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Research Papers

Influence of temperature on the responses of the guinea-pig hypogastric nerve-vas deferens preparation

D. DELLA BELLA, A. GANDINI AND M. PRETI

Over a temperature range of 32 to 20°, responses of the vas deferens to hypogastric stimulation at 50 shocks/sec became gradually smaller, while those to 10 shocks/sec increased gradually. A parallel study, under the same temperature conditions, of drugs known to interfere with peripheral sympathetic transmission revealed a marked reduction of the inhibitory properties of the adrenergic neurone blocking agents bretylium and xylocholine at the lower temperatures. The mechanism by which cooling modified the responses of the preparation to nerve stimulation as well as to the activity of the drugs on the vas is discussed and tentative hypotheses are advanced.

IN recent years the Huković (1961) preparation of the guinea-pig isolated vas deferens has been widely used for the study of peripheral sympathetic transmission and of its modifications by drugs. Apart from the observations of Kuriyama (1964), which concern only the junction potentials produced at different temperatures by hypogastric nerve or muscle field stimulation, we are unaware of any report on the influence of temperature either on the responses of the vas to different stimulation frequencies, or, with the exception of the work of Leach (1956), on the pharmacological activity of known drugs on the vas deferens.

The present paper describes experiments of this kind.

Methods and materials

HYPOGASTRIC NERVE-VAS DEFERENS PREPARATION

Guinea-pigs weighing 350–500 g were used. The preparation, as described by Huković (1961), was set up in a 100 ml organ bath containing Krebs solution gassed with carbon dioxide 5% and oxygen 95%. The hypogastric nerve was placed on shielded platinum electrodes submerged in the bath at a 1·5 to 2 cm distance from the vas and connected to an electronic stimulator. Rectangular pulses, 100 of 0·5 msec duration, were applied at 2 mm intervals, at the alternate frequencies of 10 and 50 shocks/sec; the voltage was supramaximal.

The temperatures quoted in the text are the temperatures, measured directly, of the perfusion fluid in which the organ was submerged.

The following drugs were used: physostigmine (eserine) sulphate, hexamethonium bromide, noradrenaline, isoprenaline hydrochloride, bretylium tosylate, guanethidine sulphate, veratrine, xylocholine bromide, atropine sulphate, mecamylamine hydrochloride, dihydroergotamine methane sulphonate, phenoxybenzamine hydrochloride, phentolamine methane sulphonate, ephedrine sulphate.

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Results

INFLUENCE OF TEMPERATURE ON THE RESPONSES OF THE VAS DEFERENS TO ELECTRICAL STIMULATION

With progressive lowering of bath temperature from 32 to 20°, responses to 50 shocks/sec became gradually smaller, while those to 10 shocks/sec increased (Fig. 1). The phenomenon began in the temperature range

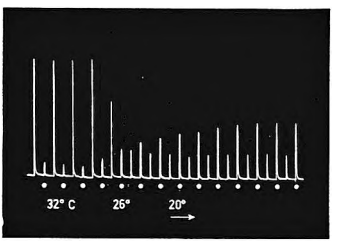

FIG. 1. Contractions of guinea-pig isolated vas deferens in response to hypogastric nerve stimulation. Each stimulation consisted of 100 shocks, applied alternately at the frequency of 50 and 10 shocks/sec (at dots), every 2 min. It may be observed that progressive lowering of bath temperature from 32 to 20° brings about a strong reduction of the responses to the high frequency stimulation, while those to low frequency become markedly increased.

28 to 25°, and became more pronounced the more the temperature was lowered. At 20°, the lowest temperature investigated, the height of contractions reached its minimum and remained constant throughout the experiment. At 20°, the responses to the higher frequency of stimulation appeared to be reduced by 60–80% compared to those at 32°, while the contractions at 10 shocks/sec were increased 5 to 10 fold. A complete and almost immediate reversal of the phenomenon was observed by raising the temperature of the bath to 32° (Fig. 2). In 5 out of 40 preparations

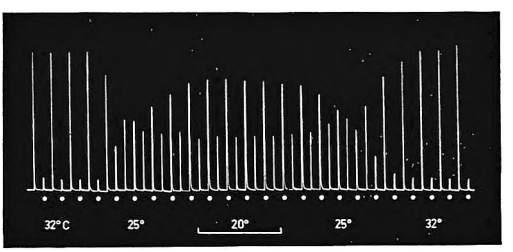

FIG. 2. Same parameters as for Fig. 1. As illustrated in Fig. 1, at 20° the responses to 10 shocks/sec are significantly potentiated and those to 50 shocks/sec greatly reduced. Note the complete reversibility of the effect occurring upon raising the temperature to 32°.

reducing the temperature caused a reduction in the high-frequency responses but did not affect those at low frequency (Fig. 3).

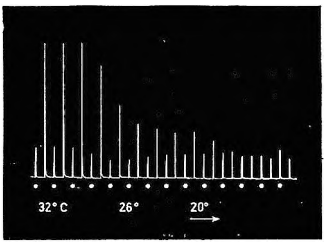

FIG. 3. Same parameters as for Fig. 1. In this preparation lowering of the temperature modifies the responses to the higher frequency of stimulation as previously shown, but leaves those to low frequency unaffected.

INFLUENCE OF TEMPERATURE ON THE RESPONSES OF THE VAS DEFERENS TO DRUGS

Noradrenaline. At 20°, in nearly all experiments the vas deferens was almost unresponsive to direct stimulation with 1–2·5 μg/ml of noradrenaline. The same concentration of noradrenaline caused a marked increase of the responses to hypogastric stimulation at both frequencies, the effect being easily and quickly reversible by washing (Fig. 4).

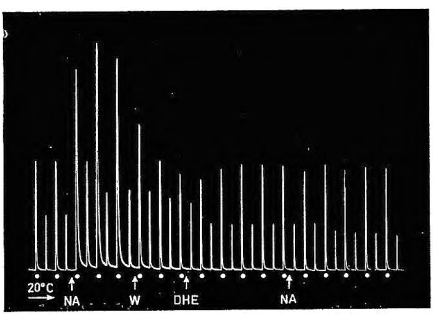

FIG. 4. Same parameters as for Fig. 1. Temperature of the bath, 20°. Addition of noradrenaline to the bath (at NA, 2·5 μg/ml) significantly enhances the responses of the vas to hypogastric stimulation at both frequencies and disappears upon washing (at W). The same dose of noradrenaline, given after dihydroergotamine (at DHE, 1 μg/ml), leaves the height of responses unimpaired.

Adrenergic blocking drugs. Dihydroergotamine, phenoxybenzamine and phentolamine at doses from 0·5 to 10 μg/ml did not reduce the responses of the preparation; in a few instances phenoxybenzamine caused a slight increase (Fig. 5).

In the presence of these drugs, noradrenaline failed to potentiate the responses to stimulation of the hypogastric nerve (Fig. 4). The antagonistic effect did not disappear upon washing out the adrenergic blocking drug; the addition of noradrenaline at this moment sometimes markedly reduced the responses of the preparation to either frequency of stimulation.

Isoprenaline. Concentrations of isoprenaline ranging between 0·25 and 1 μg/ml diminished the responses of the preparation to electrical stimulation. The degree of reduction was reversible upon washing and dependent on the dose used.

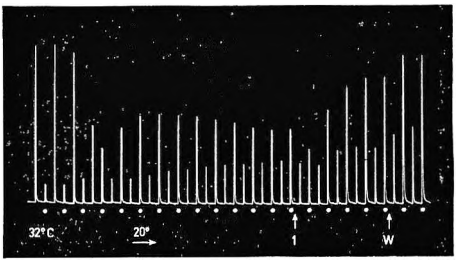

FIG. 5. Same parameters as for Fig. 1. At 20°, treatment with phenoxybenzamine (at 1, 10 μg/ml) enhances the responses of the preparation to the electrical stimulation. At W, washing of the preparation.

Ganglion blocking drugs. Mecamylamine, 5–10 μg/ml, and hexamethonium, 25–50 μg/ml, at 20°, just as at 32° (Sjöstrand, 1962; Birmingham & Wilson, 1963), strongly reduced the responses of the preparation to stimulation of the hypogastric nerve (Fig. 6). The presence of the

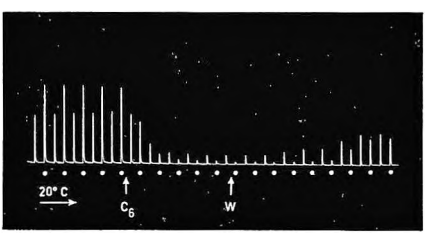

FIG. 6. Same parameters as for Fig. 1. As at 32°, hexamethonium at 20° (at C_6, 50 μg/ml) strongly reduces the responses to both frequencies of stimulation. The effect is more evident for the low-frequency responses. Note the slow reversal of the inhibitory effect of hexamethonium even after washing (at W).

ganglion blocking agent affected the responses at low frequency more than those at high frequency; this was particularly evident for hexamethonium.

Atropine. No modifications of responses occurred upon addition of atropine to the bath at doses from 0·5 to 1 μg/ml.

Physostigmine. At 20°, just as at normal temperature (Boyd, Chang & Rand, 1960; Burn & Weetman, 1963), physostigmine in concentrations ranging from 2 to 5 μg/ml gave rise to a typical enhancement of responses to either frequency of stimulation (Fig. 7). The only difference from the observations at 32° was a longer latency period.

Again as previously described for normal temperature (Della Bella, Benelli & Gandini, 1964), addition of atropine, 0·25–0·50 μg/ml, to a preparation whose responses were potentiated by physostigmine, caused an immediate strong reduction of the responses (Fig. 7).

Veratrine. Veratrine, 0·1–2 μg/ml, produced a reduction, the degree of which appeared related to the dose and which was complete at high concentrations. As with the observations at 32° (Della Bella & Benelli, 1964), no enhancement of responses was observed.

Adrenergic neurone blocking agents. In most of the experiments the responses of the preparation at 20° were reduced by guanethidine at doses

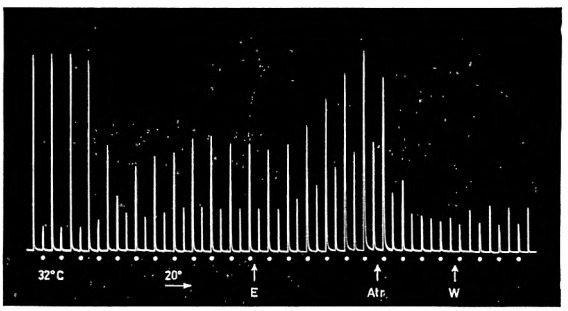

FIG. 7. Same parameters as for Fig. 1. At 20°, physostigmine (at E, 5μg/ml) enhances the responses of the vas to hypogastric stimulation. Addition of atropine (at Atr, 0·5 μg/ml) when the enhancement by physostigmine has developed, causes an immediate reduction in the responses.

of 2·5–10 μg/ml (Fig. 8). But, in three out of nine preparations, guanethidine was completely ineffective. Any reduction seen was less than that observable under the same conditions at 32° and was reversible by 5 μg/ml of ephedrine or 1–2·5 μg/ml of noradrenaline. Pretreatment

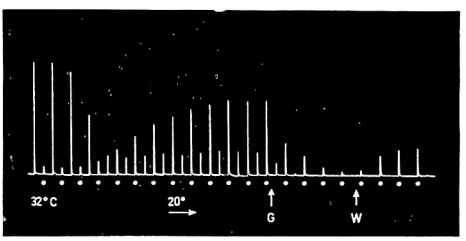

FIG. 8. Same parameters as for Fig. 1. At 20°, addition of guanethidine to the bath (at G, 5 μg/ml) reduces the responses of the preparation to either stimulation frequency strongly. The block appears slowly reversible upon washing (at W).

with the same concentrations of noradrenaline prevented the guanethidine block (Fig. 9). Xylocholine and bretylium partially reduced the responses of the preparation to both stimulation frequencies only at doses 5 to 10

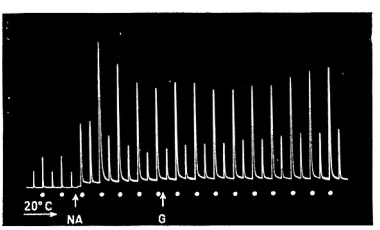

FIG. 9. Same parameters as for Fig. 1. At 20°, addition of noradrenaline to the bath (at NA, 2·5 μg/ml) induces a clear potentiation of the responses to hypogastric stimulation which are unaffected by addition of guanethidine (at G, 5 μg/ml).

times greater than those which blocked at 32°: the loss of activity appeared to be more pronounced for bretylium, which in a few instances potentiated the height of contractions (Fig. 10). Prolonged contact of the preparation

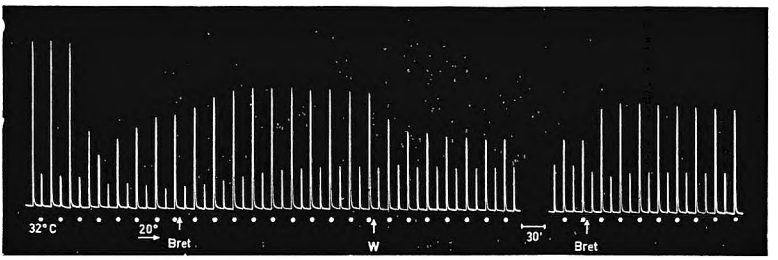

FIG. 10. Same parameters as for Fig. 1. At 20°, unlike at 32°, bretylium (at Bret, 10 μg/ml, then 20 μg/ml) fails to block the responses, which, after the second dose appear potentiated.

with high concentrations of bretylium (20–30 μg/ml) for periods of 30–60 min, did not affect the responsiveness of the vas which, when returned to 32°, responded as usual to hypogastric stimulation.

Discussion

In line with previous findings on a variety of preparations *in vivo* and *in vitro* (Brown, 1954; Malméjac, Neverre & Malméjac, 1956; Kosterlitz & Robinson, 1957; Maclagan & Zaimis, 1957; Mainwood, 1957; Li, 1958; Young, 1959; Della Bella, Gandini & Teotino, 1963), the observations made by us on the guinea-pig isolated hypogastric nerve-vas deferens preparation suggest a pronounced influence of temperature on processes connected with both ganglionic and neuromuscular transmission.

On decreasing the bath temperature from 32 to 20°, the responses to 50 shocks/sec became progressively smaller, being reduced by some 60 to 80% at 20°, and those to 10 shocks/sec were increased 5 to 10 times the initial values.

How the change in temperature produced these changes may be explained in the light of the results obtained by Kuriyama (1964) who used a microelectrode technique in his study of neuromuscular transmission in the vas deferens at different temperatures. At low temperature this worker found the rising and falling phases of junction potentials to be of relatively smaller amplitude but of much longer duration than those at higher temperature. If we consider that a longer refractory period was a consequence of these potential modifications, which in the preparation we examined occur probably at two different levels—ganglionic and neuromuscular, it seems likely that at low temperature the higher frequency of stimulation was insufficient to allow transmission, so that most stimuli were ineffective, while the lower frequency stimuli became of optimal frequency so that all stimuli were effective.

The finding that the responses at low frequency appear higher at 20° than at 32°, may be explained in the light of the following suggestions.

Firstly, if the temperature reduction acted directly on the contractile mechanism to greatly slow its operation rate, though to reduce only moderately the total amount of shortening (Keatinge, 1964), at 20° and at the frequency of 10 shocks/sec there now existed an optimal summation of the single contractions elicited by successive stimulations. It is well known that on *striated* muscle the frequency of stimulation necessary to produce tetanic fusion is markedly decreased by cooling (Maclagan & Zaimis, 1957). In addition, as suggested by Bigland, Goetzee, Maclagan & Zaimis (1958) to explain the relative insensitivity of *striated* muscle to (+)-tubocurarine at low temperature, we cannot exclude that at the lower temperature when there was enhanced height of the vas deferens contractions, there was also a slower diffusion rate of the chemical transmitter from both ganglionic and neuromuscular synaptic spaces. It is known that elimination of chemical mediator from these sites occurs either through enzymatic reactions or by simple diffusion in the medium, and both these processes are shown to possess a high temperature coefficient (Eccles & Jaeger, 1958).

Experiments now in progress in our laboratories, aiming to test the influence of temperature on the onset of direct responses of the vas deferens to noradrenaline, seem to favour this hypothesis: while at 30° noradrenaline elicits immediate responses, at low temperature the onset of contractions is characterised by a latency of 45–50 sec. In our opinion there are grounds for thinking that such a latency period may be due to the reduced diffusion rate of the drug and to delay in its reaching the action site: there would then be a phenomenon analogous to that described above, but the diffusion would be occurring in the opposite direction.

Another mechanism involved in neuromuscular and ganglionic transmission which could be affected by temperature changes, is the synthesis of chemical mediators. We know of no experiments made in this connection on noradrenaline; but in 1956, Kostial & Vouk investigated the effect of temperature on the acetylcholine output after preganglionic stimulation of the cat superior cervical ganglion at 2 shocks/sec. These authors, unlike Brown (1954) who used a frequency of 10 shocks/sec, did not find any variation for temperature values from 39 to 20°. Kostial & Vouk concluded that the slower reaction rate of the choline-acetylating process, which occurred at low temperature, and the resulting shortage of chemical mediator, were likely to be the main causes of the reduced effectiveness of the stimuli at 10 shocks/sec. This hypothesis was later substantiated by results obtained *in vitro* on the enzyme from mammalian brain (Milton, 1958).

Nevertheless, on the neuromuscular synapses of the rat vagus nerve-stomach isolated preparation, we failed to demonstrate an actual diminution of the stores of chemical mediator at low temperature. Parallel to the decrease of temperature from 32 to 20°, responses to electrical stimulation showed a progressive reduction which became a complete disappearance of motor responses. This was not seen with responses to dimethylphenylpiperazinium (DMPP) the stimulating activity of which is exerted through excitation of the ganglionic cell and the subsequent

liberation of acetylcholine at postganglionic nerve endings (Della Bella & others, 1963). Analogous results were obtained by Gillespie & Wishart (1957) in experiments directed to testing the effect of cooling on the responses of the rabbit colon to nerve and to drug stimulation.

If we now examine the behaviour of the vas deferens at 20° under drug treatment, we conclude that the preparation responds to noradrenaline, isoprenaline, adrenergic blocking agents, atropine, physostigmine, veratrine and ganglion blocking agents almost as it did at 32°.

About the effects of noradrenaline, two observations on the preparation pretreated with adrenergic blocking agents are worth stressing. The first concerns the partial and promptly reversible block of responses to noradrenaline in such conditions: a likely explanation for this inhibitory effect could be an indirect ganglion blocking activity exerted through a reduction of the cholinergic mediator output at preganglionic neurone terminals, as demonstrated by Lundberg (1952) on the superior cervical ganglion. The second relates to the complex question of the adrenergic nature of the nervous transmission mechanism at the neuromuscular junction of the vas deferens. It is known that adrenergic blocking agents are more effective against added noradrenaline than against the endogenously released amine, which is generally supposed to be responsible for the responses of the vas to hypogastric stimulation. If the enhancement by noradrenaline of the responses to electrical stimulation observed by us at 20° is to be regarded as subsequent to its uptake into storage sites and to its increased availability, as already suggested by Huković (1961) for the reserpinised preparation, we may make three postulates. These are that the inhibitory effect of adrenergic blocking agents towards such an enhancement may be due either to: (1) a selective antagonism towards the noradrenaline liberated by electrical stimulation from an easily releasable and reconstitutable pool—it is possible that several pools exist (Kuntzman & Jacobson, 1964; Potter, Axelrod & Kopin, 1962; Trendelenburg, 1961), or (2) to a prevention of the uptake of the transmitter at the storage site (Farrant, Harvey & Pennefather, 1964) or (3) to a combination of these mechanisms.

