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RESEARCH PAPERS

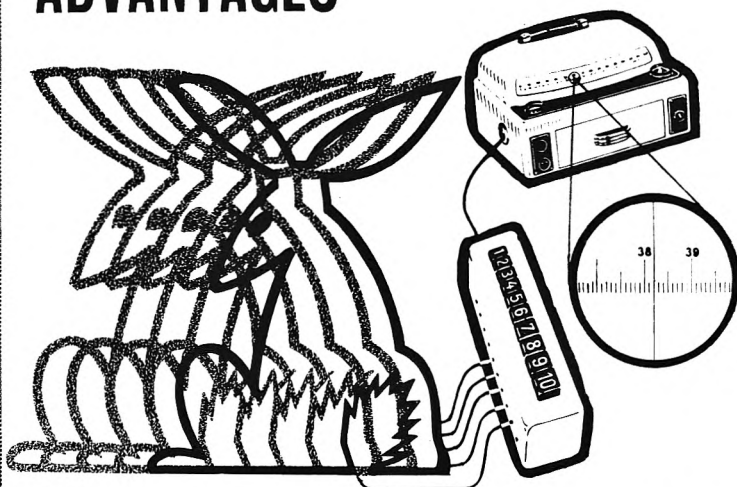## PHARMACOLOGICAL ACTIONS OF HEMLOCK (*CONIUM MACULATUM*) ALKALOIDS

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The actions of three hemlock alkaloids—coniine, *N*-methylconiine and γ-coniceine—have been examined on isolated tissues, on anaesthetised cats and hens and on conscious mice and young chicks. The most pronounced action of the alkaloids was to block spinal reflexes by an action exerted in the spinal cord. Their peripheral actions on autonomically innervated structures were mainly a consequence of an initial stimulant and a secondary depressant action on autonomic ganglia. Large doses of the alkaloids stimulated skeletal muscle and subsequently caused neuromuscular block. This blocking action differed in many respects from that produced by decamethonium or tubocurarine.

MOST of the pharmacological studies to date have been either with the juice of hemlock or with coniine (I); studies of the activity of γ-coniceine (II) and conhydrine (III) have largely been restricted to toxicity tests, while *N*-methylconiine (IV) does not appear to have been examined.

$CH_2 \cdot CH_2 \cdot Me$ (on N–H ring) (I)

$CH_2 \cdot CH_2 \cdot Me$ (on N ring) (II)

$CH_2 \cdot CHOH \cdot Me$ (on N–H ring) (III)

$CH_2 \cdot CH_2 \cdot Me$ (on N–Me ring) (IV)
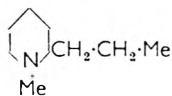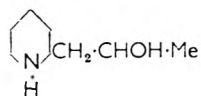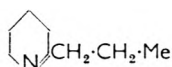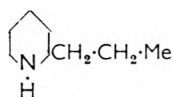
According to Sollmann (1957), "the peripheral actions of coniine are similar to those of nicotine, but it produces more pronounced paralysis of the central nervous system and of the skeletal muscle nerve endings". However, de Boer (1950) concluded that the central actions of coniine resembled those of strychnine.

The dried leaf and juice of *Conium maculatum* were official in the London and Edinburgh pharmacopoeias from 1864 to 1898 and the last official recognition of the medicinal use of hemlock in Great Britain appeared in the B.P.C. of 1934. One of the reasons for the discontinuance of the use of hemlock appears to have been the fact that different preparations varied widely in their potency. A possible explanation of the differing potency was recently provided by Fairbairn and Challen (1959).

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Using extracts of *Conium maculatum* these authors found that the alkaloidal content and composition differed widely according to the climatic conditions and even according to the time of day at which the plants were collected. It was therefore considered worthwhile to study the pharmacological actions of the individual alkaloids. Of the four main alkaloids, conhydrine occurs in the smallest proportions and, according to the limited information available, has the weakest pharmacological actions (von Oettingen, 1936). In the present experiments the actions of the remaining three alkaloids have been compared.

METHODS

*LD*50. The acute toxicity of each of the alkaloids after oral, intravenous and subcutaneous administration was determined in albino mice (A.R.C. strain) weighing between 19 and 21 g. In each test the animals were randomly distributed into 5 groups of 10 or 20 and a different dose of the alkaloid under study was administered to each group. The LD50 for each alkaloid administered by each route was calculated by the method of Litchfield and Wilcoxon (1949).

Intestinal muscle. Isolated segments of guinea-pig ileum or rabbit duodenum were suspended in continuously aerated Tyrode solution at 32° and 37° respectively. The longitudinal contractions were recorded on smoked paper. Peristalsis was recorded in isolated segments of guinea-pig ileum bathed in Tyrode solution at 32° by the method of Trendelenburg as described by Burn (1952).

Heart. Isolated rabbit hearts were perfused with McEwen's (1956) solution at 37° (gassed with 95 per cent oxygen and 5 per cent carbon dioxide) by the method described by Langendorff (see Burn, 1952).

Local anaesthetic activity. Local anaesthetic activity in small animals was studied by the guinea-pig wheal (Bülbring and Wajda, 1945), the rabbit cornea (Koppanyi and Karczmar, 1958) and the frog plexus (Burn, 1952) methods. Local anaesthetic activity in cats under chloralose anaesthesia was measured by a method similar to that described by Withrington and Zaimis (1961). Sensory impulses, initiated by regularly tapping the skin of the ankle, were recorded on a cathode ray oscilloscope by means of bipolar platinum electrodes placed on the saphenous nerve. The ability of the drugs, injected subcutaneously or intra-arterially, to abolish the sensory discharge was determined. Intra-arterial injections were made retrogradely through a needle cannula tied into the central end of the cut popliteal artery below the saphenous branch. At the moment of injection, the flow in the femoral artery was occluded, the injected fluid thereby being forced down the saphenous artery.

Skeletal muscle. Cats and hens were anaesthetised with chloralose (8 ml./kg. of a 1 per cent solution) to which pentobarbitone sodium (6 mg./kg.) was added and this mixture was injected into a subcutaneous vein of the fore-limb or into a wing vein respectively. Maximal twitches and tetani of the tibialis anterior and soleus muscles of cats and of the

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lateral head of the gastrocnemius muscle of hens were elicited with rectangular pulses of 50–100 μsec. duration and supra-maximal strength applied to the peripheral end of the cut sciatic nerves; they were recorded on a kymograph. When the muscles were stimulated directly, supra-maximal shocks of 0·5 msec. duration were applied between the tendons and the steel drill supporting the femur.

Nerve action potentials were recorded in cats by means of bipolar platinum electrodes placed on the peripheral end of the common peroneal nerve, the nerve being crushed between the recording electrodes and the muscle. Muscle action potentials were recorded from the tibialis anterior muscle by means of belly-tendon leads or concentric-needle electrodes. After differential amplification by a Tektronix (Type 122) battery driven pre-amplifier, action potentials were displayed on a Tektronix (Type 502) double beam oscilloscope and photographed on 35 mm. film. In some experiments isometric muscle tension was recorded electrically by means of a RCA 5734 mechano-electric transducer. Close-arterial injections to the tibialis anterior muscles of cats or the gastrocnemius muscles of hens were made by the methods described by Brown (1938) and Brown and Harvey (1938) respectively.

Isolated rectus abdominis muscles of frogs were suspended in aerated frog Ringer solution at room temperature and their contractions recorded on smoked paper according to the method of Chang and Gaddum (1933).

Respiration. Respiration was recorded in chloralosed cats by means of a piston recorder connected through valves to a cannula in the trachea. Only expired air moved the piston. Intra-arterial injections were made through a needle cannula tied into the cut thyroid or superior lingual artery so that the tip pointed towards the right common carotid artery.

Blood flow. The venous outflow from the skeletal muscles was recorded in the femoral vein of anaesthetised cats after ligating the skin branches. The method was similar to that described by Bowman and Zaimis (1958), except that the combined flow from all of the muscles of the lower hind-limb was recorded. When skin flow was recorded a similar method was used except that the flow in the femoral vein was restricted to that which entered it from the saphenous vein. Drugs were injected intra-venously or intra-arterially through a needle cannula tied into the central end of the gracilis branch of the femoral artery. The maximum volume administered intra-arterially was 0·01 ml. delivered from a micro-syringe. The drop chamber used was that described by Hilton (1952) and the rate of flow from muscle or skin was recorded by Gaddum or Thorpe impulse counters.

Nictitating membrane. Contractions of the nictitating membrane of cats under chloralose anaesthesia were elicited by pre- and post-ganglionic stimulation of the cervical sympathetic after sectioning the nerve centrally to the pre-ganglionic electrodes. The nerve was stimulated at a frequency of 5 or 10 per sec. for 20 or 30 sec. every 2 or 3 min. For pre-ganglionic stimulation rectangular pulses of 0·2 msec. duration and 2 V strength were used. For stimulation of the non-myelinated post-ganglionic fibres

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the pulse width was increased to 1 msec. and the strength to 10 V. Intra-arterial injections were made through a needle cannula in the cut thyroid artery.

Spinal reflexes. Reflex contractions of the quadriceps femoris or of the tibialis anterior muscles were recorded kymographically in cats under chloralose anesthesia and in cats previously decerebrated or spinalised under ether anaesthesia. In some experiments, contractions of the same quadriceps were elicited by alternately tapping the patellar tendon (Palmer automatic knee jerk hammer) and by stimulating the central end of the cut contra-lateral sciatic nerve with rectangular pulses of 1 msec. duration and strength 1–3 V at a frequency of 5–10/sec. for 1 sec. The method was similar to that described by Schweitzer and Wright (1938). In other experiments, patellar reflex contractions were recorded from the right hind leg once every 10 sec., as described above, and flexor reflex contractions of the tibialis anterior muscle were recorded from the opposite leg. Flexor contractions were elicited every 10 sec. by stimulation of the ipsilateral musculo-cutaneous branch of the peroneal nerve with single rectangular pulses of 0·5 msec. duration and 2–3 V strength. Drugs were injected intravenously, sometimes after occluding the circulation to the hind-limbs by clamping the iliac arteries (Schweitzer and Wright, 1938).

In all experiments on cats, blood pressure was recorded by means of a mercury manometer attached to a cannula in a common carotid artery or in a femoral artery. Intravenous injections were made through a cannula in a jugular or a femoral vein.

The hemlock alkaloids were extracted from the dried leaves and fruit of *Conium maculatum* by Professor J. W. Fairbairn and Mr. C. Lavender of the Department of Pharmacognosy of this School. The method of extraction for coniine and *N*-methylconiine was as described by Cromwell (1956) and that for γ-coniceine was as described by Fairbairn and Challen (1959). The alkaloids were supplied as hydrochlorides for this investigation. The optical rotations of natural coniine base and of natural *N*-methylconiine base are $[\alpha]_D^{19} = +16°$ and $[\alpha]_D^{24} = +81°$ respectively (Merck Index). The optical rotation of the coniine base used in these experiments was $[\alpha]_D^{20} = +3·6°$ showing that racemization had occurred during extraction. The *N*-methylconiine base used showed an optical rotation of $[\alpha]_D^{20} = 86·4°$ showing that it was almost entirely the (+) form. γ-Coniceine is not optically active. The doses of acetylcholine, cocaine, nicotine and the hemlock alkaloids quoted in the text, refer to the cations. Those of the other drugs refer to the salts.

RESULTS

Acute toxicity. The acute toxicity of the three hemlock alkaloids, coniine, *N*-methylconiine and γ-coniceine, after oral, intravenous and subcutaneous administration was determined in mice. The results obtained, expressed as the LD50, are presented in Table I which also shows their relative toxicities compared to coniine and the approximate

times of death after administration. The toxic symptoms were similar to those described for coniine by de Boer (1950). They included fasciculations of skeletal muscles, clonic and tonic contractions of separate limbs and convulsions of the whole animal. These signs of excessive activity were interspersed with periods of quietness, the final stage before death being one of paralysis. Respiration was first stimulated, particularly with coniine and γ-coniceine, and then depressed, the animals finally becoming cyanosed and dying apparently of respiratory failure. Micturition was frequently observed and the eyes protruded from the head.

TABLE I

ACUTE TOXICITY IN MICE

Alkaloid		Route of administration		
		I.V.	Subcut.	Oral
Coniine	LD50 (mg./kg.)	19 (16–22)	80 (72–83)	100 (97–103)
	Time of death	30 sec.	15 min.	10 min.
	Dose ratio Coniine = 1	1	1	1
N-Methylconiine	LD50 (mg./kg.)	27·5 (24·75–30·25)	150·5 (146·2–154·8)	204·5 (176–233)
	Time of death	30 sec.	16 min.	12 min.
	Dose ratio Coniine = 1	1·5	1·9	2
γ-Coniceine	LD50 (mg./kg.)	2·6 (1·95–3·25)	12 (11–13)	12 (10·4–13·5)
	Time of death	30 sec.	12 min.	8 min.
	Dose ratio Coniine = 1	0·14	0·15	0·12

The figures in brackets are the 95 per cent fiducial limits.

Smooth muscle of the intestine. Coniine (15–50 μg./ml.) and γ-coniceine (5–15 μg./ml.) caused contraction of the isolated guinea-pig ileum and rabbit duodenum. This result with coniine confirms that of others (Tamba, 1921; Hamet, 1931). The addition of atropine (1 μg./ml.) or hexamethonium (25 μg./ml.) to the bath fluid prevented this action of the alkaloids suggesting that it arose through stimulation of parasympathetic ganglia. N-methylconiine occasionally caused a weak contraction but more usually was without effect in this respect in concentrations up to the maximum used (250 μg./ml.). In concentrations of 100 μg./ml., N-methylconiine prevented the response of the smooth muscle to subsequently added nicotine but not to acetylcholine. It was not possible to demonstrate any ability of γ-coniceine or coniine to inhibit the effect of nicotine specifically, although high concentrations (200 μg./ml.) of all three hemlock alkaloids possessed a non-specific depressant effect and even after washing the tissue several times, the responses to stimulating agents such as acetylcholine, histamine, nicotine and to the alkaloids themselves, were reduced. Such concentrations also depressed the spontaneous pendular movements of the rabbit intestine. Fig. 1a

illustrates an experiment in which the potency of coniine was compared with that of nicotine on the guinea-pig ileum and Fig. 1b shows the effects of γ-coniceine, nicotine and N-methylconiine on the rabbit duodenum.
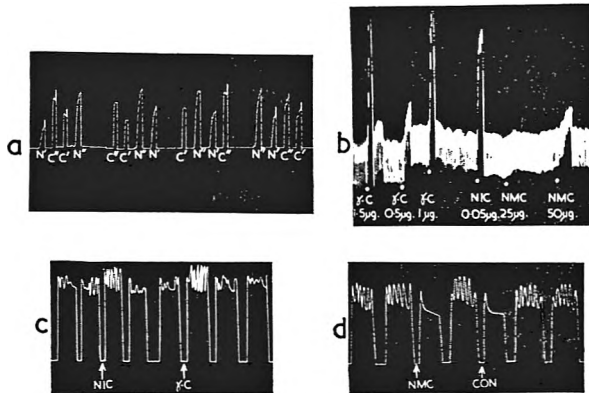
FIG. 1. *a.* Isolated guinea-pig ileum. At N′ and N″, 1 and 2 μg./ml. nicotine; at C′ and C″, 15 and 30 μg./ml. coniine. Contact time, 30 sec. Dose interval, 5 min. *b.* Isolated rabbit duodenum. At γ-C, γ-coniceine; at NIC, nicotine and at NMC, N-methylconiine. Doses in μg./ml. *c* and *d.* Peristalsis in isolated guinea-pig ileum. At NIC, 0·5 μg./ml. nicotine. At γ-C, 25 μg./ml. γ-coniceine; at NMC, 10 μg./ml. N-methylconiine and at CON, 15 μg./ml. coniine. (The lower tracings have been retouched.)

N-Methylconiine (2·5–5 μg./ml.) and coniine (15 μg./ml.) abolished the peristaltic reflex in isolated segments of guinea-pig ileum after being in contact with the tissue for 90 sec. (Fig. 1d). Neither substance accentuated peristalsis in any concentration. Weak concentrations of γ-coniceine (5–25 μg./ml.) and of nicotine (0·1–0·5 μg./ml.) augmented peristalsis (Fig. 1c) and larger concentrations depressed it.

Cardiovascular system. Minimal effective intravenous doses of coniine (0·5–2 mg./kg.) and N-methylconiine (1–4 mg./kg.) caused a small and short-lasting fall in blood pressure which was prevented by previous atropinisation (1 mg./kg.). γ-Coniceine (0·2–0·5 mg./kg.) only occasionally produced a fall in blood pressure and this was always followed by a rise. Doses of coniine slightly larger than about 2 mg./kg. produced a similar biphasic response but with very large doses both of coniine (5–10 mg./kg.) and of γ-coniceine (2–5 mg./kg.) the initial fall was absent and only a rise in blood pressure was produced. With large doses of N-methylconiine (6–8 mg./kg. and above), the initial short-lasting fall in blood pressure was often followed by a more slowly developing and longer lasting fall during which the pressor response to nicotine was reduced or abolished. When the background level of blood pressure was low, as for example in the spinal animal, the depressor response to all three alkaloids was absent and under these conditions N-methylconiine also caused a small rise in blood pressure. The pressor response to the alkaloids was prevented by the previous intravenous administration of
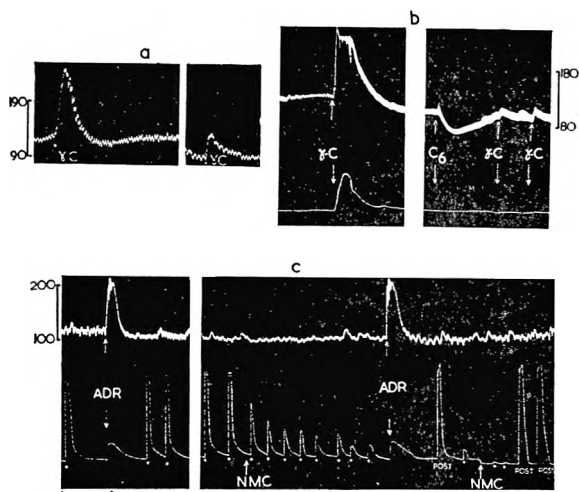
FIG. 2. Cats, chloralose anaesthesia. *a.* Arterial blood pressure responses to 3 mg./kg. γ-coniceine intravenously before and after ligating the adrenal glands. *b.* Blood pressure and nictitating membrane. At γ-C, 2 mg./kg. γ-coniceine and at C_6, 2 mg./kg. hexamethonium intravenously. *c.* Blood pressure and nictitating membrane. At the white dots, pre-ganglionic stimulation and at POST, post-ganglionic stimulation of cervical sympathetic (10/sec. for 20 sec.). At ADR, 5 μg./kg. adrenaline intravenously and at NMC, 2 mg. N-methylconiine intra-arterially. Time calibration for whole Fig., 5 min. Blood pressure in mm. Hg.

3 mg./kg. hexamethonium (Fig. 2*b*) and was markedly reduced but not abolished when the adrenal glands were excluded from the circulation (Fig. 2*a*). All three alkaloids in large doses (γ-coniceine 2 mg./kg., coniine 5 mg./kg., N-methylconiine 4 mg./kg.) administered intravenously, blocked or reduced the depressor response to stimulation of the left vagus (Fig. 3*b*).

In the isolated perfused rabbit heart all three alkaloids in large amounts (γ-coniceine 0·2 mg., coniine 2 mg., N-methylconiine 4 mg.) caused a decrease in the force of the beat, the rate of beating being unaffected. These results with coniine on the rabbit's heart confirm those of de Boer (1950) who used frog hearts.

The only effect of the hemlock alkaloids on skeletal muscle blood flow, whether injected intra-arterially (γ-coniceine, 100 μg.; coniine and N-methylconiine, 1 mg.) or intravenously (γ-coniceine. 0·5 mg./kg. and above; coniine, 2 mg./kg. and above; N-methylconiine, 4 mg./kg. and above) was a small increase in venous outflow (Figs. 3*a, b* and *c*). With intravenous injection, the pressor effects of γ-coniceine and coniine added to the local dilator action by forcing more blood through the muscle vessels. The venous outflow from the skin vessels was also increased by intra-arterial injection of the same doses of the alkaloids (Fig. 3*d*) but when administered intravenously, the pressor effects of γ-coniceine and coniine were accompanied by vasoconstriction in the skin (Fig. 3*d*). N-Methylconiine which did not usually cause a rise in blood pressure, produced only a slight vasodilatation in the skin vessels.

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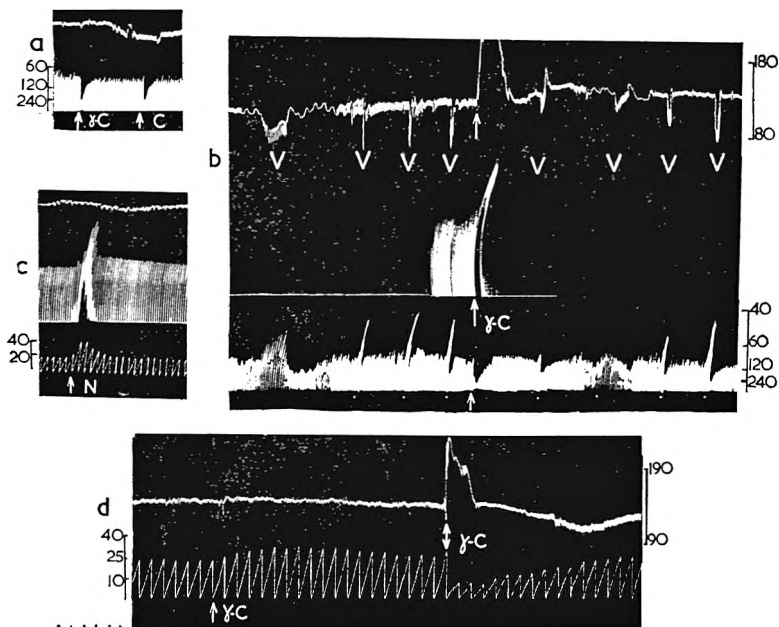
FIG. 3. Cats, chloralose anaesthesia. *a*. Blood pressure and venous outflow from hind-limb muscles. At γ-C, 200 μg. γ-coniceine and at C, 1 mg. coniine injected intra-arterially. *b*. Blood pressure, respiration and venous outflow from hind-limb muscles. Respiration was recorded only for the period shown. Artificial respiration was applied after γ-coniceine had depressed respiration. At V, the left cervical vagus was stimulated (5/sec. for 10 sec.), the kymograph speed being increased during the first and seventh period of vagal stimulation. At γ-C, 2·5 mg./kg. γ-coniceine intravenously. *c*. Blood pressure, maximal twitches of the tibialis anterior muscle elicited indirectly 1/10 sec. and venous outflow from the hind-limb muscles. At N, 0·2 mg. *N*-methylconiine intra-arterially. *d*. Blood pressure and venous outflow from the skin of the hind-limb. At γ-C, γ-coniceine 100 μg. intra-arterially and 1 mg./kg. intravenously. Time calibration in min. Note that in *a* and *b*, a Gaddum impulse counter was used to record blood flow and an increase in the height of the record means a decrease in flow. In *c* and *d*, a Thorpe impulse counter was used and an increase in the height of the record means an increase in flow. Blood flow calibration, drops/min. Blood pressure in mm. Hg.

Nictitating membrane. γ-Coniceine (50–100 μg. i.a. and 0·5–2 mg./kg. i.v.) and coniine (2 mg. i.a. and 10–15 mg./kg. i.v.) caused contraction of the nictitating membrane of the cat but *N*-methylconiine was without this effect in doses up to 5 mg. i.a. The effect of coniine and γ-coniceine was prevented by the previous administration of hexamethonium (3 mg./kg. i.v.). All three alkaloids, γ-coniceine (0·3 mg. i.a., 2 mg./kg. i.v.), *N*-methylconiine (1–2 mg. i.a., 5–8 mg./kg. i.v.) and coniine (1–2 mg. i.a., 10 mg./kg. i.v.) blocked or reduced the contractions of the nictitating membrane elicited by pre-ganglionic stimulation. During this effect, the response of the membrane to adrenaline and to post-ganglionic stimulation was unaffected. These results show that the site of both the stimulant and the blocking action of the alkaloids was the superior cervical ganglion. Fig. 2*b* and *c* illustrate some of these effects of γ-coniceine and *N*-methylconiine on the nictitating membrane.

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Respiration. Small doses of coniine (1–4 mg./kg.) and γ-coniceine (0·3–1 mg./kg.) administered intravenously, stimulated respiration but larger doses depressed it after an initial abrupt stimulation. Fig. 3*b* illustrates stimulation and block of respiration produced by intravenously injected γ-coniceine. *N*-Methylconiine did not stimulate respiration and depressed it only in doses large enough to cause neuromuscular block (see later).

When injected into the carotid artery so that they passed through the carotid sinus, small doses of coniine (20–30 μg.) and γ-coniceine (10–20 μg.) caused a slowly developing increase in respiratory rate and depth. In contrast to the hemlock alkaloids, small doses of nicotine (1–2 μg.) injected by the same route, caused an abrupt and short-lasting increase in respiration. This well known action of nicotine on chemoreceptors in the carotid body (Heymans, Bouckaert and Dautrebande, 1931) did not occur with intra-arterial injection of the hemlock alkaloids, possibly because their rate of reaction with these receptors is too slow. By this route of injection the hemlock alkaloids therefore appeared to act only directly upon the respiratory centres. Larger doses of γ-coniceine and coniine injected into the carotid artery depressed respiration and eventually caused respiratory failure. *N*-Methylconiine in doses up to 100 μg. was without effect on respiration when administered into the carotid artery. Fig. 4 compares the effects of intra-carotid injection of nicotine and of coniine on respiration.

FIG. 4. Cat chloralose anaesthesia. Blood pressure and respiration. At NIC, nicotine and at CON, coniine injected retrogradely into the thyroid artery. 10 min. elapsed between the 1st and 2nd panels and 20 min. between the 2nd and 3rd panels. The second dose of coniine was lethal. Time calibration, 5 min.

