INVESTIGATIONS INTO THE PHARMACODYNAMIC EFFECTS OF DRUGS USED IN THE TREATMENT OF PARASITIC DISEASES IN FARM ANIMALS.

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CONTENTS

INTRODUCTION

The chemotherapy and chemoprophyllaxis of parasitic disease in farm animals ranks among the more important aspects of Veterinary Pharmacology, owing to its close connexions with the economics of agriculture.

The number and variety of veterinary specific chemotherapeutic agents at present in use in the field is very large indeed. Most of the chemotherapeutic substances have systemic actions and toxicity which often limit their usefulness and among the most pharmacodynamically active compounds in use in veterinary chemotherapy is quinuronium sulphate. Until recent years this drug was unrivalled in treating British Babesiasis.

Babesiasis (piroplasmosis) is a disease of animals which has world-wide distribution. The parasite is an intra-erythrocytic protozoan which is transmitted from host to host by a species of tick. In the Continent of Europe the disease is found in cattle, sheep, horses and dogs, but in the British Isles it is only the bovine form which is recognised, and produces severe economic losses among affected herds.

The signs and symptoms of disease are similar in all species and include cachexia, fever, anaemia, jaundice and haemoglobinuria. This last named condition has given rise to the common names for the disease, namely British Redwater or Bovine Haemoglobinuria.

The advent of practical chemotherapy in the early 1930's brought considerable interest in the action of chemical agents against/

against certain protozoal infections of economic importance (The history of the development of arsenical and antimonial chemotherapy of trypanosomiasis is well-known). Veterinary research workers at this time were anxious to find a cure for babesiasis and not unnaturally many of the early trypanocidal agents were tried with only minor success, (Sergent, Donatein, Parrot and Lestoquard, 1933). It was not until 1935 that quinuronium sulphate was made available (Cernaianu, Schuldner and Magureanu, 1935) and this compound remained the principal piroplasmocidal agent for thirty years under various trade names such as Akiron, Acapron, Acaprin, Babesan, and Pirevan (Smythe (i), 1935).

From its discovery in 1935 the therapeutic use of quinuronium was always accompanied by severe toxic side-effects, and the literature makes numerous mentions of these and the fact that a concurrent subcutaneous injection of adrenaline hydrochloride did much to alleviate untoward symptoms. Despite this, fatalities were not uncommon especially in animals severely parasitised and grossly anaemic. Nevertheless quinuronium gained in popularity very rapidly indeed and in Britain the compound was described as superior to any agent so far used in the treatment of British Redwater (Smythe (ii), 1935). Among the numerous accounts of successful therapy in all domesticated species was the first accurate account of the toxic signs and symptoms in sheep (Cernaianu, Schuldner and Magureanu, 1935). These workers described an initial restlessness which was closely followed by salivation, micturition and defaecation. The more severely affected/

affected animals were seen to have generalised muscular spasm, and difficulty with respiration and a few animals would collapse and die. A further interesting point which arose in this study was that if the same sheep were given a second injection of quinuronium within about two weeks of the first, many more animals showed severe toxic signs and the mortality rate was higher than previously. This paper was the first to ascribe toxicity to cholinergic stimulation as such, but no reason was suggested by these workers for the apparent hyper-susceptibility to a second dose.

A somewhat different approach was made by Kikuth (1935) who drew attention to the resemblance between quinuronium poisoning and shock, but did not investigate this possibility.

As far as can be ascertained no precise pharmacodynamic study was made on quinuronium until 1959 (Kronfeld, 1959) when a detailed study in cats and rats revealed what was described as a "respiratory-type" death. Kronfeld reported a rise in carotid blood pressure in the cat and irregular dyspnoeic breathing. Respirations always ceased several minutes before the heart finally stopped. In unanaesthetised rats quinuronium produced a constant characteristic response, namely "excitement", coughing and face-washing movements. Gasping respirations and jumping movements ensued, ending in convulsion and death with cyanosis of the visible extremities and bulging eyes. The author ascribes this picture to anoxic excitation. No sympathomimetic amine was in any way useful in delaying or preventing death (adrenaline, ephedrine, and amphetamine/

amphetamine were tried). It seemed to Kronfeld that parasympathetic activity was but a transient phase and not the cause of death. He went on to show that quinuronium did in fact depress cellular oxygen uptake in liver and brain slices and homogenates.

In 1961 Rummler and Laue gave evidence of protection of sheep from severe quinuronium toxicity by the use of atropine (1 mgm/kilo). This is supported by the present work. However, Rummler and Laue also used 2.P.A.M. (pyridine 2-aldoxime methiodide) which is allegedly specific for poisoning by organophosphorus compounds. Rummler and Laue showed that quinuronium would reduce the circulating levels of cholinesterase in a proportion of dogs and sheep. But in other experiments designed to demonstrate enzyme-protection by pyridine 2-aldoxime methiodide (2.P.A.M.), these authors did not estimate the cholinesterase activity after premedication with 2.P.A.M. Their assessment of the value of this antagonist in protecting sheep from the effects of quinuronium poisoning was based solely on clinical observations. The results were inconclusive.

Some of the pharmaceutical companies marketing quinuronium have ascribed the toxicity of their product to "breakdown" substances from killed babesia parasites and disrupted r.b.c.s. There is doubtless some truth in this as was shown by Goodwin and Richards (1960), who described pharmacologically active compounds in the blood of rodents infected with Babesia Rhodaini. Although such compounds may be important in the symptomatology of the disease, they probably play only a minor role on the toxicity of quinuronium, since all the toxic signs described can readily be demonstrated/

demonstrated in normal unparasitised animals.

The constant search for a new babesicide with a better therapeutic index than quinuronium achieved some success in the early 1940's when a number of diamino phenyl-urea derivatives were synthesised (Gerchuk, 1941). These compounds did not differ greatly from quinuronium in respect of toxicity (Petrova, 1955). However, another substituted urea (amicarbalide) was produced, whose L.D.50. was some ten times greater than that of quinuronium. The efficacy of the new compound in field trials has secured its success and the only reported toxicity has been local oedema at the site of injection and some transient dullness and ataxia at the highest dose range (Ashley, Berg and Lucas, 1960) and (Beveridge, Thwaite and Shepherd, 1960).

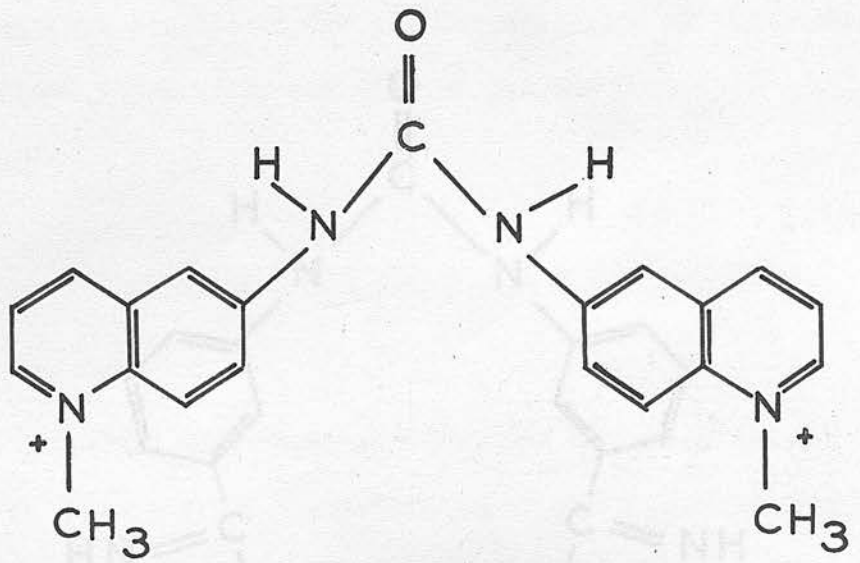
Despite the introduction of amicarbalide, quinuronium still finds favour because of its cheapness and high degree of efficacy in all forms of babesiasis, but its degree of toxicity presents many problems during clinical use.

The pharmacodynamic activity of quinuronium and the comparison with amicarbalide have thus, so far as can be ascertained, received little detailed study. Hence further investigation seemed to be important.

The experiments described in this thesis were designed to investigate the pharmacodynamic activity of quinuronium and amicarbalide, especially with regard to cholinergic activity and the release of histamine.

STRUCTURE OF QUINURONIUM SULPHATE

6:6' URYLENE - BIS - (I METHYL QUINOLINIUM)
BIS - METHYLSULPHATE.

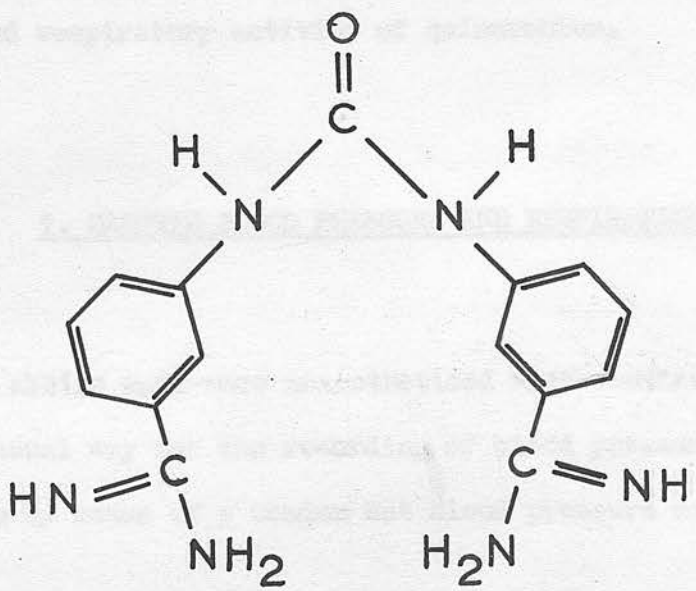


$\cdot 2CH_3SO_4^=$

This compound is a simple substituted urea. It is a yellow amorphous powder stable at room temperature and readily soluble in water to make a neutral, stable solution.

STRUCTURE OF AMICARBALIDE ISETHIONATE

3:3' DIAMINO - CARBANILIDE DIISETHIONATE.



$\bullet 2HO.CH_2.CH_2.SO_3H$

This substance is a modification of the substituted urea. It is a white amorphous powder stable at room temperature and soluble in water to produce a neutral stable solution.

SECTION I

THE INVESTIGATION OF PHARMACODYNAMIC ACTIVITY

Animals poisoned with quinuronium always show marked dyspnoea and many individuals stagger and sometimes collapse showing dilated pupils, pale mucous membranes and weak pulse. Techniques were therefore considered for the investigation of the cardio-vascular and respiratory activity of quinuronium.

METHODS.

1. CAROTID BLOOD PRESSURE AND RESPIRATION

(a) Rat.

Adult albino rats were anaesthetised with urethane and set up in the usual way for the recording of blood pressure kymographically by means of a Condon Rat Blood pressure manometer.

(b) Guinea Pig, Chicken and Rabbit.

Adult animals were anaesthetised with urethane and blood pressure was recorded kymographically with a mercury manometer.

(c) Sheep.

Adult south country cheviot sheep were anaesthetised with intravenous thiopentone sodium and maintained throughout the experiment on closed circuit cyclopropane and oxygen. Blood pressure was recorded kymographically with a mercury manometer and simultaneously a respiratory recording was made from the tracheal cannula by means of a bellows recorder.

Drugs were dissolved in isotonic saline and injected intravenously. Acetylcholine chloride, adrenaline chloride and histamine acid-phosphate were injected in order to show the effects of known compounds upon the carotid blood pressure in the various species. Quinuronium and amicarbalide were both injected for the purposes of comparison. The antagonists used were atropine sulphate and mepyramine maleate for the purpose of demonstrating respectively antimuscarine and antihistamine effects.

2. ACTIONS ON PERIPHERAL BLOOD VESSELS

A. THE ISOLATED PERFUSED RABBIT EAR.

Ears were taken from freshly killed rabbits and were either used immediately or after having been stored at 4°C overnight:- the drug responses being the same in each case.

The method is essentially that described by Rischbeiter (1913) and involved a perfusion via the central artery of a rabbit's ear of oxygenated Krebs solution (Krebs and Henseleit, 1932). The total perfusate was passed through a photo-electric drop counting unit which recorded kymographically by means of Thorpe impulse counter.

Drugs were dissolved in isotonic saline and injected into the perfusion fluid.

B. SMALL INTESTINE AND HIND LIMB PLETHYSMOGRAPHY IN SHEEP.

Plethysmography was carried out simultaneously with the recording of blood pressure and respiration in sheep. The methods are essentially those of Dale and Laidlaw (1910) and Dale (1914).

Small Intestine.

The abdomen of the sheep was opened on the right flank and a convenient loop of small intestine located. A segment of intestine some three or four inches in length was clamped at each end with bowel forceps and the ligated (producing i.e., a sausage-like isolated segment). The free cut ends of the remaining bowel were similarly ligated. The artery and vein supplying this isolated segment of bowel were cleared of mesentery and fat, and the segment was placed in an air plethysmograph made of perspex. The vessels passed to the exterior through a small notch under the lid (Fig. P.1.) which was made air tight with petroleum jelly.

Hind Limb.

With the sheep lying on its side the "upper" hind limb was taken and clipped free of wool and hair over the whole leg. The limb was greased above the stifle joint and placed inside a hollow cylinder of perspex whose distal end was closed and whose proximal end carried a rubber sleeve which inverted into the perspex tube and gripped the upper limb sufficient to allow the plethysmograph to be filled with water: the whole cylinder now being both air and water tight (Fig. P.2.). The cylinder was fitted with a side-tube for recording changes in pressure.

The gut and limb plethysmographs were connected by their

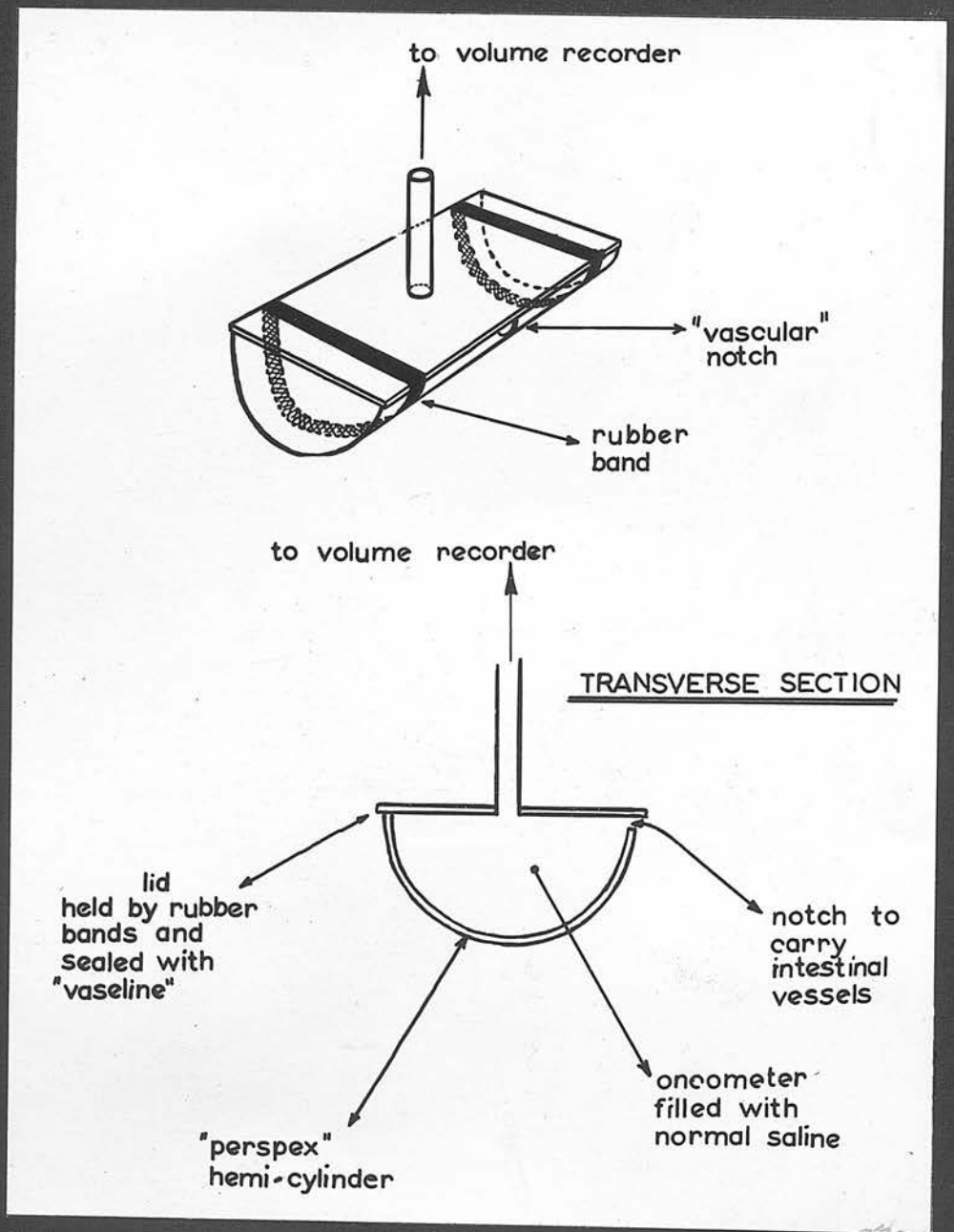


FIG. P.1.

SHEEP SMALL INTESTINE ONCOMETER.

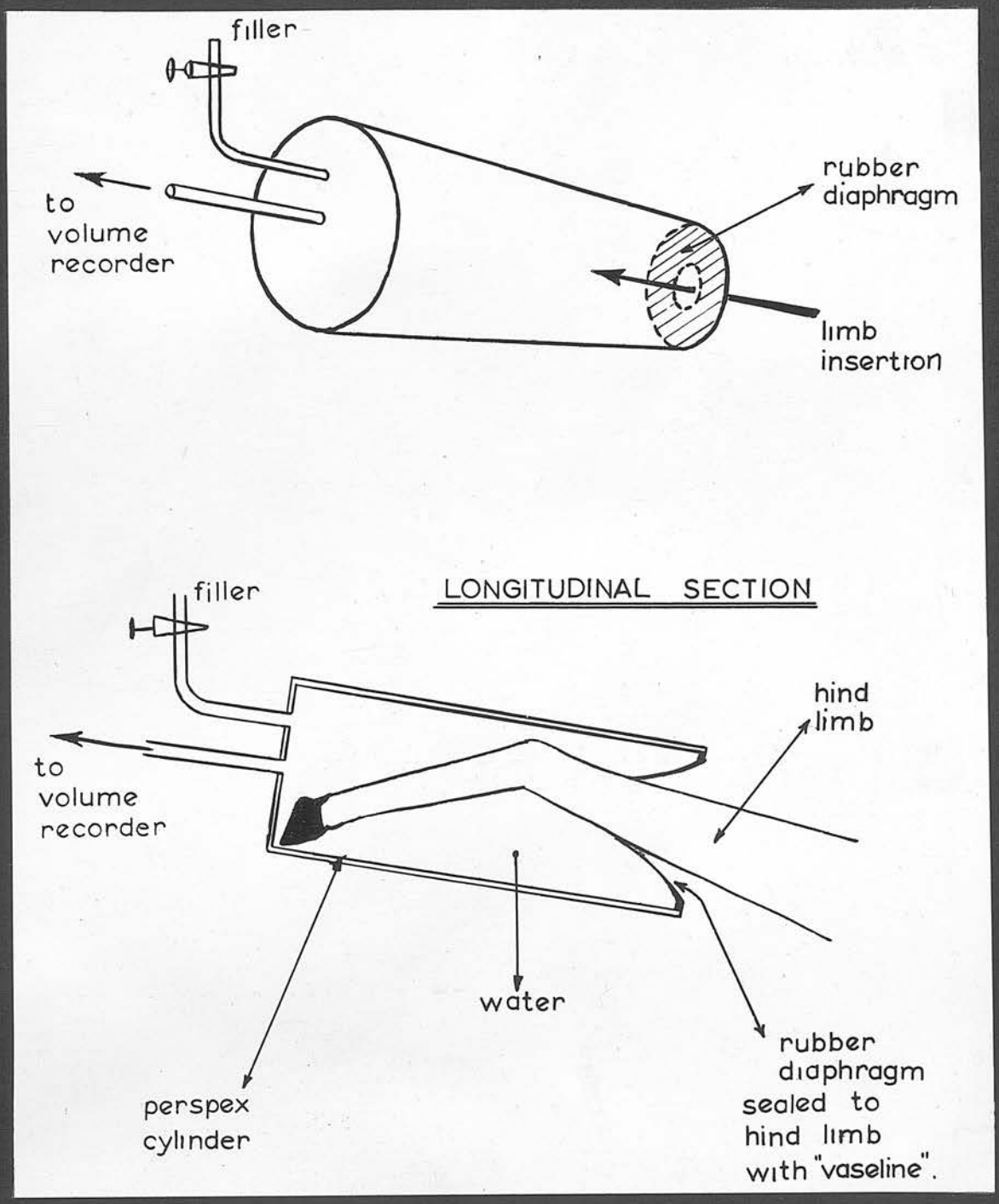


FIG. P.2.

SHEEP HIND LEG ONCOMETER.

side-tubes through transducers (Greer manometers) to a pen-recording system.

3. ISOLATED HEART OF RABBIT AND GUINEA PIG

Heart organ-bath.

The assembly is depicted in Figure C.A. and was described by Bartlet (1963). The apparatus was essentially a glass cannula fixed into the side of a perspex organ-bath. The heart coronary system was perfused via the aortic cannula with Krebs solution (Krebs and Henseleit, 1932) which had been filtered (Bleehan and Fisher, 1954) and gassed with 5% CO_2 in oxygen. The coronary perfusate accumulated in the organ-bath and was also gassed by 5% CO_2 in oxygen. Thus the totally immersed heart was perfused through its coronary vessels and the perfusate measured by displacement from the bath into a photo-electric drop counter.

The heart beat was recorded from the left ventricle by means of a thread which passed over a pulley to a spring loaded lever writing on a kymograph drum.

Drugs (contained in a small volume of saline) were injected into the perfusion fluid by means of a small rubber injection chamber just prior to the heart cannula.

Note. In the classical arrangement of the perfused isolated heart described by Langendorff (1895) severe cooling occurs especially as drug action modifies the coronary circulation. More recent methods (e.g. that described above) obviate this disadvantage.

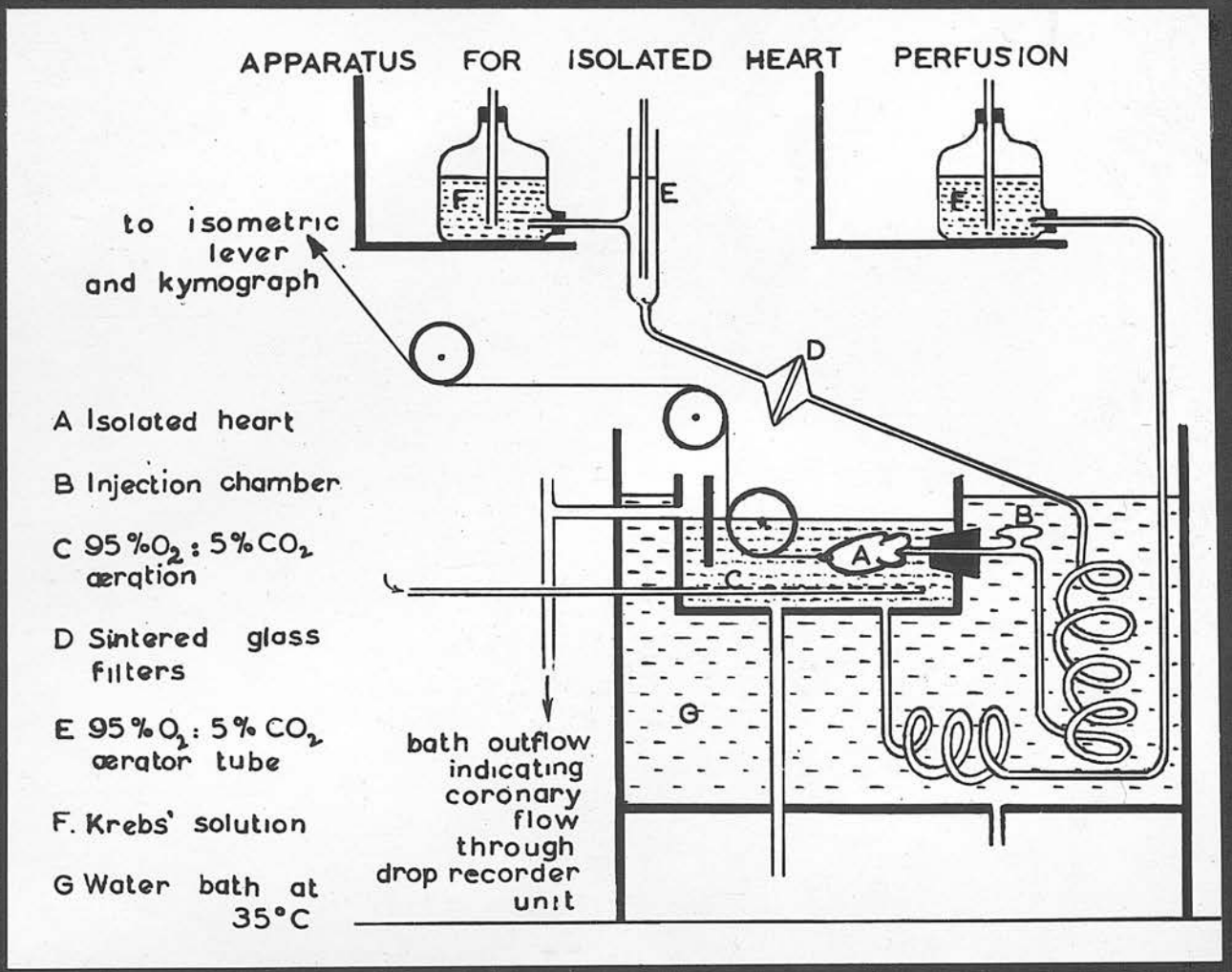


FIG. C.A.

THE ISOLATED HEART ORGAN-BATH.

4. THE ISOLATED SMOOTH MUSCLE OF THE INTESTINE AND URINARY BLADDER

Of the many clinical signs of quinuronium toxicity, micturition and defaecation are among the most obvious. It was therefore proposed to investigate the pharmacodynamics of the smooth muscle of the intestine and urinary bladder.

A. ISOLATED GUINEA PIG ILEUM.

(a) GENERAL PHARMACOLOGY

Small segments of terminal ileum were set up in the usual way in aerated Tyrode solution in an isolated organ-bath at 32°C.

Drugs. Acetylcholine, histamine, quinuronium, amicarbalide, atropine and mepyramine were used.

(b) THE TRENDELENBURG PREPARATION

On present evidence it was considered that useful information might be gained from testing the effect of quinuronium and atropine on the peristaltic activity of the guinea pig ileum. For this purpose the method of Trendelenburg (1917) was employed.

Drugs. Quinuronium (5 ug to 200 ug), carbachol (10 ug) and atropine (10 ug).

B. ISOLATED BLADDER STRIPS.

(a) Guinea Pig.

Guinea pig bladders were bisected longitudinally and each

half set up in the usual way in an isolated organ-bath in oxygenated Tyrode solution at 35°C.

(b) Sheep.

Bladders from freshly killed sheep were cut open longitudinally, washed with isotonic saline and immersed in cold Tyrode. Longitudinal strips (measuring 0.5 cm by 4 cms) were made through the whole thickness of the bladder. The mucosa was carefully removed and also all extraneous serosal attachments and each strip was set up in an organ-bath at 35°C in oxygenated Tyrode.

5. GLANDULAR SECRETION

During quinuronium therapy, in addition to signs of cardiovascular collapse and stimulation of smooth muscle, there is invariably profuse salivation in affected animals. Methods for measuring this stimulation of salivary secretion were investigated.

A. SECRETION OF PAROTID SALIVA.

Sheep were anaesthetised with intravenous thiopentone and maintained on pentobarbitone. The area of the mandible was clipped of hair and the parotid salivary duct located through a skin incision made near the angle of the jaw; (the duct lies lateral to the external maxillary artery and vein). The salivary duct was cannulated with fine bore polythene tubing.

After a space of several minutes, saliva began to flow and was passed through a photo-electric drop recorder connected to a

Thorpe impulse counter. Simultaneously, carotid blood pressure was recorded kymographically by means of a mercury manometer.

B. SECRETION OF GASTRIC ACID IN THE ANAESTHETISED RAT.

It was considered possible that the stimulation of salivary secretion by quinuronium might indicate more generalised activity on glandular secretion. Methods for investigating the effects on gastric hydrochloric acid secretion were considered:

Two strains of adult rats were used: brown-hooded and albino. Both sexes were employed in a random fashion.

The technique used was a simple modification of that described by Ghosh and Schild (1958).

All animals were anaesthetised with 25% w/v urethane at the rate of 0.7 ml per 100 gm. The trachea was cannulated and the pylorus of the stomach located through a midline incision behind the xyphisternum. A longitudinal incision was made in the duodenum near the pylorus and the end (a) of a glass tube of 4 mm diameter inserted and passed via the pylorus into the stomach and secured firmly by ligating the duodenum, carefully avoiding blood vessels (Fig. G.1.). The duodenal cannula (as shown) was clamped so that the 'U' bend (c) was inverted. This arrangement created a small back pressure into the stomach which maintained a slight degree of distension in the organ. This was found to be advantageous in stimulating a steady 'control' secretion of acid.

A polythene tube of 1.5 mm diameter and 15 cm long was passed via the mouth down the oesophagus and into the stomach. Its tip

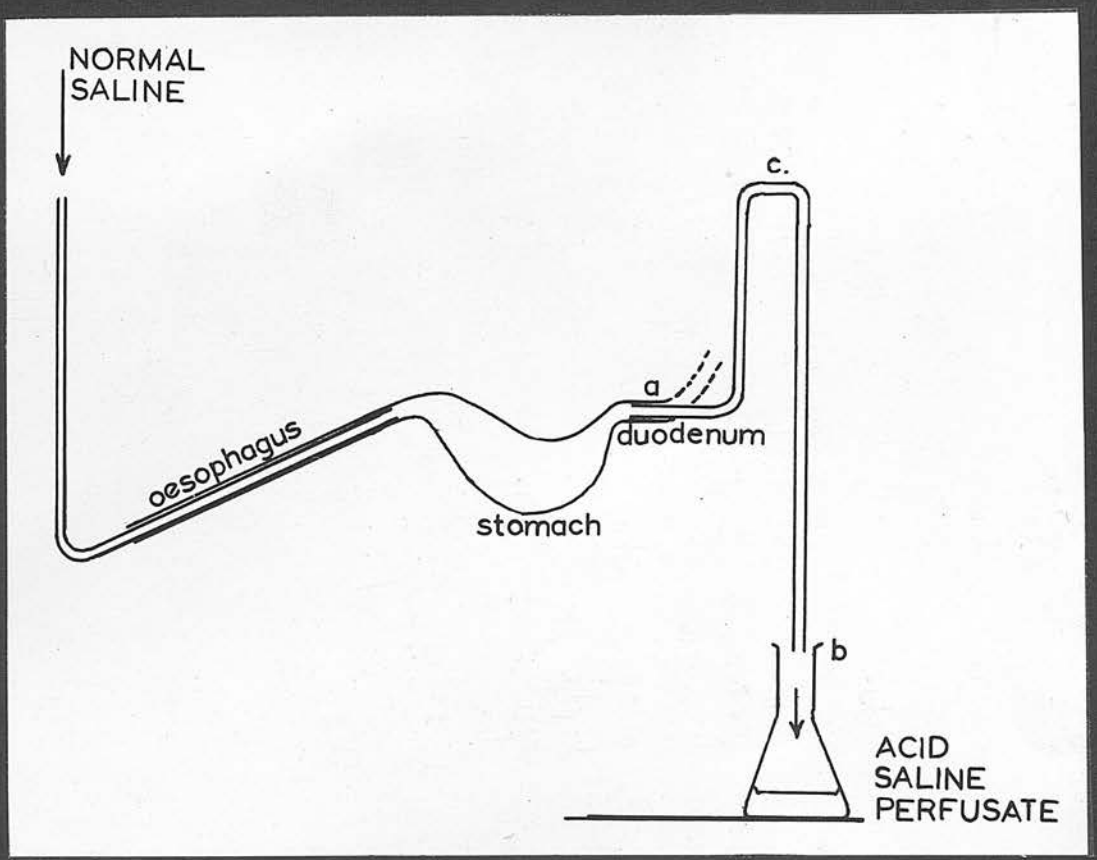


FIG. G.1.

DIAGRAM OF ROUTE OF SALINE PERFUSION OF THE STOMACH OF THE RAT.

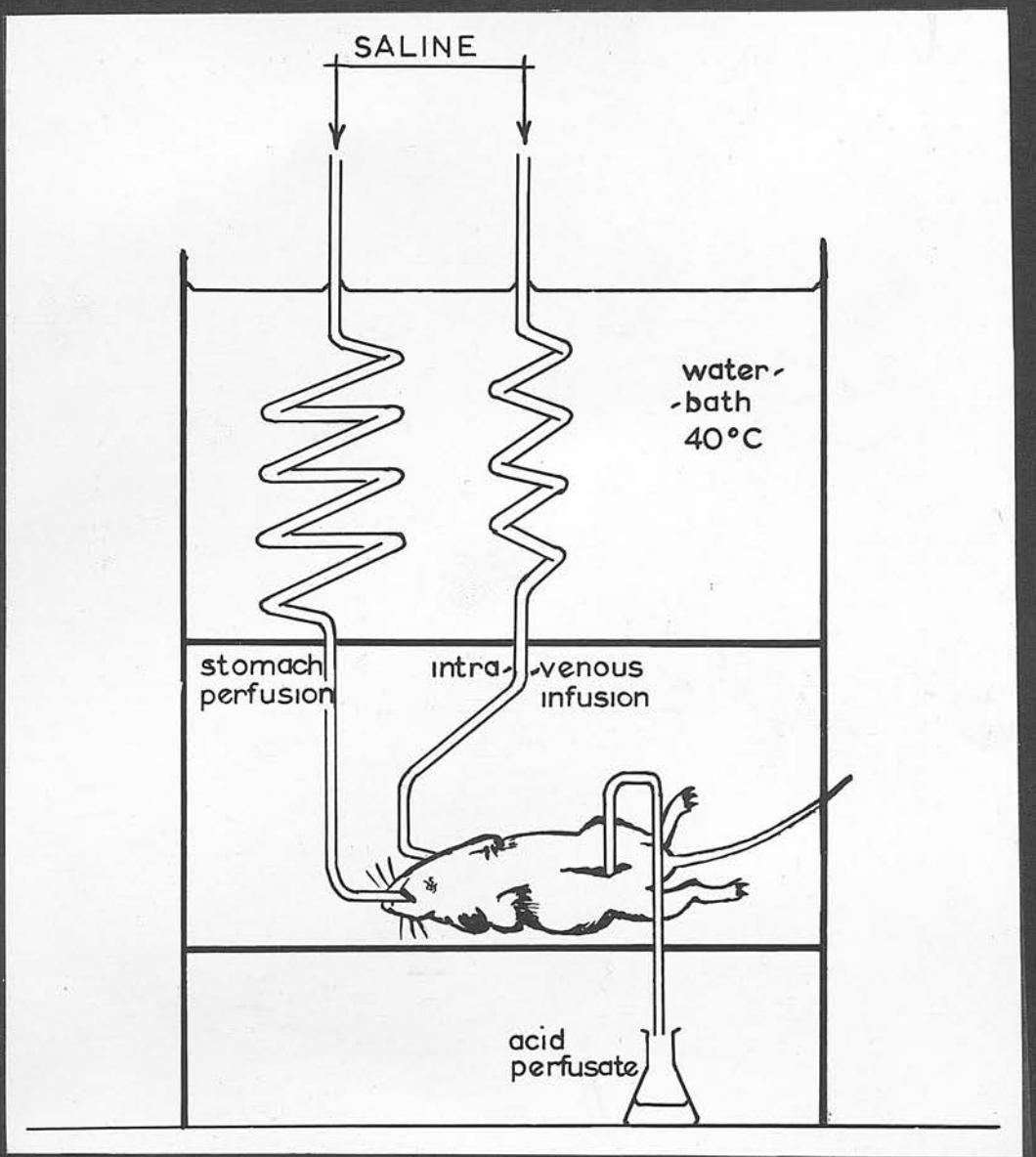


FIG. G.2.

APPARATUS FOR SALINE PERFUSION OF THE STOMACH OF THE RAT IN VIVO.

could be palpated with a finger in the abdomenal incision. When the tip of the tube had passed the cardiac sphincter, the cannula was secured by ligating the oesophagus. This oesophageal cannula was connected to an aspirator bottle of normal saline placed above the rat so that saline could flow through a water bath at 40°C to the stomach, (Fig. G.2.).

A steady flow of saline through the stomach was initiated and the organ gently palpated to dislodge and wash out food particles. By regulating a screw-clip on the saline reservoir a duodenal outflow of 0.5 cm to 1.0 ml per minute was established. This ensured that in each collection period the stomach volume had been cleared at least once.

Acid Estimation. Samples of stomach perfusate were collected over each ten minute period and immediately titrated against N/100 NaOH using methyl-red as an indicator.

Acid outflow was expressed as mls. N/100 NaOH required to neutralise each sample, or in terms of the equivalent in mls. N/100 HCl.

Pre-treatment samples were taken until three consecutive ten minute readings were approximately equal (arbitrarily within 10% of each other).

Results were plotted as histograms using acid as ordinate and time abscissa.

Drugs were administered via a cannula in the jugular vein and each compound was given over a period of five to ten minutes in small increments every minute, washed in with 0.1 ml normal saline: histamine acid phosphate; acetylcholine hydrochloride; compound

48/80; neostigmine hydrobromide; quinuronium sulphate; amicarbalide isethionate; atropine sulphate; mepyramine maleate.

The concentration of histamine is expressed in terms of base whereas all other compounds are given as their respective salts.

6. NEUROMUSCULAR TRANSMISSION

The methods employed so far have investigated the cholinergic effects of quinuronium in respect of parasympathetic (muscarinic) activity. Cholinergic substances including anticholinesterases, possess in addition to "muscarinic" actions certain "nicotinic" effects, and techniques for examining one of these (namely events at the skeletal neuromuscular junction) were now considered.

A. IN VITRO TECHNIQUES.

CHICK BIVENTER CERVICIS MUSCLE

Ginsborg and Warriner (1960) developed a method using the posterior belly of the chicken biventer cervicis muscle. The present technique is exactly as described by Ginsborg and Warriner, (1960) and the electrode assembly is depicted in Fig. NM1.

Drugs. Quinuronium sulphate; acetylcholine chloride; eserine salicylate; d-tubocurarine chloride; suxamethonium chloride. These were administered in not more than 0.2 ml normal saline and allowed to act for ten minutes or to maximum effect (whichever was shorter). Recovery was allowed over thirty minutes or to control (whichever was shorter).

B. IN VIVO TECHNIQUES.

INTRODUCTION

A method was required which would enable one to use a muscle (or muscles) with intact blood supply and attached normally to the bony skeleton in an animal under anaesthetic.

The method of recording contractions of the anterior tibial muscle in the anaesthetised cat described by Brown (1938) is well known and the following description bears some similarity in principle to this method.

(a) THE DIGITAL EXTENSOR MUSCLE GROUP OF THE CHICKEN PELVIC LIMB.

The anatomical description is given by Chamberlain (1943). By reference to Figs. NM 2, 3 and 4, the anatomical set-up can be seen. Fig. NM2 shows diagrammatically the musculature of the anterior aspect of the chicken hind limb. The two muscles involved are the tibialis anterior and the extensor digitus longus which both arise on the tibio-fibula between the lateral head of the gastrocnemius and medially the peroneus longus muscle. Fig. NM3 shows the lateral aspect of the right pelvic limb of the chicken with the skin removed, (Chamberlain, 1943). The anterior tibial muscle is inserted into the proximal end of the metatarsus whereas the extensor digitus longus makes insertions into each of the three main digits. Both muscles flex the tibiotarsal joint but only the long extensor extends the digits proper.

The bulk of the biceps femoris muscle (Fig. NM3) was carefully ligated at its insertion near the femoro-tibial joint; cut and freed (point A); (Fig. NM4). The body of the biceps muscle was carefully reflected (removing fascia) as far up towards the pelvis

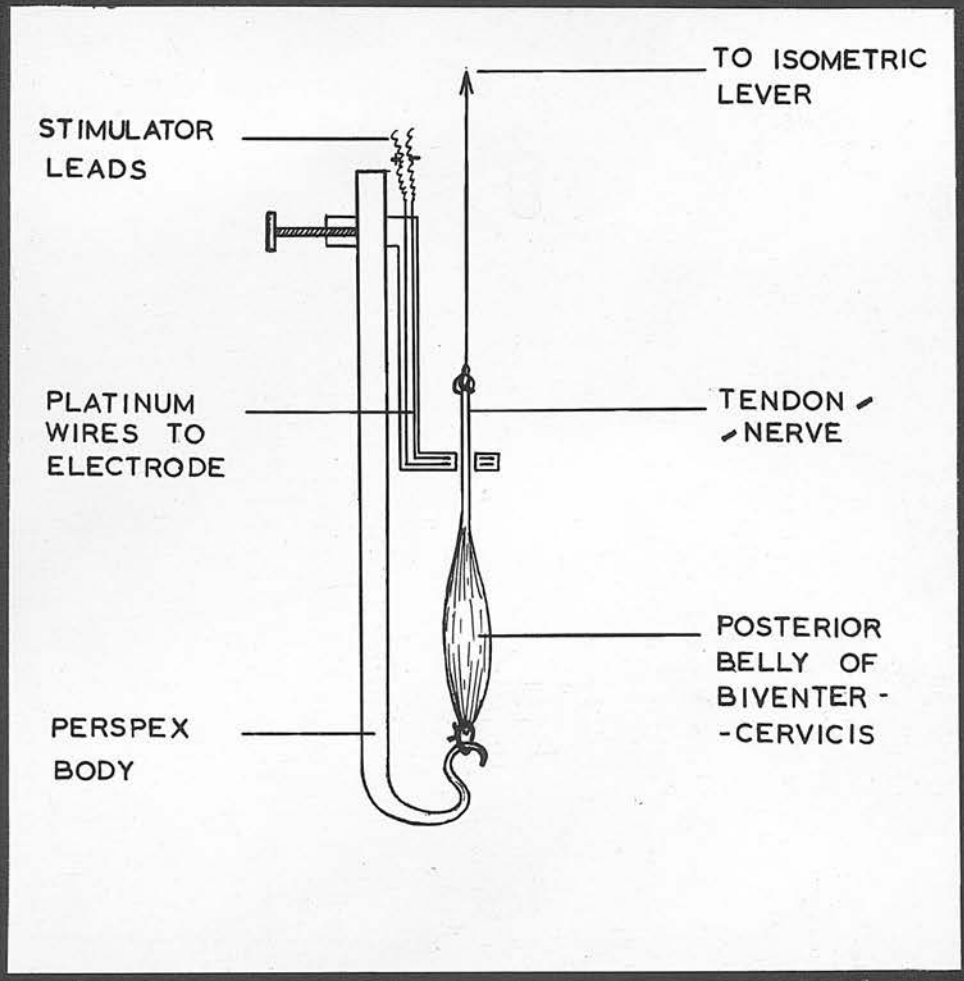


FIG. NM 1.

THE ELECTRODE ASSEMBLY FOR CHICKEN BIVENTER-CERVICIS NERVE MUSCLE PREPARATION.

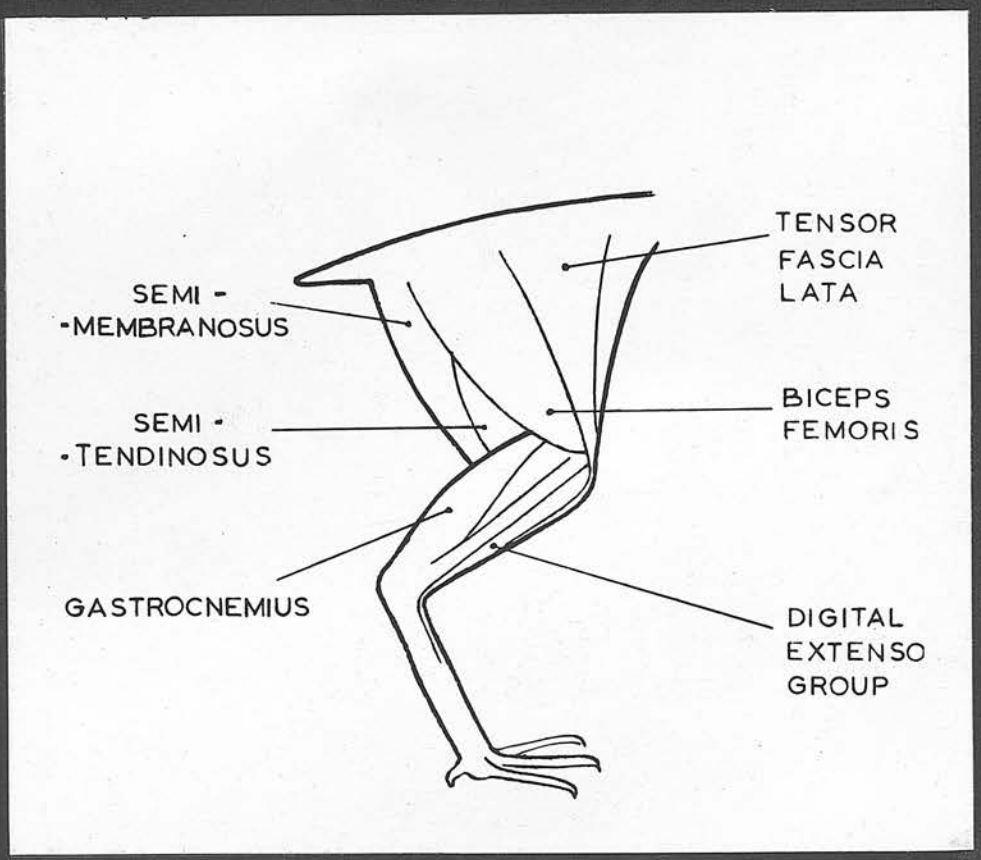


FIG. NM 3.

LATERAL ASPECT OF THE PELVIC LIMB OF THE DOMESTIC FOWL WITH SKIN REFLECTED SHOWING THE "IN SITU" MUSCLE ARRANGEMENT.