Another interesting result emerging from our study is the reduced adrenergic neurone blocking activity demonstrated at low temperatures by xylocholine and bretylium in particular. The finding, not very different from the observations on (+)-tubocurarine at *striated* neuromuscular synapses (Holmes, Jenden & Taylor, 1951) appears difficult to explain. The fact that after contact with bretylium at 20° the preparation again exhibits a normal responsiveness to the drug upon raising the temperature of the bath to 32°, leads us to suppose that at 20° the drug does not link up as usual with its action site. The failure of the drug to combine with the receptor seems rather surprising, since bretylium is known to be specifically taken up and securely bound by adrenergic fibres (Boura, Copp, Duncombe, Green & McCoubrey, 1960). A possible explanation may be advanced either on the basis of a reduced diffusion and a consequent accumulation of a hypothetical chemical mediator with which bretylium may compete, this by analogy with what is postulated for

(+)-tubocurarine by Bigland & others (1958), or, in line with the suggestions of Holmes & others (1951), it may be that there is some effect of temperature on the drug diffusion process and in particular on the reaction rate and the equilibrium conditions between the drug itself and its receptor.

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Effects of enzymes on *Yucca glauca* Nutt. and other steroid-yielding monocotyledons

G. BLUNDEN,* R. HARDMAN* AND W. R. WENSLEY

A routine assay procedure for steroidal sapogenins in plant tissue is described in which the endogenous enzymes are allowed to function before the tissue is treated with acid. Inhibition of this enzyme action either by disintegration of the tissue in acid or by autoclaving gave a low yield from the leaf, corm, rhizome, tuber or root of the genera so tested (*Agave*, *Aletris*, *Asparagus*, *Smilax*, *Trillium* and *Yucca*). Oven-dried material, in powder, needed to be incubated with water for one day for the full sapogenin-affording enzyme activity to occur. When autoclaved leaf of *Yucca glauca* Nutt. was incubated with commercial cellulase it gave about 80% of the yield of sapogenin afforded by the unheated leaf. The yield is also given when emulsin or pectinase is used instead of the endogenous enzymes.

MONOCOTYLEDONOUS plants, such as species of *Dioscorea*, *Agave* and *Yucca*, are the main source of starting material for the steroid industry. The yield of steroidal sapogenin from the tubers of *Dioscorea belizensis* Lundell, family Dioscoreaceae, is controlled by an endogenous enzyme system (Blunden & Hardman, 1963). The general applicability of such control is now demonstrated in two more families, Amaryllidaceae and Liliaceae, with leaves, roots, rhizomes and corms from six genera: *Agave*, *Aletris*, *Asparagus*, *Smilax*, *Trillium*, and *Yucca*. The extent and nature of the enzyme control is indicated by experiments with *Yucca glauca* Nutt., a member of the Liliaceae found growing in dry soil in the U.S.A. from Iowa and South Dakota to Montana, south to Missouri, Texas and Arizona (Britton & Brown, 1913). Botkin, Shires & Smith (1943) found the plant sufficiently abundant in New Mexico and its leaf yielded sufficient fibre, equal in strength to hemp, for it to serve as a possible source of hard fibre. Marker, Wagner, Ulshafer, Wittbecker, Goldsmith & Ruof (1943) showed that the leaf contained saponins which on hydrolysis yielded sarsasapogenin 0·5% of the dry weight of leaf. The highest yield of this genin isolated from leaf in the experiments described below was 1·94% of the dry weight.

Experimental

The aerial parts of *Yucca glauca* were collected from wild plants in October 1963 from southern Montana and western North Dakota, and in May 1964 from central South Dakota and eastern Wyoming. The outer dead leaves of the plants were removed and discarded. The remaining leaves were stored in a dry room at 21° for up to 6 months, and yellowed during this time. The moisture content of the leaves at the time of assay varied from 3·7–9·5%. Unless otherwise stated this was the leaf used; that it yielded sarsasapogenin was confirmed by the isolation of this steroid. The powdered leaf, after extraction with benzene, was extracted

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with 95% ethanol. The solvent was removed from the ethanolic extract and the residue treated with boiling 2N hydrochloric acid for 30 min. The acid-insoluble material was collected, washed with water, sodium carbonate solution and water until neutral, and after drying was extracted with light-petroleum (b.p. 40–60°). Yellow crystals separated from the petroleum extract; these recrystallised from acetone as colourless prismatic needles, m.p. 198–199°, giving the appropriate infrared spectrum for sarsasapogenin and a mixed m.p. of 198° with that compound.

ASSAY

The yucca leaves from one plant were cut into pieces approximately 5 mm in length and these were well mixed. From this bulk supply about 10 g was taken for each sapogenin determination and about 1 g for each of two moisture determinations. The latter was obtained by drying at 100–105° for 16 hr and the mean value used. One of the following three procedures was used for the sapogenin determination.

Procedure A. The pieces of leaf (10 g) were disintegrated for 5 min in a Waring Blendor in the presence of 70 ml water. The mixture, with water rinsings, was incubated at 37° for 24 hr in a plugged flask. After concentrated hydrochloric acid had been added to make the acid concentration 2N, the mixture was boiled for 2 hr, and cooled. The acid-insoluble material collected at the pump was neutralised by washing with water, 20% sodium carbonate solution and again with water. The residue was dried at 80° overnight, powdered and the sapogenins extracted with light petroleum (b.p. 40–60°) in a Soxhlet apparatus for 20 hr. The petroleum-soluble material (0·1–0·4 g) was acetylated with 2 ml acetic anhydride using the procedure of Wall, Eddy, McClennan & Klumpp (1952) and the benzene-soluble material was assayed in carbon disulphide at a concentration of 2·5–8·5% in a 0·422 mm cell of a Beckman I.R.8 spectrophotometer. The estimation was based on the band at 982 cm^{-1} at which pure sarsasapogenin acetate had the molecular extinction coefficient (ϵ) 5·291.

Procedure B. Procedure A was followed with the exception that 2N hydrochloric acid replaced water when the leaf was disintegrated and subsequently incubated.

Procedure C. The leaf was autoclaved at 121° for 30 min before procedure A was followed.

These assays were also applied to plant material other than *Yucca glauca*. In all instances the suitability of the actual plant specimen for these experiments was easily checked, for saponins by the haemolysis procedure (Blunden & Hardman, 1963) and for sapogenins by thin-layer chromatography (Blunden & Hardman, 1964).

Results

The yields are calculated as the genin acetate from the infrared spectra and expressed as % of the moisture-free plant material: the molecular

extinction coefficients at 982 cm^{-1} for diosgenin acetate and hecogenin acetate were 3·547 and 4·594, respectively.

Using yucca leaf there was no significant difference in the yield of sarsasapogenin when the period of boiling with 2N hydrochloric acid was changed to 1, 3 or 4 hr in assay procedure A.

From the mixed bulk supply of 5 mm pieces of yucca leaf of plant no. 1, each of seven samples were treated by one of the assay procedures in Table 1. The experiments were repeated with leaves from other plants collected

TABLE 1. YUCCA LEAF: SAPOGENIN YIELDS* AFTER VARYING ASSAY PROCEDURES

Plant No.	Month collected	Assay procedure A (Incubation 24 hr)	Assay procedure A with varied incubation periods					Assay procedure B	Increase % Assay A relative to Assay B
			None	1 hr	2 days	3 days	4 days		
1	October	1·70	1·66	1·65	1·64	1·66	1·67	0·81	110
2	October	1·91	1·94	1·94	1·94	1·94	1·92	1·14	68
3	May	0·50	0·32	0·47	0·50	0·49	0·51	0·23	117
4	May	0·90	0·78	0·88	0·89	0·90	0·91	0·62	29
5	May	1·13	1·11	1·12	1·12	1·11	1·13	0·75	51

* As % of moisture-free leaf calculated as sarsasapogenin acetate from the infrared spectra.

from the same and from different areas in October and in the following May. The results are shown in Table 1. The yields obtained by disintegrating the leaf in water were always greatly in excess of those when acid was used. While a high yield of sarsasapogenin was made available after 5 min disintegration in water at 20°, the May-collected leaves, with their lower sapogenin content, needed incubation for up to 1 day at 37° for the maximum yield of genin to be subsequently obtained. One day was chosen as the standard incubation period at 37° in assay procedure A, and adopted in procedures B and C for comparison of the results.

Besides yucca leaf, other steroidal saponin-containing plant material which had been stored at 21° was assayed by procedures A, B and C (Table 2). In every instance procedure A gave a result in excess of that afforded by procedures B and C.

TABLE 2. VARIOUS MONOCOTYLEDONS: COMPARISON OF SAPOGENIN YIELDS* BY ASSAY PROCEDURES A, B AND C

Species	Morphological part	Genin	Assay procedure			Increase % A relative to	
			A	B	C	B	C
Yucca glauca, plant no. 6	Leaf	Sarsasapogenin	1·16	0·62	0·63	87	84
Yucca glauca, plant no. 7	Leaf	Sarsasapogenin	1·31	0·83	0·82	58	60
Asparagus officinalis ..	Root	Sarsasapogenin	2·21	1·64	1·10	35	101
Smilax aristolochiaefolia	Root	Sarsasapogenin	1·65	1·26	1·40	31	18
Smilax aristolochiaefolia	Root	Sarsasapogenin	1·45	1·15	1·23	26	18
Aletris farinosa	Root and rhizome	Diosgenin	0·17	0·06	0·04	183	325
Aletris farinosa	Root and rhizome	Diosgenin	0·29	0·16	0·09	81	222
Trillium erectum	Corm	Diosgenin	0·22	0·16	0·14	37	57
Agave americana	Leaf	Hecogenin	1·94	1·00	0·92	94	111
Agave americana	Leaf	Hecogenin	2·08	1·30	1·18	60	76

* As % of moisture free plant material calculated as the acetate of the genin indicated from the infrared spectra.

For further comparison with earlier results obtained with the tubers of *Dioscorea belizensis* Lundell (Blunden & Hardman, 1963), the leaves of one yucca plant were cut up and appropriately sub-sampled as before and one sample dried for 16 hr at 80°, then powdered so that all the material passed through a No. 60 sieve, and assayed. The results are given in Table 3.

TABLE 3. YUCCA LEAF: SAPOGENIN YIELD* AFTER DRYING AT 80°

Plant No.	Leaf stored at 21°		Leaf dried at 80°, then powdered		Increase (%) when enzymes allowed to function	
	Assay procedure		Assay procedure		Column 2 relative to column 3	Column 4 relative to column 5
	A	B	A	B		
8	0·89	0·50	0·90	0·69	78	30
9	1·94	1·14	1·96	1·44	70	36

* As % of moisture free leaf calculated as sarsasapogenin acetate from the infrared spectra.

Autoclaved leaf mixed with untreated leaf from the same sample was assayed by procedure A; this procedure was also applied to a mixture of untreated leaf and the acid-insoluble material obtained by procedure C from a sub-sample of the same leaf. The results are shown in Table 4.

TABLE 4. YUCCA LEAF: SAPOGENIN YIELD* FROM AUTOCLAVED MATERIAL IN THE PRESENCE OF UNTREATED LEAF.

Plant No.	Assay procedure		Mixture used as starting material in assay procedure A						
			Autoclaved leaf mixed with untreated leaf			Acid-insoluble material by assay procedure C mixed with untreated leaf			
			Weight of leaf g		Yield from mixture	Weight of leaf g		Yield from mixture	Calculated yield if no sapogenin released from acid insoluble material by enzymes
	A	C	Autoclaved	Untreated		Autoclaved	Untreated		
6	1·16	0·63	3·972	4·694	1·16	3·207	4·264	0·94	0·93
7	1·31	0·82	3·980	3·889	1·29	3·553	3·427	1·02	1·05

* As % of moisture free leaf calculated as sarsasapogenin acetate from the infrared spectra.

Samples (10 g) of leaf were assayed by procedure A, by procedure C, and were also autoclaved at 121° for 30 min, then disintegrated in the Waring Blendor for 5 min in the presence of an enzyme (or enzyme mixture) (Table 5), followed by the addition of an equal weight of the same enzyme (or enzyme mixture) after the Blendor had been stopped, and before incubation at 37° for 3 days. All the enzymes were purchased from the Nutritional Biochemicals Corporation, Cleveland 28, Ohio, U.S.A. and were labelled as in their "Technical Specification, 1963." The total weight used for 10 g leaf (moisture content 3·7–9·5%) in each instance is given in brackets: "Cellulase (Tech) 4,000 units per g" (1·0 g); "Emulsin" (0·2 g); "Pectinase" (1·0 g); "Maltase (Standardised 600 *p*-nitrophenyl glucoside units)" (0·2 g); mixture of equal weights of "Cellulase (Tech)" and "Pectinase" (2·0 g). All the added enzymes, except the maltase, caused an increase in the yield compared with that from autoclaved leaf

TABLE 5. YUCCA LEAF: SAPOGENIN YIELD* FROM AUTOCLAVED LEAF IN THE PRESENCE OF THE COMMERCIAL ENZYME NAMED

Plant No.	Assay procedure		Assay procedure C with the named enzyme added after the leaf had been autoclaved				
	A	C	"Cellulase (Tech)"	"Emulsin"	"Pectinase"	"Maltase"	"Cellulase (Tech)" plus "Pectinase"
10	0·97	0·60	0·88 (76)†	0·77 (46)	0·71 (30)	0·63 (8)	—
11	0·50	0·23	0·41 (67)	0·42 (70)	0·32 (33)	0·22 (0)	0·43 (74)
12	0·59	0·26	0·49 (70)	—	0·40 (42)	—	0·50 (73)
13	1·32	0·75	1·11 (63)	—	1·02 (47)	—	1·25 (70)
14	1·30	0·71	—	0·90 (32)	—	0·73 (3)	—
15	1·68	1·16	—	1·41 (48)	—	1·10 (0)	—

* As % of moisture-free leaf calculated as sarsasapogenin acetate from the infrared spectra.
† Figures in brackets are the increases caused by the enzyme named (e.g. column 4 minus column 3), expressed as % of increases caused by endogenous enzyme (column 2 minus column 3).

(Table 5). The increase never brought the yield up to that given by the endogenous enzymes; "Cellulase (Tech)" was about 70% as effective as the endogenous enzymes. The mixture of "Cellulase (Tech)" and "Pectinase" gave about the same result as did "Cellulase (Tech)" alone, as also did "Cellulase (N.B.C.)", a more expensive grade, when used alone.

Discussion

The stem of *Yucca glauca* showed a higher sapogenin content than the leaf (stem 0·92 and leaf 0·41%; stem 1·74 and leaf 1·32%) in the two plants examined. This variation between stems and leaves is in agreement with the findings of Panouse & Mamlok (1963) for *Y. guatemalensis*.

Our present preliminary results indicate a seasonal variation in the sarsasapogenin content of the leaf of *Yucca glauca*. The average sarsasapogenin content of the moisture-free leaf from 10 plants collected in October was 1·1% (range 0·39–1·94%) and for 9 plants collected in the following May 0·75% (range 0·41 to 1·32%).

The leaf of *Yucca glauca* was used to assess the effects of enzyme action. The yield of sapogenin from harvested plant material when the endogenous enzymes were allowed to function (Assay Procedure A) was compared with the yield when the enzymes were prevented from functioning by acid (Assay Procedure B) or heat (Assay Procedure C) (Table 1). These assay procedures were then applied to other plants (Table 2). In each instance the genin named in Table 2 was known to occur in the plant tissue chosen (Jacobs & Simpson, 1935; Marker, Turner, Shabica, Jones, Krueger & Surmatis, 1940; Marker, Turner & Ulshafer, 1940). The results obtained with this range of plant specimens showed the general applicability of endogenous enzyme control on the yield of sapogenin from harvested material. In all instances the plant material afforded a higher yield of steroid when the enzymes were allowed to function (Table 2). The increase for *Yucca glauca* leaf in nineteen plants varied from 29 to 127 % relative to the same material in which the enzymes had been inactivated at the start of the assay.

The yield of endogenous enzyme-afforded sapogenin was not limited by the amount of enzyme activity available: a given weight of yucca leaf which

had been stored at 21° after harvesting, when added to an equal weight of the same leaf which had been autoclaved, afforded sapogenin in yield equal to that from two parts by weight of leaf which had not been autoclaved (Table 4). As with the enzymes of the tubers of *Dioscorea belizensis* (Blunden & Hardman, 1963) those of yucca leaf responsible for a part of the sapogenin yield were destroyed by autoclaving but were not permanently inactivated by being heated at 80° for 16 hr. They showed normal activity when the powder was incubated with water. Plant enzymes are often heat resistant when in the cell-wall-adsorbed state. Neither drying at 80° for 16 hr nor autoclaving at 121° for 30 min affected the sapogenin-affording "substrate"; the normal increase in sapogenin yield occurred when the dried powdered leaf was incubated with water (Table 3) or when untreated leaf was supplied as the enzyme source for autoclaved leaf (Table 4). Acid-insoluble material from the assay of autoclaved leaf (Assay Procedure C) failed to yield additional sapogenin when incubated with a sample of untreated leaf (Table 4). The sapogenin-affording enzymic process was a rapid one. It is likely that most freshly harvested plant material would require only disintegration in water for 5 min for much of the sapogenin resulting from enzyme action to be made available. Incubation with water for a day or so would be necessary when the plant tissue had been oven-dried before being powdered. During this drying process some sapogenin-affording enzyme activity may occur before the conditions become unfavourable; the yields by Assay Procedure B were lower than those obtained when the leaf was first dried at 80° before Assay Procedure B was applied (Table 3).

As observed, variations in the ratio of the sapogenin yield by Assay Procedure A to that by Assay Procedure B will be caused by varying the conditions experienced by the plant tissue from the time of harvesting to the time of commencement of the assay. The ratio of yields may also vary with the metabolic state of the plant at the time of harvesting: a sapogenin may occur in a form releasable by Assay Procedure B and also in a form only releasable by method A and the proportion of the two forms may vary with the season and with the age of the tissue as well as with the morphological part of the plant involved.

There are numerous examples of the isolation of steroidal sapogenin in the form of oligosaccharide glycosides from plant tissue and of the hydrolysis of such free glycosides to the aglycone by 2N hydrochloric acid (for example, Marker & Lopez, 1947; Krider, Branaman & Wall, 1955). Boiling 2N hydrochloric acid will also yield free sapogenin from plant tissue, as in assay procedure B above, and on several occasions it has been demonstrated that more intensive acid treatment does not increase the yield of sapogenin; the yield may be reduced (for example, Rothrock, Hammes & McAleer, 1957; Morris, Roark & Cancel, 1958; Blunden, 1962).

Harvested plant tissue affords more sapogenin if the endogenous enzymes are allowed to function before the material is treated with acid (assay procedure A, above). In these circumstances substances of greater complexity than the oligosaccharide type of saponin may be contributory

to the overall yield: for instance, where the steroid is linked to such substances as cellulose, hemicellulose and pectic substances, and also to other cell substances including the cell contents. Sapogenin may also be afforded by enzyme disruption of surfaces to which saponin or genin was formerly held by physical sorption. Some synthesis of steroid may occur in the harvested plant tissue when the numerous enzymes are caused to function.

In the sapogenin-yielding system used (Yucca leaf) the β-glycosidases, cellulase and emulsin, and the α-glycosidase, pectinase, partially replaced the endogenous enzymes in affording sapogenin (Table 5). In this, cellulase was more effective than pectinase, while maltase had no effect. Such commercial enzymes will disrupt surfaces, hydrolyse polysaccharides, release aglycones by hydrolysis, and will also yield substances such as glucose, which may contribute to synthesis of steroid. More investigation is needed to establish the contribution of these various processes to the endogenous enzyme-afforded sapogenin and to gain more information about the nature of the occurrence of steroidal compounds in plant cells.