Local anaesthetic action. With concentrations of the hemlock alkaloids up to 10 mg./ml. no local anaesthetic action could be demonstrated using the rabbit cornea or frog plexus tests. In these experiments, cocaine was used for comparison. In the guinea-pig wheal test, a weak local anaesthetic action of coniine and *N*-methylconiine was demonstrable. In this test approximately equi-potent doses by intra-dermal injection were: cocaine 150 μg., coniine 2·5 mg. and *N*-methylconiine 6 mg. γ-Coniceine was less active than coniine in this respect and no local anaesthetic potency was evident with doses up to 2·5 mg. Larger doses of this alkaloid were lethal.

Cocaine, by intra-arterial or subcutaneous injection, was markedly effective in abolishing the sensory discharge in the cat saphenous nerve elicited by tapping the skin. The hemlock alkaloids showed only a very weak effect, however, in doses up to the maximum administered (0·2 ml.

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of a 10 mg./ml. solution). Fig. 5 illustrates an experiment in which the effect of γ-coniceine was compared with that of cocaine.
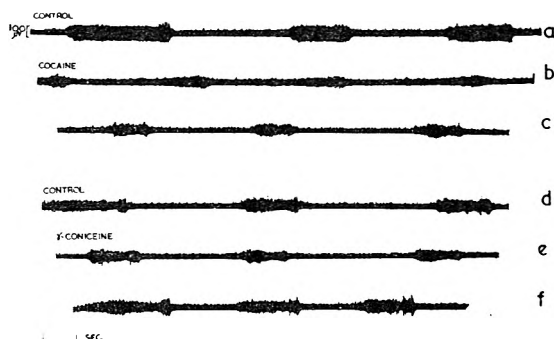
FIG. 5. Cat, chloralose anaesthesia. Oscilloscope recording of sensory discharges evoked in the saphenous nerve by tapping the same area of skin near the ankle about once every sec. *a.* Control responses at start of experiment. *b.* 10 min. after injection of cocaine (0·3 mg. in 0·2 ml.) beneath the skin at the site of stimulation. *c.* 30 min. later. *d.* Control responses 2 hr. later. *e.* 10 min. after subcutaneous injection of γ-coniceine (2 mg. in 0·2 ml.). *f.* 30 min. later. Control injections of 0·2 ml. saline were without effect on the sensory discharge.

Frog. skeletal muscle. Coniine (0·1–0·5 mg./ml.) and γ-coniceine (5–10 μg./ml.) caused contracture of the rectus abdominis muscle of the frog but N-methylconiine was without effect in concentrations up to the maximum used (1 mg./ml.). This effect of coniine and γ-coniceine was prevented by the presence of tubocurarine (1–2 μg./ml.). Providing that they were given time to act, all three alkaloids reversibly blocked or reduced the response of the muscle to acetylcholine. This effect occurred with concentrations of the alkaloids below those which caused contracture of the muscle. Thus coniine (5–10 μg./ml.), γ-coniceine (1–2 μg./ml.) and N-methylconiine (3–6 μg./ml.) blocked the response of the muscle to acetylcholine after being in contact with the tissue for 90–120 sec. After washing the tissue two or three times with fresh Ringer solution, the response to acetylcholine returned to normal. Similar small concentrations of the alkaloids prevented the contractural response to larger concentrations added subsequently.

Mammalian skeletal muscle. On close-arterial injection into the tibialis anterior muscle of the cat, all three hemlock alkaloids (coniine, 2–3 mg., γ-coniceine 25–100 μg., N-methylconiine 0·5–1 mg.) caused a quick contraction of the muscle similar to that produced by depolarising drugs such as acetylcholine, suxamethonium or nicotine (Fig. 7). Mammalian muscle therefore differed from frog muscle in which N-methylconiine was without a stimulant action in the concentrations used. Electrical recording from the tibialis anterior muscle showed that the contraction produced by the alkaloids was accompanied by an asynchronous burst of action potentials similar to that following close-arterial injection of acetylcholine (Fig. 6).

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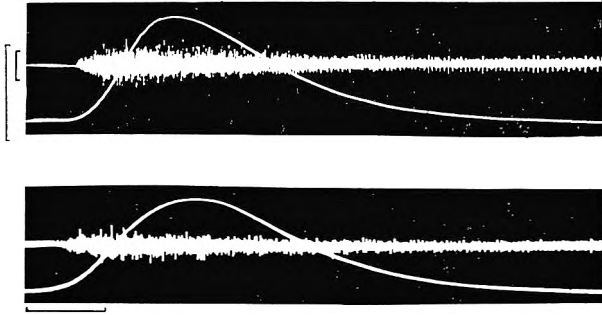
FIG. 6. Cat chloralose anaesthesia. Electromyogram (concentric needle electrode) and isometric myogram (RCA 5734 transducer valve) recorded from tibialis anterior muscle. Upper panel: response to 5 μg. acetylcholine close-arterially. Lower panel: response to 50 μg. γ-coniceine close-arterially. Voltage calibration for electromyogram, 0·5 mV. Tension calibration, 0·5 kg. Time calibration, 200 msec.

The contraction produced by the first close-arterial injection of the hemlock alkaloids was usually followed by a very slight and transient depression of the maximal twitches. This depression was followed by potentiation of the twitches but this then gradually faded and changed to a slowly developing and more pronounced reduction in twitch tension. Fig. 7c illustrates these changes produced by coniine. In striking contrast to the effect of the hemlock alkaloids, the block produced by close-arterial doses of the depolarising blocking drugs, suxamethonium (Fig. 7a) and decamethonium, was present immediately after the initial contraction which they produced. This is to be expected with these drugs since both the contraction and the block are believed to be a consequence of the

FIG. 7. Cats, chloralose anaesthesia. Maximal twitches of tibialis anterior muscles elicited indirectly once every 10 sec. except during close-arterial injections when electrical stimulation was stopped. a. At SUX, 10 μg. suxamethonium close-arterially. The horizontal bar shows the period during which electrical stimulation was stopped. b. At A, 10 μg. acetylcholine close-arterially and at TC, 0·3 mg./kg. tubocurarine intravenously. The last response to acetylcholine was recorded 40 min. after the previous one. c. At A, 5 μg. acetylcholine and at CON, 3 mg. coniine close-arterially. The last response to acetylcholine was recorded 20 min. after the previous one.

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same mechanism, namely, depolarisation of the motor end-plates, and the overall depolarisation must be greatest during the drug-induced contraction. In view of these considerations, it seems likely that only the initial transient and slight depression of the twitches immediately following the contraction produced by the hemlock alkaloids, was a consequence of depolarization block. With the second and subsequent injections of the hemlock alkaloids the initial contraction was smaller and the onset of depression of the twitches followed more rapidly. In fact with the third and fourth dose, the initial contraction was absent and block ensued immediately. Successive doses of γ-coniceine and coniine showed a cumulative paralyzing effect when administered at intervals of 2-3 hr. or less but with N-methylconiine, tachyphylaxis was evident.

Distant arterial injection of sub-blocking doses of the alkaloids did not cause contraction of the non-stimulated muscle but produced powerful fasciculations and potentiation of the maximal twitch. This effect of N-methylconiine is illustrated in Fig. 3c.

Intravenous administration of the alkaloids (coniine 15 mg./kg., N-methylconiine 10 mg./kg., γ-coniceine 1 mg./kg.) caused an initial small potentiation of the indirectly excited maximal twitches of the
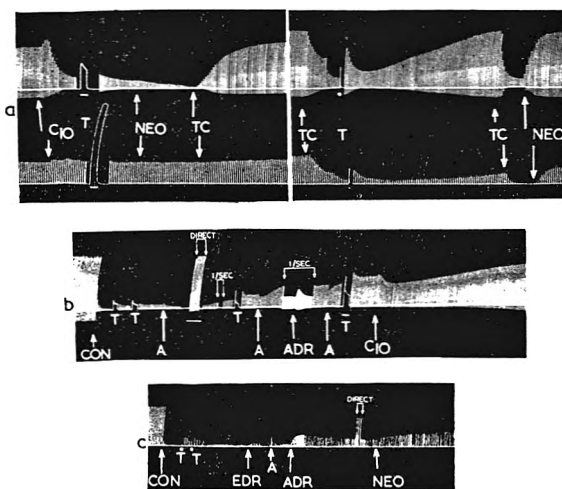
Fig. 8. Cats chloralose anaesthesia. Maximal twitches of muscles elicited indirectly once every 10 sec. except where otherwise stated. a. Tibialis anterior (upper record) and soleus muscles (lower record). At C_{10}, 35 μg./kg. decamethonium and at NEO, 50 μg./kg. neostigmine. At TC in the left-hand panel, 100 μg./kg. and in the right-hand panel 0·4 and 0·3 mg./kg. tubocurarine injected intravenously. b. Tibialis anterior muscle. At CON, 2 mg. coniine and at A, 200 μg. acetylcholine close-arterially. At ADR, 10 μg./kg. adrenaline and at C_{10}, 10 μg./kg. decamethonium intravenously. c. Tibialis anterior muscle. At CON, 5 mg. coniine, at A, 200 μg. acetylcholine and at EDR, 5 μg. edrophonium close-arterially. At ADR, 20 μg./kg. adrenaline and at NEO, 100 μg./kg. neostigmine intravenously. At T in all experiments, a tetanus was elicited by stimulation of the motor nerve (50/sec. for 10 sec.). At DIRECT, the muscle was stimulated directly. Similar responses were obtained in other experiments when the effects of each drug were studied separately.

the tibialis anterior and soleus muscles of the cat which was followed by a slowly developing reduction in twitch tension to about 50 per cent of the original level. Larger doses caused more complete paralysis. The paralysis of the soleus muscle was at least as great and sometimes slightly greater than that of the tibialis anterior muscle. The sensitivity of the two muscles to the blocking action of the alkaloids therefore resembled that to tubocurarine rather than that to decamethonium (Paton and Zaimis, 1952 and see Fig. 8a). During complete paralysis of the indirectly excited maximal twitches produced by the alkaloids, the muscle responded normally to direct electrical stimulation (Fig. 8b and c) and the shape and size of action potentials recorded from the nerve remained unchanged. These results therefore locate the site of the blocking action at the neuro-muscular junction, either on the motor end-plates or at the fine nerve terminals.

After the administration of a blocking dose of one of the alkaloids, the twitch-like response to close-arterially injected acetylcholine was reduced or abolished and this occurred even during the latent period between injection and the onset of the depression of the twitches (Fig. 7c) showing that even at this stage the sensitivity of the motor end plates was depressed. At the height of the block, the response to acetylcholine was completely abolished even when the dose of alkaloid was such that the maximal twitches were only partially depressed (Fig. 7c). These results show that a depression of motor end-plate sensitivity plays a large part in the blocking action of the alkaloids. However, the response to injected acetylcholine reappeared sooner than it does after a comparable degree of block produced by tubocurarine (Fig. 7b). In fact, after recovery from block produced by coniine, the response to acetylcholine was often greater than the control responses before coniine (Fig. 7c). The block produced by the hemlock alkaloids differed both from that produced by curare-like drugs such as tubocurarine and gallamine, and from that produced by depolarising drugs such as decamethonium or suxamethon-ium. Curare-like drugs and depolarising drugs can be shown to be mutually antagonistic in the tibialis anterior muscle of the cat (Paton and Zaimis, 1952; Hutter and Pascoe, 1951; Dellamagne and Phillipot, 1952; and see Fig. 8a). However, the blocking action of the hemlock alkaloids added to that of both types of neuromuscular blocking drug and this occurred no matter in what order the drugs were administered. Thus a small dose of a hemlock alkaloid, administered at the height of a partial block produced by tubocurarine or decamethonium, caused a further increase in the paralysis. Similarly, decamethonium or tubocurarine increased the block when administered during the effect of the hemlock alkaloids. Experiments were carried out in which maximal twitches of both tibialis anterior muscles were recorded simultaneously. A constant dose of tubocurarine was injected intravenously at intervals of 90 min. During one of the intervals between tubocurarine injections, a single dose of a hemlock alkaloid was injected close-arterially to one muscle only. The block produced in this muscle by the subsequent intravenous dose of tubocurarine was much greater than that produced in the control muscle

of the opposite leg which merely showed the normal cumulative effect of tubocurarine (Fig. 9b). A similar potentiation of decamethonium block was obtained when this drug was used in place of tubocurarine (Fig. 9a).

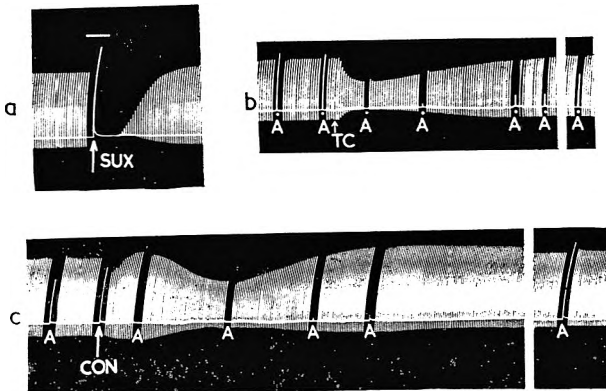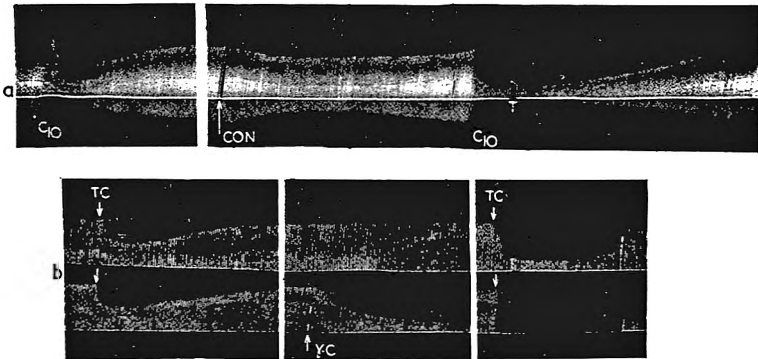FIG. 9. Cats, chloralose anaesthesia. Maximal twitches of tibialis anterior muscles elicited indirectly once every 10 sec. a. Right tibialis only. At C_{10}, 25 μg./kg. decamethonium intravenously (90 min. between each dose). At CON, 2 mg. coniine close-arterially. The block produced by decamethonium was greater after coniine. b. Left (upper) and right (lower) tibialis muscles. At TC, 0.3 mg./kg. tubocurarine intravenously (150 min. between doses). At γ-C, 25 μg. γ-coniceine close-arterially to right tibialis only. The block produced by tubocurarine was greater in the right tibialis after γ-coniceine.

A motor nerve tetanus, edrophonium, neostigmine, acetylcholine and adrenaline all possess marked decurarising actions but are without effect or slightly increase block produced by decamethonium in the tibialis anterior muscle of the cat (Paton and Zaimis, 1952). During a partial block produced by the hemlock alkaloids these tests produced responses which were intermediate between those described above. Tetanic tension was less well sustained than it is during decamethonium block but was better sustained than the brief twitch-like response characteristic of tubocurarine paralysis. The post-tetanic twitches were slightly increased in tension. Edrophonium and neostigmine produced only weak antagonistic actions and the effect of close-arterially injected acetylcholine was variable. Sometimes a small antagonistic action resulted while on other occasions the block was increased. Adrenaline exerted an antagonistic action which was roughly equal to its anti-curare effect; it was a more powerful antagonist than neostigmine or edrophonium of block produced by hemlock alkaloids. Fig. 8 illustrates experiments carried out under the same conditions, in which some of these tests were applied during blocks produced by tubocurarine, coniine and decamethonium.

Avian skeletal muscle. On intravenous injection into the conscious chick, the smallest effective doses of coniine (70–80 μg./10 g.) often, but not invariably, caused flaccid paralysis similar in appearance to that produced by tubocurarine. The effect differed from that produced by tubocurarine, however, in that pinching the foot still evoked a flexor reflex. Minimal effective doses of nicotine (1–2 μg./10 g.), produced flaccid paralysis in all chicks tested. In the same chicks, and in all

others tested, larger doses of all three hemlock alkaloids (coniine and N-methylconiine, 100–120 μg./10 g.; γ-coniceine, 5–10 μg./10 g.) and of nicotine (5–10 μg./10 g.) caused a spastic paralysis resembling that produced by decamethonium. However, this response to the hemlock alkaloids, and to nicotine, differed from that to decamethonium as follows. With lethal doses of decamethonium, the chicks died in spastic paralysis which persisted for about half an hour after death. With sublethal doses, recovery was abrupt and as the spasticity wore off, the chick stood up and was apparently normal (Buttle and Zaimis, 1949). The spasticity produced by the hemlock alkaloids and by nicotine, on the other hand, was always followed by a further period of flaccid paralysis during which death occurred or from which recovery took place.
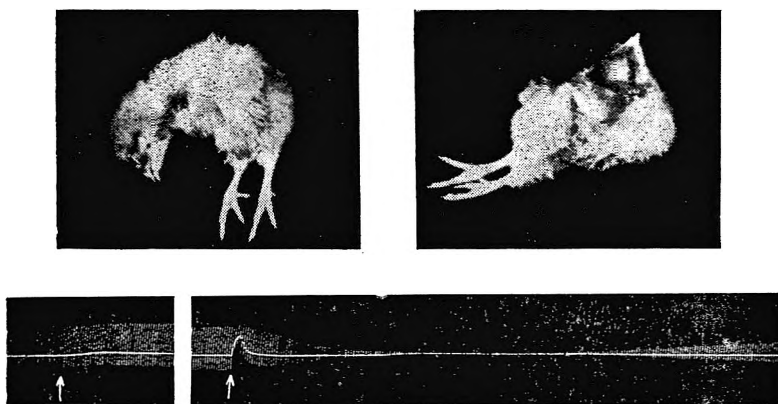
FIG. 10. The whole figure illustrates experiments on the same chick. Upper panels: before anaesthesia. Lower panels: maximal twitches of gastrocnemius muscle elicited indirectly once every 10 sec. during anaesthesia with pentobarbitone sodium (12 mg. intraperitoneally). Left-hand panels: 75 μg./10 g. coniine intravenously caused flaccid paralysis of the conscious chick but did not block the maximal twitches of its gastrocnemius muscle. Right-hand panels: 110 μg./10 g. coniine caused spastic paralysis (illustrated) followed by flaccid paralysis in the conscious chick and contracture followed by block of the gastrocnemius muscle.

It appeared unlikely that the flaccid paralysis often produced by the smallest effective doses of coniine and of nicotine could be due to depressed excitability of the muscle, since larger doses, given immediately afterwards, caused contracture. The initial flaccid paralysis could, however, be explained if the most powerful action of these substances were a depressant one in the central nervous system. This possibility was tested by anaesthetising one of the chicks, which had responded by flaccid paralysis to small doses of coniine. and recording maximal twitches of its gastrocnemius muscle elicited by stimulation of the sciatic nerve. The results obtained from this chick, both before and after it was anaesthetised, are shown in Fig. 10. They show that a small dose of coniine (75 μg./ 10 g.) which produced flaccid paralysis in the conscious chick, was completely without effect on the indirectly excited maximal twitches of the gastrocnemius muscle. This result indicated that the initial flaccid paralysis in the conscious chick had been a consequence of an action in

the central nervous system and this led to the experiments on spinal reflexes to be described later. A larger dose (120 μg./10 g.) which had caused spastic paralysis followed by flaccid paralysis in the conscious chick, produced contracture followed by a block of the maximal twitches of the gastrocnemius muscle.
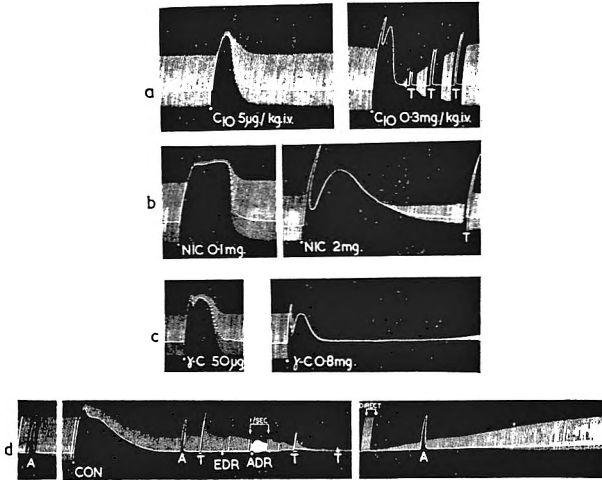
FIG. 11. Hens, chloralose anaesthesia. Maximal twitches of gastrocnemius muscles elicited indirectly once every 10 sec. *a.* At C_{10}, decamethonium intravenously and at T, tetanus (50/sec. for 10 sec. on faster kymograph). Each panel depicts a different experiment. *b.* At NIC, nicotine close-arterially. Each panel depicts a different experiment. *c.* At γ-C, γ-coniceine close-arterially. Both panels are from the same experiment. *d.* At A, 5 μg. acetylcholine; at CON, 4 mg. coniine and at EDR, 10 μg. edrophonium injected close-arterially. At ADR, 10 μg./kg. adrenaline intravenously. At T, tetanus (50/sec. for 10 sec.). At DIRECT, direct stimulation of the muscle.

In the anaesthetised adult hen, all three hemlock alkaloids and nicotine caused a sustained contracture of the gastrocnemius muscle on intravenous or close arterial injection. With small doses (γ-coniceine and nicotine, 50–100 μg.; coniine and *N*-methylconiine, 1–2 mg. close arterially) the maximal twitches following the contractures were not depressed below the pre-injection level but with larger doses the subsequent twitches remained depressed after the contracture (Fig. 11*b* and *c*). With the hemlock alkaloids, the doses necessary to produce a biphasic response were about three times greater than those which caused only a contracture, while with nicotine the ratio was about 10:1. Decamethonium was also capable of producing a biphasic response (Fig. 12*a*) but with this drug the ratio of the two doses was of the order of 50:1. Tridecamethonium in which the two quaternary nitrogens are separated by a chain of 13 methylene groups, produces a biphasic response in all effective doses and the secondary depression of the maximal twitches shows the characteristics of block produced by tubocurarine (Zaimis, 1953). Zaimis used the term "dual block" to describe this type of paralysis in which both depolarising and curare-like phases are present. With large doses of decamethonium, the secondary depression of the twitches did not resemble a tubocurarine

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block. Neostigmine was without antagonistic action, tetanic tension was well sustained and the course of the block was not altered by the tetanus (Fig. 11*a*). This secondary effect of large doses of decamethonium was probably a consequence of membrane inexcitability outlasting the depolarisation. Burns and Paton (1951) showed in the tenuissimus muscle of the cat, that the block produced by depolarising substances is a direct consequence of an electrical inexcitability of the muscle membrane to which prolonged depolarisation gives rise in the region surrounding the motor end-plates.

The secondary block of the maximal twitches produced by the hemlock alkaloids differed from that produced either by tubocurarine or by large doses of decamethonium. In most respects, it resembled that produced by the alkaloids in the skeletal muscles of the cat. During the paralysis, direct stimulation of the muscle produced normal contractions; tetanic tension was poorly sustained and the post-tetanic twitches were slightly increased in tension; neostigmine or edrophonium produced only very small antagonistic effects; tubocurarine or decamethonium administered during the block caused a further increase in the depth of paralysis; adrenaline exerted some antagonistic action. The effect differed from that in the cat in that during the paralysis the response of the muscle to close-arterially injected acetylcholine was only slightly reduced. Fig. 11*d* illustrates some of these responses during block produced by coniine.
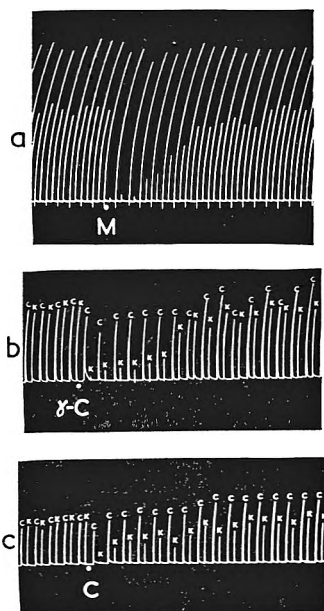
FIG. 12. Cats, light chloralose anaesthesia. Alternate crossed extensor and patellar reflex contractions of the quadriceps femoris muscle every 30 sec. (i.e. 1 min. between reflex contractions of the same type). *a.* Patellar reflex contractions are the larger deflections. At M, 10 mg./kg. mephenesin. *b* and *c.* C = crossed extensor reflex and K = patellar reflex (knee jerk). At γ-C, 300 μg./kg. γ-coniceine and at C, 4 mg./kg. coniine. All injections intravenously.

Spinal reflexes. In these experiments similar results were obtained in anaesthetised cats and in cats previously decerebrated or spinalised. Therefore no distinction is made in the following description. Small intravenous doses of the hemlock alkaloids and of nicotine blocked the response of the quadriceps muscle to tapping the patellar tendon (patellar reflex) or stimulating the central end of the contra-lateral sciatic nerve (crossed-extensor reflex). With the first injection of each hemlock alkaloid the patellar reflex was often more affected than the crossed extensor reflex. Figure 12*b* and *c* illustrates experiments in which γ-coniceine and coniine caused complete block of the patellar reflex while leaving the crossed extensor reflex almost unaffected. The contrast between this effect and that of the spinal relaxant, mephenesin, which selectively abolishes the crossed extensor reflex, is also illustrated (Fig. 12*a*). With subsequent injections of the hemlock alkaloids, both reflexes were roughly equally depressed. Nicotine always blocked both reflexes to a similar extent. Doses of the hemlock alkaloids and of nicotine sufficient to cause complete paralysis of the patellar and crossed extensor reflexes were without effect on the response of the tibialis anterior muscle to stimulation of the central end of the musculocutaneous branch of the ipsilateral peroneal
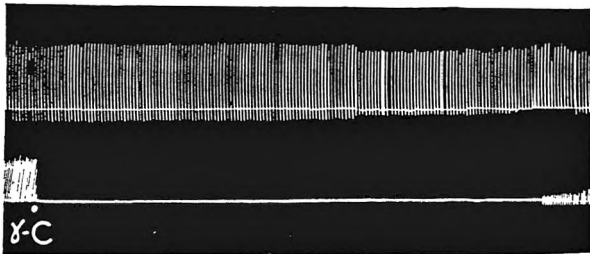
FIG. 13. Cat decerebrate. Upper record: flexor reflex contractions of right tibialis anterior muscle once every 10 sec. Lower record: patellar reflex contractions of left quadriceps femoris muscle, once every 5 sec. At γ-C, 300 μg./kg. γ-coniceine intravenously.

nerve (flexor reflex). Fig. 13 illustrates this effect of γ-coniceine. When the limb, from which the patellar and crossed extensor reflexes were recorded, was deprived of its circulation by clamping the ipsilateral iliac artery just below the aorta, the hemlock alkaloids and nicotine still abolished these reflexes when administered intravenously. Recovery occurred after the expected time showing that the lack of circulation had not contributed to the block. These results rule out the neuromuscular junction and the muscle spindles as the site of this action and show that the effects were a consequence of an action in the spinal cord.