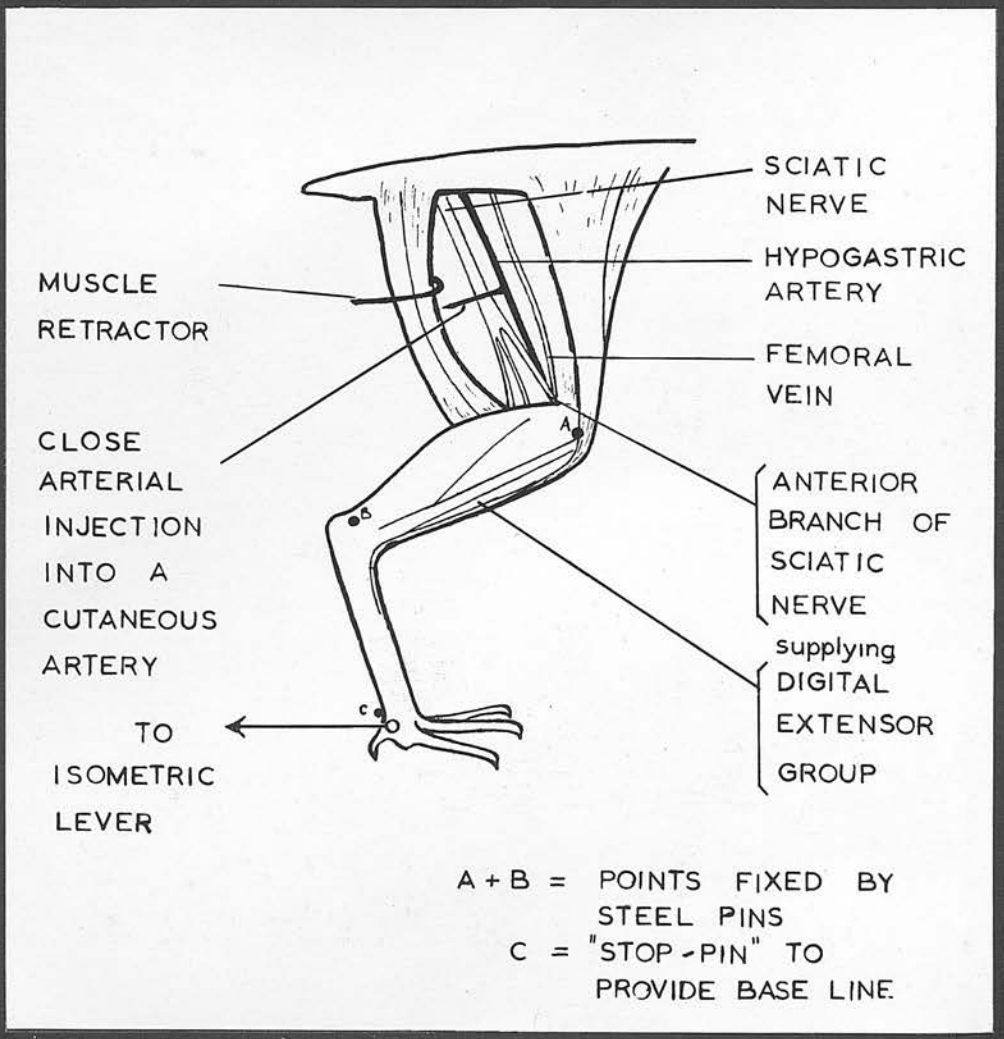


FIG. NM 4.

DEEP DISSECTION OF THE PELVIC LIMB OF THE DOMESTIC FOWL WITH BICEPS FEMORIS REFLECTED AND SEMI-TENDINOSUS RETRACTED TO REVEAL THE SCIATIC NERVE AND FEMORAL BLOOD VESSELS.

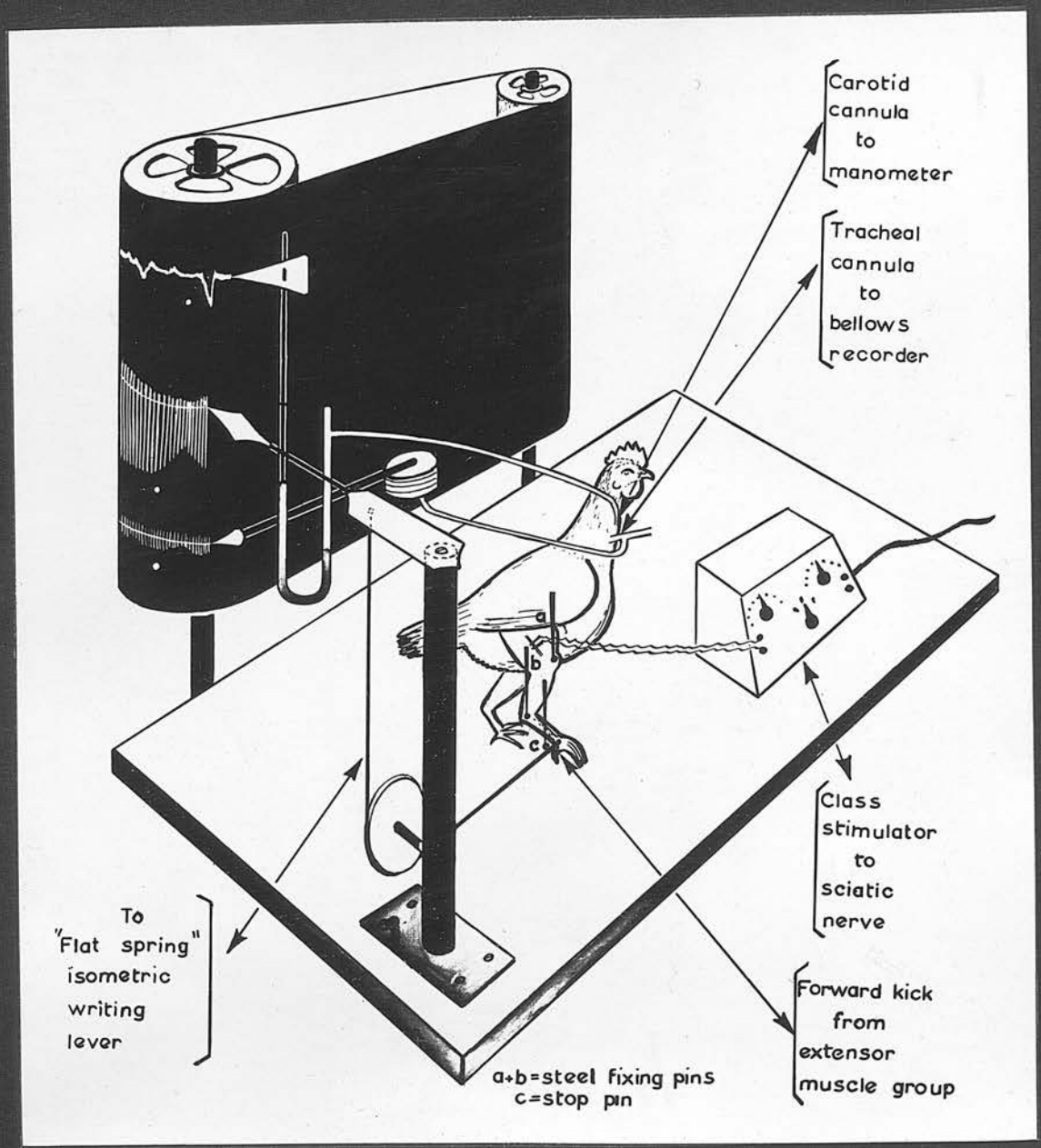


FIG. NM. 5.

APPARATUS FOR RECORDING CONTRACTIONS OF THE EXTENSOR MUSCLE GROUP OF THE PELVIC LIMB OF THE DOMESTIC FOWL, WITH SCIATIC NERVE STIMULATION.

as possible. The muscle was then cut across near to the pelvis and its body completely removed. Bleeding points were picked up with artery forceps and tied off individually, as it was virtually impossible to apply an efficient mass ligature on so little residual tissue. The complete removal of the biceps femoris greatly facilitated the following procedures. With a small muscle retractor, the bodies of the semi-membranosus and semi-tendinosus muscles were withdrawn posteriorly as shown in Fig. NM4. This procedure clearly revealed the hypogastric artery, the femoral vein and the sciatic nerve which were seen lying on the "floor" of the cavity created by the removal of the biceps muscle.

/ The sciatic nerve lies in the most posterior position in this cavity and divides before reaching the femorotibial joint into three branches, the most anterior of which is the nerve trunk which supplies the digital extensor muscles. Lying close to this is the main hypogastric artery which supplies all the lower limb. From the main artery arise numerous branches. One of these is a large cutaneous vessel shown in Fig. NM4 and another is the gastrocnemius artery which arises near the femorotibial joint. Either vessel is suitable for giving close arterial injections into the hypogastric trunk and may be cannulated with 1 mm diameter polythene tubing or alternatively a small size (sawn off) intravenous needle. These cannula may then be connected to a 1 ml all glass "tuberculin" syringe by means of flexible tubing, for the purpose of giving intra-arterial injections. /

The anterior branch of the sciatic nerve was freed of fascia

and the electrode passed underneath. [The electrode comprised two silver wires which were curved in a half circle and set in perspex or other insulating material.] When the artery was cannulated and the electrode fixed in position under the nerve the "muscle cavity" was filled with medicinal paraffin and the skin closed over the site.

In order to free the movement of the tibiotarsal joint all muscle tendon attachments were severed save those of the tibialis and long extensor. Basically this amounted to "ham-stringing" the limb.

All this completed and the animal lying on its side, the limb was fixed rigidly in a semi-extended position by the use of intra-medullary surgical pins A. and B., which passed respectively through the femorotibial and the region of the tibiotarsal joints (shown in Figs. NM4 and NM5): pin B. avoided the actual joint in order to allow the free movement of the latter. Both pins A. and B. were clamped above the limb and their pointed lower ends passed into the soft wood of the base board.

A strong linen thread was attached to the foot near the small digit and the thread passed over a pulley to an isometric "spring" lever which inscribed on a smoked kymograph drum, (Fig. NM5).

The "kick" of the limb was in a forward direction and so a third pin - C.- was driven into the base board behind the limb and acted as a "stop" for the resting limb, i.e. provided a firm and constant base line for the kymograph tracing.

Stimuli were supra-maximal from a "Palmer" Student-Class stimulator at a rate of 12 short rectangular wave impulses per

minute.

In addition to this procedure, the blood pressure of the animal was taken from the carotid artery and recorded kymographically via a mercury manometer.

Simultaneously, respiratory movements were recorded from a tracheal cannula connected with a writing bellows recorder.

Also a jugular intravenous cannula enabled drug-sulutions to be injected by the alternative route.

Drugs. Quinuronium sulphate; acetylcholine chloride; d-tubocurarine chloride. These were injected in a maximum of 0.2 ml normal saline and allowed to act for ten minutes. Recovery was indicated by a return to normal, or after thirty minutes, whichever was shorter.

(b) THE DIGITAL EXTENSOR MUSCLE GROUP OF THE RABBIT HIND LIMB.

This experiment involved similar techniques to those described above for the chicken. Adult rabbits of approximately 3 kilo weight were anaesthetised with urethane and the neuromuscular preparation made as described for the chicken.

Respiratory movements were recorded by a thread attached to the rib-cage, connected via a pulley wheel system to a spring loaded lever.

Drugs. Quinuronium sulphate; d-tubocurarine chloride, suxamethonium chloride.

RESULTS.

1. CAROTID BLOOD PRESSURE AND RESPIRATION

(a) Rat.

Acetylcholine (0.2 to 1.0 ug) invariably produced hypotension (Dale, 1914) and adrenaline (0.5 ug) typically produced a rise in blood pressure. It was noticable that histamine (0.5 to 1.0 ug) produced a variable effect. Of the five rats recorded, histamine showed a pressor response in two, and a depressor response in three animals. Quinuronium (2 to 10 ug) always had the same effect as histamine whether pressor or depressor. Atropine sulphate (500 ug) reversed the hypotensive effects of acetylcholine (Figs. BP. 1 & 2) and of quinuronium. However, mepyramine maleate (200 ug) completely blocked the action of both histamine and quinuronium (Fig. BP 3). Amicarbalide had no measurable effect on carotid blood pressure in this species.

(b) Chicken, Guinea Pig and Rabbit.

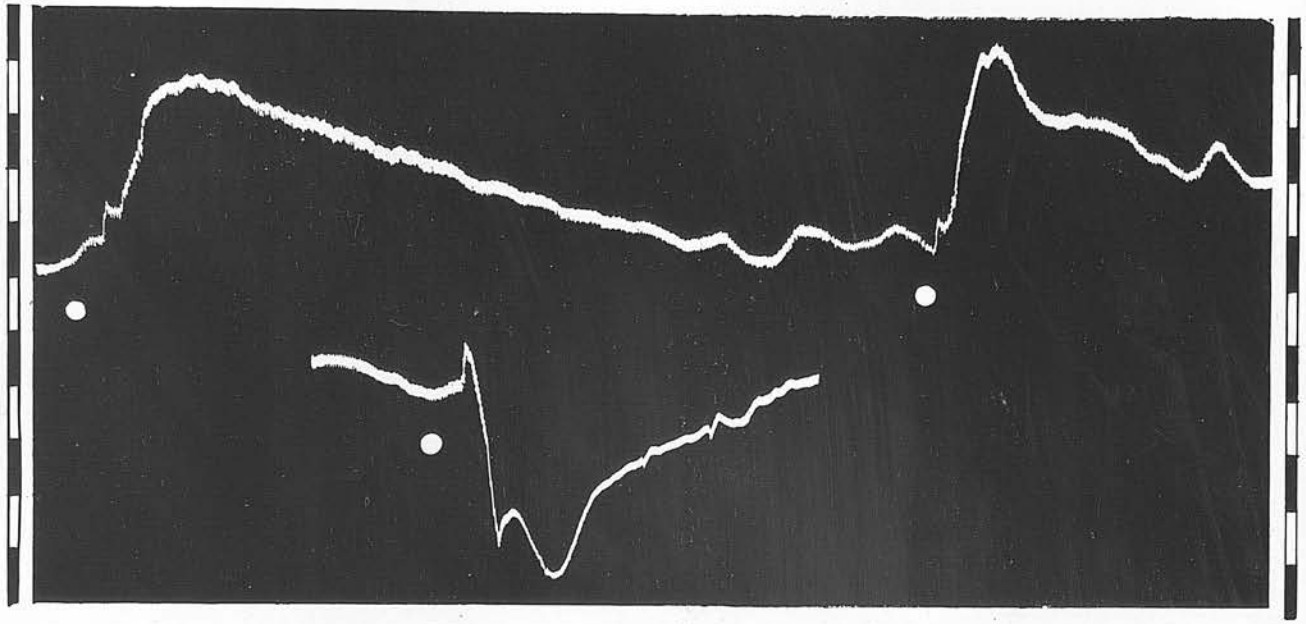
In these species quinuronium always produced a fall in blood pressure which could not be modified significantly by either atropine or mepyramine in the case of guinea pig and chicken; but in the rabbit, atropine afforded some protection.

(c) Sheep.

In sheep, acetylcholine (50 to 100 ug), histamine (75 to 100 ug) and quinuronium (50 ug to 50 mgm) produced falls in blood pressure, whereas adrenaline (20 ug) was typically pressor throughout the experiments.

In Figures BP 4 (a) and (b) it can be seen also that

Rat blood pressure



HIST 0.35µg ACH 0.75µg P 5.0µg

FIG. BP 1.

CAROTID BLOOD PRESSURE OF THE RAT. HIST. = HISTAMINE BASE; ACH. = ACETYLCHOLINE CHLORIDE; P. = QUINURONIUM SULPHATE.

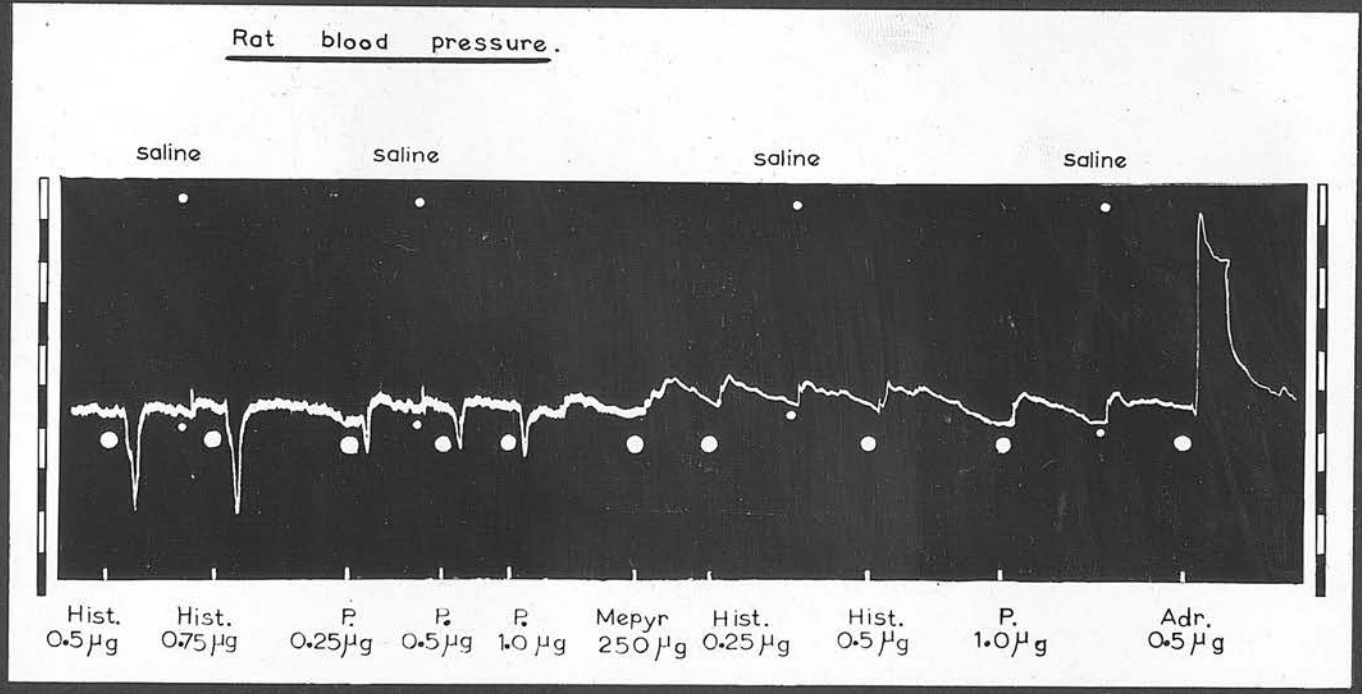


FIG. BP 3.

CAROTID BLOOD PRESSURE OF THE RAT. HIST. = HISTAMINE BASE; P. = QUINURONIUM SULPHATE; MEPYR. = MEPYRAMINE MALEATE; ADR. = ADRENALINE TARTARATE.

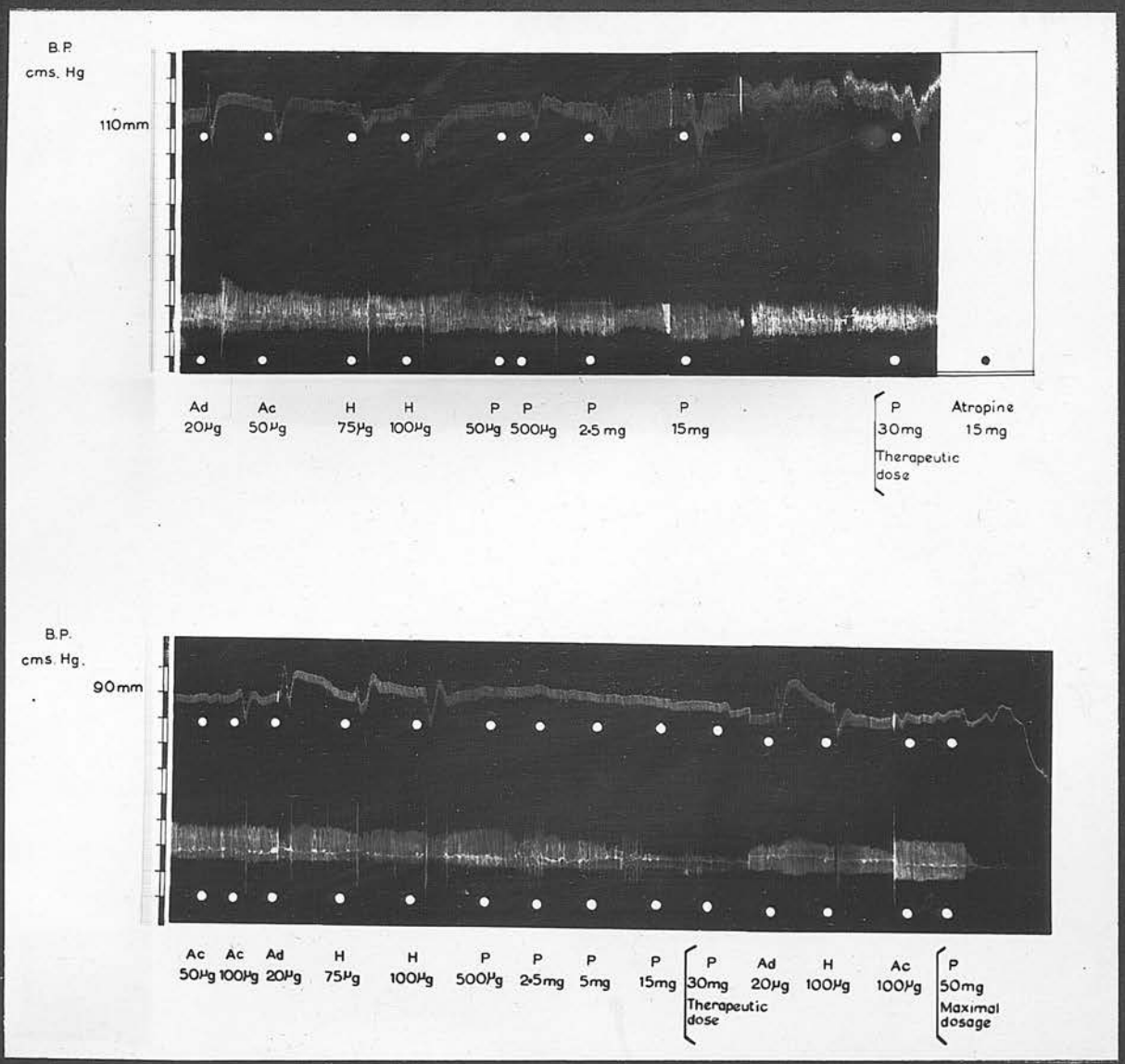


FIG. BP 4 (a)

CAROTID BLOOD PRESSURE OF SHEEP. Ad = ADRENALINE TARTARATE; Ac = ACETYLCHOLINE CHLORIDE; H = HISTAMINE BASE; P = QUINURONIUM SULPHATE.

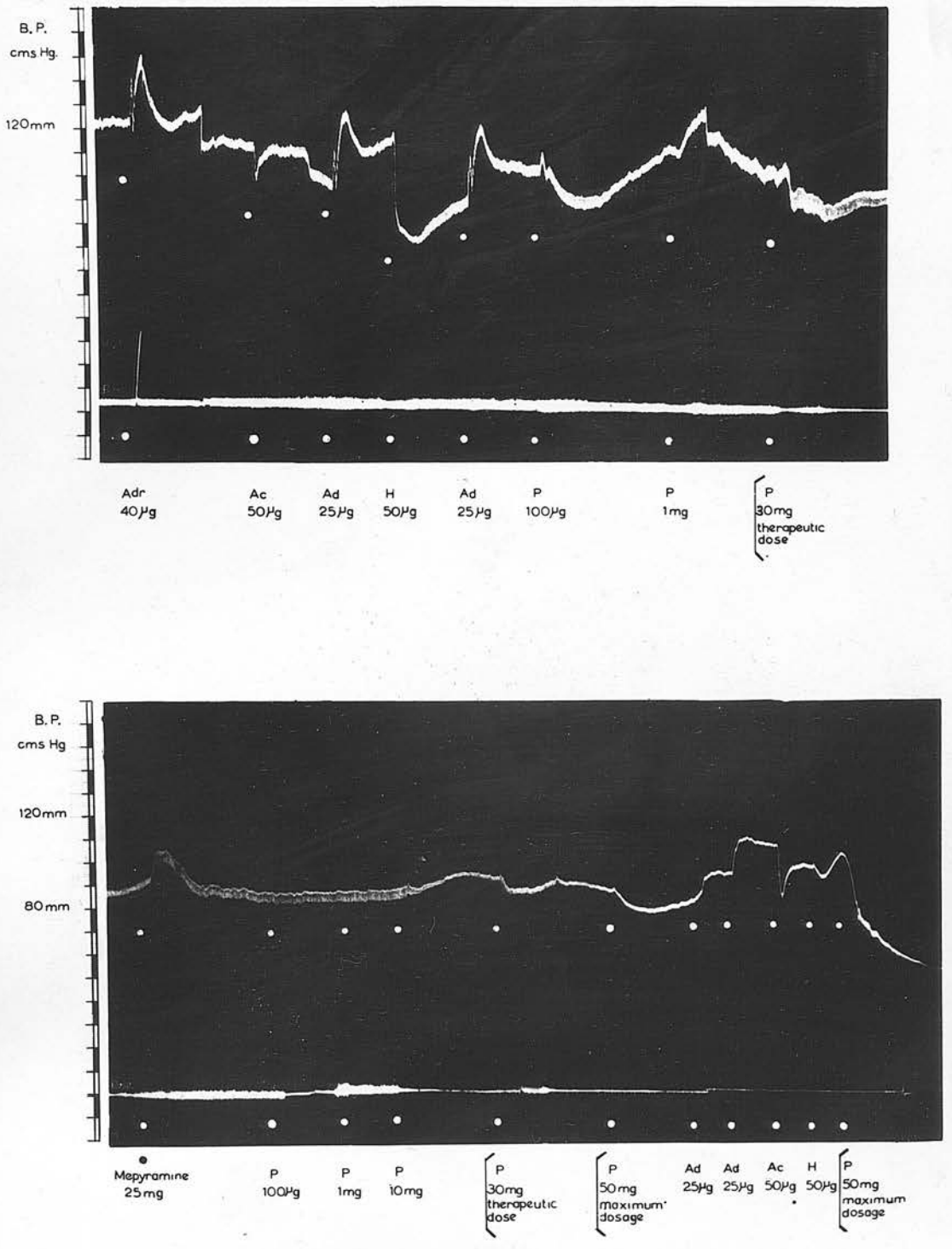


FIG. BP 4 (b)

CAROTID BLOOD PRESSURE OF SHEEP. Adr/Ad = ADRENALINE TARTARATE; Ac = ACETYLCHOLINE CHLORIDE; H = HISTAMINE BASE; P = QUINURONIUM SULPHATE.

quinuronium produced a reduction both in rate and amplitude of respiration. A therapeutic dose of quinuronium (10 to 30 mgm) reduced the tidal volume by 50% and larger doses (e.g. 50 mgm) produced temporary cessation of respiratory activity.

Atropine (1 mgm per kilo) effectively blocked the action of quinuronium up to a dose of 20 to 30 mgm (1 to 1.5 mgm per kilo) but failed to protect sheep from death following an intravenous dose of 50 mgm quinuronium (Fig. BP 4 a.). Mepyramine blocked the depressor effect of histamine and antagonised quinuronium up to 10 mgm (see Fig. BP 4 b.). A 30 mgm dose of quinuronium was much reduced in its action, whereas 50 mgm of quinuronium on the first injection produced prolonged hypotension and a second 50 mgm killed the sheep.

Atropine and mepyramine did not modify the effect of quinuronium on respiration.

Amicarbalide had no effect on the blood pressure of the sheep.

2. ACTIONS ON PERIPHERAL BLOOD VESSELS

A. THE ISOLATED PERFUSED RABBIT EAR.

Adrenaline (0.25 ug), acetylcholine (10 ug), histamine (10 ug) and quinuronium (1 mgm) were approximately equally vasoconstrictor, (Figs P3 a. & b. and P4); however, quinuronium had a much more prolonged effect than any other agent used. After atropinisation of the ear (1 mgm) acetylcholine failed to show a response, whereas quinuronium continued to cause vasoconstriction for some time, but over a period of approximately two hours the response to quinuronium gradually diminished but

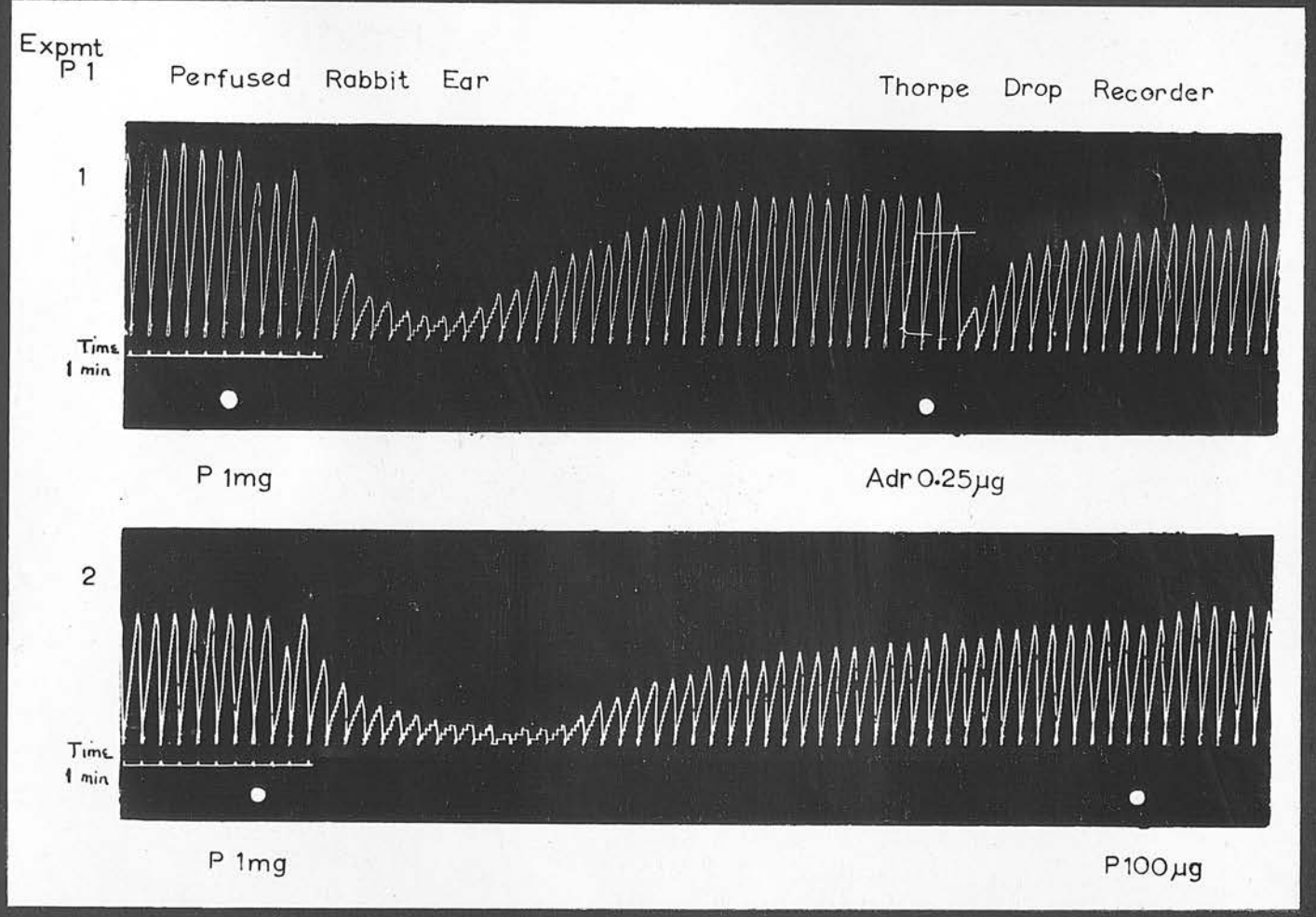


FIG. P.3 (a).

VENOUS OUTFLOW FROM ISOLATED SALINE-PERFUSED RABBIT EAR.
P. = QUINURONIUM SULPHATE; ADR. = ADRENALINE TARTARATE.

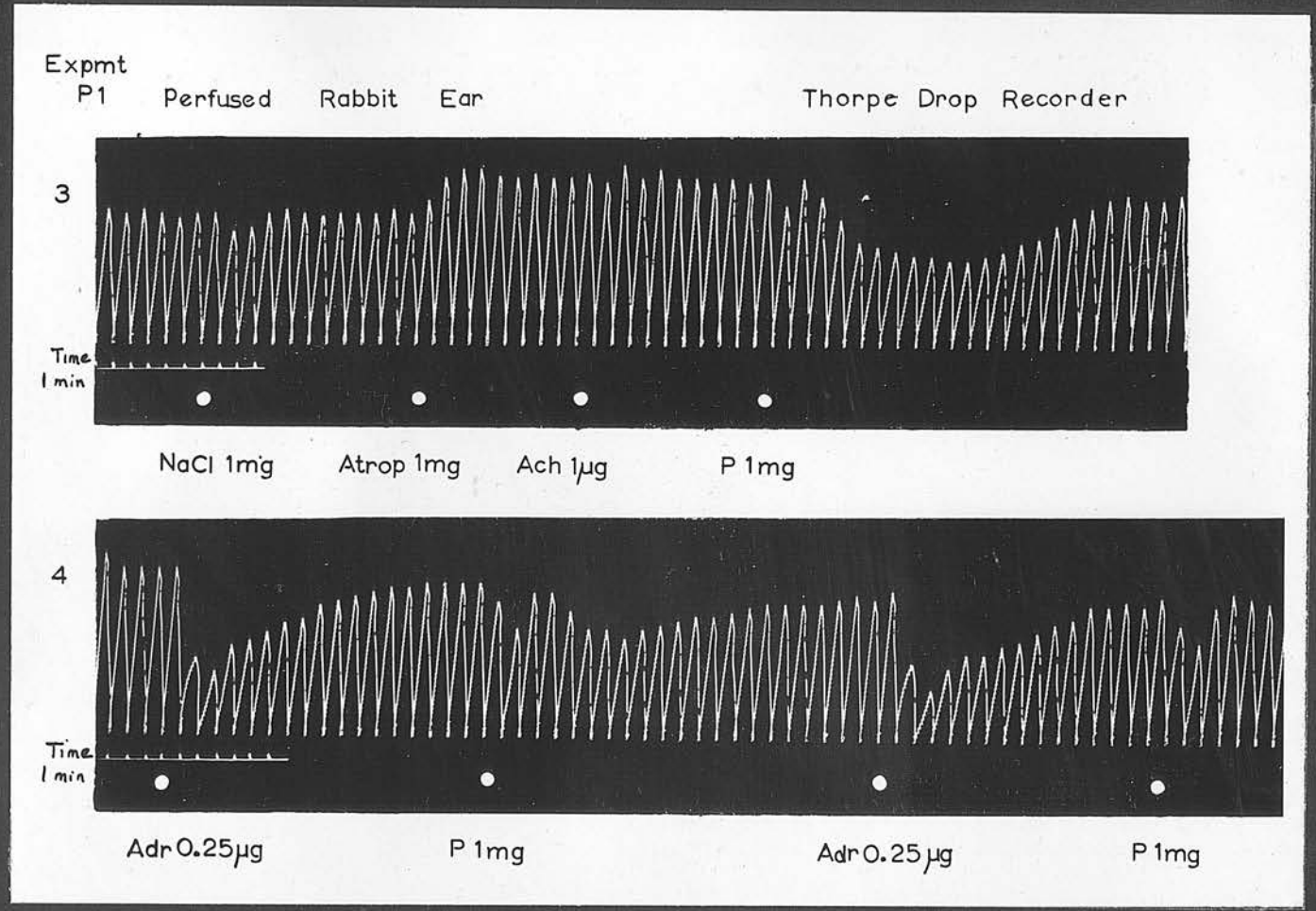


FIG. P.3 (b).

VENOUS OUTFLOW FROM ISOLATED SALINE-PERFUSED RABBIT EAR.
NaCl = SODIUM CHLORIDE; ACH. = ACETYLCHOLINE CHLORIDE;
ADR. = ADRENALINE TARTARATE; P. = QUINURONIUM SULPHATE;
ATROP. = ATROPINE SULPHATE.

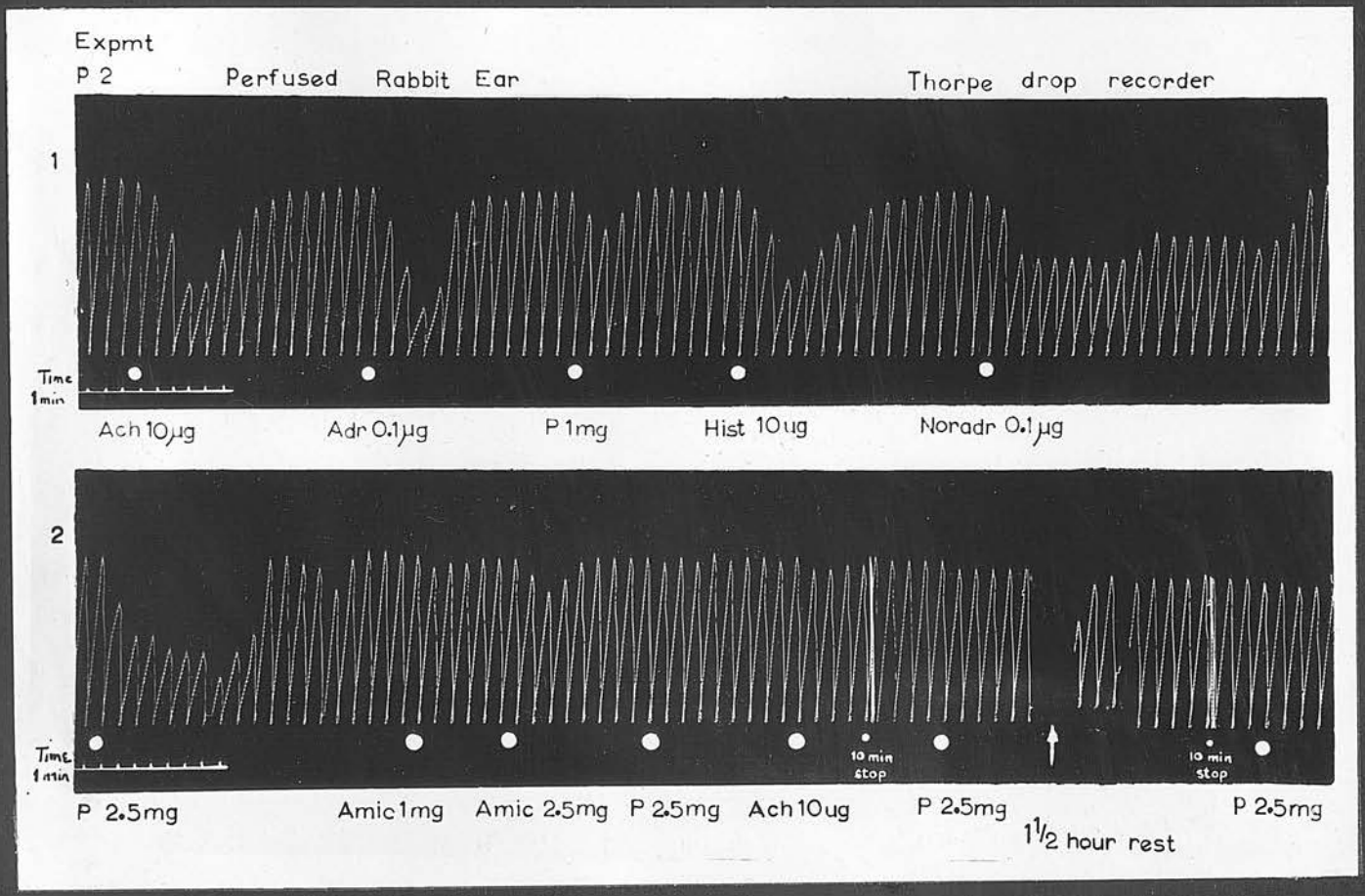


FIG. P.4.

VENOUS OUTFLOW FROM ISOLATED SALINE-PERFUSED RABBIT EAR. ACH. = ACETYLCHOLINE CHLORIDE; ADR. = ADRENALINE TARTARATE; NORADR. = NORADRENALINE; HIST. = HISTAMINE BASE; P. = QUINURONIUM SULPHATE; AMIC. = AMICARBALIDE ISETHIONATE.

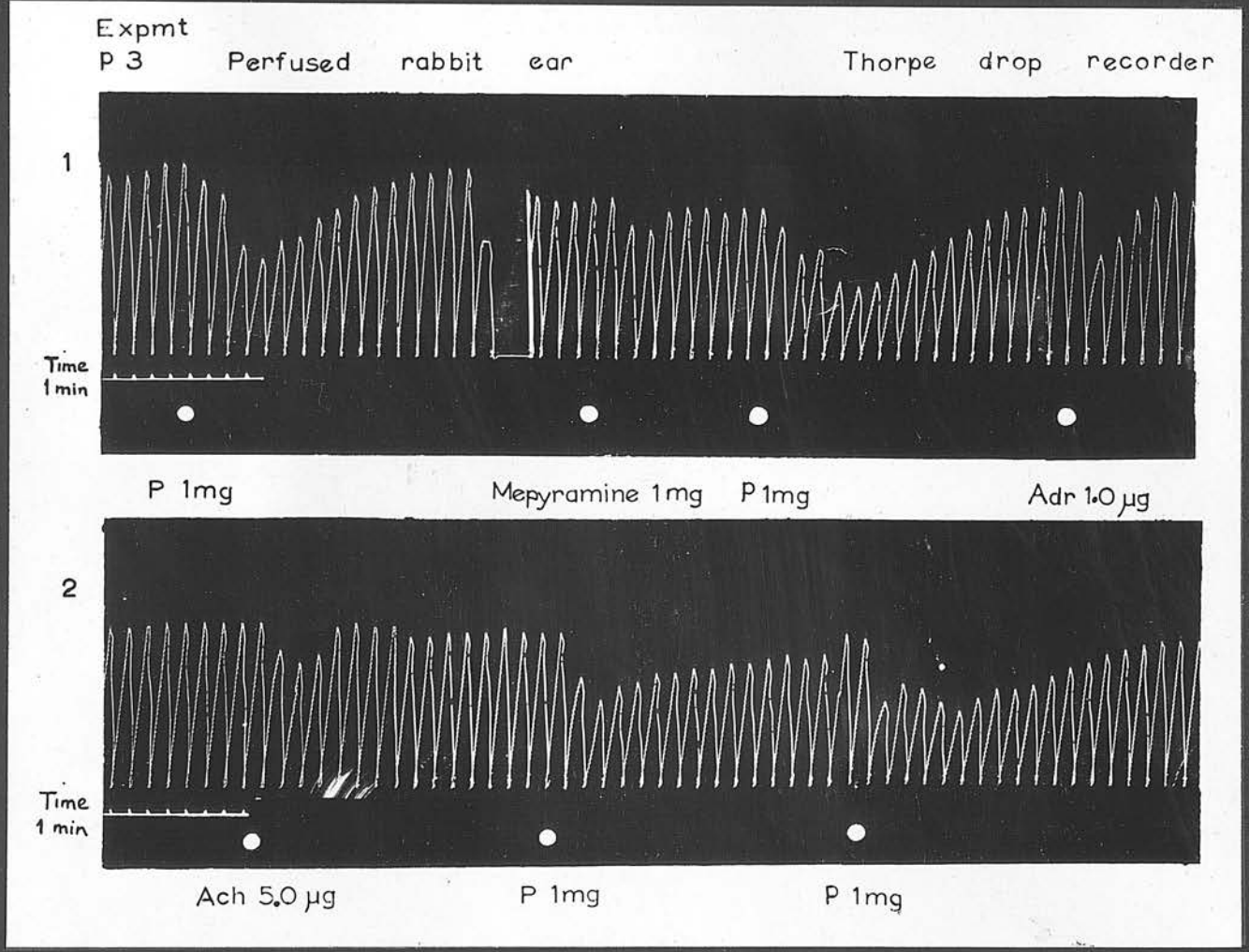


FIG. P.5.

VENOUS OUTFLOW FROM ISOLATED SALINE-PERFUSED RABBIT EAR. ACH. = ACETYLCHOLINE CHLORIDE; ADR. = ADRENALINE TARTARATE; P. = QUINURONIUM SULPHATE.

was never completely abolished. In another experiment mepyramine was injected (1 mgm) and this failed to show any antagonistic effect on the action of quinuronium (Fig. P5). In each experiment, adrenaline produced the same effect throughout.

Amicarbalide failed to elicit any response in the ear, but after amicarbalide (Fig. P4) quinuronium and acetylcholine failed to show responses.

B. SMALL INTESTINE AND HIND LIMB PLETHYSMOGRAPHY IN SHEEP.

(i) Gut.

Acetylcholine (50 ug), adrenaline (25 ug) and histamine (50 ug) all produced a distinct but transient constriction in the vascular volume of the bowel segment. Quinuronium (100 ug to 10 mgm) tended to produce very slight and sometimes prolonged dilation of the vascular bed of the bowel, which remained unaffected after mepyramine (25 mgm). Atropine (10 mgm) on the other hand, tended to abolish the quinuronium response, (see Fig. P6).

(ii) Limb.

Acetylcholine (50 ug) produced slight vasoconstriction of short duration followed by a marked vasodilation. This effect was simulated by quinuronium (100 ug to 10 mgm) which also showed slight initial constriction followed by slight dilation and increase in pulse volume. Histamine (50 ug) produced a vasoconstriction in the limb and decreased pulse volume; whereas adrenaline showed strong vasodilation and increased pulse volume. Mepyramine (25 mgm) caused abolition of quinuronium vasodilation

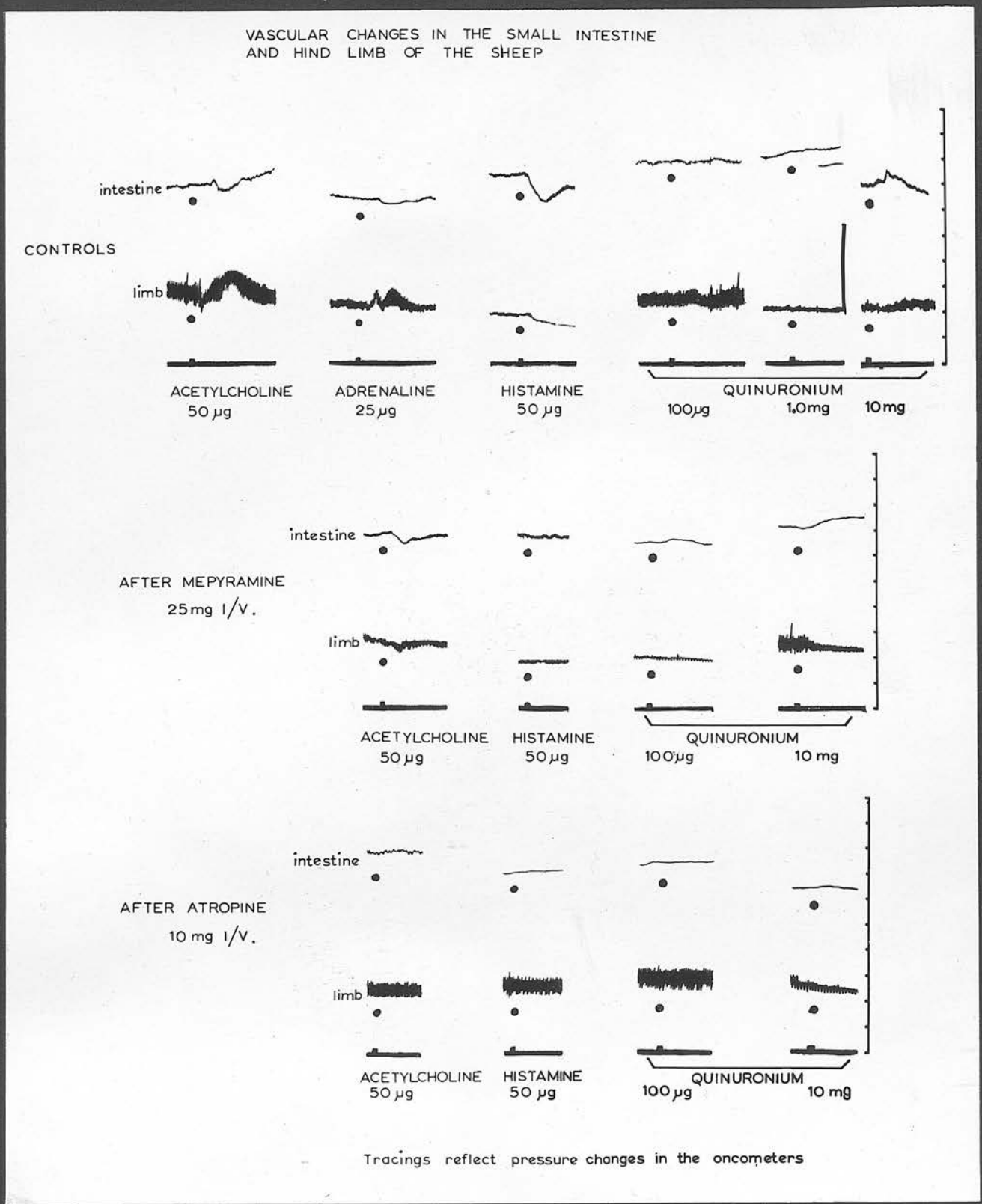


FIG. P.6.

