from the bladder 30 min after finishing the infusion. With hippuric acid 70% of the dose is recovered in the urine collected after 1 h.

With compounds in Group II relatively large amounts (50–80% of dose) could be recovered in the bile within 30 min of stopping the retrograde biliary infusion (Table 1). These compounds, compared to those of Group I, appear to be relatively poorly

Table 1. *Recovery of some organic compounds from the bile of rats after either intravenous administration or retrograde biliary infusion.* The compounds (20 μmol/kg) were administered to biliary-cannulated female rats either (*a*) intravenously, in which case the renal pedicles were ligated or (*b*) by retrograde biliary infusion using an "Agla" micrometer syringe joined to a short polythene cannula inserted into the common bile duct. In the latter case the dose (0·1 ml) was washed in with 0·1 ml of an NaCl solution (0·9% w/v), the syringe being held in place for 1 min after the injection of the saline before bile collection was commenced. In some experiments the bladder urine was totally removed at 0·5 or 1 h after the infusion. Results are the means of three or more animals; ranges are given in parentheses.

Compound	Molecular weight	(a) Intravenous administration % dose excreted in bile in 1 h	3 h	(b) Retrograde biliary infusion % dose recovered in bile at 5 min	15 min	30 min
Group I						
[^{14}C]Hippuric acid	179	—	5·4 (4·6–5·8)	12 (11–13)	14 (12–15)	14 (13–14)
3-Aminophenylsulphate	189	—	1·0 (0·5–1·5)	5·5 (2·6–10)	6·7 (2·7–12)	7·0 (3·0–14)
Succinylsulphanilamide	272	—	10 (5·5–17)	7·8 (3·4–15)	9·0 (4·2–17)	9·8 (4·8–18)
2-Aminophenylglucoronide	285	—	4·0 (3·5–4·8)	12 (10–14)	13 (11–16)	14 (12–17)
Group II						
[^{14}C]Stilboestrol glucuronide	445	71 (61–80)	92 (89–94)	43 (35–50)	68 (59–76)	78 (66–93)
Phenolphthalein disulphate	479	60 (55–66)	76 (66–79)	31 (16–35)	47 (33–67)	65 (50–87)
Phenolphthalein glucuronide	495	65 (57–72)	96 (87–98)	29 (20–23)	41 (37–60)	65 (56–81)
Indocyanine green	752*	58 (53–68)	78 (68–86)	39 (31–47)	44 (39–53)	53 (39–73)

* Value for anion.

absorbed from the biliary tract for the following reasons. Firstly, after retrograde biliary infusion, only small amounts (less than 2% of dose) of stilboestrol glucuronide, phenolphthalein disulphate and phenolphthalein glucuronide were found in the urine collected at 60 min. Secondly, relatively large amounts (30–40% of dose) can be recovered in the bile within 5 min after stopping the infusion. However, it appears that some absorption from the biliary system followed by excretion of the Group II compounds may occur since the amounts recovered in bile following retrograde biliary infusion slowly increase with time, whereas for those compounds in Group I the amounts found in bile were approximately the same for bile samples collected at 5, 15 and 30 min.

Consideration of the two groups of compounds in terms of the criteria previously suggested for extensive biliary excretion of foreign compounds to occur in the rat points to molecular size and polarity as being important factors in determining the extent of biliary excretion (Millburn & others, 1967a). Large organic anions (molecular weight > 300) are usually extensively excreted in the bile of the rat whereas smaller ones (molecular weight < 300) are not and are eliminated mainly in the urine. The compounds in Group II occur as anions at physiological pH and have molecular weights between 445 and 752 and are extensively excreted in bile. It may be therefore of significance, in relation to their extensive biliary excretion, that these compounds of relatively large molecular weight are comparatively poorly absorbed from the biliary tract. On the other hand all the compounds of Group I exist as polar anions at body pH and have molecular weights less than 300. These relatively small anions are poorly excreted in the bile of the rat and they appear to be extensively absorbed from the biliary tract.

These preliminary observations suggest that the extensive biliary excretion of compounds of relatively high molecular weight may be a reflection of their poor reabsorption from the primary bile. Conversely, organic anions of lower molecular weight can be readily absorbed from the biliary system of the rat and their low

biliary excretion may be a consequence of their extensive absorption from primary bile.

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REFERENCES

ABOU-EL-MAKAREM, M. M., MILLBURN, P., SMITH, R. L. & WILLIAMS, R. T. (1967). *Biochem J.*, **105**, 1269–1274.

ANDREWS, W. H. H. (1955). *Lancet*, **2**, 166–169.

BRAUER, R. W. (1959). *J. Am. med. Ass.*, **169**, 1462–1466.

GOLDFARB, S., SINGER, E. J. & POPPER, H. (1963). *J. Lab. clin. Med.*, **62**, 608–615.

LEVINE, W. G., MILLBURN, P., SMITH, R. L. & WILLIAMS, R. T. (1970). *Biochem. Pharmac.*, **19**, 235–244.

MILLBURN, P., SMITH, R. L. & WILLIAMS, R. T. (1967a). *Biochem. J.*, **105**, 1275–1281.

MILLBURN, P., SMITH, R. L. & WILLIAMS, R. T. (1967b). *Ibid.*, **105**, 1283–1287.

SMITH, R. L. (1966). *Prog. Drug. Res.*, **9**, 299–360.

SPERBER, I. (1959). *Pharmac. Rev.*, **11**, 109–134.

WHEELER, H. O. (1968). In: *Handbook of Physiology, Section 6: Alimentary Canal*, Vol. 5, pp. 2409–2431. Editor: Code, C. F. Washington D.C.: American Physiological Society.

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