Larger doses of hemlock alkaloids and of nicotine blocked the flexor reflex. The block was usually preceded by potentiation of the contractions and powerful fasciculations of the muscle (Fig. 14). The latter were more pronounced after nicotine than after the hemlock alkaloids. After large doses, fasciculations of the quadriceps muscle sometimes occurred even though the elicited reflex contractions of this muscle were completely abolished. This stimulant activity was reduced but not abolished on

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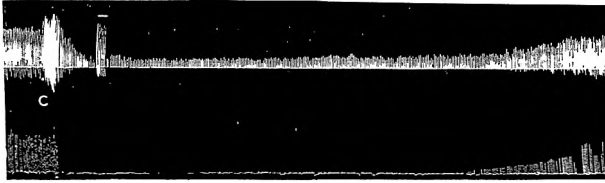
FIG. 14. Spinal cat. Upper record: Flexor reflex contractions of right tibialis anterior muscle once every 10 sec. During period marked by horizontal bar, maximal twitches of tibialis anterior muscle were elicited by stimulation of the motor nerve. Lower record: Patellar reflex contractions of left quadriceps femoris muscle once every 10 sec. At C, 7·5 mg./kg. coniine intravenously.

sectioning the sciatic-nerve. An action of the drugs on the intrafusal fibres of the muscle spindles was unlikely to have contributed to the stimulant action. The muscle spindles are the sensory receptors involved in the patellar reflex and the doses required to produce fasciculations were greatly in excess of those which completely abolished this stretch reflex.

TABLE II

APPROXIMATE INITIAL INTRAVENOUS DOSES (MG./KG.) NECESSARY TO CAUSE COMPLETE BLOCK OF REFLEX CONTRACTIONS AND THE DURATIONS OF THE EFFECTS

Alkaloid	Patellar	Crossed extensor	Flexor
Coniine	4 mg. 25–35 min.	4–5 mg. 25–35 min.	10 mg. 15–30 min.
N-Methylconiine ..	4 mg. 5–15 min.	4–5 mg. 5–15 min.	10 mg. 10–20 min.
γ-Coniceine	0·12 mg. 25–35 min.	0·15 mg. 25–35 min.	0·8 mg. 15–30 min.
Nicotine	0·1 mg. 25–35 min.	0·1 mg. 25–35 min.	0·6 mg. 30–50 min.

The fasciculations were therefore probably partly due to motor end-plate stimulation and partly due to a central stimulant action. Table II shows the doses of the alkaloids necessary to cause block of the three types of reflex contraction and the approximate durations of the effects. The duration of the effect of N-methylconiine was always shorter than that of the other alkaloids. Fig. 15 illustrates the short duration of action of N-methylconiine compared with that of coniine. The doses of the

FIG. 15. Cat decerebrate. Patellar reflex contractions of quadriceps femoris once every 10 sec. At NMC, 4 mg./kg. N-methylconiine and at C, 4 mg./kg. coniine. 5 min. before the second injection of coniine, 20 mg./kg. mephenesin (M) was injected. All injections intravenously.

alkaloids necessary to block the patellar and crossed-extensor reflexes were much smaller than those necessary to cause peripheral neuromuscular block but the doses necessary to block flexor reflex contractions were only slightly smaller. However, at the time of maximal block of the flexor reflex, it was always possible to elicit twitches by motor nerve stimulation and such twitches were frequently not reduced below the control level (Fig. 14).

The previous administration of mephenesin reduced the ability of small doses of the hemlock alkaloids to block the patellar reflex. Fig. 15 illustrates the reduction in the action of coniine when injected after mephenesin. Furthermore, when administered during a partial block of the patellar reflex produced by the hemlock alkaloids, mephenesin caused an immediate increase in the size of the contractions (Fig. 16). However, this antagonistic effect of mephenesin was limited and could be demonstrated only when the extent of the block was small.

FIG. 16. Cat decerebrate. Patellar reflex contractions of quadriceps femoris elicited once every 5 sec. At γ-C, 200 μg./kg. γ-coniceine; at M, 20 mg./kg. mephenesin and at NMC, 2 mg./kg. N-methylconiine intravenously. Time calibration, 5 min.

Strychnine, administered intravenously in doses of 20–50 μg./kg. caused a powerful increase in the tension of the reflex contractions, particularly in the flexor and crossed-extensor reflexes. All three hemlock alkaloids in doses necessary to block the reflexes, temporarily reduced the potentiation produced by strychnine. When administered during block of all three types of reflex contraction produced by the hemlock alkaloids, strychnine caused a powerful increase in the tension of the partially blocked contractions (Fig. 17).
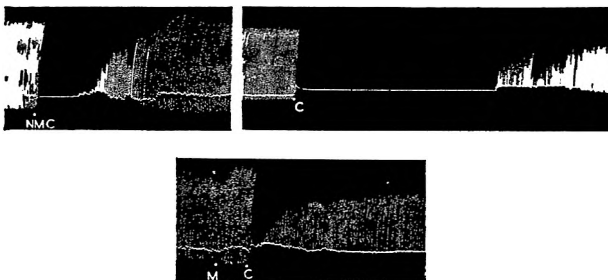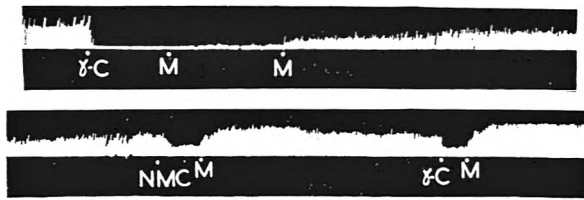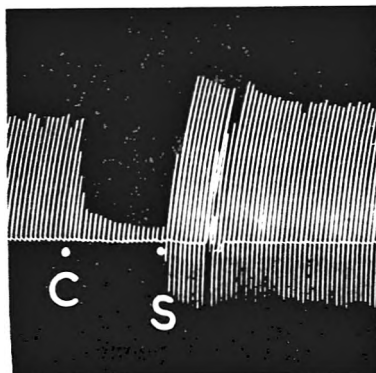

FIG. 17. Cat, decerebrate. Patellar reflex contraction of quadriceps femoris elicited once every 10 sec. At C, 6 mg./kg. coniine and at S, 60 μg./kg. strychnine intravenously.

DISCUSSION

The most pronounced action of the three hemlock alkaloids and of nicotine was shown to be their ability, in relatively small doses, to block the crossed extensor reflex and the knee-jerk by an action exerted in the spinal cord. This action of nicotine on the knee-jerk was first demonstrated by Schweitzer and Wright (1938). With small doses, these effects occurred without any evidence of a stimulant action. The results do not, therefore, support the conclusion of de Boer (1950) that the action of coniine on the spinal cord resembles that of strychnine. In fact, the actions of the hemlock alkaloids and of strychnine were shown to be mutually antagonistic. As with most drugs possessing a central depressant action on spinal reflexes, the flexor reflex was much more resistant to the hemlock alkaloids and to nicotine than the other reflexes studied. The large doses necessary to depress this reflex initially potentiated the contractions and caused fasciculations of the muscle. This stimulant action appeared to be partly central and partly peripheral in origin.

Small doses of the hemlock alkaloids, particularly the first injection, often blocked contractions of the quadriceps muscle elicited by tapping the patellar tendon while leaving contractions of the same muscle, elicited by stimulating the central end of the contra-lateral sciatic nerve, almost unaffected. This apparently selective action is difficult to account for since the patellar reflex has been shown to be monosynaptic (Lloyd, 1952) and the final path, that is the large α-neurons to the extra-fusal muscle fibres, is common to both reflexes. Such a selective action might be explained if there is a difference in the synaptic gaps in the two reflex arcs. It may be that the boutons of the afferent fibres from the muscle spindles make closer contact with the anterior horn cells than those of the interneurons involved in the crossed extensor reflex. Alternatively, the two sets of boutons might release different transmitters and the alkaloids may preferentially block the actions of one of them. However, perhaps the most likely explanation of the selective action is that it was an artifact arising from the different methods of initiating the reflexes. The afferent sensory discharge from the muscle spindles elicited by tapping the patellar tendon may well be weaker than that induced by electrical stimulation and so the knee jerk may appear to be selectively blocked. Further work is therefore necessary to determine whether the selective action is a true one.

The action of the hemlock alkaloids in the spinal cord is complicated by the fact that both inhibitory and excitatory neurons may be affected. Both mephenesin and strychnine antagonised the depressant action of the alkaloids on the patellar reflex. This similar effect of two substances which themselves are mutually antagonistic might be explained if the alkaloids depress the patellar reflex, initially at least, by stimulating inhibitory neurons rather than by blocking excitatory ones. Although the patellar reflex arc is monosynaptic there is evidence that its central synapse is controlled by polysynaptic inhibitory pathways. Mephenesin acts preferentially on spinal interneurons (Tavener, 1952) and might,

therefore, antagonise the effect of the alkaloids by blocking the inter-neurons of the inhibitory pathways. There is also evidence that strychnine exerts its effects at least partly by antagonising the actions of inhibitory transmitters in the spinal cord (Bradley, Easton and Eccles, 1953; Eccles, Fatt and Koketsu, 1954).

The peripheral effects of the hemlock alkaloids on involuntary structures appeared to be mainly a consequence of a stimulant, and with larger doses, a blocking action in both parasympathetic and sympathetic ganglia. With N-methylconiine the blocking action predominated, the stimulant phase of the action being transient, or, in some tissues, non-existent. With γ-coniceine, on the other hand, the stimulant phase was always pronounced while the blocking action was relatively difficult to demonstrate. The action of coniine fell between those of γ-coniceine and N-methylconiine; in most experiments it resembled γ-coniceine and produced powerful stimulant effects but in others, the stimulant phase was absent and only the blocking action could be demonstrated. The pressor effects of coniine and γ-coniceine were shown to be a consequence both of stimulation of sympathetic ganglia and of the release of catecholamines from the adrenal medullae. The results obtained in experiments on local blood flow were in accordance with these findings. When injected intra-arterially into muscle or skin, only a weak vasodilatation was produced. With this route of injection, the drugs do not reach the sympathetic ganglia and therefore do not cause vasoconstriction. When injected intravenously, vasoconstriction occurred in the skin but an increase in flow still occurred in the muscles. The muscle vessels of the cat differ from those of the skin in that they possess a dual innervation-sympathetic cholinergic vasodilator fibres (Folkow and Uvnäs, 1948b, 1950) and sympathetic vasoconstrictor fibres which liberate noradrenaline (Folkow and Uvnäs, 1948a). The effects of stimulation of both types of fibre must counteract each other and the resulting change in calibre will therefore be small. Both adrenaline and noradrenaline released from the adrenal medullae may cause vasodilatation in skeletal muscles (Bowman, 1959) and the rise in blood pressure caused by vasoconstriction in other areas probably forces more blood through the muscle vessels whatever the slight change in their calibre, so that only an increase in flow is recorded.

The ability of the alkaloids to cause a quick contraction on close-arterial injection into the tibialis anterior muscle of the cat and a slow contracture of the frog rectus abdominis and gastrocnemius muscles of the hen may be taken as evidence of a depolarising action. It was of interest that in the mammal, N-methylconiine was more powerful than coniine in stimulating skeletal muscle but considerably less powerful in stimulating autonomic ganglia.

Although the alkaloids appeared to possess a depolarising action, the neuromuscular block which they produced differed in many respects from that produced by the depolarising blocking drugs, decamethonium and suxamethonium. With the first close-arterial injection of coniine and γ-coniceine there was a considerable latent period between the immediate contraction they produced and the development of neuromuscular block.

With decamethonium and suxamethonium, both the contraction and the block are known to be a consequence of the same mechanism, namely depolarisation of the motor end-plates. With these drugs therefore neuromuscular block is always present immediately after the initial contraction. For persistent depolarisation to occur, the affinity of the drug for the acetylcholine receptors must presumably be high so that the drug remains in dynamic equilibrium with the receptors and keeps the end-plates depolarised. It seems likely that the hemlock alkaloids have a low affinity for the receptors. When injected close-arterially in high concentration they are able to react with the receptors to cause a sudden but fleeting depolarisation and consequent contraction, after which they probably dissociate rapidly and are quickly diluted to subthreshold strength. It appears therefore that their blocking action may be unrelated to their ability to cause end-plate depolarisation. The block produced by the hemlock alkaloids also differed from block produced by tubocurarine and from the "dual block" which is sometimes produced by depolarising drugs and which was first described by Zaimis (1953).

In the experiments on the cat, the alkaloids blocked the response of the muscle to close-arterially injected acetylcholine showing that at least part of their blocking action is post-junctional. The alkaloids are secondary and tertiary amines and they are well absorbed after oral or subcutaneous administration, penetrating the blood brain barrier to exert central actions. These facts imply that the alkaloids can penetrate cell membranes readily and suggest that their blocking action at the motor end-plate might be a consequence of an intracellular action which renders the end-plate inexcitable by acetylcholine. del Castillo and Katz (1955) have shown that the acetylcholine receptors are located only on the external surface of the motor end-plate and reaction with these receptors would account for the initial contraction produced by large amounts of the alkaloids. The slow onset of the subsequent block might then be explained if the alkaloids require time to penetrate the cell membrane. An intracellular mechanism of action would also account for the finding that in the rectus abdominis muscle of the frog, concentrations of the alkaloids, smaller than those necessary to cause contracture, blocked the response of the muscle to acetylcholine providing they were left in contact with the tissue for a sufficient time. Bennett, Tyler and Zaimis (1957) postulated a similar intracellular mechanism of action for the secondary amine mecamylamine.

Although, in the cat, the response to close-arterially injected acetylcholine was blocked by the hemlock alkaloids, it returned sooner than it did following a similar degree of block produced by tubocurarine. In the hen, the response of the muscle to acetylcholine was only slightly reduced by the alkaloids even at the time of maximum depression of the indirectly excited twitches. These results suggest that the failure in neuromuscular transmission produced by the alkaloids is partly due to an action on the nerve endings through which the amount of acetylcholine released by a nerve impulse is reduced. The local anaesthetic, procaine, is known to cause neuromuscular block through both pre- and

post-junctional mechanisms (Harvey, 1939; Straughan, 1961) and this action is usually attributed to its local anaesthetic action stabilising the cell membranes and preventing the abrupt changes in permeability which are necessary for depolarisation of both nerve terminals and motor end-plates to occur. The local anaesthetic activity of the hemlock alkaloids was tested in the present experiments but did not appear powerful enough to account for their neuromuscular blocking actions although it may have contributed to the effect.

The blocking action of N-methylconiine was quickest in onset after close-arterial injection. This alkaloid was the least readily absorbed after oral administration and these findings may indicate that its blocking action has a large extra-cellular component in it. This might be expected since at body pH, the tertiary amine will carry the strongest positive charge. The finding that, unlike the other two alkaloids, N-methylconiine was without effect on respiration when injected into the carotid artery may also reflect an extracellular action.

The mechanism of action of the alkaloids on neuromuscular transmission is obviously complex and may well involve more than one site of action. This is not unlikely with substances of this type which combine with acetylcholine receptors but whose action, unlike that of quaternary ammonium compounds, is not confined to extra-cellular sites.

In general, Sollman's (1957) statement that the effects of coniine resemble those of nicotine both centrally and peripherally, was confirmed. With few exceptions, N-methylconiine and γ-coniceine had qualitatively similar actions. With the possible exception of the effects of small doses of the alkaloids on the spinal cord their actions are clearly too widespread to be of real therapeutic use. Indeed it seems unlikely that hemlock could ever have been administered in doses sufficient to produce the peripheral actions for which it was recommended in the older clinical literature. The central depressant action of the alkaloids is of interest, that on the knee-jerk and crossed extensor reflex occurring with doses which produced only minor additional actions. This action of the alkaloids was obviously the basis of the use of hemlock in spastic states in man, and not, as was believed by many, the peripheral neuromuscular blocking action which requires much larger doses. The central depressant action was much longer lasting and occurred with smaller doses than that produced by mephenesin, and the alkaloids might therefore serve as a starting point for the synthesis of more specific and less toxic spinal relaxants.

PHARMACOLOGICAL ACTIONS OF HEMLOCK ALKALOIDS

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SYNTHESIS AND PHARMACOLOGICAL PROPERTIES OF SOME DERIVATIVES OF p-AMINOPHENYLETHANOLAMINE

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Synthesis of a series of N-alkyl derivatives of 2-amino-1-p-aminophenylethanolamine has been described, and the results of pharmacological screening reported. The antagonistic action exhibited at the adrenergic β-receptors by the p-nitro- and p-amino-N-isopropyl derivatives and the outstanding spasmolytic and bronchodilator activity of 1-p-aminophenyl-2-s-butylaminoethanol have been pointed out.

In chemical and pharmacological investigations made in this laboratory on variously substituted phenylethanolamine derivatives, a series of p-nitrophenylamines was prepared (Teotino, Polo Friz, Steis and Della Bella, 1961).

In studying these compounds we prepared some hitherto unreported p-amino-derivatives of the structure I. These have received little attention

CH(OH)·CH₂NRR'
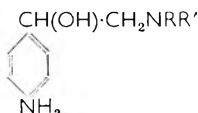
I   Where R and R' = H, Alkyl, Alkylaryl

NH₂

from a biological point of view, although some interesting modifications of the pharmacological properties of sympathomimetic phenylalkylamines caused by the amino-group in the nucleus have been pointed out. Thus, in a comparison of bronchodilator activity in perfused guinea-pig lungs, p-aminoephedrine is reported to be twice as active as ephedrine and half as toxic (Tainter, 1933; Tainter and others, 1934), while p-aminophenethyldimethylamine was shown to be a hypertensive agent 10 times as potent as dimethylphenethylamine, but acting through a nicotinic mechanism (Bovet and Benoit, 1942).

Derivatives of 2-amino-1-m-aminophenylethanol have been investigated by Lands (1952), who reported that a pressor activity was exhibited by some derivatives, but no bronchodilator effects on guinea-pigs were observed with either the nor-derivative or 1-(m-aminophenyl)-2-isopropylaminoethanol.

A series of 2-alkylamino-1-(m- and p-aminophenyl)ethanols has been synthesised by Russian workers (Serghievskaja and Sventsitskaja, 1956) through 2-alkylbenzylamino-m- and p-nitroacetophenones. Catalytic hydrogenation of the latter compounds produced a simultaneous reduction of the nitro-group to an amino-group and of the keto-group to a secondary alcohol group as well as debenzylation of the original amino-group.

Another Russian study (Syrneva, 1957) reports the activity of some alcohols of the structure: *m*- and *p*-H₂N·C₆H₄·CH(OH)·CH₂·N(Me)R (R = from Me to C₅H₁₁). The sympathomimetic activity of these alcohols was shown to diminish on increasing R until when R = C₅H₁₁ the derivative became sympatholytic.

We prepared the derivatives I (Table I) by reducing in ethanol the corresponding 2-mono- and -di-alkylamino-1-*p*-nitrophenylethanols at room temperature and atmospheric pressure using Pd over C as catalyst. The *p*-nitro-derivatives were obtained by reacting *p*-nitrostyrene oxide with primary or secondary amines (Teotino and others, 1961). The reduction usually proceeds quite rapidly (40–50 min.) until the theoretical quantity of hydrogen has been adsorbed. The corresponding mono-hydrochlorides of the aniline derivatives should be protected from air and light during isolation, to prevent excessive darkening.

The corresponding bases of the monoalkyl derivatives were obtained as solid substances readily purified by crystallisation from benzene; bases of dialkyl derivatives are mostly low-melting solids.

1-*p*-Aminophenyl-2-benzylaminoethanol monohydrochloride was obtained by reducing 2-benzylamino-1-*p*-nitrophenylethanol hydrochloride at room temperature and atmospheric pressure with Pd over C as catalyst; the product was subjected to hydrogenation again with Pd as catalyst and at 50–60° was debenzylated to give 2-amino-1-*p*-aminophenylethanol (Teotino and others, 1961).

The derivative of I where R = R′ = Me (1-*p*-aminophenyl-2-dimethylaminoethanol) was quaternised with ethyl iodide to obtain the corresponding ethiodide.

<div align="center">EXPERIMENTAL</div>

Reduction of 2-*mono- and -di-alkylamino*-1-p-*nitrophenylethanols.* Reductions were effected at atmospheric pressure in a hydrogenation apparatus.

Example: 2-mono- or di-alkylamino-1-*p*-nitrophenylethanol hydrochloride (0·0735 mole) is dissolved or suspended in absolute ethanol (300 ml.). Pd over C (10 per cent 0·7 g.) is added and the mixture hydrogenated at room temperature. The reduction ceases when 0·218 mole of hydrogen has been absorbed. The catalyst is filtered off and the filtrate evaporated to dryness *in vacuo* in a current of nitrogen with protection from light. The residue solidifies on standing and is crystallised from a suitable solvent (Table I). Yield: 80–90 per cent of 2-mono- or di-alkylamino-1-*p*-aminophenylethanol monohydrochloride. The corresponding bases are obtained from aqueous solutions of the mono-hydrochlorides by the addition of alkali and successive extractions with a nonpolar solvent (diethyl ether or benzene).

1-p-*Aminophenyl*-2-*dimethylaminoethanol ethiodide.* 1-*p*-Amino-phenyl-2-dimethylaminoethanol (1 g., 5·56 mmole) is dissolved in acetone (5 ml.). Ethyl iodide (0·86 g., 5·56 m mole) is added at room temperature. After about 10 min. crystals begin to separate. After 2 hr. the mixture is filtered: yield 1·4 g. (76 per cent), m.p. 195–6°. After recrystallisation from methanol m.p. rises to 198–9° (decomp.).

TABLE 1

$$NH_2 \text{---} \diagdown \text{---} CH \cdot CH_2 \cdot NRR'$$
$$\overset{|}{OH}$$
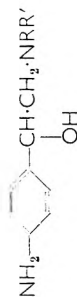
R	R'	Derivative	Crystal solvent	m.p. °C	Found per cent				Formula	Required per cent				References
					C	H	Cl	N		C	H	Cl	N	
H	H	Monohydrochloride	Ethanol	150-2	50·81	6·81	18·64	14·75	$C_9H_{13}ClN_2O$	50·93	6·95	18·80	14·85	C.A., **48**, 10537 (1954)
H	Me	Base	Ethanol	124-5	64·90	8·41	—	16·70	$C_9H_{14}N_2O$	65·03	8·49	—	16·85	C.A., **51**, 5047 (1957)
H	Et	Base	Isopropanol	134·5	66·55	8·89	—	15·61	$C_{10}H_{16}N_2O$	66·63	8·95	—	15·54	C.A., **51**, 5047 (1957)
H	Prⁱ	Base Monohydrochloride	Water Ethanol	138·9 157	67·54 57·34	9·37 8·22	15·27	14·37 12·31	$C_{11}H_{18}N_2O$ $C_{11}H_{19}ClN_2O$	68·00 57·25	9·34 8·30	15·37	14·42 12·14	C.A., **51**, 5047 (1957)
H	Buⁿ	Base		90	69·07	9·57	—	13·37	$C_{12}H_{20}N_2O$	69·19	9·68	—	13·45	C.A., **51**, 5047 (1957)
*H	Buˢ	Base Monohydrochloride	Benzene Isopropanol	73-4 112·3	69·25 59·01	9·64 8·52	14·37	13·35 11·27	$C_{12}H_{20}N_2O$ $C_{12}H_{21}ClN_2O$	69·19 58·88	9·68 8·65	14·48	13·45 11·45	—
H	Buᵗ	Monohydrochloride	Isopropanol	185·6	58·36	8·34	14·62	11·50	$C_{12}H_{21}ClN_2O$	58·88	8·65	14·48	11·45	—
H	$C_6H_5 \cdot CH_2$	Monohydrochloride	Ethanol	155·6	64·53	6·61	12·31	10·54	$C_{15}H_{19}ClN_2O$	64·62	6·87	12·73	10·05	—
Me	Me	Ethiodide	Methanol	198·9	42·95	6·25	I = 38·08	8·30	$C_{11}H_{21}IN_2O$	42·86	6·31	I = 37·75	8·33	—
Et	Et	Monohydrochloride	Isopropanol	141·2	58·99	8·72	14·23	11·41	$C_{14}H_{23}ClN_2O$	58·88	8·65	14·48	11·45	—

* For this compound a patent application was filed in England under No. 23696/61 on June 30, 1961.

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PHARMACOLOGICAL PROPERTIES OF THE COMPOUNDS EXAMINED

A preliminary screening of the pharmacological properties of the *p*-aminophenylethanolamines was effected *in vivo* on the blood pressure of the rat and cat, with simultaneous recording of nictitating membrane responses to electrical preganglionic stimulation, and *in vitro* on the rabbit and guinea-pig intestine responses to histamine, acetylcholine and barium chloride. At the same time the corresponding *p*-nitro-compounds (Teotino and others, 1961) were tested.

Except for the nor and *N*-methyl derivatives, which exhibited a slight hypertensive activity (1,000 to 1,500 times less potent than adrenaline), the *N*-alkylated derivatives with a higher alkyl substituent were all shown to possess some degree of hypotensive activity. This activity of both *p*-nitro- and *p*-amino- compounds increases as follows: propyl < iso-propyl < t-butyl < n-butyl < s-butyl; the *p*-amino-derivatives proved, however, much more active than the corresponding *p*-nitro derivatives.