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Twitch responses with acetylcholine in the isolated innervated and chronically denervated rat diaphragms and their modification by neuromuscular blocking agents

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The increased sensitivity to acetylcholine after chronic denervation was investigated by retrograde injection of the acetylcholine into the venous drainage of the isolated diaphragm of the rat set up in Krebs solution at 38°. Supersensitivity of the twitch response was observed 3 days after nerve section and developed further to reach a peak at the eighth to tenth day after denervation and then slowly declined with atrophy of the muscle. At the time of the onset of supersensitivity the degenerating peripheral nerve stump failed to respond to electrical stimulation and fibrillation of the muscle was observed. The muscle also responded to acetylcholine and other depolarising drugs by giving a contracture. After denervation, the response to injected acetylcholine was more sensitive to block by depolarising neuromuscular blocking agents but not to block by competitive blocking drugs. Tubocurarine did not cause a stimulation on injection into denervated muscle, while decamethonium gave an acetylcholine-like response. The significance of the observations is discussed.

ISOLATED mammalian muscle does not under normal conditions give a twitch response to the application of acetylcholine to the fluid bathing the external surface of the muscle. With low concentrations (5×10^{-6} to 2×10^{-5} w/v) there is some potentiation of responses to nerve stimulation and with higher concentrations (10^{-4} w/v) this gives way to neuromuscular block. With none of these concentrations is a twitch response obtainable as a result of the action of the acetylcholine. The reason may lie in the rate of change of concentration of acetylcholine at the motor end-plate.

It is well known that if acetylcholine is injected into the vascular supply of a skeletal muscle, *in vivo*, a twitch can be produced (Brown, Dale & Feldberg, 1936). Similarly electrophoretic application of acetylcholine by micropipette is also capable of inducing a twitch response. With "close-arterial" injection, the twitch has been shown to be a short asynchronous tetanus (Brown, 1937). "Close-arterial" injection is impracticable in the isolated rat diaphragm muscle because of its diffuse arterial supply. However, the venous drainage of the right hemidiaphragm affords a suitable channel for retrograde injection into the muscle. This was developed as a technique by Burgen, Dickens & Zatman (1949) in their studies on the mode of action of botulinum toxin. The method lends itself well to the study of the twitch responses to acetycholine before and after denervation and for the investigation of substances modifying these twitch responses.

Experimental

Albino rats, 150–250 g, were stunned and bled. The entire diaphragm with the right phrenic nerve and the thoracic inferior vena cava and

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hepatic vein intact was dissected into cold Krebs solution gassed with 95% oxygen and 5% carbon dioxide. The right phrenic nerve was separated from the thoracic inferior vena cava and the abdominal aspect of the diaphragm cleared of fascial attachments to give access to the abdominal inferior vena cava. This vein was then tied just below the diaphragm, care being taken to leave the right phrenic vein patent. The liver was then dissected to leave the tied stump of the abdominal inferior vena cava. The veins draining the left hemidiaphragm and the crura were then tied and these muscles dissected. A polythene cannula was tied into the thoracic inferior vena cava and the preparation set up in Krebs solution at 38° and gassed with 95% oxygen and 5% carbon dioxide (Fig. 1).

Drugs were injected through the cannula into the muscle as follows: the cannula and its attached length of tubing was filled by injecting sufficient of the drug solution to fill the dead space (about 0·15 to 0·2 ml); 0·15 ml quantities of the solution could then be displaced rapidly into the right phrenic vein. Further 0·15 ml quantities of the solution were then injected at 3 min intervals in normal muscle and 2 min intervals in denervated muscle.

The injection of acetylcholine into the muscle gives rise to a tetanic twitch of short duration and tension dependent upon the dose. With constant speed of injection a uniform response to a given dose can be obtained, providing adequate time for recovery between doses is given.

The uniformity of the response to injected acetylcholine is maintained for up to 8 hr provided the bath fluid is changed after a series of injections is made. The diffusion of the drug into the bath after injection gives a a negligible final concentration with the smaller doses used and has no discernible effect on the responses to subsequent injections. Between injections the responses to nerve stimulation in normal muscle and direct stimulation in denervated muscle were recorded. Frequency of stimulation was 5 per min.

DENERVATION

Adult rats 150–300 g were anaesthetised with diethyl ether and the right phrenic nerve exposed where it crosses the brachial plexus. After section, the distal end of the nerve was avulsed slowly. In most animals the nerve broke at the level of the right atrium. The rats were then allowed to recover and were killed at intervals after nerve section. The denervated diaphragm was then set up in the same way as for the innervated preparation. The success of the denervation can be judged at the time of operation by the absence of diaphragmatic respiration on the right side and by the presence of fibrillation when the muscle was subsequently prepared for recording.

STIMULATION

In the innervated preparation, the nerve was stimulated by square wave pulses of 10 or 30 μsec duration and 4 to 5 V at a frequency of 5/min delivered from a Multitone stimulator.

The denervated muscles were stimulated between a platinum wire

on the Perspex rod to which the central tendon margin of the diaphragm was attached, and a stainless-steel wire tied into the costal margin which served both as an electrode and as a connecting link to the lever system (Fig. 1). Pulses were 0·3 to 1 msec in duration and 120 V.

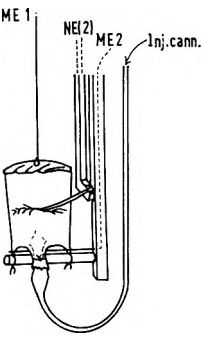

FIG. 1. Electrode and injection assembly for intravenous injection into the isolated rat diaphragm. ME1: thin gauge stainless steel wire acting as one electrode for muscle stimulation and for attachment to spring lever. ME2: second muscle electrode; platinum wire making contact with central tendinous margin of the diaphragm. NE (2): two platinum wires over which the phrenic nerve, if present, is laid for stimulation. Inj. cann: small bore polythene tubing distended where it inserts into the right phrenic vein. Injections are made through this into the venous drainage of the muscle.

Twitches were recorded on a smoked drum with a semi-isometric spring lever.

Results

SENSITIVITY TO ACETYLCHOLINE

Normal diaphragm. The responses of the innervated diaphragm to injected acetylcholine are shown in Fig. 2. A small injection artifact was obtained when 0·15 ml of Krebs solution was injected through the phrenic vein. With 0·32 μg of acetylcholine the response on injection was no different from the artifact; with 1·25 μg, there was a twitch about 50% of that of nerve stimulation and with 10 μg there was a twitch about equal to that of the nerve-induced twitch.

Diaphragm denervated 6 days previously (Fig. 2). Again, control injections of 0·15 ml of Krebs solution gave small artifacts. After denervation much less acetylcholine was required for a given height of contraction than in the normal diaphragm. After the larger doses (100 ng to 1 μg; Fig. 2) the denervated muscle also showed a contractural response accompanied by a decrease in the response of the muscle to direct stimulation, from which it recovered rapidly.

RELATION OF SUPERSENSITIVITY TO ACETYLCHOLINE TO THE LENGTH OF TIME AFTER NERVE SECTION

Log dose-response curves were obtained at various lengths of time after nerve section. Results are shown graphically in Fig. 3. Three days

after denervation the muscle began to show increased sensitivity to injected acetylcholine. Before this time no supersensitivity could be detected. Three days was also the earliest time at which fibrillation of the muscle was observed during dissection. At this time the muscle failed to respond to electrical stimulation of the peripheral end of the degenerating nerve. Thereafter the sensitivity increased to reach a peak at the eighth to tenth day after denervation and then declined slowly as atrophy of the muscle progressed. Eighty-four days after denervation the sensitivity of one muscle was still about one hundredfold greater than that of an innervated muscle. The maximum twitch tension in this muscle was very much reduced because of atrophy.

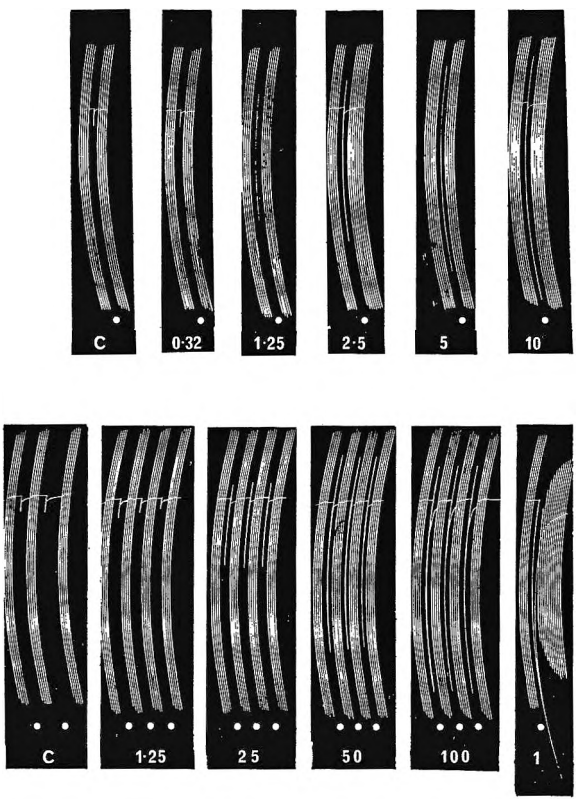

FIG. 2. Responses of innervated and denervated diaphragm to retrograde intravenous injection of acetylcholine. Responses are shown between blocks of nerve stimulation or direct stimulation (rate 5/min) of the muscles. Upper row: normal diaphragm; Responses to 0·15 ml Krebs (C) and 0·32, 1·25, 2·5, 5 and 10 μg Ach in 0·15 ml Krebs. Lower row: diaphragm denervated for 6 days; Responses to 0 15 ml Krebs (C) and 12·5, 25, 50, 100 ng, and 1 μg Ach in 0·15 ml Krebs.

EFFECTS OF NEUROMUSCULAR BLOCKING AGENTS ON THE TWITCH RESPONSE TO ACETYLCHOLINE

Neuromuscular blocking agents added to the bathing fluid reduced the responses to injected acetylcholine in both normal and denervated muscles.

In the innervated muscle, tubocurarine in a concentration of 10^{-6} reduced to a greater extent the response to acetylcholine than the response to a single nerve volley (Fig. 4A). This concentration of tubocurarine caused a similar reduction of the response to acetylcholine in a diaphragm denervated 7 days previously (Fig. 4B). After washing, the responses to acetylcholine returned slowly to normal in both preparations. The difference in blockade between the endogenous and exogenous acetylcholine probably lies in the difference in nature of the two responses, one being a single impulse phenomenon and the other a tetanic response. It is known that tetani are more easily reduced than single nerve volley responses. Blockade of the acetylcholine response by gallamine is also

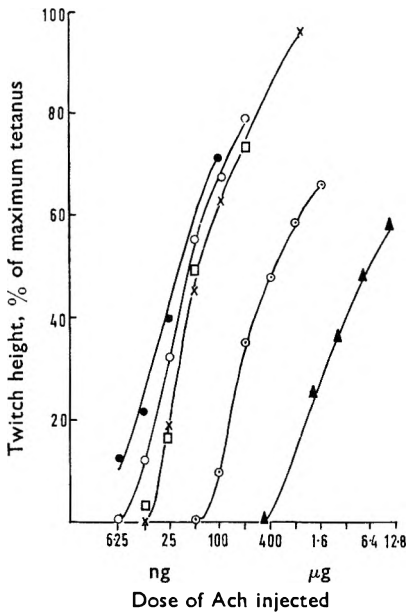

FIG. 3. Log. dose-response curves of rat diaphragms, innervated and at varying stages of denervation. Abscissa: dose of Ach injected in 0·15 ml Krebs, log range from 6·25 ng to 12·8 μg. ▲ Normal diaphragm. ○ Denervated 3 days. X Denervated 6 days. ○ Denervated 7 days. ● Denervated 18 days. □ Denervated 84 days.

unchanged after denervation (Fig. 5). If anything, both tubocurarine and gallamine are slightly less effective after denervation.

Depolarising blocking agents on the other hand, have a greater effect on the response to acetylcholine after denervation. In the innervated diaphragm, about 80% block of the response to acetylcholine was obtained with suxamethonium (10^{-6}), whereas in the denervated preparation complete block of its action was recorded with 2×10^{-7} suxamethonium (Fig. 6).

Decamethonium (Fig. 7) in a concentration of 10^{-5} caused a block of responses to acetylcholine in the normal diaphragm comparable with that of a concentration of 10^{-6} in the denervated muscle. Similar results

were obtained with carbachol where the block of responses to acetylcholine of the normal diaphragm with $2{\cdot}5 \times 10^{-6}$ was equivalent to a block with $2{\cdot}5 \times 10^{-7}$ in a muscle denervated 14 days previously. In Figs 7C, 8, 9, a further characteristic of the actions of depolarising agents is shown. In all of these instances the response to direct stimulation of the denervated diaphragm is depressed. This is seen to be maintained in Figs 8, 9, and can be reversed on washing. It is easily reversible (Fig. 7C) by hexamethonium 10^{-5} and by tubocurarine 5×10^{-7} whereas the response to injected acetylcholine still remains depressed.

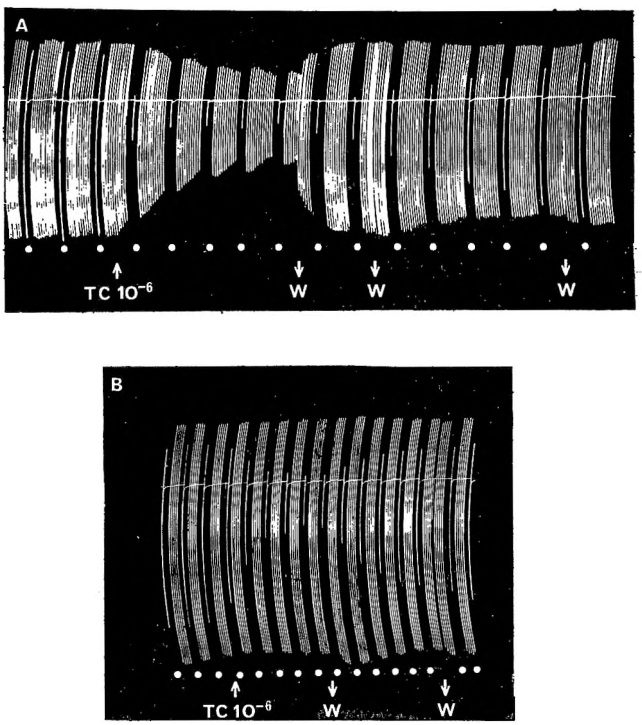

FIG. 4. Blockade of injected Ach by (+)-tubocurarine. (A) Normal diaphragm. At dots, 5 μg Ach was injected every 3 min between blocks of responses to nerve stimulation. At TC, (+)-tubocurarine was added to bath to give a final concentration of 10^{-6}. The drug was washed out at W. (B) Diaphragm denervated 7 days previously. At dots, 50 ng Ach was injected every 2 min between blocks of direct stimulation of the muscle. At TC, (+)-tubocurarine was added to the bath to give a final concentration of 10^{-6}.

SENSITIVITY OF DENERVATED MUSCLE TO RETROGRADE INTRAVENOUS INJECTION OF SUBSTANCES OTHER THAN ACETYLCHOLINE

Decamethonium and potassium chloride both gave twitch and contractural responses on injection into the phrenic vein of a diaphragm denervated 21 days previously (Fig. 10). However, even in large doses (1 mg) tubocurarine did not cause contraction, contracture or depression of direct stimulation.

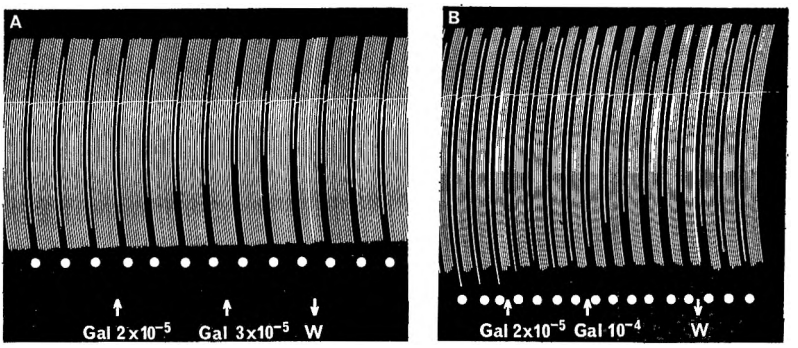

FIG. 5. Blockade of injected acetylcholine by gallamine. (A) Normal diaphragm. At dots, 2 μg Ach was injected every 3 min between blocks of responses to nerve stimulation. At Gal, gallamine was added to the bath to give final concentrations shown, 5×10^{-5} *in toto*. (B) Diaphragm denervated 8 days previously. At dots, 25 ng Ach was injected every 2 min between blocks of responses to direct stimulation. At Gal, gallamine was added to the bath to give final concentrations shown, $1{\cdot}2 \times 10^{-4}$ *in toto*.

EFFECT OF NEOSTIGMINE ON TWITCH AND CONTRACTURAL RESPONSES TO ACETYLCHOLINE

Fig. 11 shows responses to acetylcholine before and after treatment with a concentration of neostigmine (10^{-7}) which potentiates considerably the response to nerve stimulation in the innervated diaphragm. The twitch response was little affected but the contractural action was enhanced and prolonged.

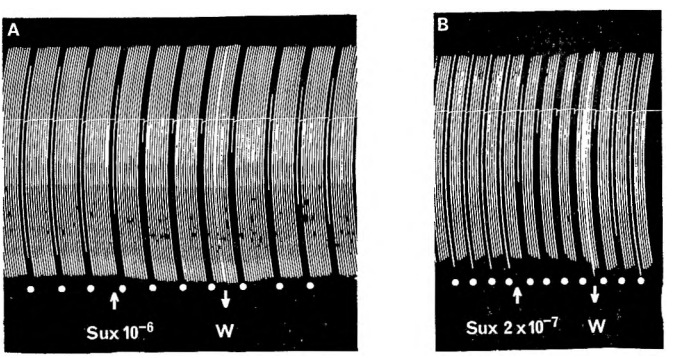

FIG. 6. Blockade of injected acetylcholine by suxamethonium. (A) Normal diaphragm. At dots, 2 μg Ach was injected every 3 min between blocks of responses to nerve stimulation. At Sux, suxamethonium was added to the bath to give a final concentration of 10^{-6}. (B) Diaphragm denervated 21 days previously. At dots, 25 ng Ach was injected every 2 min between blocks of responses to direct stimulation. At Sux, suxamethonium was added to the bath to give a final concentration of 2×10^{-7}.

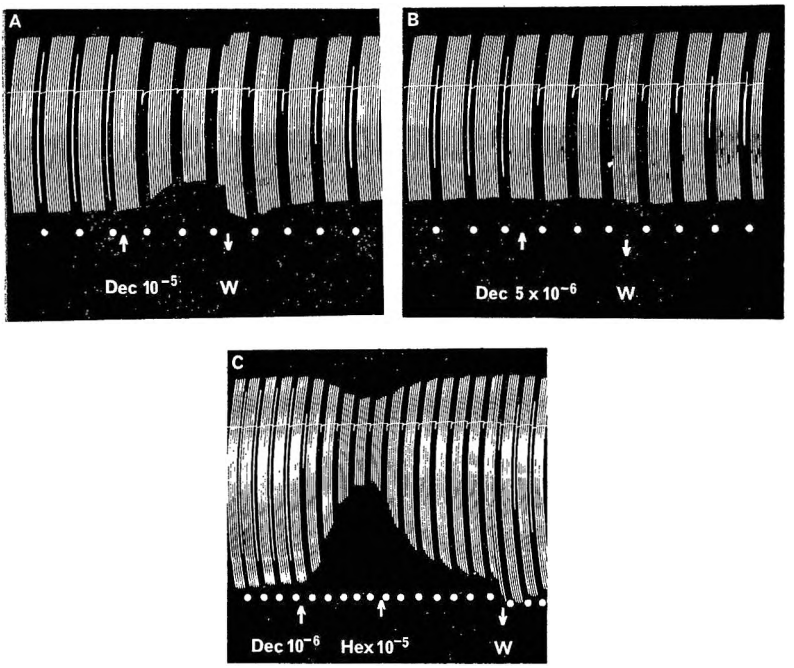

FIG. 7. Blockade of injected acetylcholine by decamethonium. (A) and (B) Normal diaphragm. At dots, 2·5 μg Ach was injected every 3 min between blocks of responses to nerve stimulation. (A) At Dec 10^{-5}, decamethonium was added to the bath to give a final concentration of 10^{-5}. (B) At Dec 5×10^{-6}, decamethonium was added to the bath to give a final concentration of 5×10^{-6}. (C) Diaphragm denervated 18 days previously. At dots, 25 ng Ach was injected every 2 min between blocks of responses to direct stimulation. At Dec, decamethonium was added to the bath to give a final concentration of 10^{-6} and at Hex, hexamethonium to give a final concentration of 10^{-5}.