CHANGES IN VASCULAR VOLUME IN SMALL INTESTINE AND HIND LIMB OF SHEEP.

TABLE C1.

THE RESPONSE OF RABBIT HEARTS TO PERFUSION OF ACETYLCHOLINE, ADRENALINE, NORADRENALINE, HISTAMINE, QUINURONIUM AND AMICARBALIDE, AND THE INFLUENCE OF ATROPINE AND MEPYRAMINE

DRUG	DOSE ug	PER CENT CHANGE IN AMPLITUDE OF CONTRACTION		
		DRUG ALONE	AFTER ATROPINE 1 mgm	AFTER MEPYRAMINE 1 mgm
ACETYLCHOLINE	1.0	-29 (± 7)	0	
ADRENALINE	5.0	+25 (± 6)		
NORADRENALINE	1.0	+118 (±42)		
HISTAMINE	50.0	+49 (±21)		+24 (± 8)
QUINURONIUM	100.0	+30 (±10)		
	500.0	+78 (±28)	+55 (±16)	+76 (±23)
AMICARBALIDE	2 mgm	0		

The values are means (4 experiments); and standard errors are shown in brackets.

in the low dose range, however 10 mgm quinuronium still produced a vasoconstriction. Mepyramine (25 mgm) followed by atropine (10 mgm) abolished all vaso-activity of quinuronium up to and including a dose of 10 mgm (Fig. P6).

3. ISOLATED HEART OF RABBIT AND GUINEA PIG

(i) Rabbit Heart.

Acetylcholine (1 ug) produced a typical reduction in rate and amplitude of the beating heart. Adrenaline (1 to 5 ug), noradrenaline (1 to 5 ug), histamine (10 to 50 ug) and quinuronium (100 to 500 ug) increased the amplitude of contraction, see Table C.1. and Figs. C1 & C2. Amicarbalide was without effect.

Effect of antagonists. The doses of agonist compounds were adjusted to produce approximately equal responses.

Atropine (1 mgm) itself tended to depress the amplitude of contraction of the heart. It was thus difficult to assess the degree of change in amplitude. This was however measured as the percentage change in amplitude of contraction. Atropine did not alter the increase in amplitude induced by quinuronium, although the effect of acetylcholine was abolished completely, (Table C.1.).

Mepyramine (1 mgm) itself depressed the amplitude of the heart by 60%, but did not modify the effect either of quinuronium or histamine (see Table C.1.).

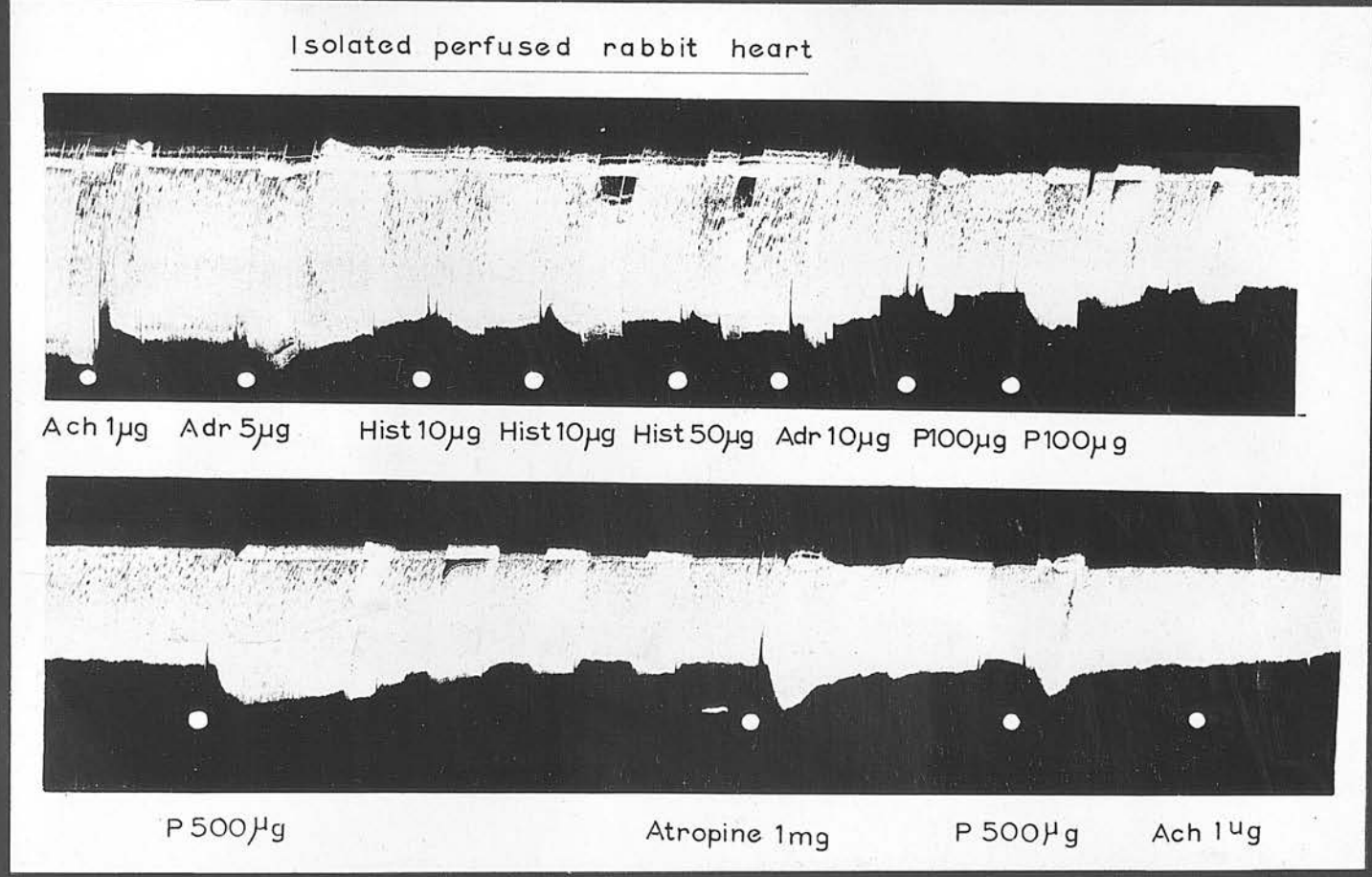


FIG. C.1.

RABBIT HEART. (DRUGS WERE INJECTED INTO THE PERFUSION FLUID). ACH. = ACETYLCHOLINE CHLORIDE; ADR. = ADRENALINE TARTARATE; HIST. = HISTAMINE BASE; P. = QUINURONIUM SULPHATE.

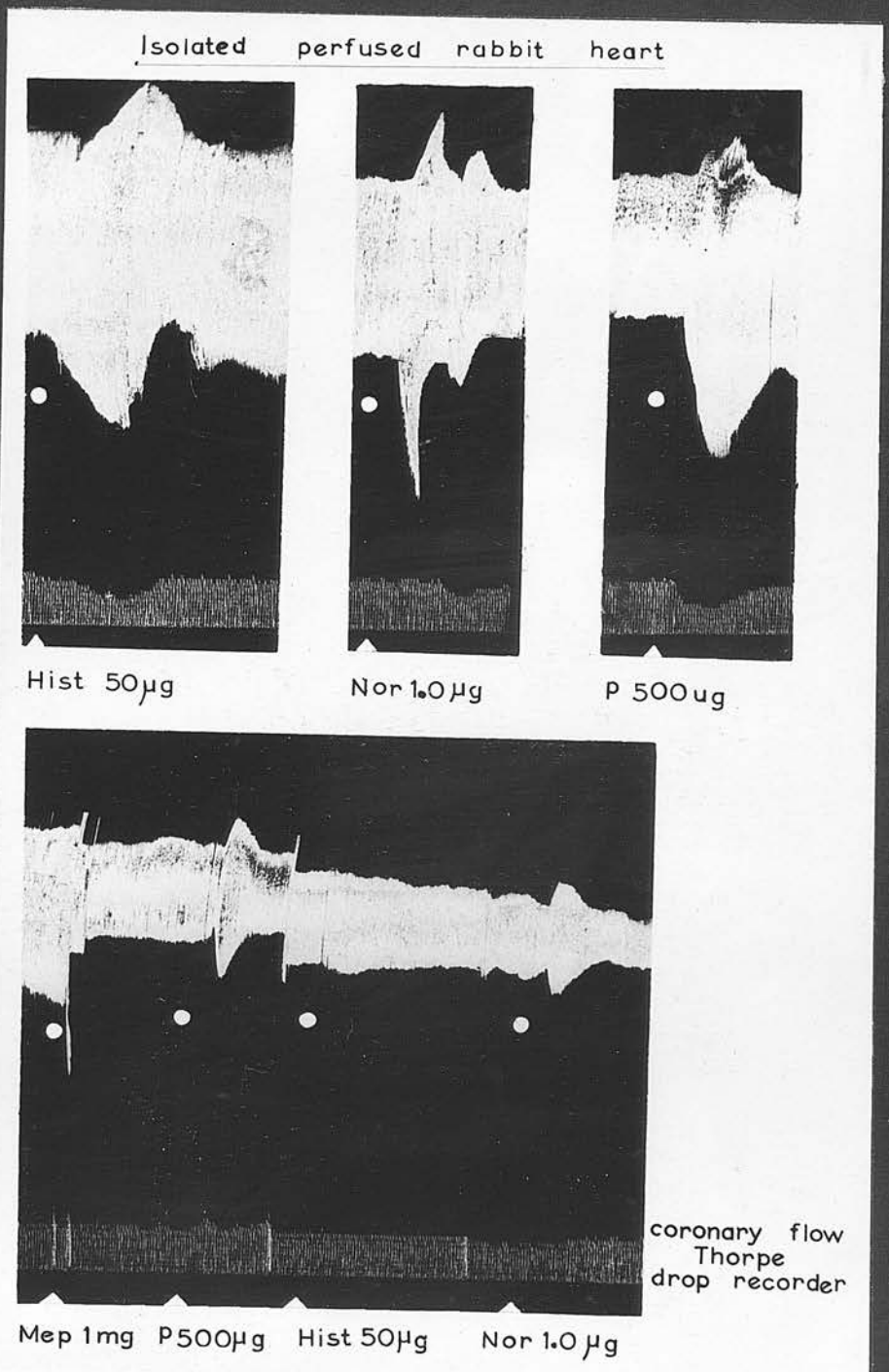


FIG. C.2.

RABBIT HEART. LOWER TRACING REPRESENTS CORONARY FLOW. HIST. = HISTAMINE BASE; NOR. = NORADRENALINE; P. = QUINURONIUM SULPHATE; MEP. = MEPYRAMINE MALEATE.

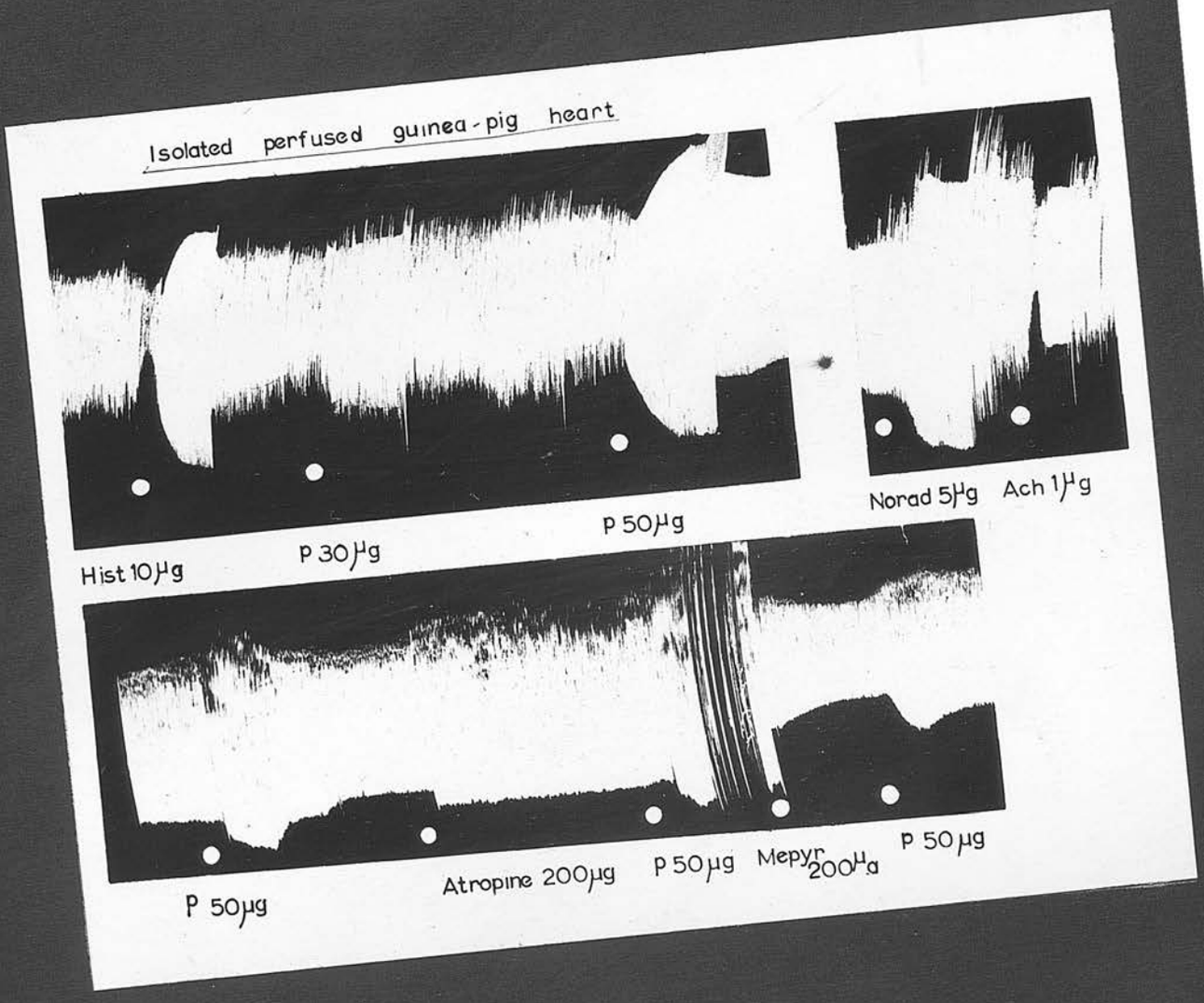


FIG. C.3.

GUINEA PIG HEART. HIST. = HISTAMINE BASE; P = QUINURONIUM SULPHATE; Norad = NORADRENALINE; Mepyr = MEPYRAMINE MALEATE; Atropine = ATROPINE SULPHATE.

TABLE C2.

THE RESPONSE OF GUINEA PIG HEARTS TO PERFUSION OF ACETYLCHOLINE, NORADRENALINE, HISTAMINE, QUINURONIUM AND AMICARBALIDE, AND THE INFLUENCE OF ATROPINE AND MEPYRAMINE

DRUG	DOSE ug	PER CENT CHANGE IN AMPLITUDE OF CONTRACTION		
		DRUG ALONE	AFTER ATROPINE 200 ug	AFTER MEPYRAMINE 200 ug
ACETYLCHOLINE	1.0	-50 (±13)	-5 (± 3)	
NORADRENALINE	5.0	+86 (±23)		
HISTAMINE	10.0	+60 (±18)		+48 (±15)
QUINURONIUM	30.0	+27 (± 9)		
	50.0	+55 (± 5)	+57 (±10)	+60 (±17)
AMICARBALIDE	200.0	0		

The values are means (4 experiments); and standard errors are shown in brackets.

(ii) Guinea Pig Heart.

Histamine (10 ug) produced a transient depression followed by a strong increase in amplitude. Quinuronium (50 ug) increased the amplitude of contraction, whereas amicarbalide had no effect, see Fig. C3.

Effect of antagonists. Atropine (200 ug) produced an increase in amplitude of contraction but failed to modify the "stimulant" effect of quinuronium.

Mepyramine (200 ug) by itself depressed the amplitude of contraction by approximately 50% and failed to protect the heart from the effects of quinuronium, (Table C.2.).

4. THE ISOLATED SMOOTH MUSCLE OF THE INTESTINE AND URINARY BLADDER

A. ISOLATED GUINEA PIG ILEUM.

(a) GENERAL PHARMACOLOGY

Acetylcholine and histamine produced strong contractions of the guinea pig ileum. Quinuronium produced increased spontaneous gut activity and usually increased the resting tone of the muscle. After quinuronium the activity of acetylcholine was potentiated and the spontaneous activity of the organ became very marked; four to six changes of Tyrode being necessary to stabilise the preparation. Amicarbalide did not show any noticable activity on the ileum, however after amicarbalide the activity of acetylcholine was slightly inhibited.

Atropine abolished the actions of acetylcholine and quinuronium completely anddid not alter the response due to histamine.

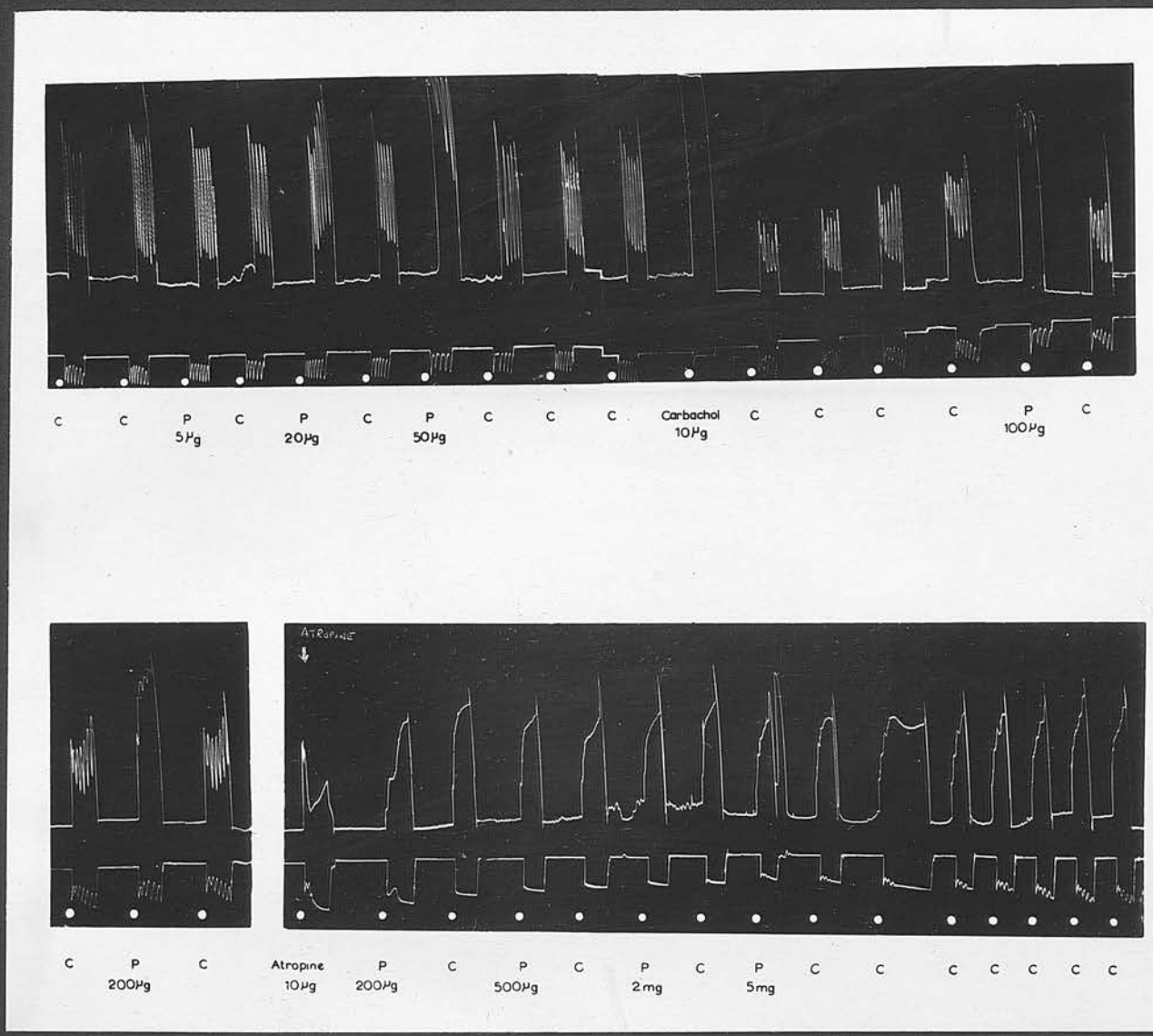


FIG. S.1.

GUINEA PIG PERISTALSIS: THE TRENDELENBURG PREPARATION OF GUINEA PIG ILEUM. UPPER TRACING SHOWS LONGITUDINAL CONTRACTIONS AND LOWER TRACING REPRESENTS CHANGES IN VOLUME. C = CONTROL RESPONSE; P = QUINURONIUM SULPHATE.

(b) THE TRENDELENBURG PREPARATION

In Fig. S1, the upper tracing represents longitudinal contractions and the lower tracing changes in volume. Quinuronium (5 ug) did not influence the response, whereas doses of 20 ug or more increased the tone of the gut segment. Volume changes were smaller and peristaltic rhythm slower under the influence of quinuronium. This effect was shown also by carbachol.

Atropine abolished all reflex peristaltic activity in the ileum (Trendelenburg, 1917) and antagonised the effects of quinuronium and carbachol on peristaltic activity.

B. ISOLATED BLADDER STRIPS.

Guinea pig (Fig. S2) and sheep bladder muscle (Fig. S3) contracted strongly to acetylcholine and histamine. Quinuronium had a variable effect either producing some longitudinal contraction or enhancing spontaneous movement of the muscle. In some specimens, quinuronium alone produced no observable effect. However, in all cases after application of quinuronium to the tissue the response produced by acetylcholine was markedly enhanced, whereas that due to histamine was unaltered.

Amicarbalide generally produced no observable effect on the bladder musculature, but in some sheep preparations the drug produced a small contraction. It was possible to demonstrate that amicarbalide inhibited slightly the action of acetylcholine without altering the response to histamine, (see Figs. S2 and S3).

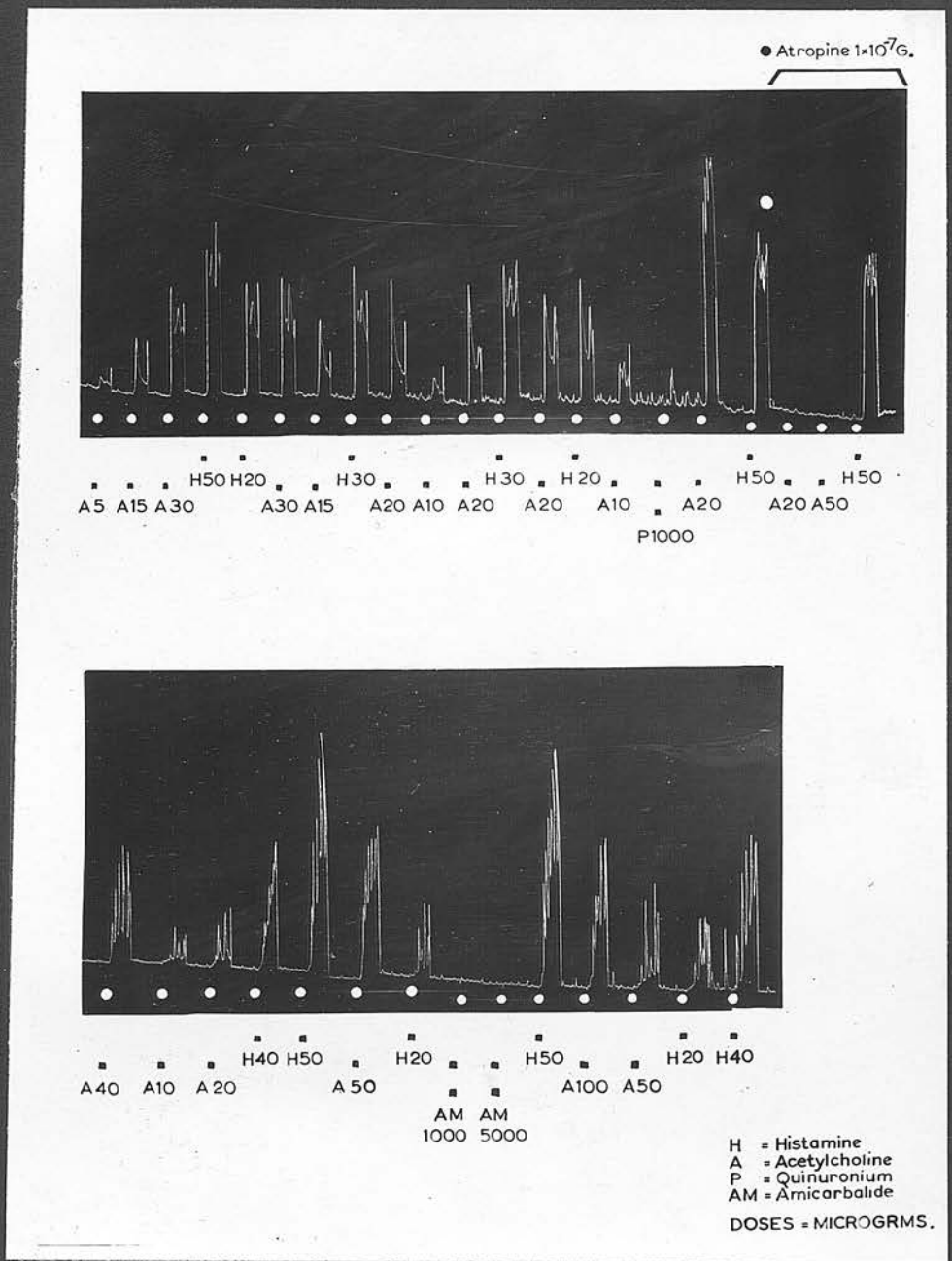


FIG. S.2.

CONTRACTIONS OF THE SMOOTH MUSCLE OF GUINEA PIG URINARY BLADDER.

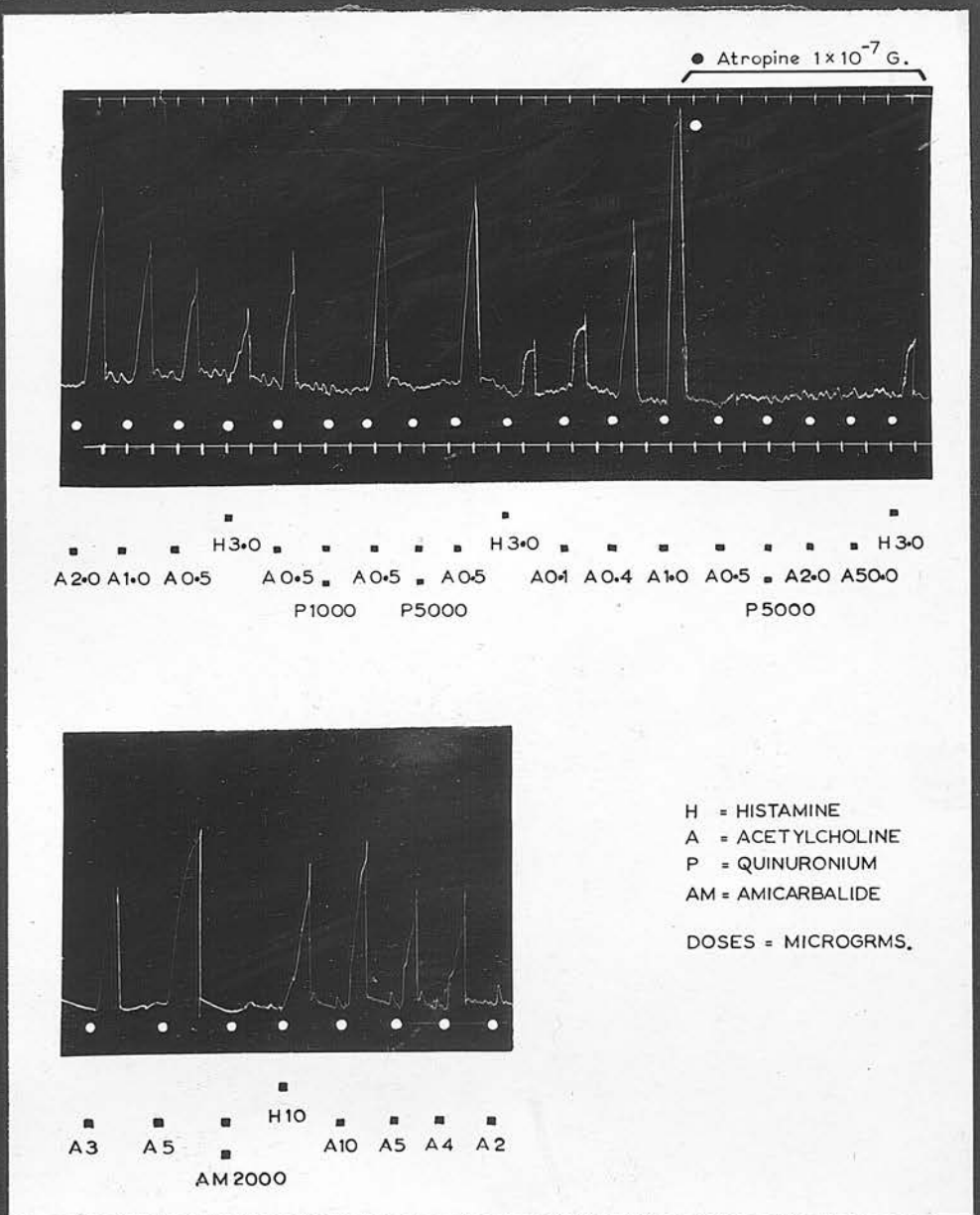


FIG. S.3.

SMOOTH MUSCLE CONTRACTIONS OF THE SHEEP URINARY BLADDER.

5. GLANDULAR SECRETION

A. SECRETION OF PAROTID SALIVA IN SHEEP.

Acetylcholine increased the flow of saliva and lowered the carotid blood pressure; but the effect was very transient, only lasting for one minute. A dose of histamine which was equi-active with acetylcholine in lowering the blood pressure had either a very small effect or produced no change in salivary flow. A hypertensive dose of adrenaline produced a distinct but transient increase in salivary secretion. Quinuronium in doses less than 1 mgm produced no change in salivation, whereas doses of 1 mgm to 5 mgm induced a large and prolonged increase in salivary flow. The initial very marked effect lasted between ten and fifteen minutes and then decreased slowly but did not return to the original (control) flow within forty-five minutes of the last injection of quinuronium. When doses of acetyl-choline were superimposed upon this steady phase of enhanced secretion, the activity of acetylcholine on both salivation and blood pressure was potentiated, (See Fig. S.4 and Table S.1). Amicarbalide had no effect on salivation.

The upper tracing on Fig. S.4 indicates the flow of saliva as recorded by a Thorpe impulse counter working on a one minute cycle. Calibration of the drop counting device showed that each "step" on the tracing indicated 0.1 ml of saliva. On this basis the values shown in Table S.1. were evolved. The figures therefore indicate the <u>increase</u> in mean salivary outflow per minute induced by intravenous drug injections.

The sheep were injected with atropine (1.0 mgm per kilo),

which did not reduce the rate of salivation to its initial resting (control) flow. Nevertheless atropine effectively blocked the action of both acetylcholine and quinuronium on blood pressure and salivary secretion, whereas the action of histamine on blood pressure remained unaffected.

TABLE S.1.

THE ACTION OF ADRENALINE, HISTAMINE, ACETYLCHOLINE AND QUINURONIUM ON THE BLOOD-PRESSURE AND SALIVATION OF SHEEP.

DRUG	DOSE	INCREASE IN MEAN SALIVARY FLOW: (mls per minute)		
		Drug Alone	After Quinuronium	After Atropine
ADRENALINE	25 ug	0.35	-	-
HISTAMINE	40 ug	0.00	-	0.00
	60 ug	0.00	-	0.00
ACETYLCHOLINE	10 ug	-	0.51	-
	20 ug	-	0.60	-
	40 ug	0.50	1.04	0.00
	150 ug	-	-	0.00
QUINURONIUM	250 ug	0.00	-	-
	1.0 mgm	0.32	-	-
	5.0 mgm	1.66	-	0.00
	10.0 mgm	-	-	0.00

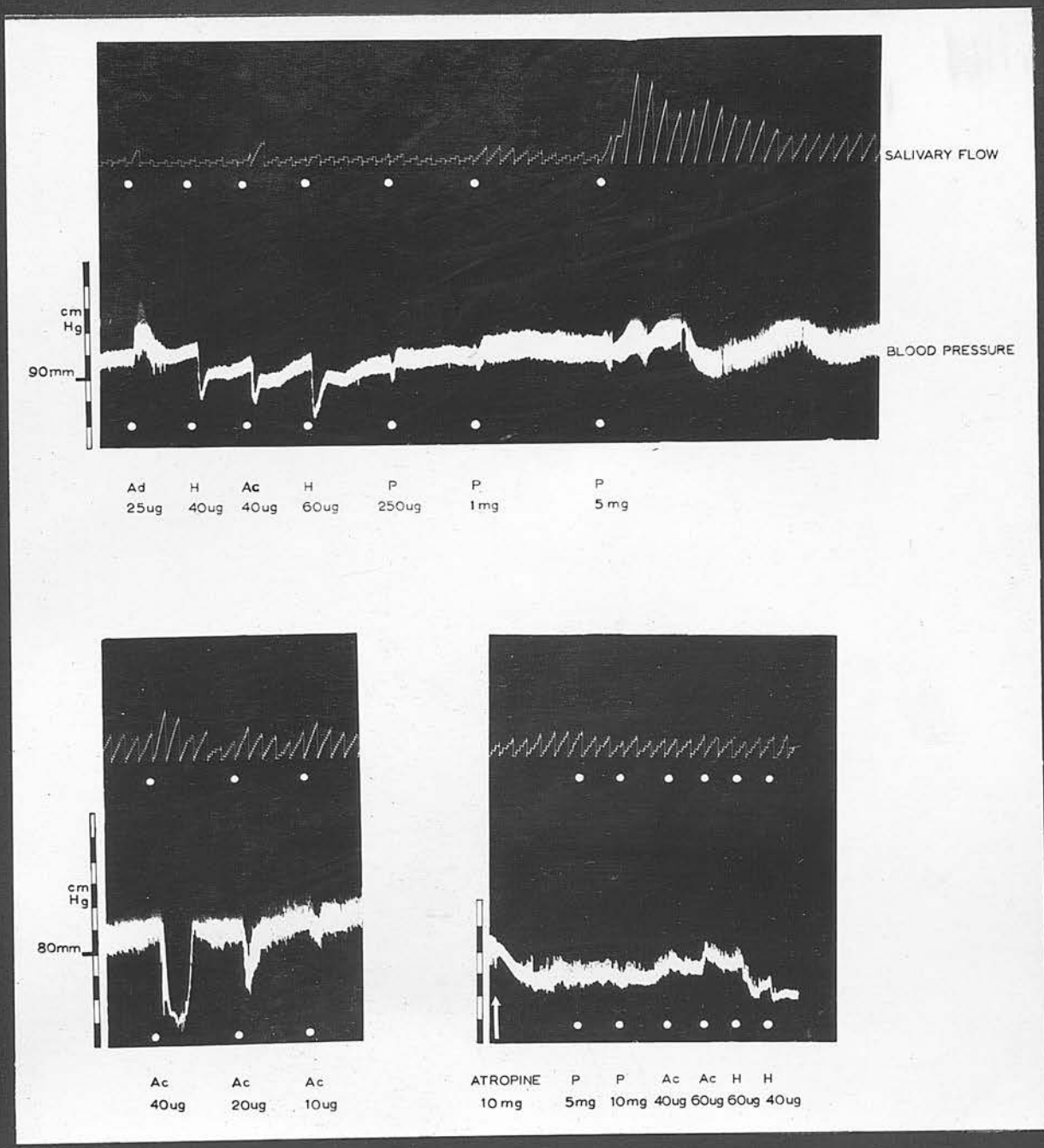


FIG. S.4.

SECRETION OF PAROTID SALIVA IN THE ANAESTHETISED SHEEP. UPPER TRACING SHOWS RATE OF SALIVARY FLOW AND LOWER TRACING CAROTID BLOOD PRESSURE.
AD. = ADRENALINE; H. = HISTAMINE;
AC. = ACETYLCHOLINE; P. = QUINURONIUM.

B. SECRETION OF GASTRIC ACID IN THE RAT.

Histamine (25 ug per min for 10 mins) and acetylcholine (10 ug per min) increased gastric acid secretion (Ghosh and Schild, 1958). Quinuronium (30 ug/min) produced a marked and prolonged increase in acid secretion. However, if at the maximum rate of acid secretion by quinuronium, 2 mgm atropine was given intravenously the acid outflow was immediately reduced. A second dose of quinuronium given thirty minutes after atropine gave a distinct but markedly reduced acid output. Atropine inhibited the acetylcholine-induced acid secretion, but did not modify that stimulated by histamine, (see Fig. G3 a. & b., and Table G.1.).

Mepyramine did not antagonise gastric acid secretion induced by histamine, acetylcholine or quinuronium (Table G.2.).

Neostigmine (20 ug per min for 10 mins) increased the gastric acid outflow. Not only was neostigmine active in inducing gastric acid secretion, but the response to acetylcholine was potentiated after neostigmine (Table G.3.). Compound 48/80 (20 ug per min for 10 mins) induced gastric acid secretion, (Table G.3.) but failed to potentiate acetylcholine induced secretion, whereas from Fig. G.4. it is clear that quinuronium produces a significant potentiation of acetylcholine.

The effect of amicarbalide on gastric secretion was investigated. As can be seen in Table G.4. amicarbalide produced a small amount of gastric acid secretion. Neither mepyramine nor atropine showed any significant antagonism for this secretion. It was noted that after amicarbalide, the acid secretion induced by acetylcholine was greatly reduced (Table G.5.).

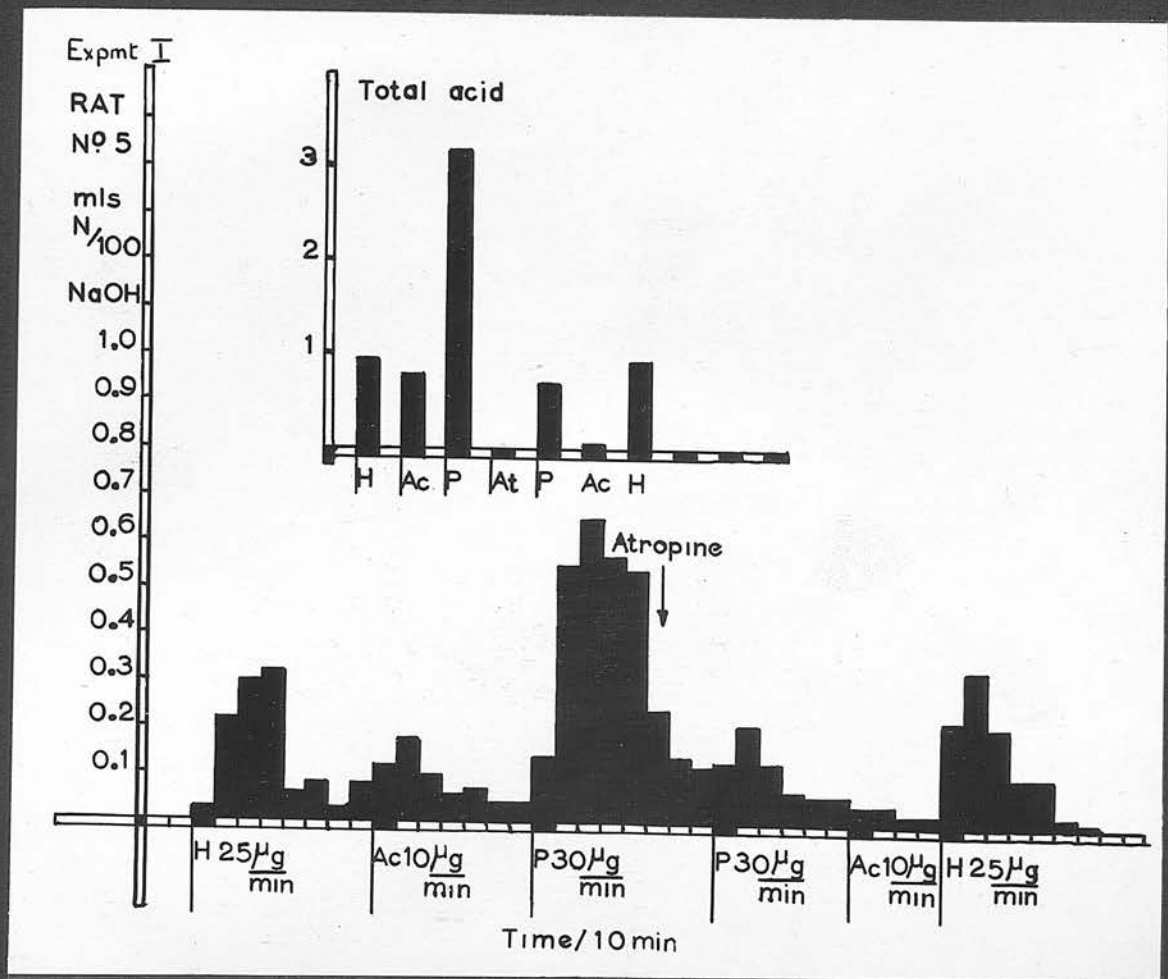


FIG. G.3. (a).

SECRETION OF GASTRIC HYDROCHLORIC ACID IN THE ANAESTHETISED RAT INDUCED BY:- H = HISTAMINE BASE; Ac = ACETYLCHOLINE CHLORIDE; P = QUINURONIUM SULPHATE. (DRUGS INJECTED EVERY MINUTE OVER A TEN MINUTE PERIOD). ORDINATE = mls. N/100 ACID; ABSCISSA = TIME.

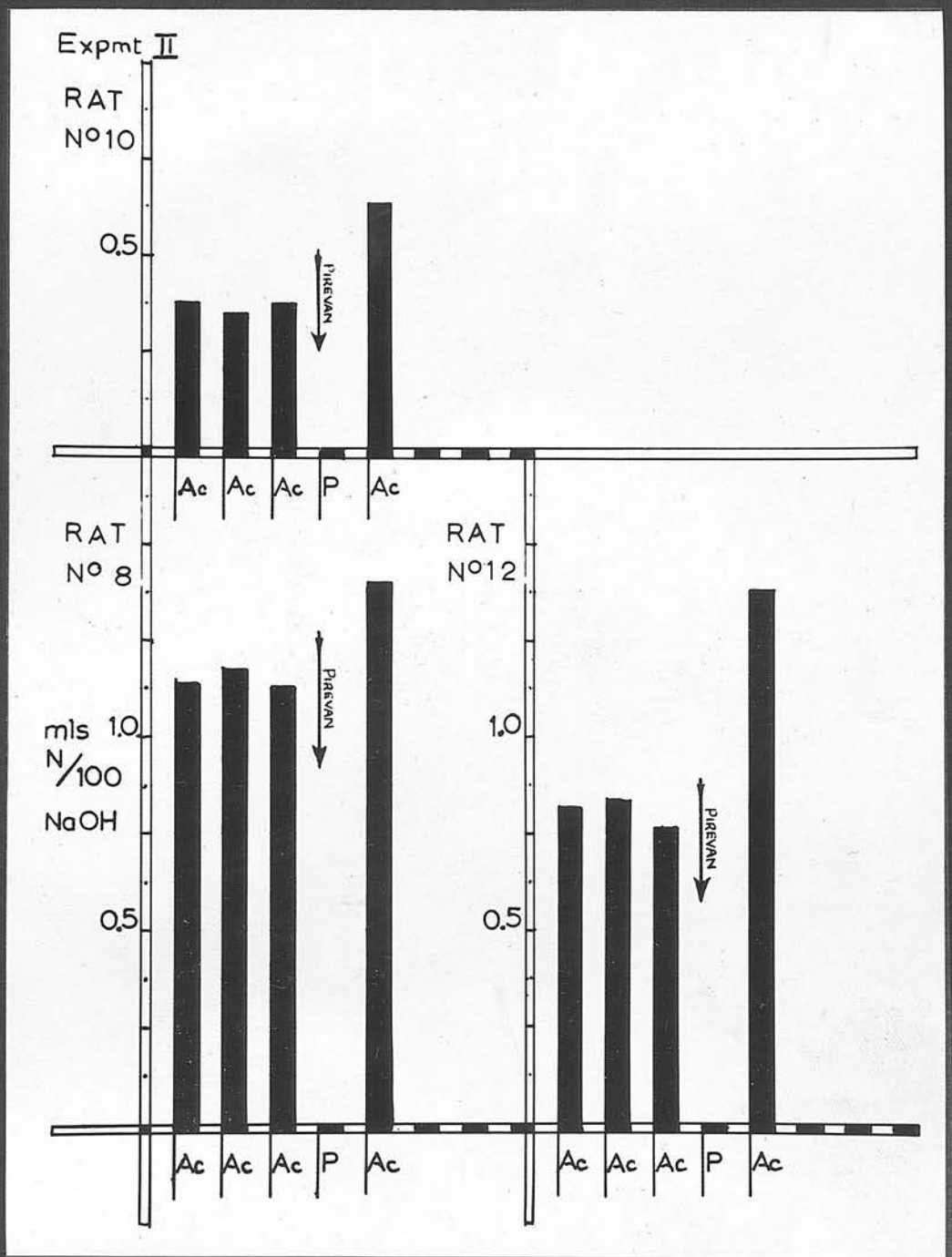


FIG. G.4.