FIG. 1. Rat, 220 g., amylobarbitone anaesthesia (200 mg./kg., i.p.). Carotid blood pressure. Pretreatment (60 min. before) with 5 mg./kg. of phentolamine, intravenously. It can be seen that the characteristic hypotensive responses to adrenaline (0·25 µg./kg.) at (1) and to isoprenaline (0·16 µg./kg.) at (2), are abolished after administration of 10 mg./kg. of the *p*-nitro-*N*-isopropyl derivative intravenously at (3).
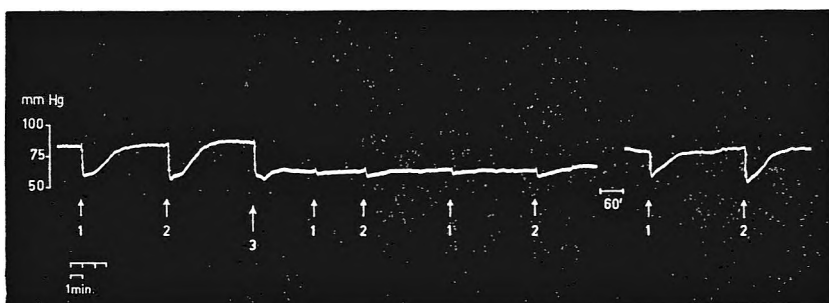
FIG. 2. Rat, 250 g., amylobarbitone anaesthesia (200 mg./kg., i.p.). Carotid blood pressure. Pretreatment (60 min. before) with 5 mg./kg. of phentolamine, intravenously. It can be seen that the characteristichypotensive responses to adrenaline (0·25 µg./kg.) at (1) and to isoprenaline (0·16 µg./kg.) at (2) are abolished after administration of 10 mg./kg. of *p*-amino-*N*-isopropyl derivative intravenously at (3).

The hypotensive property of the derivatives was not affected by pre-treatment of the test preparation with atropine. The response of the nictitating membrane was not appreciably influenced by any of the compounds tested, and its response to both adrenaline and noradrenaline remained practically unmodified.

The pressor responses to *N*-ethyl derivatives were inconsistent, being hypertensive in some experiments and hypotensive in others. Of particular interest were the results obtained with the two *N*-isopropyl compounds, which exhibited an antagonistic action at the adrenergic β-receptor level. This property, illustrated in Figs. 1 and 2, was shown by both the *p*-amino- and the *p*-nitro-compounds in equal degrees: they inhibited both the reversed response to adrenaline after phentolamine and the response to isoprenaline. An experiment with dichloroiso-prenaline effected under the same experimental conditions has also been reported for comparison (Fig. 3).
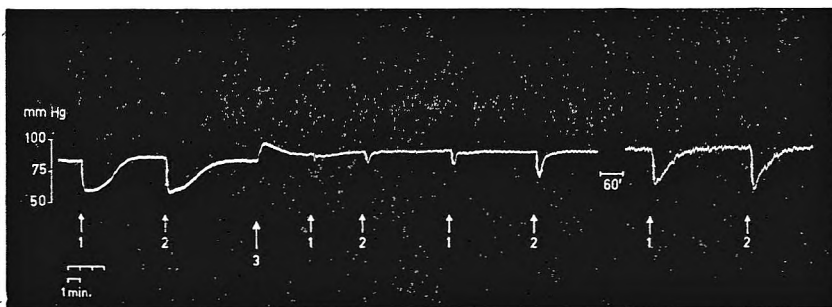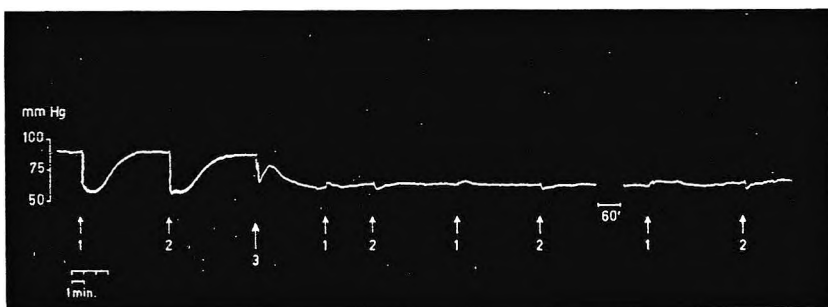

FIG. 3. Rat, 205 g., amylobarbitone anaesthesia (200 mg./kg., i.p.). Carotid blood pressure. Pretreatment (60 min. before) with 5 mg./kg. of phentolamine, intravenously. It can be seen that the characteristic hypotensive responses to adrenaline (0·25 μg./kg.) at (1) and to isoprenaline (0·16 μg./kg.) at (2) are abolished after administration of 10 mg./kg. of dichloroisoprenaline intravenously at (3).

The Figs. 1 and 2 show also the different pressor effects induced by the two derivatives after pretreatment of the test preparation with an adrenergic blocking agent: with the *p*-amino-derivative a constant hypotension is observed, while the hypotensive effect of the *p*-nitro-compound in the whole animal is reduced or even reversed. A more detailed study of the properties of the two compounds, which seem very similar to those of dichloroisoprenaline, will be reported. No significant effects were noted in the study of the *NN*-dialkyl derivatives. The quaternary ethiodide of the *NN*-dimethyl derivative, assayed on the rabbit isolated intestine with intact sympathetic nerve supply according to Finkleman, lacked any adrenergic nerve blocking activity. The *in vitro* experiments on the guinea-pig intestine response to histamine, acetylcholine and barium revealed that among the compounds tested those with a higher alkyl substituent were capable of antagonistic action. No appreciable differences were observed in this test between the *p*-amino- and the

p-nitro-compounds, but the compounds with a higher alkyl substituent, for example the *N*-isopropyl derivatives, and above all the *N*-butyl isomers, proved more active.

The influence of these latter derivatives on the isolated intestine of the rabbit is worthy of note. Among the *p*-amino-*N*-butyl-substituted isomers, only the s-butyl isomer exhibited a marked spasmolytic property. The corresponding *p*-nitro-derivatives instead proved effective stimulants of the rhythmic activity of the isolated organ. Figs. 4 and 5 show the qualitative as well as the quantitative differences observed between the two series of compounds.
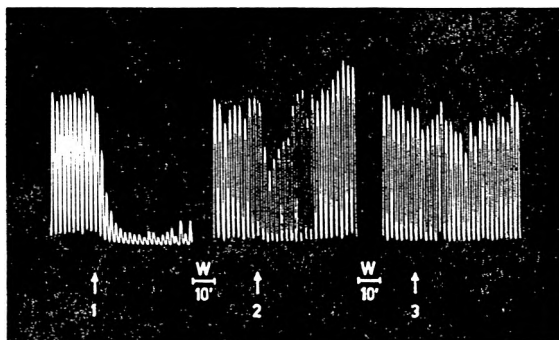
FIG. 4. Isolated rabbit duodenum in Tyrode solution. Differences in the influence exerted by *N*-butyl isomers of the *p*-amino-substituted series are shown. While the *N*-s-butyl derivative at (1), at a concentration of 5 μg./ml. (time of contact 2 min.), markedly inhibits the motor activity, both the *N*-t-butyl at (2) and the *N*-n-butyl derivative at (3), at concentrations of 10 μg./ml., proved almost inactive.
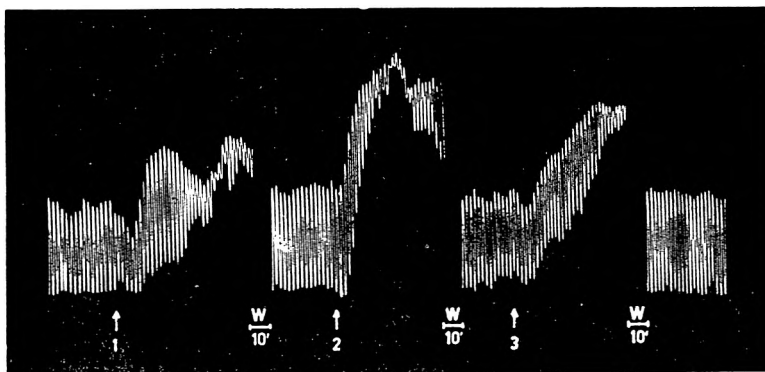
FIG. 5. Isolated rabbit duodenum in Tyrode solution. The properties of the *N*-butyl isomers of the *p*-nitro-substituted series were found to be different from the properties of the isomers of the *p*-amino-substituted series. The *N*-s-butyl at (1), the *N*-n-butyl at (2) and the *N*-t-butyl derivative at (3), at a concentration of 10 μg./ml., proved effective stimulants of intestinal motility.

Further Investigations

The results of preliminary *in vivo* and *in vitro* experiments reported prompted us to investigate further the properties of 1-*p*-aminophenyl-2-s-butylaminoethanol.

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Acute toxicity. This derivative proved of low toxicity: the LD50 in rats was about 120 mg./kg. i.v. and 650 mg./kg. orally. On oral administration of 25 mg./kg. daily for 90 days there was no evidence of weight loss in the animals nor observable morphological changes in the blood or parenchymal organs (liver, kidney, spleen, bone marrow and gastric mucosa) were noted. Doses ranging from 5 to 50 mg./kg. i.v., in a single dose, produced in rats and cats a slight hypotension (10–20 mm. Hg at higher doses) of some duration.

Autonomic actions. No appreciable modifications have been observed on vagal and sympathetic peripheral synapses. The pressor responses to both adrenaline and noradrenaline remained unmodified and the responses to acetylcholine were slightly reduced. In the rabbit the electrocardiographic tracing, recorded at the moment of intravenous introduction of 10–20 mg./kg. of the compound, remained within normal limits and no change in pulse frequency was observed. The compound antagonised the effects of both acetylcholine and histamine on the guinea-pig intestine and also inhibited the motility of the rabbit intestine (Fig. 4). The responses to barium were abolished at a concentration of 20–30 μg. ml. of the compound. The responses of guinea-pig tracheal rings to histamine and acetylcholine were inhibited by the compound.

Spasmolytic activity. This derivative was successively tested for spasmolytic activity *in vivo* by observing its influence on the intestinal transit of wood charcoal introduced by a gastric tube and also on the

TABLE II

EFFECT OF INTRAPERITONEAL 1-*p*-AMINOPHENYL-2-S-BUTYLAMINOETHANOL, OF PAPAVERINE AND OF EPHEDRINE ON THE INTESTINAL TRANSIT OF WOOD CHARCOAL IN THE RAT

Treatment and dose, mg. kg.	No. of rats	Per cent of inhibition
Controls	10	0
Papaverine 10	10	49
Ephedrine 10	8	41
1-*p*-Aminophenyl-2-s-butylaminoethanol 25	12	42
" " 50	12	59

TABLE III

TIME OF BRONCHOSPASM ONSET INDUCED BY AN AEROSOL OF A SOLUTION CONTAINING ACETYLCHOLINE (0·06 PER CENT), HISTAMINE (0·03 PER CENT) AND 5-HT (0·03 PER CENT) IN GUINEA-PIGS PRETREATED, ORALLY, BY 1-*p*-AMINOPHENYL-2-S-BUTYLAMINOETHANOL, EPHEDRINE AND DIPROPHYLLINE

(Mean results obtained in groups of 6 guinea-pigs are indicated)

Treatment and dose, mg./kg.		Time of bronchospasm onset after aerosol given A, 60, B 120 and C 240 min. after treatment with drug		
		A	B	C
Controls	45″			
Ephedrine 10		2′	1′ 40″	50″
Diprophylline 100		1′ 27″	1′ 53″	53″
1-*p*-Aminophenyl-2-s-butylaminoethanol		1′ 40″	3′ 14″	over 5′

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onset of bronchospasm induced in the guinea-pig by an aerosol of a solution containing acetylcholine, histamine and 5-hydroxytryptamine. The effects of the compound upon the speed of peristalsis in the rat are illustrated in Table II. The results obtained with papaverine and ephedrine are shown for comparison.

Table III shows the results of experiments made to study protection against bronchospasm induced by an aerosol. As can be seen, the compound possessed an evident protective action, even more marked than that obtained by corresponding doses of diprophylline[7-(2,3-dihydroxy-propyl)theophylline] and by ephedrine.

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INHIBITIONS BY TETRACYCLINE AND OXYTETRACYCLINE OF THE CONSUMPTION OF PYRUVATE BY *AEROBACTER AEROGENES*

BY J. G. JONES AND G. A. MORRISON

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The effects of tetracycline and oxytetracycline on the production of pyruvate and on its utilisation by a strain of *Aerobacter aerogenes* in glucose-mineral salt media, have been examined. Both antibiotics inhibit the production of formate from pyruvate by non-proliferating suspensions of cells in unaerated, but not in aerated media. The only acidic material accumulated during growth is formic acid and the cessation of growth coincides with the concentration of undissociated formic acid reaching 0·2 mM and 0·3 mM respectively in unaerated and aerated media. At the end of the growth phase of unaerated cultures the reproduction of cells is replaced by the production of lactic acid. The possibility of a common primary site of inhibition being responsible for inhibition of pyruvate and mode II inhibition of growth (Jones and Morrison, 1962) is discussed.

THE molecular forms of both tetracycline and oxytetracycline decrease the rate of growth of unaerated cultures of *A. aerogenes* in glucose-mineral salt media by interfering with the hydrogen-transfer mechanisms (Mode II, Jones and Morrison, 1962). Since the glucose of the media is the source of both the hydrogen and of its ultimate acceptor, the chain of reactions producing this acceptor from glucose may contain the site of origin of mode II inhibition, or its overall functioning be affected by mode II. It is possible also that some reaction in this chain is inhibited independently but not sufficiently severely to make it the rate-controlling reaction of growing cultures.

EXPERIMENTAL METHODS AND MATERIALS

The organism, medium and many of the procedures have been described previously (Jones and Morrison, 1962). Pyruvic acid was released in solution as required from pure lithium pyruvate hydrate (prepared from freshly distilled pyruvic acid) by passing through a column of "Amberlite" IR 120 (H).

Preparation of suspensions of cells. Cells were obtained from fully grown cultures, observed by nephelometry, in which the extent of growth had been limited by the amount of glucose present initially to two-thirds of the maximum obtainable in cultures starting at pH 7·00. They were washed twice in iso-osmotic aqueous buffer, in which they were re-suspended to form a stock suspension which was incubated at 37° for 1 hr. Aliquots of fresh stock suspensions were used in individual experiments.

Concentrations of acids: All acids together. 3·00 ml. of the test liquor was allowed to percolate slowly through 5 cm. of well-washed "Amberlite" C.G. 120 (H) in columns of 1 cm. diameter, the eluate and 12 ml. of

washings with demineralised water being collected. The combined eluate and washings were titrated with 0·100 N NaOH (carbonate-free) from a micro-burette using thymolphthalein as the indicator. The equivalence of the mineral acids released from the buffering salts was determined from samples of the medium and subtracted from the results. The accuracy was ±0·5 m-equiv./litre.

Volatile Acids

The micro-diffusion method was used (Conway, 1961a). The accuracy was ±0·13 m-equiv./litre.

Keto-acids. Differential colorimetric methods based on the reaction with dinitrophenylhydrazine (Friedemann and Haugen, 1943) were used to determine the total concentration of keto-acids, the concentration of pyruvic acid, and hence by difference, the concentration of keto-acids other than pyruvic. The accuracy was ±10 μM, and suspensions of cells produced as described above did not produce measureable concentrations of keto-acids within 3 hr. unless a suitable substrate was added.

Lactic acid. The modification (Hullins and Noble, 1953) of the method devised by Barker and Summerson (1941) was used. The accuracy was ±10 μM.

Malic acid. An E.I.L. direct reading fluorimeter was applied to the method devised by Hummel (1949). Since large concentrations of glucose are troublesome, media containing graded concentrations of glucose were made up and the malate determined in each just as the glucose had been consumed completely. The accuracy was ±2 μM.

RESULTS

Net Rates of Production of Keto-acids and Concentration of Antibiotics

Pyruvate is an essential intermediate in the utilisation of glucose for growth by the test organism (Dagley, Dawes and Morrison, 1951) and small concentrations of it can be measured. It thus provides an opportunity for the examination in two portions of the pathway of reactions in which it takes part between glucose and cellular material. Inhibition of either its production or of its utilisation could decrease the rate of growth, but inhibition of a reaction preceeding the formation of pyruvate must result in a smaller steady-state concentration of pyruvate whereas inhibition of a reaction subsequent to its formation must result in a larger steady-state concentration of it. Preliminary experiments with growing cultures showed that a small constant steady state concentration of other keto-acids was present during the logarithmic phase of growth and consequently they also provide a similar opportunity in the pathway(s) in which they take part.

The test media contained: 12 g. glucose, 5·4 g. KH$_2$PO$_4$, 0·082 or 6·56 mmol. magnesium, and 0 to 0·25 μmol. antibiotic, per litre and were adjusted to pH 7·00.

Variation in the concentration of the magnesium or in the concentration of either antibiotic had no effect on the net rate of production of either pyruvate or of keto-acids other than pyruvic by aerated suspensions. Similarly the antibiotics did not affect the net rate of production of keto-acids other than pyruvic by unaerated suspensions, but the net rate of production of pyruvate increased with increasing concentration of anti-biotic or with decrease in concentration of magnesium which combines with the antibiotic to form a non-inhibitory complex (Jones and Morrison, 1962). The experimental results for tetracycline are presented in Fig. 1.
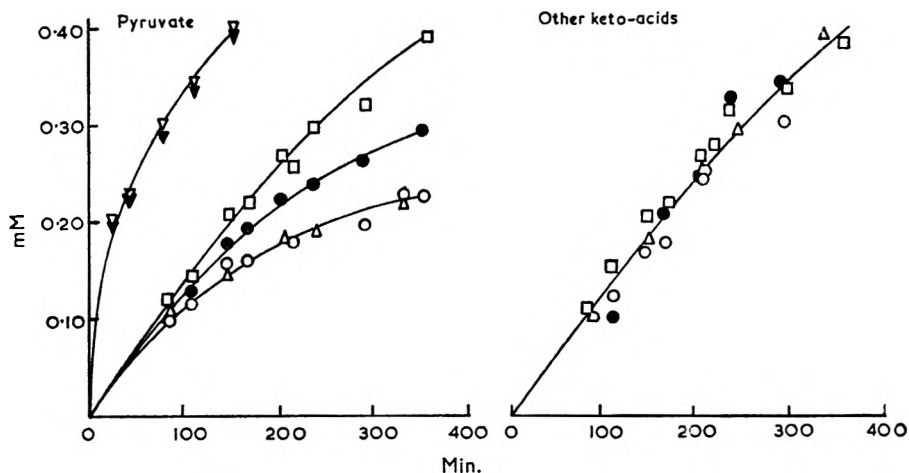

FIG. 1. Effect of tetracycline on the production of keto-acids by suspensions of A. aerogenes (0·115 g. (dry) cells per litre).

Concentration of magnesium (mM)	..	0·082	0·082	0·082	6·56	0·082	0·082
Concentration of tetracycline (μM)	..	0·000	0·125	0·250	0·125	0·000	0·300
Concentration of formate (N) ..	..					0·46	0·46
		○	●	□	△	▽	▼

Thus the utilisation of pyruvate is inhibited more severely than is its production, and th utilisation and production of the other keto-acids are affected equally: since two separate reactions are very unlikely to be equally inhibited at more than one concentration of inhibitor, it is concluded that neither the production nor the utilisation of other keto-acids is inhibited.

Rate of Consumption of Pyruvate and Concentration of Antibiotic

The inhibitions of the consumption of pyruvate by tetracycline and oxy-tetracycline were examined in test media in which approximately 0·8 mM pyruvate replaced the glucose of the previous experiments. The results are presented in Fig. 2.

Both antibiotics inhibit the consumption of pyruvate in unaerated media but not in aerated media. This inhibition cannot be the origin of mode I inhibition which is effective in aerated conditions (Jones and Morrison, 1962). Also it is not the origin of mode II.

0·204 μM is the critical concentration of tetracycline at which control of the rate of growth of unaerated cultures passes from mode I to mode II,
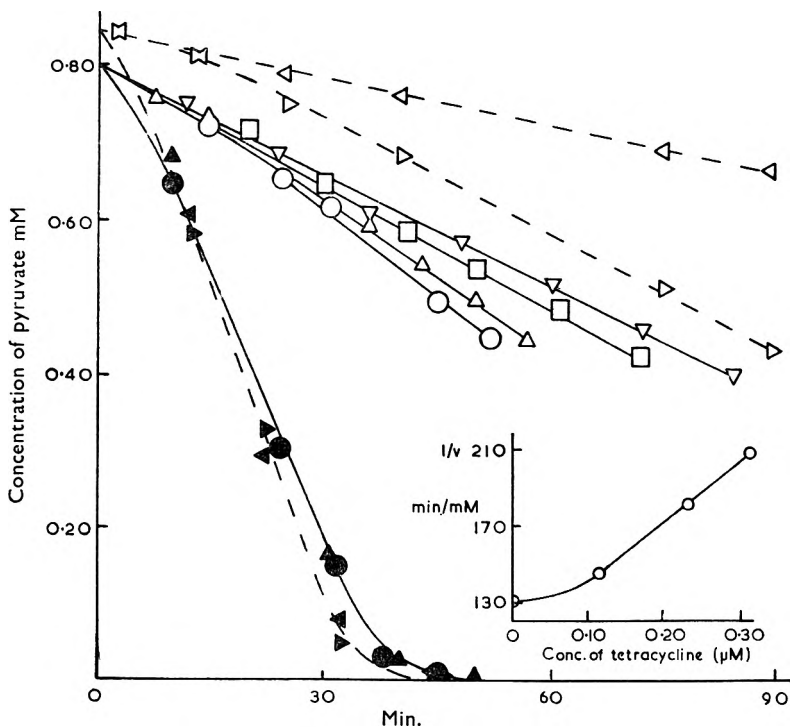
FIG. 2. Consumption of pyruvate by suspension of *A. aerogenes*.
Unaerated media containing 0·100 g. (dry) cells per litre: concentration of tetra-
cycline (μM): 0, ◖ ; 0·014, △ ; 0·228, □ ; 0·34, ▽.
Unaerated media containing 0·061 g. (dry) cells per litre: concentration of oxy-
tetracycline (μM): 0, ▷ ; 0·340, △.
Aerated media containing 0·275 g. (dry) cells per litre: concentration of tetra-
cycline (μM): ●, 0; 0·250, ▲.
Aerated media containing 0·325 g. (dry) cells per litre: concentration of oxytetra-
cycline (μM): 0, ▶; 0·340, ◀.

the mean generation time being $84 \pm 2·5$ min. (Jones and Morrison,
1962). At this concentration the reciprocal, $1/v$, of the rate of consump-
tion of pyruvate is 174 min./mM and this reciprocal increases linearly with
increase of concentration of tetracycline (Fig. 2). Thus, if inhibition of
the rate of consumption of pyruvate is the cause of mode II, the mean
generation time, m, should be given by $(84/174)(1/v)$. In Table I, com-
parison of the mean generation times at higher concentrations of tetra-
cycline calculated from this expression, with those obtained empirically,

TABLE I

TEST OF INHIBITION OF UTILISATION OF PYRUVATE AS ORIGIN OF INHIBITION OF GROWTH.
V IS THE RATE OF CONSUMPTION OF PYRUVATE

Concentration of tetracycline (μM)	$1/v$ for consumption of pyruvate	Mean generation times (min.)	
		Calculated	Measured
0·204	0·174	84	84
0·250	0·188	91	109
0·300	0·203	98	135

shows that inhibition of the consumption of pyruvate by tetracycline does not increase rapidly enough with increase of concentration of tetracycline to account for inhibition of growth by mode II.

A similar test for inhibition of consumption of pyruvate by oxytetracycline gives the same result.

In these experiments the total concentrations of keto-acids were measured and found to be indistinguishable from the concentrations of pyruvate. Thus pyruvate itself is not an intermediate in the production of other keto-acids from glucose. The liquors were examined to establish the nature of the products of consumption of pyruvate: the original concentration of pyruvate (*ca.* 0·8 mM) was replaced by 0·8 ± 0·13 m-equiv./litre of volatile acid. This volatile acid reduced alkaline permanganate and gave a single spot which ran concurrently with formate during partition chromatography on paper using a number of solvent mixtures (Lederer and Lederer, 1957). Standard tests for acetate failed to yield positive results, and neither lactate, nor acetyl phosphate (Lipman and Tuttle, 1945) could be detected. It is concluded therefore that the volatile acid is formic.

The experiments of the previous section in which glucose was the primary substrate in unaerated media were repeated in the presence of added formate: small concentrations increased the net rate of production of pyruvate and decreased the difference between the rates of production in media containing 0 and 0·3 μM tetracycline. The presence of 0·46 N formate in media at pH 7·00 eliminated the effect of the tetracycline (included in Fig. 1) and lactate was produced. It is concluded that the consumption of pyruvate is by a reversible reaction of the type $MeCO \cdot COOH + Y \rightleftharpoons HCOOH + X$, where X is not a volatile acid, and that this reaction is inhibited either directly, or indirectly by inhibition of the utilisation of X, and that tetracycline does not inhibit the production of pyruvate from glucose.

Attempts to follow the consumption of pyruvate by measuring changes in concentration of volatile acid produced using larger initial concentrations of pyruvate, were frustrated by the intrusion of another reaction. When the initial concentration of pyruvate was 8 mM the rate of consumption of pyruvate by 1 g. of cells per litre was 3·6 times as fast as when the initial concentration of pyruvate was 0·8 mM until the concentration of pyruvate had decreased to less than 1 mM. This intruding reaction was not inhibited by tetracycline. The pyruvate was replaced eventually by 14 mM volatile acids including formic, and during the experiments lactate appeared, attained a concentration of 0·1 mM, and then declined to zero. This intruding reaction was not investigated further at the time since it is not affected by tetracycline.

Production of Acids during Growth

Growth of *A. aerogenes* in the glucose-mineral salt medium is associated with the production of acid, and there is a maximum population which is attained before any of the nutrients have been consumed completely, but which depends on the initial pH of the medium (Hinshelwood, 1946).

Measurements of the concentration of total formate produced have lead to the suggestion that this waste product at critical concentrations stops the production of cells, and this suggestion is supported by the fact that additions of formate decreased the extent of growth (Dagley, Dawes and Foster, 1953). Additions of malate, lactate or succinate also decrease the extent of growth, and the dependence of the extent of growth on the initial pH and the ionic strength of the medium can be explained if it is assumed that the undissociated form of an acid produced during growth is the inhibitor (Morrison, 1953).