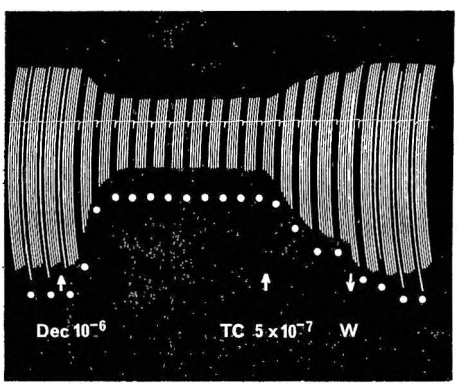

FIG. 8. Reversal by tubocurarine of depression of direct stimulation with decamethonium. Diaphragm denervated 23 days previously. At dots, 25 ng Ach injected every 2 min between blocks of direct stimulation. At Dec, decamethonium was added to the bath to give a final concentration of 10^{-6}. At TC, (+)-tubocurarine was added to give a final concentration of 5×10^{-7}.

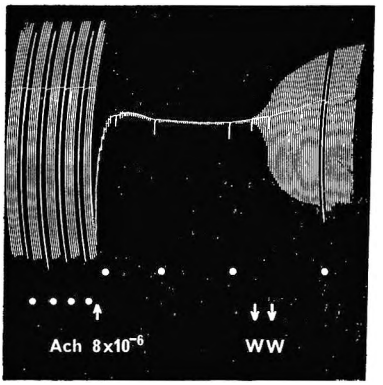

FIG. 9. Blockade of responses to injected acetylcholine by acetylcholine added to the bath. Diaphragm denervated 21 days previously. At dots, 25 ng Ach was injected intravenously into the muscle. At Ach, acetylcholine was added to the bath to give a final concentration of 8×10^{-6}.

Discussion

The chronically denervated diaphragm increases in sensitivity to acetylcholine injected intravascularly; the increase is gradual and becomes evident on the third day after denervation, that is, at a time when the degenerating stump of the phrenic nerve fails to elicit a twitch response to electrical stimulation. At the same time contractural properties develop and the contraction of the muscle on direct stimulation is depressed. No abrupt change occurs from the low sensitivity of the innervated muscle to the much greater sensitivity after denervation. Instead, a gradual transition takes place, which although rapid in the first few days after nerve degeneration, is nevertheless progressive up to the eighth to tenth day after nerve section. Reid & Vaughan Williams (1949) in their experiments on the denervated tibialis anterior muscle of the cat, found that sensitivity of the twitch response to acetylcholine increased rapidly on the sixth and seventh days after denervation while the contractural response appeared earlier and continued to develop after increased sensitivity had been established. In the rat gastrocnemius muscles, sensitisation and contractural responses to acetylcholine appeared between the second and third days after section of the nerve; both these increased to a maximum at 7 days after denervation. The onset of supersensitivity to acetylcholine after nerve section seemed to depend, to some extent, on the length of the peripheral nerve stump left after cutting the nerve. Luco & Eyzaguire (1955) noted that it appeared earlier when the nerve was cut nearer the motor end-plate. In the present experiments the length of nerve that remained after avulsion was about 2 to 2·5 cm since at phrenectomy the nerve broke at the level of the right atrium.

The reason for the supersensitivity is not certain, but one main factor has emerged and has gained considerable support in recent years (Ginetzinsky & Shamarina, 1942, Axelsson & Thesleff, 1959, Miledi, 1960a).

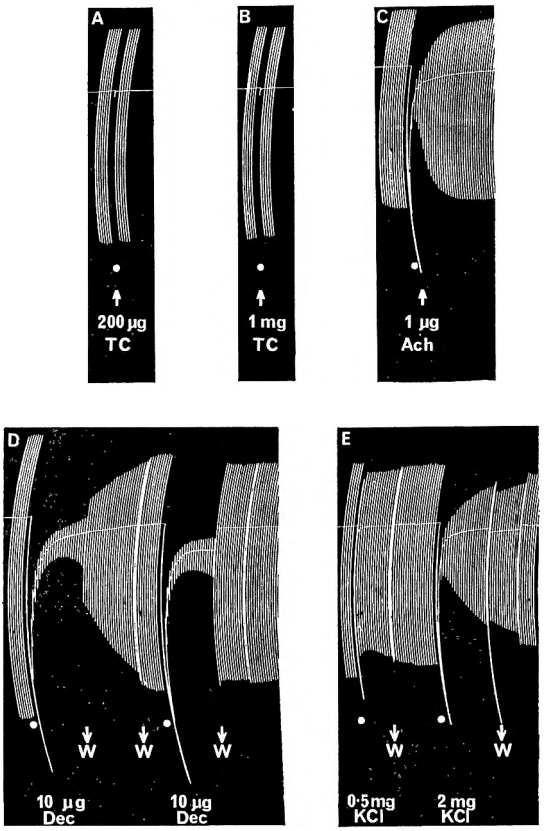

FIG. 10. Effects of injections of substances other than acetylcholine into the phrenic vein of a diaphragm denervated 21 days previously. TC, (+)-Tubocurarine. Ach, Acetylcholine. Dec, Decamethonium. KCl, Potassium chloride.

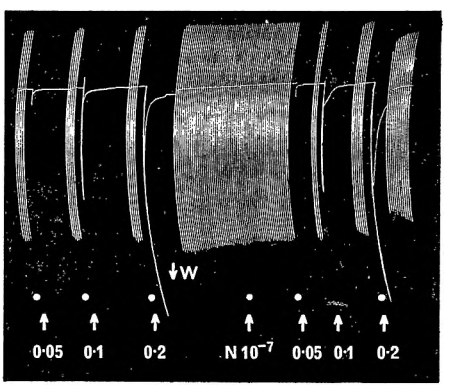

FIG. 11. Effect of neostigmine on the responses to injected acetylcholine. Diaphragm denervated 6 days previously. At 0·05, 0·1 and 0·2, injections of Ach were made through the cannula of 0·05 μg in 0·05 ml Krebs, 0·1 μg in 0·1 ml Krebs and 0·2 μg in 0·2 ml Krebs respectively. At N, neostigmine was added to the bath to give a final concentration of 10^{-7}.

This is the concept that after nerve section and degeneration, extra-junctional receptors appear, spreading with time after denervation over the entire surface of the muscle. The end-plate area still seemed to show a greater sensitivity than did the rest of the fibre (Miledi, 1962) but the existence of the extra-junctional receptors appeared to explain the overall contractural effects and the depression of direct stimulation seen with depolarising drugs. It is probable that in the same way this accounted for the increased sensitivity for the twitch response to injected acetylcholine. The mechanism by which these changes are brought about is also open to dispute. Thesleff (1960, 1961) believed that the absence of the transmitter is the governing factor since blockade of release of acetylcholine from nerve terminals with botulinum toxin induced changes indistinguishable from denervation. His concept received some support from experiments by Emmelin & Stromblad (1956) on salivary glands in which they induced supersensitivity with a muscarinic blocking agent which prevented the effects of acetylcholine released by nerve action. On the other hand, Miledi's experiments (Miledi 1960a, c; Katz & Miledi, 1961, 1964) have led him to conclude that a substance other than the transmitter is responsible for the effect, this substance exerting a controlling influence on the spread of receptors from the end-plate. Whatever the reason for the induction of the extra-junctional receptors, their presence altered the response of the muscle to a state which in many ways resembles that of foetal muscle (Paterson, 1957a; Diamond & Miledi, 1959, 1962).

The changes shown in response to acetylcholine are also seen with other depolarizing drugs and this is reflected in three ways. These drugs can induce contracture in denervated muscle (Muscholl & Lüllman, 1955; Paterson, 1957a, b). Depression of the effects of direct electrical stimulation and blockade of the action of injected acetylcholine are also seen. There is no evidence that these effects are not facets of the same action.

When the blockade of injected acetylcholine by depolarising blocking agents is compared in normal and denervated muscles, much less of the depolarising drug is required to block in the denervated than in the innervated muscle. There is no such difference in the action of competitive blocking agents. Maclagan & Vrbova (1964) described an increase in sensitivity of reinnervated motor-end plates to depolarisation blockade. Sensitivity was greatest immediately after reinnervation and gradually returned to normal during 9 weeks. This was seen although no difference in receptor area could be detected between normal and reinnervated muscles even when the period of denervation had been 3 weeks. They ascribed the increased sensitivity to the effects of denervation. The change may be the result of altered efficacy of the depolarising blocking drugs since in some instances, although not in the present experiments, tubocurarine and gallamine induced contractural effects in denervated muscle (McIntyre, King & Dunn, 1945; Jarcho, Berman, Eyzaguirre & Lilienthal, 1951; Bülbring & Depierre, 1949). Bowman (1964) could find no contractural or twitch-depressant action of 0·5 mg/kg

tubocurarine on a cat tibialis muscle denervated 14 days previously. It is surprising that Waser (1962), using radioactive curarine, was unable to detect by autoradiographic means the spread of receptors from the end-plate region in denervated mouse diaphragms, since competitive drugs can block all of the effects of acetylcholine seen after denervation.

Tubocurarine and hexamethonium reversed the depression by decamethonium of direct stimulation in the denervated muscle. This would suggest that the receptors which appeared after denervation had qualitatively similar properties to those of the normal motor end-plate, an observation which agrees with the findings of other groups of workers (Axelsson & Thesleff, 1959; Miledi, 1960a; Klaus, Kuschinsky, Lüllman & Muscholl, 1959; Jenkinson, 1960; Letley, 1960). It also means that the sustained depression of contraction caused by the decamethonium is the result of a sustained specific reaction of the decamethonium with these receptors and not the result of the ionic changes which accompany the contracture caused by depolarisation of the muscle membrane.

The sustained depression with decamethonium seen in Fig. 8 emphasises one difference between the response of innervated and denervated muscle *in vitro*. On isolated innervated muscle, depolarising blocking agents have a diphasic action, where the neuromuscular block develops in about 10 min (phase I block), but is not maintained. A reversal of block appears which then continues into a further block (phase II block) (Jenden, Kamijo & Taylor, 1954; Jenden 1955; Dillon & Sabawala, 1959; Maclagan, 1962; Huskisson & Paterson, 1965, in preparation). During Phase I block there is a concomitant reduction in the response of the muscle to direct stimulation. This transitional action of the depolarising blocking drugs *in vitro* has led Zaimis (1962) and Maclagan (1962) to urge caution in the comparison of the actions of depolarising drugs *in vitro* and *in vivo*. No transitional effect is seen in Fig. 8 and this suggested that this action of decamethonium was different from that seen during phase I in the innervated muscle.

The suggestion that acetylcholine receptors have a role in conduction along muscles (Hinterbuchner & Nachmansohn, 1960) must also be examined in the light of these results. If the depression of conduction by depolarising drugs were to arise from the ability of the drug to cause a reduction in the activity of endogenous acetylcholine, then the same should be true for any competitive blocking agent reaching the same site. That this is not so, when taken together with the inability of tubocurarine to excite or depress the muscle on intravenous injection of large doses, suggests that acetylcholine is not concerned in impulse conduction in this muscle. Also, Hebb, Krnjević & Silver (1964) have shown that in the denervated diaphragm of the rat acetylcholine was practically undetectable in that part of the muscle, which in the innervated diaphragm was designated to be "nerve-free". They also found that choline acetyltransferase activity fell in the denervated diaphragm to 4% of normal values; even this residuum of activity might be accounted for by Schwann cell activity in denervated muscle (Birks, Katz & Miledi, 1960). In agreement with the conclusion that acetylcholine has no role in conduction

are the results of Cooke & Grinnell (1964) who, investigating the effects of tubocurarine on frog sartorii and rat intercostal muscle, were unable to demonstrate any significant difference between action potentials of normal and denervated muscle fibres in Ringer solution with or without tubocurarine up to 10^{-3} M. The depression of direct stimulation by acetylcholine, suxamethonium, carbachol and decamethonium might be thought to contradict the hypothesis (Grundfest, 1957, 1963) that acetylcholine sensitive areas of muscle were electrically inexcitable, but Grundfest (1961, 1964) proposes an explanation which supposed chemosensitive areas to develop in patches after denervation, but not to cover the entire membrane. The results of the present experiments agree with this supposition when we take into consideration the sustained transmitter-like action of the depolarising drugs and the ease by which it is reversed by competitive blocking drugs. It would be difficult to envisage a "short-circuit" transmitter-like action which involved all of the active membrane and yet was sustained for prolonged periods of time.

Neostigmine did not enhance the twitch caused by acetylcholine in the denervated diaphragm, but did prolong the contracture. The temporal difference between the two forms of response may be one explanation for this observation since there is still much cholinesterase activity in the rat diaphragm for up to 42 days after denervation (Lüllmann & Muscholl, 1955). Since the contractural action relies more on a sustained ambient concentration than does the twitch response, protection from hydrolysis would be expected to prolong the contracture. The twitch response being tetanic would remain little changed. Miledi (1962) found a reduction by edrophonium of the effect of ionophoretically-applied acetylcholine at the end-plate region in a frog sartorius fibre 58 days after denervation, so it would seem that at that stage little if any cholinesterase remained.

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Chemical fractionation of phosphorus-32 labelled cells of *Micrococcus lysodeikticus* treated with chlorhexidine

R. M. RYE AND DAVID WISEMAN

A method for the chemical fractionation of *Micrococcus lysodeikticus* into "cold trichloroacetic acid soluble", alcohol soluble, ribonucleic acid and residual fractions is described. The results of applying this method to untreated and chlorhexidine-treated ^{32}P labelled cells are discussed. In untreated cells the ^{32}P content of the "cold trichloroacetic acid soluble" fraction, which constitutes the metabolic pool, increases at a rate dependent on temperature increase and there is a corresponding decrease in the ^{32}P content of the ribonucleic acid fraction. At the highest temperature, 40°, the increase of ^{32}P in the pool is followed by a decrease due to leakage from the pool. The release of ^{32}P from chlorhexidine-treated cells maintained at 1°, 20°, 30° and 40° has been measured after 0·5, 2 and 13 hr. There is an initial rapid release which is entirely from the "cold trichloroacetic acid soluble" fraction and a slower secondary release from the ribonucleic acid fraction. This secondary release is almost completely inhibited at 1° and by high (64 and 128 μg/ml) chlorhexidine concentrations.

THE release of radioactive compounds from phosphorus-32 labelled cells of *Micrococcus lysodeikticus* treated with chlorhexidine occurs in two stages. The initial rapid release (1–2 hr) has been attributed to cell membrane damage allowing the leakage of small molecules from the cells, and the slower secondary release to autolytic breakdown in the damaged cells (Rye & Wiseman, 1964). The antibacterial action of chlorhexidine has now been further investigated by studying the effect of temperature on the patterns of release and by a chemical fractionation of untreated and chlorhexidine-treated cells.

Experimental

Materials. The materials, conditions of culture and harvesting were as previously described (Rye & Wiseman, 1964) except that the culture medium used was 1% tryptone agar containing 0·5 μc/ml of sodium phosphate-^{32}P.

Suspending medium. M/15 phosphate buffer pH 7·2 or M/40 tris-hydroxymethylaminomethane (Tris)-HCl buffer pH 7·2.

Reaction mixtures. Bacterial suspensions and equal volumes of buffer or chlorhexidine solutions were maintained at the required temperature for 1 hr before mixing. The final cell concentrations were $1{\cdot}5 \times 10^{10}$/ml.

CHEMICAL FRACTIONATION OF CELLS AND MEASUREMENT OF RADIOACTIVITY

1. *Release of ^{32}P into the suspending medium.* 4 ml samples from each reaction mixture were taken at various time intervals and centrifuged at 5000 rpm for 5 min. The supernatant fluids were removed, recentrifuged and retained.

From the Department of Pharmacy, Bradford Institute of Technology, Bradford.

2. *Cold trichloroacetic acid soluble fraction.* (*"Cold TCA soluble" fraction*). The walls of the tubes from stage 1 were dried with cellulose tissues to remove traces of liquid and the residual cells suspended in 5 ml of 10% TCA and maintained at 4° for 30 min. The tubes were centrifuged and the supernatant fluids recentrifuged and retained.

3. *Alcohol soluble fraction.* The residues from stage 2 were washed with buffer, centrifuged, suspended in 5 ml of 75% ethanol and maintained at 40° for 30 min. After centrifuging, the residues were resuspended in 4 ml of a mixture of equal volumes of ether and 75% ethanol for 15 min at 40° and recentrifuged. The ethanol and ethanol-ether extracts were then pooled and retained.

4. *Ribonucleic acid (RNA) fraction.* The residues from stage 3 were suspended in 4 ml of M sodium hydroxide for 90 min at 30° and then acidified with 5 ml of M hydrochloric acid. After standing for 15 min at room temperature, the tubes were centrifuged and the supernatant fluids retained.

5. *Residual fraction.* The residues from stage 4 were washed, centrifuged and resuspended in buffer.

The radioactivities of the residual fraction and of 2 ml samples of each of the fractions collected in stages 1–4 were measured and calculated as a percentage of the total radioactivity present. The method used was that described by Rye & Wiseman (1964).

Results

EFFECT OF TEMPERATURE ON THE RELEASE OF ^{32}P FROM UNTREATED AND CHLORHEXIDINE-TREATED CELLS

Table 1 shows the percentage of ^{32}P released from untreated and chlorhexidine-treated cells maintained at 1°, 20°, 30° and 40° after 0·5, 2 and

TABLE 1. RELEASE OF ^{32}P FROM *Micrococcus lysodeikticus* TREATED WITH CHLORHEXIDINE AT VARIOUS TEMPERATURES, EXPRESSED AS PERCENTAGE OF TOTAL CELLULAR ACTIVITY. CELL CONCENTRATION $1·5 \times 10^{10}$/ml. SUSPENDING MEDIUM M/15 PHOSPHATE BUFFER (pH 7·2).

	1° Chlorhexidine concentration µg/ml					20° Chlorhexidine concentration µg/ml				
Time	0	8	16	32	128	0	8	16	32	128
30 min	0·7	1·2	3·3	7·8	10·8	0·4	0·7	3·1	10·5	14·8
2 hr	1·0	1·8	4·7	8·1	12·7	0·6	1·2	8·5	15·7	15·7
13 hr	1·3	2·0	5·8	11·0	12·6	2·3	3·1	12·3	32·0	17·3
	30° Chlorhexidine concentration µg/ml					40° Chlorhexidine concentration µg/ml				
Time	0	8	16	32	128	0	8	16	32	128
30 min	0·5	1·0	4·9	13·7	15·5	1·7	3·3	10·6	31·2	30·0
2 hr	1·2	1·9	8·5	22·5	17·4	3·4	4·7	15·8	35·0	30·0
13 hr	6·1	7·8	20·2	34·8	16·8	20·4	23·4	46·5	56·0	36·0

13 hr. There is a gradual release of ^{32}P from untreated cells at all temperatures. This release is small except after 13 hr at 30° and 40°. Treatment of cells with chlorhexidine increases the amount of ^{32}P released. At 1°, 20° and 30°, the extent of release after 30 min increases with increasing concentrations of chlorhexidine and at 20° and 30° the subsequent secondary release is similar to that previously described (Rye & Wiseman, 1964). At 1° the secondary release is almost completely inhibited. At 40° both the initial release and the secondary release are greater than at the other temperatures.