TOTAL INCREASE IN GASTRIC ACID SECRETION INDUCED BY ACETYLCHOLINE (Ac) BEFORE AND AFTER QUINURONIUM (PIREVAN).

ORDINATE = mls N/100 ACID.

TABLE G1.

THE STIMULATION OF GASTRIC ACID SECRETION IN THE RAT BY ACETYLCHOLINE, HISTAMINE AND QUINURONIUM, AND THE INFLUENCE OF ATROPINE

DRUG	DOSE ug per min (10 min)	PER CENT INCREASE IN MEAN GASTRIC ACID OUTFLOW, PER MINUTE	
		DRUG INDUCED	
		BEFORE ATROPINE	AFTER ATROPINE (2 mgm)
ACETYLCHOLINE	10	444 ± 63 (6)	0 (4)
HISTAMINE	25	580 ± 35 (4)	553 ± 20 (4)
QUINURONIUM	30	3305 ±220 (4)	320 ± 50 (4)

Values are means with standard errors, number of animals involved is shown in parenthesis.

TABLE G2.

THE STIMULATION OF GASTRIC ACID SECRETION IN THE RAT BY ACETYLCHOLINE, HISTAMINE AND QUINURONIUM, AND THE INFLUENCE OF MEPYRAMINE

DRUG	DOSE ug/min for 10 min	PER CENT INCREASE IN MEAN GASTRIC ACID OUTFLOW, PER MINUTE	
		DRUG INDUCED	
		BEFORE MEPYRAMINE	AFTER MEPYRAMINE
ACETYLCHOLINE	10	490 ± 42 (4)	561 ± 65 (4)
HISTAMINE	25	526 ± 45 (6)	580 ± 28 (6)
QUINURONIUM	30	2944 ±180 (4)	3606 ±350 (4)
MEPYRAMINE	2 mgm	349 ±110 (6)	

Values are means with standard errors, number of animals used is shown in parenthesis.

TABLE G3.

THE STIMULATION OF GASTRIC ACID SECRETION IN THE RAT BY ACETYLCHOLINE, AND THE INFLUENCE OF NEOSTIGMINE AND 48/80

DRUG	DOSE ug/min for 10 min	PER CENT INCREASE IN MEAN GASTRIC ACID OUTFLOW, PER MINUTE		
		DRUG INDUCED		
		DRUG ALONE	AFTER NEOSTIGMINE	AFTER 48/80
ACETYLCHOLINE	10	590 ± 83 (8)	910 ± 46 (4)	450 ± 45 (4)
NEOSTIGMINE	2.0	626 ± 185 (4)		
COMP. 48/80	20	575 ± 48 (4)		

Values are means with standard errors.
Number of estimations is shown in parenthesis.

TABLE G4.

THE STIMULATION OF GASTRIC ACID SECRETION IN THE RAT BY AMICARBALIDE, AND THE INFLUENCE OF ATROPINE AND MEPYRAMINE

DRUG	DOSE ug/min for 10 min	PER CENT INCREASE IN MEAN GASTRIC ACID SECRETION, PER MINUTE		
		DRUG INDUCED		
		AMICARBALIDE ALONE	AFTER MEPYRAMINE	AFTER ATROPINE
AMICARBALIDE	200 ug	433 ± 86 (5)	540 ± 123 (4)	517 ± 103 (4)

Values are expressed as means with standard errors.
Number of estimations is shown in parenthesis.

TABLE G5.

THE INFLUENCE OF AMICARBALIDE ON GASTRIC ACID SECRETION INDUCED BY ACETYLCHOLINE AND HISTAMINE

DRUG	DOSE ug per min for 10 mins	PER CENT INCREASE IN MEAN GASTRIC ACID SECRETION	
		BEFORE AMICARBALIDE	AFTER AMICARBALIDE
ACETYLCHOLINE	10	505 ± 28 (6)	273 ± 33 (6)
HISTAMINE	25	596 ± 33 (4)	545 ± 41 (4)

Values are means with standard errors.
Number of animals is shown in parenthesis.

6. NEUROMUSCULAR TRANSMISSION

A. IN VITRO - CHICK BIVENTER CERVICIS.

Quinuronium (5 - 10 ug) produced a potentiation of the muscle twitch which was comparable with that induced by eserine (150 ug). It was noticeable with quinuronium that after the initial potentiation of the twitch amplitude there was a rapid return to normal or even reduced amplitude of contraction. With eserine the potentiation was maintained and the preparation was restored to normal only by washing. Large doses of quinuronium (1 to 2 mgm) produced marked reduction in the height of contraction. This response is outwardly typical of the effect of competitive blocking agents such as curare, (see Figs. NM 6 a., b., & c.). The action of quinuronium however, did not seem to be reversible by eserine alone.

Acetylcholine (20 ug) in the eserinised muscle produced a typical muscle contracture which was indicative of muscle depolarisation. This state of contracture was rapidly reversed by the addition of tubocurarine 1 mgm (a competitive inhibitor of acetylcholine). Likewise the depolarisation produced by acetylcholine was equally rapidly reversed by 2 mgm of quinuronium. Conversely the competitive blockade induced by d-curare (300 mgm) could be overcome by the addition of eserine and acetylcholine and the apparently competitive neuromuscular block shown by quinuronium was also overcome by adding eserine (50 ug) and acetylcholine (300 ug). Suxamethonium (succinyl-choline) (40 ug) induced neuromuscular block and contracture in the musculature,

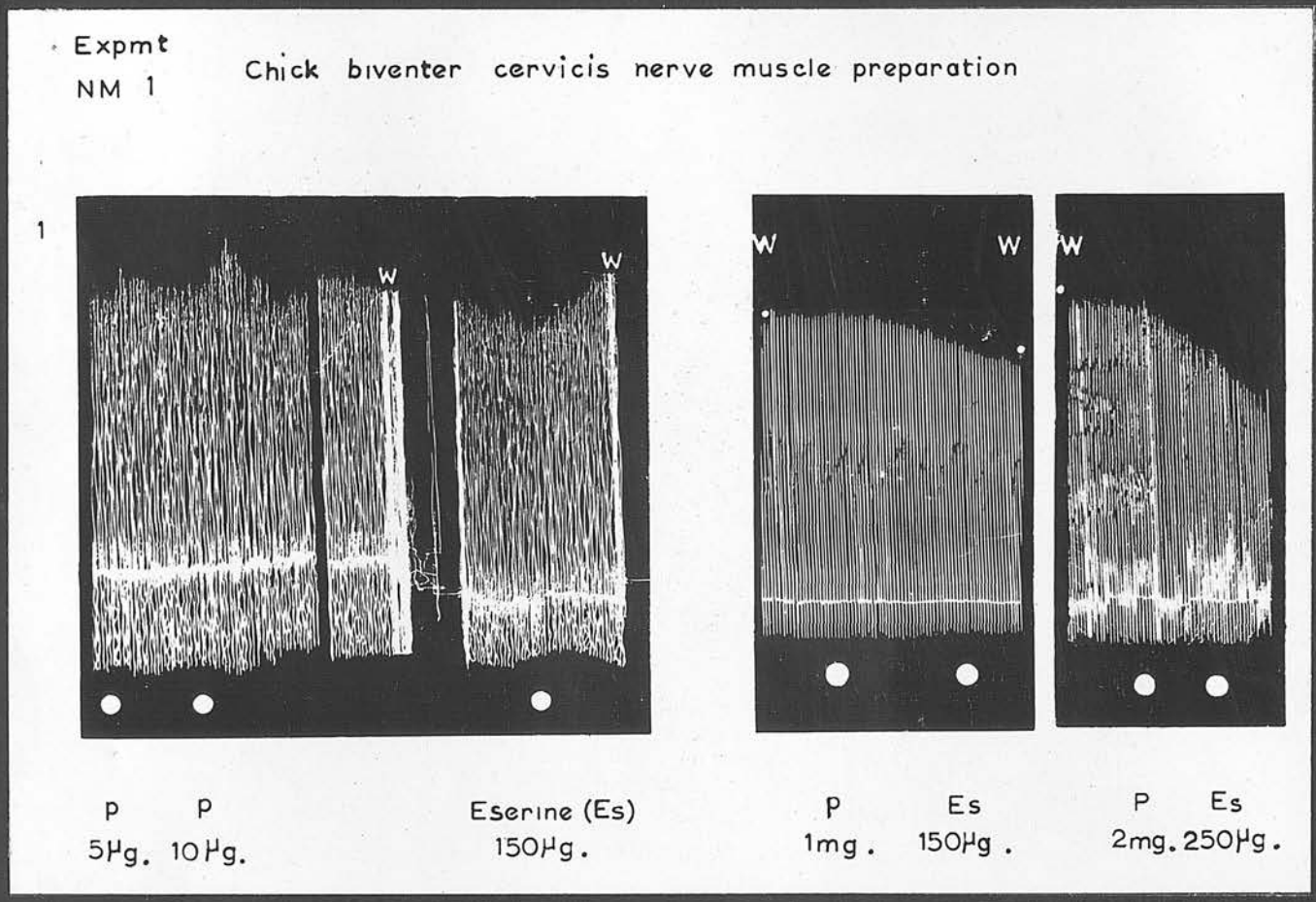


FIG. NM 6 (a).

CHICK BIVENTER CERVICIS MUSCLE. P. = QUINURONIUM SULPHATE; ES. = ESERINE SALICYLATE.

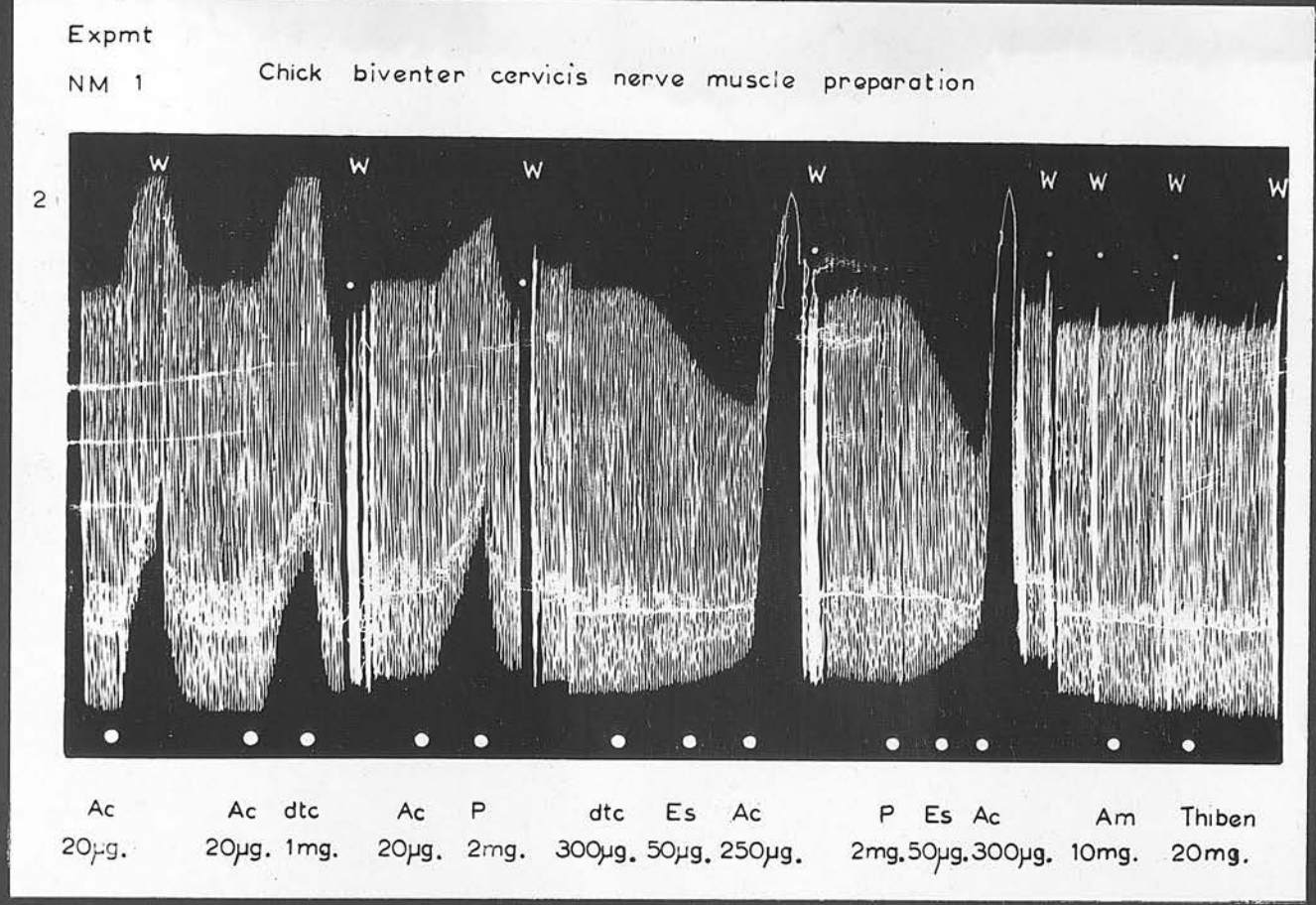


FIG. NM 6 (b).

CHICK BIVENTER CERVICIS MUSCLE. AC. = ACETYLCHOLINE CHLORIDE; dTC. = d-TUBOCURARINE CHLORIDE; P. = QUINURONIUM SULPHATE; ES. = ESERINE SALICYLATE.

/ AM. = AMICARBALIDE; THIBEN. = THIBENDAZOLE - both of which were tried with no effect. /

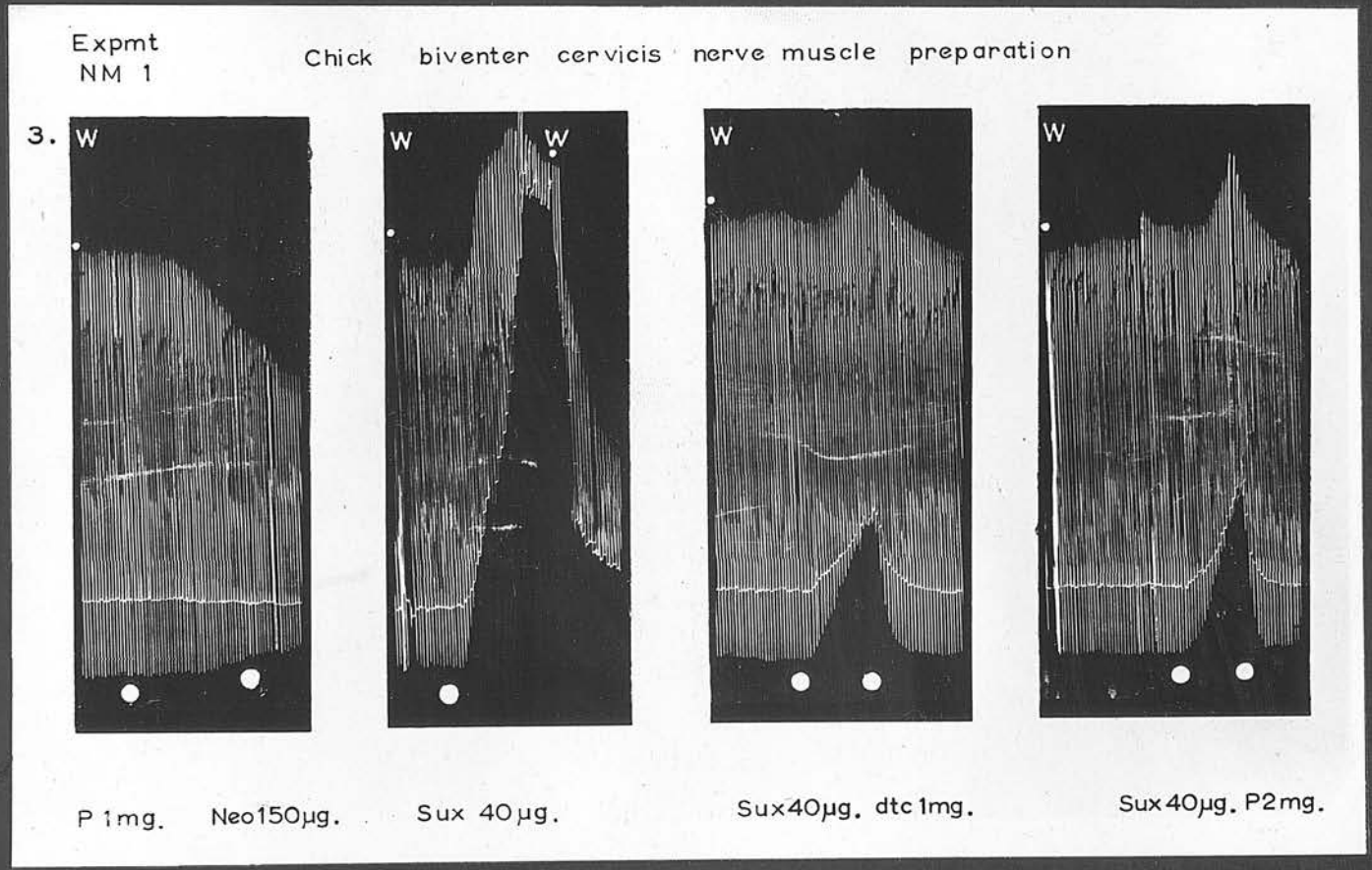


FIG. NM 6 (c).

CHICK BIVENTER CERVICIS MUSCLE. P. = QUINURONIUM SULPHATE; NEO. = NEOSTIGMINE BROMIDE; SUX. = SUCCINYLCHOLINE CHLORIDE.

which effect could only be reversed by repeated washing of the preparation. This suxamethonium-induced depolarisation could readily be reversed by the addition of d-curare (1 mgm) which evidently competed with the suxamethonium at the receptors of the post-junctional end-plate region of the muscle. Quinuronium (2 mgm) also readily reversed the depolarisation produced by succinyl-choline. Amicarbalide (10 mgm) was without effect in this preparation.

B. IN VIVO.

(a) DIGITAL EXTENSOR MUSCLES OF CHICKEN PELVIC LIMB.

(Figure NM7). The drugs used had similar effect on neuromuscular transmission whether they were administered by the close arterial route or intravenously. Smaller doses were effective intra-arterially and these did not display generalised systemic effects on blood pressure and respiration. The tracings reproduced here-under are of drug effects by the intravenous route, as such observations are much more informative as regards the use of therapeutic doses of drugs and the effects on blood pressure and respiration.

Quinuronium at less than 50 microgram dose was without effect on neuromuscular transmission or respiration, although a slightly delayed fall in blood pressure was produced. Doses of quinuronium 100 to 500 micrograms produced a distinct and, at the latter dose, very marked potentiation of the muscle contraction. In addition the respiratory volume was markedly reduced. Administration of

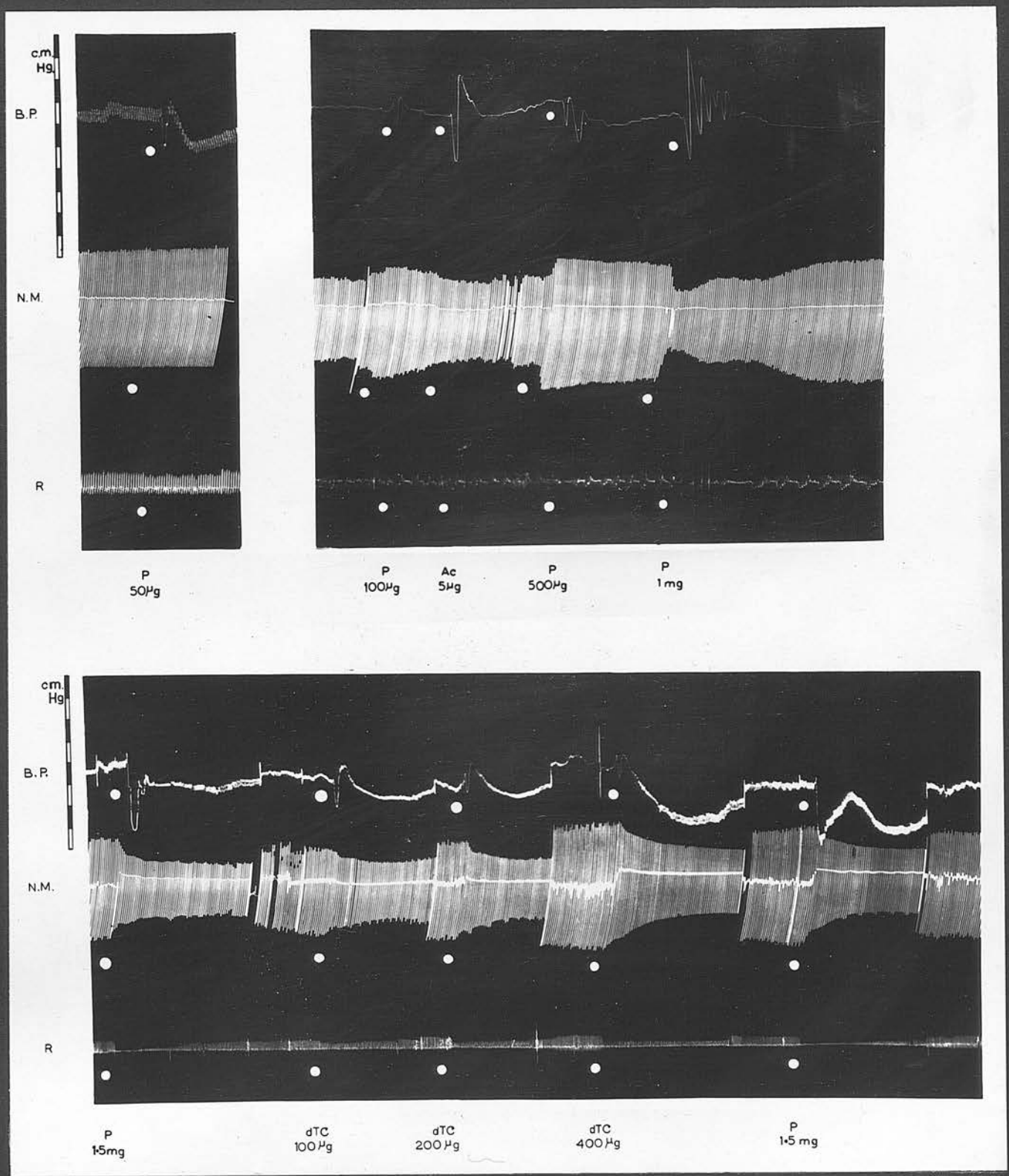


FIG. NM 7.

IN VIVO RECORDING OF: (1) CAROTID BLOOD PRESSURE - TOP TRACING; (2) CONTRACTIONS OF DIGITAL EXTENSOR MUSCLES - MIDDLE TRACING; (3) RESPIRATION - BOTTOM TRACING, IN THE ANAESTHETISED DOMESTIC FOWL. dTC. = d-TUBOCURARINE CHLORIDE; AC. = ACETYL-CHOLINE CHLORIDE; P. = QUINURONIUM SULPHATE.

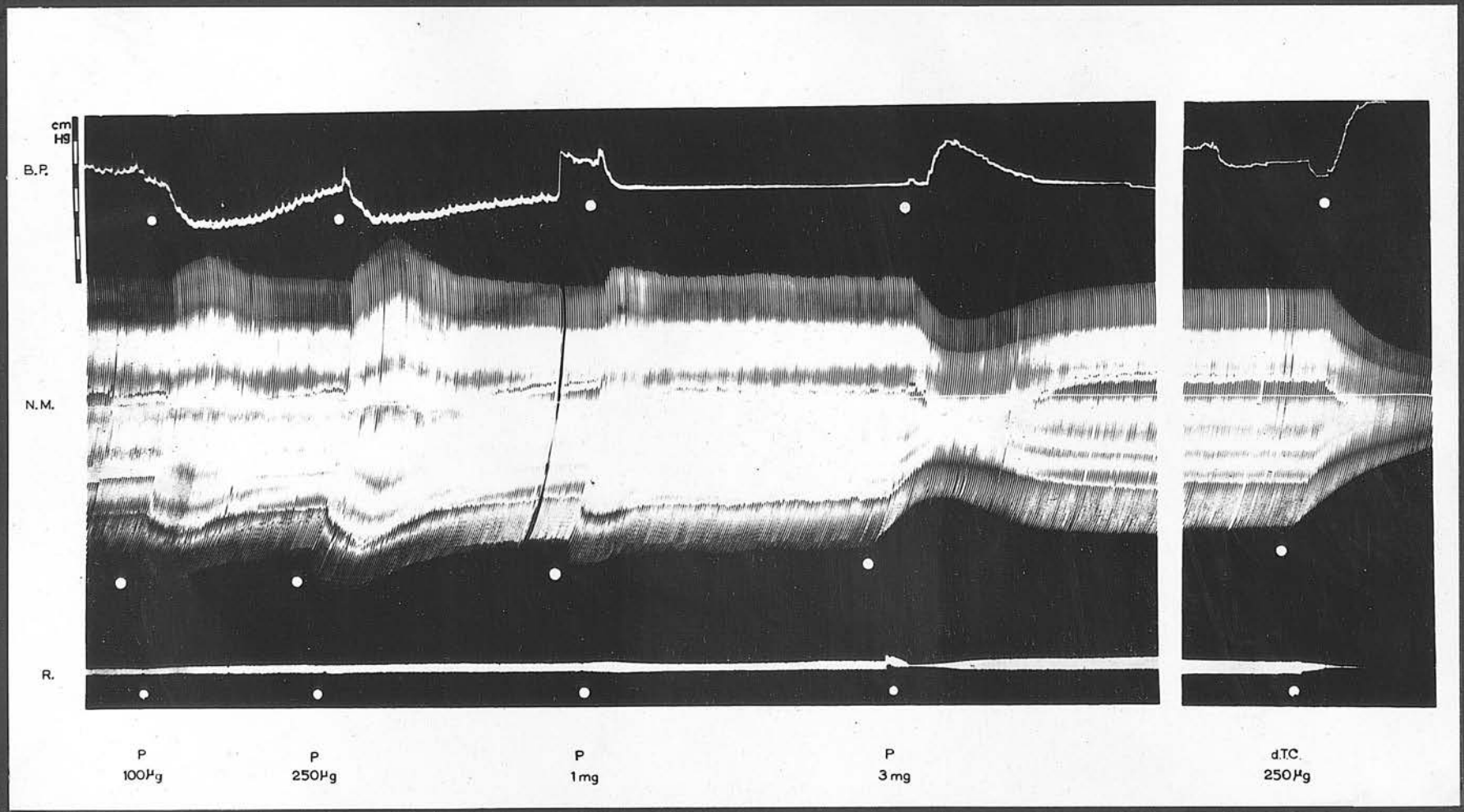


FIG. NM 8.

IN VIVO RECORDING OF: (1) BLOOD PRESSURE - TOP TRACING; (2) CONTRACTIONS OF HIND LIMB DIGITAL EXTENSOR MUSCLES - MIDDLE TRACING; (3) RESPIRATION - BOTTOM TRACING, IN THE ANAESTHETISED RABBIT. dTC. = d-TUBOCURARINE CHLORIDE; P. = QUINURONIUM SULPHATE.

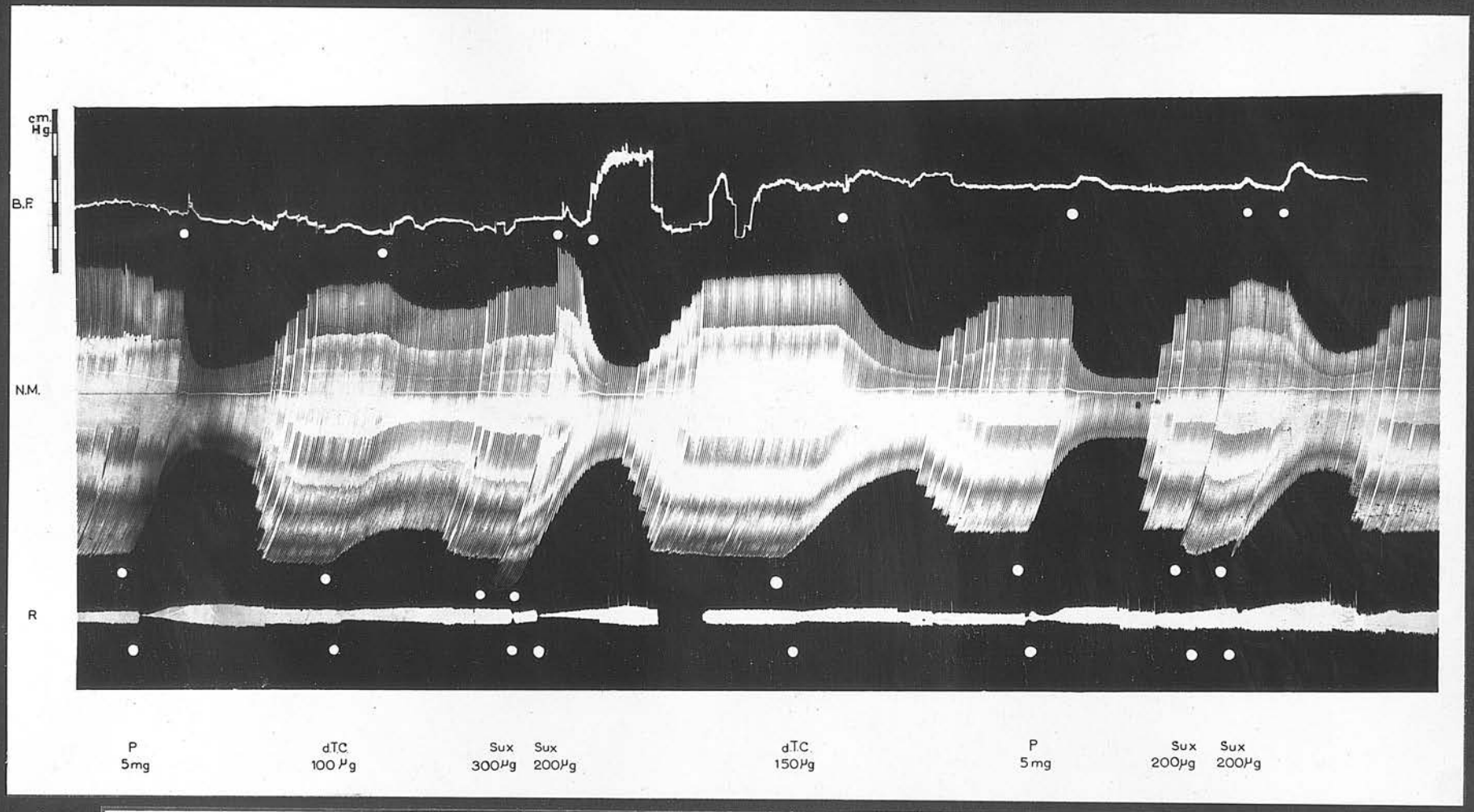


FIG. NM 9.

IN VIVO RECORDING OF: (1) BLOOD PRESSURE - TOP TRACING; (2) CONTRACTIONS OF HIND LIMB DIGITAL EXTENSOR MUSCLES - MIDDLE TRACING; (3) RESPIRATION - BOTTOM TRACING, IN THE ANAESTHETISED RABBIT. P. = QUINURONIUM SULPHATE; dTC. = d-TUBOCURARINE CHLORIDE; SUX. = SUCCINYLCHOLINE CHLORIDE.

TABLE NM1.

A COMPARISON OF QUINURONIUM AND d-TUBOCURARINE ON NEUROMUSCULAR TRANSMISSION AND RESPIRATION IN THE CHICKEN

DRUG	DOSE	NEUROMUSCULAR TRANSMISSION % INHIBITION	RESPIRATION	
			PER CENT REDUCTION IN RATE	PER CENT REDUCTION IN TIDAL VOLUME
QUINURONIUM (i)	500 ug	(POTENTIATED)	- 14% (± 8)	- 45% (± 9)
QUINURONIUM (ii)	1.5 mg	- 48% (±10)		- 100%
d-TUBOCURARINE	400 ug	- 51% (±12)	- 35% (±12)	- 52% (± 7)

Figures are mean percentages ± S.E. of 4 animals

quinuronium at the therapeutic dose level (1 mgm per kilo) produced marked neuromuscular blockade, approaching 50% inhibition of contraction. There was a fall in blood pressure and cessation of respiration for a few seconds followed by a much slower respiratory movement with greatly reduced tidal volume. d-Tubocurarine invariably produced prolonged hypotension, neuromuscular block and slight reduction in both respiratory rate and tidal volume, which appeared to be in proportion to the degree of neuromuscular block.

(b) DIGITAL EXTENSOR MUSCLES OF RABBIT HIND LIMB.

Small doses of quinuronium (100 to 1 mg) injected intravenously produced a potentiation of the muscle contraction. Larger doses (3 - 5 mgm) caused neuromuscular blockade without any initial potentiation. Curare (250 ug) produced a competitive-type block, (see Fig. NM8). Suxamethonium (300 ug) produced an enhancement of muscle contraction. If this dose was then followed by another injection (e.g. 200 ug) neuromuscular block ensued.

In this species, again it was clear that while 150 ug curare was about equipotent with 5 mgm quinuronium (full therapeutic dose) in respect of the inhibition of neuromuscular transmission: quinuronium at this dosage caused virtual cessation of respiration for a short period; an effect which was more severe than that corresponding to d-tubocurarine, (see Table NM2 and Figs. NM8 & 9).

In both species small doses of quinuronium produced potentiation of muscle contraction in the supramaximally stimulated

TABLE NM2.

A COMPARISON OF QUINURONIUM AND d-TUBOCURARINE ON NEUROMUSCULAR TRANSMISSION AND RESPIRATION IN THE RABBIT

DRUG	DOSE	% INHIBITION OF NEUROMUSCULAR TRANSMISSION	% INHIBITION OF RESPIRATORY TIDAL VOLUME
QUINURONIUM (i)	500 ug	(POTENTIATED)	- 30% ± 15 (4)
QUINURONIUM (ii)	1.5 mgm	- 69% ± 6 (4)	- 90% ± 9 (4)
d-TUBOCURARINE	150 ug	- 60% ± 10 (4)	- 45% ± 12 (4)

Figures are mean percentages (± S.E.).
Number of animals is shown in parenthesis.

muscle and simultaneously depressed both rate and tidal volume of respiration. Larger doses of quinuronium produced partial neuromuscular blockade and temporary cessation of respiration followed by a profoundly reduced tidal volume. Curare on the other hand always produced neuromuscular blockade paralleled by degree of respiratory inhibition, (see Tables NM1 & NM2) which was in proportion to the neuromuscular block.

DISCUSSION.

The investigation of pharmacodynamic activities revealed that quinuronium produced marked hypotension. In the rat the complete antagonism of this hypotension by mepyramine suggested the possibility of its being caused in part by histamine release. However, in rabbits atropine seemed to be a good antagonist to the hypotension, which suggested a muscarinic type of action. In sheep, both atropine and mepyramine gave partial protection; the former drug being almost a complete antagonist to quinuronium. Neither atropine nor mepyramine produced any significant alleviation of the respiratory inhibition which occurred with the hypotension. These results emphasised the importance of analysing more completely the mode of action of quinuronium in producing hypotension.

In the blood vessels of the rabbit's ear, quinuronium produced vasoconstriction which was slowly antagonised by atropine, which taken together with the fact that mepyramine consistently failed to antagonise the vasoconstrictor action seemed to indicate that histaminic effects were not greatly involved in the action of quinuronium in this species. This observation was consistent with the failure of mepyramine to antagonise hypotensive effects in the rabbit.

While amicarbalide showed no vascular activity itself, the compound appeared to antagonise the action of acetylcholine and quinuronium in the rabbit ear.

Quinuronium invariably produced an increase in the vascular

volume of the gut and increased the vascular and pulse-volume in the limbs of sheep. Only the lowest doses of quinuronium were antagonised by mepyramine whereas atropine abolished the vaso-activity of quinuronium in both gut and limb. The findings were consistent with the fall in carotid blood pressure in sheep and it seemed reasonable to infer that part of the mode of action of quinuronium in inducing hypotension may be on the vascular beds of the intestine and limb, and that this action is largely muscarinic.

In the isolated heart, quinuronium always produced an increase in amplitude of contraction which was unaffected by atropine; which suggested that the action on the heart was not cholinergic. Mepyramine did not antagonise the stimulant effects of either quinuronium or histamine.

In 1914, Dale and Laidlaw showed that histamine stimulated the heart of cat and rabbit, and Went and Lissack (1935) found that histamine also stimulated the heart of guinea pig. Recently several authors have reported that the common anti-histamine agents do not antagonise histamine on the heart of guinea pig, (Lockett and Bartlet, 1956; Trendelenburg, 1960). Although Mannaioni (1960) reported that diphenhydramine antagonised the cardiac action of histamine - this could not be confirmed, (Bartlet, 1963). It is concluded, therefore, that the cardiac action of quinuronium could possibly involve some histamine-like activity. In the absence of an antagonist this could not be established or denied. However, the fact that quinuronium stimulated the heart in these circumstances suggested that its

cardiac action probably played little part in inducing hypotension, and that the primary cause of hypotension was a peripheral vasodilation. These experiments take no account of the situation in the live animal where homeostatic reflexes may be involved.

The effect of quinuronium on the smooth muscle of the intestine and bladder was inconsistent. On some preparations there was no visible action and on others there was increased spontaneous activity. In some experiments a contraction of the smooth muscle occurred and this was usually seen when a dose of quinuronium followed one of acetylcholine. This might be explained in terms of an anticholinesterase action of quinuronium which was acting on a small residual amount of acetylcholine. This idea was supported by the fact that in all these experiments, pre-treatment with quinuronium resulted in a marked potentiation of all subsequent doses of acetylcholine which seemed to be a possible manifestation of anticholinesterase activity. Similar results were obtained when recording the flow of saliva from the parotid duct of sheep. In this preparation large doses of quinuronium alone always induced a marked increase in salivary flow. Atropine effectively blocked (i.e. antagonised) the activity of acetylcholine and quinuronium on the smooth muscle of the intestine and bladder and on salivation.

These results provided confirmation of the clinical observation that quinuronium stimulated the acts of defaecation, salivation and micturition: all of which could be prevented by premedication with atropine. Amicarbalide was itself inactive

but showed partial antagonism for the action of acetylcholine and quinuronium on smooth muscle. The observation supported the atropine-like activity of amicarbalide which was observed on the blood vessels of the rabbit's ear.

Quinuronium produced a very marked and prolonged stimulation of gastric acid secretion in rats, which was never completely antagonised by atropine. Atropine has been reported to be an incomplete antagonist to gastric secretion,(Gray and Ivy, 1937; Code, 1951). Nevertheless, in view of the fact that in these experiments atropine appeared completely to block the action of acetylcholine, it seemed likely that the atropine-resistant factor in the action of quinuronium was probably not cholinergic. Mepyramine failed to antagonise the gastric secretion induced by histamine, quinuronium or acetylcholine, and in fact secretion tended to be enhanced, (Table G.2.). Mota (1960) showed that mepyramine released histamine in vitro and many authors have reported that antihistaminic agents themselves may stimulate gastric secretion and do not antagonise the gastric effects of histamine,(Paton and Schachter, 1951; Loew, 1950; Ashford, Heller and Smart, 1949). Hence in the absence of a histamine antagonist it was not possible to show whether the gastric secretion stimulating component of quinuronium not antagonised by atropine, was histamine. Further support of anticholinesterase action was the specific potentiating action of quinuronium upon the action of acetylcholine on gastric secretion. Neostigmine also potentiated acetylcholine, but compound 48/80 which acts by releasing histamine, (Paton and Schachter, 1951; Smith, 1952)

did not.

The stimulant effect of amicarbalide on gastric secretion and the failure of either atropine or mepyramine to antagonise this could not be explained on the evidence so far available. However, it was interesting to observe that amicarbalide was partially antagonistic towards the action of acetylcholine which supported the data on the atropine-like activity of this drug in smooth muscle.

Quinuronium was shown to have a dual role in affecting neuromuscular transmission. Small doses potentiated the supra-maximally stimulated nerve muscle preparation whereas bigger doses produced neuromuscular blockade. When compared with the action of curare, it appeared that the action of quinuronium at the neuromuscular junction could not be distinguished from the former compound. It has been established that the isolated chicken biventer muscle is especially useful as a method for detecting depolarising agents which produce sustained contracture of the muscle, (Ginsburg and Warriner, 1960). Eserine by virtue of its anticholinesterase action produces neuromuscular blockade and sustained contracture. Most anticholinesterase compounds have such an effect at the neuromuscular junction, (Modell and Krop, 1946; Holmes and Robbins, 1955; Brown et al, 1957), and furthermore they have the ability to reverse the competitive block produced by curare, (Blaschko, Bülbring and Chou, 1949). Quinuronium like curare antagonised the contracture produced by eserine and acetylcholine or succinylcholine, and conversely

eserine and acetylcholine readily reversed the competitive blockade of curare or quinuronium. It seemed, therefore, that quinuronium stimulated neuromuscular transmission at low doses, possibly by an anticholinesterase action, and at higher doses produced neuromuscular block which was probably a result of competitive antagonism for the nicotinic actions of acetylcholine and not associated with anticholinesterase activity.

Acute experiments in the chicken and rabbit, in which the relative effects of quinuronium on respiratory activity and neuromuscular transmission were compared, showed that this agent had a more profound effect on respiration than on neuromuscular transmission. When comparing doses of quinuronium and d-tubocurarine which are equi-active at the neuromuscular junction it was clear that quinuronium had a more marked effect on respiratory movement. Moreover, the smallest dose of quinuronium produced a potentiation of neuromuscular transmission concurrent with respiratory inhibition. It is suggested, therefore, that at therapeutic doses of quinuronium, neuromuscular blockade plays a significant part in producing respiratory inhibition, but that a large part of the depression of respiratory movement is independent of neuromuscular block and may be partly the result of histamine or muscuranic bronchoconstriction. The central depression as a result of anticholinesterase activity, (Modell and Krop, 1946; Holmes and Robbins, 1955), and the inhibition of oxygen metabolism, (Kronfeld, 1959) are also possible influences.

SECTION II

ANTICHOLINESTERASE PROPERTIES

INTRODUCTION.

Despite the long-recognised side effects of quinuronium, the precise elucidation of the mode of toxicity of this compound did not attract much attention until recent years, (Kronfeld, 1959), (Rummler and Laue, 1961). The last named authors showed that in a proportion of dogs and sheep treated with quinuronium sulphate, a fall in circulating cholinesterase activity was detectable. These authors had anticipated such activity on the grounds that quinuronium was a substituted urethane.

Several considerations must be made of Rummler and Laue's work before attempting further investigations. These authors used twelve dogs and six sheep initially. Animals had venous blood samples withdrawn before and after injection of quinuronium (5% aqueous up to 0.1 ml per kgm.). Whole blood was subjected to manometric (Warburg) estimation of cholinesterase activity. Four dogs and four sheep failed to show any reduction in enzyme activity. In the first instance, therefore, it seemed important to repeat this investigation both in vitro and in vivo in a number of species.

Rummler and Laue (1960) proceeded to test a number of antidotes and to assess their efficacy on clinical grounds: no estimation of cholinesterase activity being carried out on these "premedicated" animals.

The antidotes used were:

1. Pyridine 2-aldoxime methiodide
2. Atropine sulphate
3. An unspecified analeptic.

Their results may be tabulated as follows:

(a.) All animals injected with quinuronium at a dose rate of 5 mgm per kilo died if unpremedicated.

(b.) One sheep was premedicated with PAM alone and died.

(c.) Two sheep which received PAM and an analeptic likewise died. It has been reported that although the neuromuscular effects of anticholinesterase toxicity (both organophosphorus and quaternary salts) are readily reversed by oximes, the muscarinic and central neural symptoms are not appreciably influenced by oxime administration, (Grob and Johns, 1958). In the sheep in question, therefore, it is conceivable that they died in part as a result of central depression of respiration and muscarinic action on the heart.

(d.) Of the four animals pretreated with atropine alone, one died and the other three showed toxic signs; whereas of the twelve sheep premedicated by these authors with atropine and 2.P.A.M. combined, only two died and all others showed definite toxic signs which were said to be clinically less severe than in those which had received atropine alone. One cannot conclude positively from such an experiment that a combination of atropine and PAM offers significant advantages over atropine alone.

No investigation was made by these workers to correlate the premedicant therapy with directly determined cholinesterase

activity.

It therefore seemed important:

(i) To confirm the anticholinesterase activity of quinuronium in vitro and in vivo in a wide variety of species.

(ii) To test directly the action of 2.P.A.M. in sheep which had received a large dose of quinuronium up to 2 mgm per kilo. The dosage rate used by Rummler and Laue is thought to have been too great (5 mgm per kilo) in view of the fact that the drug manufacturers recommended dose rate is of the order of 1 mgm per kilo and the L.D.50 is of the order of 2 to 4 mgm per kilo, (Kronfeld, 1959). It was therefore decided to adopt 1 to 2 mgm per kilo as the experimental dose range in the living unanaesthetised sheep and to scale this down to similar proportions for use in in vitro investigations.

(iii) To compare quinuronium and amicarbalide on cholinesterase enzyme preparations.

(iv) To try to determine the mechanism of action of quinuronium sulphate and to compare this with a known standard, such as eserine.

METHODS.

CHOLINESTERASE ESTIMATIONS.

The method of choice from the point of view of accuracy of determination has been the Warburg manometric method.

For convenience, and for efficiency in handling large numbers of tissue samples from a variety of animals it was decided to use the colorimetric method for determining carboxylic

esters first described by Feigl, Anger and Frehden (1934) as modified by Hestrin (1949). The method was applied to the study of anticholinesterases by Katsch (1955)and by Fleisher, Pope and Spears (1955). These authors published details of the estimations and the degree of precision which could be expected. Augustinsson (1954) described the colorimetric method as convenient for small quantities of tissue and applicable to a wide variation in pH and enzyme concentration in almost any buffer solution. The limits of measurement were from 5.0 to 0.04 micromole. The accuracy was less than with either manometric or titrimetric methods and some 20% to 30% of the ester had to be hydrolysed in order to give the best results.

A good index of activity of a cholinesterase inhibitor has been shown to be an expression of 50% activity. This is the logarithm of the reciprocal of the molar concentration of inhibitor causing 50% inhibition of enzyme activity; i.e. pI_{50} (Blaschko, Bülbring and Chou (1949)), (Bergmann et al (1950)), and(Myers, 1952).

THE CHEMICAL REACTION.