Unaerated cultures. The concentrations of cells, the pH and the concentrations of (*a*) all acids produced taken together, (*b*) volatile acids, (*c*) malic acid, (*d*) pyruvic acid, and (*e*) lactic acid, were measured at intervals during the growth of cultures starting at different pH values. The results illustrated in Figs. 3 and 4 are:
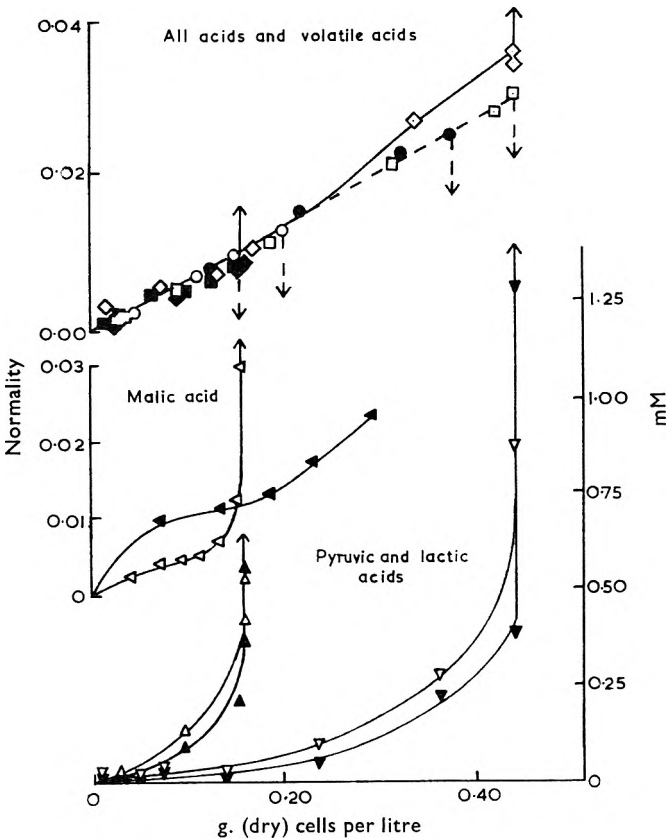

FIG. 3. Production of acids in unaerated cultures of *A. aerogenes.*

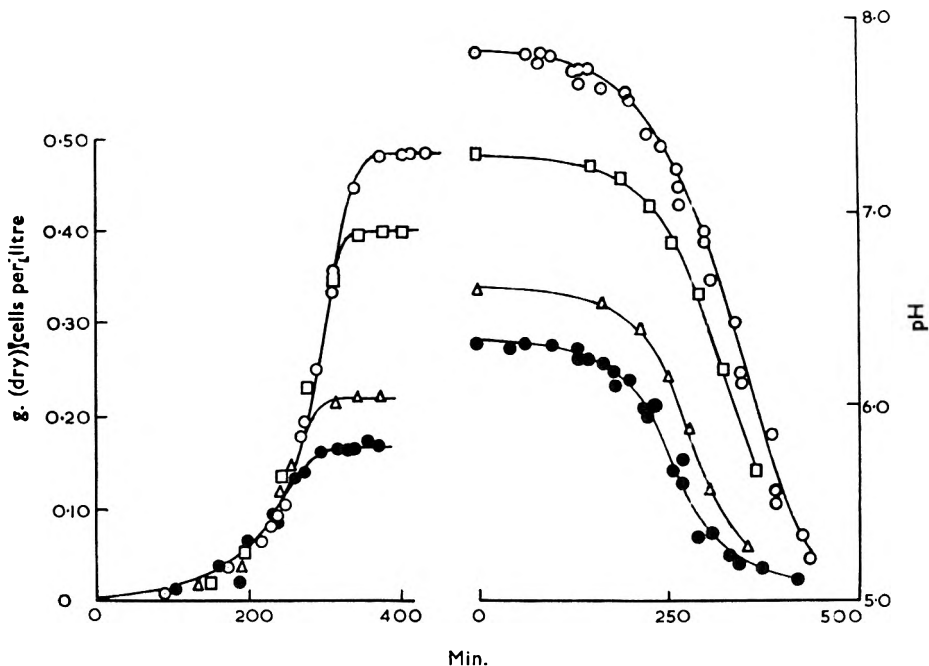| Initial pH of culture	Concentration of	all acids	Volatile acids	Malic acid	Pyruvic acid	Lactic acid
7·82		◇	□	◀	▽	▼
7·32			●			
6·60			○			
6·32		◆	■	◁	△	▲

FIG. 4. Cell populations and pH of unaerated cultures of *A. aerogenes*. Initial pH of the culture: 7·82, ○ ; 7·30, □ ; 6·60, △ ; 6·32, ●.

Malate: accounts for only 1/1000th of the total acid produced during growth. Early in the growth period it is almost constant in concentration and hence is probably an intermediate, but at the end of growth it appears to be a waste product to a small extent as its concentration increases.

Pyruvate and lactate: during the early logarithmic phase these acids also account for about 1/1000th of the total acid produced, but later their concentrations increase until they make a decided contribution to the total acidity at the end of growth. After growth stops the production of acids continues and lactate becomes the main product. Clearly growth does not stop because of an inability to dispose of hydrogen since this continues with the formation of lactate.

Volatile acids: throughout the growth phase these acids account for practically all the acidic material produced, and the production of them and the production of cells have an almost constant ratio to each other. Exhaustive examination by standard methods detected only formate and repeated paper chromatography of the ammonium salts using various combinations of alcohols, ammonia and water for development, gave single spots at the R_F expected for formate which ran concurrently with known samples of formate but not with those of acetate. It is concluded that formate is the main acidic waste product during growth.

40

After growth stops the concentration of volatile acids (formate) decreases slowly with time (Fig. 3) and from this plot the peak concentration of formate, that at the end of growth, can be determined for cultures starting at different pH values. From the first part of Fig. 4 the time at which growth ends can be determined to within 10 min. and the value used to establish from the second part of Fig. 4 the pH within a narrow range of the culture as growth ends. Since the dissociation constant for formic acid is 1.74×10^{-4} (*Handbook of Chemistry and Physics*, 1959) the concentrations of formate ion and of undissociated formic acid present just as growth stops in cultures starting at different pH values can be calculated. Table II gives the results.

TABLE II

CONCENTRATIONS OF FORMATE (ACID PLUS FORMATE IONS) AND UNDISSOCIATED FORMIC ACID IN UNAERATED CULTURES AS GROWTH STOPS

			As growth stops			
Initial pH	Time from inoculation (min.)	pH	Cell concentration (g. dry cells/litre)	[HCOOH] + [HCOO⁻] (mM)	[HCOOH] (mM) calculated	Ratio of concentrations of cells and acid produced (g./m.mol.)
7·82	370 ± 5	5·94 ± 0·06	0·460	30·0	0·20 ± 0·03	0·015
7·32	345 ± 5	5·91 ± 0·05	0·387	25·2	0·18 ± 0·02	0·015
6·60	305 ± 5	5·52 ± 0·03	0·207	12·2	0·20 ± 0·02	0·017
6·32	300 ± 10	5·40 ± 0·06	0·155	9·0	0·20 ± 0·03	0·017

The active phase of growth in all cultures ends when the concentration of undissociated formic acid reaches 0·2 mM. Thus attributing inhibition of growth to undissociated formic acid accounts for the dependence of the extent of growth on the initial pH of the medium. Adding formate to media at different pH values in sufficient quantity to give 0·2 mM undissociated formic acid initially prevented all growth for 24 hr. whereas smaller quantities restricted the extent of growth.

Aerated cultures. Changes with time of the concentrations of cells and volatile acids, and of the pH were determined in cultures initially having different pH values. As with unaerated cultures only formate could be detected in the volatile acid produced during growth and for each culture there was a linear relation between the production of cells and the production of volatile acid (formic). The ratio of cells produced to formate produced, expressed in g./litre/mM, however, varied from 0·028 in cultures initially at pH 7·65 and 7·20, to 0·047 in cultures initially at pH 6·20 (Table III below) compared with a value of 0·015 to 0·017 for all unaerated cultures (Table II).

Further, after the active growth phase in aerated cultures a very slow growth could be detected and the rate of consumption of formate was greater than in unaerated cultures. The higher ratio for aerated cultures can be accounted for by an appreciable rate of consumption of formate during aerated growth and the even higher ratio in the case of the culture initially at pH 6·20 may be due to loss of formic acid in the stream of air. The concentrations of undissociated formic acid at the end of the active growth phase calculated as before, are given in Table III.

TABLE III

CONCENTRATIONS OF FORMATE (ACID PLUS FORMATE IONS) AND UNDISSOCIATED FORMIC
ACID IN AERATED CULTURES AS GROWTH STOPS

Initial pH	Time from inoculation (min.)	pH	Cell concentration (g. dry cells/litre)	[HCOOH] + [HCOO⁻] (mM)	[HCOOH] (mM) calculated	Ratio of concentrations of cells and acid produced (g./m.mol.)
7·65	355 ± 5	5·78 ± 0·02	0·860	31·0	0·29 ± 0·02	0·028
7·20	345 ± 5	5·67 ± 0·03	0·575	20·9	0·26 ± 0·02	0·028
6·20	245 ± 5	5·08 ± 0·05	0·320	6·9	0·32 ± 0·03	0·047

Thus the active growth phase of aerated cultures ends when the un-
dissociated formic acid produced attains a concentration of approximately
0·3 mM.   Adding sufficient formate to media having different pH values
to give an initial concentration of 0·3 mM undissociated formic acid
prevented all growth for 6–12 hr. whereas normal cultures complete their
active growth phase within 4 hr.

DISCUSSION

The production of formate from pyruvate, which was studied with
suspensions of *A. aerogenes,* and the production of cells during growth of
this bacterium, respond in parallel ways to changes of conditions: both
rates increase when the media are aerated, both productions are stopped
by the same critical concentration of undissociated formic acid (0·46N
formate at pH 7·00—i.e., 0·2 mM undissociated acid), and the inhibition
of the pyruvate to formate reaction and mode II inhibition of growth
(Jones and Morrison, 1962) by the two tetracyclines are exhibited in
unaerated media but not in aerated.   This parallelism is emphasised
by the linear relation in growing cultures between the production of cells
and the production of formate which is the main acidic waste product
during growth.   Since pyruvate is known to be an essential intermediate
for the growth of this strain of *A. aerogenes* in the medium used (Dagley,
Dawes and Morrison, 1951), it is probable that the pyruvate to formate
reaction is an essential step in the utilisation of pyruvate for growth, i.e.,
the second product, X, of the reaction $MeCOCOOH + Y \rightleftharpoons HCOOH +$
X, is an essential intermediate for growth.

The reversibility of this proposed reaction accounts for the increased
rate of production of formate in aerated media: if aeration enhances the
rate of consumption of X, the concentration of X when the system is in
its steady state will be smaller, and this must stimulate the consumption
of pyruvate and the production of formate.   Further, since the rate of a
reversible reaction depends on the concentrations of all its products, the
smaller concentration of X in aerated media will permit a larger concen-
tration of undissociated formic acid before the reaction, and hence, growth,
is brought to rest.

Similarly, inhibition of the consumption of X (e.g., by the tetracyclines,
mode II) will cause the system to have a steady state in which the concen-
tration of X is larger and its rate of production, and hence the concomitant

rate of production of formate, smaller. Thus the inhibition of the reaction by the two tetracyclines in unaerated media is indirect, which is in accord with the quantitative results that $1/v$, where v is the rate of consumption of pyruvate, bears a linear relation to mean generation time of the cultures as the concentration of inhibitor is increased but that $1/v$ does not increase rapidly enough with increase of concentration of inhibitor to account for mode II inhibition of growth as a consequence of inhibition of the reaction.

A reaction of this type has been proposed for a coliform organism, an *Escherichia coli*, by Chantrenne and Lipman (1950):

$$\text{Me·CO·COOH} - (\text{Co-enzyme A} - \text{apo-enzyme}) \rightleftharpoons \text{HCOOH} +$$
$$(\text{Me·CO} - \text{enzyme})$$
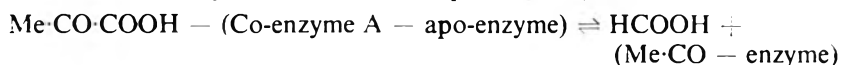
here the enzyme, "formotransacetylase", combines with co-enzyme A and then accepts the acetyl group from pyruvic acid (acetyl-formate). Thus the co-enzyme A - enzyme complex acts as a carrier for acetyl groups. Some years earlier another reaction was proposed for the same organism

$$\text{Me·CO·COOH} + \text{H}_3\text{PO}_1 \rightleftharpoons \text{HCOOH} + \text{Me·CO·OPO}_3\text{H}_2$$
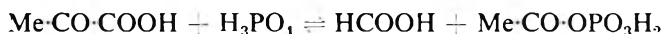
(Utter, Lipman and Werkman, 1945). This reaction followed by hydrolysis of the acetyl-phosphate could be the reaction which intruded at higher concentrations of pyruvate and which produced twice as much volatile acid as pyruvate consumed.

Since both the inhibition of the pyruvate to formate reaction and mode II inhibition of growth are manifested solely in unaerated media, a common primary site of inhibition may affect a hydrogen-transfer reaction which is by-passed in aerated media. The organism is known to utilise the sequence oxalo-acetate $\rightleftharpoons$ malate $\rightleftharpoons$ fumarate $\rightleftharpoons$ succinate and probably to be able to produce succinate from acetate by an aerobic mechanism (Dagley, Dawes and Morrison, 1951). The sequence of reactions can only operate from left to right under reducing conditions and thus fits the requirements for the involvement of a hydrogen-accepting reaction in a utilisation of X in unaerated media. When two chains of reactions join, in this case the hydrogen-transfer chain and the production of the ultimate acceptor of hydrogen, the kinetic consequences of additions of intermediates to the medium cannot be predicted unequivocally since an excess concentration must inhibit one chain whilst overcoming a shortage for the other: none-the-less, the fact that addition of fumarate to the medium decreases the severity of mode II inhibition (Jones and Morrison, 1962) suggests that the primary site of inhibition is in a chain of reactions which produces fumarate from X or is the fumarate to succinate reaction.

The literature provides some support since inhibition by tetracyclines of carbohydrate oxidation at the tricarboxylic acid level and a sensitiveness of acetate oxidation to tetracyclines, have been noted (Eagle and Saz, 1955). The precise identification of the common site of the inhibition of the consumption of pyruvate by suspensions and of mode II inhibition of growth, or establishing that the phenomena are distinct but coincidentally affected by undissociated formic acid and by the two tetracyclines, requires

detailed investigation of the effects of the inhibitors on the isolated reactions. The kinetic studies however, have directed attention to the parts of metabolism which should be so investigated.

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PRESSOR EFFECTS OF ADRENALINE, NORADRENALINE AND REFLEX VASOCONSTRICTION SENSITISED BY LOW CONCENTRATIONS OF GANGLION BLOCKING DRUGS

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In cats, rats and pigeons, concentrations of hexamethonium, tetra-ethylammonium and pentolinium ions too low to influence transmission in autonomic ganglia potentiate the pressor effects of adrenaline, noradrenaline and of reflex vasoconstriction. These effects of the ganglion blocking drugs are not modified by spinalization or by acute adrenalectomy but are absent after pretreatment of an animal with reserpine or of a tissue by postganglionic sympathetic denervation. These concentrations of ganglion blocking drug increase the uptake of noradrenaline by the adrenal medulla, increase the total amine and the proportion of it present as adrenaline in this gland, fail to antagonise adrenal medullary depletion of catecholamine by reserpine and can reverse a pre-existing potentiation of the pressor effects of adrenaline and noradrenaline induced by cocaine.

PAGE and Taylor (1947) first drew attention to potentiation of the pressor actions of adrenaline and noradrenaline by ganglion blocking drugs, an observation confirmed by others (St. Clair and Stone, 1951; Bartorelli, Carpi and Cavalca, 1954). In general, the concentration of ganglion blocking drugs has been sufficient to prevent transmission in autonomic ganglia. Indeed, Bartorelli and others (1954) found no evidence of potentiation in dogs until blocking concentrations of tetraethylammonium were used.

Our interest in this phenomenon was aroused by a chance observation made in the cat. A single intravenous dose of tetraethylammonium ions, sufficient to produce no more than a 50 per cent reduction in the sub-maximal response of a nictitating membrane to preganglionic stimulation of the ipselateral ascending cervical sympathetic chain potentiated the pressor actions of adrenaline or noradrenaline. Moreover, this potentiation persisted for more than an hour after all trace of the ganglion blocking action of the tetraethylammonium ions had disappeared.

METHODS

The male or female wistar rats weighed 300 to 450 g. unless otherwise stated and were killed either by a blow on the head or were anaesthetised by the intraperitoneal injection of 1 ml. 10 per cent urethane in 0·9 per cent sodium chloride per 100 g. body weight, or were made spinal under ether anaesthesia. Records of arterial pressure were taken from a carotid artery and intravenous injections were made through a polythene cannula inserted into an external jugular vein. Cats varied in weight

from 0·75 to 3·2 kg.; anaesthesia was induced with ether and was main-
tained by the intravenous injection of 8·0 ml. 1·0 per cent chloralose in
0·9 per cent sodium chloride per kg. body weight. Records of arterial
pressure were taken either from a carotid or from a femoral artery;
intravenous injections were made through cannulae inserted into femoral
veins. Heparin was used as anticoagulant. Cocaine hydrochloride,
dissolved in 0·9 per cent sodium chloride, was either injected intravenously,
1 mg./kg. or subcutaneously 5 mg./kg. Reserpine, dissolved in 20 per
cent aqueous ascorbic acid, was injected intramuscularly, 5 mg./kg.,
48 and 24 hr. before experiments. Postganglionic denervation of nicti-
tating membranes was effected by aseptic removal of the corresponding
superior cervical ganglia under pentobarbitone anaesthesia 8 to 10 days
before experimental use.

*Measurement of the effect of blocking agents on pressor responses to
adrenaline and noradrenaline.* Three, occasionally two, different doses
either of (−)-adrenaline or of (−)-noradrenaline, submaximal in effect,
were administered to rats in the order of Latin Squares until the resting
blood pressure and responses to individual doses were uniform and had
remained so for more than 30 min. Intravenous injection of a small
quantity of a ganglion blocking drug was then made; the doses selected
were too small to depress the resting arterial pressure and failed to reduce
submaximal effects of injections of nicotine acid tartrate introduced
intravenously. When absence of ganglionic effect had been demonstrated,
the dose effect curve for the catecholamine was redetermined. The
procedure was repeated with increase in the dose of the ganglion blocking
agent until potentiation of the pressor effects of the catecholamine was
well developed. Potentiation was then shown; it was reversible with
time. The procedure in experiments made on cats differed in that the
effects of ganglion blocking agents were examined simultaneously on the
pressor actions of adrenaline and noradrenaline. Absence of ganglion
block was evidenced by failure to reduce the submaximal effects of
preganglionic stimulation of a vagus nerve on the mean arterial pressure
and of stimulation of an ascending cervical sympathetic chain on a
nictitating membrane.

*Measurement of the effect of small repeated doses of ganglion blocking
agents on adrenal medullary amines in rats.* Litter mates of both sexes,
weighing 180–205 g., were evenly distributed, and one animal from each
group began a 10-day period of treatment on each successive day. All
animals were killed on the eleventh day for the preparation of extracts of
adrenal glands from which the amines were separated chromatographically,
eluted and assayed biologically. No significant differences were detected
in the adrenal medullary amines of the two sexes.

*Measurement of the effect of low concentrations of ganglion blocking
agents on the uptake of noradrenaline by the adrenal glands and aortae of
rats.* Litter mates of both sexes were divided evenly between groups to
which separate treatments were assigned for use in subsequent acute

experiments. Thereafter one animal from each group was used daily in a series of experiments made according to a standardised procedure. First. an intramuscular injection was made of a ganglion blocking agent or of saline alone. Immediately afterwards, anaesthesia was induced with urethane, and a polythene cannula was inserted into an external jugular vein. Through this, intravenous injection was made either of saline or of saline containing a blocking agent. This was at once followed by the continuous infusion of 80 μg. noradrenaline in 2 ml. 0·9 per cent NaCl at constant rate over 40 min. The adrenals were removed for the preparation of extracts 5 min. after the infusion ended. Amines were separated chromatographically from these extracts and were assayed biologically.

Extracts of adrenal glands. Adrenal glands were carefully cleaned of fatty tissue immediately after removal and were stored in the refrigerator at 0° C. for 2 hr. before weighing and grinding in 0·1N HCl with silver sand. The extract and the mortar washings were combined, heated in a boiling water bath for 2 min., cooled and centrifuged. Thereafter the chromatographic separation of adrenaline and noradrenaline was effected, and the amines were eluted and prepared for biological assay as described by Lockett (1954). Assays of adrenaline were made on the rat uterus or colon (Gaddum, Peart and Vogt, 1949) and of noradrenaline on the rat colon or on the mean arterial pressure of pithed rats.

RESULTS

Potentiation of the pressor effects of adrenaline or noradrenaline and of reflex activity in the sympathetic nervous system. Experiments on rats, pigeons and cats have shown that concentrations of three different ganglion blocking agents, too small to influence transmission in autonomic ganglia, increase the pressor effects of intravenous adrenaline and noradrenaline and of reflex activity in vasoconstrictor fibres of the sympathetic nervous system. The ganglion blocking agents studied were tetraethylammonium, hexamethonium and pentolinium ions. The criteria accepted as evidence of absence of ganglion action were unchanged submaximal vascular responses to nicotine in the rat and the pigeon and unchanged submaximal effects of preganglionic stimulation of the peripheral end of a divided vagus on arterial pressure and of the ascending cervical sympathetic on the nictitating membrane in the cat. The increased pressor effects of fixed doses of adrenaline and of noradrenaline induced by a concentration of tetraethylammonium ions too small to influence transmission in autonomic ganglia (Fig. 1) are of variable duration; in general, it may be expected to last from 40 min. up to several hours in cats, from 20 to 60 min in rats and from 10 to 25 min. in pigeons: it was always reversible. The increase in the pressor effects of intravenous adrenaline and noradrenaline was accompanied by increase in the pressor component of the carotid mechanoreceptor reflex (Fig. 2). The sensitisation was induced with equal ease in anaesthetised animals

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(Figs. 1 and 2) and in those made spinal, vagotomised and acutely adrenalectomised (Fig. 3). An analysis of the changes occurring in the log dose effect curves for the pressor actions of adrenaline and noradrenaline under the influence of sub-blocking doses of tetraethylammonium and of hexamethonium ions was made in rats under urethane
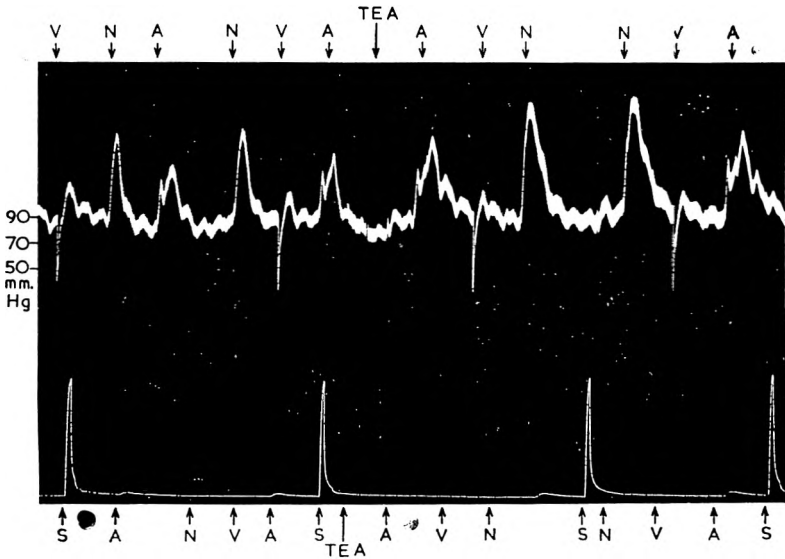

Fig. 1. Low concentrations of a ganglion blocking drug potentiate pressor effects of adrenaline and noradrenaline. Records of mean arterial pressure (above) and the contractions of a nictitating membrane (below), taken from a cat 2·2 kg. under chloralose anaesthesia. Intravenous injections: A, 3 μg. (−)-adrenaline; N, 1 μg. (−)-noradrenaline; TEA, tetraethylammonium chloride, 1 mg./kg. V signifies rectangular pulses to R. peripheral vagus, 250 μsec., 20/sec. 4 V. S, rectangular pulses to ascending cervical sympathetic, 250 μsec., 5/sec. 2 V. for 30 sec. in each case.
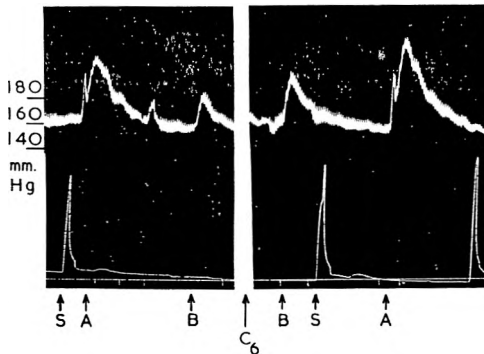
Fig. 2. Low concentrations of a ganglion blocking drug potentiate reflex vasoconstriction. Records of mean arterial pressure and of contractions of a nictitating membrane from a cat, 2·3 kg. under chloralose anaesthesia. Intravenous injections: A, 2·5 μg. (−)-adrenaline; C_6, hexamethonium bromide, 1 mg./kg. Other procedures: B, occlusion of the L. carotid for 30 sec., S as in Fig. 1.
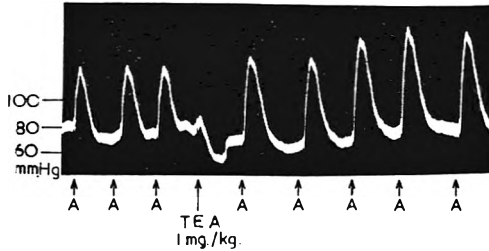
FIG. 3. Low concentrations of a ganglion drug potentiate the pressor effects of adrenaline in a spinal, vagotomized and acutely adrenalectomised cat, 1·9 kg., responding to i.v. injection of 4 μg. (−)-adrenaline at intervals of 4 min. TEA as in Fig. 1.

anaesthesia. The results of these experiments are summarised in Table I. In every experiment the dose-effect curve for the catecholamine shifted to the left without change in its slope.