EFFECT OF TEMPERATURE ON THE ^{32}P RELEASE AND ^{32}P CONTENT OF THE "COLD TCA SOLUBLE" FRACTION IN UNTREATED CELLS

Fig. 1A shows (*a*) the amount of ^{32}P released into the suspended medium from untreated-cells maintained at 20° and (*b*) that remaining in the "cold TCA soluble" fraction which constitutes the metabolic pool of these cells.

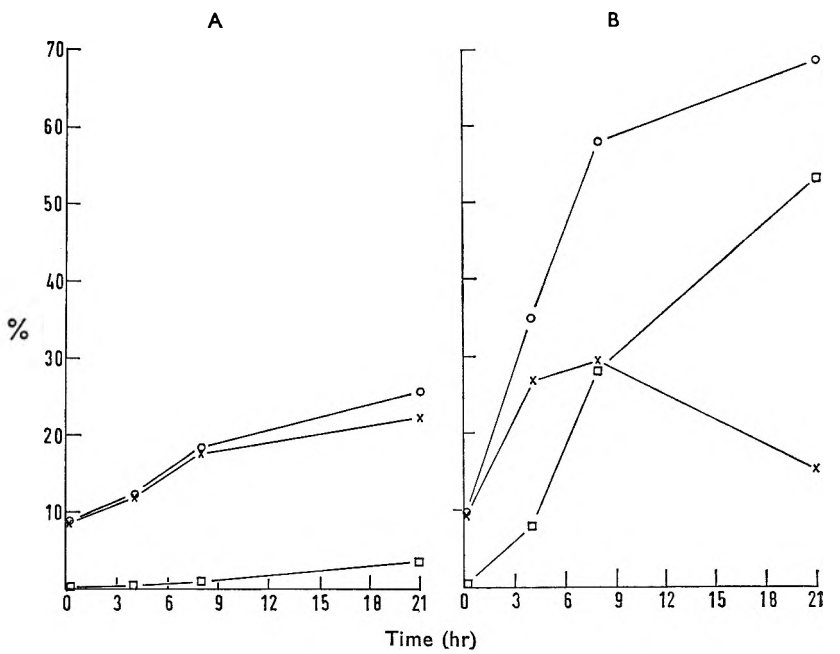

FIG. 1. Changes (%) in the amount of ^{32}P present in the cold TCA soluble fraction and released from *Micrococcus lysodeikticus* on storage in phosphate buffer (pH 7·2) at 20° (A) and 40° (B). □ Release (*a*), × Cold TCA soluble (*b*), ○ Sum of (*a*) and (*b*).

Both (*a*) and (*b*) gradually increase over 21 hr. Fig. 1B shows the results of similar measurements at 40°. Considerable release of ^{32}P occurs and the ^{32}P content of the metabolic pool at first increases but then decreases due to leakage from the cells.

EFFECT OF CHLORHEXIDINE ON THE DISTRIBUTION OF ^{32}P IN THE CELL FRACTIONS

Fig. 2A shows (*a*) the amount of ^{32}P released from cells treated with chlorhexidine for 15 min at 20° and (*b*) that remaining in the "cold TCA soluble" fraction of these cells. As the concentration of chlorhexidine

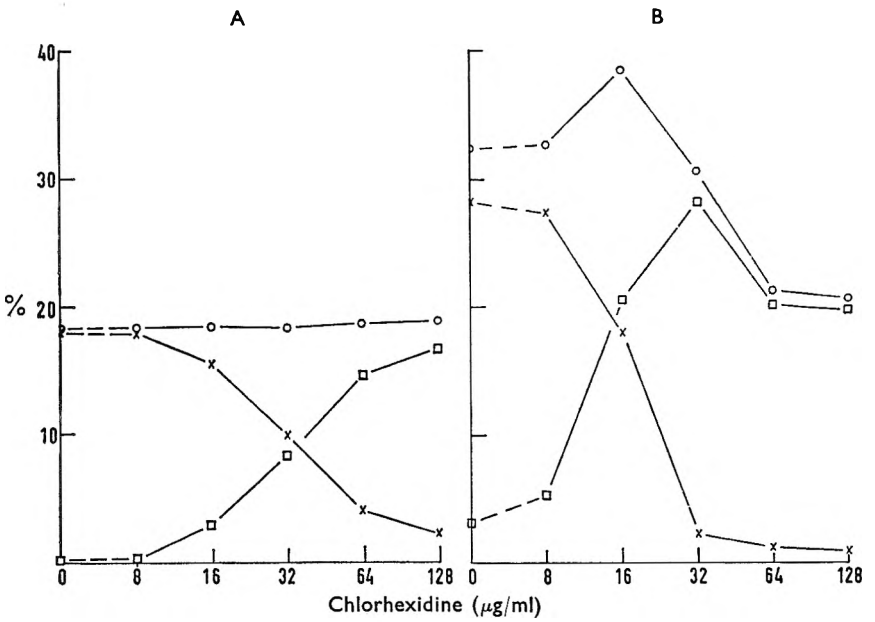

FIG. 2. Distribution of ^{32}P between the released material and the cold TCA soluble fraction of *Micrococcus lysodeikticus* treated with chlorhexidine in Tris buffer (pH 7·2) at 20° for 15 min (A) and for 22 hr (B). □ Release (*a*), × Cold TCA soluble (*b*), ○ Sum of (*a*) and (*b*).

is increased, (*a*) increases and there is a corresponding decrease in (*b*), the sum of (*a*) and (*b*) remaining constant. After 22 hr at 20° an increase occurs in the sum of (*a*) and (*b*) at low chlorhexidine concentrations but not at 64 and 128 μg/ml (Fig. 2B). The rate of increase of the sum of (*a*) and (*b*) at 30° is greater than at 20°. The results at 30° between 15 min and 22 hr are shown in Fig. 3.

The alcohol-soluble fraction was found in all our experiments to contain between 2–3·5% of the total ^{32}P content of the cells.

The ^{32}P content of the RNA fraction decreases to varying extents over a period of 22 hr in both untreated and chlorhexidine-treated cells. Results obtained after 22 hr at 30° are shown in Fig. 4. At 64 and 128 μg/ml of chlorhexidine, little change in the ^{32}P content of the RNA fraction occurs, but at lower concentrations (16 and 32 μg/ml) the decrease in the ^{32}P content of this fraction is significantly greater than that occurring in untreated cells. Fig. 4 also shows that a decrease in the ^{32}P content of the RNA fraction is accompanied by a corresponding increase in the total of ^{32}P released and ^{32}P present in the "cold TCA soluble" fraction, and that the residual fraction remains unchanged.

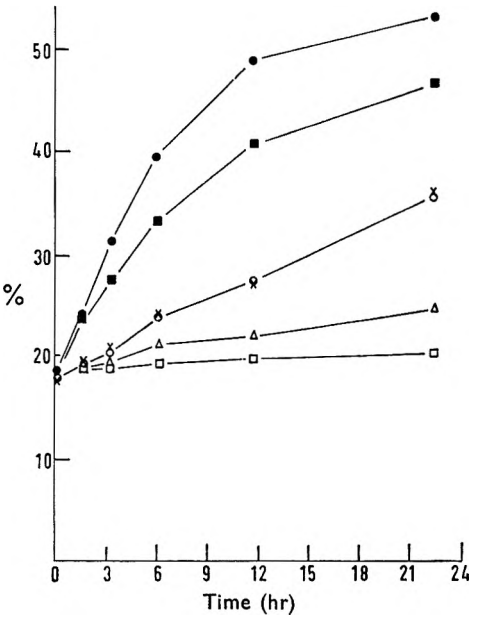

FIG. 3. Effect of chlorhexidine on the rate of change of the sum of the amount (%) of ^{32}P released and that present in the cold TCA soluble fraction of *Micrococcus lysodeikticus* suspended in Tris buffer (pH 7·2) at 30°. Chlorhexidine concentrations, ○ 0, × 8, ● 16, ■ 32, △ 64, □ 128 μg/ml.

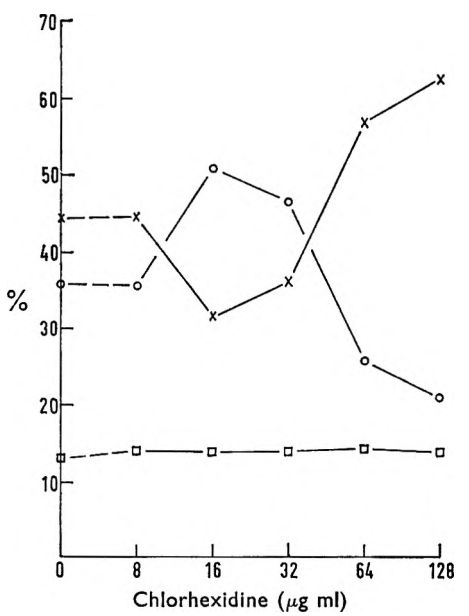

FIG. 4. The distribution of ^{32}P in *Micrococcus lysodeikticus* suspended in Tris buffer (pH 7·2) at 30° after 22 hr treatment with chlorhexidine. × RNA fraction, □ Residual fraction, ○ Sum of ^{32}P release and that present in the cold TCA soluble fraction.

Discussion

FRACTIONATION TECHNIQUES

The distribution of phosphorus among various broad classes of compounds present in bacteria can be determined after chemical fractionation of the cells. The fractionation methods reported in the literature, although similar in principle, have varied in detail (Hutchison & Munro, 1961). We selected the concentrations of reagents and conditions of reaction used in each stage after studying the effect of varying these factors upon the rate and extent of extraction from *M. lysodeikticus*.

Treatment of cells with cold TCA extracts the metabolic pool (Britten & McClure, 1962) and in several species of bacteria the phosphorus-containing compounds in this pool have been shown to consist of inorganic phosphate and nucleoside phosphates (Roberts, Abelson, Cowie, Bolton & Britten, 1957; Franzen & Binkley, 1961; Smith & Maaloe, 1964). We found that when using 10% TCA at 4° the percentage of ^{32}P extracted from labelled cells of *M. lysodeikticus* remained constant between 5 and 60 min. The radioactivity extracted by a second treatment with 10% TCA was approximately 6% of that removed by the first treatment; this can be attributed to the intercellular liquid remaining from the first extraction.

The alcohol-soluble fraction was found to contain only 2–3·5% of the total ^{32}P and no conclusions could be drawn from the variations observed under different conditions of treatment.

RNA is extracted from cells by sodium hydroxide solution, and after investigating the rate of this extraction we selected 90 min treatment at 30° with M sodium hydroxide followed by acidification for the separation of RNA from deoxyribonucleic acid and protein which thus comprise the residual fraction.

INITIAL RELEASE OF ^{32}P FROM CHLORHEXIDINE-TREATED CELLS

The ^{32}P released from cells treated with chlorhexidine for 15 min at 20° comes entirely from the metabolic pool and the proportion of this pool that is released depends on the chlorhexidine concentration (Fig. 2A). Thus under conditions where breakdown of other bacterial constituents does not take place, the maximum release of ^{32}P which can occur is governed by the content of this pool. Leakage of the pool from only a proportion of the cells may account for the incomplete release of ^{32}P from the "cold TCA soluble" fraction at low chlorhexidine concentrations.

RNA BREAKDOWN IN WASHED CELLS

During endogenous metabolism in starved bacteria, RNA can be utilised as a substrate, being degraded into smaller molecules which are subsequently oxidised (Dawes & Ribbons, 1964). This process results in the release of degradation products from the cells (Strange, Wade & Ness, 1963) and in an accumulation of material in the metabolic pool (Holden, 1958; Gronlund & Campbell, 1963). Figs 1A and B suggest that breakdown of RNA occurs in washed cells of *M. lysodeikticus* suspended in buffer. The rate of this breakdown is temperature dependent, being

negligible at 1° and occurring at between 6–10% per hr at 40° during the first 8 hr. Rapid breakdown of RNA during the preincubation of cells at 40° accounts for the high initial release (Table 1) on subsequent treatment with chlorhexidine at this temperature. In untreated cells at 20°, RNA degradation products accumulate in the "cold TCA soluble" fraction with little ^{32}P-containing material being released (Fig. 1A). At 40° an extensive release of ^{32}P into the suspending medium occurs, probably due to cell membrane damage. The ^{32}P content of the "cold TCA soluble" fraction thus depends both on the rate of RNA breakdown and on the rate of release of ^{32}P from the metabolic pool (Fig. 1B).

SECONDARY RELEASE OF ^{32}P FROM CHLORHEXIDINE-TREATED CELLS

The secondary release of ^{32}P from chlorhexidine-treated cells results from the breakdown of RNA and subsequent leakage of degradation products into the medium. The rate of this breakdown in chlorhexidine-treated cells differs from that in untreated cells (Fig. 3). At concentrations of 16 and 32 μg/ml chlorhexidine accelerates this breakdown and the consequent leakage, but at 64 and 128 μg/ml the breakdown is inhibited and virtually no secondary release occurs. *M. lysodeikticus* has been shown to contain the enzyme polynucleotide phosphorylase which can degrade RNA to nucleoside diphosphates, the activity of this enzyme depends on the Mg^{++} concentrations (Olmsted & Lowe, 1959). The differences in the rates of breakdown of RNA in chlorhexidine-treated cells compared with the rate in untreated cells may be due to a reduction in the availability of Mg^{++} in the treated cells. The different patterns of release of ^{32}P from labelled cells thus result from the dependence of RNA breakdown upon temperature, time and chlorhexidine concentration.

Our results also show that in experiments designed to study the release of material from cells treated with bactericides, the conditions before and during treatment should be strictly controlled and the extent of the release related to the content of the metabolic pool and to the rate of breakdown of cellular constituents in untreated cells.

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The mechanism underlying potentiation of the pressor action of noradrenaline by some drugs which depress sympathetic tone

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The potentiation of the action of noradrenaline on the blood pressure of spinal cats by intravenously injected hexamethonium or bretylium and intrathecally injected procaine, all of which depress sympathetic tone, was accompanied by an increase in cardiac output but no significant change in total peripheral resistance. Acute cardiac sympathectomy itself caused increases in cardiac output and in the pressor action of noradrenaline which were similar to those produced by the drugs. After cardiac sympathectomy or after treatment of spinal cats with pronethalol, the blood pressure responses to noradrenaline were not further modified by bretylium, hexamethonium, or intrathecal procaine. It is concluded that potentiation of the pressor action of noradrenaline by these drugs in the spinal cat results from inhibition of cardiac sympathetic tone.

THE potentiation of blood pressure responses to noradrenaline by a variety of drugs which affect sympathetic tone is well recognised. It is known to occur after administration of adrenergic neurone blocking drugs such as bretylium and guanethidine (Maxwell, Plummer, Schneider, Povalski & Daniel, 1960; Laurence & Nagle, 1963); after ganglion blocking agents (Corcoran & Page, 1947; Paton, 1951; Page & Taylor, 1950); and after total spinal anaesthesia (Hilton & Reid, 1956). While the increased sensitivity to adrenaline and noradrenaline following the administration of ganglion blocking drugs has been attributed by some early workers to blocking of compensatory baroreceptor reflexes (Moe, 1948; Page & Taylor, 1950); other investigators have realised that such a potentiation cannot be attributed only to the abolition of normal compensatory nervous mechanisms since it occurs after bilateral vagotomy (Page & Taylor, 1950); after denervation of the carotid sinus and aortic arch baroreceptors (Maengwyn-Davies, Walz & Koppanyi, 1958); and also after section of the spinal cord at a high level (Bartorelli, Capri & Cavalca, 1954).

According to Wilber & Brust (1958), and Hodge & Whelan (1962), potentiation of the pressor action of noradrenaline by ganglion blocking drugs is not accompanied by a change in peripheral resistance. Similar findings with bretylium have been reported by Laurence & Nagle (1963).

The present investigation was designed to determine whether the cardiac stimulant action of noradrenaline is involved in the potentiation of its pressor action by drugs and procedures which lower the sympathetic tone.

Experimental

Cats were anaesthetised with ether and rendered spinal by Dale's method as described by Burn (1952). Blood pressure was recorded from

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a cannulated femoral artery by means of a mercury manometer. Drugs were injected via a cannulated femoral vein.

The chest was opened, when necessary, at the beginning so that the whole experiment was made under uniform conditions.

The routine was as follows. Three doses of noradrenaline, all of which produced sub-maximal effects, were administered at 5 min intervals until responses had become constant. Intravenous injection of bretylium (5 mg/kg); hexamethonium (10 mg/kg); or procaine hydrochloride (2·5 ml/kg of 2% procaine hydrochloride), injected intrathecally between fourth and fifth lumber vertebrae was then made and 30 min later responses to noradrenaline redetermined. In some experiments the above routines were followed after injecting pronethalol (5 mg/kg) or after cardiac sympathectomy by removing the sympathetic chain from the stellate ganglia to the fifth thoracic vertebra, or by cutting all the postganglionic cardiac sympathetic nerves.

The effect of noradrenaline on the cardiac output in spinal cats was estimated in open chest cats under positive artificial respiration by the Fick method. Noradrenaline, 0·5 μg/kg/min was infused until the increased arterial blood pressure had attained constant level. Mixed venous blood samples were withdrawn into slightly heparinised syringes through a fine bore polythene cannula placed into the right atrium via the right external jugular vein. Arterial blood samples were obtained from a polythene cannula inserted in a carotid artery. The oxygen content of arterial and mixed venous blood samples was determined by the method of Roughton & Scholander (1943). The rate of oxygen consumption was measured with a Benedict-Roth spirometer filled with 100% oxygen and running for at least 5 min before sampling.

Total Peripheral Resistance, T.P.R. was calculated using the following formula.

$$\text{T.P.R.} = \frac{\text{Arterial blood pressure in mm Hg}}{\text{Cardiac output (ml/kg/min)}}$$

Cardiac Output (C.O.) was calculated using the following formula.

$$\text{C.O.} = \frac{\text{Oxygen consumption ml/min}}{\text{A-V oxygen difference ml/litre}}$$

Drugs used. (−)-Noradrenaline bitartrate; bretylium tosylate; hexamethonium bromide; procaine hydrochloride; pyrogallol; pronethalol.

Results

THE EFFECT OF DRUGS AND PROCEDURES WHICH ABOLISH THE SYMPATHETIC TONE ON THE PRESSOR ACTION OF NORADRENALINE IN SPINAL CATS

The result of the first series of experiments, in which the effects of bretylium (5 mg/kg), hexamethonium (10 mg/kg), spinal anaesthesia (2·5 ml/kg of 2% procaine hydrochloride), and of cardiac sympathetic denervation on the pressor responses to three different doses of noradrenaline were investigated, are presented in Fig. 1. All these drugs and

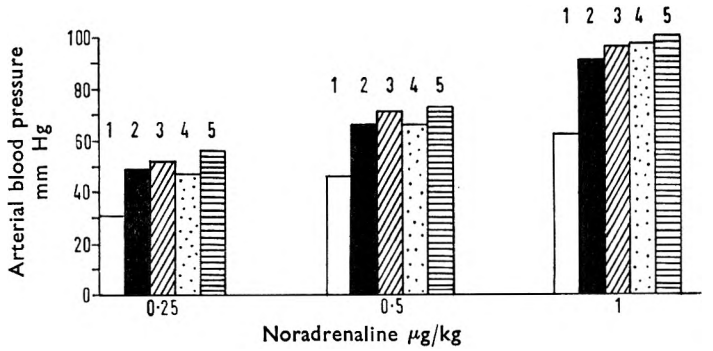

FIG. 1. The effects of hexamethonium (10 mg/kg), bretylium (5 mg/kg), total spinal anaesthesia (procaine 2·5 ml/kg, 2% solution), and acute cardiac sympathetic denervation on the pressor responses to noradrenaline (0·25, 0·5 and 1·0 μg/kg) injected intravenously in spinal cats. 1. Control. 2. Hexamethonium. 3. Bretylium. 4. Spinal anaesthesia. 5. Acute cardiac sympathetic denervation. Control columns represent the means of 16 animals. All other columns represent the means of 4 animals. Hexamethonium, bretylium, spinal anaesthesia and cardiac sympathetic denervation significantly potentiated the pressor actions of all doses of noradrenaline.