The basic chemical reaction described by Feigl et al (1934) is as follows:-

The ester reacts in alkaline solution with hydroxylamine to form acethydroxamic acid:

(i) $(CH_3)\cdot\overset{+}{N}\cdot\cdot CH_2\cdot CH_2\cdot O\cdot COCH_3 + NH_2OH$

$\longrightarrow (CH_3)_3\cdot\overset{+}{N}\cdot CH_2\cdot CH_2\cdot OH + CH_3CO\cdot NHOH$

The hydroxamic acid so formed reacts in acid solution with ferric chloride to form a soluble red complex:

(ii) $CH_3CO \cdot NHOH + FeCl_3 \longrightarrow$

$$\left[CH_3 \cdot C = O \right]_3 Fe \quad + 3HCl$$
$$\left[\quad\quad NH - O \right]$$

The intensity of colour is proportional to concentration of acetylcholine present.

CHEMICAL PROCEDURES AND ENZYME PREPARATION TECHNIQUES.

1. Acetylcholine standardisation

(a) Aliquots of 0 (control) 0.2, 0.4, 0.6, 0.8, and 1.0 ml of buffer acetylcholine solution are pipetted into centrifuge tubes and phosphate buffer added to each to give a final volume of 1.0 ml.

(b) 2 ml of alkaline hydroxylamine is added to each tube which is shaken vigorously.

(c) After at least one minute, 1.0 ml of 33% HCl solution is added (attains final pH of approx 1.2) and 1.0 ml of ferric chloride solution added. N.B. Vigorous shaking is undertaken after each addition to avoid any bubble formation during colour absorption readings.

(d) Absorbance of colour in each tube is measured at 540 mu using a control tube to adjust the instrument to 100. Absorbance can be plotted against the known acetylcholine concentrations and the curve is used thereafter as a standard for all

determinations.

2. Procedure for plasma [or other enzyme preparation (e.g. tissue homogenate.)]

(a) A quantity of plasma [volume 0.05 to 0.5 ml depending on species and experimental conditions (Table CH1)] is expelled into 1.0 ml of buffer acetylcholine solution. The pipette is rinsed three times in the buffer. The time is noted and the tube immersed in water at 37°C after thorough mixing.

(A standard of 1.0 ml buffer acetylcholine solution without plasma and a control of 1.0 ml buffer without Ach are also made up.)

(b) After an exact time (20-30 minutes depending on the species) 2.0 ml of alkaline hydroxylamine is added to all tubes and the acetylcholine estimation proceeds as described above after centrifugation to remove suspended particles.

(c) Plasma is added to the standard and control immediately after the hydroxylamine. The standard allows one to correct the readings for non-enzymatic hydrolysis of acetylcholine and the control allows one to adjust the colorimeter to zero.

3. Procedure for erythrocytes.

(a) The packed red cells are washed three times with an equal volume of normal saline, centrifuged and decanted after each washing.

(b) One volume of washed red cells is pipetted into five volumes of water to haemolyse the cells; rinsing the pipette thrice in the solution.

(c) A volume of haemolysate (Table CH1) is pipetted into 1.0 ml buffer Ach.

(d) The estimation proceeds exactly as for plasma using standard and control tubes with centrifugation in all cases to remove suspended matter.

4. Procedure for whole blood.

(a) Heparinised whole blood is mixed with 0.01% saponin solution at the rate of one volume of blood to four of saponin.

(b) A volume of haemolysate 0.5 - 1.0 ml (Table CH1) is added to 1.0 ml acetylcholine buffer at zero time.

(c) At a predetermined time (Table CH1) the reaction is stopped by adding alkaline hydroxylamine and the procedure carried out exactly as described above.

The standard curve was used to determine the amount of acetylcholine in each sample after incubation with the enzyme (with or without inhibitor). The cholinesterase activity is expressed as micromoles of acetylcholine hydrolysed by 1 ml of plasma, erythrocytes or whole blood in one hour. (The relationship between acetylcholine concentration and colorimeter reading was linear over the range of concentrations used).

TABLE CH1.

CONDITIONS USED FOR THE DETERMINATION OF BLOOD CHOLINESTERASE ACTIVITY

	WHOLE BLOOD		PLASMA		ERYTHROCYTES	
	VOLUME	INCUBATION TIME	VOLUME	INCUBATION TIME	VOLUME	INCUBATION TIME
HORSE	0.1 ml	20 mins	0.05 ml	30 mins	0.01 ml	30 mins
SHEEP	0.1 "	20 "	0.25 "	30 "	0.05 "	30 "
COW	0.1 "	30 "	0.25 "	30 "	0.05 "	30 "
PIG	0.1 "	30 "				
RABBIT	0.1 "	30 "				
GUINEA PIG	0.1 "	30 "				
RAT	0.1 "	30 "				
CHICKEN	0.2 "	30 "				
DOG	0.1 "	30 "				

THE ANTICHOLINESTERASE ACTIVITY OF QUINURONIUM SULPHATE AND AMICARBALIDE ISETHIONATE

1. IN VITRO.

(a) PLASMA AND R.B.C.'s OF HORSE, COW AND SHEEP.

Blood samples were taken from horses and sheep by jugular venepuncture using a wide bore needle and collecting blood in a heparinised stoppered test tube. Cattle blood was collected at slaughter from the severed jugular and carotid.

The drug compound was added in concentrated solution to the tissue fluid to give the appropriate final concentration as shown below. The mixture was then incubated at 37°C for one hour, after which an aliquot of drug incubated tissue fluid was added to 1 ml of acetylcholine buffer solution and this mixture was incubated at 37°C for thirty minutes (see Table CH1).

Drugs. Quinuronium sulphate in phosphate buffer approximated to blood concentration followed a therapeutic dose, i.e. 20 ug per ml of plasma or r.b.c. haemolysate; amicarbalide isethionate on a similar basis approximated to 100 ug per ml of plasma or r.b.c.'s.; eserine salicylate was used as a control at a concentration of 20 ug per ml.

(b) WHOLE BLOOD DETERMINATIONS OF CHOLINESTERASE.

Nine species were involved in this investigation and whole blood haemolysates were made from blood samples obtained as indicated in Table CH3. Aliquots were taken into a series of stoppered test tubes and to each was added a quantity of inhibitor

compound so covering a range of drug concentrations as shown below in Table CH2.

TABLE CH2.

ESERINE	0,	5,	10,	25,	50,	100 ug/ml
QUINURONIUM	0,	5,	10,	25,	50,	100 ug/ml
AMICARBALIDE	0,	5,	10,	25,	50,	100, 500 ug/ml and 1 mgm/ml

At each drug concentration, estimations were always carried out in pairs and the mean of two readings taken.

All the tubes now containing the required concentration of releaser compound were incubated for one hour at 37°C in order that enzymic inhibition could take place. After one hour had elapsed, 1 ml of buffer acetylcholine was added to each tube and all tubes were again incubated at 37°C for a further twenty to thirty minutes (see Table CH1) after which time the reaction was arrested by adding 2 ml of alkaline hydroxylamine to each tube and the estimation carried out as described previously using a non-acetylcholine tube (blank), and an unreacted acetylcholine tube (standard).

The degree of enzymic inhibition was determined at each dose level for the three compounds, eserine, quinuronium and amicarbalide, in the nine species, horse, sheep, cow, pig, dog, chicken, rabbit, guinea pig and rat.

TABLE CH3

METHODS OF BLOOD SAMPLING

SPECIES	METHOD OF OBTAINING WHOLE BLOOD SAMPLE
HORSE, SHEEP	Jugular venepuncture into heparinised tube
COW, PIG	Mixed arterial - venous blood at slaughter into heparinised tube
DOG	Heart puncture at the time of euthanasia
CHICKEN	Wing vein into heparinised tube
RABBIT	Ear vein into heparinised tube
GUINEA PIG, RAT	Whole animal heparinised (intraven.), killed by stunning and mixed A.-V. blood collected from the neck

(c) THE EFFECT OF INCREASED SUBSTRATE CONCENTRATION UPON THE ENZYME INHIBITION INDUCED BY QUINURONIUM SULPHATE AND ESERINE SALICYLATE.

The techniques employed are similar to those described above for the in vitro activity of the inhibitors on whole blood haemolysate.

1. Sheep blood was haemolysed by mixing with four volumes of 0.1% saponin solution.

2. 0.5 ml of this blood solution was added to 2 ug of quinuronium or 1 ug eserine - each contained in 0.5 ml phosphate buffer solution.

3. The blood and inhibitor were incubated together at 37°C for one hour; after which varying quantities of acetylcholine were added to a duplicated series of tubes (each of which now contained the 1 ml of blood - inhibitor mixture) as follows:-

Acetylcholine 1, 2, 4, 8, 20 + 40 u moles.

4. The acetylcholine was reacted for twenty minutes at 37°C with the blood enzyme - inhibitor system and the hydrolysis was arrested after twenty minutes by the addition of 2 mls alkaline hydroxylamine to each tube. N.B. In the case of the tubes containing 8, 20 and 40 u moles, the contents of each tube was diluted by factors of 2, 5 + 10 respectively to bring the potential acetylcholine concentration into the range of the standard curve which is employed to calculate the acetylcholine concentrations from the colorimetric readings (see above). Before the final calculation these results had to be multiplied by the dilution factor.

5. Together with this series of tubes; another containing the range of unreacted acetylcholine concentrations was employed as standard and control Ach reactions (uninhibited) were also estimated. The colorimeter was set to zero using a "non-acetylcholine" tube which contained all the reagents and solutions excluding the substrate.

Drugs. Quinuronium and eserine were investigated.

2. IN VIVO.

(a) ANTICHOLINESTERASE STUDIES IN SHEEP.

In view of the work carried out by Rummler and Laue (1961) on the living animal, in which lowering of circulating cholinesterase activity was shown after injection of quinuronium; it was thought necessary to investigate more fully the events taking place in the blood of sheep injected with a therapeutic dose of a number of anticholinesterase compounds. The work of Rummler and Laue (1961) had suggested an easily reversible inhibition by quinuronium, and it was shown in the previous experiments that in fact quinuronium tended to resemble the mode of action of eserine and to be of the competitive type (substrate reversal).

Technique. The sheep was chosen as the experimental animal both for convenience and to compare with Rummler and Laue's work. The animals were all South Country Cheviots of approximately one year old which had been housed in the department for several weeks before experimentation began. The animals were of mixed sexes (including castrated males) taken at random.

The first blood sample from each sheep was taken immediately prior to an injection of atropine which was thirty minutes before the injection of the suspected anticholinesterase agent. Thus after this lapse of half an hour (to allow for full atropinisation, 1 mgm per kilo) a further blood sample was taken; immediately after which the animal received the anticholinesterase drug. Blood samples were taken at five, ten, fifteen, thirty and sixty minutes; two, three, four, six and twenty-four hours; and again on the seventh and fourteenth days post injection (Table CH7),

and were assayed for cholinesterase activity by the colorimetric method already described.

Drugs. Eserine salicylate; quinuronium sulphate; amicarbalide isethionate; octamethylpyrophosphoramide (O.M.P.A.) and atropine sulphate.

(b) THE EFFECT ON CIRCULATING CHOLINESTERASE OF A SECOND DOSE OF QUINURONIUM.

In the same manner as already described, sheep were bled and injected intravenously with atropine (1 mgm per kilo). After thirty minutes a blood sample was taken and immediately followed by a subcutaneous injection of quinuronium. Further blood samples were taken at five, fifteen, thirty and sixty minutes; at two, three, four, six and twenty-four hours, and again on the seventh and fourteenth days, as before. On the fourteenth day, the process of injection and blood sampling was repeated exactly as described above (see Table CH8 and also Appendix Fig. K).

(c) STUDIES WITH PYRIDINE 2-ALDOXIME METHIODIDE IN SHEEP.

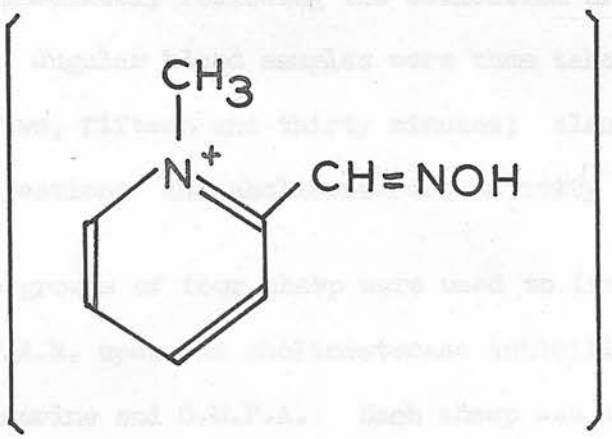
Rummler and Laue (1961) were the first workers in this field to conclude that quinuronium sulphate was an anticholinesterase compound although many authors had referred to some form of cholinergic stimulation for almost thirty years before this publication. The "symptoms" described by certain authors: (Sergent, et al, 1933; Smythe, 1935; Cernaianu, et al, 1935; and Kikuth, 1935) as early as 1935 were to some extent consistent with what we now recognise in cases of acute acetylcholine

poisoning following the administration of a potent anticholinesterase substance such as D.F.P. or the insecticides Malathion and Parathion to a mammal. As noted previously, Rummler and Laue (1961) suspected that quinuronium might have anticholinesterase properties in view of the similarity of its molecule to certain urethanes known to possess this activity.

These authors thought it possible that the recently introduced idea of organophosphorus cholinesterase inhibition being relieved by PAM (pyridine 2-aldoxime methiodide) (Wilson and Ginsburg, 1955), (Hobbiger, 1955, 1956, 1957 and 1959), and (Rutland, 1958), might be an indication that premedication of an animal (in this case sheep) with PAM could have beneficial effects in preventing the onset of acetylcholine poisoning as a result of therapy with quinuronium. In fact, useful prophylaxis had been reported in 1957 by Brown, Kunkel et al, in the case of organophosphorus poisoning by the use of a combination of atropine and PAM.

Formula.

PYRIDINE 2-ALDOXIME METHIODIDE (2.P.A.M.)



This form of prophylaxis was administered by Rummler and Laue (1961) to sheep prior to quinuronium therapy as has been explained above, the outcome was appraised purely on the result of clinical observation which could not (according to published data) necessarily be interpreted as a significant alleviation of toxicity by PAM.

The observations on Rummler and Laue's publication led naturally on to a direct study of cholinesterase activity circulating in the blood of sheep which had been premedicated with atropine and 2.P.A.M.

Techniques.

1. In view of the observation that 2.P.A.M. may in its own right bring about interference with acetylcholine metabolism, (Grob, 1961); possibly inhibiting the cholinesterase enzyme as such, it was decided to investigate the levels of circulating cholinesterase in sheep which were given a full dose (20 mgm per kilo) of 2.P.A.M. intravenously.

Three sheep were so treated. The animals each received 20 mgm per kilo of pyridine 2-aldoxime methiodide into the jugular vein immediately following the collection of a control blood sample. Jugular blood samples were then taken at intervals of five, fifteen and thirty minutes; also one and two hours after injection; and cholinesterase activity estimated.

2. Three groups of four sheep were used to investigate the effects of 2.P.A.M. upon the cholinesterase inhibition induced by quinuronium, eserine and O.M.P.A. Each sheep was injected with

TABLE CH4.

PER CENT INACTIVATION OF PLASMA AND R.B.C. CHOLINESTERASE IN VITRO BY INHIBITOR COMPOUNDS

CONDITIONS		PER CENT ENZYMIC ACTIVITY (MEAN ± S.E.)					
DRUG	conc. ug/ml	HORSE		SHEEP		COW	
		PLASMA	R.B.C.	PLASMA	R.B.C.	PLASMA	R.B.C.
NONE	-	100	100	100	100	100	100
ESERINE	20	12.0±2.3	31.0±4.7	10.0±2.0	34.0±3.5	32.0±5.0	15.0±3.0
QUINURONIUM	20	39.0±9.0	32.0±2.7	15.0±3.4	30.0±1.3	14.0±2.3	15.0±1.4
AMICARBALIDE	100	84.0±3.0	69.0±4.1	70.0±3.8	88.0±3.0	83.0±2.4	94.0±2.0

Number of animals in all cases is 4.

atropine sulphate (1 mgm per kilo) intravenously. After thirty minutes a control blood sample was taken and PAM (20 mgm per kilo) injected intravenously. At the same time, quinuronium, eserine or O.M.P.A. was injected subcutaneously and venous blood samples were taken as previously described at five, fifteen and thirty minutes; also at one, two, four, six and twenty-four hours after injection, and again on the seventh day.

RESULTS.

THE ANTICHOLINESTERASE ACTIVITY OF QUINURONIUM AND AMICARBALIDE.

1. IN VITRO.

(a) PLASMA AND ERYTHROCYTES OF HORSE, COW AND SHEEP.

Eserine was confirmed as being a potent inhibitor of cholinesterase activity and quinuronium was approximately equipotent with eserine. Both drugs had an approximately equal action on each blood fraction. Amicarbalide had a very weak action on cholinesterase activity. (Results are shown in Table CH4 and Figs. CH1, 2 and 3).

(b) WHOLE BLOOD DETERMINATIONS OF CHOLINESTERASE.

In all species, curves were plotted of the percentage inactivation of the enzyme by the drug (ordinates); against the concentrations of drug used (abscissa) and results are shown in Fig. CH4 (eserine, sheep), CH5 (quinuronium) and CH6 (amicarbalide.)

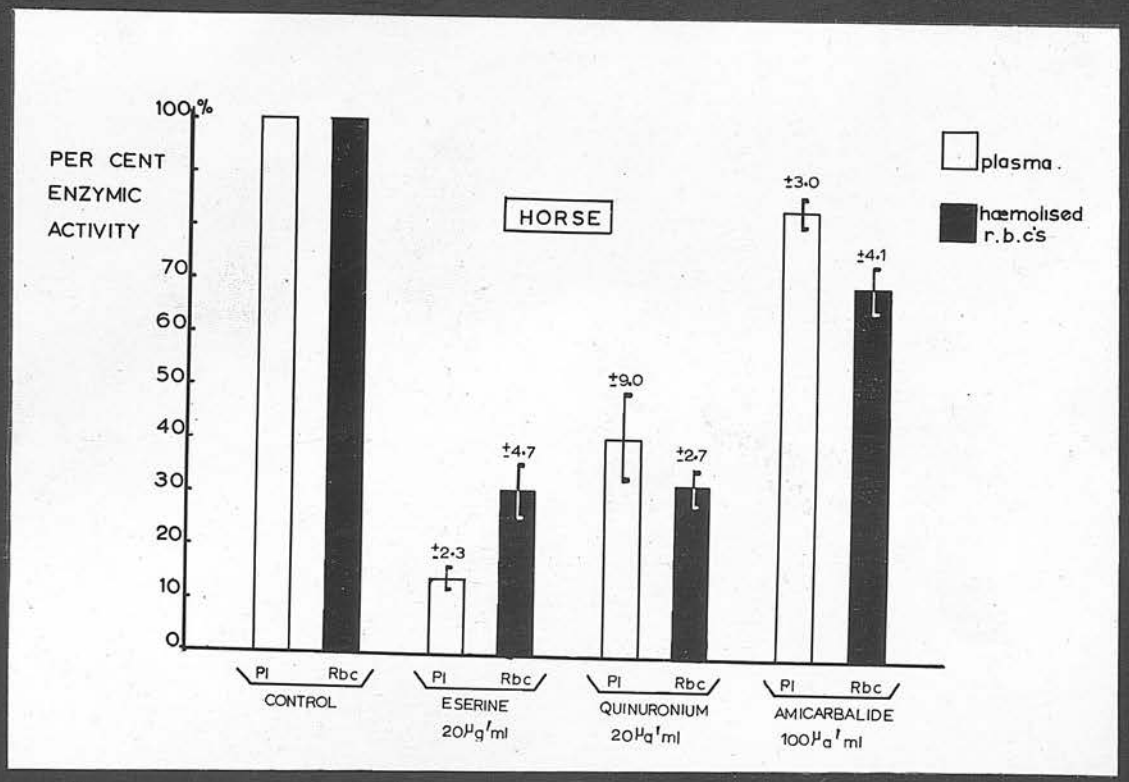


FIG. CH. 1.

THE INHIBITION BY ESERINE, QUINURONIUM AND AMICARBALIDE IN VITRO, OF PLASMA AND ERYTHROCYTE (r.b.c.) CHOLINESTERASE ACTIVITY IN THE HORSE.
ORDINATE = % CHOLINESTERASE ACTIVITY.
VALUES ARE MEANS OF FOUR ANIMALS ± S.E.

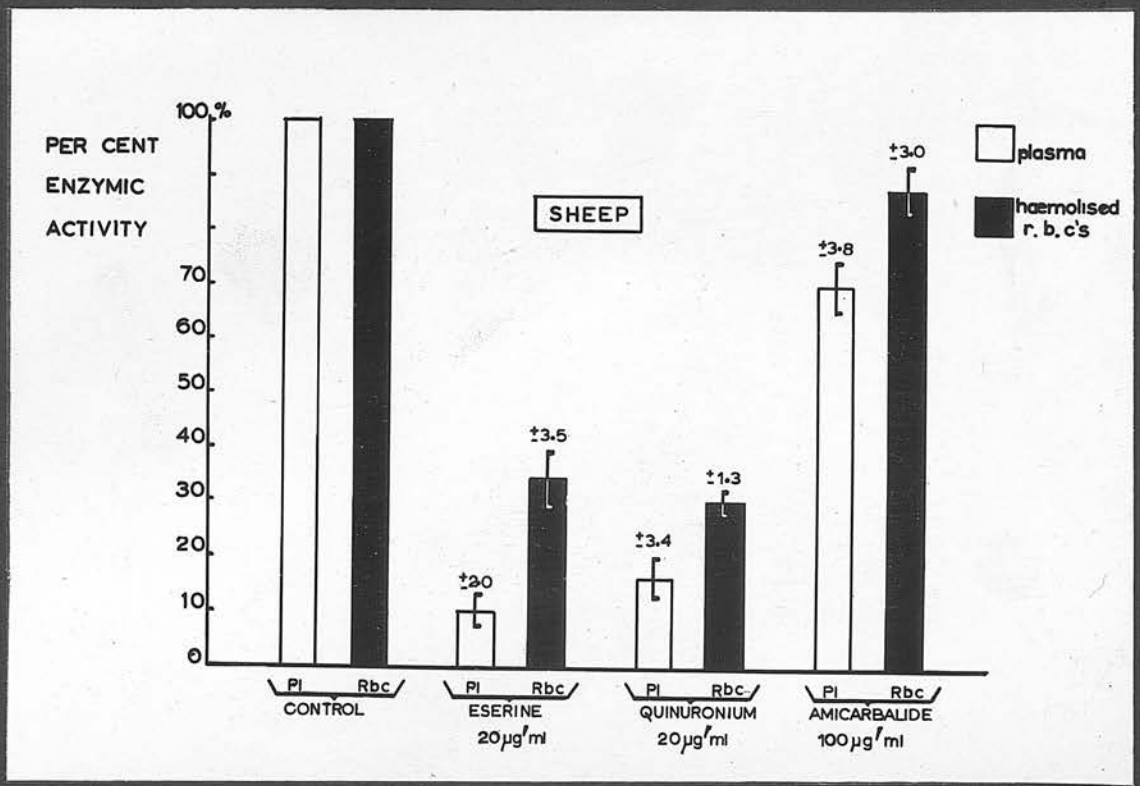


FIG. CH. 2.

THE INHIBITION BY ESERINE, QUINURONIUM AND AMICARBALIDE IN VITRO, OF PLASMA AND ERYTHROCYTE (r.b.c.) CHOLINESTERASE ACTIVITY IN SHEEP.

ORDINATE = % CHOLINESTERASE ACTIVITY.

VALUES ARE MEANS OF FOUR ANIMALS ± S.E.

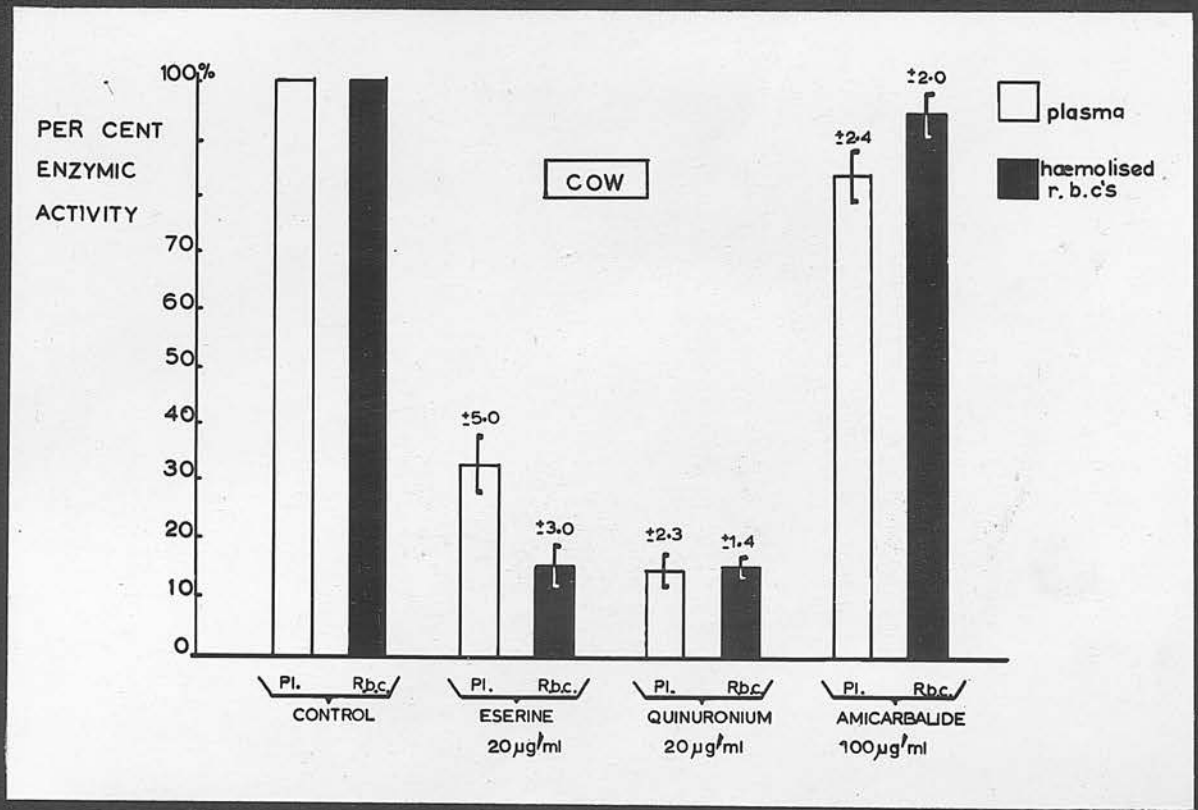


FIG. CH. 3.

THE INHIBITION BY ESERINE, QUINURONIUM AND AMICARBALIDE IN VITRO, OF PLASMA AND ERYTHROCYTE (r.b.c.) CHOLINESTERASE ACTIVITY IN CATTLE.
ORDINATE = % CHOLINESTERASE ACTIVITY.
VALUES ARE MEANS OF FOUR ANIMALS ± S.E.

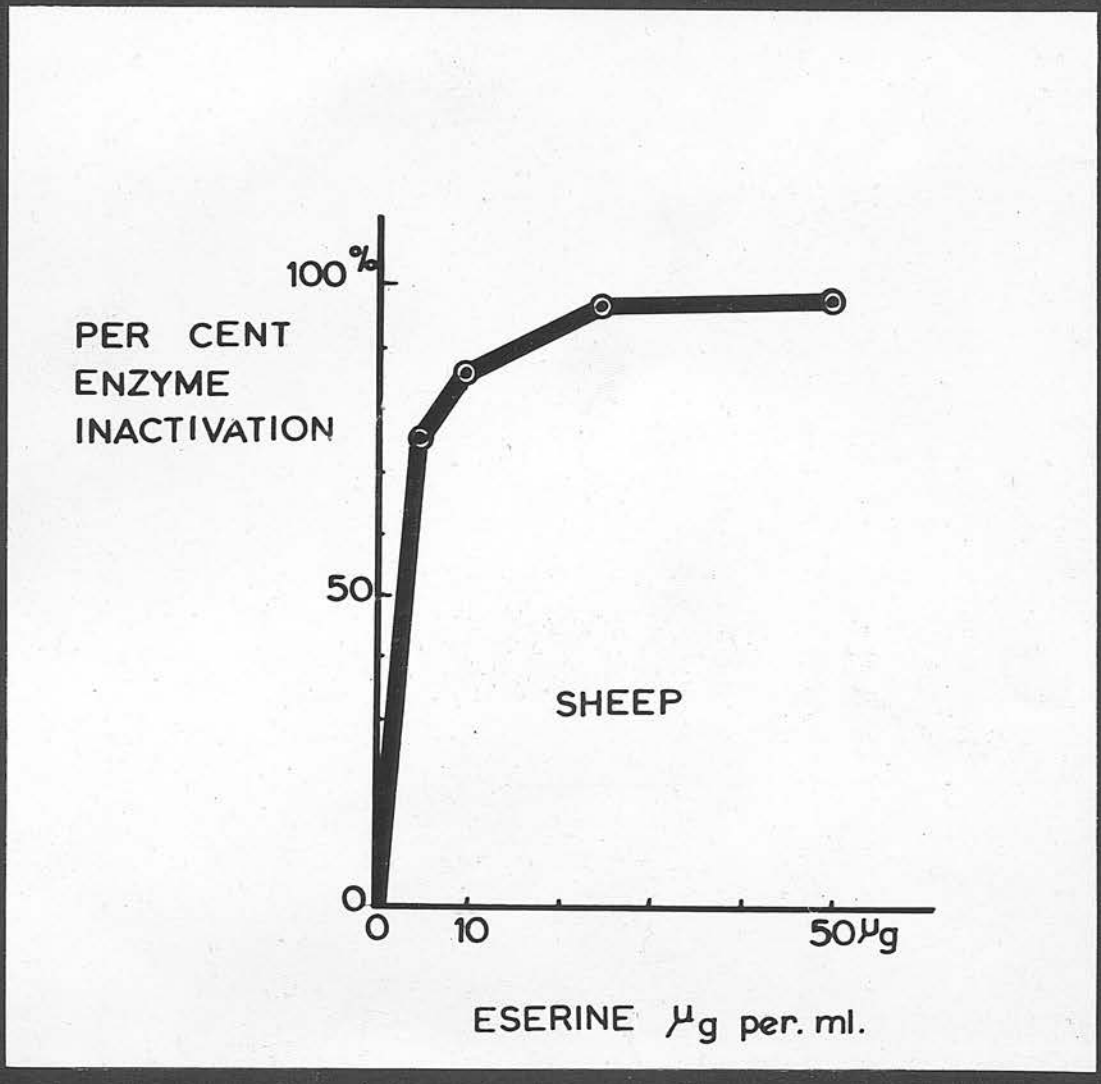


FIG. CH. 4.

IN VITRO INHIBITION OF WHOLE BLOOD CHOLINESTERASE ACTIVITY BY ESERINE, MEASURED BY THE COLORIMETRIC METHOD. (THE CURVE REPRESENTS THE SITUATION IN ALL SPECIES, WHICH MAY BE SEEN IN THE APPENDIX, FIGS. A - J. incl.)

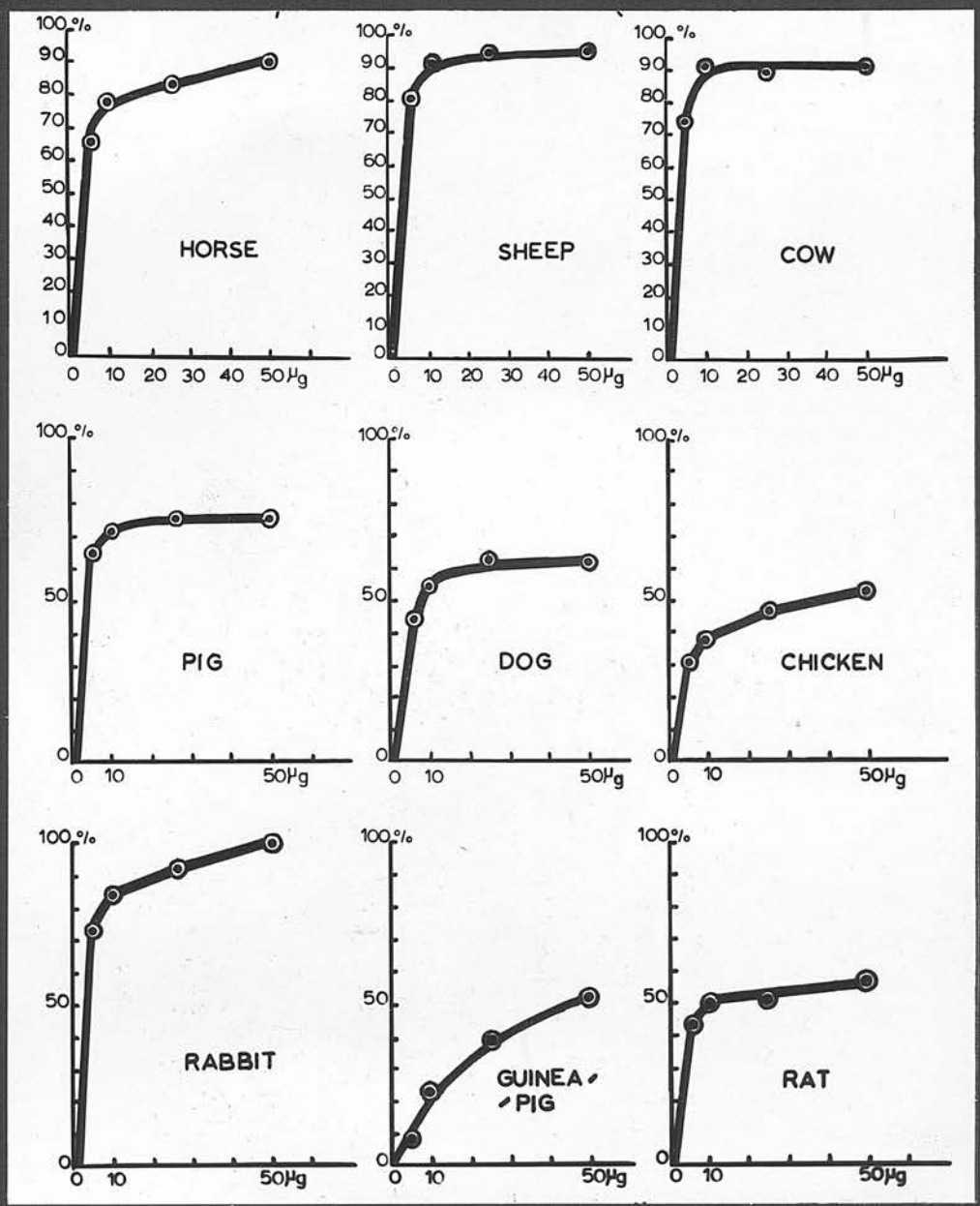


FIG. CH. 5.

SPECIES VARIATION IN INHIBITION OF WHOLE BLOOD CHOLINESTERASE ACTIVITY BY QUINURONIUM SULPHATE, MEASURED BY THE COLORIMETRIC METHOD.

ORDINATES= % ENZYME INACTIVATION.

ABSCISSAE = MICROGRAM. QUINURONIUM PER ML BLOOD.

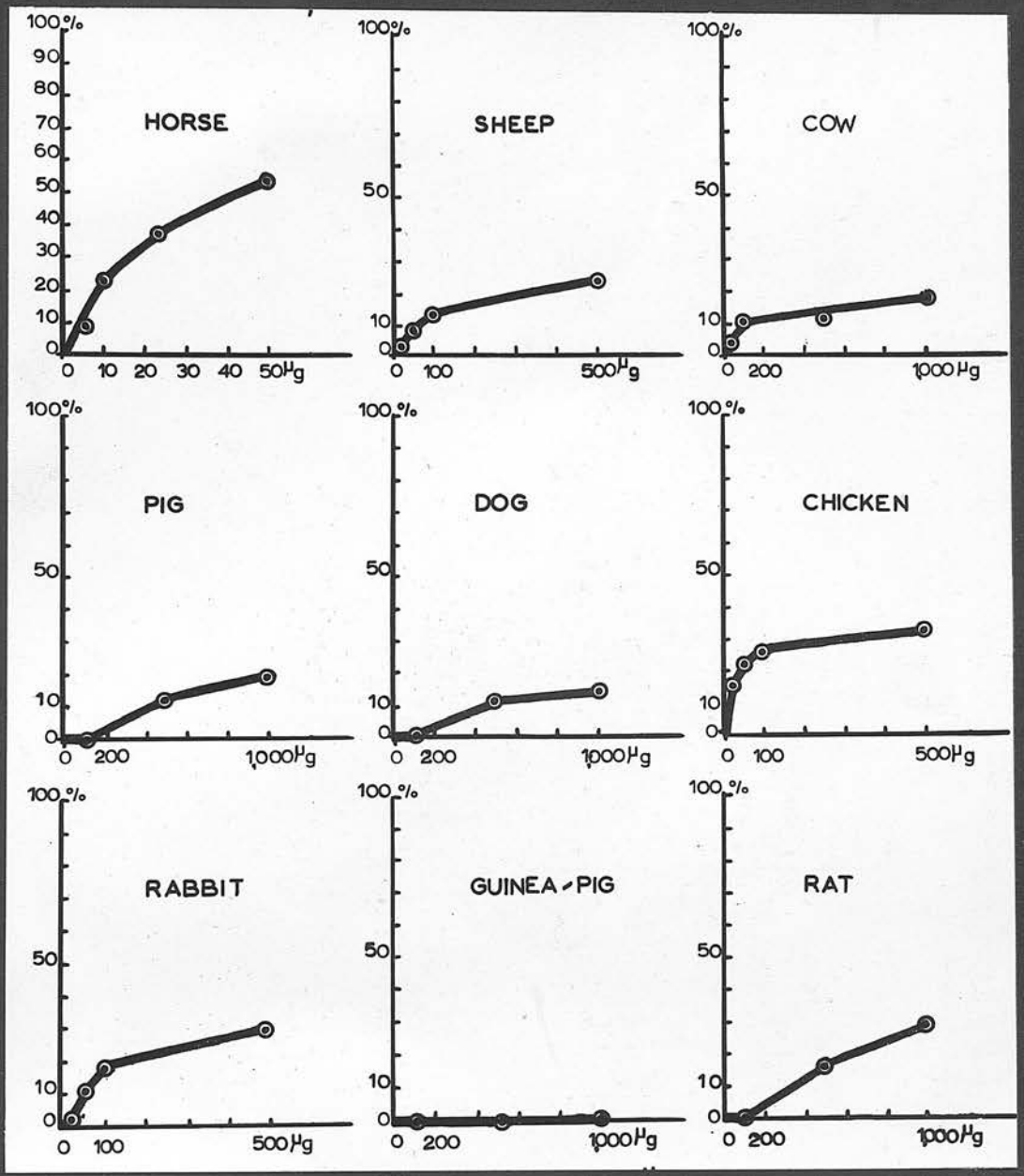


FIG. CH. 6.

SPECIES COMPARISON OF INHIBITION OF WHOLE BLOOD CHOLINESTERASE ACTIVITY BY AMICARBALIDE ISETHIONATE, MEASURED BY THE COLORIMETRIC METHOD.
ORDINATES = % ENZYME INACTIVATION.
ABSCISSAE = MICROGRAM. AMICARBALIDE PER ML BLOOD.

TABLE CH5.

EVALUATION OF WHOLE BLOOD ANTICHOLINESTERASE ACTIVITY OF ESERINE, QUINURONIUM AND AMICARBALIDE BY THE COLORIMETRIC METHOD

SPECIES	ANTICHOLINESTERASE ACTIVITY pI_{50} VALUE		
	ESERINE SALICYLATE	QUINURONIUM SULPHATE	AMICARBALIDE ISETHIONATE
HORSE	5.3	5.2	5.1
SHEEP	5.1	5.3	4.1
COW	5.1	5.2	3.4
RABBIT	5.0	5.2	3.7
PIG	5.0	5.2	3.4
RAT	5.3	4.8	3.6
GUINEA PIG	5.1	4.2	-
CHICKEN	5.1	4.3	3.0
DOG	5.1	4.9	3.4

It is seen from Fig. CH_4 (which is the curve produced by incubating a range of concentrations of eserine with sheep blood), that such a curve begins at very low dose levels and rises very steeply. The dose range from 1 to 5 ug/ml covers 70 to 80% enzyme inhibition in all nine species in which the shape of the curve for eserine was virtually identical in all species, as can be seen in Appendix Figures A to J (incl.).

The curve of cholinesterase inhibition by quinuronium (Fig. CH5) was steep and similar to eserine in horse, sheep, cow, pig and rabbit. In dog, chicken and rat the curve was steep but rapidly achieved a maximum (equilibrium under the prevailing conditions) at about 50% inhibition. In the guinea pig, the slope of the curve was least of all nine species.

A comparison of pI_{50} figures (i.e. logarithm of reciprocal molar inhibitor concentration required to produce 50% enzyme inhibition: Blaschko, Bülbring and Chou (1949)), indicated that in the whole blood of horse, cow, sheep, rabbit and pig quinuronium had the same degree of activity as eserine; whereas in rat, guinea pig, chicken and dog quinuronium was less active than eserine in respect of cholinesterase inhibition under the experimental conditions (Table CH5).

In the case of amicarbalide very little inhibition could be produced in any species save the horse in which a fairly steep curve was obtained, indicating some 50% inhibition at a dose level of 50 ug/ml. In the guinea pig no inhibition by amicarbalide could be detected in the conditions of the experiment and in the seven other species, varying slight degrees of inhibition were

TABLE CH6.

THE EFFECT OF INCREASING SUBSTRATE CONCENTRATION UPON CHOLINESTERASE INHIBITION PRODUCED BY ESERINE AND QUINURONIUM

SUBSTRATE CONCENTRATION (u moles Ach.)	PER CENT ENZYME INHIBITION AFTER INCUBATION WITH:-	
	ESERINE (1 ugm)	QUINURONIUM (2 ugm)
1	79.0±1.5	86.0±6.00
2	89.0±3.0	80.0±6.40
4	81.0±4.5	94.0±7.80
8	75.0±4.9	87.0±5.20
20	30.0±5.6	21.0±7.30
40	15.0±4.5	10.0±6.4

Figures are expressed as means with standard error.
Number of estimations for each mean = 4.

produced from 10% to 30% at a concentration of amicarbalide ranging from 100 ug to 1 mgm per ml. A comparison of pI_{50} figures showed that in horse whole blood amicarbalide had anti-cholinesterase activity which was of the same order as eserine and quinuronium. In sheep whole blood amicarbalide activity was rather less than eserine, but in rabbit, pig, rat, chicken and dog the activity was very much less (see Table CH5). In appendix Figures A to J (incl.) the three respective curves (corresponding to inactivation of whole blood of each species by the three inhibitor compounds) are drawn on the same diagram to allow for a comparison of activity of the three agents in each individual species.

(c) THE EFFECT OF INCREASED SUBSTRATE CONCENTRATION UPON ENZYME INHIBITION INDUCED BY QUINURONIUM AND ESERINE.

Results were calculated as the percentage inactivation produced by a constant amount of inhibitor (eserine or quinuronium) in the presence of varying quantities of substrate (Ach).

In the case of both eserine and quinuronium it was shown that an increase in the concentration of acetylcholine up to 40 u moles per ml resulted in a diminished ability to inhibit the enzyme cholinesterase. Up to and including 8 u moles per ml the percentage inhibition remained fairly constant and thereafter fell markedly, see Table CH6 and Figs. CH7 and 8.

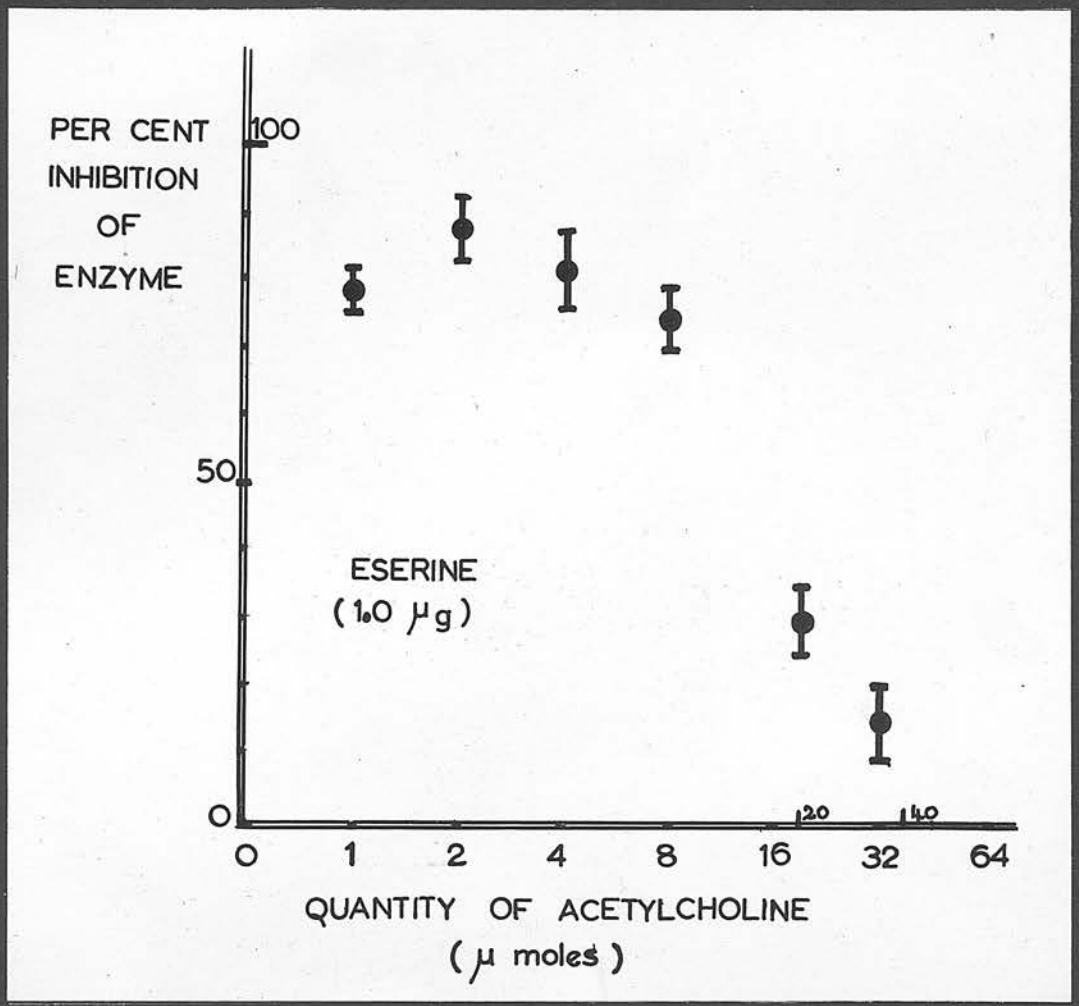


FIG. CH. 7.

IN VITRO SUBSTRATE (ACETYLCHOLINE) COMPETITION OF THE INHIBITION OF WHOLE BLOOD CHOLINESTERASE ACTIVITY BY ESERINE.