TABLE I

Concentrations of hexamethonium and tetraethylammonium ions too low to depress activity in autonomic ganglia, shift the log-dose effect curves for the pressor actions of adrenaline and noradrenaline in rats under urethane anaesthesia to the left, but do not influence their slope

| | Log-dose effect curves | | | |
| | Adrenaline | | Noradrenaline | |
Ganglion blocking drug	Value of b	mm.Hg rise, 0·4 μg.	Value of b	mm.Hg rise, 0·4 μg.
Tetraethylammonium μg./kg. i.v.				
0	35·7 ± 1·08	28·7 ± 1·65	37·7 ± 2·77	29·0 ± 1·53
125	37·0 ± 1·41	34·0 ± 1·00*	36·3 ± 3·37	34·3 ± 1·54*
250	41·0 ± 3·22	39·3 ± 2·01**	41·0 ± 2·18	38·7 ± 1·83**
500	37·0 ± 2·52	44·3 ± 0·91**	41·0 ± 2·05	43·3 ± 2·83**
Hexamethonium μg./kg. i.v.				
0	36·5 ± 3·48	13·0 ± 1·23		
200	36·0 ± 4·01	20·2 ± 2·24**		
400	37·5 ± 5·49	25·4 ± 20**		

Three rats per group. Significance of effective alteration in a mean as a result of the blocking agent was tested by 't' test, and is shown by asterisk; one, P = 0·05, two, P = <0·01.

How do low concentrations of ganglion blocking drugs increase the pressor effects of intravenous adrenaline and noradrenaline? Sensitisation to the pressor effects of adrenaline and of noradrenaline may follow from changes in the tissue chromaffin stores, from inhibition of enzyme systems capable of metabolising these catecholamines or from an increase in the efficiency of the response mechanisms. Fig. 4 shows that whereas a partially blocking concentration of tetraethylammonium caused an increase in the effects of adrenaline on mean arterial pressure almost immediately, sensitisation of the normal nictitating membrane was more gradual; there was no sensitisation in the denervated membrane. Whereas tetraethylammonium, in low concentration, readily potentiates the pressor effects of adrenaline and noradrenaline in spinal rats, it fails to do so if the tissue chromaffin stores have been depleted by pretreatment with reserpine (Fig. 5).
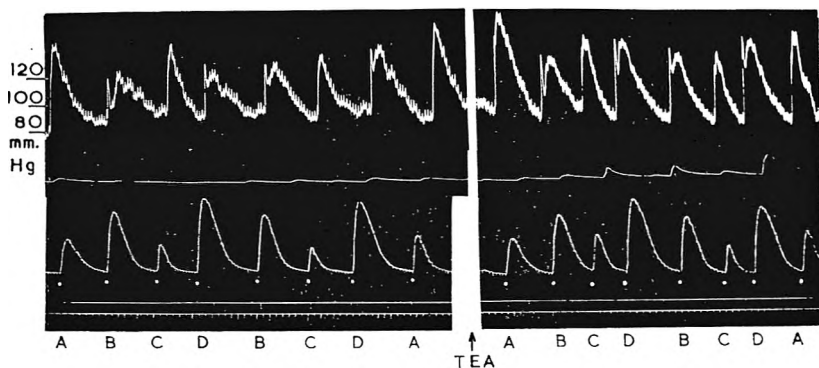
FIG. 4. Low concentrations of a ganglion blocking drug potentiate the effects of adrenaline on a normal but not a denervated nictitating membrane. Spinal cat, 3·2 kg. Records, from above, downward: mean arterial pressure, normal nictitating membrane, membrane 10 days after postganglionic denervation. Intravenous injections: A, 2 μg. and C, 1 μg. (−)-noradrenaline; B, 2 μg. and D, 4 μg. (−)-adrenaline. TEA as in Fig. 1 between the two sets of tracings, in 3 min. interval.
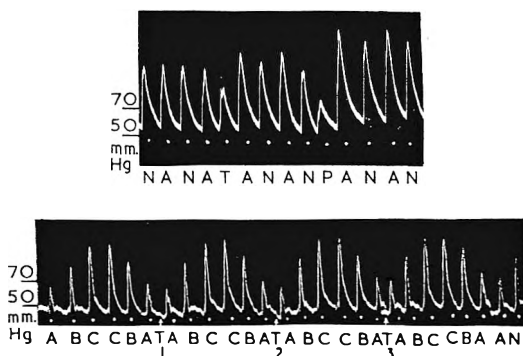

FIG. 5. Absence of potentiation in reserpine-treated animals. Record of mean arterial pressure from a spinal rat, 450 g. above, and a spinal reserpine-treated rat 490 g., below. Injections, i.v. (above), N, 50 ng. (−)-noradrenaline; A, 200 ng. (−)-adrenaline; T, TEA 100 μg./kg.; P, pyrogallol, 25 mg./kg. (below), A, B and C, 40, 80 and 160 ng. (−)-noradrenaline respectively. T_1, T_2 and T_3, 50, 100 and 200 μg. TEA respectively.

Potentiation of the pressor effects of adrenaline and noradrenaline by cocaine, 1 mg./kg., i.v., or 5 mg./kg. subcut., lasts for more than an hour in spinal rats; it is, however, rapidly reversed shortly after its onset by concentrations of these ganglion blocking drugs too small to influence transmission in autonomic ganglia (Fig. 6). Whereas pyrogallol, 25 mg./kg. given intravenously further increases sensitisation to the pressor effects of adrenaline and noradrenaline when this has been caused by low concentrations of ganglion blocking drug, these same concentrations of the blocking drugs fail to augment the pressor effects of the catecholamines in the presence of a pre-existing submaximal potentiation produced by pyrogallol (Fig. 7).
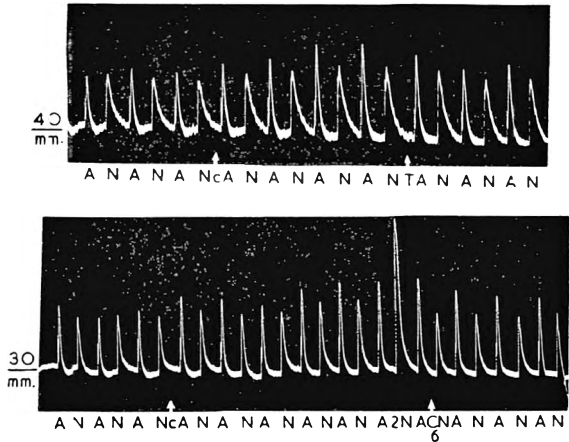
FIG. 6. Reversal of cocaine potentiation of pressor responses to adrenaline and noradrenaline by low concentrations of ganglion blocking drugs. Records of mean arterial pressure from two spinal rats, 400 and 350 g. responding to the following intravenous injections : A, (−)-adrenaline, 100 ng. N, (−)-noradrenaline, 50 ng. Cocaine administered subcutaneously 5 mg./kg. TEA and C_6 as in Figs. 1 and 2 respectively.
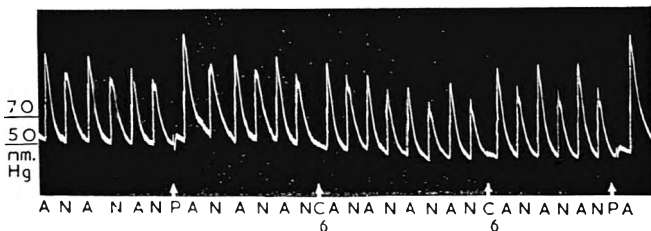

FIG. 7. Interactions between potentiations caused by pyrogallol and low concentrations of ganglion drugs in pressor responses to adrenaline and noradrenaline. Record of the mean arterial pressure of a spinal rat 450 g. responding to the following intravenous injections: A, 0·1 μg. (−)-adrenaline, N, 0·05 μg. (−)-noradrenaline; P. pyrogallol, 25 mg./kg. C_6 as in Fig. 2.

Concentrations of these ganglion blocking drugs too small to influence transmission in autonomic ganglia, which increased pressor responses to adrenaline and noradrenaline, failed to alter pressor responses to tyramine (Fig. 8).
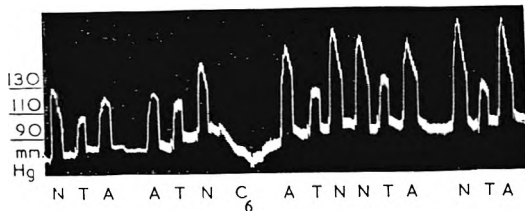

FIG. 8. Low concentrations of a ganglion blocking drug fail to potentiate the pressor effects of tyramine. Record of the mean arterial pressure of a cat, 1·5 kg., under chloralose anaesthesia. Intravenous injections: A, 3 μg. (−)-noradrenaline; T, 100 μg. tyramine hydrochloride. C_6 as in Fig. 2.

TABLE II

THE EFFECT OF TETRAETHYLAMMONIUM IONS, 1 OR 10 MG./KG. AND OF ATROPINE SULPHATE 5 MG./KG. GIVEN SUBCUTANEOUSLY TWICE DAILY FOR 10 DAYS, AND OF COCAINE HYDROCHLORIDE 1 MG./KG. FOR 2 DAYS, ON THE ADRENAL MEDULLARY HORMONES OF RATS AND CATS

Treatment	Weights		µg. Amine per mg. adrenal gland				Number of animals
	Body, g.	Adrenal, mg.	Noradrenaline	Adrenaline	Total pressor amine	Adrenaline noradrenaline	
Rats							
None	203 ± 4·5	45 ± 3·4	0·15 ± 0·020	0·65 ± 0·065	0·80 ± 0·671	4·4 ± 0·64	8
Hexamethonium, 1 mg./kg.	186 ± 6·6	44 ± 2·1	0·07 ± 0·008*	1·16 ± 0·106*	1·25 ± 0·081*	17·7 · 0·04**	8
Tetraethylammonium, 1 mg./kg.	200 ± 5·0	43 ± 3·0	0·09 ± 0·011*	1·28 ± 0·141*	1·36 ± 0·143*	16·8 ± 0·31**	8
None	188 ± 2·1	45 ± 3·0	0·14 ± 0·015	0·56 ± 0·061	0·70 ± 0·063	4·3 ± 0·87	8
Hexamethonium, 10 mg./kg.	196 ± 4·6	49 ± 3·7	0·09 ± 0·019*	1·03 ± 0·130*	1·12 ± 0·122*	14·8 ± 0·33**	8
Tetraethylammonium, 10 mg./kg.	197 ± 1·7	49 ± 4·4	0·08 ± 0·013*	0·085 ± 0·118	0·95 ± 0·105	16·0 ± 0·40**	8
None	273 ± 4·5	53 ± 6·4	0·13 ± 0·015	0·67 ± 0·339	0·80 ± 0·040	7·2 ± 4·4	4
Cocaine-HCl, 1 mg./kg.	273 ± 4·2	48 ± 5·1	0·20 ± 0·010*	1·21 ± 0·108**	1·41 ± 0·061*	8·1 ± 3·7	4
None	284 ± 6·5	43 ± 4·4	0·07 ± 0·093	0·34 ± 0·049	0·41 ± 0·047	4·9 ± 1·18	8
Atropine, 5 mg./kg.	296 ± 6·1	47 ± 2·1	0·07 ± 0·007	0·30 ± 0·033	0·37 ± 0·033	4·3 ± 0·85	8
Cats	kg.						
None	1·3 ± 0·16		0·15 ± 0·03	0·32 ± 0·09	0·47 ± 0·08	2·6 ± 0·01	4
Hexamethonium, 1 mg./kg.	1·2 ± 0·02		0·07 ± 0·02*	0·62 ± 0·18	0·69 ± 0·02*	10·4 ± 3·14**	4

Significance of differences between means was examined by 't' test and is indicated by asterisks: one, $P < 0·05$; two, $P < 0·01$.

TABLE III

THE EFFECT OF GANGLION BLOCKING DRUGS AND COCAINE ON THE DEPLETION OF STORES OF ADRENALINE AND NORADRENALINE IN THE RAT ADRENAL MEDULLA BY RESERPINE 5 MG. PER KG. DAILY FOR 2 DAYS

Ganglion blocking agent		μg. Catecholamine per mg. adrenal gland				Rats		
Drug	mg./kg. 12 hrly subcut. for 2 days	Noradrenaline	Adrenaline	Total	Adrenaline noradrenaline	Body wt. g	Adrenal wt. mg.	No.
None		0.02 ± 0.003	0.09 ± 0.008	0.12 ± 0.012	6.2 ± 1.24	390 ± 5.8	45 ± 2.8	16
Tetraethylammonium	1.0	0.05 ± 0.006*	0.10 ± 0.025	0.13 ± 0.025	2.6 ± 0.28*	420 ± 7.9	45 ± 1.8	8
...	5.0	0.04 ± 0.012*	0.11 ± 0.013	0.15 ± 0.010	4.5 ± 0.14	421 ± 9.0	54 ± 4.7	6
Hexamethonium	1.0	0.03 ± 0.005	0.10 ± 0.009	0.19 ± 0.047	1.1 ± 0.64	399 ± 4.5	43 ± 2.6	8
...	5.0	0.04 ± 0.012*	0.13 ± 0.020	0.17 ± 0.020	6.3 ± 0.51	397 ± 7.8	52 ± 5.2	6
None		0.008 ± 0.001	0.08 ± 0.005	0.09 ± 0.018	7.2 ± 4.4	274 ± 5.2	53 ± 5.5	4
Cocaine hydrochloride	1.25	0.095 ± 0.014*	0.325 ± 0.078*	0.42 ± 0.087*	8.1 ± 3.7	273 ± 4.7	53 ± 4.6	4

The significance of differences in the action of reserpine caused by the presence of a second drug is indicated by an asterisk where P = 0.05.

TABLE IV

THE EFFECT OF TETRAETHYLAMMONIUM AND HEXAMETHONIUM IONS ON THE UPTAKE OF NORADRENALINE BY THE ADRENAL GLANDS OF NORMAL AND RESERPINISED RATS

Pretreatment		Infusion	Weight		μg. Amine per mg. adrenal gland			Adrenaline noradrenaline	No. of rats
Drug	mg./kg. both i.m. and i.v.		Body g.	Adrenals mg.	Noradrenaline	Adrenaline	Total		
Normal rats									
None	nil	noradrenaline	295 ± 5.6	58 ± 3.7	0.14 ± 0.023††	0.52 ± 0.045	0.67 ± 0.054††	4.8 ± 1.23	8
None	nil	0.9% NaCl	297 ± 11.6	62 ± 5.1	0.06 ± 0.006	0.51 ± 0.044	0.57 ± 0.043	5.0 ± 1.11	8
Tetraethylammonium	1.0	noradrenaline	297 ± 5.4	63 ± 5.2	0.74 ± 0.052*	1.45 ± 0.413*	1.91 ± 0.039*	6.3 ± 1.18	8
...	10.0	noradrenaline	259 ± 5.0	66 ± 4.5	0.36 ± 0.081*	2.00 ± 0.473**	2.25 ± 0.501	6.5 ± 2.89	8
Hexamethonium	10.0	noradrenaline	282 ± 7.4	68 ± 4.8	0.07 ± 0.004	0.47 ± 0.044	0.58 ± 0.040	8.3 ± 2.23	8
...	1.0	noradrenaline	263 ± 6.7	64 ± 4.2	0.35 ± 0.056*	1.61 ± 0.438*	1.98 ± 0.375*	3.6 ± 0.51	8
...	10.0	0.9% NaCl	259 ± 5.0	68 ± 3.1	0.24 ± 0.036*	1.38 ± 0.262**	1.62 ± 0.258**	3.8 ± 0.85	8
Reserpinised rats									
None	nil	noradrenaline	273 ± 3.7	72 ± 4.7	0.047 ± 0.018†	0.05 ± 0.005	0.10 ± 0.011†	1.0 ± 0.06††	6
None	nil	0.9% NaCl	270 ± 3.9	71 ± 3.5	0.019 ± 0.008	0.06 ± 0.011	0.07 ± 0.017	3.5 ± 0.17	6
Tetraethylammonium	1.0	noradrenaline	273 ± 3.5	73 ± 5.6	0.061 ± 0.023*	0.08 ± 0.033	0.14 ± 0.010*	1.4 ± 0.28*	6
Hexamethonium	1.0	noradrenaline	272 ± 2.3	68 ± 2.7	0.063 ± 0.021*	0.08 ± 0.081	0.14 ± 0.013*	1.4 ± 0.22*	6

Significance of differences between means was examined by '*t*' test and is shown by crosses for the effect of infusions of noradrenaline alone, and by asterisks for the effect of a ganglion blocking drug:—one, P = 0.05; two, P = <0.01.

The influence of ganglion blocking agents on the adrenal medullary amines of rats and cats. Subcutaneous injections of as little as 1 mg. per kg. of either hexamethonium or tetraethylammonium ions, given twelve hourly for 10 days, increase the total stores of pressor amine and the proportion present as adrenaline and decrease of noradrenaline in the adrenal medullae of rats and cats (Table II). These same subcutaneous doses of the ganglion blocking drugs fail effectively to antagonise depletion of the chromaffin stores in the adrenal medulla by reserpine, as, by contrast, cocaine does (Table III). Given intramuscularly these small amounts of blocking drug augment the rate of uptake of noradrenaline and its conversion to adrenaline by the adrenals of normal and reserpine treated animals (Table IV).

DISCUSSION

Bartorelli, Carpi and Cavalca (1954) were unable to demonstrate potentiation of the pressor effects of adrenaline and noradrenaline by concentrations of tetraethylammonium ions that failed to block transmission in autonomic ganglia. Their work was done in dogs. By contrast, in rats, cats and pigeons (Figs. 1 to 8) concentrations of hexamethonium, tetraethylammonium and pentolinium ions too low to influence transmission in autonomic ganglia, increased the pressor effects of intravenous adrenaline and noradrenaline and of reflex vasoconstriction. It is probable that changes in the chromaffin stores in tissues are concerned with this sensitisation since low concentrations of ganglion blocking drugs do not increase the pressor effects of adrenaline and noradrenaline when the chromaffin stores have degenerated after postganglionic sympathectomy (Fig. 4 and Schmitterlow, 1948) or have been depleted by reserpine (Fig. 5 and Burn and Rand, 1957). Burn and Rand have suggested that cocaine potentiates the responses to adrenaline and noradrenaline by "sealing" the chromaffin stores and that the effect of the "sealed stores" is equivalent to the disappearance of chromaffin tissue after postganglionic denervation: cocaine certainly makes tissue stores more resistant to depletion by reserpine (Table III), and probably hinders the rate of release of catecholamine from the adrenal gland (Table II). But, the ganglion blocking drugs studied also sensitise to the pressor effects of adrenaline and noradrenaline by an action on the tissue stores but they do not "seal" these stores. Indeed, they increase the rate of uptake of noradrenaline by the adrenal gland and accelerate the conversion of noradrenaline to adrenaline in some cells (Tables IV and II, and Eränkö, 1955). Since low concentrations of these ganglion blocking drugs also antagonise a pre-existing sensitisation by cocaine (Fig. 6) to the pressor affects of adrenaline and noradrenaline, both drugs may share a common site of action, not necessarily in the tissue stores. One possibility is that both cocaine and the ganglion blocking drugs inhibit the *o*-methyl transferase (Fig. 7). The resulting potentiation of the pressor effects of adrenaline and noradrenaline would then sum with the action of cocaine on tissue stores and mask the opposing action of

the ganglion blocking drugs at the same site, if the hypothesis concerning the "sealing of stores," advanced by Burn and Rand, holds.

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IN VITRO AND IN VIVO PROPERTIES OF ALOXIPRIN: A NEW ALUMINIUM DERIVATIVE OF ACETYLSALICYLIC ACID

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The rates of solution of aspirin from aloxiprin, an aluminium derivative of acetylsalicyclic acid, in buffer solutions of pH 2–8 were found to be lower than those of aspirin B.P., particularly in the more acid buffers.

The excretion of salicylate in the urine of 11 human subjects who received aloxiprin was more delayed than that from a corresponding dose of aspirin B.P. Little difference was found in the total amount of salicylate excreted in 24 hr.

THE name Aloxiprin has recently been selected as the Approved Name to describe a "polymeric condensation product of aluminium oxide and aspirin." This new compound results from the interaction of aluminium isopropoxide and aspirin, and corresponds approximately to the formula $Al_3O_2(C_6H_4(O \cdot OCMe)-COO)_5$.

Aloxiprin has recently been the subject of favourable clinical comment (Wheatley, 1962, and Wood, Harvey-Smith, and Dixon, 1962).

The literature provides many references to various compounds under the general designation, aluminium acetylsalicylate. These compounds have been claimed to approximate in composition to one or other of the structures I, II or III (French Patent 1951, 734,754; U.S. Patent 1951, 2,698,332; 1955, 2,918,485; German Patent 1958, 1,076,703):
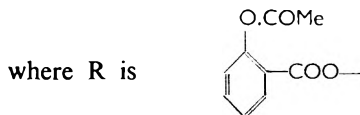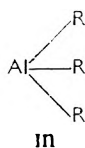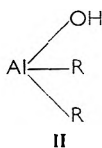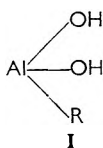
Aluminium acetylsalicylate acid is the subject of a monograph in the National Formulary, U.S.A., and structure II is assigned to this compound Aluminium aspirin (N.F.) possesses a high free salicylic acid content ("not more than 7·5 per cent"), a feature shared by some and probably all other aluminium acetylsalicylates which are prepared in aqueous solution. Such compounds have also generally been observed to be powders of low density which are not readily amenable to the pharmaceutical formulation of acceptable dosage forms.

Aloxiprin differs from other aluminium acetylsalicylates hitherto described. It is characterised by a relatively low free salicylic acid content and also by the fact that the powder has a much greater bulk density.

Before aloxiprin was used clinically, it was considered desirable to examine the *in vitro* and the *in vivo* rate or release of aspirin from this compound and also to seek information of the availability of its salicylate content after oral administration.

In the present investigation, the rate of solution of aspirin from aloxiprin in buffer solutions of pH 2-8 at 37° has been compared with that of aspirin B.P. and the rate of excretion of salicylate in human subjects has been estimated after the oral administration of equivalent doses of aloxiprin and aspirin B.P.

EXPERIMENTAL

Materials

Aloxiprin. The material prepared as reported above, was used for the determination of the solution rates after being screened through a No. 120 B.S.S. sieve. This material complied with the specification: total acetylsalicylic acid, not less than 76 per cent; free acetylsalicylic acid, not more than 2·5 per cent; free salicylic acid, not more than 0·4 per cent; aluminium, not less than 7·2 per cent; bulk density, 205–215 ml./100 g.

Tablets containing 400 mg. ($\pm$20 mg.) aloxiprin equivalent to 5 grains of aspirin, were used for the excretion studies.

Aspirin. Aspirin B.P. powder passing a No. 120 B.S.S. sieve and aspirin tablets B.P. were used in the corresponding investigations.

Buffer solutions. The buffer solutions were prepared by mixing the following in the proportions given and adjusting to the required pH measured with a glass electrode. A, 0·1M disodium hydrogen citrate; B, 0·1M hydrochloric acid; C, 0·2M disodium hydrogen phosphate; D, 0·1M citric acid.

Buffer pH 2, 3·0 vol. A with 7·0 vol. B; pH 4, 5·5 vol. A with 4·5 vol. B; pH 6, 6·3 vol. C with 3·7 vol. D; pH 8, 9·7 vol. C with 0·3 vol. D.

METHODS

The Rate of Solution of Aspirin B.P. and of the Aspirin from Aloxiprin at 37° in Buffer Solutions of pH 2, 4, 6 and 8, measured as Total Salicylate

The aloxiprin powder (0·5 g.) was weighed directly into each of six plastic bottles of approximately 100 ml. capacity. Buffer solution (100 ml.) at 37° was added to each and after a brief shaking, the bottles were mechanically rotated in a water bath at 37°. Each bottle contained a piece of glass rod to facilitate mixing.

One bottle was removed from the water bath after 15, 30, 45, 60, 90 and 120 min., timed from the moment of the addition of the buffer solution. The solution was rapidly filtered and an aliquot of the filtrate diluted with 4 parts of water. The total salicylate content was determined by adding N NaOH (1 ml.) to the diluted filtrate (1 ml.) in a stoppered tube, heating in a boiling water bath for 30 min., cooling,

acidifying with concentrated HCl and following the procedure described by Brodie, Udenfriend, and Coburn (1944).

The rate of solution of aspirin B.P. (0·4 g.) was measured under identical conditions, except that it was found unnecessary to continue the incubation for more than 30 min.

The Rates of Salicylate Excretion after the Oral Administration of 650 mg. of Aspirin B.P. and the Equivalent of Aloxiprin

Eleven healthy male volunteers took part. At 9 a.m. urine was voided and discarded. At 9.30 a.m. each subject took two aspirin tablets B.P. with 100 ml. of water, and urine collections were made after 0, 0·5, 1, 2, 3, 5 and 7 hr. The urine samples collected at each time interval were pooled, the total volume measured and an aliquot was analysed for total salicylate.

The urine (4 ml.) was hydrolysed by boiling with 12N H_2SO_4 (2 ml.) under a reflux condenser for 3 hr. and the hydrolysate diluted to 25 ml. with water. This solution (2–4 ml.) was used for the determination of the "total salicylate" by the method of Brodie and others (1944).

One week later the same procedure was followed with the same 11 individuals except that each received two aloxiprin tablets.

In a separate study the salicylate excretion was followed for 24 hr. after the oral administration of 1 g. of aspirin B.P. and an equivalent dose of aloxiprin to 5 individuals. No restrictions were placed on the diet or fluid intake.