Significance of difference between means (control and treated) was examined by '*t*' test. The difference between the treated and the control responses was significant in all instances ($P = <0{\cdot}05$).

procedures significantly potentiated the effect of noradrenaline on the blood pressure of cats. Each value in Fig. 1 represents a mean of four experiments except for the control values which represent a mean of 16 experiments. There is no statistically significant difference between degrees of potentiation produced by bretylium, hexamethonium, spinal anaesthesia and cardiac sympathectomy.

SPINAL CATS WITH SYMPATHETICALLY DENERVATED HEART

Sympathetic denervation of the heart itself potentiated responses to noradrenaline (Figs 1 and 3) while bretylium (5 mg/kg), hexamethonium (10 mg/kg) or spinal anaesthesia then failed to produce any further potentiation of the pressor action (Figs 2 and 3). In spinal cats previously injected with pronethalol (5 mg/kg), sympathetic denervation, as well as hexamethonium, bretylium and spinal anaesthesia failed to potentiate the pressor response to noradrenaline (Fig. 4).

In cats with denervated hearts and in pronethalol-pretreated cats pyrogallol potentiated the pressor action of noradrenaline (Fig. 5).

CARDIAC OUTPUT MEASUREMENTS

The effect of infusion of 0·5 μg/kg/min of noradrenaline on the cardiac output, before and after intravenous injections of bretylium (5 mg/kg), hexamethonium (10 mg/kg), spinal anaesthesia or cardiac sympathetic denervation, was investigated in open-chest cats under artificial respiration. Before the potentiating drug or procedure, the infusion of noradrenaline produced an increase in arterial blood pressure with an increase in peripheral resistance and no change in cardiac output. After administration of bretylium, hexamethonium, cardiac sympathectomy or after

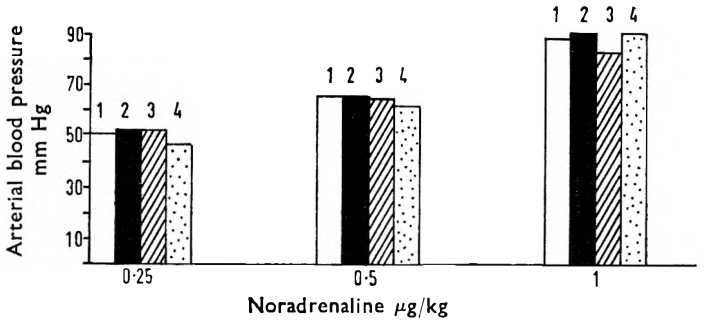

FIG. 2. The effect of drugs and procedures as described in Fig. 1 on spinal cats with sympathetically denervated hearts. 1. Control. 2. Hexamethonium. 3. Bretylium. 4. Spinal anaesthesia. Control columns represent the means of 12 animals. All other columns are the means of 4 animals. Hexamethonium, bretylium and spinal anaesthesia did not significantly potentiate the pressor actions of any doses of noradrenaline. Significance of difference between means (control and treated) was examined by '*t*' test; P = <0·05.

spinal anaesthesia, the pressor action of the same dose of noradrenaline was potentiated; the increase in peripheral resistance remained the same but there was now an increase in cardiac output (Table 1).

Administration of pyrogallol also potentiated the pressor action of noradrenaline but in this case the potentiation was accompanied by an increase in peripheral resistance without any change in cardiac output. Detailed results of these experiments are given in Table 1.

TABLE 1. THE EFFECT OF INFUSION OF NORADRENALINE (NA; 0·5 μg/min) ON THE ARTERIAL BLOOD PRESSURE, CARDIAC OUTPUT AND TOTAL PERIPHERAL RESISTANCE IN SPINAL CATS BEFORE AND AFTER BRETYLIUM, HEXAMETHONIUM, SPINAL ANAESTHESIA OR CARDIAC SYMPATHETIC DENERVATION

Treatment	Average arterial blood pressure mm Hg		Average cardiac output ml/kg/min		Total peripheral resistance		
	Before NA	Change produced by NA	Before NA	Change produced by NA	Before NA	Change produced by NA	% change
Control	85	+22·5	153	+2	0·55	+0·14	25·4
After bretylium 5 mg/kg i.v.	70	+50·0*	117*	+38*	0·60	+0·17	28·0
Control	80	+20·0	165	+5	0·48	+0·10	20·8
After hexamethonium 10 mg/kg i.v.	65	+30·0*	117*	+24*	0·55	+0·12	21·8
Control	90	+29·0	140	−4	0·64	+0·23	35·9
After spinal anaesthesia ..	47	+41*	88*	+32*	0·53	+0·20	37·7
Control	100	+25	170	0	0·59	+0·14	23·7
After cardiac sym. denerv.	80	+51*	106*	+36*	0·75	+0·17	22·6
Control	90	+28	138	+3	0·65	+0·19	29·2
After pyrogallol	95	+48*	136	0	0·70	+0·35*	50·0*

(+) Indicates an increase and (−) a decrease in cardiac output. Each value in the table represents a mean of four experiments. Significance of differences between means was examined by "*t*" test and is indicated by asterisk; P = <0·05.

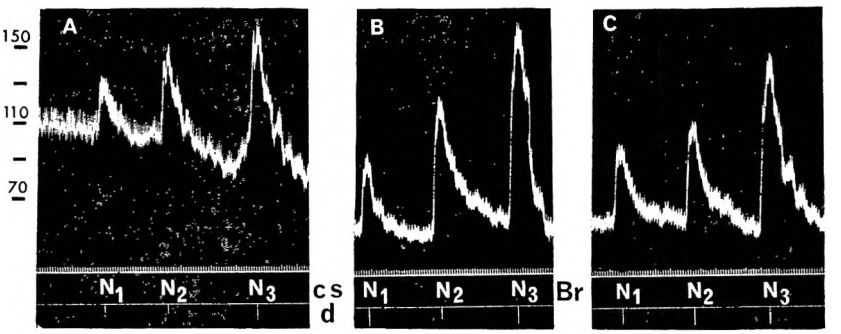

FIG. 3. The effect of cardiac sympathetic denervation followed by bretylium on the pressor responses to intravenous noradrenaline in the spinal cat (2·8 kg). A. Responses to noradrenaline 0·25 (N_1) 0·5 (N_2) 1·0 (N_3) μg/kg. B. 15 min after acute sympathetic cardiac denervation (csd). C. 30 min after the intravenous bretylium (Br) (5 mg/kg). Time trace: 10 sec intervals.

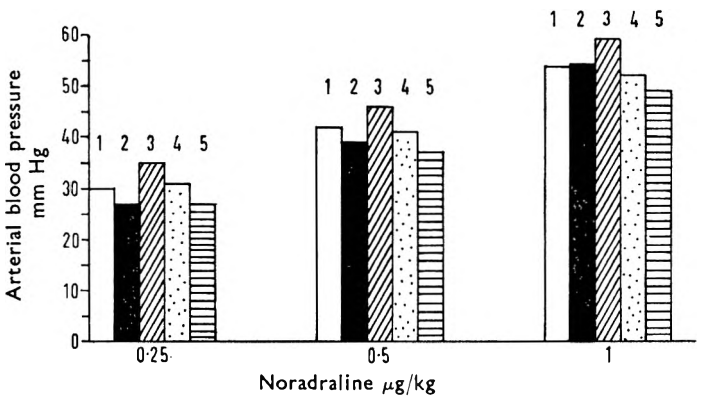

FIG. 4. Spinal cats pretreated with pronethalol (5 mg/kg i.v.) injected 15 min before the beginning of the experiments. Drugs and procedures and control and test animals as described in Fig. 1. Hexamethonium (2), bretylium (3), spinal anaesthesia (4) or cardiac sympathetic denervation (5) did not potentiate the pressor action of any doses of noradrenaline. Significance of difference between means (control and treated) was examined by 't' test; $P = <0{\cdot}05$.

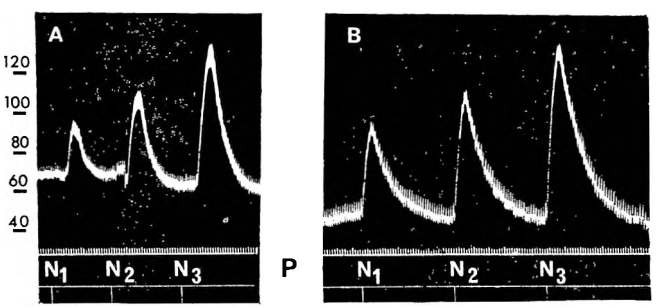

FIG. 5. The effect of pyrogallol (10 mg/kg i.v.) on the pressor responses to intravenous noradrenaline (0·25 (N_1), 0·5 (N_2) and 1·0 (N_3) μg/kg i.v.) in a spinal cat (2·2 kg) with a sympathetically denervated heart. Acute cardiac sympathectomy was made 15 min before beginning the experiment. A. Responses to noradrenaline. B. 15 min after the administration of pyrogallol (P). Time trace: 10 sec interval.

Discussion

The degree of potentiation produced by bretylium, hexamethonium, spinal anaesthesia and cardiac sympathetic denervation was similar in all experiments. These results agree with those of Hilton, Arellano & Fenner (1963) who found that ganglion blocking drugs and spinal anaesthesia produced equal potentiation of the pressor action of adrenaline in dogs. When potentiation was produced by one drug or procedure, the other three drugs or procedures failed to increase the pressor action of noradrenaline any further. These results indicate a similar mode of action for bretylium, hexamethonium, cardiac sympathectomy and spinal anaesthesia in potentiating the increase in blood pressure produced by noradrenaline and suggest that the drugs act by producing a "chemical sympathectomy." Pyrogallol, which potentiates the pressor action of sympathomimetic catecholamines by protecting them from enzymatic destruction (Bacq, 1936a, b), further potentiated the pressor action of noradrenaline after bretylium, hexamethonium, cardiac sympathectomy or spinal anaesthesia.

However, Mantegazza, Tyler & Zaimis (1958) have shown potentiation of the vasoconstrictor action of noradrenaline, and the potentiation of the actions of adrenaline, noradrenaline and of post ganglionic sympathetic nerve stimulation on the nictitating membrane of the cat has been demonstrated by Shimamoto, Kanauchi & Uchizuim (1955). According to these authors such a potentiation does not involve the abolition of the sympathetic tone.

By whichever of the four methods the potentiation was produced, it was accompanied by an increase in cardiac output with no further increase in peripheral resistance. These findings support those of Laurence & Nagle (1963) who have shown that the pressor potentiation of noradrenaline by bretylium and guanethidine in man is not due to increased peripheral resistance. Hodge & Whelan (1962) and Wilber & Brust (1958) have reported similar findings for the potentiation of noradrenaline by ganglion blocking drugs.

Eckstein & Horsley (1961) and Zimmerman, Brody & Beck (1960) have shown that removal of sympathetic tone to the heart causes a reduction in heart rate, cardiac output and ventricular work. Thus it is hardly surprising that noradrenaline injected under these conditions increases cardiac output. The question as to why noradrenaline does not increase cardiac output in animals with intact sympathetic innervation is difficult to explain although it has been demonstrated before. Thus according to Eckstein & Horsley (1961), in the presence of intact sympathetic innervation, the heart fails to increase its rate and output when atrial pressure is increased, whereas after denervation it responds to an increase in filling pressure by increasing its rate and output.

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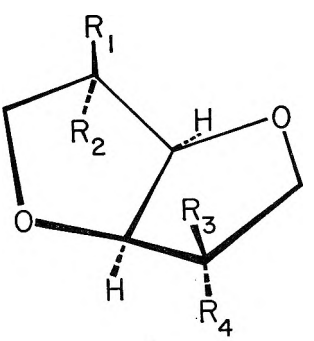
Pharmacological properties of isoidide dinitrate

J. E. HALLIDAY AND S. C. CLARK

Isoidide dinitrate is a more active vasodepressor agent in anaesthetised animals than either of its geometric isomers, isosorbide dinitrate and isomannide dinitrate. Isoidide dinitrate also has the greatest vasodilator action of the three isomers on the vascular system of the dog hind leg and is a more potent hypotensive agent than isosorbide dinitrate in hypertensive rats. The greater activity of isoidide dinitrate may be attributable to its higher oil : water partition coefficient or enhanced drug-receptor interaction resulting from the spatial positions of the nitrate groups or both factors. No evidence was found for involvement of adrenergic receptors in responses of vascular or intestinal smooth muscle to isoidide dinitrate.

MANY nitrate esters have been examined for vasodilator activity including 1,4:3,6-dianhydro-D-mannitol 2,5-dinitrate (isomannide dinitrate; IMDN) and its geometric isomer 1,4:3,6-dianhydro-D-sorbitol 2,5-dinitrate (isosorbide dinitrate; ISDN; Carvasin; Isordil) (Krantz, Carr, Forman & Ellis, 1939a & 1939b). These two esters were the subjects of a comparative study by Goldberg (1948) who found that isosorbide dinitrate was a more active vasodepressor and coronary vasodilator than isomannide dinitrate. He also reported the former to be effective in lowering the blood pressure in hypertensive patients and of value in angina pectoris and intermittent claudication.

More recently Jackson & Hayward (1960) have prepared 1,4:3,6-dianhydro-L-iditol 2,5-dinitrate (isoidide dinitrate; IIDN) thus making three known isomeric dianhydrohexitol dinitrates. These three esters have their nitrate groups attached to the same carbon atoms and differ only in the spatial arrangement of these groups. The configurational formula for the 1,4:3,6-dianhydrohexitol (isohexide) molecule with the various positions of the nitrate groups has been represented as shown by I.



I

The molecule possesses two fused five-membered rings having a *cis* arrangement of the ring junction. The rings, being nearly planar, are inclined to one another at an angle of about 120°. Substituents on the

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carbon atoms at positions 2 and 5 can either be *exo* (R_2R_4) or *endo* (R_1R_3). The conformation of the ring system is rigid (Jackson & Hayward, 1959).

We have compared the pharmacological properties of isoidide dinitrate with its two geometric isomers and investigated possible mechanisms of action for the compound.

Experimental

BLOOD PRESSURE IN ANAESTHETISED ANIMALS

Arterial blood pressure was recorded in 6 cats and 5 dogs anaesthetised with pentobarbitone sodium and in 4 rabbits anaesthetised with allobarbitone and urethane. Respiration was recorded simultaneously in some animals. The nitrates, as a 1% solution in 20% ethanol, were administered intravenously in doses ranging from 0·1 to 0·5 mg/kg depending on the responsiveness of the animal. In the 5 dogs the nitrates were administered intraduodenally as a warmed aqueous suspension containing 5 mg of the nitrate per ml with acacia as a suspending agent. Administration was preceded and followed by 5 ml of warm water by the same route. The order in which the nitrates were given was changed from animal to animal each nitrate being administered at least on one occasion, first, second and last in order.

HYPERTENSIVE RATS

Male Wistar rats, 250–300 g weight, were made hypertensive by the method of Grollman (1944). Within 4 to 6 weeks most of the rats had blood pressures ranging from 170 to 230 mm of mercury (Photoelectric Tensometer, Metro Scientific, Inc.). Before taking blood-pressure readings the rats were fasted for 16 hr then given 50 mg/kg of phenobarbitone sodium intraperitoneally. Control blood-pressure levels were determined 1½ to 2 hr after administration of phenobarbitone and then isosorbide dinitrate or isoidide dinitrate, 10 mg/kg, was given by stomach tube as a 0·1% solution in 10% ethanol. Blood-pressure readings were continued at 5 to 10 min intervals until control levels were restored. A cross-over comparison was made by testing both nitrates in 14 hypertensive rats; at least 3 days were allowed between the administration of drugs to the same rat. Following the same procedure, the effect of the solvent alone was then determined in 8 of the hypertensive rats.

PERIPHERAL BLOOD FLOW

The effect of the three nitrates on the blood flow in the hind limb was determined in 4 dogs using a bubble flowmeter to intercept the flow through the femoral artery. The drugs were injected into the return tube and each nitrate, 0·1 mg in 0·1 ml of 20% ethanol, was tested at least twice in each dog. Glyceryl trinitrate, in the same solvent, was sometimes used for comparison. The dogs were anaesthetised with pentobarbitone sodium and given 3 mg/kg of heparin before making measurements. Supplementary heparin, 1 mg/kg, was given at hourly intervals through a cannula in the external jugular vein. Arterial blood pressure was recorded from the opposite femoral artery.

WATER SOLUBILITY AND OIL : WATER PARTITION COEFFICIENT

The water solubility of the nitrates was determined at 20°. The oil : water partition coefficient for each nitrate was found by dissolving 10 mg in 50 ml of maize oil and shaking with an equal volume of water for 90 min at 20°. After separation the nitrate content of the aqueous layer was determined by the method of Whalen (1930).

MECHANISM OF ACTION

Male Wistar rats, 300–500 g, were anaesthetised with pentobarbitone sodium or urethane and prepared for recording arterial blood pressure by a technique similar to that of Dekanski (1952). Heparin, 1·5 mg/100 g, was injected intravenously before cannulation of the carotid artery with polythene tubing (PE50). Pentolinium tartrate, 0·125 mg/rat was injected initially and the animal left for about 1 hr to allow blood-pressure levels to stabilise. This procedure, recommended by Gillis & Nash (1961), minimised fluctuations in blood pressure during the experiment. Rectal temperatures were maintained at 33°. Isoidide dinitrate was administered intravenously in doses of 0·05 mg/rat. Some rats were given 2 mg/kg of reserpine the day before the experiment to deplete tissue catecholamines.

Segments of rabbit duodenum were suspended in a 25-ml bath containing Tyrode solution at 38° and gassed with oxygen 95% and carbon dioxide 5%. The lever was lightly weighted so that the longitudinal muscle maintained a high level of tone and exhibited good responses to relaxant drugs. To deplete catecholamines, reserpine 0·5 mg/kg was administered subcutaneously to some rabbits 18 hr before the experiment.

DRUGS

Samples of crystalline isomannide dinitrate, isosorbide dinitrate and isoidide dinitrate were provided by Dr. L. D. Hayward and a commercial sample of powdered isosorbide dinitrate with 75% lactose was also used. Other drugs used were glyceryl trinitrate (hypodermic tablets Parke-Davis), adrenaline hydrochloride, isoprenaline hydrochloride, phenylephrine hydrochloride, tyramine hydrochloride, dichloroisoprenaline hydrochloride (DCI), pronethalol hydrochloride, phenoxybenzamine hydrochloride, guanethidine, papaverine, histamine diphosphate and diphenhydramine hydrochloride. Concentrations of the salts are expressed as such. Reserpine (Serpasil) was used as the parenteral solution.

Results

BLOOD PRESSURE IN ANAESTHETISED ANIMALS

Intravenous administration of small doses of the three nitrates produced a transient fall in blood pressure : in rabbits and dogs, 0·1 mg/kg was usually sufficient; in cats, doses of 0·2 mg/kg or more were required. The response to isoidide dinitrate was always greater and persisted longer than the responses to either of its isomers. The responses to isosorbide dinitrate were sometimes slightly greater than those to isomannide dinitrate

but in most instances were similar (Fig. 1). In terms of average fall in blood pressure the potency exhibited by isoidide dinitrate was 1·7 to 2·1 times that of isomannide dinitrate and 1·4 to 2·1 times that of isosorbide dinitrate.

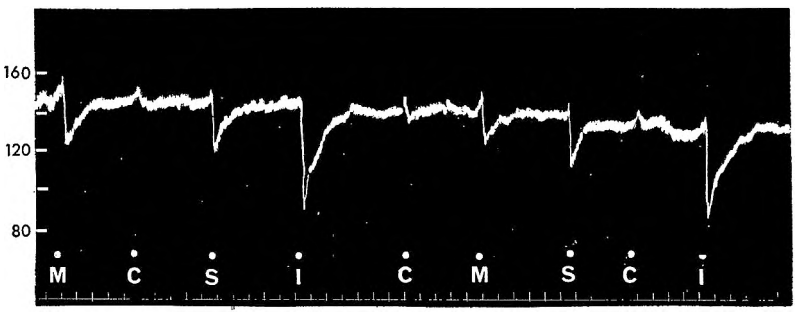

FIG. 1. Effects of isohexide dinitrates on carotid arterial pressure of an anaesthetised cat. Intravenous injection of 0·2 mg/kg of isomannide dinitrate (M), isosorbide dinitrate (S) and isoidide dinitrate (I). Control injections of solvent at C. Time, 1 min.