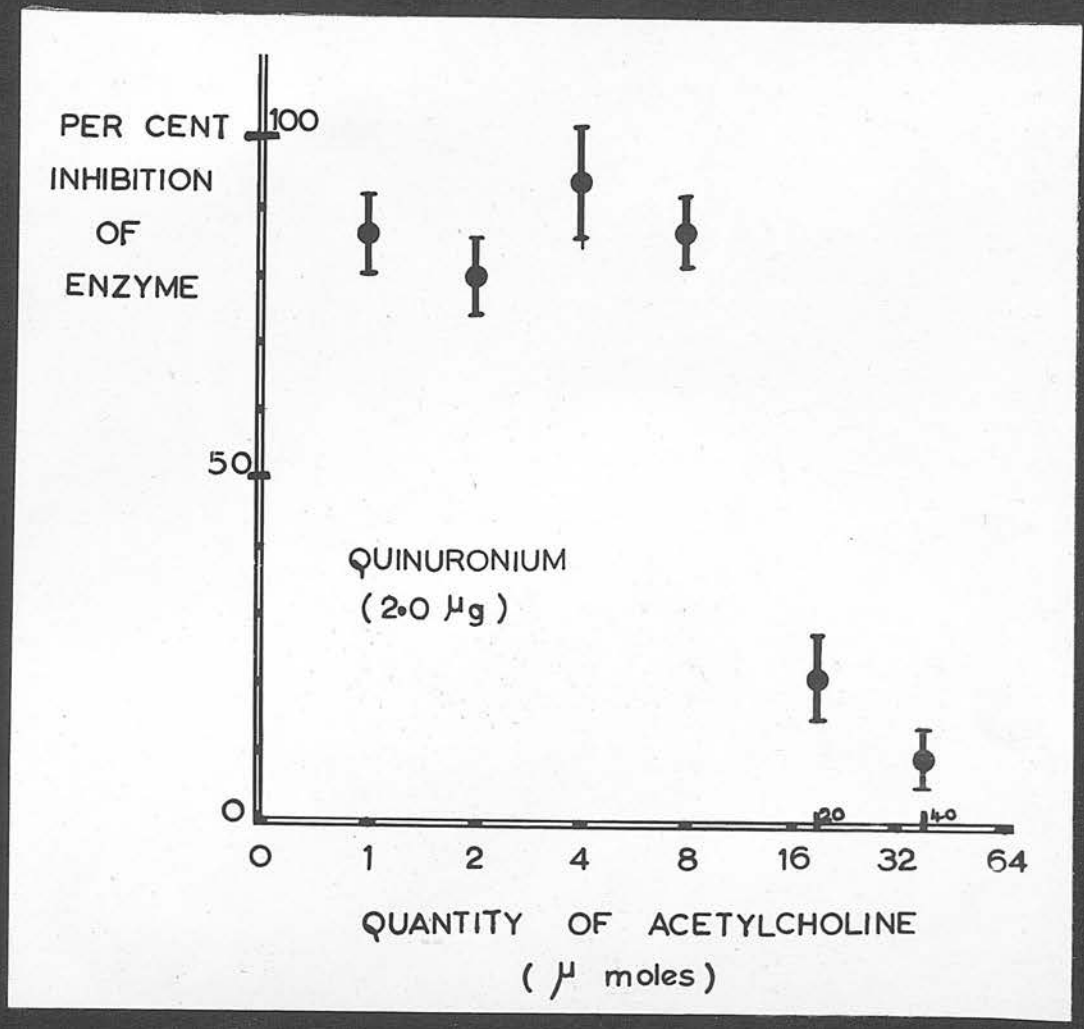


FIG. CH. 8.

IN VITRO SUBSTRATE (ACETYLCHOLINE) COMPETITION OF THE INHIBITION OF WHOLE BLOOD CHOLINESTERASE ACTIVITY BY QUINURONIUM.

TABLE CH7.

THE ANTICHOLINESTERASE ACTIVITY OF ESERINE, OCTAMETHYLPYROPHOSPHORAMIDE, QUINURONIUM AND AMICARBALIDE IN WHOLE BLOOD OF LIVE SHEEP

DRUG DOSE	Mean percentage cholinesterase activity in sheep whole blood in vivo after injection of the inhibitor drugs															
	CONTROLS				Time after drug injection											
					Minutes					Hours					Days	
	1		2		0	5	15	30	60	2	3	4	6	24	7	14
ESERINE s/c 20 mgm	100 -	ATROPINE i/v 1mgm per kilo	100 -	INJECT INHIBITOR	100 -	79 ±5.4	66 ±8.2	88 ±10.8	37 ±9.6	50 ±8.3	61 ±6.6	68 ±6.1	84 ±8.0	95 ±4.3	103 ±7.2	105 ±10.0
OCTAMETHYLPYRO--PHOSPHORAMIDE i/v 100 mgm	100 -		100 -		100 -	68 ±7.7	52 ±5.1	26 ±4.5	20 ±8.0	15 ±9.1	15 ±6.5	15 ±5.4	20 ±7.3	42 ±12.0	63 ±6.7	93 ±9.3
QUINURONIUM s/c 30 mgm	100 -		100 -		100 -	83 ±7.3	57 ±9.2	41 ±4.7	44 ±2.2	56 ±8.0	66 ±7.2	70 ±3.8	68 ±7.0	88 ±7.7	95 ±4.1	92 ±4.8
AMICARBALIDE s/c 200 mgm	100 -		100 -		100 -	87 ±5.1	71 ±6.4	67 ±3.4	72 ±5.2	77 ±3.7	88 ±5.8	92 ±7.6	89 ±6.1	98 ±2.0	98 ±2.0	102 ±6.3

Values are mean percentages with standard errors.
Number of animals = 4 in all cases.

2. IN VIVO.

(a) ANTICHOLINESTERASE STUDIES IN SHEEP.

(Table CH7 and Figure CH9)

Eserine salicylate 20 mgm (4 sheep).

Eserine produced a rapid and profound fall in blood cholinesterase activity. Enzymic activity fell to a mean of 37% (±9) within an hour. Thereafter activity gradually returned to normal: within six hours enzymic activity was 84% (±4) of normal and at twenty-four hours was 95% (±4) of normal. At the seventh and fourteenth days, the activity of the enzyme was respectively 103% and 105% of normal.

Quinuronium sulphate 30 mgm (4 sheep).

Injection of the chemotherapeutic agent was followed by a very rapid fall in blood cholinesterase activity to a mean of 41% (±4) within thirty minutes, after which the enzyme activity returned to normal more slowly than with eserine. At six hours the activity was 68% (±7), and at twenty-four hours 88% (±7). The activity of the blood on the seventh and fourteenth days respectively was 95% (±4) and 92% (±4) which could not be regarded as significantly different from normal. However, despite lack of statistical significance, the blood enzyme activity of these four sheep appeared to remain depressed over the two weeks when compared with the four sheep treated with eserine.

Amicarbalide isethionate 200 mgm.

Amicarbalide reduced the blood cholinesterase activity to only about 70%. At the fourth and sixth hours the enzyme levels were respectively 92% (±7) and 89% (±6) which were well within

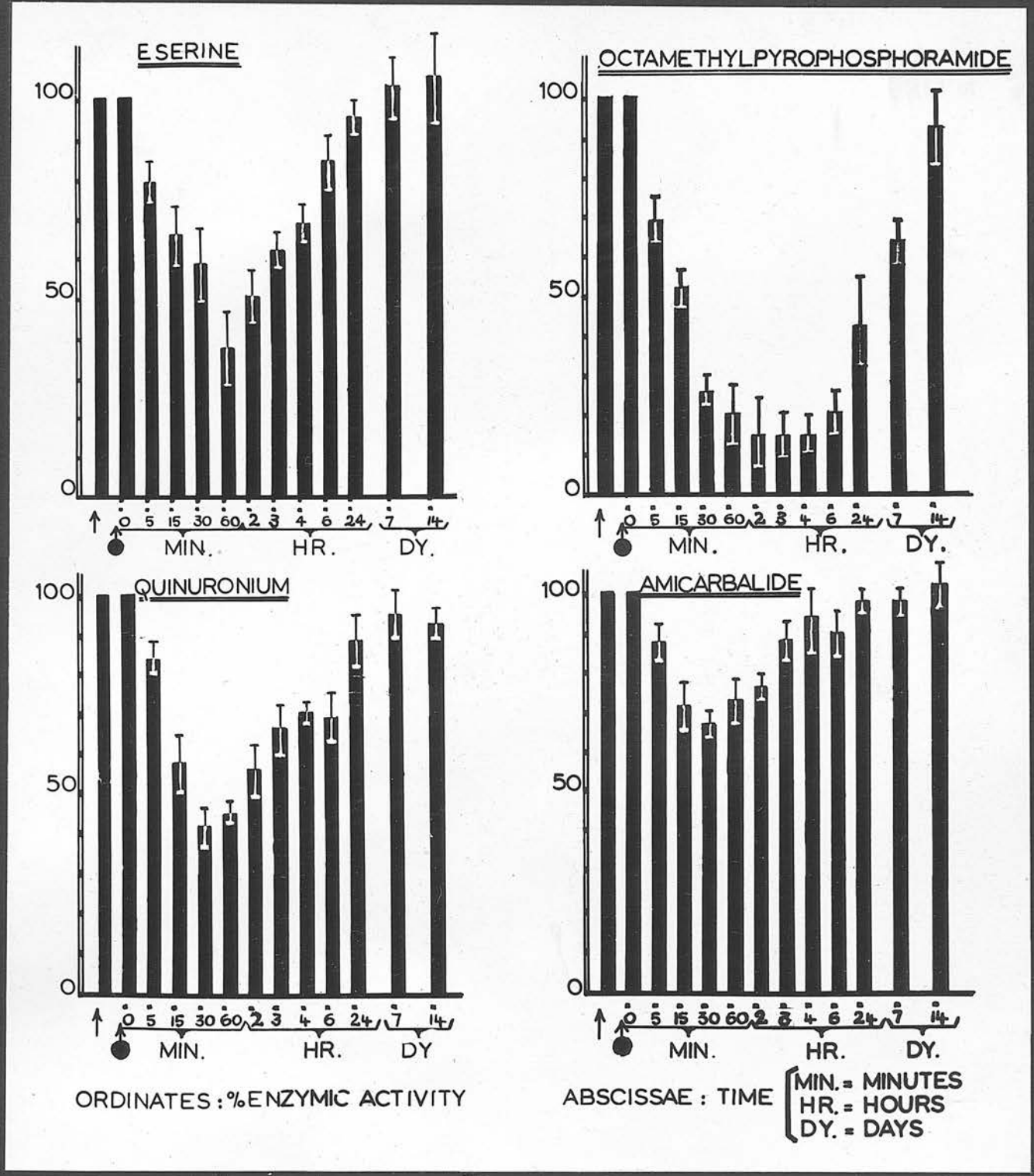


FIG. CH 9.

THE INHIBITION OF WHOLE BLOOD CHOLINESTERASE ACTIVITY IN ATROPINISED SHEEP IN VIVO BY SUB-CUTANEOUS INJECTION OF: (1) ESERINE SALICYLATE; (2) OCTAMETHYL PYROPHOSPHORAMIDE; (3) QUINURONIUM SULPHATE; (4) AMICARBALIDE ISETHIONATE.

TABLE CH8.

THE EFFECT OF A SECOND DOSE OF QUINURONIUM ON THE WHOLE BLOOD CHOLINESTERASE ACTIVITY OF SHEEP

DRUG DOSE	Mean percentage cholinesterase activity of whole blood of sheep after injection of quinuronium, repeated after two weeks															
	CONTROLS				Time after injection											
					Minutes					Hours					Days	
	1		2		0	5	15	30	60	2	3	4	6	24	7	14
QUINURONIUM s/c 30 mgm FIRST INJECTION	100 -	ATROPINE i/v	100 -	QUINURONIUM s/c	100 -	66 ±5.6	57 ±2.0	48 ±4.2	42 ±2.8	52 ±5.1	63 ±3.7	71 ±2.9	80 ±3.0	96 ±3.6	93 ±5.4	91 ±8.3
QUINURONIUM s/c 30 mgm SECOND INJECTION	91 ±8.0		90 ±6.7		- -	72 ±3.3	61 ±7.0	49 ±9.1	39 ±7.3	31 ±3.2	39 ±3.1	- -	51 ±9.4	80 ±6.5	86 ±10.1	94 ±4.5

Values are mean percentages with standard errors.
Number of animals = 4.

normal limits and remained so over the following two weeks.

Octamethylpyrophosphoramide 100 mgm.

According to DuBois, Doull & Coon (1950) the L.D.50 of this compound varies from 5 to 17 mgm per kilo depending on the species and route of administration. 5 mgm per kilo was chosen as an initial sheep dose. This compound was slow in affecting the cholinesterase activity; the lowest reading (15 - 20%) being attained after one and a half to two hours. Thereafter, recovery was very slow. At the sixth hour enzymic activity remained depressed at 15 - 20% and at twenty-four hours enzyme activity was only 40% of normal. By the fourteenth day post injection however, the activity of the enzyme was within a few per cent of normal.

(b) THE EFFECT OF A SECOND DOSE OF QUINURONIUM.

Within one hour of the second injection of quinuronium (day 14) the level of circulating cholinesterase was a mean of 29% normal which was 10% lower than the enzymic activity following the first injection, (Table CH8), and in spite of the high dose of atropine which had been used, the sheep showed more marked toxicity than after the first dose of quinuronium, (see also Appendix K).

(c) STUDIES WITH PYRIDINE 2-ALDOXIME METHIODIDE IN SHEEP.

1. 2.P.A.M. alone injected intravenously in sheep produces a very slight but nevertheless positive lowering of blood cholinesterase (Table CH9). The effect was very transient, and

TABLE CH9.

THE INFLUENCE OF PYRIDINE 2-ALDOXIME METHIODIDE (2 P.A.M.) ON THE ANTICHOLINESTERASE ACTIVITY IN SHEEP BLOOD OF ESERINE, QUINURONIUM AND OCTAMETHYLPYROPHOSPHORAMIDE (O.M.P.A.)

DRUG DOSE	Percentage cholinesterase activity in sheep whole blood														
	CONTROLS				Time after injection										
					Minutes					Hours					Day
	1		2	-	5	15	30	60	-	2	3	4	6	24	7
2-PAM i/v 20 mgm per kilo	100 -	INJECT ATROPINE	100 -	- -	86 -	79 -	100 -	100 -	- -	- -	- -	- -	- -	- -	- -
ESERINE s/c 20 mgm	100 -		100 -	Inject 2-PAM + ESERINE	80 ±0.9	39 ±1.9	37 ±4.1	37 ±3.2	- -	36 ±9.1	- -	67 ±6.6	92 ±8.0	99 ±6.4	104 ±7.8
QUINURONIUM s/c 30 mgm	100 -		100 -	Inject 2-PAM + QUINURONIUM	97 ±6.7	49 ±10.5	31 ±8.9	31 ±6.9	Inject i/v 2-PAM	48 ±4.7	52 ±6.2	64 ±5.0	90 ±2.2	93 ±4.8	96 ±8.4
O.M.P.A. i/v 5 mgm per kilo	100 -		100 -	Inject 2-PAM + O.M.P.A.	81 ±3.6	65 ±8.8	53 ±5.2	27 ±9.7	Inject i/v 2-PAM	18 ±6.0	56 ±4.4	54 ±4.7	20 ±3.4	51 ±10.0	93 ±2.7

Values are mean percentages with standard errors.
Number of animals used was 3 with 2-PAM alone, and 4 with each of QUINURONIUM, ESERINE and O.M.P.A.

normal enzyme activity was recorded after 30 minutes post PAM.

2. From Table CH9, it can be seen that premedication with PAM intravenously immediately prior to quinuronium therapy did not have any significant effect on the lowering of cholinesterase enzyme activity. A further injection of 2.P.A.M. just prior to blood sampling at two hours did not alter the course of events, which was in fact no different from that in sheep to which PAM had not been administered (Table CH7). Blood cholinesterase activity was some 95% normal at the sixth hour and remained similar at twenty-four hours. By the seventh day the enzyme activity was within a few per cent of normal.

2.P.A.M. likewise failed to protect the sheep enzyme in any way against eserine, (Kewitz, Wilson and Nachmahnsohn).

However, in the case of O.M.P.A., 2.P.A.M. showed a definite tendency to slow the rapidity of onset of enzyme inhibition, but the ultimate minimum activity (18%) was not significantly different from that in sheep which had not received 2.P.A.M.

A further injection of 2.P.A.M. immediately prior to the blood sampling at two hours post-O.M.P.A. showed a significant "recovery" in the enzyme, so that over the next two hours, cholinesterase activity was about 55% of normal. By the sixth hour activity had fallen to 20% of normal and at twenty-four hours was 51%. After the lapse of one week the enzyme activity was 93% of the original control reading. (Compare Table CH7 with CH9).

DISCUSSION.

Mendel and Rudney (1943) and Mendel, Mundell and Rudney (1943) showed that two distinct cholinesterase enzymes existed, both capable of hydrolysing acetylcholine and both sensitive to the effects of eserine. The two enzymes were called "true" and "pseudo" cholinesterase. True cholinesterase as found in brain, and in human and horse erythrocytes was most active in hydrolysing choline esters most closely resembling acetylcholine and is now usually referred to as specific acetylcholinesterase. Pseudo-cholinesterase has greatest activity towards esters closely resembling butyrylcholine and is referred to as non-specific or butyryl-cholinesterase.

It is known that the non-specific cholinesterase may be almost completely inhibited without an animal showing signs of acetylcholine poisoning (Hawkins and Gunter, 1946; Mazur and Bodansky, 1946), but inhibition of specific acetylcholinesterase by some 60 to 70% leads to acute acetylcholine poisoning, (Hawkins and Mendel, 1947 and 1949; Nachmahsohn and Feld, 1947). Experiments have shown that there is a correlation between the inhibition of specific cholinesterase and the pharmacological effects of cholinesterase inhibitors, (Blaschko, Bülbring and Chou, 1949; Kordik, Bülbring and Brown, 1952). No species is thought to be devoid of pseudo-cholinesterase, but ruminants do not possess measurable quantities in their plasma (Mendel et al, 1943; Gunter, 1946). Hence in the in vivo experiments reported here in sheep and cattle it is probable that specific acetyl-cholinesterase was measured in each blood fraction; and in the

horse plasma pseudo-cholinesterase was actually encountered. Fleisher, Pope and Spears (1955) showed in several species that there was a direct relationship between inhibition of the erythrocytic enzyme, which is the critical one, and inhibition of whole blood cholinesterase activity. On this evidence and in view of the fact that in the experiments here, there was no significant difference in the affinity of the inhibitor compounds for the two respective blood fractions, it was considered that whole blood estimations would yield satisfactory results and it should be possible to infer relative potencies of inhibitors from in vitro pI_{50} figures for whole blood haemolysates.

Whole blood pI_{50} figures (using acetylcholine as substrate) showed a wide species variation on the anticholinesterase action of quinuronium and amicarbalide, whereas eserine was constant. Assuming that quinuronium is a competitively reversible inhibitor, the degree of inhibition will depend on equilibrium of both substrate and inhibitor with enzyme receptors, and it may be possible that an inhibitor such as quinuronium has different equilibrium constants with the enzyme receptors of different species - this could produce a differential species susceptibility to the agent.

An important investigation now considered was the determination of the relationship of eserine and quinuronium with the substrate and enzyme. Burgen (1949) showed that the inhibitory power of a competitive anticholinesterase diminishes in the presence of increasing substrate concentration, and also that the enzyme itself is inhibited by excessive quantities of its own

substrate; substrate inhibition. In the investigations to determine the reversibility of quinuronium, samples containing inhibitor were compared with similar samples containing no inhibitor. Thus relative enzymic activities of uninhibited and inhibited cholinesterase were compared at each concentration of acetylcholine. The relative potencies of inhibitors could be calculated by subtracting the activity of inhibited from uninhibited enzyme and expressing the difference as a percentage. Quinuronium was seen to be similar to eserine; i.e. subject to substrate reversal of activity.

The plateau of inhibition over the lower range of acetylcholine concentrations does not agree with the usual expotential curve produced by other authors (Burgen, 1959). However it may be explained by the fact that the lowest concentrations of acetylcholine fall near to or above the upper limit of linearity of the acetylcholine - colorimeter calibration curve which may produce falsely low values at low Ach concentrations.

The experiments described here showed that eserine and quinuronium were competitively reversible by acetylcholine. However it was not possible to give a quantitative expression by this method of assay. Other authors, e.g. Burgen (1949), describe Warburgian estimations which are suitable for the wide range of values involved.

In the living sheep, cholinesterase assays following treatment with cholinesterase inhibitors showed that eserine had a marked but transient effect. Octamethylpyrophosphoramide (O.M.P.A.) had a profound action which was slow in onset:-

80-85% inhibition of whole blood activity occurred after a period of one and a half to two hours. DuBois *et al* (1950) ascribe this slowness of onset to the fact that O.M.P.A. is metabolised in the liver to produce a strong anticholinesterase compound, as O.M.P.A. itself is almost inactive *in vitro*. According to DuBois *et al*, the serum of rats receiving 5 mgm O.M.P.A. per kgm weight did not recover enzyme activity for one week after injection. It was seen that sheep behaved similarly.

Quinuronium had a marked effect on whole blood cholinesterase activity: inhibition was rapid in onset and prolonged. It was shown *in vitro* that quinuronium was a competitively reversible antagonist; nevertheless, Cernaianu *et al* (1935) found that sheep which received a second dose of quinuronium within two weeks of the first dose showed greater toxicity. It seemed important to investigate this directly and try to show whether repeated doses of quinuronium had a cumulative effect. It was shown that a second dose of quinuronium did appear to reduce enzyme activity to a lower level than the first dose, which would support the clinical observations of Cernaianu *et al* (1935). However, using only four animals the standard deviations were so large that the difference was not significant. Nevertheless, clinical signs of toxicity in these four sheep were much more marked after the second dose of quinuronium and it might be inferred that a more exact method of determining cholinesterase activity would be necessary to establish this particular point. A cumulative effect would in any event be difficult to explain in view of the evidence for the competitive reversible nature of quinuronium! It is

possible nevertheless that part of the action of quinuronium on cholinesterase may not be reversible or that other factors produce a cumulative effect which may or may not be associated with anti-cholinesterase activity; for instance, detoxication/excretion about which, as far as can be ascertained, little precise information is available.

Amicarbalide had only a very slight inhibitor effect on circulating cholinesterase and this would not be regarded as clinically significant.

The evidence of Rummler and Laue (1961) that pyridine 2-aldoxime methiodide (2.P.A.M.) significantly alleviated quinuronium poisoning could not be confirmed. 2.P.A.M. is recognised as an agent which protects or reactivates cholinesterase, after the enzyme has been inhibited by organo-phosphorus compounds, (Wilson and Ginsburg, 1955). Enzyme inhibition by organo-phosphorus agents has been described as being the result of phosphorylation of active groups on the enzyme surface, (Bergman, Wilson and Nachmahnsohn, 1950; Wilson, 1954; Hobbiger, 1954) and alleviation of toxicity is said to be brought about by the oxime splitting the phosphate linkages, (Hobbiger, 1955, 1956 & 1957; Hobbiger and Sadler, 1959; Rutland, 1958; Childs, Davies, Green and Rutland, 1955). However, the oximes will produce some protection and alleviation of human cholinesterase inhibited by quaternary ammonium compounds such as neostigmine, but <u>not</u> eserine, (Grob and Johns, 1958; Kewitz, Wilson and Nachmahnsohn, 1956). It must follow, therefore, that oximes reverse cholinesterase inhibition by means other than splitting the phosphate linkages.

High concentrations of 2.P.A.M. have produced reversible block to acetylcholine in isolated frog muscle, (Fleisher, Corrigan and Howard, 1958) so it may be that one of the actions of oximes is to antagonise acetylcholine.

In sheep 2.P.A.M. itself showed slight inhibition of whole blood cholinesterase which agreed with earlier observations in other species (Grob, 1961). However, the oxime afforded no protection against the inhibitory activity of eserine, (Grob and Johns, 1958) or quinuronium: (in the latter case, 2.P.A.M. was useless prophyllactically and therapeutically). Nevertheless, the oxime protected onset of inhibition by O.M.P.A. and relieved inhibition during the period of depression of enzyme activity. Such relief was short-lived owing to the short life of 2.P.A.M. which has a half clearance of 0.9 hour; the molecule being removed by the kidney (Grob, 1961). These biochemical findings showed that 2.P.A.M. did not alleviate the inhibition of whole blood cholinesterase activity by quinuronium in sheep. The results however do not necessarily invalidate the clinical improvements reported by Rummler and Laue (1961) since it is possible that 2.P.A.M. may be acting on cholinesterases in sites other than blood, or may be acting in some other capacity.

SECTION III

THE RELEASE OF TISSUE HISTAMINE

While it had been shown that most of the pharmacodynamic activity of quinuronium was parasympathetic, it was quite clear that there was some activity which could not be explained in these terms. This together with Kikuth's (1935) observations on the resemblance between quinuronium toxicity and shock, suggested the investigation of methods for determining the release of tissue histamine.

METHODS.

1. THE BLUE DYE TEST

The technique was first described by McIntosh and Paton (1949) and improved by Miles and Miles (1952) and by Feldberg and Miles (1953).

In this method a blue dye such as Evans Blue or Pontamine Blue is injected intravenously. The dye remains in circulation and does not stain the tissues appreciably. The intradermal injection of histamine or a histamine releaser, by increasing capillary permeability in the local area produces a discreet blue patch. Histamine releasers given intravenously produce a generalised blue coloration.

(a) Guinea Pig.

White guinea pigs were used (300 - 500 gms). Hair was

roughly clipped from one flank three days prior to the experiment and the clipped area was then depilated with barium sulphide cream. The flank was washed well with soapy water, dried and powdered with talc. Three days later the animals were anaesthetised with urethane and fixed laterally. Each animal received Pontamine Sky Blue 6x (5% aqueous solution) intravenously at the rate of 1.3 ml per kgm. Small intradermal injections were made of solutions containing the stated amount of drug in 0.2 ml isotonic saline. In each animal 0.2 ml of isotonic saline was injected intradermally as a control.

(b) Sheep.

Sheep were clipped over the whole of the left flank, which was then depilated, washed and powdered with talc. After three days the animals were anaesthetised with thiopentone and maintained on closed circuit cyclopropane and oxygen.

Intravenous Pontamine Sky Blue was given as in guinea pigs and drug solutions were injected intradermally.

Drugs. Histamine; compound 48/80; quinuronium and amicarbalide.

2. IN VITRO PERFUSED RAT HIND-QUARTERS

The perfusion technique was described by Feldberg and Mongar (1954) and the following description is very similar.

White female rats were killed and cut in half at the thoraco-lumbar junction. The body was eviscerated and duodenum and rectum ligated. The posterior half of the body was perfused

with oxygenated Krebs solution (Krebs and Henseleit, 1932), (pre-heated to 40°C) via the dorsal aorta, and perfusate collected from the caval vein, (Fig. H.A.).

When the Krebs perfusate was blood-free, a control sample was collected over a period of twenty minutes, then perfusion was transferred to a second reservoir which contained a solution of the drug under test in Krebs solution. Perfusate from the drug treated tissue was collected for approximately one hour.

Both the control sample and the 'drug' perfusate were treated according to the method of Feldberg and Talisnik (1953); i.e. hydrochloric acid (conc.) was added to the perfusion fluids to produce an acid concentration of 20% v/v HCl. These mixtures were then boiled for ten minutes and neutralised with strong sodium hydroxide solution (using universal indicator paper) and diluted to the required volume with Tyrode solution. Histamine assays were performed on guinea pig ileum in atropinised Tyrode (1×10^{-8}G. atropine) using the matching assay technique.

To exclude any non-histamine component the solutions were re-assayed after the antagonism of histamine by mepyramine maleate (1×10^{-7}G.).

NOTE. After completion of tissue perfusion in each case, Indian ink was injected into the perfusing fluid and the coloration of the tissue indicated the efficiency with which the perfusion had been carried out. In all cases recorded, this was virtually 100%.

Drugs. Quinuronium sulphate, 500 ug/ml; amicarbalide isethionate, 2 mgm/ml; compound 48/80, 100 ug/ml.

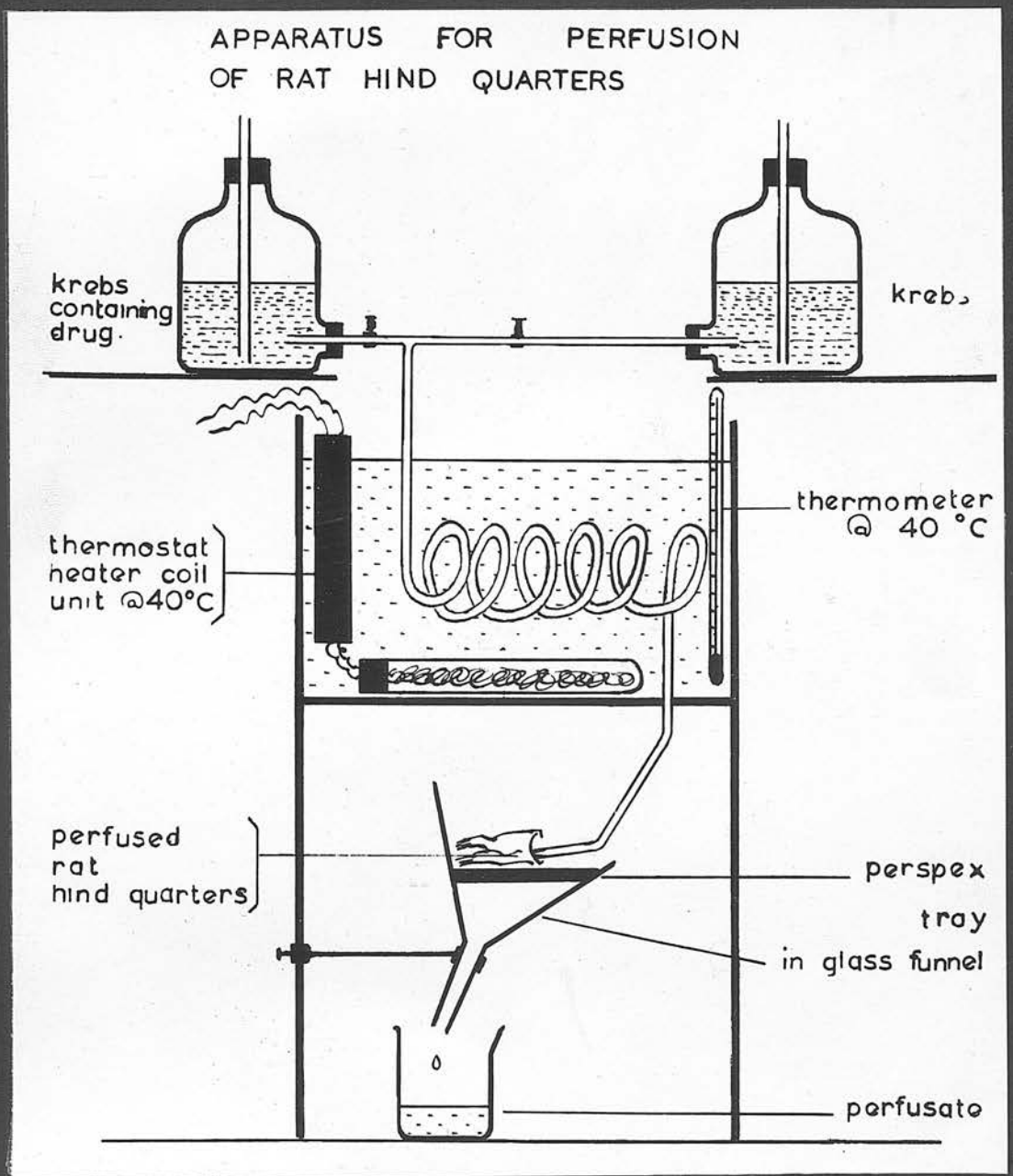

APPARATUS FOR PERFUSION
OF RAT HIND QUARTERS
krebs containing drug.
kreb
thermostat heater coil unit @40°C
thermometer @ 40 °C
perfused rat hind quarters
perspex tray in glass funnel
perfusate

FIG. H.A.

3. IN VITRO RELEASE OF HISTAMINE FROM SHEEP DIAPHRAGM STRIPS

The diaphragm of the rat has been used as a standard technique for demonstrating histamine release (Rocha e Silva and Schild, 1949). These authors incubated hemi-diaphragms of freshly killed rats in saline containing potential histamine releasing agents and assayed both the bathing fluid and the tissue for histamine.

Tissue: A small area of diaphragm was taken from a freshly killed sheep. The area chosen was at the junction of the muscular and tendinous portions of the organ. From the peritoneal side of this muscle, a thin sheet was prepared by splitting the musculature with a clean safety razor blade to leave the peritoneum and a thin layer of attached diaphragmatic muscle about 3 mm thick. This thin sheet was then divided into a number of strips approximately 1 cm wide and 3 cms in length. The strips were carefully "washed" in warm (37°C) normal saline for one minute, after which they were carefully dried between sheets of cellulose tissue and weighed.

Sixteen such strips were prepared and each was placed in a tube containing 8 mls of Krebs saline (Krebs and Henseleit, 1932). Four tubes remained untreated as controls and the second, third and fourth groups of four respectively contained quinuronium, 500 mgm per ml; amicarbalide, 2 mgm per ml; 48/80, 100 mgm per ml. All tubes were incubated for half-an-hour in a constant temperature water bath at 37°C.

After exactly thirty minutes, the tissue was carefully removed from each tube, to each of which was added a sufficient quantity

of concentrated HCl (i.e. 2 ml) to make the resultant solution approximately 20% w/v. All tubes were heated in a boiling water bath for thirty minutes, then neutralised with sodium hydroxide solution. The volume of fluid in each tube was measured and its histamine content assayed as described; before and after mepyramine.

4. IN VIVO HISTAMINE RELEASE IN MICE

Experiments were designed according to the recommendations of Riley (1959) for rats and mice during mast cell studies and investigation of histamine release. A modification of Riley's "sub-acute" experiment was adopted.

The investigation involved groups of mice (six - eight) into which therapeutic doses of the babesicides were injected intraperitoneally over a period of several days. A "standard" group was injected with 48/80, and another group received an equivalent volume of normal saline. In addition to the assay of histamine in the animal tissues after chemotherapy, microscopy was planned in order to investigate the effect of babesicidal chemotherapy on the mast cell population.

Drugs.

(1) Quinuronium sulphate.

A therapeutic dose is of the order of 1 mgm per kilo. According to Kronfeld (1959), mice will not tolerate 2 mgm/kilo over two or three days, whereas rats may tolerate up to 10 mgm/kilo. The following dosage routine was adopted:

day 1: 1 ug/Gm (= 1 mgm/kilo); day 2: 1.5 ug/Gm; day 3: 2 ug/Gm.

(2) Amicarbalide isethionate.

This compound is active at a dose rate of 5 mgm/kilo and is well tolerated in all species up to 20 mgm per kilo, (Beveridge et al 1960). A dose rate as high as 40 mgm/kilo is toxic but not lethal. On this basis the following routine was made: day 1: 20 ug/Gm; day 2: 25 ug/Gm; day 3: 30 ug/Gm.

(3) Compound 48/80.

According to Riley, (1959) the dose rate approximated to 2 mgm per kilo so the dosage routine was: day 1: 2 ug/Gm; day 2: 2.5 ug/Gm; day 3: 3 ug/Gm (see Table H3).

TABLE H3

SUMMARY OF DOSAGE ROUTINE IN MICE

GROUP	DAY 1	DAY 2	DAY 3
	10.0 a.m.	10.0 a.m.	9.0 a.m.
CONTROL	0	0	0
QUINURONIUM	1.0 ug/Gm	1.5 ug/Gm	2.0 ug/Gm
AMICARBALIDE	20.0 " "	25.0 " "	30.0 " "
48/80	2.0 " "	2.5 " "	3.0 " "

The mice used were of mixed strains and sexes, chosen at random (weights varied from 20 to 40 grammes). On the third day

of injection, (three hours after the final injection), 4 mice from each group were killed and skinned completely, including the ears. A representative portion of skin was taken, chopped up finely, weighed and added to a small volume of 20% w/v HCl. This was then boiled gently for ten minutes and filtered through Watman No. 1 filter paper, and washed with three volumes of distilled water. The clear filtrate was boiled to a small volume and either assayed immediately (where possible) or stored deep frozen until convenient. (Each batch of samples was treated identically; either all being assayed as soon as possible after extraction or all being stored an equal length of time.) Before assay the extracts were neutralised with sodium hydroxide and diluted if required with Tyrode solution and assay was carried out as described above according to the method of Feldberg and Talisnik (1953).

Histology. In addition to tissue extraction for histamine; fresh tissue samples of subcutaneous fascia were taken and spread thinly on a microscope slide for histological treatment after the method of Riley (1953) for investigating the mast cell population.

Staining method.

a. Smears fixed in 80% alcohol for 24 hours.

b. Excess fat trimmed from smear.

c. Immersed in xylol for 1 hour.

d. Immersed in 80% alcohol for 1 hour.

e. Stained with 1% toluidine blue (1:40 alcohol) for 1 to 3 minutes.

(i) washed in 80% alcohol.

(ii) rinsed in absolute alcohol.

(iii) cleared in xylol.

(iv) mounted in D.P.X. medium.

THE EFFECT ON MAST CELLS OF MASSIVE ACUTE DOSES OF COMPOUND 48/80 AND QUINURONIUM.

Further groups of six mice were taken concurrently with the experiment previously described, in order to investigate the effect of large single doses of quinuronium and 48/80.

The two compounds were injected intraperitoneally, each at a dose rate of 5 ug per gm. Tissue spreads from the mesentery were fixed and stained as described previously and examined for mast cells.

5. IN VIVO HISTAMINE RELEASE IN RATS

(See earlier note on methods in mice.)

The experiments were repeated using white female rats. Compound 48/80 was not included in these investigations and it was decided to increase slightly the dose of amicarbalide, (see Table H4). Histamine assay and histological examination was undertaken as before.

TABLE H4

SUMMARY OF DOSAGE ROUTINE IN RATS

GROUP	DAY 1	DAY 2	DAY 3
	10.0 a.m.	10.0 a.m.	9.0 a.m.
CONTROL	0	0	0
QUINURONIUM	1.0 ug/Gm	1.5 ug/Gm	2.0 ug/Gm
AMICARBALIDE	30.0 " "	35.0 " "	40.0 " "

6. MAST CELLS OF SHEEP

It is convenient to consider here a histological study of the mast cells of the same group of sheep which were used for recording of carotid blood pressure in Section I.

Technique. Sheep were anaesthetised with thiopentone and cyclopropane. The animals received intravenous injections of small quantities of histamine (100 ug), acetylcholine (100 ug), and adrenaline (20 ug) and in a number of injections over a period of one hour received a total of 150 to 200 mgms of quinuronium in doses ranging from 50 ug to 50 mgm, (see Fig. BP 4 (i) and (ii): SectionI.) This dose amounted to approximately five times the therapeutic dose spread over the one hour period. The animals received atropine (1 mgm per kilo) midway through the experiment which was designed to investigate the antagonism of atropine for quinuronium. Sheep which had received mepyramine as an

TABLE H5.

THE INTRADERMAL BLUE WHEAL TEST

DRUG	QUANTITY IN 0.2 ml SALINE	OBSERVATION
HISTAMINE	100 ug 250 ug 500 ug	Blue ring - Blue ring - Intensely blue disc -
48/80	200 ug 500 ug 1 mgm	Blue ring - Intensely blue disc -
QUINURONIUM	500 ug 1 mgm 5 mgm	Bright blue narrow ring - Intense blue band -
AMICARBALIDE	1 mgm 5 mgm 10 mgm	Small pale blue ring - Blue ring - Deeper blue ring -
SALINE	0.2 ml	Tiny blue spot indicating injection site

antagonist were not used in the present connexion owing to the possible interference of this compound with histamine release, (Mota, 1960). After blood-pressure recording was completed the sheep were either killed by overdosing with cyclopropane or were already dead.

"Control" tissues were obtained from sheep which had been killed by an overdose of thiopentone sodium and into which no other drug had been injected. At post mortem, tissue spreads were taken from the peritoneum. Liver capsule also was carefully stripped off, placed with the serosal surface on to a clean microscope slide and excess liver parenchyma scraped off with clean knife blade. Both tissues were fixed and stained as described earlier for mice and rats.

RESULTS.

1. THE BLUE DYE TEST

(a) Guinea Pig.

The drug injections and the observed effects are tabulated in detail in Table H5 and may be summarised:- histamine, 48/80, and quinuronium all produced a bright blue ring at the lowest dose level within one to two minutes, which became more intense and of wider diameter within the succeeding few minutes. The highest dose levels used produced intensely blue rings very rapidly indeed, which quickly spread over an area of several square centimetres. At post mortem the "reverse" side of the skin was examined and this presented a similar picture to the

outer surface and one could clearly make out the cutaneous blood vessels which had been outlined in blue also.

Amicarbalide at its lowest level produced only a very faint small blue ring which was slow in appearing (five minutes). In higher doses a somewhat deeper ring was obtained but its formation was slower than that of the other drug induced rings (three to four minutes) and was not of the same intensity of colour.

An equal volume of normal saline injected intradermally as a control in each animal produced no blue ring or wheal but merely a tiny blue spot which marked the site of entry of the hypodermic needle.

(b) Sheep.

From Fig. H0 it can be seen that the relatively large dose of histamine produced the most marked response. With histamine an intense blue wheal appeared within half-a-minute of injection and spread rapidly and widely. The two histamine wheals merged after several minutes and yielded a large elongated blue patch of some six to eight square inches in area.

Compound 48/80 produced discreet but intensely blue wheals of the order of one square inch area, as did quinuronium. With quinuronium there was a yellowish green colour at the centre of the wheal due to the yellow colour of the drug; and an intense blue could be seen at the periphery of the wheal. Amicarbalide at the lower dose produced only a very faint small wheal after a lapse of five minutes or more, whereas the larger dose yielded a more rapid response and a larger wheal which was not so intensely coloured as those due to other drugs.

FIG. H.O.

EXTRA-VASCULAR DIFFUSION OF PONTAMINE BLUE IN THE SKIN OF SHEEP AFTER INTRADERMAL INJECTIONS OF: HIST. = HISTAMINE BASE; 48/80 = COMPOUND 48/80; PIR. = QUINURONIUM SULPHATE "PIREVAN"; AMIC. = AMICARBALIDE ISETHIONATE; SAL. = ISOTONIC SALINE.

TABLE H6

QUANTITIES OF INTRADERMAL INJECTIONS IN SHEEP

DRUG	INTRADERMAL INJECTION DOSE IN 0.2 ml N SALINE	
	1.	2.
HISTAMINE	100 ug	500 ug
48/80	200 ug	1 mgm
QUINURONIUM	500 ug	2.5 mgm
AMICARBALIDE	500 ug	2.5 mgm
NORMAL SALINE	0.2 ml	

2. IN VITRO PERFUSED RAT HIND QUARTERS

Assays indicated that control samples contained no histamine. Quinuronium and amicarbalide at their respective dose rations (1:4) were approximately equi-potent in releasing histamine, whereas compound 48/80 was about three times as potent, (Fig. H1).

/ Histamine from the tissue was expressed as microgram base per ml. perfusate (see also Appendix Table L)./

3. IN VITRO RELEASE OF HISTAMINE FROM SHEEP DIAPHRAGM STRIPS

Figure H2 shows that an appreciable quantity of histamine appeared in the control tubes, however in one tube there was

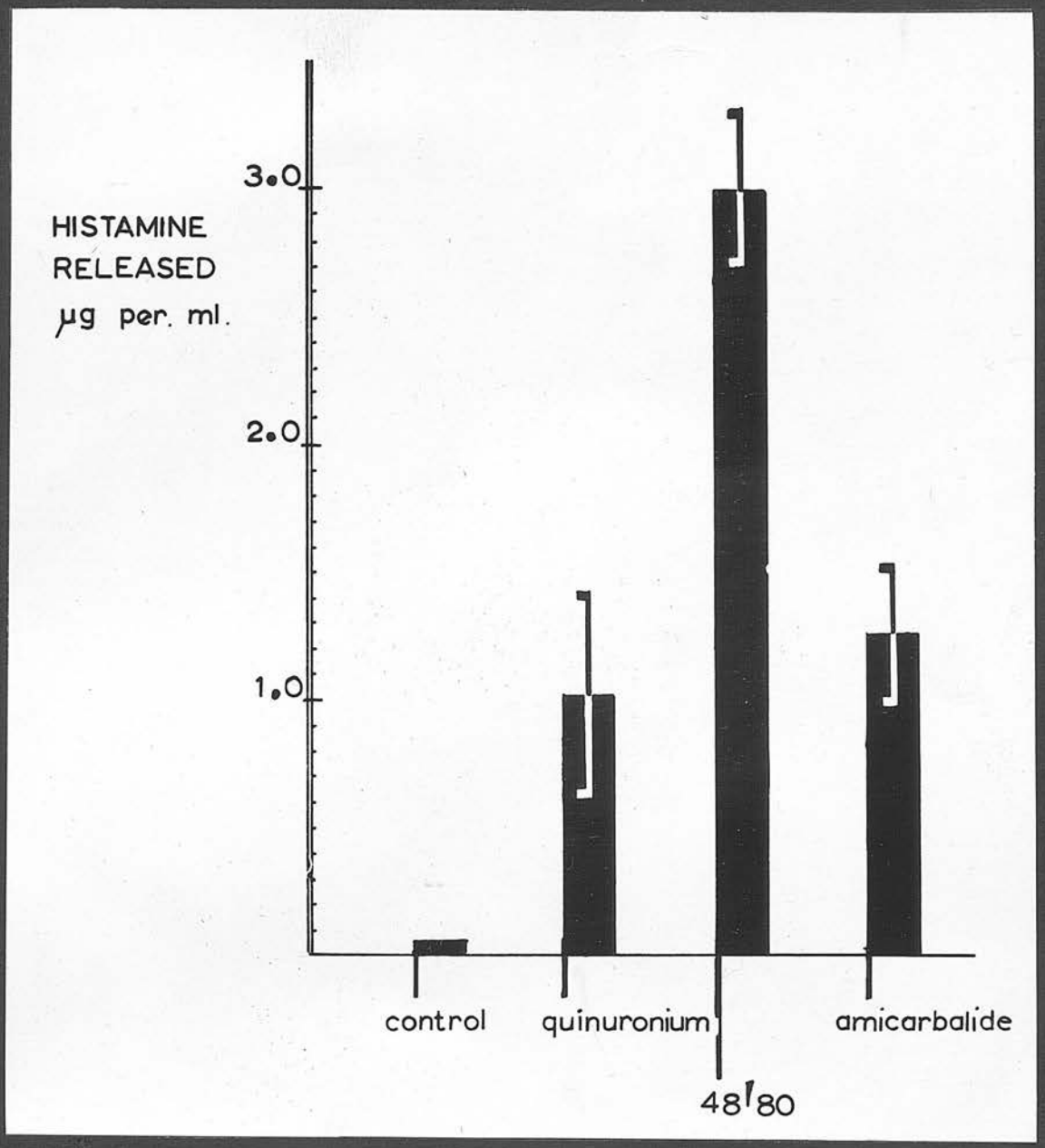


FIG. H.1.