<div align="center">RESULTS</div>

Solution Rate Studies

The rates at which aspirin dissolved from aloxiprin in buffer solutions of pH 2, 4, 6 and 8 respectively at 37°, and the corresponding results obtained with aspirin B.P., are given in Fig. 1.
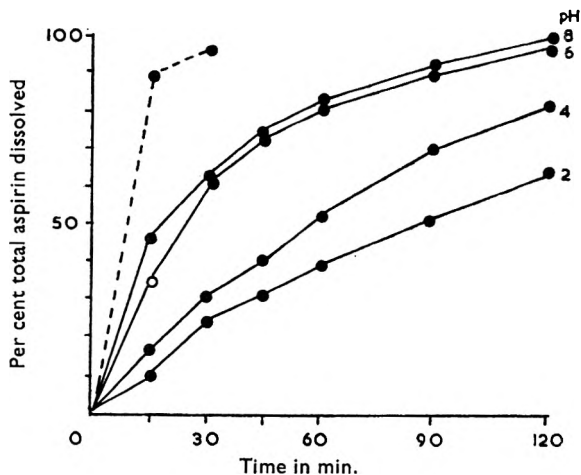

FIG. 1. The rate of solution of aspirin from ●—● aloxiprin (0·5 g.) and ● – – ● aspirin B.P. in 100 ml. of buffer solutions.

The aspirin of aloxiprin dissolves at an appreciably slower rate than aspirin B.P. in all the buffer solutions used. The solution rate of aspirin from aloxiprin increased with increasing pH, so that during the first 60 min. 2–4 times as much aspirin dissolved at pH 8 as at pH 2, whilst changes in pH had little effect upon the solution rate of aspirin powder, under the experimental conditions used.

Excretion Rate Studies

The total amounts of salicylate found in the pooled urines of 11 individuals after the ingestion of 0·65 g. aspirin B.P. and an equivalent dose of aloxiprin are given in Table I and Fig. 2. The results show that less

TABLE I

THE MEAN SALICYLATE EXCRETION AFTER THE ORAL ADMINISTRATION OF ASPIRIN B.P.
AND ALOXIPRIN TO ELEVEN SUBJECTS (DOSE ≡ 0·648 MG. ASPIRIN)

| Period of urine collection hr. | Salicylate excreted* | | | | | | Ratio A — B |
| | Aloxiprin | | | Aspirin B.P. | | | |
	Amount mg. (A)	Cumulative dose per cent	Excretion rate mg./30 min.	Amount mg. (B)	Cumulative dose per cent	Excretion rate mg./30 min.	
0·5	0·6	0·12	0·6	5·0	1·0	5·0	0·12
1	5·0	1·1	5·0	15·5	4·2	15·5	0·32
2	25·0	6·2	12·5	43·0	12·8	21·5	0·58
3	37·5	13·9	18·8	42·0	21·2	21·0	0·89
5	77·0	29·6	19·3	77·0	36·8	19·3	1·00
7	74·5	44·8	18·6	74·0	51·7	18·5	1·01

* Expressed as salicylic acid.
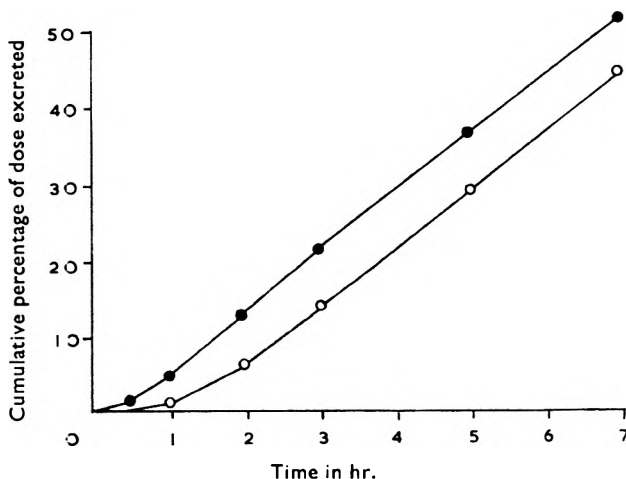
FIG. 2. The mean salicylate excretion after oral administration of 0·65 g. aspirin B.P. (●—●) and the equivalent of aloxiprin (○—○) to 11 subjects.

salicylate is excreted during the first 3 hr. after the administration of aloxiprin than after aspirin B.P., but thereafter the rates of excretion are similar (Fig. 3).
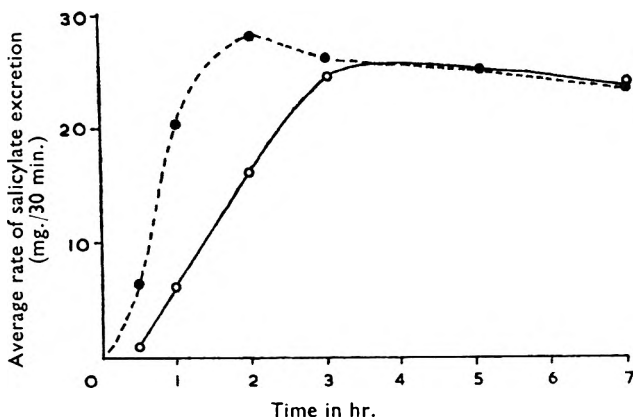
FIG. 3. The average rate of salicylate excretion after the oral administration of 0·65 g. aspirin B.P. (● – – ●) and the equivalent of aloxiprin (○—○) to 11 subjects.

The results obtained by following the salicylate excretion for 24 hr. after the ingestion of 1 g. of aspirin B.P., or the equivalent of aloxiprin in 5 subjects, are given in Table II.

TABLE II

THE SALICYLATE EXCRETION IN 24 HR. AFTER AN ORAL DOSE* OF ASPIRIN B.P. AND ALOXIPRIN IN 5 SUBJECTS

	Salicylate excreted‡			
	Aloxiprin		Aspirin B.P.	
	Total mg.	Dose per cent	Total mg.	Dose per cent
A	603	81·7	598	80·2
B	650	88·5	595	80·0
C	556	75·5	595	80·0
D	552	75·0	660	88·5
E	603	81·7	—	—
Mean	593	80·5	612	81·2

* Dose administered: Aspirin B.P. = 972 mg. (50 mg.); Aloxiprin = 960 mg. (50 mg.) Aspirin.
‡ Expressed as salicylic acid.

Thus, while the salicylate excretion after the administration of aloxiprin is initially lower than that after aspirin B.P. there is little difference in the total amount of salicylate excreted in 24 hr.

DISCUSSION

The results in Table I show that comparable quantities of the two salicylate preparations were excreted at different time intervals. Amounts of total salicylate in the urine corresponding to approximately 1, 5 and 13 per cent of the ingested doses were excreted during periods of 30 min., 1 hr. and 2 hr. after the oral administration of aspirin and during 1, 2 and 3 hr. after aloxiprin. Urinary excretion of salicylate after aloxiprin

therefore shows a time lag of between 30 min. and 1 hr. This lag in excretion could be due to comparatively little of the aspirin of aloxiprin being absorbed whilst it remains in the acid environment of the stomach.

This supposition is supported by the results of the solution rate investigations (Fig. 1) which show that the aspirin of aloxiprin dissolves appreciably more slowly in acid solution (pH 2) than aspirin B.P. and thus would be expected to dissolve less readily in gastric fluid. It has been previously reported that the rate of absorption of aspirin from the gastrointestinal tract is limited by its rate of solution (Edwards (1951), Nelson and Schaldemose (1959), Levy (1961), Levy, Gumtow, and Rutowski (1961)).

The results further suggest that in the intestinal tract, aspirin is absorbed at comparable rates from the two formulations. If this were not so, the plots of the respective amounts of salicylate excreted against time (Fig. 2) would give diverging lines; in fact, two parallel lines are obtained, which are separated by an interval corresponding to approximately 45 min.

It has been demonstrated (Table II) that the total amount of salicylate excreted over 24 hr. from aspirin and from aloxiprin does not differ appreciably, thus indicating that the whole of the aspirin of aloxiprin becomes available.

Thus, aloxiprin may provide a form of aspirin suitable for clinical conditions recuiring the prolonged administration of relatively large doses of aspirin. Since it would seem that, as the aspirin of aloxiprin is relatively slowly released in the stomach, this compound could well cause fewer gastric disturbances than aspirin B.P., a view which is supported by the recent work of Wood and others (1962).

The excretion rates of the two preparations have been compared on the basis of the salicylate content of the pooled urines from a number of subjects. In view of the fact that a cross-over design was employed and that information of variation between subjects was not specifically required, analysis of the pooled urines, rather than of individual urines, was considered adequate. A consequence of this procedure is that the excretion characteristics revealed by the mean of several individuals, may well exhibit a pattern which is quite atypical of that for any individual. If, for example, the subjects show peak levels at different times, then the peak of the mean will be lower than that of most of the individuals and will be sustained over a longer period, so as to show a plateau effect as an artefact. The shape of the salicylate excretion curve as plotted from the mean values will not therefore necessarily portray the excretion pattern normally encountered in an individual.

Levy and Sahli (1962) have recently compared the gastrointestinal absorption of aluminium aspirin (National Formulary) and of aspirin (U.S.P.). They reported that the total apparent salicylate excretion from aluminium aspirin (N.F.) is markedly less than that from aspirin (U.S.P.), and concluded that the aluminium aspirin in the form which they used, was incompletely absorbed.

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A NERVOUSLY-MEDIATED ACTION OF ANGIOTENSIN IN ANAESTHETISED RATS

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In rats under chloralose anaesthesia, angiotensin in large doses caused a nervously-mediated vasoconstriction in the vascularly-isolated innervated hind limb, whereas in small doses it caused a nervously mediated peripheral vasodilatation. Noradrenaline over an equipressor dose range caused only a nervously-mediated peripheral vasodilatation. These peripheral responses were abolished by section of the nerves leading to the hind limb.

ANGIOTENSIN, a pressor polypeptide formed *in vivo* by the action of renin on plasma globulins, has been prepared in a pure form (Rittel, Iselin, Kappeler, Riniker and Schwyzer, 1957), resulting in renewed interest in its pharmacology, Experimental studies using the biologically prepared and, more recently, synthetic material have elucidated many of its reactions (Braun-Menendez, 1956; Page and Bumpus, 1961).

During a comparative study of the actions of synthetic and biologically prepared angiotensin and noradrenaline on rats (Laverty, 1960) it was found that large doses of angiotensin caused a nervously-mediated vaso-constriction in peripheral blood vessels. This observation, here investigated further, suggests some modification of the usual view that "angiotensin has a strong peripheral vasoconstrictor action and no action on the central nervous system" (Page and Bumpus, 1961). A comparable effect of angiotensin on the central nervous system of dogs has also been observed recently (Bickerton and Buckley, 1961).

METHODS

The hind limb of a rat (300–380 g.) anaesthetised with chloralose (60 mg./kg.) was isolated from the circulation of the remainder of the animal at the level of the inguinal ligament by cutting the muscular tissue, leaving only the femur, the femoral and sciatic nerves and the femoral vein intact (Field and Laverty, 1958). This isolated innervated hind limb was then perfused through the femoral artery, with blood taken from the opposite femoral artery, by means of a constant output pump (Field, de Graaf and Wallis, 1958). In the experiments investigating the nervously-mediated effects of drugs given to the remainder of the animal, on the hind-limb peripheral resistance, a delay coil of 6 ml. volume was interposed between the animal and the pump, to separate the neurogenic effects from direct effects of the blood-borne drug on the hind-limb blood vessels.

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In some experiments the isolated innervated hind limb was perfused with blood taken from a separate donor rat (Field and Laverty, 1958) instead of from the animal that supplied the hind limb. This ensured that drugs given to the remainder of the recipient animal could only exert an action on the blood vessels of the isolated hind limb through the nervous system.

The trachea and jugular vein of the recipient rat were cannulated in all experiments. The drugs being tested were given intravenously by the jugular vein into the main circulation of the animal or directly into the blood perfusing the hind limb by injection into the femoral cannula. Drugs used were noradrenaline bitartrate (Levophed, Winthrop; dose given in terms of the free base), synthetic angiotensin (CIBA prep. 19990a, hypertensin-val$_5$-amide; dose as pure compound) and biologically prepared angiotensin (Angiotonin, Lilly; dose in units).

Pressures were measured by small-volume manometers. Since the output of the perfusion pump was constant (Field and others, 1958), the peripheral resistance of the perfused hind limb was measured directly as the perfusion pressure.

RESULTS

Small doses of angiotensin (0·1–0·5 μg.) were administered intravenously into the main circulation of a rat anaesthetised with chloralose. The rises in the animal's blood pressure due to the angiotensin were accompanied simultaneously by small falls in the peripheral resistance of the
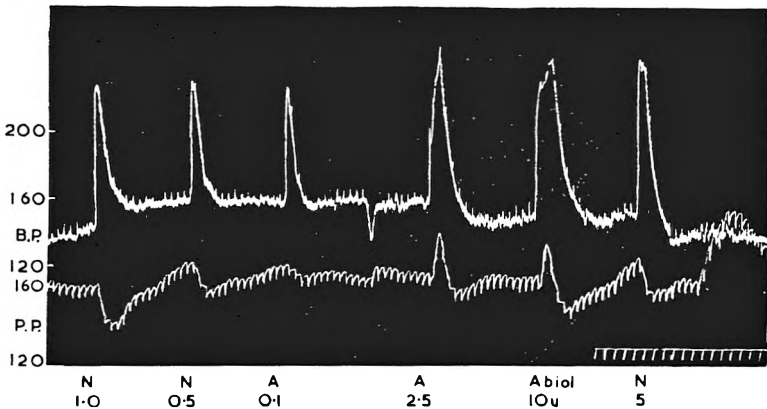
FIG. 1. Effects of synthetic angiotensin (A), noradrenaline (N) and biologically prepared angiotensin (A biol) given intravenously in varying doses, on the blood pressure (B.P.) and the hind-limb perfusion pressure (P.P.) of a rat anaesthetised with chloralose. The innervated vascularly-isolated hind-limb was perfused with blood at a constant rate so that changes in perfusion pressure directly represent changes in peripheral resistance. There was at least a 6 min. delay between the time the drug was given intravenously and the time the drug reached the hind limb in the perfusing blood. Time in min.; dosages in μg. or biological units.

Increasing the dose of angiotensin converted the immediate effect on the hind limb from vasodilatation to vasoconstriction; increasing the dose of noradrenaline merely increased the immediate vasodilatation.

blood vessels of the blood-perfused innervated hind limb, as shown by falls in the hind-limb perfusion pressure. An increase in the dose of angiotensin to 1 μg. or more changed the response in the hind limb to an immediate vasoconstriction (Fig. 1). Small doses of noradrenaline (0·5–1·0 μg.) caused slight vasodilatation in the hind limb; increasing the dose of noradrenaline caused only a slightly increased vasodilatation (Fig. 1). In all instances, the hind-limb effect was apparent within 5 min. of the drug being given to the remainder of the animal, i.e., before the drug could reach the hind limb in the perfusing blood. The immediate vasoconstriction produced by angiotensin was sometimes accompanied by respiratory depression and increased voluntary muscular movement.
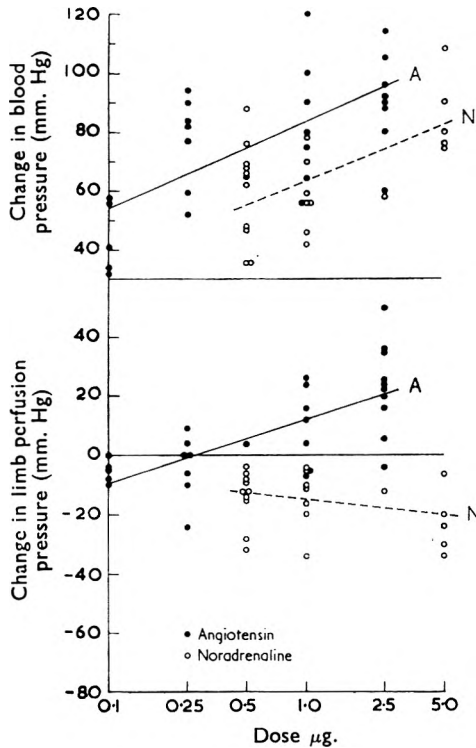
FIG. 2. Dose-response curves for the direct blood pressure effects and the nervously-mediated effects on the hind-limb perfusion pressure of angiotensin (A) and nor-adrenaline (N) for 14 experiments.

Fig. 2 summarizes the results of 14 experiments, showing the mean values, in each rat, of the response of the blood pressure and the response of the hind-limb perfusion pressure to differing doses of angiotensin and noradrenaline administered to the main circulation of the rat. The regression coefficients of the lines shown in Fig. 2 are statistically significant except for the regression with dose of the hind-limb response to noradrenaline. There is a highly significant difference statistically between the

slopes of the regression lines of the hind-limb effects of angiotensin and noradrenaline. The results demonstrate a marked difference between angiotensin and noradrenaline in their indirect actions on the blood vessels of the perfused hind limb. Biologically prepared angiotensin was also capable of producing a similar immediate vasoconstriction (Fig. 1).

Section of the nerves connecting the hind limb to the remainder of the animal totally abolished the immediate effects of both angiotensin and noradrenaline (Fig. 3), showing that these immediate effects were mediated solely through the nervous system.
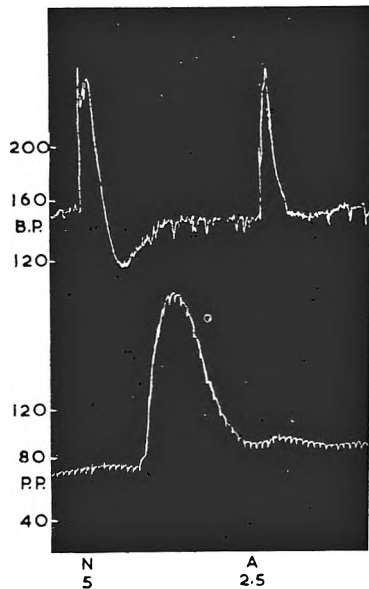
FIG. 3. Effect of synthetic angiotensin and noradrenaline on the blood pressure (B.P.) and the perfusion pressure (P.P.) in the *denervated* vascularly-isolated hind limb of a rat anaesthetised with chloralose. Time in min.: dosages in μg.

Drugs were given intravenously and reached the hind limb in the perfusing blood approximately 6 min. later. Thus the rise in blood pressure preceded the direct effect on the hind limb. There was no immediate effect on the limb after the nerves supplying it had been cut (compare Fig. 1).

Clamping the carotid arteries in three single animal experiments reduced only slightly the neurogenic vasodilatation produced by noradrenaline and did not prevent the nervously-mediated peripheral vasoconstriction induced by large doses of angiotensin.

In five experiments the hind limb of one rat was perfused with blood from a second animal to ensure the complete isolation of the circulation through the hind limb. Angiotensin and noradrenaline were given into the main circulation of the first animal as a prolonged infusion. Noradrenaline thus administered caused a nervously-mediated vasodilatation in the hind limb during the infusion, whereas angiotensin caused a small vasodilatation or a vasoconstriction in the hind limb (Fig. 4).
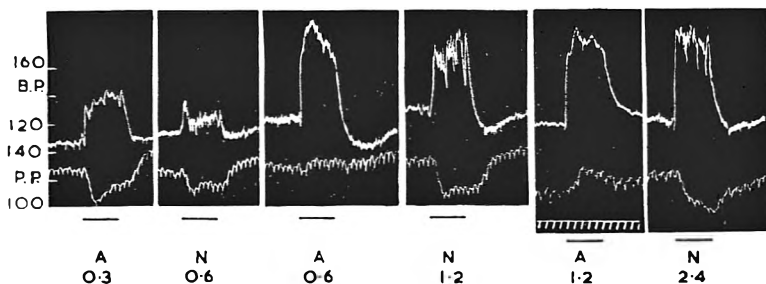
FIG. 4. Effects of prolonged intravenous infusions of synthetic angiotensin (A) and noradrenaline (N) on the blood pressure (B.P.), and on the perfusion pressure (P.P.) in the innervated vascularly-isolated hind limb, of a rat anaesthetised with chloralose. In this experiment the blood perfusing the hind limb came from a separate donor rat, thus preventing any drug given to the recipient animal from reaching the hind limb. Time in min.; dosages in μg./min.

Increasing the dose of angiotensin converted the nervously-mediated effect on the hind-limb perfusion pressure from vasodilatation to vasoconstriction, whereas increasing the dose of noradrenaline merely increased the vasodilatation.

DISCUSSION

The ability of angiotensin to produce a nervously-mediated peripheral vasoconstriction represents a new aspect of its pharmacology in rats, and suggests that angiotensin may have other actions than those of a purely peripheral vasoconstrictor. The vasoconstriction was abolished by nerve section (Fig. 3) which strongly suggests that it was mediated through the nervous system. Attempts to show vascular anastomoses through the femur or other tissues by means of dye were unsuccessful; if the vasoconstriction were due to anastomotic leaks to the hind limb from the remainder of the recipient animal, it would be expected that a similar vasoconstriction would occur with noradrenaline. In fact, it is the marked difference between the neurogenic effects of angiotensin and noradrenaline, both drugs being regarded mainly as peripheral vasoconstrictors, that is of great interest. Bickerton and Buckley (1961) have shown that angiotensin administered to the isolated head of a dog causes a neurogenic vasoconstriction in the vascularly-isolated remainder of the animal, which suggests that this nervously-mediated action of angiotensin is not restricted to one species.

The nervously-mediated peripheral vasoconstriction following angiotensin administration is not due to the rise in systemic blood pressure, as it was not observed following corresponding blood pressure rises due to noradrenaline (Fig. 2). However, angiotensin may cause a more marked cerebral vasoconstriction with consequent anoxia, since angiotensin and noradrenaline differ in their relative potencies in different vascular beds (Gross and Turrian, 1960). The muscular and respiratory effects of large doses of angiotensin may be consistent with this, though Mandel and Sapirstein (1962) have recently shown that angiotensin in low dosage had little effect on cerebral blood flow in rats. Another possible site of the difference between the actions of angiotensin and noradrenaline is the

carotid sinus; noradrenaline causes reflex hypotension when applied directly (Heymans and Delaunois, 1953) whereas angiotensin does not (McCubbin, Page and Bumpus, 1957; Bianchi, de Shaepdryver, de Vleeschhouwer and Preziosi, 1960). Attempts to alter any carotid sinus effects by carotid occlusion were inconclusive.

Thus it appears that angiotensin in large doses through an action probably on the central nervous system causes a nervously-mediated peripheral vasoconstriction. The determination of the exact site of action needs further experiments, preferably on larger animals than the rat.

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A NOTE ON THE RELATION BETWEEN THE RESTING RELEASE OF ACETYLCHOLINE AND INCREASE IN TONE OF THE ISOLATED GUINEA-PIG ILEUM

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When the Magnus preparation of the guinea-pig ileum treated with diisopropylphosphorodiamidic fluoride (Mipafox) was suspended in Krebs solution, the increase in tone with time was found to be proportional to the acetylcholine output. Use may be made of this phenomenon to monitor the state of the preparation in experiments involving the resting release of acetylcholine.

THE isolated ileum of the guinea-pig has little inherent tone compared with that of the rabbit. The "tone" of an ileal segment suspended for periods of time in Krebs solution increases and the magnitude of this increase depends on the duration of incubation.

The experiments reported in this paper were made to discover a possible connection between this increase in tone and the resting release of acetylcholine.

METHODS

Adult guinea-pigs were killed and the ileum was excised. The terminal ileum was cut into 3 cm. segments which, along with a 10 cm. length from the middle region, were incubated for 75 min. in 200 ml. aerated Krebs solution containing the organophosphorus anticholinesterase diisopropylphosphorodiamidic fluoride (Mipafox) in a concentration of 1×10^{-5}.

A 3 cm. segment from the middle region so treated was suspended in a 3 ml. organ bath of Krebs solution aerated with a mixture of 95 per cent oxygen and 5 per cent carbon dioxide and arranged to record longitudinal contractions isotonically. The lever had a magnification of ten times with a load of about 250 mg.

The bathing fluid was changed by means of a 3-way tap and samples for assay were obtained by removing the entire bath volume in a 5 ml. syringe. Samples were removed after incubating for 5, 10, 20 and 40 min.

The resting release of acetylcholine was assayed by the method described by Birmingham (1961) on the terminal ileum.

IDENTIFICATION

The spasmogenic substance released was inhibited by hyoscine, destroyed by boiling with normal sodium hydroxide but was not affected by boiling with normal hydrochloric acid. These tests eliminated histamine, 5-hydroxytryptamine and adrenaline as possible agents and it was concluded that the substance was an ester of choline with an order of potency identical to acetylcholine.

The Krebs solution used throughout the experiments contained choline in a concentration of 1×10^{-6} (Bligh, 1952).

RESULTS

Increase in Tone

Throughout the periods of incubation in Krebs solution there was a progressive increase in tone of the ileum which was recorded as a slow contraction. The magnitude of the response depended on the duration of incubation (Fig. 1). The responses were tabulated as per cent of the 40 min. response (Table I). Cooling to 25° and reduction of the calcium
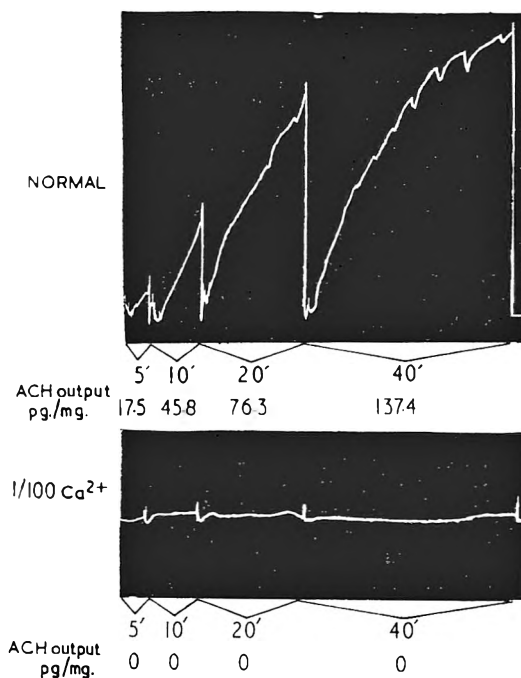

NORMAL

ACH output pg./mg.	5′	10′	20′	40′
	17·5	45·8	76·3	137·4

1/100 Ca²⁺

ACH output pg/mg.	5′	10′	20′	40′
	0	0	0	0

FIG. 1. Upper trace. The relation between increase in tone and acetylcholine output in pg./mg. with time, for the resting ileum in Krebs solution at 37°.
Lower trace. The effect of a hundred-fold reduction in the calcium content of the Krebs solution.
The reduction in calcium ions inhibits the resting release of acetylcholine, and also prevents the increase in tone.