Introduction of the nitrates into the duodenum of dogs was followed by a depressor response indicating that absorption readily occurred. The fall in blood pressure after isoidide dinitrate was always greater than that after either of its isomers, regardless of the order of administration (Fig. 2).

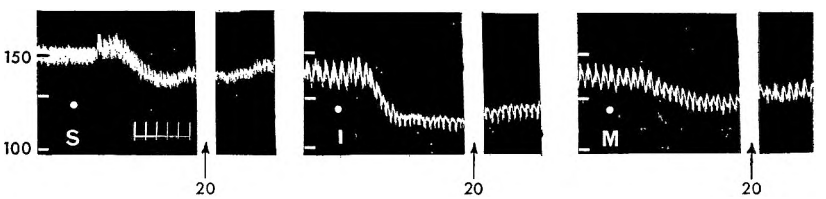

FIG. 2. Effect of isohexide dinitrates on carotid artery blood pressure of anaesthetised. dog. Intraduodenal administration of 4 mg/kg of isosorbide dinitrate (S), isoidide dinitrate (I) and isomannide dinitrate (M) in order shown. Intervals between giving drugs 2½ hr. Interval between consecutive traces 20 min. Time, 1 min.

HYPERTENSIVE RATS

Oral administration of 10 mg/kg of isosorbide dinitrate or isoidide dinitrate caused a decline in blood pressure which became maximal at 20 to 25 min. No hypotensive effect was produced in the rats which received the solvent alone. The mean fall % in blood pressure produced by isoidide dinitrate was significantly greater than that produced by isosorbide dinitrate. The activities of the isomers did not differ significantly in onset or duration of action. The results are summarised in Table 1.

PERIPHERAL BLOOD FLOW

Injection of 0·1 mg of any of the isomers into the femoral artery of the dog caused a transient increase in rate of blood flow through the hind limb.

TABLE 1. EFFECT OF ISOHEXIDE DINITRATES ON BLOOD PRESSURE OF HYPERTENSIVE RATS. ORAL ADMINISTRATION OF 10MG/KG

Ester	Mean fall in blood pressure % ± s.e.	Time to maximum effect (min) ± s.e.	Duration* (min) ± s.e.
Isosorbide dinitrate ..	14·0 ± 1·5	21·7 ± 0·97	34·8 ± 3·47
Isoidide dinitrate ..	20·9 ± 1·98	23·5 ± 0·94	47·4 ± 5·56
	P < 0·01	P > 0·2	P > 0·05

* Time until return to control levels

No difference was demonstrated between the effects of isomannide dinitrate and isosorbide dinitrate on blood flow. The mean response to isoidide dinitrate was significantly greater than the response to either of the other isomers, but much less than the response to glyceryl trinitrate. (Table 2). Intra-arterial injection of 0·1 mg of any of the isomers had no effect on systematic blood pressure.

TABLE 2. EFFECT OF ISOHEXIDE DINITRATES ON BLOOD FLOW IN THE DOG HIND LEG. EXPRESSED AS INCREASE % IN FLOW AFTER INTRA-ARTERIAL INJECTION OF 0·1 MG

Dog No.	Isomannide dinitrate	Isosorbide dinitrate	Isoidide dinitrate	Glyceryl trinitrate
1	61·0	65·8	89·3	
	87·5	85·0	131·0	209·0
2	34·9	37·4	66·7	
	45·8	78·2	138·8	
	47·0	59·4	90·0	
3	59·2	50·0	89·0	299·0
	39·0	36·5	95·0	204·0
4	80·2	64·3	112·5	
	80·2	88·7	143·3	240·0
Average ± s.e.	59·4 ±6·46	62·8 ±6·36	109·2* ±7·68	238·0 ±22·8

* Significantly different from the mean values for isosorbide and isomannide dinitrates (P < 0·001).

WATER SOLUBILITY AND OIL : WATER PARTITION COEFFICIENTS

The results, each based on 5 determinations, are shown in Table 3. The water-solubility values found for isomannide dinitrate and isosorbide dinitrate correspond closely to those reported (1·7 mg/ml for isomannide dinitrate, Krantz, Carr, Forman & Ellis, 1939b; 1·08 mg/ml for isosorbide dinitrate, Sherber & Gelb, 1961).

TABLE 3. WATER SOLUBILITY AND OIL: WATER PARTITION COEFFICIENTS OF ISOHEXIDE DINITRATES

Ester	Water solubility* mg/ml	Oil/water coefficient*
Isomannide dinitrate	1·67 ± 0·3	81·42 ± 2·15
Isosorbide dinitrate	1·08 ± 0·2	112·0 ± 8·80
Isoidide dinitrate	0·845 ± 0·02	158·4 ± 7·50

* average of 5 values ± s.d.

STUDIES ON MECHANISM OF ACTION

The tone of isolated rabbit intestine was reduced when any of the isomers was added to the bath at a concentration of 2 mg%. The relaxant

effect of isoidide dinitrate was greater than that of either of the other isomers. The effect produced by isoidide dinitrate was not prevented by dichloroisoprenaline, pronethalol, phenoxybenzamine or a combination of pronethalol and phenoxybenzamine. Exposure of the gut for 90 min to guanethidine, or pre-treatment of the rabbit with reserpine did not prevent the response to isoidide dinitrate.

When addition of any one of the nitrates was repeated several times, tachyphylaxis developed to all of the isomers, but the gut still responded to isoprenaline, adrenaline or papaverine. The vasodepressor effect of isoidide dinitrate in the rat was not reduced by previous administration of pronethalol, and in rats pretreated with reserpine responses were unaltered. The depressor effect produced by isoidide dinitrate was not prevented by diphenhydramine.

Discussion

The three isomeric dinitrate esters are structurally identical, except for the spatial orientation of their nitrate groups. Alteration in the position of these groups results in different oil: water partition coefficients and different pharmacological potencies. The isomer most active pharmacologically, isoidide dinitrate, also has the highest oil: water coefficient. For some series of nitrate esters an increase in oil: water partition coefficient seems to be responsible for increased pharmacological activity (Krantz, Carr, Forman & Cone, 1940). But it does not seem likely that this physical property has a direct influence in determining the potency of the isohexide dinitrates, since isosorbide dinitrate, which has a considerably higher oil: water coefficient than isomannide dinitrate, did not differ from it in potency. The position of the nitrate groups probably has a more direct effect on the activity of these compounds. Both of the nitrate groups of isoidide dinitrate are in the *exo*-position (see 1) and may thus combine most readily with tissue receptors. The nitrate groups of isomannide dinitrate, both being in the *endo*-position, may approach receptors the least readily because of hindrance by the isohexide skeleton. The arrangement of only one of the nitrate groups in the *exo*-position, as in isosorbide dinitrate, does not significantly increase potency and this seems to indicate that combination of both groups with tissue receptors is necessary for activity.

Relaxation of smooth muscle, the most characteristic effect of nitrate esters, is associated physiologically with adrenergic stimulation, particularly of β-receptors. The present availability of a variety of pharmacological tools for studying adrenergic mechanism prompted us to see if any evidence could be found for the involvement of adrenergic receptors in responses to isoidide dinitrate. However, though the responses of the preparations to this drug resembled those resulting from stimulation of β-receptors by agents such as isoprenaline, or with the gut, from the results of stimulation of both types of receptors by adrenaline, there was no indication that either an action on adrenergic receptors or a release of noradrenaline contributed to its action.

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Action of the neurohypophysial hormones on the vascular system of the male monkey and the rooster

R. R. CHAUDHURY AND T. K. TARAK

Oxytocin caused a fall in the blood pressure of the normal, oestrogen- and progesterone-treated male monkeys. Vasopressin always caused a rise of blood pressure in similarly treated monkeys and oestrogen and progesterone treatment did not enhance the pressor response. Oxytocin also caused a fall in the blood pressure of the normal, oestrogen- and progesterone-treated rooster.

IN 1959, Lloyd demonstrated that the hypotensive effect of oxytocin on the rat was converted to a hypertensive effect by treating the animal with oestrogen. The pressor effect of vasopressin was exaggerated in these animals. We were interested to see whether this phenomenon would occur in the primate and in the rooster, in which animal the oxytocin-induced fall in the blood pressure is the parameter used for its assay and standardisation (Coon 1939).

Experimental

Eleven male rhesus monkeys weighing 1·4 to 3 kg were anaesthetised with pentobarbitone sodium (40 mg/kg) and the carotid blood pressure recorded. Injections of oxytocin, 1·0 unit, and vasopressin, 0·5 unit, were made through the femoral vein. Oestradiol dipropionate, 100 μg/kg, was injected subcutaneously daily for 3 days before the experiment in four monkeys. Progesterone, 1 mg/kg, was injected subcutaneously daily for 3 days before the experiment in four monkeys. Progesterone, 1 mg/kg, was injected subcutaneously daily for 3 days before the experiment in another four monkeys. Three monkeys served as controls, Ten roosters weighing 1·3 to 2·8 kg were anaesthetised with pentobarbitone sodium (35 mg/kg) and the carotid blood pressure recorded. Injections of oxytocin, 0·5 unit, were made through the subclavian vein. Four roosters served as controls while four were injected daily with oestradiol dipropionate, 100 μg/kg, subcutaneously for 3 days before the experiment. Two birds were injected subcutaneously daily for 3 days with progesterone, 1 mg/kg, before the experiment.

Results

Table 1 shows the fall in the blood pressure when 1·0 unit of oxytocin was injected intravenously into normal, oestrogen- and progesterone-treated monkeys. It also indicates the rise in the blood pressure caused by the intravenous administration of 0·5 unit vasopressin in these animals. Fig. 1 shows the results in a typical series of experiments in the monkeys where oxytocin caused a fall in the blood pressure of normal, oestrogen- and progesterone treated-animals. In the rooster, oxytocin at a dose 0·5 unit caused a fall in the blood pressure in all birds (Table 2).

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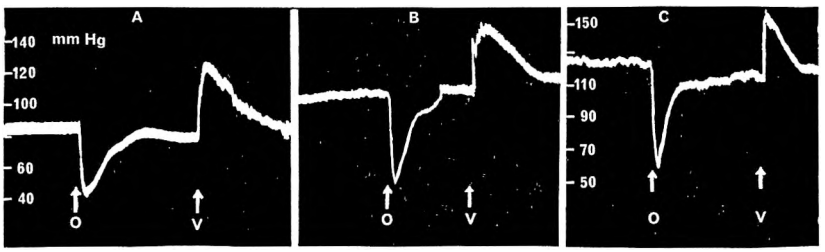

FIG. 1. Action of 1 unit of oxytocin (O) and 0·5 unit of vasopressin (V) on the blood pressure of A, normal monkey; B, monkey pretreated with oestradiol dipropionate, 100 μg/kg/day for 3 days; C, monkey pretreated with progesterone, 1 mg/kg/day for 3 days.

TABLE 1. THE EFFECT OF OXYTOCIN AND VASOPRESSIN ON THE BLOOD PRESSURE OF THE MONKEY

	Fall in B.P. due to 1 unit of oxytocin (mm Hg)	Rise in B.P. due to 0·5 unit of vasopressin (mm Hg)
Normal animals	20, 40, 70	34, 44, 38
Oestradiol dipropionate treated animals (100 μg/kg/day for 3 days)	53, 22, 54, 52	26, 40, 34
Progesterone treated animals (1 mg/kg/day for 3 days)	68, 63, 56, 54	24, 40, 32, 28

TABLE 2. THE EFFECT OF OXYTOCIN ON THE BLOOD PRESSURE OF THE ROOSTER

	Fall in B.P. due to 0·5 unit of oxytocin (mm Hg)
Normal Animals	64, 92, 42, 60
Oestradiol dipropionate treated animals (100 μg/kg/day for 3 days)	58, 50, 60, 50
Progesterone treated animals (1 mg/kg/day for 3 days)	68, 56

Discussion

Our results indicate that oxytocin caused a fall in the blood pressure of the normal monkey and the same result was obtained when these animals were pre-treated for 3 days with 100 μg/kg of oestrogen or 1 mg/kg of progesterone. In no experiment did we observe a hypertensive effect of oxytocin. Roosters treated with oestrogen or progesterone behaved similarly. Lloyd & Pickford (1962) have demonstrated that oestrogen treatment in dogs did not reverse the blood pressure responses to oxytocin but that in some vascular beds the vasodilator effect was converted to a constrictor effect. The same phenomenon may occur in monkeys and roosters. The fall in the blood pressure, however, was clearly seen in all the monkeys and roosters with prior oestrogen or progesterone treatment and in the doses used these ovarian hormones did not alter the overall hypotensive effect of oxytocin.

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Potential reserpine analogues. Derivatives of reduced isoquinolines and of meconine

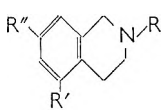
NOORUL HAQ KHAN AND L. K. SHARP

The syntheses of a series of eight 1,2,3,4-tetrahydro-2-methylisoquinolines substituted in positions 5 and 7 are described. In addition meconine-3-acetanilide and meconine-3-acetmorpholide have been prepared. None of the compounds caused any appreciable potentiation of barbiturate hypnosis in mice. Other tests for reserpine-like activity were not made.

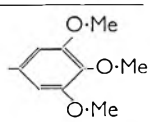
IN the belief that the pharmacological activity of reserpine may be connected with the presence of an isoquinoline fragment (rings D & E), 1,2,3,4-tetrahydro-5-methoxycarbonyl-2-methyl-7-(3,4,5-trimethoxybenzamido)isoquinoline [I; R = Me; R′ = COOMe; R″ = $NHCOC_6H_2(OMe)_3$] was prepared and subjected to pharmacological tests.

In addition, following the work of Chodnekar, Karim, Linnell & Sharp (1960, 1962) on substituted benzamides as potential reserpine analogues, meconine-3-acetanilide (II) and meconine-3-acetmorpholide (III) were also prepared, and tested.

None of the above compounds showed any appreciable potentiation of barbiturate hypnosis in mice and therefore other pharmacological tests, i.e., those for the depletion of the 5-hydroxytryptamine content of brain and spleen and for hypotensive action, were not made.



(I)

Compound	R	R′	R″
	Me		
IV	Me	CN	H
V	Me	CN	NO_2
VI(HCl)	Me	CO_2H	NO_2
VII	Me	CO·O·Me	NH_2
VIII	Me	CO·O·Me	OH
IX	Me	$CH_2·NH_2$	—
X	Me	$CO·NH·NH_2$	NO_2

The D and E rings of reserpine consist of a substituted decahydroisoquinoline; these isoquinoline derivatives should carry the same substituents at positions 5 and 7 as are present in reserpine at the corresponding positions 16 and 18. The methoxyl group at position 17 in reserpine is not important as it was shown that 17-desmethoxydeserpidine

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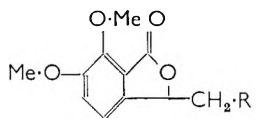
has hypotensive and sedative properties comparable with those of reserpine.

Onda, Kawanishi & Sasamoto (1956) prepared some tetrahydroisoquinolines with various substituents at positions 6 and 7. More recently Chodnekar, Sharp & Linnell (1962) have reported a series of benzamide derivatives; their work was based on the previous work of Karim & others (1960) with an attempt to combine the pharmacologically active trimethoxybenzanilide with other favoured fragments of reserpine. Their preparation of amides of 4-ethoxycarbonyloxy-3,5-dimethoxybenzoic acid followed the work of Lucas, Kudhne, Ceglowski, Dzieman & Macphillamy (1959) on syrosingopine.

The observation of Karim & others (1960) and the report by Borsy (1960) that the morpholide of 3,4,5-trimethoxybenzoic acid is neurosedative prompted the preparation and testing of the compounds II and III.



(II) R = CO·NHPh

(III) R = CO·N O

Experimental

5-Cyano-2-methylisoquinolinium iodide. 5-Cyanoisoquinoline (0·2 g) was dissolved in excess of methyl iodide (5 ml) by warming on a water-bath until a clear solution was obtained. It was kept at room temperature for 4 hr. Ether was added and the crystalline methiodide thus obtained was filtered off and washed with ether. Yield 0·29 g (50%), m.p. 275–76° (decomp.). Found: C, 44·6; H, 3·0; N, 9·7. $C_{11}H_9IN_2$ requires C, 44·6; H, 3·05; N, 9·5%.

5-Cyano-1,2,3,4-tetrahydro-2-methylisoquinoline (IV). 5-Cyano-2-methylisoquinolinium iodide (0·9 g) in methanol (30 ml) was treated with potassium borohydride (1 g), added slowly with shaking and gentle warming on a water-bath. The reaction mixture was left overnight, the solvent removed at the water pump, and the residue taken up in little water. The solution was basified with 2% sodium hydroxide solution and extracted with chloroform. The extract was washed with water, dried over Na_2SO_4 (anhydrous), filtered, and the solvent removed *in vacuo*. The residue on distillation yielded a liquid (b.p. 75°/0·1 mm) which solidified to a crystalline solid melting at 40–42°, 52° after crystallisation from ether. Found: C, 76·6; H, 7·15; N, 16·0. $C_{11}H_{12}N_2$ requires C, 76·7; H, 6·9; N, 16·3%.

5-Cyano-1,2,3,4-tetrahydro-2-methyl-7-nitroisoquinoline (V). To 5-cyano-1,2,3,4-tetrahydro-2-methylisoquinoline (0·9 g) dissolved in concentrated sulphuric acid (d = 1·84) (4 ml) was added dropwise with

continuous stirring a solution of sodium nitrate (0·9 g) in concentrated sulphuric acid (d = 1·84) (4 ml) at 0°. The reaction mixture was kept at room temperature for 2 hr, and then poured onto crushed ice. The solution obtained was basified with sodium hydrogen carbonate (solid) and extracted with chloroform. The chloroform solution was washed with water, dried over anhydrous sodium sulphate and filtered. Removal of the solvent at the water pump at low temperature yielded the nitro-compound, which crystallised from ethyl acetate and light petroleum (b.p. 40–60°), m.p. 111–12° to yield 0·9 g (70%). Found: C, 60·9; H, 5·1; N, 18·5. $C_{11}H_{11}N_3O_2$ requires C, 60·8; H, 5·1; N, 19·35%.

The location of the nitro-group at position 7 was established by nuclear magnetic resonance spectrum of the compound V as follows.

τ	Number of protons	Appearance	Assignment
7·44	3	Singlet	N – Me
7·13	2	Distorted triplet J = 5 c/s	Protons on position 3
6·83	2	Distorted triplet J = 5 c/s	Protons on position 4
6·26	2	Singlet	Protons on position 1
1·73	1	Singlet ⟩ meta splitting at top	Proton on position 6
1·53	1	Singlet ⟩ of peak J = 2·5 c/s	Proton on position 8

The spectrum was run in deuterochloroform with tetramethylsilane as an internal standard. The spectrum tabulated above has been totally assigned and firmly supports the suggested structure. The alternative structure with nitro-group on position 8 is thus ruled out.

1,2,3,4-*Tetrahydro*-2-*methyl*-7-*nitroisoquinoline*-5-*carboxylic acid hydrochloride* (VI). 5-Cyano-1,2,3,4-tetrahydro-2-methyl-7-nitroisoquinoline (0·55 g) and concentrated hydrochloric acid (6 ml) were heated in a sealed Carius tube in a muffle furnace at 150° for 8 hr. The tube was then left to cool to room temperature and the yellowish crystalline slurry transferred with the aid of the least amount of water to an evaporating dish and evaporated on a water-bath. The golden-yellow solution obtained by dissolving the residue in water and charcoaling, deposited crystals which melted at 239–42° (frothing and decomp.). These on recrystallisation from MeOH/ether yielded needles, having the same melting-point. Found: C, 48·1; H, 4·7; Cl, 13·2; N, 10·1. $C_{11}H_{13}ClN_2O_4$ requires C, 48·4; H, 4·8; Cl, 13·0; N, 10·3%.