QUANTITY OF HISTAMINE RELEASED FROM ISOLATED SALINE-PERFUSED HIND QUARTERS OF THE RAT BY QUINURONIUM SULPHATE, COMPOUND 48/80 AND AMICARBALIDE ISETHIONATE.

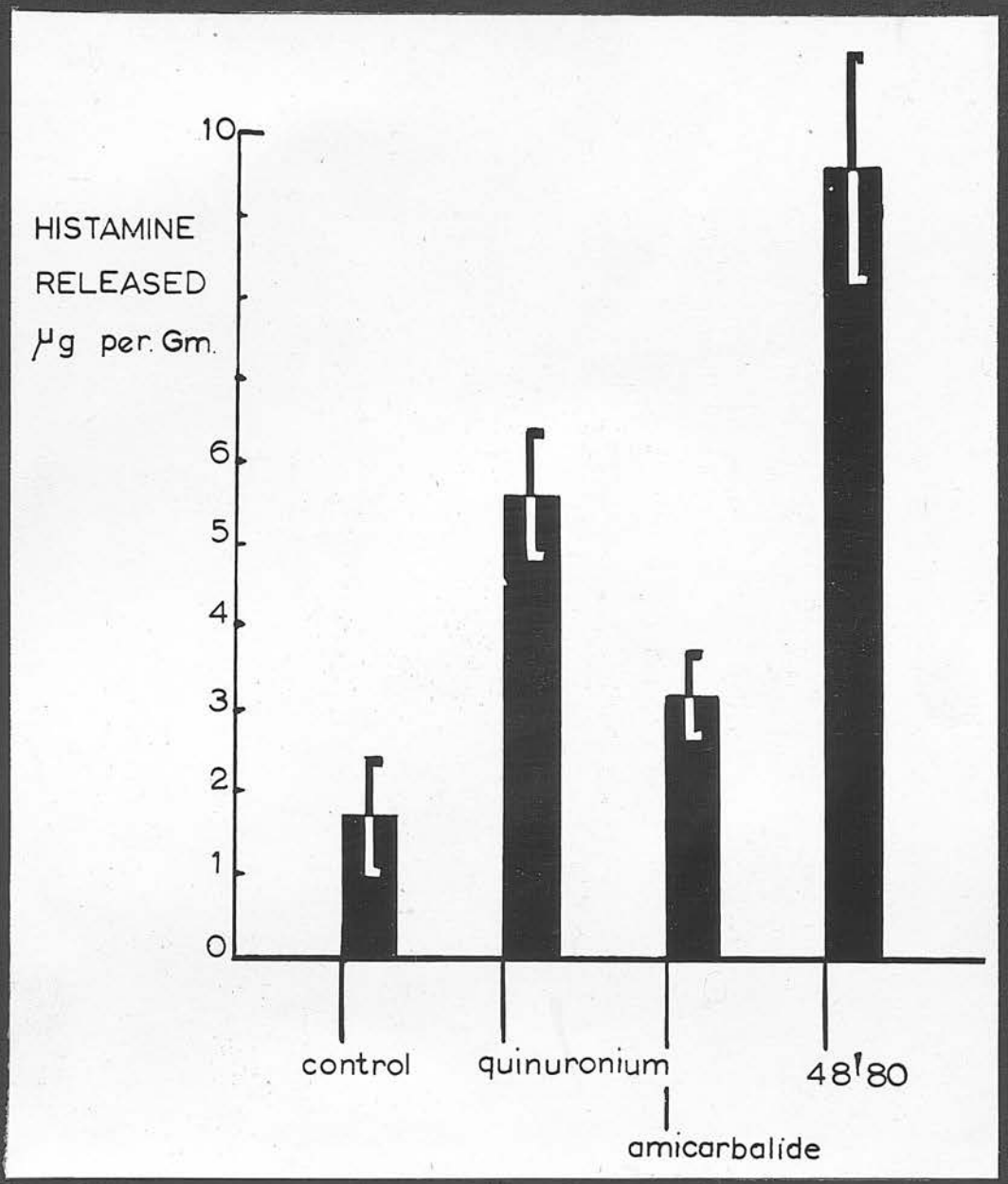


FIG. H.2.

QUANTITY OF HISTAMINE LIBERATED FROM ISOLATED STRIPS OF SHEEP DIAPHRAGM (IN VITRO) BY QUINURONIUM SULPHATE, AMICARBALIDE ISETHIONATE AND COMPOUND 48/80.

virtually none (mean 1.63). This gave rise to the large standard error in the control reading ($\pm$0.6).

Compound 48/80 released 9.60 microgram of histamine per gram tissue. Quinuronium released a mean 5.62 $\pm$ 0.66, whereas amicarbalide released an amount of histamine which was not significantly different from the controls, (see also Appendix Table M).

4. IN VIVO RELEASE OF HISTAMINE IN MICE

Within five minutes of the intraperitoneal injection of quinuronium or compound 48/80, the mice became restless and showed signs of dyspnoea and cyanosis of the extremities. Mice which had received quinuronium were more markedly affected and eventually lay quite still before recovering. Over the period of three days the mice appeared to acquire tolerance to 48/80 and by the third day showed little signs of distress. Mice did not seem to have tolerance to the action of quinuronium. The group of animals which received amicarbalide showed little effect but became slightly restless.

(i) Histamine Assay.

Figure H3 shows that compound 48/80 and quinuronium reduced the skin histamine content by approximately 50%, whereas amicarbalide failed to show any significant release of histamine. Repetition of the experiment on a number of occasions produced the same results. (See also Appendix N).

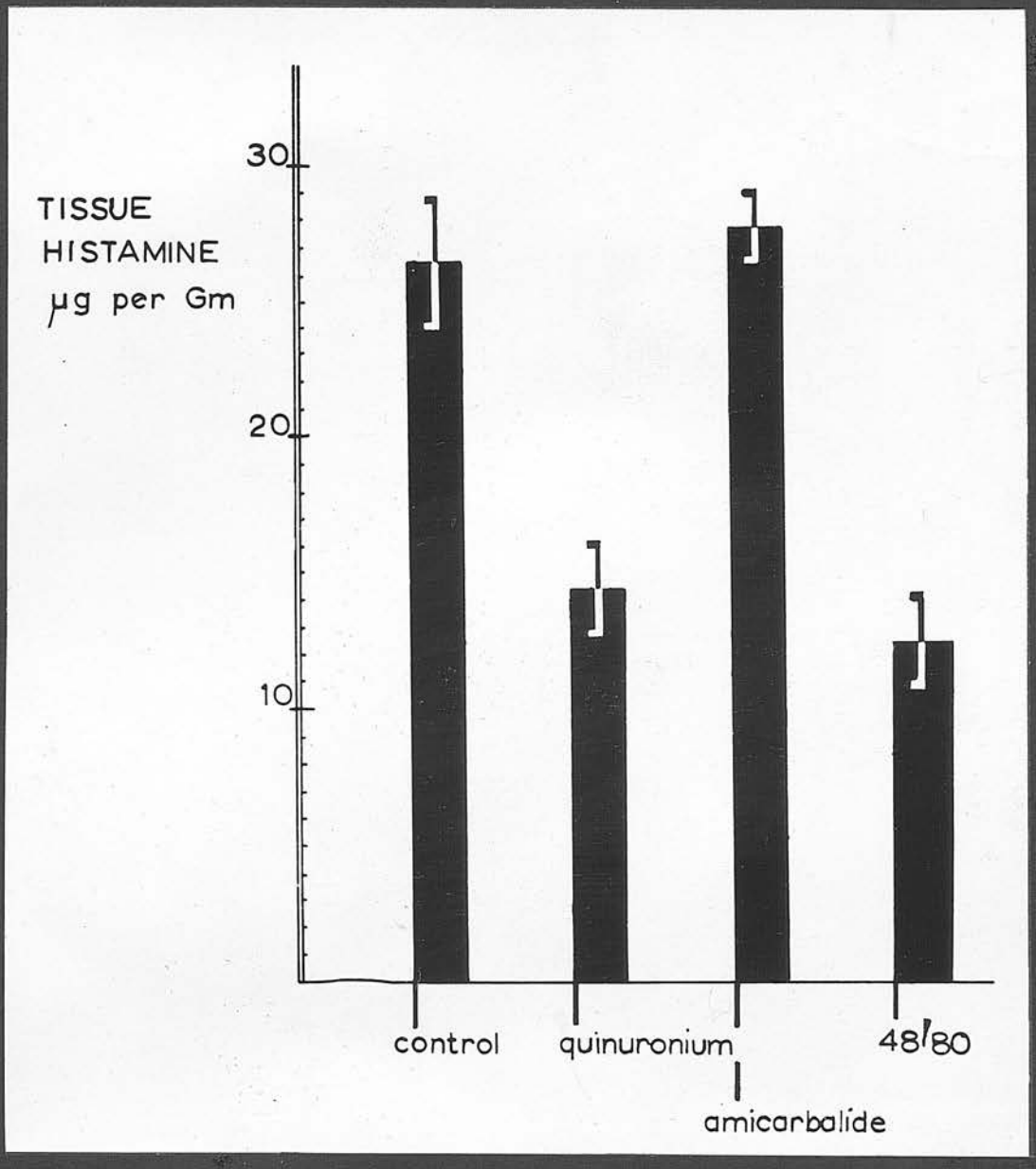


FIG. H.3.

TISSUE HISTAMINE CONTENT OF THE SKIN OF MICE (IN VIVO) TREATED WITH QUINURONIUM SULPHATE, AMICARBALIDE ISETHIONATE AND COMPOUND 48/80.

(ii) Histology.

It was thought to be fairly satisfactory to observe relative degrees of mast cell change rather than to make counts as described by Riley and West (1952 & 1953) and Riley (1953). Significance was taken from obvious reduction in cell numbers and from degranulation and metachromasia of cells.

Photomicrographs of subcutaneous tissue stained for mast cells are reproduced under Appendix P. There was generally good qualitative correlation between the histamine content and the number and character of the mast cells of the tissue.

Normal mice (having received only isotonic saline) showed many mast cells of an intact dark-blue staining character. Subcutaneous tissue from quinuronium treated mice contained fewer mast cells which were pale-staining (metachromatic) and non-granular. After compound 48/80 it was difficult to find any intact mast cells. Amicarbalide had not altered the mast cell picture very markedly, but there appeared to be fewer cells in any field of the microscope and many of these cells seemed to be swollen and possibly in a state of disruption. This may or may not have been significant, (see Appendix PI to IV incl.).

THE EFFECT ON MAST CELLS OF MASSIVE ACUTE DOSES OF COMPOUND 48/80 AND QUINURONIUM.

In all groups of mice receiving 5 microgram per gram of quinuronium there was usually 100% death rate, but occasionally one mouse out of six would survive. Symptoms were severe and included marked dyspnoea, bulging eyes and cyanosis with collapse

and death. The mice treated with compound 48/80 invariably survived although they showed severe toxic symptoms as above. Histological investigation was undertaken in four mice from each group of six.

Toluidine blue stained mesentery spreads showed only a few intact mast cells. There had been widespread disruption of cells by both 48/80 and quinuronium and many blue and red staining granules were scattered over the whole field. (Representative pictures are shown under Appendix PV a. and b.).

5. IN VIVO HISTAMINE RELEASE IN RATS

Within a few minutes of the injection of quinuronium the rats began to show signs of respiratory distress, frenzied movements and cyanosis.

(i) Histamine assay.

Figure H4 demonstrates that quinuronium and amicarbalide at these their maximum therapeutic dose rates are approximately equi-active in reducing the amount of tissue histamine in the skin of rats.

The experiment was repeated several times and gave similar results: the actual figures for the first experiment are given in Appendix O.

(ii) Histology.

Subcutaneous tissue spreads from rats which received only isotonic saline showed a population of dense dark-blue staining mast cells. In rats which had received quinuronium, mast cells

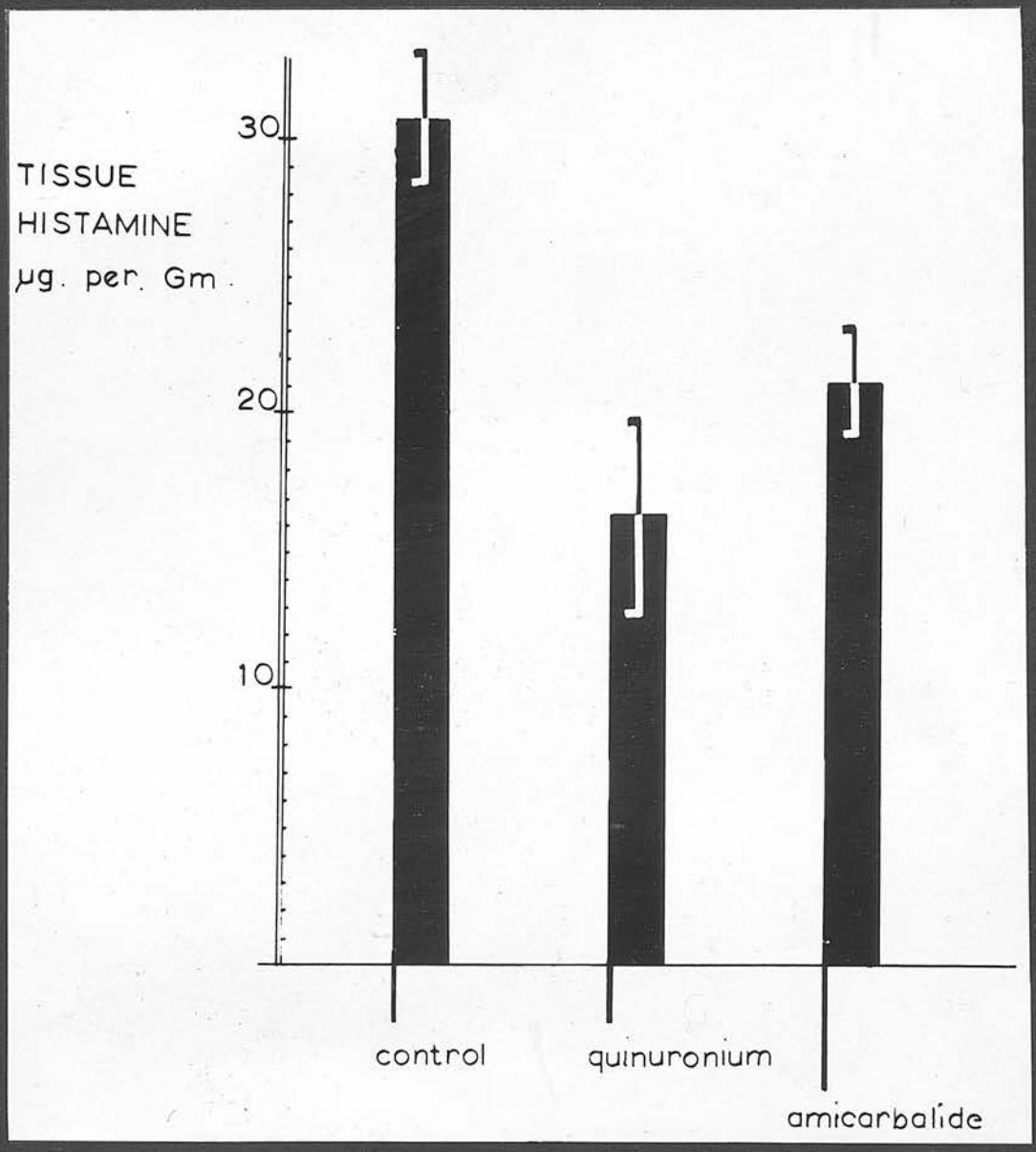


FIG. H.4.

TISSUE HISTAMINE CONTENT OF THE SKIN OF RATS (IN VIVO) TREATED WITH QUINURONIUM SULPHATE AND AMICARBALIDE ISETHIONATE.

were very few in number and in some fields of the microscope there were scattered metachromatic granules. Amicarbalide also reduced the number of mast cells present and those which remained were either pale and non-granular or appeared to be swollen and possibly in a state of disruption. (See Appendix PVI, VII & VIII).

6. MAST CELLS OF SHEEP

The effects of quinuronium in the anaesthetised sheep were reported in Section I and included dyspnoea and hypotension.

Histology.

Peritoneum. Sheep which had received no drug other than an aqueous solution of thiopentone showed tissues rich in mast cells which were dark-blue staining. Tissues from sheep which had received large doses of quinuronium acutely seemed to have fewer mast cells present but the most obvious change was the pale metachromasia of the "treated" cells and the presence of metachromatic granules over the tissue preparation.

Liver capsule. Here quinuronium produced marked depopulation of tissue mast cells although metachromasia in the remaining cells was not marked.

(See Appendix PXI & XII).

DISCUSSION.

Evidence from experiments with intravenous pontamine blue and intradermal drug injections has been shown to be good though indirect evidence of histamine release, and has been used as a screening test for histamine liberating properties of new drugs. In the guinea pig,(Miles and Miles, 1952), intradermal histamine or 48/80 did not produce a true wheal as in man. In sheep in the experiments described here it was possible to detect a slight but positive wheal although the colour reaction was the most obvious response. Miles and Miles (1952) also found that many influences modified the reaction, in particular body temperature and depth of anaesthesia. Although an experiment such as this cannot be truly quantitative and does not constitute complete proof that histamine is the agent responsible, it gives a good indirect indication of the potential histamine releasing activity of a drug: compound 48/80, quinuronium and amicarbalide all produced distinct evidence of histamine release, in guinea pigs and sheep.

It was clear that in order to produce rigorous proof of histamine release by quinuronium and amicarbalide it would be necessary to do direct determinations of the tissue histamine content before and after drug treatment. Hence the direct methods of assay were adopted.

In vitro evidence from perfused rat hind quarters and isolated sheep diaphragm indicated that quinuronium was a potent histamine liberator. In the rat, amicarbalide was equi-potent with quinuronium, whereas in sheep diaphragm, amicarbalide had no

apparent action. A difficulty which arose in the sheep diaphragm experiments was a very high and variable spontaneous release of histamine. Rocha e Silva and Schild (1949) reported this also in rat hemi-diaphragms. These authors were incubating tissues with d-tubocurarine in oxygenated Tyrode at 37°C, whereas in the sheep experiments described here, Krebs solution was unoxygenated. This may have represented a significant difference in technique. Other factors which may have contributed to the spontaneity and variability were (1) uneven thickness of the strips; (2) the absolute thickness, 3mm, making oxygen and drug diffusion less efficient; (3) trauma in the preparation of the samples. Nevertheless, evidence was obtained for the histamine releasing activity of quinuronium but not for amicarbalide.

In vivo experiments in small rodents (rats and mice) were more informative. In mice, compound 48/80 and quinuronium released similar quantities of histamine, and this histamine release was reflected in the respective degrees of mast cell degranulation and disruption. Amicarbalide failed to release any significant amount of histamine from mouse skin although the mast cells appeared to be of an abnormal character and fewer in number. These findings were not exactly reproducible in the live rat where it was shown that amicarbalide and quinuronium liberated similar amounts of histamine and reduced the number of mast cells. The symptoms of toxicity in mice showed that although quinuronium and 48/80 released similar quantities of histamine, quinuronium was distinctly more toxic. Also in rats where quinuronium and amicarbalide were equi-active in liberating histamine, quinuronium produced more severe symptoms. This suggested that quinuronium

had toxic properties other than the release of histamine which is in agreement with the experimental evidence of Sections I and II of this thesis.

Riley (1959) observed that in mice the skin histamine could not be depleted by more than 50% and only a proportion of subcutaneous mast cells were correspondingly affected. It would seem that the toxicity of 48/80 in mice is less dependent upon histamine release than in rats. The current experiments confirmed these observations and showed that quinuronium released in the region of 50% histamine. However, the very extensive, almost complete destruction of mast cells seemed to be out of proportion to the 50% histamine liberation. This discrepancy was difficult to explain. As Riley (1959) pointed out, not all histamine is held in mast cells and it does not all respond to the action of chemical liberators. These facts may explain this anomaly.

Experiments in mice with large single doses of compound 48/80 and quinuronium showed very marked mast cell damage in the mesentery. There were a few intact cells which appeared to be swollen, and scattered around them were pale metachromatic granules. The patchy distribution of mast cell degranulation is typical of the action of 48/80 in mice as reported by Riley (1959). It seems clear that there is more than one way of releasing histamine from the mast cell granules: chemical, electrical and osmotic effects are among the more obvious. Chemical liberation seems to be brought about by one of two means; either by chemical displacement of histamine from the mast cell granule or by destruction of the cell and liberation of granules,

McIntosh and Paton (1949), Riley (1959). The evidence of the experiments reported here did not allow one to infer in which capacity quinuronium was acting, since the histological picture also depended to some extent on the dose of the chemical agent used.

The observations on the acute degranulation of sheep mast cells are incomplete in themselves as they are not supported by histamine assay. They do however correlate with other results in sheep and rodents and add another piece of evidence on the histamine liberating properties of quinuronium. From the results of other experiments in this department on sheep and standard conditions for histamine release; and from the experiences of Sanford (personal communication) it appeared that the normal variation in sheep tissue histamine was so great as to make assays difficult or impossible to interpret. It was found to be extremely difficult to deplete sheep in vivo of histamine by standard methods which are employed successfully in other species; for example, 48/80 in rodents, (Riley, 1959) and in the dog, (Paton and Schachter, 1951). Sheep invariably died during the course of sub-acute or acute dosage with 48/80 without showing any significant difference in histamine content of tissues. It seemed that as with mice, the toxicity of 48/80 depended more on some toxic factor not associated with histamine liberation.

It is quite clear that there is a large variation in the response of tissue to histamine releasing chemicals even between closely similar species and, therefore, evidence from one cannot necessarily be applied directly to another species. The present

experiments yielded ample evidence both direct and indirect for the histamine releasing activity of quinuronium; especially in rodents, but also in sheep. Evidence for histamine release by amicarbalide is negative in mice, strongly positive in rats and positive though less significant in sheep. Only in the rat did histamine release seem to play a primary role in amicarbalide toxicity and this provided a possible explanation of the non-cholinergic action of amicarbalide in stimulating gastric secretion.

SUMMARY OF THE PHARMACODYNAMIC ACTIVITY OF QUINURONIUM AND AMICARBALIDE

1. QUINURONIUM SULPHATE.

(a) Hypotension was produced by quinuronium in rat, guinea pig, chicken, rabbit and sheep. In the rat this hypotension was antagonised by mepyramine; in rabbits only by atropine; in sheep partially by both atropine and mepyramine. In guinea pig and chicken neither antagonist was effective.

(b) Experiments on the blood vessels of the rabbit's ear showed a muscarinic effect of quinuronium, whereas plethysmographic studies on the hind-limb and small intestine of the sheep showed that both histaminic and muscarinic factors were involved in the vascular actions, with the latter predominating.

(c) Quinuronium stimulated both the rate and amplitude of contraction of the isolated heart of guinea pig and rabbit. The effect was not muscarinic, but in the absence of a suitable antagonist it was not possible to determine the extent this might have been due to histamine or to histamine-like activity. The stimulant effect of quinuronium on the isolated heart suggested that such a cardiac action could play little part in inducing hypotension and it was concluded that peripheral vaso-dilatation was probably the more important factor.

(d) The contractions of the smooth muscle of the intestine and bladder by quinuronium appeared to be muscarinic and the

specific potentiating effect of this agent on the action of acetylcholine was evidence of anticholinesterase activity. The findings supported clinical evidence of defaecation and urination produced by quinuronium and antagonised by atropine.

(e) Excessive salivation after quinuronium appeared to be attributable to muscarinic effects, whereas the stimulation of gastric secretion by quinuronium was not entirely muscarinic. The potentiating action of quinuronium on acid production induced by acetylcholine was indicative of anticholinesterase activity. It was clear that part of the action of quinuronium on gastric acid secretion in rats was not muscarinic, but in the absence of antagonists of the gastric effects of histamine it was not possible to show whether or not this other action was histaminic.

(f) Small doses of quinuronium potentiated the contraction of supra-maximally stimulated skeletal muscle in chicken and rabbit, whereas larger doses produced a neuromuscular block apparently similar to that produced by d-tubocurarine. The effect of quinuronium in depressing respiration in these species was much greater than that of d-tubocurarine at doses which were equi-potent in producing neuromuscular block. This suggested that respiratory depression of quinuronium was only partially accounted for by neuromuscular blockade.

(g) Anticholinesterase activity was shown in whole blood <u>in vitro</u> in a wide variety of species and was confirmed in the live sheep. There was evidence of substrate reversal of the action of quinuronium <u>in vitro</u>, whereas in the living sheep the cholinesterase

enzyme did not return to completely normal activity for two weeks, which suggested a partial irreversibility. Sheep were hypersusceptible to a second dose of quinuronium when this was given within two weeks of the first dose. A number of explanations were proposed. Studies with the cholinesterase "reactivator" pyridine 2-aldoxime methiodide indicated that this agent did not protect or alleviate whole blood cholinesterase inhibition by quinuronium.

(h) Quinuronium was shown to liberate histamine from rat skin *in vitro* and from the skin of rats and mice *in vivo*. In the rat the evidence for histamine-induced hypotension and gastric acid secretion was substantiated by direct evidence for histamine release; and it seemed that released histamine was at least as important as muscarinic activity in this species. Direct evidence of histamine release from sheep diaphragm *in vitro* and indirect evidence from increased capillary permeability ("blue-dye" test) and from mast cell disruption and degranulation indicate that histamine release is an important part in the overall pharmacodynamic activity of quinuronium in sheep.

2. AMICARBALIDE.

(a) Experiments with the isolated rabbit ear showed that amicarbalide antagonised the action of acetylcholine on the blood vessels.

(b) Further evidence of an atropine like action was obtained from studies on the smooth muscle of the bladder and intestine.

(c) Amicarbalide stimulated the secretion of gastric acid in the anaesthetised rat and neither atropine nor mepyramine antagonised this. Amicarbalide partially antagonised the action of acetylcholine on gastric acid secretion which was further evidence of an atropine like action.

(d) The anticholinesterase activity of amicarbalide was weak by comparison with quinuronium. This result was probably not clinically significant.

(e) The release of histamine by amicarbalide was most marked in rat tissue, absent in mice and weak in sheep in vitro and in vivo. The evidence suggests that in the rat, histamine release is a major factor in the toxicity of amicarbalide and in the sheep, histamine release is positive but of less significance.

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Finally/

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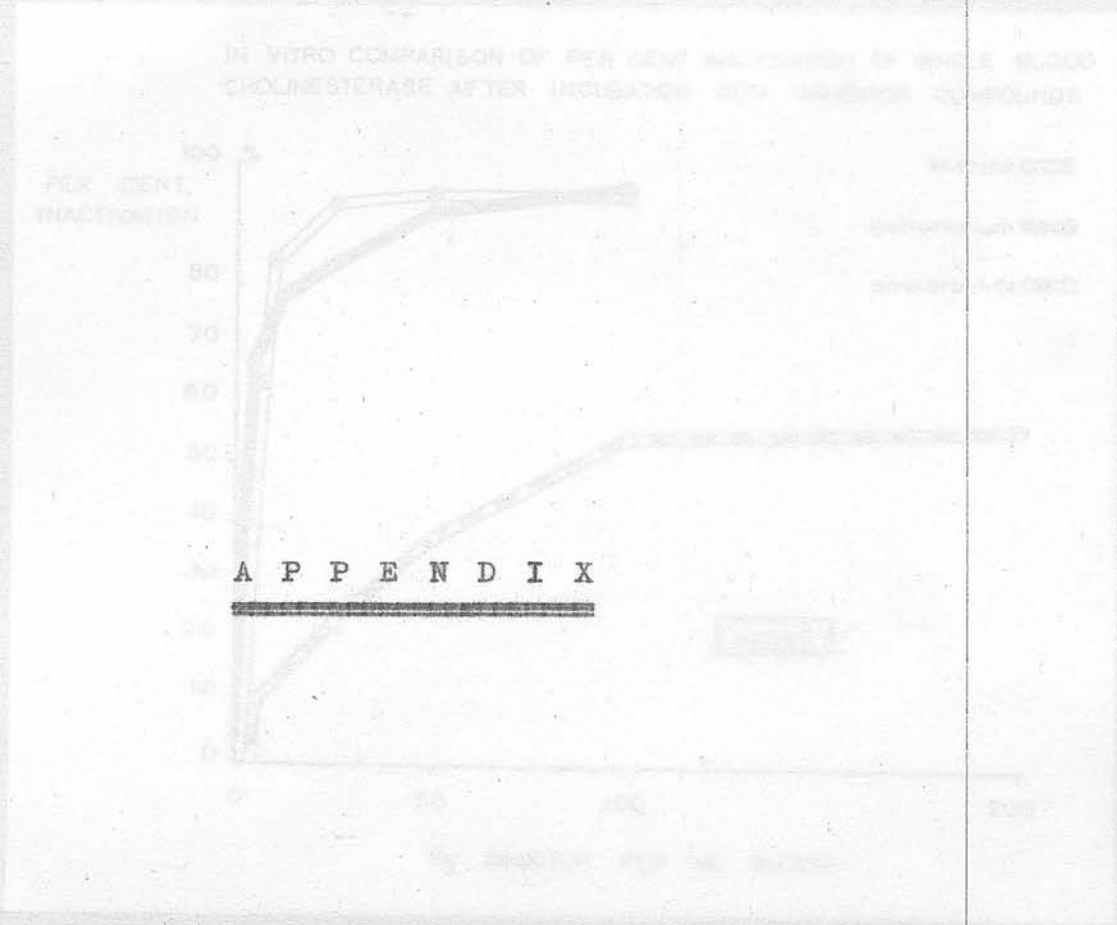

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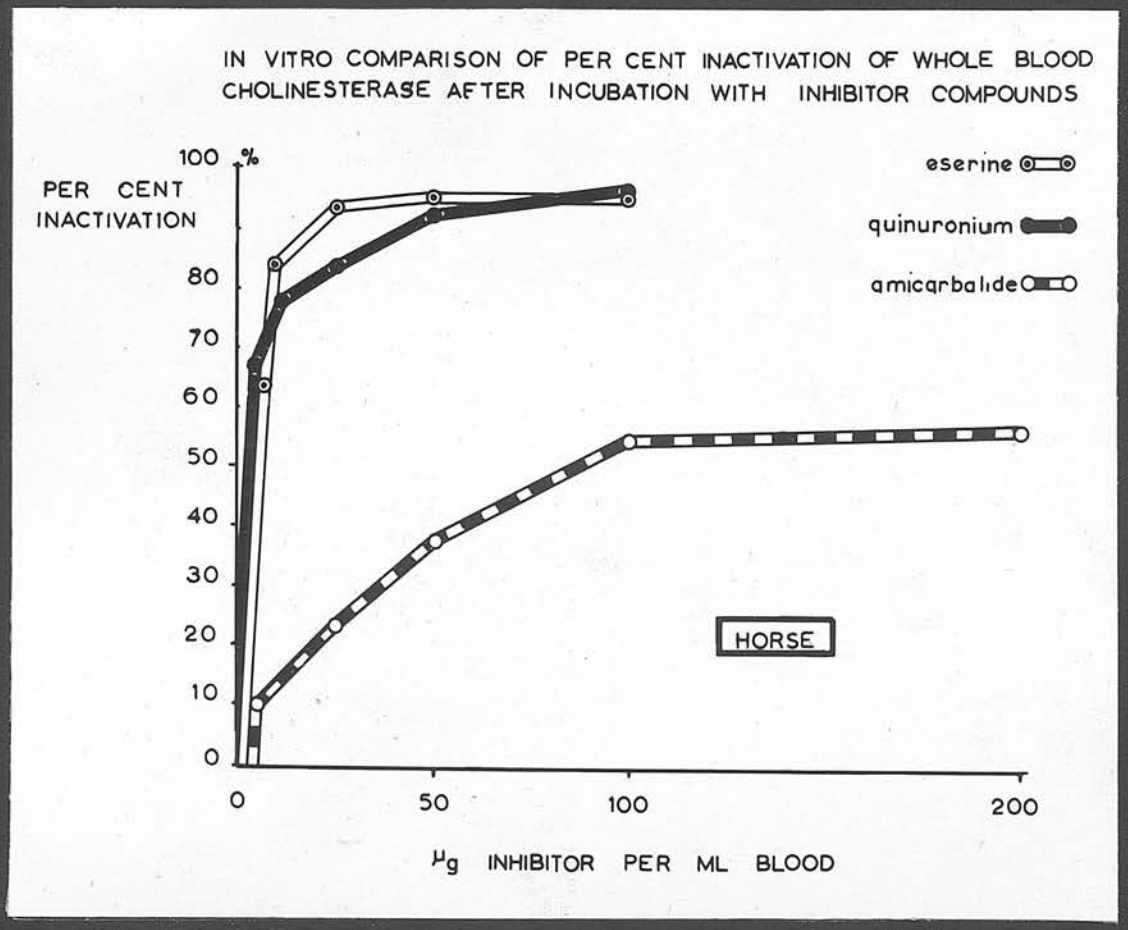

IN VITRO COMPARISON OF PER CENT INACTIVATION OF WHOLE BLOOD CHOLINESTERASE AFTER INCUBATION WITH INHIBITOR COMPOUNDS
PER CENT INACTIVATION
%
100
80
70
60
50
40
30
20
10
0
eserine
quinuronium
amicarbalide
HORSE
0
50
100
200
µg INHIBITOR PER ML BLOOD

FIG. A

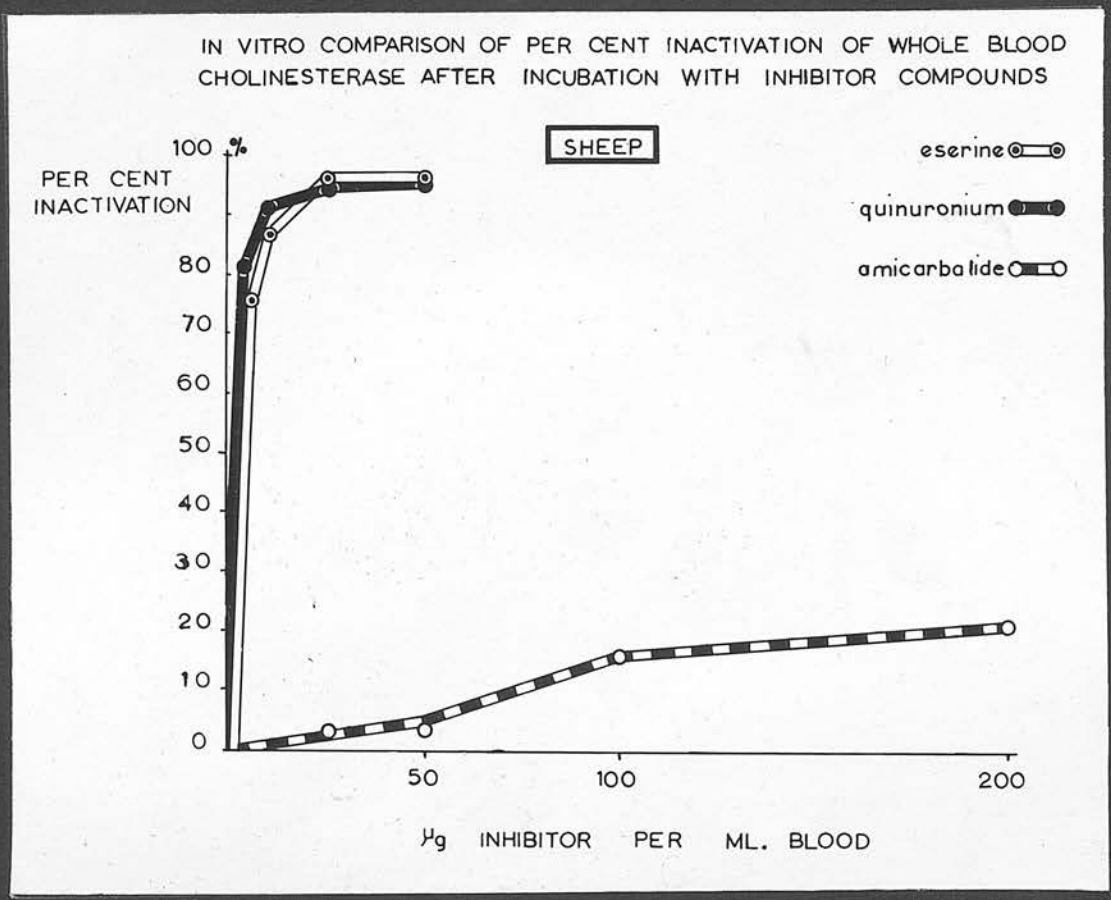

IN VITRO COMPARISON OF PER CENT INACTIVATION OF WHOLE BLOOD CHOLINESTERASE AFTER INCUBATION WITH INHIBITOR COMPOUNDS
SHEEP
eserine
quinuronium
amicarbalide
PER CENT INACTIVATION
100 %
80
70
60
50
40
30
20
10
0
50
100
200
µg INHIBITOR PER ML. BLOOD

FIG. B

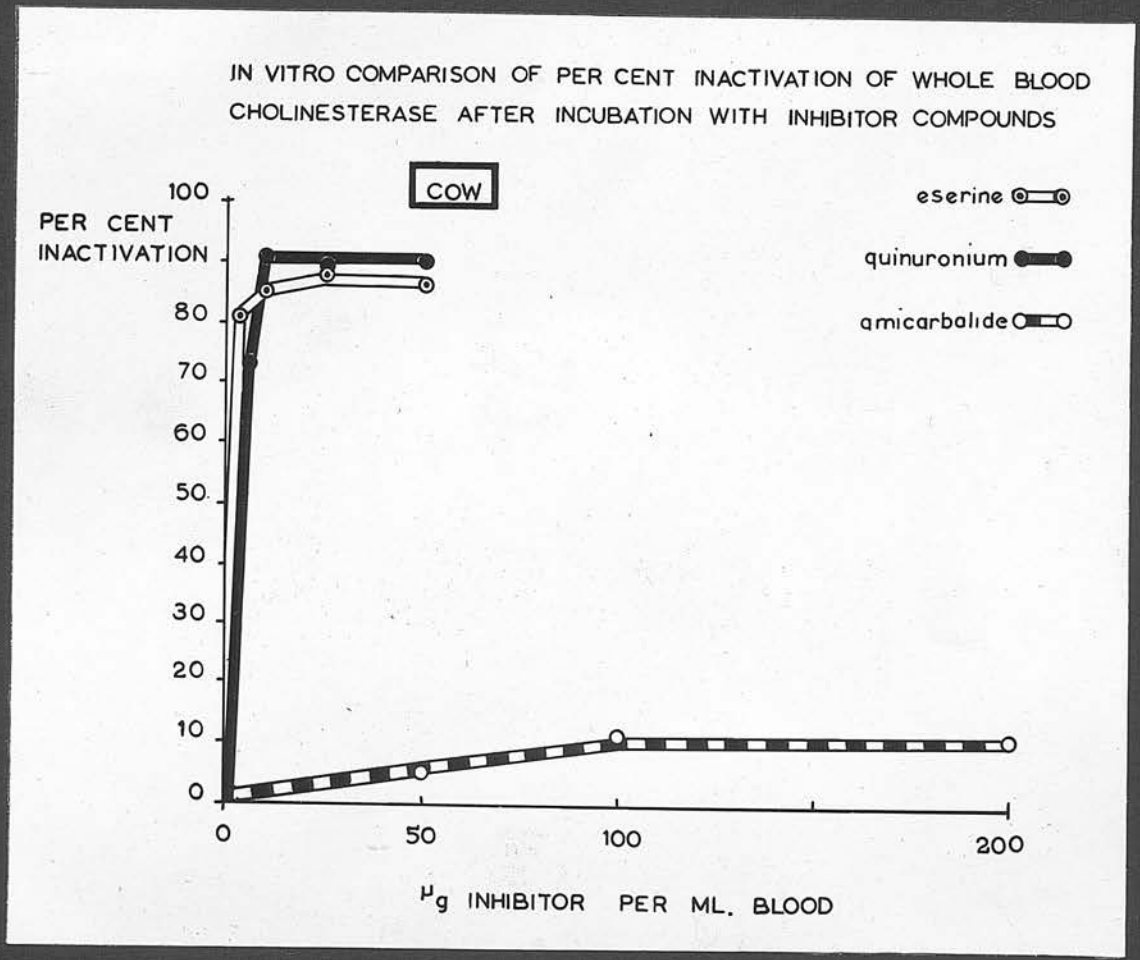

IN VITRO COMPARISON OF PER CENT INACTIVATION OF WHOLE BLOOD CHOLINESTERASE AFTER INCUBATION WITH INHIBITOR COMPOUNDS
COW
eserine
quinuronium
amicarbalide
PER CENT INACTIVATION
100
80
70
60
50
40
30
20
10
0
0
50
100
200
μg INHIBITOR PER ML. BLOOD

FIG. C

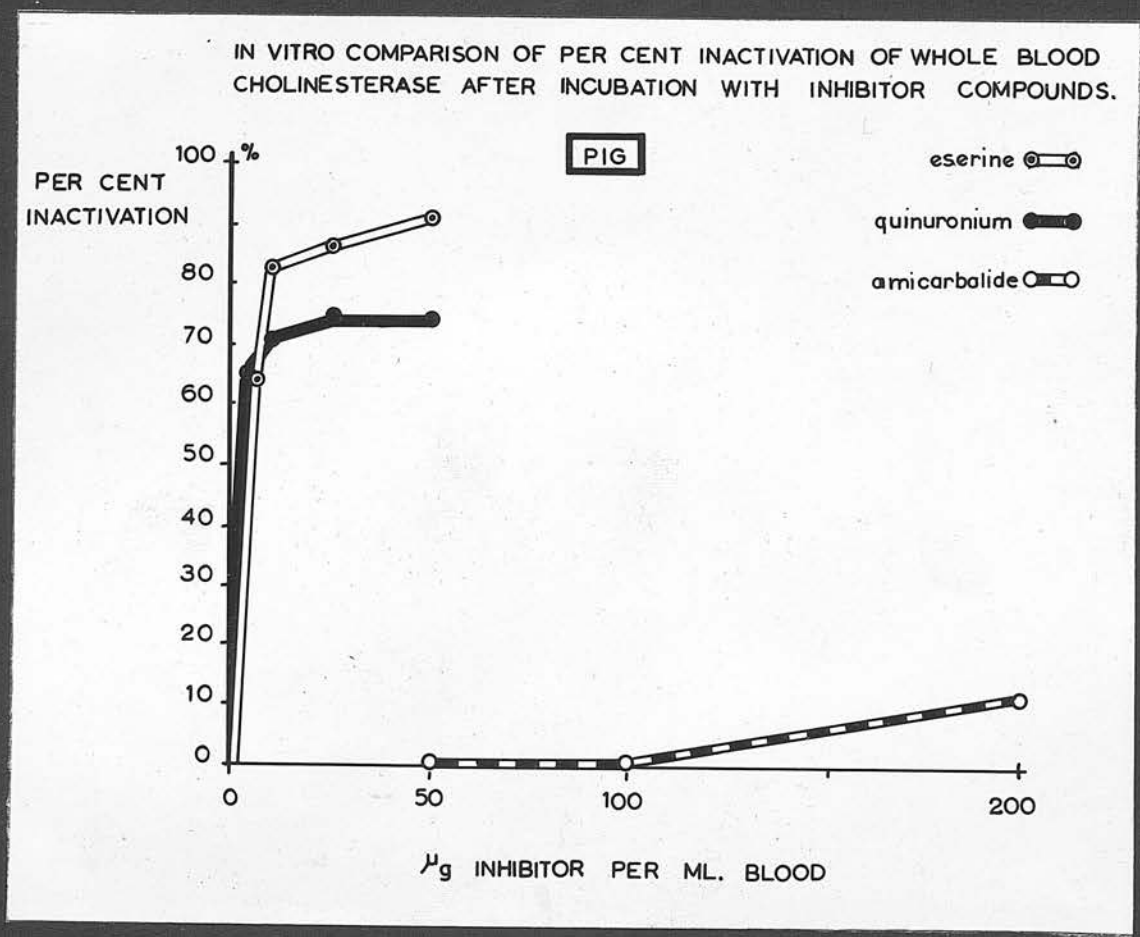

IN VITRO COMPARISON OF PER CENT INACTIVATION OF WHOLE BLOOD CHOLINESTERASE AFTER INCUBATION WITH INHIBITOR COMPOUNDS.
PIG
PER CENT INACTIVATION
100 %
80
70
60
50
40
30
20
10
0
0
50
100
200
μg INHIBITOR PER ML. BLOOD
eserine
quinuronium
amicarbalide

FIG. D

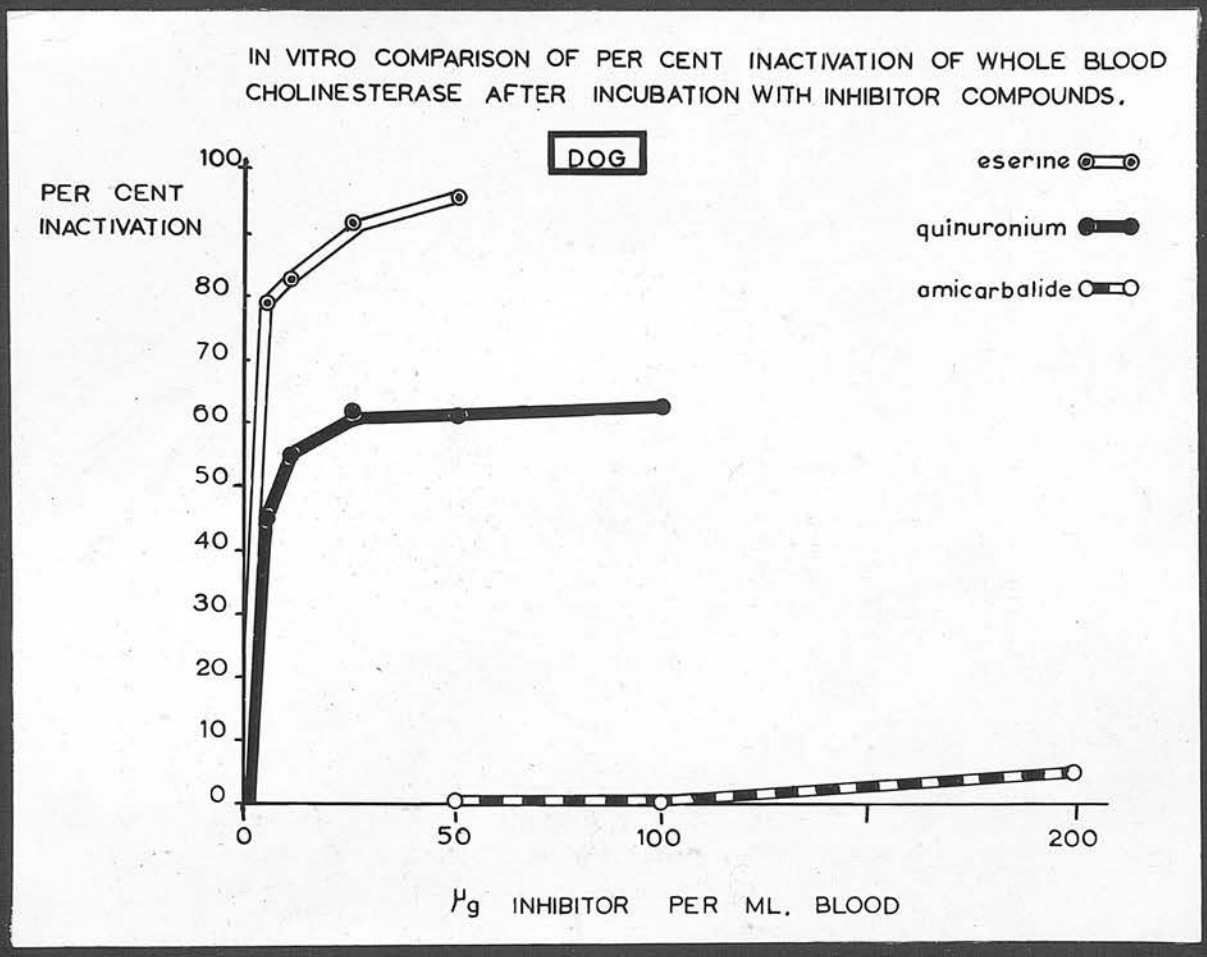

IN VITRO COMPARISON OF PER CENT INACTIVATION OF WHOLE BLOOD CHOLINESTERASE AFTER INCUBATION WITH INHIBITOR COMPOUNDS.
DOG
eserine
quinuronium
amicarbalide
PER CENT INACTIVATION
100
80
70
60
50
40
30
20
10
0
0
50
100
200
μg INHIBITOR PER ML. BLOOD