TABLE I

THE INCREASE IN TONE OF THE GUINEA-PIG ILEUM RECORDED DURING PERIODS OF 5, 10, 20 AND 40 MIN. AS PER CENT OF THE 40 MIN. PERIOD. THE AMOUNT OF ACETYLCHOLINE FOUND BY ASSAY FOR THESE FOUR PERIODS IS GIVEN IN PG./MG. OF WEIGHED WET ILEUM AND IS ALSO CALCULATED AS PER CENT OF THE 40 MIN. PERIOD. EACH RESULT IS A MEAN OF 10 EXPERIMENTS AND ITS STANDARD ERROR

Incubation period, min.	Increase in tone mean ± S.E. as per cent 40 min. period	Acetylcholine release, pg./mg. ± S.E.	Acetylcholine release mean ± S.E. as per cent 40 min. period
5	19·3 ± 3·7	34·3 ± 3·3	29·4 ± 2·8
10	40·1 ± 3·9	50·6 ± 4·7	43·4 ± 4·0
20	70·8 ± 4·4	70·6 ± 12·3	60·6 ± 10·6
40	100	116·5 ± 23·1	100 ± 19·8

content of Krebs solution to one hundredth of its normal value abolished the increase in tone.

Resting Release of Acetylcholine

The resting release of acetylcholine per unit weight of intestine was found to be directly proportional to the time of incubation. The results of 10 experiments are given in Table I. Fig. 2 demonstrates the correlation of the resting release of acetylcholine and the increase in tone of the preparation. The graphs are not statistically significantly different. If the experiment is made at 25° or with the calcium content of the Krebs solution reduced to one hundredth of its normal value, the resting release of acetylcholine is reduced to a level below the limit of sensitivity of the assay.
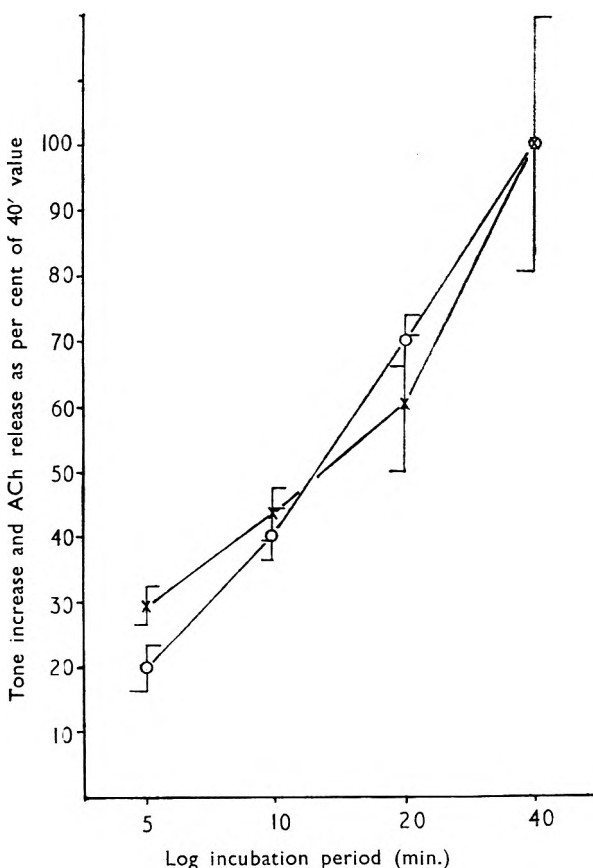
FIG. 2. The relation between the resting release of acetylcholine (x) and the increase in tone (○) for four periods of incubation. Each result is a mean of ten experiments together with the standard error. There appears to be a simple relationship between the acetylcholine release and the increase in tone; both are related to the length of the incubation period.

DISCUSSION

The property of tone in smooth muscle is ill understood and has been attributed to many factors. The rhythmic activity and tone of the longitudinal muscle of the intestine have been regarded as inherent properties of the muscle fibre on the one hand, and as dependent on the intrinsic innervation on the other hand. It is well known that acetylcholine and choline are liberated continuously from the guinea-pig ileum at rest, but no one has yet successfully related this release with tone.

The origin of the resting release of acetylcholine has been the subject of much discussion; Feldberg and Lin (1949, 1950) favoured the release from non-nervous structures and Schaumann (1957) claimed that it was released mostly from nerve endings.

My results demonstrate that the increase in tone of the resting ileum is directly proportional to the release of acetylcholine. When the intestine is cooled to 25° or its Krebs solution replaced by that containing a reduced calcium content (Fig. 1), there is no increase in tone at rest, and no measurable release of acetylcholine. This means that in experiments made to investigate the resting release of acetylcholine the tone of the ileum can be used to indicate the state of the preparation, an increase in tone signifying an increase in the output of acetylcholine.

Feldberg and Lin (1949) accepted the muscular contractions produced by eserine on the intestine as evidence for the spontaneous release of acetylcholine. Harry (1962) showed that eserine induced contractions of both circular and longitudinal muscle of the guinea-pig ileum, and considered this to be a muscarinic effect. This means that the effects of eserine do not result only from cholinesterase inhibition. The responses in Fig. 1 are slow contractions (unlike those of Feldberg and Lin (1949) which developed after a few seconds) and represent the effects of a gradual increase in the resting output of acetylcholine.

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NEW APPARATUS

A MODIFIED APPARATUS FOR REINSCH'S TEST

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A modified method for the rapid identification of antimony, arsenic and mercury is described.

STANDARD procedures for the determination of arsenic (Analytical Methods Committee, 1960; Methods of Analysis A.O.A.C., 1960a) and mercury (Methods of Analysis, A.O.A.C., 1960b), although excellent in themselves, are time consuming and require considerable experience to obtain reproducible results. There is, therefore, still a need for some simple qualitative test of reasonable delicacy and of such simplicity that it may be made rapidly by any trained chemist with adequate laboratory facilities. Although it must be emphasised that irreplaceable materials should always be left for the expert attention of the forensic laboratory, there are many occasions when a rapid qualitative identification may be of the greatest value.

It is the purpose of this communication to describe a modification of Reinsch's test (1841), a reaction which has already been much investigated and variously modified (Gettler, 1937; Gettler and Kaye, 1950; Stolman, 1961; Umberger, 1960).

EXPERIMENTAL METHOD

The first stage of the test is made in the conventional way. 20 g. of the suspected material is placed in a 250 ml. conical flask fitted with a long air condenser and 2N HCl (50 ml.) added together with a piece of thin copper foil (1 cm. × 0·5 cm.) which has been cleaned by rubbing with fine emery paper, immersing in 4N HNO_3 and washing in distilled water. The mixture is boiled gently for 30 min. the copper is then removed, washed thoroughly with water, dried between sheets of filter paper, washed in ethanol and ether, and examined. Discoloration may be due to antimony, arsenic, bismuth, mercury, selenium, silver, sulphur or tellurium. Even if no change is visible to the naked eye, the second stage of the test should be made. It is to this second stage, which consists of the sublimation of the deposit and microscopical examination of the sublimate, that the present modification applies.

The copper, which must be thoroughly dry, is folded and placed in the bottom of a Pyrex ignition tube (Fig. 1), into the neck of which is inserted a piece of thick walled capillary tubing 3 cm. long (A). This is surrounded by a brass collar (B) held in place by a piece of curved watch

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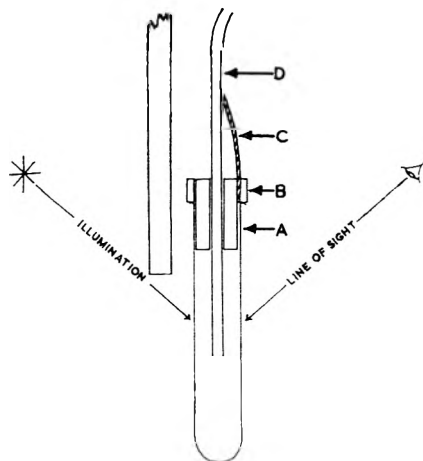
Fig. 1

spring (C), notched at its upper end to retain the melting-point tube, 10 cm. $\times$ 0·1 cm. internal diameter (D). The top of the melting-point tube is connected by a piece of polythene tubing to an aspirator which enables a slow current of air (20–30 ml./min.) to be drawn up the tube. A metal strip, with a suitable hole drilled in it, forms a convenient support for the apparatus.

The bottom of the ignition tube is now heated in a small micro-bunsen flame. The deposit on the copper volatilises and is drawn into the melting-point tube where it is redeposited. The presence of this deposit is best seen by indirect illumination as shown in the Figure. It should be noted that considerably more heat is required for the volatilisation of antimony, selenium and tellurium than for either arsenic or mercury. Silver, which gives a whitish deposit on the copper, and bismuth, which gives a mechanically unstable black deposit, do not sublime.

As soon as the deposit begins to move up the tube the air flow is stopped and the flame removed. After being allowed to cool. the melting-point tube is removed and the deposit examined under low magnification ($\times$60–$\times$100). Arsenic forms a crystalline deposit of As_2O_3, antimony an amorphous deposit of Sb_2O_3. while mercury gives globules of the metal. Selenium gives a sublimate easily seen by indirect light, but which is very difficult to see under the microscope. When seen, it has the appearance of a liquid. Tellurium has sometimes a crystalline form (rods or needles), but is often too finely divided to be distinguished from antimony.

In most instances the appearance of the sublimate will be distinctive enough for identification. It is as well, however. to carry out confirmatory tests as follows: (i) for arsenic, seal the lower end of the melting-point tube and replace the tube in the apparatus. Heat the outer tube strongly until the sublimate disappears. The thermal capacity of the thick-walled capillary tubing prevents the loss of the sublimate. Allow the apparatus to cool slowly until the outer tube is cool to the touch. Remove the melting-point tube and again examine under the microscope. A few

large crystals of As_2O_3, tetrahedral or octahedral, will be found. (ii) For antimony proceed in the same way; the sublimate will remain amorphous though it may become invisible. Cut off the sealed end of the tube, draw a small drop (10 μl.) of concentrated HCl into and along the tube and expel it on to a white tile. Add a microdrop (0·1 μl.) of a 10 per cent solution of sodium nitrite, and then a microdrop of an 0·1 per cent aqueous solution of rhodamine B. A mauve colour shows the presence of antimony. It is important that HCl be in excess. This test is not given by any other element under consideration.

Selenium may be identified by drawing a small drop of a 1 per cent solution of morphine in 2N acetic acid into and along the melting-point tube, expelling it on to a white tile, evaporating to dryness and moistening the residue with concentrated H_2SO_4 when a green colour is obtained. Under similar conditions tellurium gives a light brown which is not very distinctive.

(iii) For mercury, remove the copper from the ignition tube, add a few small crystals of iodine and warm carefully with the air flow turned on so that the iodine vapour is carried up the melting-point tube. As it passes the mercury an orange colour, due to the formation of mercuric iodide. will be seen. Examination under the microscope will show a mixture of both red tetrahedra and yellow rhomboids, the latter showing brilliantly under polarised light. Should the appearance not be distinctive the tube is sealed at one end, reheated. and allowed to cool slowly as for arsenic. The resublimation and slow cooling will give larger crystals. It is essential that the apparatus and iodine be completely dry as traces of water vapour may prevent the formation of typical crystals.

The sensitivities, given conservatively, are 10–20 μg. for arsenic and antimony and 100–200 μg. for mercury. 100 μg. of bismuth, silver, selenium and tellurium may discolour the copper, but the two latter will not sublime unless present in milligram quantities.

It must be noted that certain organic compounds of mercury used for seed dressings, such as ethyl mercuric chloride, are not found by Reinsch's test. This is due to the stability of the carbon-mercury bond (Calfruny, 1961).

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LETTERS TO THE EDITOR

The Effect of *Haemophilus pertussis* Vaccine on Lymphoid Tissue in the Rat

Sir,—The hypothesis has been put forward by Miller (1961) that during embryonal and early neonatal life the thymus gland in the mouse produces the progenitors of immunologically competent cells which mature and migrate to other sites. More recently (1962), he has reported that the thymus in adult mice may be essential for complete recovery of the immune mechanism after sub-lethal irradiation. In fact, "the thymus may provide the environment wherein cells can differentiate and acquire the capacity for immunological competence by direct cell-to-cell interaction or by the elaboration of some specific humoral-like maturation factor by non-lymphoid thymic cells."

It is well known that rats (like mice) are highly resistant to histamine but they can be made sensitive by treatment with drugs such as thyroxine (Parratt and West, 1960), by removal of the adrenal glands (Spencer and West, 1962), or by a single dose of *Haemophilus pertussis* vaccine (see Sanyal and West, 1959). When given together with another antigen, *H. pertussis* vaccine also raises the blood antibody titre of that antigen so that fatal anaphylactic shock results when the challenge is made with the specific antigen. The vaccine presumably stimulates the reticulo-endothelial system of the rat (particularly the spleen and thymus) to allow a more effective production of antibody. A study has therefore been made of the effect of the vaccine on the thymus gland of the rat.

It was essential, before carrying out the study, to establish control values for the weight of the two lobes of the thymus gland at different ages. These are shown in Figs. 1 and 2, each value being the mean of 5 rats at each body weight. Although the thymus increases in size from birth until the rat weighs about 150 g. and is sexually mature, the rate of its growth in relation to the body weight reaches a maximum just about weaning time (21 days, body weight about 40 g.). This result in the rat is similar to that already reported in the mouse (Kay, Playfair, Wolfendale and Hopper, 1962).

Groups of 5 Wistar albino rats (about 150 g.) were given either *H. pertussis* vaccine (20,000 million bacteria) and killed at various times up to 17 days, or the same dose of vaccine with antigen (horse serum, 1 ml.) and challenged
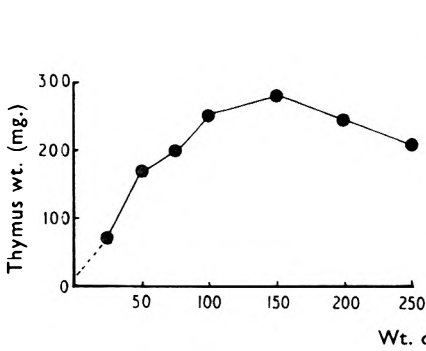
Wt. of rat (g.)

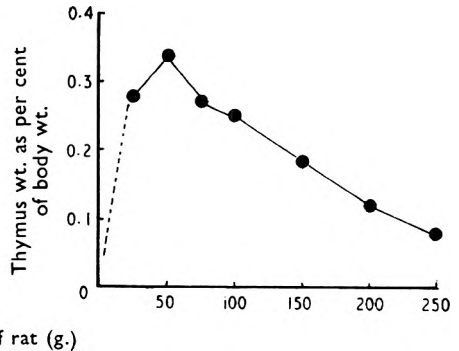Fig. 1. The relationship between thymus weight and body weight in rats. Note the peak at about 150 g. body weight.

Fig 2. The relationship between thymus weight expressed as a percentage of body weight and the weight of the animal. Note the peak at about weaning time (body weight about 40 g.).

intravenously with horse serum (1 ml.) 10–14 days later. Other groups of rats were thymectomized 7 days before treatment with the vaccine or with the vaccine and antigen. Anaphylactic shock was severe whether the thymus was present or not and all these rats died within 4 hr. of the challenge; on dissection, the thymus glands were found to be of normal size in the unoperated animals. The thymus glands were also of normal size in unoperated rats receiving only the vaccine. But in both instances, enlargement of lymph nodes lying just above the thymus was noted. This "extra thymus" consists of a varying number (4 to 8) of nodes and is easy to dissect if haemorrhage is kept to a minimum; in control rats, it never weighs more than 40 mg. (the value reached when the rat weighs 150 g.). After *H. pertussis* vaccine, it rapidly increased in size to reach a maximum at 14 days when its weight almost equalled that of the two thymus lobes (see Fig. 3). This six-fold increase in weight of the "extra thymus" occurred equally well in the thymectomised rats receiving either the vaccine or the vaccine and antigen. A small number of rats were then splenectomised or splenectomised and thymectomised; after *H. pertussis* vaccine, these two showed grossly enlarged "extra thymus" tissue.
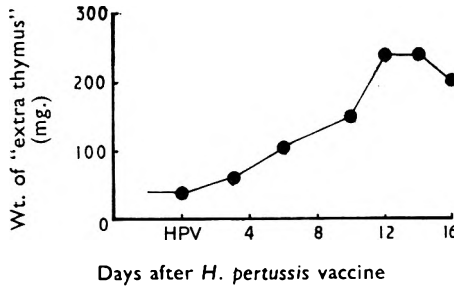
Fig. 3. The effect of a single dose of *H. pertussis* vaccine (HPV) on the weight of the "extra thymus" in rats of 150 g. body weight. Note the peak after about 14 days.

In the adult rat, therefore, the thymus and the spleen may not be important in initiating immunogenesis, and other lymphoidal tissue may be activated by procedures such as the single injection of *H. pertussis* vaccine.

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December 1, 1962

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Association of Histamine and 5-Hydroxytryptamine with the Inflammatory Processes

SIR,—It was reported that mast cells undergo characteristic changes during aseptic inflammation in the rat (Sanyal, 1959). As histamine and 5-hydroxytryptamine (5-HT) may be associated with these cells, at least in the rat and the mouse, we have studied the tissue levels at intervals after the production of aseptic inflammation, and also the effect of previous depletion of either amine on the production of inflammatory exudate or development of fibrous tissue.

Accordingly groups of 6 rats were anaesthetised with ether, and a surgical incision was made on the back with aseptic precautions. The skin flaps were mobilised and sutured. Animals anaesthetised and then allowed to recover served as controls. Control animals, and those subjected to surgical trauma, were killed, 1, 2, 3, 4, 6, 8, 12, 16 and 26 days after the operation, for extraction and assay of histamine and 5-HT (Parratt and West, 1957a) of the skin subjected to trauma, an adjacent area, and an area away from the site of injury, namely, the legs. Skin samples from all the animals in one group were pooled for extraction and assay. The values obtained from the operated group were compared with those obtained from control animals at comparable sites. There was a 25 per cent reduction in the values for histamine obtained from the operated area, in the first 24 hr.; these values returned to control level by 4th day, and thereafter showed a progressive rise to 170 per cent by the 12th day, finally returning to the control levels by 16-26th day. The 5-HT values showed a progressive rise from the beginning, reaching a maximum of about 300 per cent of the control levels by the end of one week, thereafter returning to control levels by the 16th day. In the adjacent and distal areas, values for both histamine and 5-HT began to rise in the first 24 hr.; they reached a maximum of 200–300 per cent of the control levels in 6–8 days and then returned to control levels in 16–26 days. Results from mice were similar.

The exudative phenomenon was studied by the granuloma pouch method and the development of fibrous tissue by the cotton wool pellet method (Finney and Somers, 1958). Control animals, animals depleted of either histamine by repeated injections of polymixin B or 5-HT by injections of reserpine (Parratt and West, 1957b) were anaesthetised and either a granuloma pouch was produced by creating an air pocket in the back into which a little croton oil in arachis oil was injected or 8 weighed cotton wool dental pellets were inserted in a subcutaneous pocket. After about one week the animals were killed. The amount of exudate in the pouch was most in the control animals; in histamine depleted animals the values were about 35 per cent of control whereas values of 20 per cent of control were obtained in animals depleted of 5-HT. The latter group showed least inflammation, though in some animals the overlying skin had become parchment-like and necrotic. The groups in which cotton wool pellets were implanted were also killed after one week. The pellets were cleaned of extraneous tissues and dried in an oven to constant weight. They showed an increase in weight of about 28.18 ± 2.05 mg. in control animals, the increase in the polymixin B treated animals being 11.89 ± 1.09 mg., and in the reserpine treated animals, 7.92 ± 0.60 mg. These changes were statistically significant, all having confidence limits greater than 99 per cent. Thus the maximum reduction in the development of fibrous tissue was in the reserpine-treated animals. This effect could not be due to the depletion of catecholamines, since carbachol which also causes the excretion of catecholamines did not reduce the development of fibrous tissue. In a comparative study, the action of reserpine was only slightly inferior to prednisolone (20 mg./kg. per day for 3 days) in

preventing the development of fibrous tissues. Thus both histamine and 5-HT may be involved in both exudative and reparative stages of the inflammatory response. Recently it has been suggested that histamine liberated from mast cells may prepare many more connective tissue cells than are normally available to receive heparin or heparin containing granules, which may be used in preparing ground substances (Riley, 1962), and that 5-HT may possibly, particularly in the rat and mouse, act similarly (West, 1962). The anti-inflammatory effect of histamine and more particularly 5-HT depletion, lend support to this view.

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Demonstration of Interaction between Pairs of Antibacterial Agents

SIR,—It has been recognised that the antibacterial action of pairs of antibiotics may be that of simple addition of their separate effects, or one may enhance the activity of the other, or there may be mutual antagonism. These phenomena have been demonstrated by a number of techniques, one of which uses paper strips loaded with the compounds and laid at right angles on a seeded agar plate. Zones of inhibition are produced after incubation and the pattern of the growth between the strips gives information about the mutual effect, if any, of the pairs of compounds (Dye, 1955–56; Maccaro, 1961).

In a problem concerning the formulation of eye drops it was required to find if pairs of compounds used as bacteriostatic agents were more or less effective than each one alone or if no interaction between them occurred, and the method applied to antibiotics quoted above was investigated. In effect, the interaction of 28 combinations of antibacterial substances from the following list, phenylmercuric nitrate, 2-phenylethanol, chlorocresol, thiomersalate, chlorhexidine, benzalkonium chloride, chlorbutol and Eye-drop Solution B.P.C. were tested against *Pseudomonas aeruginosa*, NCTC 7244, *Streptococcus pyogenes*, NCTC 8708, *Staphylococcus aureus*, NCTC 4163, *Escherichia coli*, NCTC 86, *Bacillus subtilis*, NCTC 8236 and *Proteus vulgaris*, NCTC 4636.

With all six bacterial species, no antagonism was demonstrated between any pair of bacteriostats listed; there was evidence of mutual enhancement of activity between 2-phenylethanol and the organic mecurials. Antagonism between calcium thioglycollate and phenylmercuric nitrate and between chlorhexidine and lecithin can be strikingly demonstrated by this method.

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LETTERS TO THE EDITOR

Dilutions of Sulphuric Acid

SIR,—Dilutions of sulphuric acid defined as reagents in the B.P. and B.P.C. are used mainly in simple qualitative tests and for this purpose reagents prepared by mixing specified proportions by volume of acid and water should generally be satisfactory. Some simplification of the preparation of these reagents is therefore possible on the lines suggested by Betts, (1962) but the data he has put forward for conversion of strengths from w/w and w/v to v/v are stated in its source to be approximate only (Handbook of Chemistry and Physics, 1961–62). We have verified experimentally that the corresponding strengths he proposes are satisfactory, except that 80 per cent v/v sulphuric acid does not necessarily correspond to 80 per cent w/w. We prepared 80 per cent v/v acid by mixing 4 volumes of concentrated acid (assaying 99·5 per cent w/w of H_2SO_4) with 1 volume of water as directed in the B.P., and found by titration of a weighed sample that the dilution contained about 88 per cent w/w of H_2SO_4. An 80 per cent v/v acid prepared from a sample of sulphuric acid which assayed only 97 per cent w/w of H_2SO_4 (the minimum B.P. limit) would, of course, correspond more closely than this to 80 per cent w/w. Sulphuric acid 80 per cent w/w is used in the B.P.C. in tests to distinguish cellulose from other fibres, and as the 80 per cent v/v acid we prepared is rather strong for this purpose it would seem to be more convenient to use a simple (2 + 1) dilution (66 per cent v/v) as recommended by Wallis (1960) and to indicate the precautions which should be taken to prevent undue dilution of the acid while the tests are being carried out.

In the next edition of the British Pharmaceutical Codex it is intended to replace 25 per cent w/v sulphuric acid by 14 per cent v/v (1 + 6 dilution), and 80 per cent w/w sulphuric acid by 66 per cent v/v. In order to eliminate 50 per cent w/w acid entirely the text will be modified. Dilute sulphuric acid (about 5 per cent v/v) will be retained as this strength is available in commerce.

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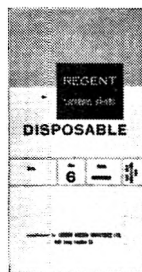
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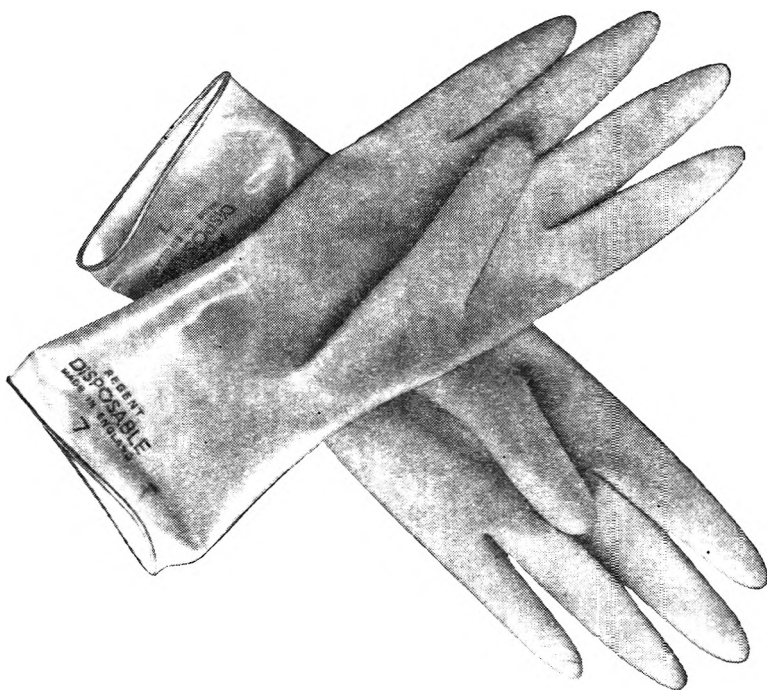
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