7-*Amino*-1,2,3,4-*tetrahydro*-5-*methoxycarbonyl*-2-*methylisoquinoline* (VII). (*a*) 1,2,3,4-Tetrahydro-2-methyl-7-nitroisoquinolin-5-carboxylic acid hydrochloride (0·2 g) in thionyl chloride (2·0 ml) was heated on a water-bath until all the thionyl chloride had evaporated off. Absolute methanol, at the water-bath temperature, was carefully added until solution was obtained. After removal of the methanol, the residue was washed with ether and crystallised from methanol/ether yielding the hydrochloride of the required product melting at 195–198° (decomp. sealed tube), yield 0·15 g (71%).

(*b*) 1,2,3,4-Tetrahydro-5-methoxycarbonyl-2-methyl-7-nitroisoquinoline hydrochloride (0·07 g) was dissolved in the minimum quantity of water and ammonia solution was added until the solution was just alkaline.

This at once deposited a pale yellow precipitate, which after extracting with ethyl acetate, washing the ethyl acetate extract with water, drying over Na_2SO_4 (anhydrous) and removing the solvent, was crystallised from ethyl acetate/light petroleum (40–60°) yielding the free base m.p. 129–30°.

(*c*) 1,2,3,4-Tetrahydro-5-methoxycarbonyl-2-methyl-7-nitroisoquinoline (0·4 g) was hydrogenated in ethanol (50 ml) with palladium charcoal (30%, 0·9 g) for 2½ hr. After 1 hr the absorption of hydrogen seemed to be complete. The reaction mixture was filtered, and the solvent removed from the filtrate *in vacuo*. The residue, on crystallisation from Me OH/ether yielded the product (VII), m.p. 144–46°. Found: C, 65·3; H, 7·5; N, 12·8. $C_{12}H_{16}N_2O_2$ requires C, 65·45; H, 7·3; N, 12·7%.

The *base hydroiodide* melted at 219°. *Acetyl derivative:* m.p. 93–95°.

1,2,3,4-*Tetrahydro*-5-*methoxycarbonyl*-2-*methyl*-7-(3,4,5-*trimethoxybenzamido*)*isoquinoline* (I). 3,4,5-Trimethoxybenzoic acid (0·34 g) was dissolved in redistilled thionyl chloride (5 ml) and the solution evaporated on a water-bath. The residue was again treated similarly. Benzene (5 ml) was added and evaporated. To the 3,4,5-trimethoxybenzoyl chloride left behind, was added a solution of 7-amino-1,2,3,4-tetrahydro-5-methoxycarbonyl-2-methylisoquinoline (0·3 g) in dry pyridine (5 ml) whereupon a precipitate appeared at once. The mixture was evaporated to dryness on the water-bath. Water (least amount) was added to the residue and the solution was again evaporated. The residue was again dissolved in the least amount of water, the solution just made alkaline by addition of a dilute sodium hydrogen carbonate solution, then extracted with benzene, washed with water, dried over sodium sulphate (anhydrous) and the solvent removed. The residue (0·3 g) was crystallised from ethyl acetate, m.p. 170–71°. Found: C, 63·9; H, 6·3; N, 6·6. $C_{22}H_{26}N_2O_6$ requires C, 63·8; H, 6·3; N, 6·8%.

1,2,3,4-*Tetrahydro*-7-*hydroxy*-*methoxycarbonyl*-2-*methylisoquinoline* (VIII). A cooled solution of 7-amino-1,2,3,4-tetrahydro-5-methoxycarbonyl-2-isoquinoline (0·4 g) in dilute sulphuric acid (2 ml of concentrated acid in 6 ml water) was diazotised by the dropwise addition with continuous stirring of sodium nitrite solution (0·15 g in 4 ml water). The temperature of the mixture was kept at 0°. The diazotised solution was poured slowly into a boiling saturated copper sulphate solution (15–20 ml) whereupon brisk evolution of nitrogen occurred. Boiling was continued for 5 min. The solution was cooled, basified with concentrated ammonia solution, and extracted with ethyl acetate. The extract was washed with water, dried over sodium sulphate (anhydrous) and filtered. The solvent was then removed from the filtrate. The residue was the crystalline phenolic compound (0·2 g 50%), m.p. 168–70°. 180–181° after crystallising from ethyl acetate. Found: C, 64·7; H, 6·9; N, 6·15. $C_{12}H_{15}NO_3$ requires C, 65·15; H, 6·8; N, 6·3%.

5-*Aminomethyl*-1,2,3,4-*tetrahydro*-2-*methylisoquinoline* (IX). 2-Cyano-1,2,3,4-tetrahydro-2-methylisoquinoline (1 g) was dissolved in sodium-dried ether (24 ml). Lithium aluminium hydride (1 g) was added carefully and the mixture refluxed for 6 hr. The cooled reaction mixture was

decomplexed carefully by addition of water. The base thus liberated was extracted with an additional amount of ether. The ether extract was washed with water, dried over sodium sulphate (anhydrous), filtered and the solvent removed from the filtrate. The residue failed to crystallise. This was therefore dissolved in the least amount of pyridine and an excess of acetic anhydride was added. The mixture was then evaporated in a crystallising dish on a water-bath. The excess of acetic anhydride was destroyed by the repeated addition of water followed by evaporation on a water-bath. The residue, 5-acetamidomethyl-1,2,3,4-tetrahydro-2-methyl isoquinoline, thus obtained was crystallised from ethyl acetate and light petroleum (b.p. 40–60), and melted at 126–126·5°. Found: C, 71·2; H, 8·4; N, 12·7. $C_{13}H_{18}N_2O$ requires C, 71·6; H, 8·3; N, 12·8%.

1,2,3,4-*Tetrahydro-2-methyl-7-nitroisoquinoline-5-carboxyhydrazide* (X). 1,2,3,4-Tetrahydro-5-methoxycarbonyl-2-methyl-7-nitroisoquinoline (0·1 g) was dissolved in methanol, a slight excess of hydrazine hydrate (0·4 g) was added, and the mixture left overnight, whereupon crystals appeared. These were recrystallised from water, m.p. 185°. Found: C, 52·5; H, 6·0; N, 22·6. $C_{11}H_{14}N_4O_3$ requires C, 52·8; H, 5·6; N, 22·4%.

Meconine-3-acetanilide (II). To a solution of aniline (1·8 g) in ether (10 ml) was added meconine-3-acetyl chloride (2·58 g) and left at room temperature for 24 hr. The precipitate was filtered off, washed with water and crystallised from ethanol m.p. 199°–200°. Found: C, 66·1; H, 5·1; H, 4·2. $C_{18}H_{17}NO_5$ requires C, 66·05; H, 5·2; N, 4·3%.

Meconine-3-acetmorpholide (III). To a solution of morpholine (1·74 g) in ether (10 ml) was added meconine-3-acetyl chloride (2·58 g) slowly at 5°. It was kept at room temperature for 24 hr. The precipitate was filtered off and washed with water and crystallised from ethanol m.p. 175–177°. Found: C, 59·55; H, 5·75; N, 4·3. $C_{16}H_{19}NO_6$ requires C, 59·8; H, 5·9; N, 4·4%.

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Letters to the Editor

Vincamajine, the major alkaloid of leaves of *Rauwolfia mannii* Stapf

SIR,—The alkaloidal composition of a number of species of *Rauwolfia* has been studied in recent years (Saxton, 1960). The species of which we know least is *R. mannii* Stapf, an undershrub or small tree 2 to 30 ft high of the thick forests on the borders of Eastern Nigeria and Western Cameroon, also found in New Guinea, Gabon and the Congo. It was previously known as *R. preussii* K. Schum. (Thistelton-Dyer, 1904), also as *R. rosea* K. Schum. (Boutique & Monseur, 1955) in Tanganyika, Kenya and Mozambique (Bisset, 1958). Monseur (1957) found 0·002% of reserpine in the roots of *R. mannii* and Kaiser & Popelak (1959) reported the absence of quaternary bases from the roots. Korzun (1957) examined the weak bases from the roots of the East African material (syn. *R. rosea*) and showed the probable presence of reserpine, rescinnamine, deserpidine, δ-yohimbine and reserpiline. There is no published work on the alkaloids of the leaves of the species.

During a visit to the Oban Forest Reserve in Eastern Nigeria, one of the authors (M.B.P.) collected a small quantity of leaves from plants 2 to 5 ft high, bearing flowers and fruits and taxonomically identified as *R. mannii*.

The powdered, dried leaves (200 g) were extracted by percolation to exhaustion with 5 litres of ethanol containing 1% acetic acid. Solvent was removed from the extract by evaporation at low temperature under reduced pressure, the residue was taken up in 300 ml of 5% tartaric acid solution and filtered. The filtrate was washed several times with light petroleum (b.p. 60–80°), made alkaline with ammonium hydroxide (0·88) and extracted with ether (6 × 150 ml). The combined ether extracts were evaporated to yield 0·95 g of crude base.

Ether-soluble bases (950 mg), dissolved in chloroform (6 ml), were chromatographed on 50 g of kieselgel. Elution was carried out successively as follows, residues from each fraction being examined by thin layer chromatography (kieselgel G Merck Alkaline, solvent: dichloromethane containing 2% methanol, reagent: ceric sulphate):

(*a*) Chloroform: acetone (94: 6; 750 ml) yielded 43 mg, containing at least three alkaloids with Rf values higher than that of vincamajine.

(*b*) Chloroform: acetone (94: 6; 750 ml) yielded 380 mg, mainly vincamajine (see (*c*) below).

(*c*) Chloroform: methanol (95: 5; 500 ml) yielded 185 mg, mainly vincamajine. This residue, combined with that from (*b*) above (565 mg) crystallised readily from methanol to give vincamajine (200 mg; 1 g/kg of dried leaves), m.p. 221°, giving no depression on admixture with an authentic specimen. $[\alpha]_{58}^{23}$ −16° (chloroform containing 0·5% ethanol; c, 1; vincamajine gives −18° under same conditions). Infrared, ultraviolet and mass spectra of the alkaloid and authentic vincamajine were each identical. Found: C, 71·9; H, 7·5; N, 7·7. $C_{22}H_{26}O_3N_2$ requires C, 72·1; H, 7·15; N, 7·65.

(*d*) Chloroform: methanol (95: 5; 1,000 ml) yielded 210 mg, containing two alkaloids with Rf values lower than that of vincamajine.

Vincamajine is thus the principal alkaloid of the leaves of *R. mannii*. It has been previously isolated from *Vinca major* L. (Janot & Le Men, 1955), *Vinca difformis* Pourr. (Gabbai, 1958) and *Alstonia longifolia* (A. DC.) Pichon (= *Tonduzia longifolia* (A. DC.) Markg.) (Goodwin & Horning, 1956), all of

the family Apocynaceae. Its structure is that of a carbomethoxy-16-tetraphyllicine (Gosset, Le Men & Janot, 1961; Janot, Le Men, Gosset & Levy, 1962; Janot, Le Men & Garnier-Gosset, 1965). It is known that tetraphyllicine and its derivatives occur in many species of *Rauwolfia* (Saxton, 1960), but this is the first time, so far as we are aware, that vincamajine has been found in the genus *Rauwolfia*.

Because of the small amount of leaf sample available, we have been unable to study the five other bases which are present in concentrations much smaller than vincamajine but none of them was found to correspond to authentic reserpine.

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Spectral slit width and the absorption of light by substances in the Addendum 1964 to the British Pharmacopoeia 1963

SIR,—The previous surveys (Rogers, 1959, 1964) of slit-width effects have now been extended to those substances that are subject to spectrophotometric assay in the Addendum 1964 to the B.P. 1963. On this occasion, a Hilger and Watts Uvispek H.700 and a Unicam SP. 700 spectrophotometer have been used.

Table 1 lists the drugs examined and shows on the left of the third column the widest instrumental half-intensity spectral slit width h that may safely be used. Prednisolone trimethylacetate has been included in the Table; its assay was changed from a colorimetric to a direct ultraviolet spectrophotometric procedure by an amendment official from January, 1964. Levorphanol tartrate was the subject of a spectrophotometric assay in the B.P. 1963, and so was reported previously (Rogers, 1964), but the figures are repeated here because the injection is the subject of a spectrophotometric assay in the Addendum 1964.

Examination of the Table shows that special care in selecting a sufficiently narrow slit width is needed with levallorphan tartrate and levorphanol tartrate.

TABLE 1. EFFECT OF CHANGE OF SPECTRAL SLIT WIDTH ON THE SPECTROPHOTOMETRIC DETERMINATION OF EXTINCTION

Substance	λ max (mμ)	Max. h (mμ) for extinction error of		
		0·2%	1%	2%
Dichlorophen	304	1·3	1·8	
Edrophonium chloride	273	1·1	1·5	2·2
Ethosuximide	248	1·5		
Levallorphan tartrate	279	0·9	1·6	2·3
Levorphanol tartrate	279	0·7	1·5	2·1
Paracetamol	257	1·7		
Phenolphthalein	550	1·0		
Phenylephrine hydrochloride	273	1·1	1·5	2·2
Prednisolone trimethylacetate	242	1·2		
Spironolactone	238	1·2		

Solutions were prepared as directed by the Addendum 1964 to the B.P. 1963.

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Antagonism of analgesics by amine-depleting agents

SIR,—The antagonism of the analgesic effect of morphine in mice by reserpine was first reported by Schneider (1954) and confirmed more recently by Takagi, Takashima & Kimura (1964). The analgesic response of morphine in reserpinised mice could be returned to control levels by administration of 3,4-dihydroxyphenylalanine, 5 hydroxytryptophan or a combination of the two compounds (Takagi, & others, 1964). They suggested that a possible mechanism for this antagonism of morphine analgesia by reserpine might arise in the need for catecholamines and 5-hydroxytryptamine (5-HT) in the mediation of the action of morphine. The depletion of these amines by reserpine would be expected to result in the failure of morphine to produce analgesia.

The present investigation was undertaken to elucidate further the mechanism of reserpine antagonism of morphine and to discover whether reserpine also antagonised analgesics of the salicylate type.

Male albino mice (Harlan Industries), 18–22 g, were used, and analgesia measured by the ability of morphine or acetylsalicylic acid to antagonise the writhing response which was induced by the intraperitoneal injection of 10 ml/kg of 0·1% hydrochloric acid solution (Eckhardt, Cheplovitz, Lipa & Govier, 1958). ED50 values were calculated by the method of Litchfield & Wilcoxon (1949).

In Table 1 are shown the effects of reserpine, α-methyldopa, and α-methyl-*m*-tyrosine on the ED50 of morphine and acetylsalicylic acid. Reserpine significantly elevated the ED50 values of morphine and acetylsalicylic acid indicating an antagonism of their analgetic effects. Unexpectedly, neither α-methyldopa nor α-methyl-*m*-tyrosine altered the ED50 values of morphine or acetylsalicylic acid. Nor had α-methyldopa or α-methyl-*m*-tyrosine any effect on the writhing response by themselves.

TABLE 1. THE EFFECT OF VARIOUS AMINE DEPLETORS ON THE ANALGESIC RESPONSE TO MORPHINE AND ACETYLSALICYLIC ACID

Treatment	Route	Time[1]	Dose mg/kg	ED50[2] Morphine SO_4 mg/kg s.c.	ED50[2] Acetylsalicylic acid mg/kg s.c.
Control	—	—	—	0·89 (0·71–1·11)	49·5 (42·0–58·4)
Reserpine	s.c.	24	5	2·48 (2·20–2·81)	200·0 (160·0–250·0)
α-Methyldopa	i.p.	4	400	0·88 (0·67–1·16)	35·0 (28·7–42·7)
α-Methyl-*m*-tyrosine	i.v.	4	400	0·61 (0·41–0·91)	42·0 (36·9–47·9)
	i.v.	24	400	1·13 (0·96–1·32)	50·1 (38·6–65·6)

[1] Time of pretreatment
[2] ED50 (Confidence limits)

If depletion of catecholamines or 5-HT or both were responsible for the reserpine antagonism of morphine or acetylsalicylic acid analgesia, then both α-methyldopa (Smith, 1960) and α-methyl-*m*-tyrosine (Hess, Connamacher, Ozaki,& Udenfriend, 1961) which deplete catecholamines and 5-HT should also antagonise this analgesia. Since neither of these two brain amine-depleting agents significantly elevated the ED50 values of morphine or acetylsalicylic acid, it is conceivable that the antagonism of analgesia by reserpine is due to some intrinsic property of reserpine other than its effect on brain amines. This

conclusion is supported by earlier work which suggested that the reserpine antagonism of the anticonvulsant effect of diphenylhydantoin (Chen, Ensor & Bohner, 1954) was competitive and not the result of brain amine depletion (Gray, Rauh & Shanahan, 1963).

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Rapid release of ^{3}H-metaraminol induced by combined treatment with protriptyline and reserpine

SIR,—Two different amine uptake and concentrating mechanisms of the adrenergic neurone have been demonstrated, namely, the amine transport mechanism of the cell membrane, "the cell membrane pump", and the uptake mechanism of the specific storage granules (Carlsson, Hillarp & Waldeck, 1962; Hamberger, Malmfors, Norberg & Sachs, 1964; Hillarp & Malmfors 1964; Malmfors, 1965; Carlsson & Waldeck, 1965a, b). Either of these mechanisms can be selectively blocked by drugs. Thus protriptyline and desipramine were found to block the former, reserpine and prenylamine the latter mechanism.

In the present investigation the effect of simultaneous blockade of the two mechanisms, or of either mechanism alone, was investigated, using ^{3}H-metaraminol as an indicator. Mice were given ^{3}H-metaraminol 0·02 mg/kg intravenously, followed after 15 min by protriptyline 10 mg/kg i.v., or reserpine 0·5 mg/kg i.v., or a mixture of both. The animals were killed 15 or 45 min after the administration of the inhibitors. Determination of ^{3}H-metaraminol in heart was performed as described earlier (Carlsson & Waldeck, 1965a). Given alone protriptyline or reserpine caused a moderate reduction of ^{3}H-metaraminol in the heart (Table 1). In combination, however, the two drugs caused a rapid and pronounced decrease of the amine. Within 15 min, 80% of the ^{3}H-metaraminol had disappeared, and 45 min after the drug mixture had been given only 5% was left. Preliminary experiments where reserpine was replaced by prenylamine gave essentially the same result.

Analogous results were obtained in experiments where the ^{3}H-metaraminol had been given 3 days before protriptyline and reserpine, alone or in combination.

TABLE 1. RELEASE OF ^{3}H-METARAMINOL BY PROTRIPTYLINE AND RESERPINE FROM THE HEARTS OF MICE

Treatment at zero time*	^{3}H-metaraminol in ng/g tissue	
	after 15 min	after 45 min
Control	52	43
	52	45
Protriptyline	34	27
	35	28
Reserpine	28	29
	46	25
Protriptyline + reserpine	11	3
	12	2

The values are single values, obtained from 6 pooled hearts.

* ^{3}H-metaraminol 0·02 mg/kg i.v. 15 min before zero time.

Inhibition of the two uptake mechanisms probably results in unmasking of physiological release mechanisms (Carlsson 1965). Blockade of the cell membrane pump will thus unmask amine release through this membrane. The comparably slow release induced by blockade of this mechanism alone probably indicates that the concentration of free amine in the neurone cytoplasm is low. Blockade of the uptake mechanism of the storage granules will unmask amine release from these granules into the cytoplasm. The most probable reason why blockade of this mechanism alone does not result in rapid loss of amine, is that the cell membrane pump is able to cope fairly successfully with the amine released into the cytoplasm.

Experiments are in progress to investigate the effect of the drug combination on labelled and endogenous noradrenaline.

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