FIG. E

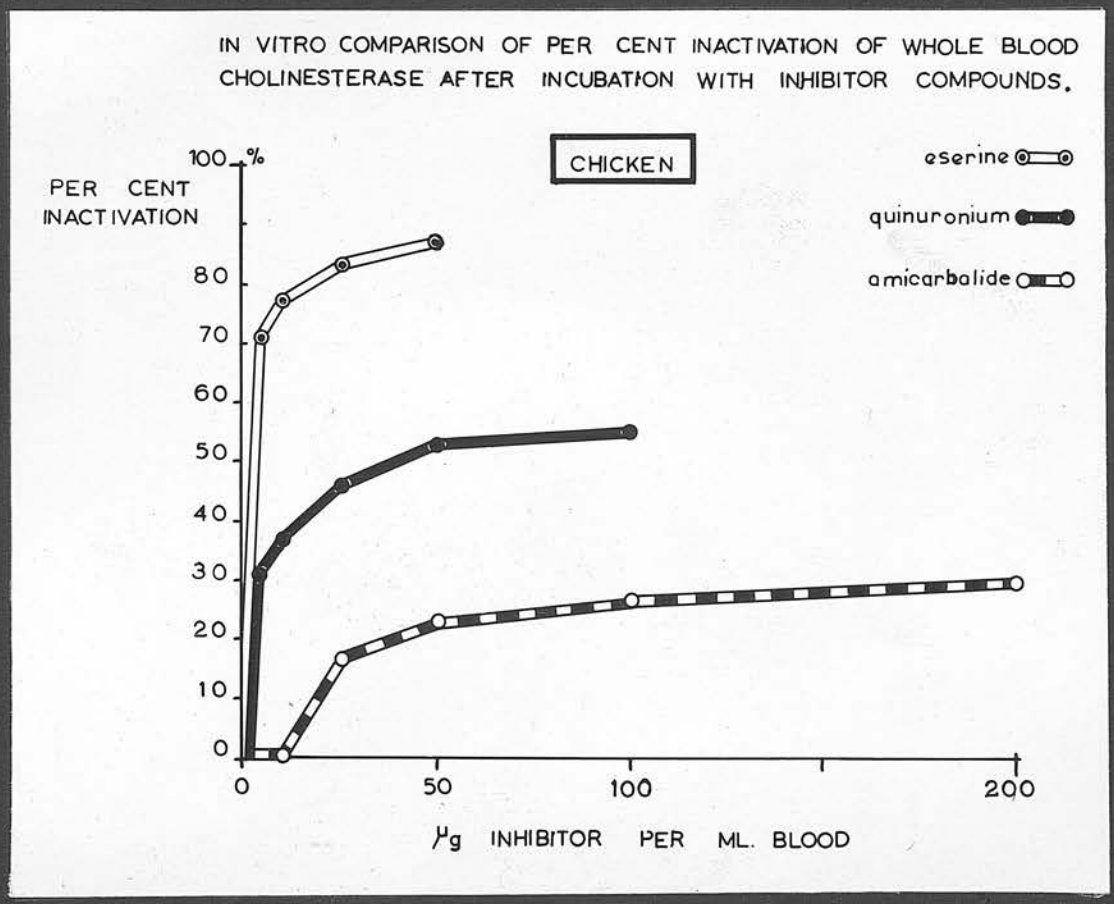

IN VITRO COMPARISON OF PER CENT INACTIVATION OF WHOLE BLOOD CHOLINESTERASE AFTER INCUBATION WITH INHIBITOR COMPOUNDS.
CHICKEN
eserine
quinuronium
amicarbalide
PER CENT INACTIVATION
100 %
80
70
60
50
40
30
20
10
0
0
50
100
200
µg INHIBITOR PER ML. BLOOD

FIG. F

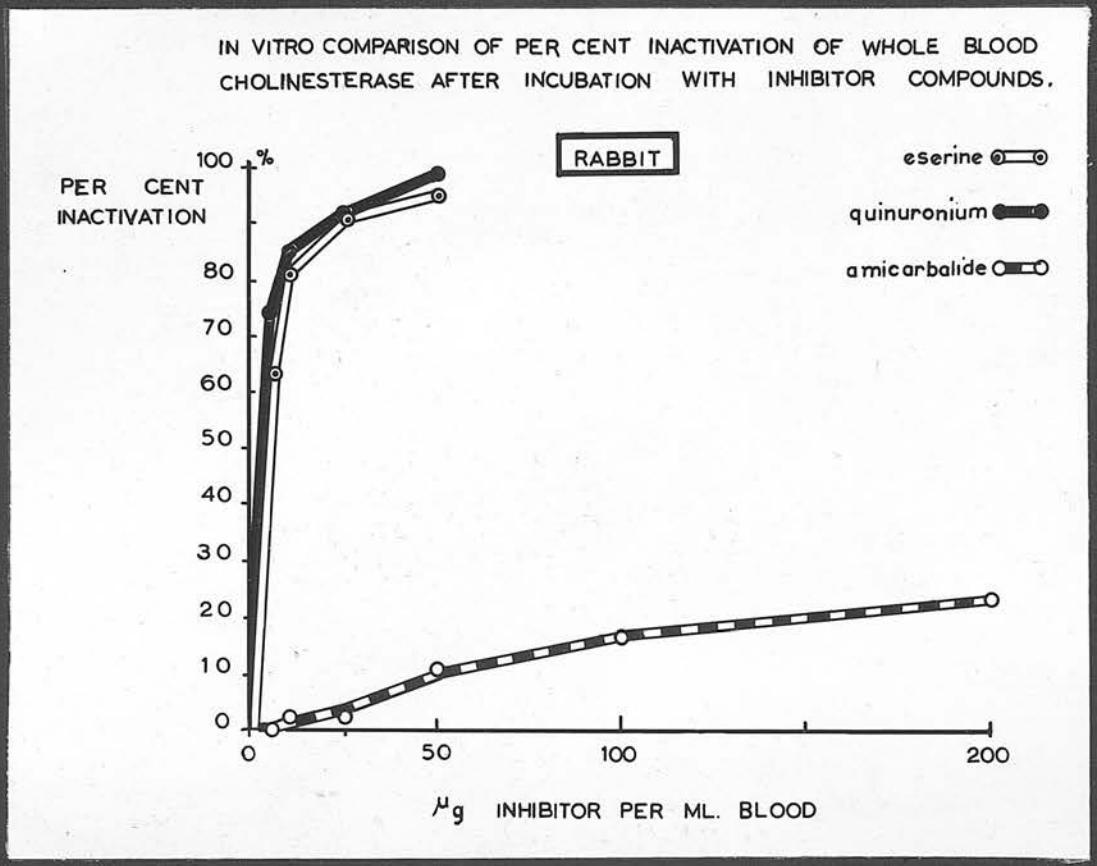

IN VITRO COMPARISON OF PER CENT INACTIVATION OF WHOLE BLOOD CHOLINESTERASE AFTER INCUBATION WITH INHIBITOR COMPOUNDS.
RABBIT
eserine
quinuronium
amicarbalide
PER CENT INACTIVATION
100 %
80
70
60
50
40
30
20
10
0
0
50
100
200
μg INHIBITOR PER ML. BLOOD

FIG. G

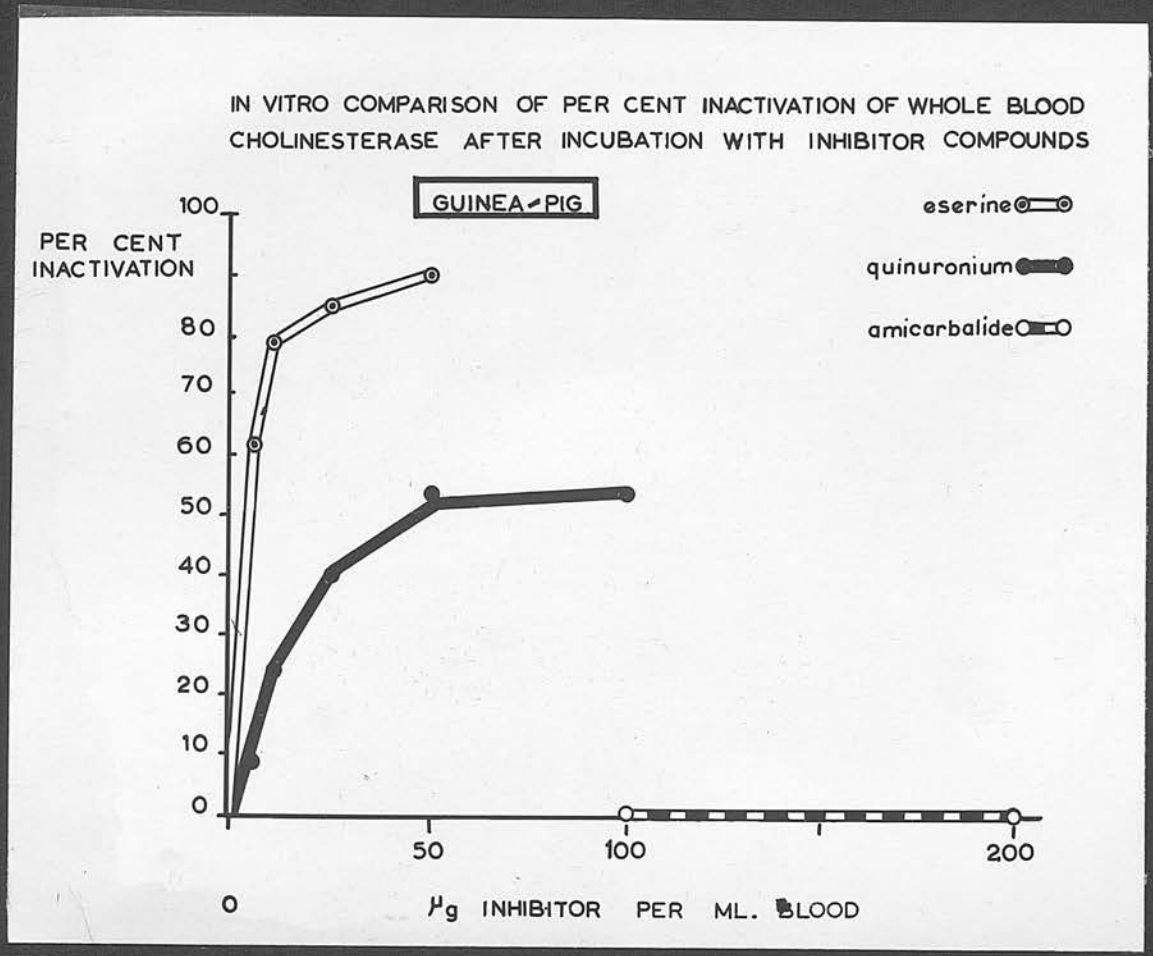

IN VITRO COMPARISON OF PER CENT INACTIVATION OF WHOLE BLOOD CHOLINESTERASE AFTER INCUBATION WITH INHIBITOR COMPOUNDS
GUINEA-PIG
PER CENT INACTIVATION
100
80
70
60
50
40
30
20
10
0
eserine
quinuronium
amicarbalide
50
100
200
0
μg INHIBITOR PER ML. BLOOD

FIG. H

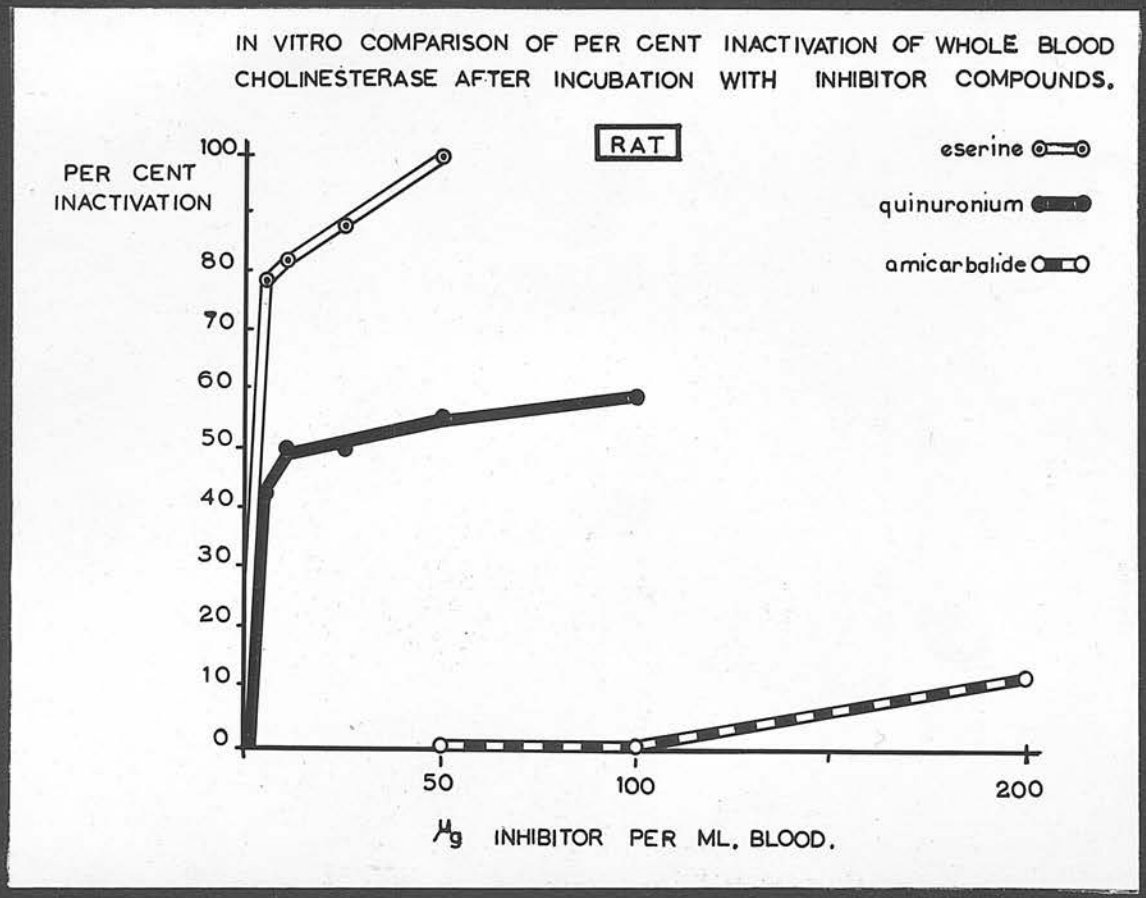

IN VITRO COMPARISON OF PER CENT INACTIVATION OF WHOLE BLOOD CHOLINESTERASE AFTER INCUBATION WITH INHIBITOR COMPOUNDS.
RAT
eserine
quinuronium
amicarbalide
PER CENT INACTIVATION
100
80
70
60
50
40
30
20
10
0
50
100
200
μg INHIBITOR PER ML. BLOOD.

FIG. J

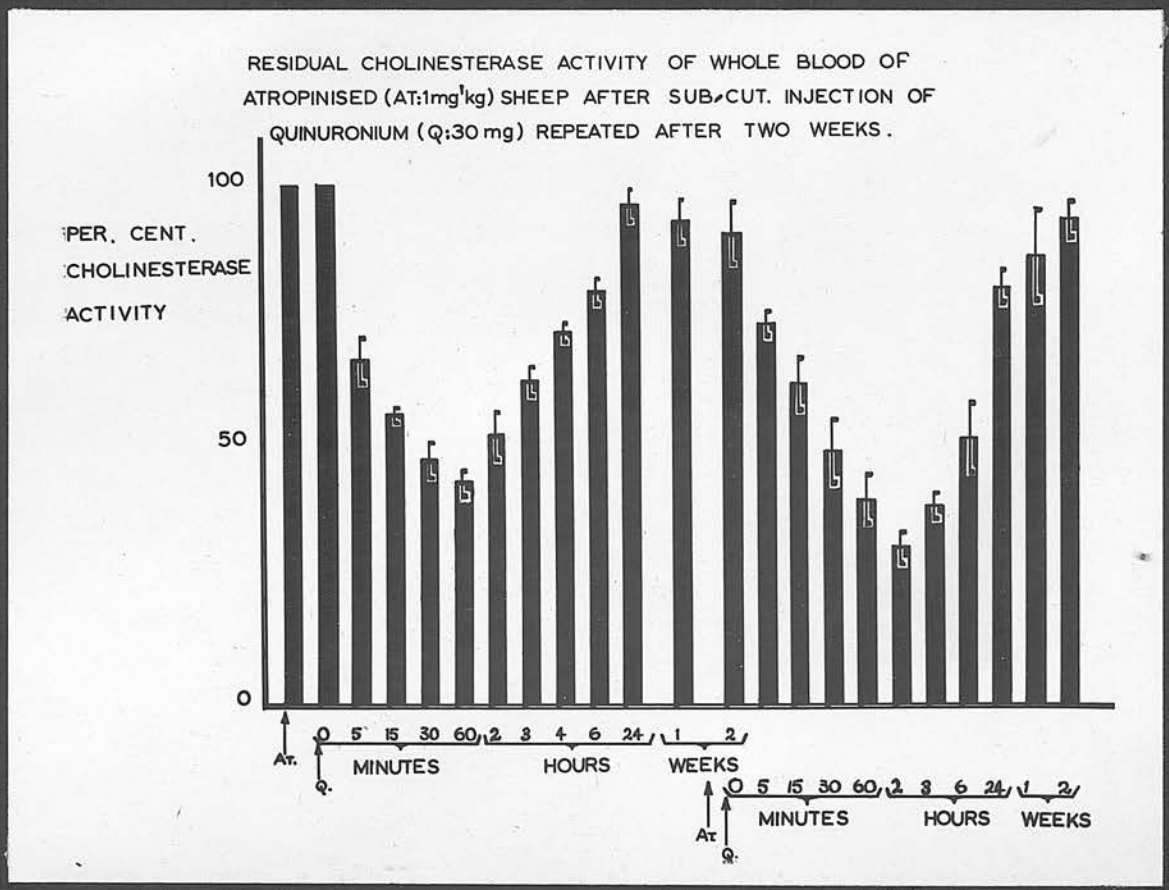

RESIDUAL CHOLINESTERASE ACTIVITY OF WHOLE BLOOD OF ATROPINISED (AT:1mg/kg) SHEEP AFTER SUB-CUT. INJECTION OF QUINURONIUM (Q:30 mg) REPEATED AFTER TWO WEEKS.
PER. CENT. CHOLINESTERASE ACTIVITY
100
50
0
At.
Q.
0 5 15 30 60 2 3 4 6 24 1 2
MINUTES
HOURS
WEEKS
At
Q.
0 5 15 30 60 2 3 6 24 1 2
MINUTES
HOURS
WEEKS

FIG. K

APPENDIX L.

THE RELEASE OF HISTAMINE FROM PERFUSED RAT HIND-QUARTERS

DRUG	MEAN HISTAMINE RELEASE (ug/ml) ± S.E.
QUINURONIUM 500 ug per ml	1.01 ± 0.40 (4)
48/80 100 ug per ml	2.98 ± 0.32 (4)
AMICARBALIDE 2 mgm per ml	1.25 ± 0.26 (4)

Number of samples is in parenthesis.

APPENDIX M.

THE RELEASE OF HISTAMINE FROM SHEEP DIAPHRAGM IN VITRO

DRUG TREATMENT	ug/ml DOSE	MEAN HISTAMINE RELEASE (ug/Gm ± S.E.)
NONE = CONTROL	0	1.63 ± 0.60 (4)
QUINURONIUM	500	5.62 ± 0.66 (4)
AMICARBALIDE	2000	3.11 ± 0.44 (4)
48/80	100	9.60 ± 1.45 (4)

Number of samples is in parenthesis.

APPENDIX N.

THE RELEASE OF TISSUE HISTAMINE FROM MICE IN VIVO

DRUG	TISSUE (SKIN) HISTAMINE (ug/Gm) MEAN ± S.E.
NONE (CONTROL)	26. 1 ± 2.27 (4)
QUINURONIUM	14. 1 ± 1.85 (4)
AMICARBALIDE	27.35 ± 1.25 (4)
COMP. 48/80	12.31 ± 1. 9 (4)

Number of animals is shown in parenthesis.

APPENDIX O.

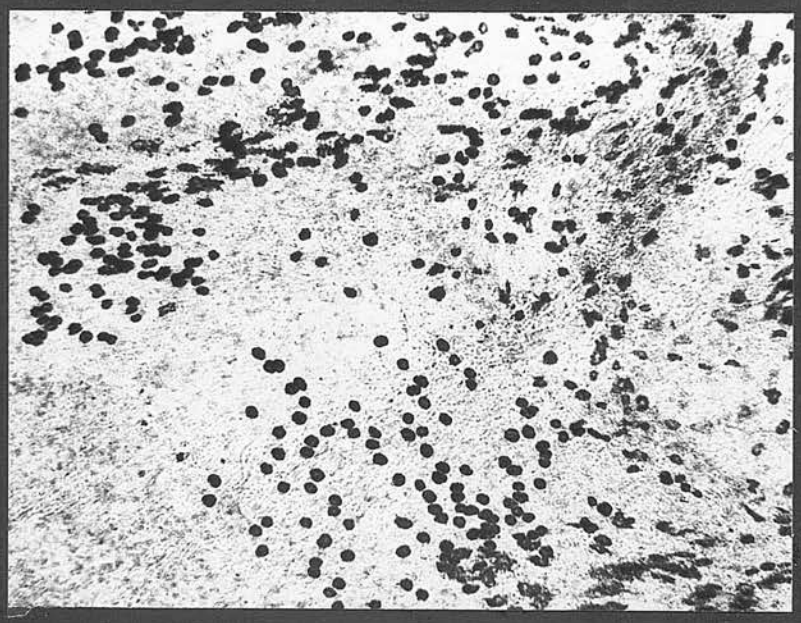
THE RELEASE OF TISSUE HISTAMINE FROM RATS IN VIVO

DRUG	TISSUE (SKIN) HISTAMINE (ug/Gm) MEAN ± S.E.
NONE (CONTROL)	31.4 ± 2.4 (4)
QUINURONIUM	16.1 ± 37.6 (4)
AMICARBALIDE	21.0 ± 2.0 (4)

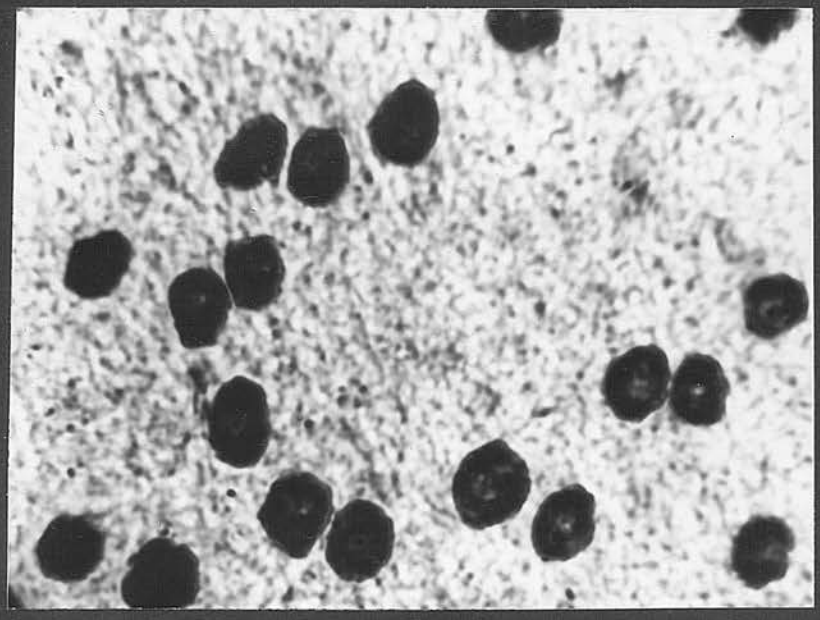
Number of animals is shown in parenthesis.

APPENDIX P

Under appendix P are representative histological preparations stained by toluidine blue from mice, rats and sheep which show the effects on mast cells of compound 48/80, quinuronium sulphate and amicarbalide isethionate.



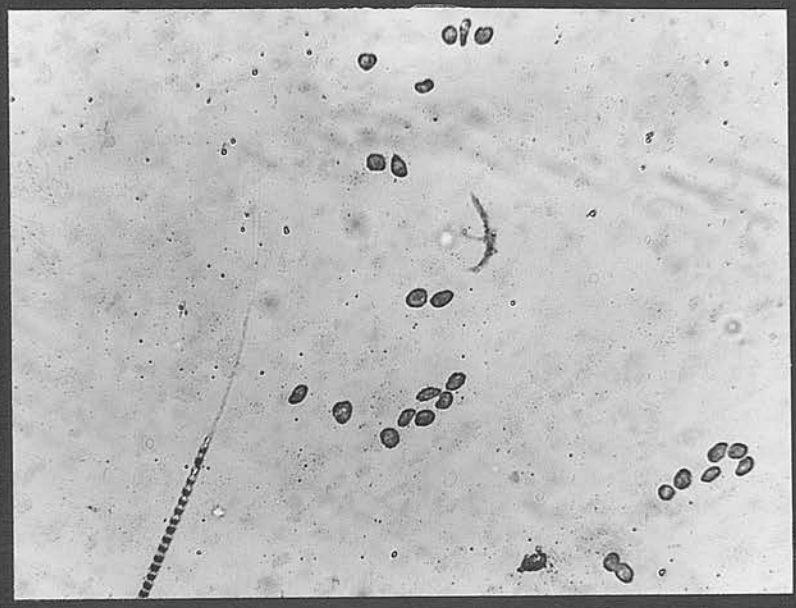
×200



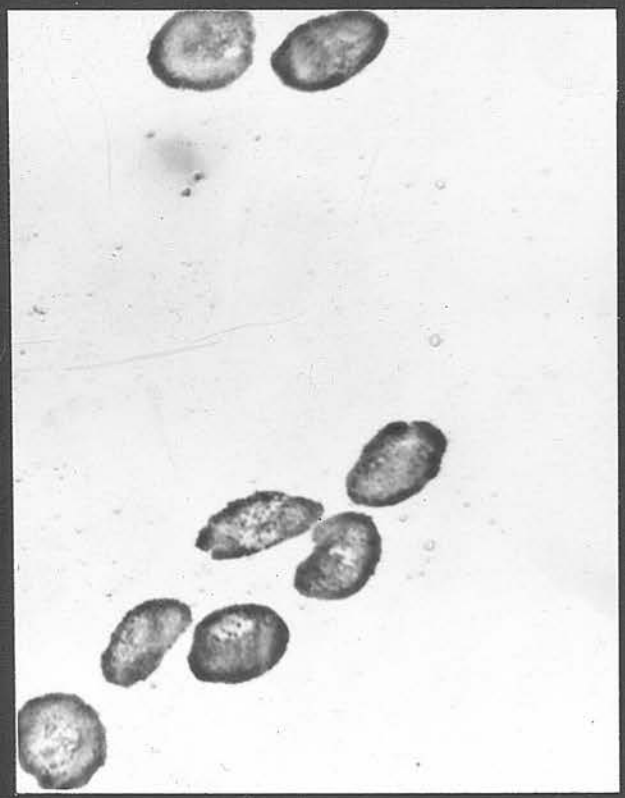
×900

FIG. P.1.

SUB-CUTANEOUS TISSUE OF NORMAL MOUSE.



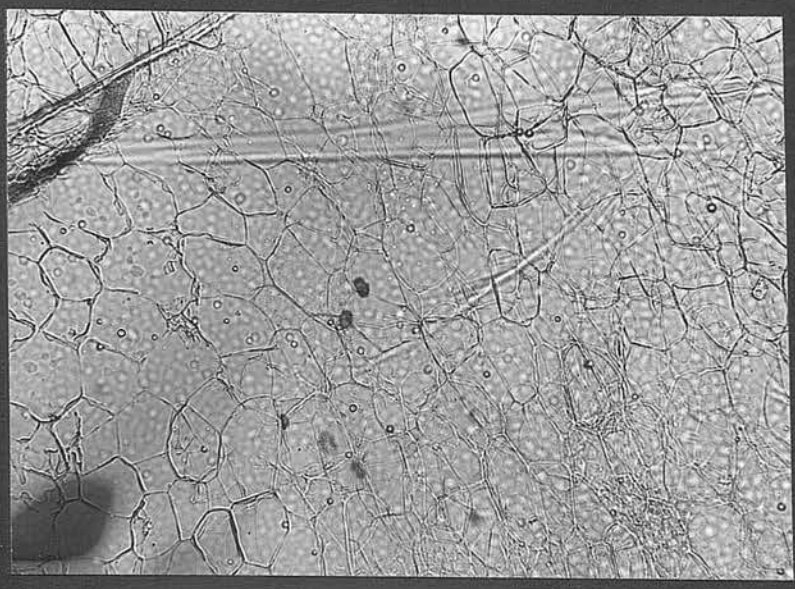
×210



×900

FIG. P.2.

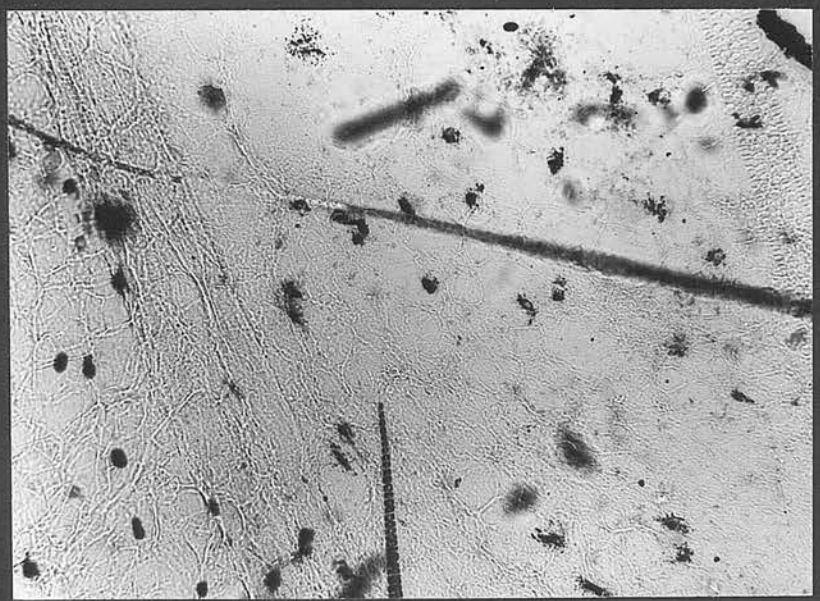
SUB-CUTANEOUS TISSUE OF MOUSE TREATED FOR THREE DAYS WITH QUINURONIUM.



×210

FIG. P.3.

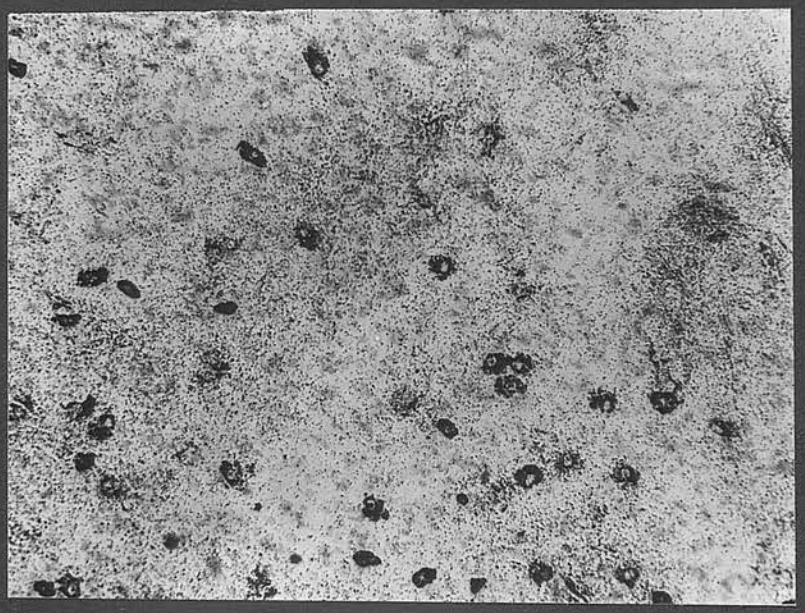
SUB-CUTANEOUS TISSUE OF MOUSE TREATED FOR THREE DAYS WITH COMP. 48/80.



×210

FIG. P.4.

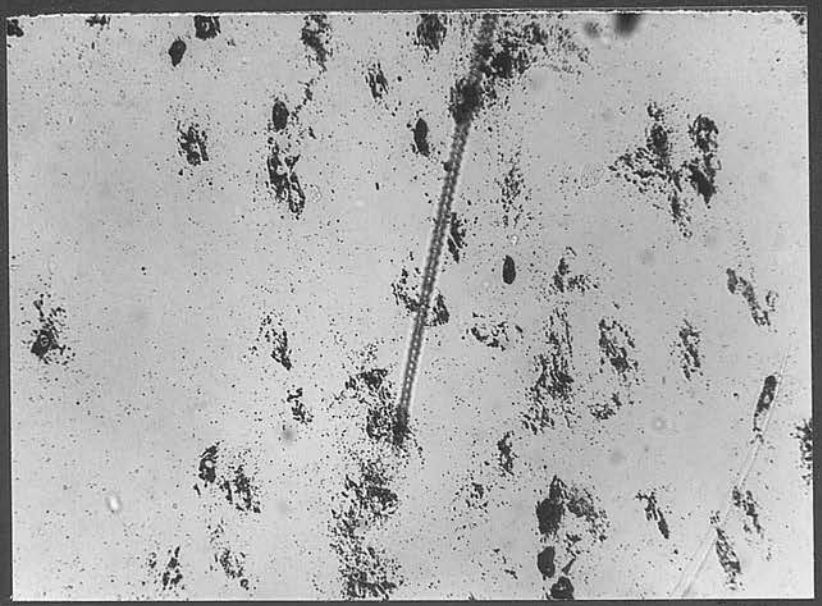
SUB-CUTANEOUS TISSUE OF MOUSE TREATED FOR THREE DAYS WITH AMICARBALIDE.



×240

FIG. P.5 (a).

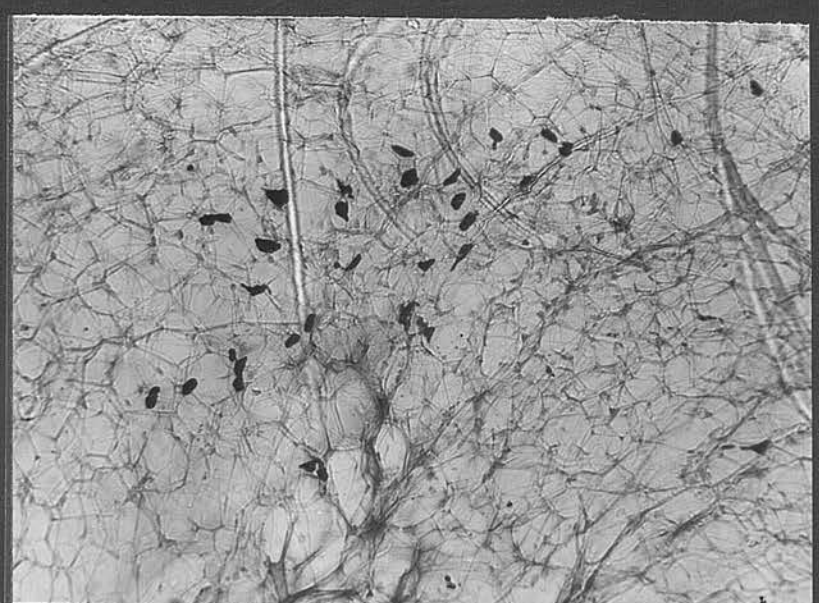
MESENTERY OF MOUSE WHICH HAD RECEIVED ONE LETHAL DOSE OF QUINURONIUM.



×240

FIG. P.5 (b).

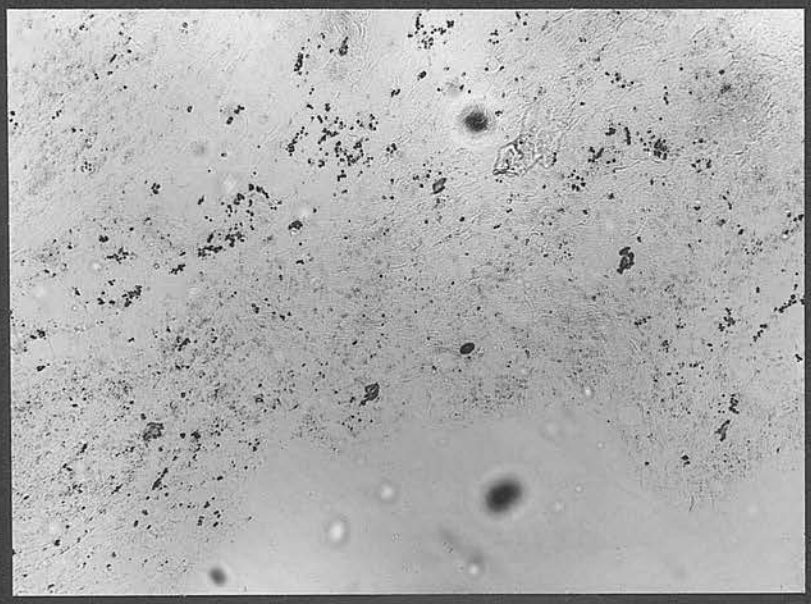
MESENTERY OF MOUSE WHICH HAD RECEIVED ONE LARGE DOSE OF 48/80.



×210

FIG. P.6.

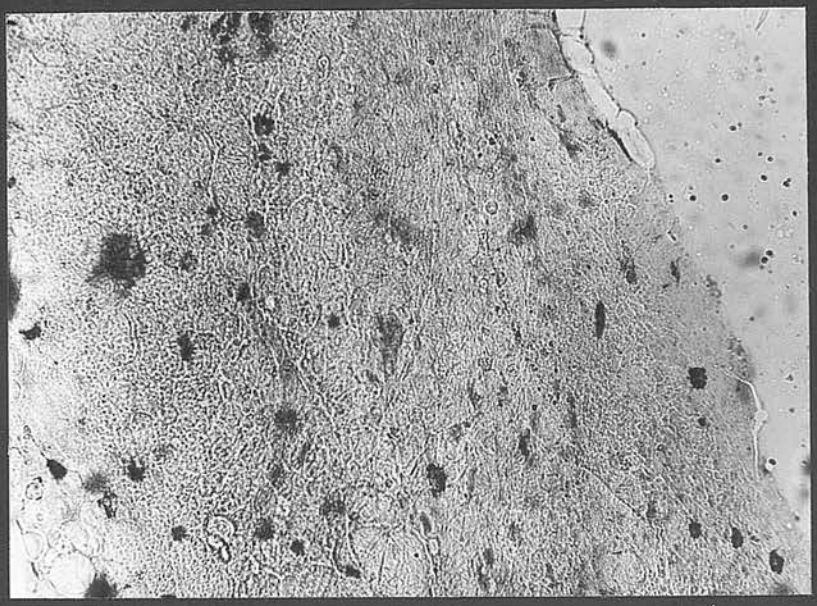
SUB-CUTIS OF NORMAL RAT.



×210

FIG. P.7.

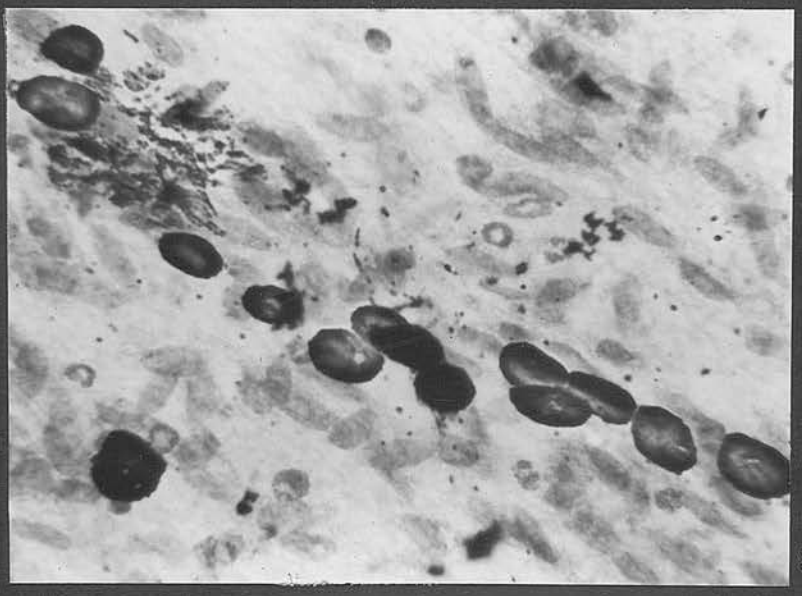
SUB-CUTIS OF RAT TREATED WITH QUINURONIUM FOR THREE DAYS.



×210

FIG. P.8.

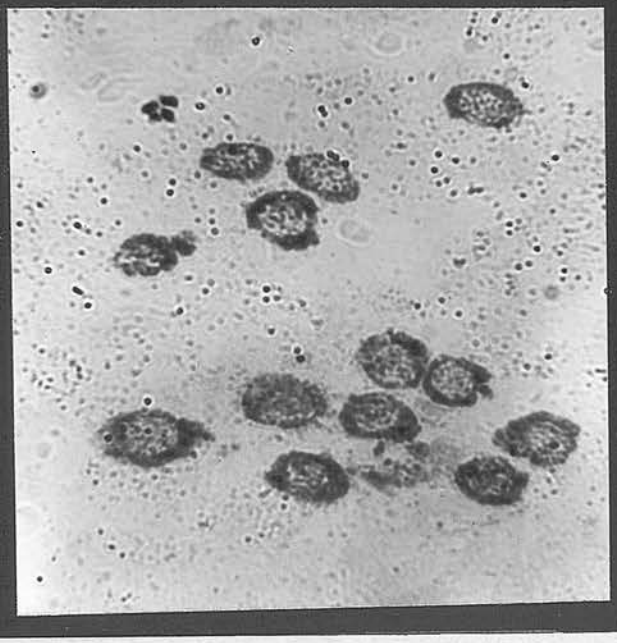
SUB-CUTIS OF RAT TREATED WITH AMICARBALIDE FOR THREE DAYS.



×850

FIG. P.9.

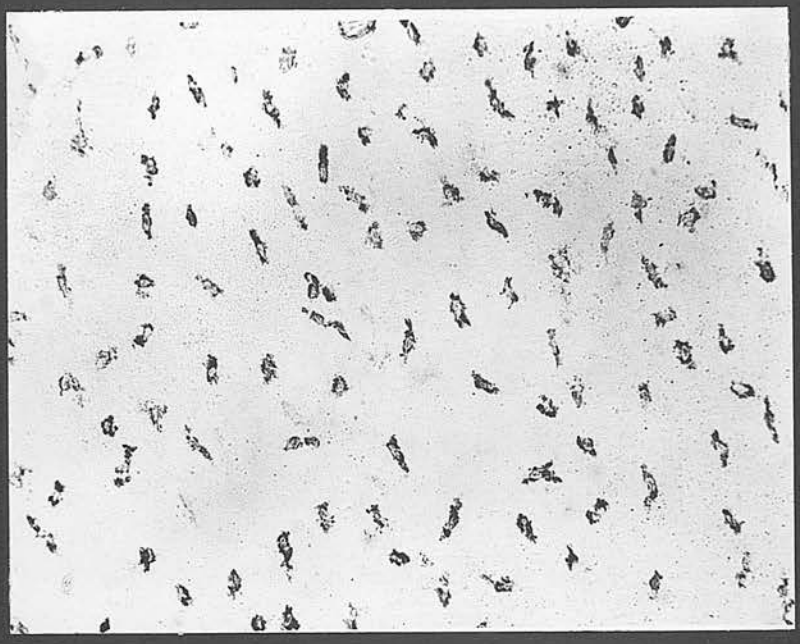
PERITONEUM OF SHEEP KILLED BY THIOPENTONE.



×850

FIG. P.10.

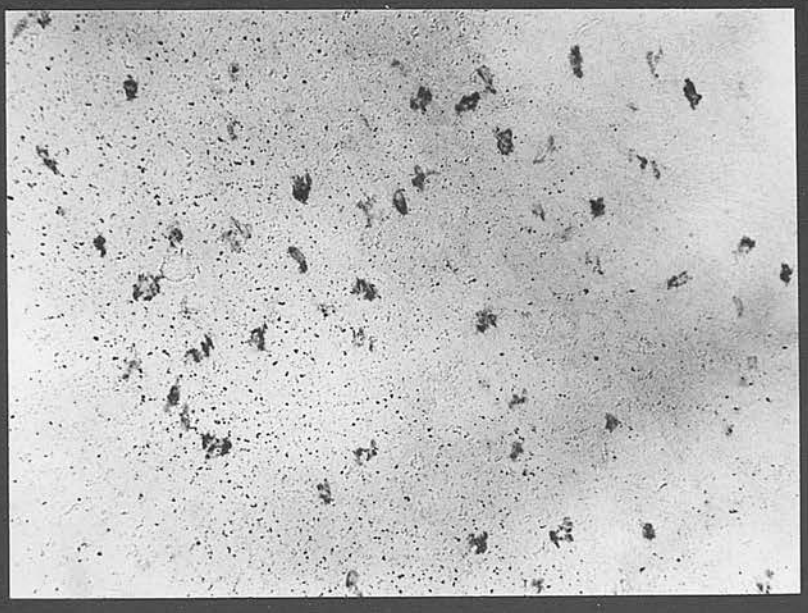
PERITONEUM OF SHEEP KILLED BY QUINURONIUM.



×210

FIG. P.11.

LIVER CAPSULE OF SHEEP KILLED BY THIOPENTONE.



×210

FIG. P.12.

LIVER CAPSULE OF SHEEP KILLED BY QUINURONIUM.